Plant Biotechnology in Ornamental Horticulture

Yi Li, PhD
Yan Pei, PhD
Editors

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Pre-publication REVIEWS, COMMENTARIES, EVALUATIONS . . .

"Although many books have been published dealing with plant biotechnology and transgenic plants, the ones that focus on ornamental plants are rare. This book FILLS THE GAP, and should be A VERY USEFUL REFERENCE FOR GRADUATE STUDENTS, FACULTY, AND INDUSTRY SCIENTISTS working in and outside the field. The chapters are well written by experts all over the world in various research directions of the field and provide the updated knowledge in biotechnology of ornamental plants. . . . In addition to the chapters that deal with the current knowledge, such as biotic and abiotic stresses, that can be applied to all or most crops, the book has some quite unique chapters that deal with issues that only apply to ornamentals, such as manipulating flowering time, and the color and scents of flowers, which are very interesting to read. . . . Provides a view from industry regarding the importance and concerns of biotechnology to the horticultural industry, as well as a discussion on economics of horticultural biotechnology. Another item I found very useful is the tables at the end of many chapters that list all the approaches reported to alter a specific trait, and the references."

Dr. Rongda Qu
Associate Professor
Department of Crop Science
North Carolina State University

More pre-publication
REVIEWS, COMMENTARIES, EVALUATIONS . . .

"AN EXTRAORDINARY COLLECTION. . . . Each chapter is WELL WRITTEN by a world authority on the subject matter. The editors have done an excellent job keeping a tight focus on each subject. Of particular note is the chapter on plant pigment engineering by Rosati and Simoneau and the chapter on the engineering of scents by Dudareva and Pichersky. Both of these areas will likely have far-reaching benefits in the years ahead. But whether they do or not depends on public acceptance and this topic is more than adequately examined in the chapter by Klingeman and Hall with supporting chapters by Meilan and by Alston et al. This book is A MUST HAVE by anyone working in the area of horticulture biotechnology and by regulators and policy makers whose decisions will impact everyone everywhere in the coming decade."

Michael E. Horn, PhD
Director
Cell & Molecular Biology
Phyton Biotech Inc.

"A THOROUGH REVIEW OF THE RESEARCH, which employs genetic engineering strategies and biotechnology for ornamental crop improvement. . . . Of interest to any researcher involved in improving horticultural crops, such as gene introduction and increasing resistance to pests and environmental stress. Chapters also include reviews of research with the goal of manipulating developmental and aesthetic characters of ornamental crops. Researchers with projects having applied objectives will find the chapters on 'The Economics of Horticultural Biotechnology,' 'Challenges to Commercial Use of Transgenic Plants' and 'Risk, Trust and Consumer Acceptance of Plant Biotechnology: Implications for Genetically Modified Ornamental Plants' insightful and helpful as their projects and plants near fruition. These last chapters discussing responsibilities, commercialization and marketing of genetically engineered crops will be IMPORTANT FOR RESEARCHERS AND STUDENTS TO READ."

Alan G. Smith, PhD
Associate Professor
Department of Horticultural Science
University of Minnesota
St. Paul, MN

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Plant Biotechnology in Ornamental Horticulture

CONTENTS

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Yi Li, PhD, is an Associate Professor of horticultural/ornamental molecular biology and biotechnology in the Department of Plant Science at University of Connecticut, Storrs, Connecticut, USA. Dr. Li received his BA degree from Department of Forestry, Beijing Forestry College (now Beijing Forestry University), Beijing and his PhD degree from Department of Biology, College of Environmental Science and Forestry, State University of New York, Syracuse, New York. He had his postdoctoral training from Department of Biochemistry, University of Missouri, Columbia, Missouri.

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Preface

The ornamental horticulture industry is one of the fastest growing segments of agriculture worldwide in recent decades. Transgenic plant technology is rapidly emerging as a powerful tool to improve ornamental plants. This volume, *Plant Biotechnology in Ornamental Horticulture*, provides an overview of the key scientific and technical advances, issues and challenges in the area of ornamental plant biotechnology. The volume contains 19 topics, beginning with an introduction of the importance of biotechnology to the horticultural and ornamental plant industry by Harriman et al., followed with Brand's summary of the recent advances in genetic transformation of ornamental plants. Handa et al. then provide a detailed description of molecular phylogeny-assisted breeding of ornamental plants. Because abiotic and biotic stresses cause enormous economic and quality losses to the ornamental plant industry and to consumers, this volume offers a series of reviews emphasizing the current status in the use of molecular tools to create horticultural and ornamental plants that exhibit increased tolerance to low temperatures (Park and Chen), drought (Shen and Wang), and diseases (Hammond et al).

Directed alterations in plant growth and development including rooting, flowering, reproduction and plant architecture are of major importance for the ornamental plant industry. Cheng et al. review the use of transgenic technologies to enhance adventitious root formation in difficult-to-root woody species. Giovannini outlines the progress in characterizing the genetic basis of signaling pathways that regulate the transition from vegetative growth to flowering using *Arabidopsis* plant as a model system and discusses molecular strategies to alter flowering

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and flower architecture of ornamental plants. Kim describes the genetic regulation of leaf shape, from the perspective of the spatial and temporal balance between cell division, cell enlargement, and cell differentiation. Subsequently, Franklin and Whitelam provide a detailed review for the recent progress in characterization of the phytochrome gene family and examples of transgenic manipulation of phytochrome gene expression to influence a variety of phenotypic traits such as plant height, lateral branching and harvest yield to meet the market needs. This volume also offers a review to discuss problems and challenges of invasive ornamental plants and possible transgenic strategies to neutralize the invasiveness of exotic ornamental plants (Li et al.).

Although traditional breeding methods have been used to alter floral color and to enhance fragrance of major ornamental plants, impressive progress has been made in the molecular cloning and characterization of genes important in biosynthesis of secondary metabolites that may regulate floral color and scent. Rosati and Simoneau examine the recent advances in the area of plant pigment formation, from the pioneering stages to fundamental and applied research with an emphasis on flavonoids and carotenoids. Dudareva and Pichersky provide a detailed review of the latest discoveries of genes and enzymes involved in the synthesis of scent compounds, and their potential use for enhancing fragrance of ornamental plants.

Synthetic growth regulators have been widely used in the ornamental plant industry to produce desirable traits, but exogenous applications of these regulators are often expensive and environmentally problematic. Progress has been made in recent years regarding genes that can alter the concentration of, or the plant response to plant hormones. Duan et al. outline biosynthetic and catabolic genes for auxin, cytokinin, and abscisic acid and their potential applications in ornamental crops. Mino et al. offer a summary for molecular biology of the metabolism and signal transduction of gibberellins and possible applications to crop improvement. Finally, Shibuya and Clark provide a thorough review on current status and future directions of using transgenic techniques to manipulate ethylene biosynthesis and signaling for improvement of flower longevity of ornamental crops.

Economics, government regulations, public perception and acceptance of transgenic plants will shape the commercial development of transgenic plants for ornamental uses. Alston et al. discuss economical aspects of commercialization of transgenic plants and their implications for ornamental crops. Meilan outlines the federal regulations and the process to obtain permission to release transgenic plants for experimen-

tal field trials and for commercial purposes with a specific discussion on ornamental crops. Finally, Klingeman and Hall summarize our current knowledge about the anticipated rewards, potential risks, and reality surrounding the public perception of transgenic plants.

We wish to thank the authors for their excellent contribution to this collection. Their time and effort are gratefully acknowledged. We also appreciate the scientists who reviewed the manuscripts. Their comments and suggestions were very helpful and highly appreciated. We would like to take this opportunity to thank Mr. William Smith, Mr. Wei Deng, Mr. Litang Lu and Mr. Chandra Thammina for reading the manuscripts and converting them into the appropriate format.

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Importance of Biotechnology to the Horticultural Plant Industry

Robert W. Harriman
Jyothi Prakash Bolar
Franzine D. Smith

SUMMARY. Horticulture continues to provide variety, color and flavor to the foods we eat, enriches our lives with many aesthetically appealing products, and adds to sustaining a healthy environment. Biotechnology is introducing new tools to advance the many benefits of horticulture. This review explores some of the benefits of horticulture and provides examples of successes being realized in agricultural biotechnology. The review also introduces some of the exciting work that may some day be available for consumers. A future that includes more nutritious fruits and vegetables; fragrant and more colorful flowers; a broader selection of indoor plants; and thicker, greener lawns that require less mowing and water are quickly becoming more than just a vision. We hope after reading this text, you feel the same way. doi:10.1300/J411v17n01_01 *[Article copies available for a fee from The Haworth Document Delivery Service: 1-800-HAWORTH. E-mail address: <docdelivery@haworthpress.com> Website: <http://www.HaworthPress.com>*

Robert W. Harriman is Vice President of Technology Development, Jyothi Prakash Bolar is Scientist and Franzine D. Smith is Director of Ornamental Biotechnology, Biotechnology Research and Development Group, The Scotts Company, Marysville, OH.

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INTRODUCTION

Horticulture is a branch of agriculture concerned with the cultivation of garden crops, generally fruits, vegetables, flowers and ornamentals such as plants used for landscaping (Encyclopedia Britannica, 1990). Horticulture is a large industry that supplies consumers throughout the world with edible and ornamental plants as well as the most popular leisure activity (gardening) in the U.S. The horticulture industry is financially big and logistically complex. The Food and Agriculture Organization lists 160 fruits and vegetables with an export value of $57 billion for the top 30 nations that it tracks in international trade (Huang et al., 2004). The global market for cut flowers is estimated at US$27 billion (Chandler, 2003). The U.S. turfgrass industry is estimated to be a US$40 billion business. Market size in 2003 for the floriculture industry represented sales of approximately $5 billion at the wholesale level (USDA Floriculture Crops 2003 Summary). Overall covered production area for production of flowers and plants comprised 929 million square feet and overall ground usage covered 47,000 acres managed by 11,913 growers.

The horticultural market has expanded dramatically due to the many benefits that plants, flowers, fruits and vegetables provide to enhancing human health and the environment. Fruits and vegetables are the primary source of essential minerals and vitamins. As our awareness of the health protecting importance of anti-oxidants continue to grow, the demand for fresher, better tasting, more varied and nutritious fruits and vegetables will continue to expand. Gardening will continue to play a vital role in preserving our mental health, providing therapeutic and physical value to our lives while promoting spiritual and physical healing. Flowers are the gift of choice for lifting the spirits of loved ones in the hospital, consoling grieving friends on the loss of a family member or to celebrate the birth of a child. Gardening is the number 1 outdoor leisure activity in the U. S. with 74 million American households participating. The urban and suburban sanctuaries provide food and shelter for birds and insects, and stress release for our fast-paced society. Flowering and foliage potted plants and fresh cut flowers allow us to bring indoors many of the emotional benefits provided by garden plants.

In our residential and commercial landscapes, public parks, gardens and athletic fields, turfgrass not only provides an emotional enhancement to our lives, it is often the foundation for many of our most popular sports. Turfgrass protects our environment. Lawns limit soil erosion and runoff. Grass blades filter pollen and dust, 12 million tons of dust per year. Turfgrass effectively sequesters carbon through carbon dioxide conversion and protection of fixed carbon from erosion, and it is an important source of oxygen; only 25 square feet provides enough oxygen for one person for one day. Turfgrass reduces the use of energy; an average lawn has the cooling effect of about ten tons of air conditioning (Beard and Green, 1994).

Most varieties of agricultural and horticultural crops have been developed by plant breeding. For centuries humans have been selecting and breeding plants for food, fiber and aesthetics. The principles of plant genetics, first established by Gregor Mendel in the 19th century, laid the foundations for traditional plant breeding, in which desirable traits are combined from different plants. These techniques resulted in the introduction of hybrids that led the way to green revolution and improving the efficiency of agriculture production throughout the world. Biotechnology or recombinant DNA technology is the latest stage in the development of plant breeding. Biotechnology is a process of selective breeding that involves identification of one or a few known genes responsible for a particular trait and to precisely insert, delete or modify them in a plant. This extends the range of traits at the breeder's disposal, and enables specific genes to be expressed in a crop plant without the uptake of unwanted characteristics, which must then be selected out (Prakash and Conko, 2004).

Agricultural biotechnology is a young science that has demonstrated the opportunity to reduce pesticide use, reduce soil erosion and develop plants with improved nutrition (Shelton et al., 2002; Herrera et al., 2005). Ten years ago, the Flavr Savr™ tomato was commercialized. During this time, biotechnology has become a key element of several crops in many countries. In 2004, 8.25 million farmers in 17 countries planted 200 million acres of biotech crops (James, 2004). During the 2004 growing season, 86% of soybeans, 76% of cotton and 46% of corn planted in the US were biotech-enhanced varieties (Runge and Ryan, 2004). These market penetration numbers clearly show that farmers have recognized the benefits of biotechnology. However, these benefits go far beyond value to agriculture. Soybeans developed to be resistant to the herbicide Roundup™ resulted in a six hundred million dollar reduction in the use of chemicals. Canola farmers who grow herbi-

cide-tolerant crops saved 14 million gallons of fuel a year due to fewer herbicide applications. Bt crops contains a gene obtained from *Bacillus thuringiensis* that protects the plants from lepidoptoran pests. Bt cotton and corn reduced insecticide use by 4.5 million pounds. Bt protected corn has reduced insect pest damage and the resulting molds and mycotoxins that are harmful to livestock and humans. No-till and conservation tillage is saving 1 billion tons of precious topsoil per year. These increases in production, decreases in pesticides, elimination of tractor runs, preservation of land and topsoil, and reduction in toxins benefits us as consumers and the environment we all depend upon.

In spite of the size and importance of the horticultural market and the astounding success of biotechnology in most of the world, the application of biotechnology for horticultural crop improvement has lagged significantly behind when compared to agricultural biotechnology products. Unfortunately, we have only a few examples of biotechnology application in horticulture, such as virus-resistant squash, Flavr Savr™ tomato with increased shelf life, a blue colored carnation, insect-resistant potatoes and transgenic papaya with enhanced resistance to papaya ringspot virus. While the Flavr Savr™ tomato and insect-resistant potatoes are no longer being commercialized, biotechnology-enhanced carnations, squash and papaya are in the market and performing well (Sankula and Blumenthal, 2004). Products that are in the pipeline include bentgrass that is resistant to glyphosate developed by The Scotts Company and Monsanto, and a blue-colored rose by Suntory.

Why has horticultural biotechnology not had a higher level of success? Although horticultural crops are of high economic importance, each crop represents only a small segment of a market that consists of hundreds of cultivars representing many different species. The lower economic value of each horticultural crop has limited investment in research for biotech improvements as compared to large acreage field crops like corn, soybean, cotton, etc. (Alston, 2004; also reviewed by Alston et al. and Meilan in this volume). While obvious traits are still unexploited due to the lack of effective technology, proven technology exists to develop biotechnology-enhanced products that would have real producer and consumer value. However, the cost to develop a regulatory petition and commercial launch delays are particularly difficult for those trying to develop specialty crops due to smaller market size. Thus, the high cost of biotechnology (Alston, 2004) and smaller market returns for individual crops make investing in horticulture biotechnology a more difficult business decision. As mentioned in this review, several highly efficacious and market-worthy products have already

gained regulatory clearance, but are not commercially available. Concerns of consumer acceptance may have influenced market launch of such biotechnology products as Bt potato, Bt apple and Bt sweet corn. Transgenic papaya with enhanced resistance to papaya ringspot virus has been enthusiastically adopted by farmers and stably integrated into consumer markets in Hawaii, Canada, and the U.S. mainland (Gonsalves et al., 2004). The success of transgenic papaya demonstrates that consumers appear ready to buy biotechnology products that improve performance and result in more sustainable production. Industry needs committed champions to bring the benefits of biotechnology to the marketplace.

While market complexity, technology, regulatory costs and concerns of public acceptance may be slowing product introductions (reviewed by Klingeman and Hall in this voume), a tremendous amount of highly, promising research is underway. This volume reviews many of these areas of discovery that may have commercial application in the future. Researchers are elucidating genes, defining pathways and developing molecular techniques that can improve horticultural crops. With the substantial number of plant categories and vast number of improvement potentials, this review will focus on introducing areas of biotechnology research that could result in the development of superior, value-added plants for the marketplace. While the long-standing agricultural enhancement targets such as disease resistance, insect resistance, stress tolerance and herbicide tolerance are also important opportunities for horticulture, there are many unique opportunities to provide plants with improved performance not applicable to row crops. Flowering ornamental plants with more fragrance, unique color or petal form, modified plant architecture or leaf structure could generate heightened demand by satisfying the core desire for "new and unusual" plants. More nutritious fruits and vegetables with more color and better shelf-life are achievable in the future. Also, thicker, greener lawns that require less water, fertilization and mowing will improve the quality of our lives and the environment around us. This text lays the foundation for an exciting look into the future value that biotechnology will add to horticultural products.

THE TOOLS OF BIOTECHNOLOGY

Biotechnology has evolved to include three interdependent applications important to horticulture: (1) cloning of elite germplasm using

techniques of micropropagation and regeneration; (2) genetic improvement of existing germplasm using molecular techniques of gene insertion, and (3) use of molecular assisted breeding to identify specific genes for selecting desirable phenotypes (reviewed by McCown, 2003).

One of the early applications of biotechnology in the horticulture industry has been the micropropagation of crops. Several techniques have been developed based on the source and/or type of explant tissue. Also, disease-free healthy plant propagules are produced for use as starting material. In addition, androgenesis or haploid plant generation, which has an advantage of producing homozygous lines in one generation, has found limited use in the breeding of asparagus and some woody crops (Ferrie, 2002). The techniques of somatic hybridization have had limited impact on the commercialization of new cultivars due to complex cell manipulations, change in ploidy, and the necessity for further extensive breeding (Johnson and Veilleux, 2001).

In recent years, the term biotechnology has become synonymous with genetic transformation or the process of inserting DNA into the host cell. One of the most widely used tissue culture techniques for plant transformation is the regeneration of whole plants from individual cells. Regeneration exploits the fundamental physiology of plant cells "totipotency," where any plant cell, given the right set of conditions, has the inherent genetic capacity to develop into a whole plant. Therefore, a reliable regeneration system is a key component for successful plant transformation. The process of introducing genes into plant cells can be grouped into vector-mediated gene transfer and direct gene transfer (Lorence and Verpoorte, 2004). *Agrobacterium* is the most commonly used vector for gene transfer (Gelvin, 2003). Several methodologies have been developed for direct DNA delivery (Rakoczy, 2002), the most common being microprojectile bombardment (Taylor and Fauquet, 2002). Plant transformation systems will be further reviewed by Brand in this volume. Regeneration and transformation techniques are now routinely being used for many crops. However, since there are many species and cultivars that have economic value in the horticulture and ornamental sector, it becomes a challenge to develop an efficient system for each one of these crops.

Advances in molecular biology have rapidly changed the techniques that are used for plant breeding. DNA marker technologies and genome analysis has increased the precision and efficiency in the manipulation of traits by facilitating the tagging of genes. Several techniques have evolved, including amplified fragment-length polymorphisms (AFLPs) that may give the largest number of markers while restriction fragment

length polymorphism (RFLP) and simple sequence repeats (SSRs) can be used to tie together the loci into a single map (Abbott, 2002). DNA marker technologies may be useful as marker-assisted selection, particularly for qualitative traits. Mapping of quantitative trait loci (QTLs) has been used to identify locations of chromosomes of loci having major effects on crop performance. Although most of these technologies have been developed with non-horticultural crops, they have immense potential for genetic improvement of horticultural crops (McCown, 2003). The current status in molecular assisted breeding of ornamental crops has been reviewed by Handa et al. in this volume.

The availability of entire genomic sequences of *Arabidopsis* and rice has strongly boosted plant molecular biology research. DNA microarray and other high-throughput technologies have enabled whole-genome expression analysis (Zhu, 2003). Bioinformatics is an integral aspect of plant and crop science research. Developments in data management and analytical software are reviewed with an emphasis on applications in functional genomics. Bioinformatics facilitates the analysis of genomic and post-genomic data and the integration of information from related fields of transcriptomics, proteomics, metabolomics, and phenomics. Such integration enables the identification of genes and gene products and can elucidate the functional relationships between genotype and observed phenotype (Edwards and Batley, 2004). Knowledge gained from functional analysis of *Arabidopsis* and rice sequences promises to lead to major advances in our understanding of desirable traits in horticultural crops. The development of technologies that allow the introduction and functional expression of heterologous genes in plant cells has extended to the production of transgenic plants with improved insect and disease resistance, seeds and fruits with enhanced nutritional qualities, and plants that are better adapted to adverse environmental conditions (Herrera et al., 2005).

ABIOTIC STRESS

Increasing tolerance to abiotic stresses, such as drought, heat, freezing, salt and low light, would improve the performance of many horticultural plants. Abiotic stress is the primary cause of crop loss worldwide, reducing the average yields for most crop plants by more than 50% (Boyer, 1982; Wang et al., 2003). The consumers desire to grow a wider selection of ornamentals has driven the industry to look for species and cultivars that have better adaptability to a range of temperature, moisture

and light conditions. Extensive research is being conducted to elucidate molecular mechanisms involved in plant response to environmental stress, which would result in developing plants with better adaptability to these stresses. Several reviews highlight recent studies on gene expression in response to environmental stress and on the signaling pathway that are either common or specific to the stress response (Seki et al., 2003; Wang et al., 2003; Park and Chen; Shen and Wang in this volume). Stress-inducible genes have been classified into two groups: (1) genes that protect against environmental stress and (2) genes for the regulation of expression and signal transduction in the stress response. Since several of these genes interact with each other and are part of different networks of response, engineering pathways may be necessary rather than single gene regulation.

It would be hard to argue that the rate-limiting element for productivity and quality is the availability of water. Restrictions on plant type and watering days are being enacted in several parts of the U. S. Development of low water requiring ornamental and turfgrass plants would be beneficial to the environment. Fruit and vegetable growers would benefit from varieties that maintain yield under water deficit conditions. Our understanding of the process underlying plant response to drought, at the molecular and whole-plant level has progressed rapidly. Hundreds of genes that are induced under drought have been identified. Several new tools that operate at molecular, plant and ecosystem levels are being used to study the specific function of these genes and their role in plant acclimation or adaptation to water deficit (reviewed by Chaves et al., 2003).

Soil salinity is a major abiotic stress in plant agriculture, strongly influencing plant productivity worldwide. Drought and salinity are becoming particularly widespread in many regions, and may cause serious salinization of more than 50% of all arable lands by the year 2050 (Wang et al., 2003). Soil salinity can also be an issue in horticulture. For example, golf courses and large landscapes that rely on use of reclaimed water could benefit from having salt tolerant plants. Frequent irrigation and dry climates could result in salt buildup in the soil. Improving salt tolerance would increase the adaptability of these horticultural crops. Research towards improving the salt tolerance in transgenic plants can be grouped into five categories: synthesis of osmolytes, protection of cell integrity, oxidative stress, ion homeostasis, and transcription factors (reviewed by Borsani et al., 2003). Under drought or salt stress, glycine betaine is accumulated in cells of some higher plants and animals as an osmoprotectant. Transgenic turfgrass overexpressing the

gene encoding betaine aldehyde dehydrogenase (BADH) had increased tolerance to salinity and drought conditions compared to the controls (Chen et al., 2004). Overexpressing AtNHX1, a vacuolar Na(+)/H(+) antiport from *Arabidopsis thaliana* significantly improved the salinity tolerance of transgenic *Brassica napus* plants (Zhang et al., 2001). Advances in mechanisms and applications leading to drought and salt tolerance in plants will be further reviewed by Shen and Wang in this volume.

Ongoing research on the genetic mechanisms associated with plant response to low temperature has resulted in identification of several genes. Hsieh et al. (2002) improved chilling tolerance in tomato by constitutively expressing an *Arabidopsis* C-repeat/dehydration responsive element-binding factor 1 (CBF1). The CBF1 transgenic tomato plants also exhibited considerable tolerance against oxidative stress. Park et al. (2004) transformed tomato with a chloroplast-targeted codA gene of *Arthrobacter globiformis*, which encodes choline oxidase to catalyze the conversion of choline to glycinebetaine (GB). These GB-accumulating plants were more tolerant to chilling stress than their wild-type counterparts. Alpha-galactosidase is involved in many aspects of plant metabolism, including hydrolysis of the alpha-1,6 linkage of raffinose oligosaccharides during deacclimation. Down-regulating alpha-galactosidase resulted in an increase in freezing tolerance in transgenic petunia plants (Pennycooke et al., 2003). Malik et al. (1999) demonstrated the ability to both increase and decrease thermotolerance in carrot by manipulation of expression of a single small heat shock protein gene, hsp17. 7. Recent advances in engineering cold tolerance for a number of plant species are summarized by Park and Chen in this volume.

Low-light or shady conditions and infrared signals from neighboring plants often lead to a significant reduction in plant performance, and under higher shade competition, it can prevent growth. Over-planted flower beds often have stretched plants or plants that have been knocked over by the elements. Color opportunities for shady landscapes are limited to a few species such as hostas, impatiens, camellias and hydrangea. One of the most common questions we get at The Scotts Company concerns growing turfgrass in shady environments, such as the northern exposure of a home or under trees. A number of genes have been identified that control plant response to light quantity and duration (Fankhauser and Staiger, 2002). In the future, will we have a greater selection of horticultural specimens to plant around our homes and lawns that survive the summer competition from neighboring trees and shrubs? Franklin

and Whitelam (this volume) outline encouraging research that would certainly suggest these products will be developed.

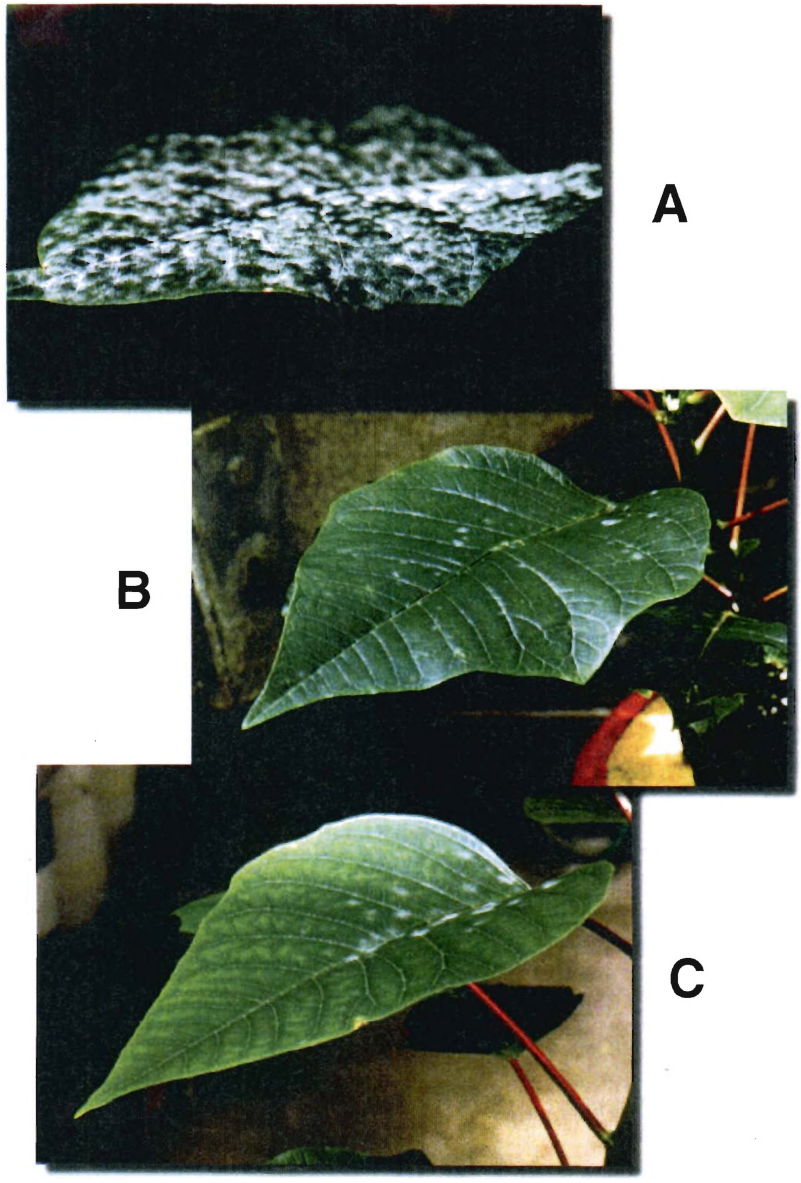
DEVELOPMENT OF DISEASE RESISTANCE

Plant disease resistance from pathogens, such as viruses, bacteria or fungi is one of the major factors that influences not only the yield of horticultural plants, but also the quality and aesthetic appeal. Apples with scab or strawberries with *Botrytis* are rejected by most U. S. consumers. The constant challenge of controlling black spot (*Diplocarpon rosea*) is turning consumers away from growing garden roses. Commercially, golf course superintendents aggressively manage dollar spot, brown patch and snow mold to provide true playing surfaces for their clients.

Several disease control options are available to growers and consumers, such as resistant germplasm, chemical control and cultural practices. Routinely, the horticulture plant industry uses many aspects of plant disease control. Breeders select germplasm not only for aesthetic value but also for outdoor performance, which can include disease resistance or reduced incidence of diseases. Many germplasm companies produce disease free stock through the use of micropropagation techniques, e.g., *Xanthomonas*-free geranium stock material and virus-free strawberry plants. Growers apply cultural practices that favor plant growth without favoring plant pathogens, i.e., clean greenhouse conditions, steam-sterilized potting media, plant spacing, controlled environmental conditions, etc.

Starting with clean propagation material that has inherent genetic resistance to a pathogen would be beneficial. However, genetic resistance to diseases is not available in all the key commercial varieties of horticultural crops. Biotechnology offers another tool to improve disease resistance. To date, horticultural crops with genetically engineered pest resistance are limited (e.g., virus resistant squash, papaya and melons; Gonsalves, 2002; Fermin et al., 2005; Fuchs et al., 2004). All of the examples of transgenic improvement of horticultural crops to other diseases are either in the research phase in labs or field trials. For instance: an antimicrobial peptide (AMP) gene, Ace-AMP1, was introduced into *Rosa hybrida* cv. Carefree Beauty. These transgenic lines showed enhanced resistance to powdery mildew in both a detached-leaf inoculation assay and an *in vivo* greenhouse whole-plant assay (Li et al., 2003) (Figure 1). Similarly, transgenic *Impatiens wallerana* expressing a

FIGURE 1. Powdery mildew on the most heavily infected poinsettia leaf 21 days after inoculation. Non-transgenic control (A), MagII transgenic line AN-03-15 (B) and MSI99 transgenic line AN-04-25 (C).



magainin analog had increased resistance to *Botrytis cinerea* infection (Knapp and Brand, 2003).

In the above examples, single effector molecules were utilized. Recent work has included identification of regulatory genes that influence the control of a battery of disease resistance genes. In addition, in-depth studies on host-pathogen interactions, genetics of diseases, plant genomic, proteomic and bioinformatic studies of several key pathogens of important crops have led to a better understanding of the molecular mechanisms of plant resistance (Jones, 2001; Martin et al., 2003; Michelmore, 2003). Knowledge accumulated from these areas of research can be used to enhance resistance using marker assisted breeding and plant transformation techniques. Specific genes can be moved across species or genera, and native genes can be regulated within the same crop. This area of research as applicable to horticulture has been extensively reviewed by Hammond et al. in this volume.

PROTECTION FROM INSECTS

Not surprisingly, insect protection has been extremely successful in agricultural biotechnology. In the late 1990s, Dry Creek Lab developed Bt-apples to control cottling moths. This product would not only have been highly effective at reducing pesticides for apple production, but could have also reduced pesticides on sentinel plants for almond and walnut production. Unfortunately, this product is currently not in active development. Other impressive Bt-products not on the market include Bt-potato for control of Colorado potato beetles and Bt-sweet corn for protection from a variety of lepidopteron pests. Bt-technology has increased yields, reduced pesticide levels and energy usage (Shelton et al., 2002).

Does insect control have a role in horticultural crops? The need is clear and present. With improvements in public perception and regulatory approval, we would expect to see these products and more making their way back into the marketplace.

HERBICIDE TOLERANCE

As noted in the introduction, herbicide tolerance has been a boon to farmers and the environment. Crops resistant to broad-spectrum herbicides, such as glyphosate and glufosinate, increase the efficacy and

flexibility of weed control. The result is less energy needed to grow the crop, fewer herbicide applications, more food and fiber per acre of production and adoption of topsoil-saving methods such as no-till farming.

As weeds reduce yields, increase pesticide usage and threaten quality, many horticultural crops would also benefit from the introduction of herbicide tolerance. One such product under development is creeping bentgrass, *Agrostis stolonifera*, which is resistant to glyphosate. Creeping bentgrass is a low-growing, fine-textured, dense turfgrass that tolerates low mowing and is well adapted to handle the rigors of mowing at 1/8th of an inch imposed by golf course superintendents and golfers. While glyphosate resistant bentgrass would allow the effective removal of several weeds, elimination of annual bluegrass, *Poa annua*, will provide the greatest impact (Figure 2). Because of the lack of products to remove annual bluegrass, golf course superintendents are forced to co-manage the desirable bentgrass along with the undesirable annual bluegrass or risk significant die-backs from biotic and abiotic stresses. Without the need to co-manage, superintendents may reduce the amount

FIGURE 2. Weed management in glyphosate resistant bentgrass. The right plot shows infestation of annual bluegrass while the left plot is weed free following an application of glyphosate.

of fungicides (11.6 pounds/acre), growth regulators (0.7 pounds/acre), water and fertilization while improving the quality of their course (USDA petition, docket #03-104-01p).

CONTROLLING FLOWERING

The shortage of labor in California during World War II led to the development of determinate flowering varieties of processing tomatoes to allow for mechanical harvesting. Poinsettia's success during the Christmas season was enabled by the use of black cloth to protect developing plants from light exposure at night. Our winter months have become brighter with pots bursting with color from bulb plants of tulips and daffodils due to artificial vernalization known in the industry as forcing.

Timing of production of a particular stage of the plant is crucial for the marketing success at the retail level. The timing of flowering is of great importance especially for ornamental crops. In most of these crops, the product appeal to the consumer is the flower. For the breeders, the product is often flowers or the result of flowering, i.e., fruit or seed production. The demand for certain ornamentals increases exponentially during particular times of the year, such as roses during Valentine's Day and poinsettia during Christmas. Several environmental factors interact and influence the developmental stage or transition from vegetative to reproductive stages of plants. Artificial manipulation of growing conditions including expensive greenhouse facilities is frequently used to meet this consumer need. Therefore, understanding the molecular basis of flowering-time control could lead to strategies to regulate flowering by genetic manipulations that modify the timing of flowering (Amasino et al., 2004; further reviewed by Giovannini of this volume). By regulating flowering-time, we could synchronize flowering to generate a more dramatic floral display or aid in harvesting. We may also be able to extend the growing season for crops in a region where the season is otherwise too short or the conditions do not induce flowering.

The transition of plants from vegetative growth to flowering is the major developmental switch in the plant life cycle. The timing of flower initiation is critical for the reproductive success of plants, and most plant species have evolved systems to precisely regulate flowering time. Genetic analysis has identified genes in several species that affect the timing of flowering. In *Arabidopsis* over 80 genes that regulate flower-

ing time have been identified (Simpson et al., 1999). Regulation of flowering in *Arabidopsis* is mediated by two main pathways in response to environment (photoperiod/long-day and temperature/vernalization pathways) and two pathways that function independently of environmental cues: (1) the autonomous pathway, which promotes flowering under all conditions, and (2) the gibberellins pathway, which is needed for flowering under non-inductive short-day conditions. These pathways converge in the induction of floral meristem identity genes and the floral transition (Blazquez et al., 2001). It is likely that the same genes identified as controlling flowering in *Arabidopsis* are involved in controlling flowering time in other plant species and hence would allow us to genetically regulate their reproductive traits.

A molecular understanding of the genes and pathways responsible for perceiving and transducing environmental stimuli could be used to manipulate the plant response. Several photoreceptors play important roles in the transduction of light to endogenous clock and flowering-time genes, each being activated by specific qualities and quantities of light and their interactions with each other. Overexpression of phytochrome A (*PHYA*) in transgenic aster shortened the critical day length for inflorescence development from 14 to 8 h. Overexpression of *PHYB* shortened the length of the night break needed to induce inflorescence development from 2h to 15 min. Under commercial conditions, from autumn through spring, the overexpression of either *PHYA* or *PHYB* in transgenic asters substantially increased the yield of flowering shoots (Wallerstein et al., 2002). This potential application of manipulation of the phytochrome family of plant photoreceptors has been reviewed by Franklin and Whitelam in this volume. Alteration of the phytochrome genes can modulate not only the timing of reproductive development, but also plant architecture.

MODIFYING PLANT ARCHITECTURE

To satisfy consumer's desire for something 'new,' breeders are constantly challenged to produce novel ornamentals with different shapes and sizes for the whole plant and flower or fruit. Traditionally, this has been achieved mainly by selective breeding. Also, exogenous applications of compounds that regulate plant hormones and help change or modify plant architecture are being used in production. However, these compounds are not commonly used at the consumer level.

In the chrysanthemum industry, one of the major goals is to produce small, well-branched compact pot plants. Removing apical meristems to promote branching (eliminates apical dominance) and the use of growth retardants are used to promote branching and to control height, respectively. Gibberellin biosynthesis inhibitors applied to plants restricts stem elongation. Production costs are increased and timing is critical in the use of the chemicals. Recently, a number of GA-insensitive genes have been cloned, including *spindly (spy)*, *short internodes (shi)*, and *GA insensitive (gai)* (Teixeira da Silva and Nhut, 2003). Petty et al. (2003) introduced the *gai* gene into the chrysanthemum cultivar 1581 and reported that the majority of the transgenics had reduced height and more compact growth habit. Reducing height of turfgrasses could result in reduced water use and reduced mowing. Growth regulators, such as paclobutrazol, have been used to increase turfgrass color and density, reduce mowing and clipping production and enhance shade tolerance. The Scotts Company and Monsanto are developing bluegrass and St. Augustinegrass cultivars that reduce the level of active GA. When approved for commecialization, these grasses will reduce mowing time, yard waste and emissions, and likely reduce water and fertilizer usage, while increasing the color, density and product quality.

Another method to control excessive internode elongation in greenhouses is to use photoselective filters that selectively attenuate far-red wavelengths. As a genetic approach, Zheng et al. (2001) ectopically expressed in chrysanthemum a tobacco phytochrome B1 (*Phy-B1*) gene resulting in growth reduction, which has the same effect as commercial growth retardants. Since light rich in red wavelengths inhibits stem elongation, the expression of *Phy-B1* (a sensor for red light) probably increased the sensitivity to red wavelength resulting in inhibition of stem elongation. Kim's review in this volume describes on-going research towards modifying the shape of plants.

Several commercially important traits were altered in transgenic petunia by the introduction of the rolC gene from *Agrobacterium rhizogenes*: reduction in plant height, leaf and flower size, a break in apical dominance and decreased male and female fertility (Winefield et al., 1999). Time to flowering was also reduced in these transgenic plants. Similarly, Mercuti et al. (2001) observed desirable ornamental traits such as dwarfness and early flowering in transgenic limonium carrying the rol A, B and C genes. An *A. rhizogenes* based transformation of *Angelonia salicariifolia* plants with wild type strains of the bacterium resulted in plants exhibiting dwarfness and smaller leaves. There were no apparent alterations observed in the number, shape and size of the

flowers. Pollen fertility of the transformed plants was 60-80% (Koike et al., 2003). Boase et al. (2004) reported altered phenotype including reduction in plant height, leaf area, petal area and corolla length in transgenic regal pelargonium cv. Dubonnet expressing the rolC gene. Trangenics flowered 22 days earlier than non-transformed plants. Constitutive expression of rolC gene in transgenic carnation (*Dianthus caryophyllus* L. 'White Sim') resulted in an increase in the number of flowering stems and better rooting compared to the controls (Zuker et al., 2001).

It is known that auxin interacts with other signaling pathways to regulate inter-cellular developmental processes (Swarup et al., 2002). Examples of such interactions include auxin and cytokinin, auxin and ethylene, auxin and ABA, auxin and GA, as well as auxin and light. Consequently, biotechnological manipulation of auxin may have impacts on many aspects of plant development leading to novel phenotypes, which is a goal of many plant breeders. Zazimalova and Napier (2003) have further reviewed the complexity of these auxin interactions and auxin regulation. Similarly, cytokinins are involved in many processes involved in plant growth and development and act in concert with other signals (Brault and Maldiney, 1999). The importance of auxins, cytokinins and gibberellins in plant growth and development is further reviewed by Duan et al. and Mino et al. in this volume.

DELAYING SENESCENCE

Fruits and vegetables with greater shelf-life, flowers that maintain their color longer, lawns and garden plants that stay green and healthy are all desirable advantages of delaying senescence. In fact, several horticultural products have been developed to delay senescence, but only one has actually been introduced to the market, the Flavr Savr™ tomato. Controlling physiological responses to ethylene has been introduced into tomatoes, melons and various flowering ornamentals. In addition to ethylene, cytokinins have been shown to be antagonists of senescence. Leaf senescence was significantly delayed by increasing cytokinin levels with the autoregulatory expression of the isopentenyl transferase (ipt) gene using a senescence-induced promoter (Gan and Amasino, 1995).

Ethylene plays an integral role in numerous plant developmental pathways, ranging from fruit ripening to plant/pathogen interactions (Klee, 2004). As ethylene is also involved in the floral senescence of

many ornamental plants, there has been a plethora of research to dissect the processes involved (van Doorn and Stead, 1997; Have and Woltering, 1997; Sato-Nara et al., 2003). It has been shown that ethylene controls abscission of inflorescences, flowers, petals, sepals, styles and stigmas and flower buds. Consequently, the inhibition of ethylene perception should result in a delay in the initiation of these processes, with a concomitant increase in the life of the flower (Figure 3). Stearns and Glick (2003) reviewed the impact of altered ethylene biosynthesis or perception on a large number of transgenic crops. In ornamentals, floral longevity is important for bedding plants, flowering potted plants, foliage plants and cut flowers (Have and Woltering, 1997). The role of ethylene and its impact on flower longevity of horticultural crops is reviewed by Shibuya and Clark in this volume.

MODIFYING FLOWER COLOR

As consumers seek an ever-increasing range of color in their favorite species, breeders strive to develop a range of colors and variegation patterns. However, certain petal colors are not available in the germplasm of particular species due to limitations such as deficiencies in the vari-

FIGURE 3. Flower performance assay. Comparison of ETR1-1 petunia transgenic line Ph03-0024 (left) vs. the non-transgenic control (right) 12 weeks after germination in the greenhouse. Ph03-0024 has more open flowers (more color) compared to the control.

ous pigment pathways, pigment localization, and pH (Mol et al., 1999; Yamaguchi et al., 2001; Davies, 2004).

Modifying flower color has been the poster child of unique products that could be developed with biotechnology. In 1996, Florigene brought this vision to the market in the form of blue carnations under "Moon" series brand names: Florigene Moondust™ and Florigene Moonshadow™ (Tanaka et al., 2005). By introducing flavonoid genes, in particular delphinidins, from petunia, Florigene was able to produce carnations ranging in color from light purple to an intense violet that appears black. Several selections are currently being marketed in Japan, Australia, the U.K. and the U.S. Recently, Suntory Ltd., a Japanese based flower breeding company, reported the development of the world's first genetically modified blue rose (Associated Press, 2004). As with carnations, roses do not contain delphinidins. Suntory Ltd chose pansy as the source of delphinidin genes. While additional enhancement is needed to bring the performance to a commercial level, we are looking forward to the day the Blue Rose will be on the market.

A chalcone reductase (*Chr*) gene from *Medicago sativa* was used to alter chalcone biosynthesis in petunia resulting in a change from white to yellow flowers (Davies et al., 1998). The orange pigment pelargonidin is not found in petunias, as the dihydroflavonol 4-reductase (DFR) enzyme from petunia is unable to convert dihydrokaempferol into the substrate for pelargonidin, kaempferol. Researchers at Novartis were able to produce uniformly colored orange petunias by introgression of the maize *Dfr* gene into the multiflora petunia plant type (Oud et al., 1995). Elomaa et al. (1995) obtained similar results of stable orange in petunia using the gerbera *Dfr* gene.

Anthocyanins in plants make complexes with copigments such as flavones or flavonols, and thus make the flowers look more blue. Inactivation of the *Dfr* gene in torenia caused the accumulation of flavones, which made the flowers more blue (Aida et al., 2000). Davies et al. (2003) were able to redirect the flavonoid biosynthesis away from production of colorless flavonols towards the production of colored anthocyanins. They were able to change white-flowered 'Mitchell' petunia to a pink-flowered phenotype by down-regulating the flavonol synthase (*Fls*) gene. To modify flower color in carnation 'Eilat', Zuker et al. (2002) used antisense suppression to block the expression of a gene encoding flavanone 3-hydroxylase, a key step in the anthocyanin pathway. The transgenic plants exhibited flower color modifications ranging from attenuation to complete loss of their original orange/reddish color. Transgenic plants with severe modifications were more fragrant than

control plants, suggesting a possible interrelation between the pathways leading to anthocyanin and fragrance production.

Cytochrome P450 enzymes play important roles in biosynthesis of flavonoids that determine flower color. Ueyama et al. (2002) of Suntory Ltd. were able to modify flower color of torenia by regulating the P450 genes. The phenotypes of the transgenic flowers ranged from pale pink to red from the original blue color. Mori et al. (2004) expressed the flavonoid 3′,5′-hydroxylase gene of *Vinca major* (VmFH1) in transgenic *Petunia hybrida* and showed a drastic flower color alteration from red to deep red with deep purple sectors. Antisense expression of flavonol synthase gene (FLS) in transgenic purple colored lisianthus produced flowers with more red than the original untransformed plant (Nielsen et al., 2002).

Marigolds are popular ornamentals grown for their showy flowers. In addition, they are a good source of natural colorants such as carotenoid and are used as a feed in the poultry industry to intensify the yellow color of egg yolks and broiler skin. Hauptmann et al. (2003) were able to genetically alter the carotenoid compositions and ratios in marigold as a potential commercial application to the poultry feed industry. Our current understanding and future potential for enhancing flower and plant color for ornamental purposes have been further reviewed by Rosati and Simoneau in this volume.

INCREASING FRAGRANCE

Ornamental plants have been valued for flower color, architecture and plant habit. However, non-visual benefits are also commercially important, especially the fragrant volatiles emitted by both flower petals and foliage. Many plants primarily emit floral scent to attract a variety of pollinators. Nevertheless, humans find an aesthetic value in certain types of floral scent, especially of moth-pollinated flowers, which is often described as sweet smelling. A large number of commercial flower varieties have lost their scent during the selection and breeding processes due to the breeders focus on enhancing visual aesthetic values (reviewed by Dudavera and Pichersky, 2000; Vainstein et al., 2001; Blowers, 2003).

Two approaches can be used to genetically enhance flower fragrance. One is based on the introduction of heterologous genes encoding enzymes with activities that are absent in the target plant. The genes allow new branching of existing pathways or the generation of a novel path-

way. The second approach is based on regulating the expression of native genes within the plant, either by up-regulating a desirable volatile or blocking the production of an undesirable volatile (Vainstein et al., 2001). Several genes are being cloned which encode floral scent biosynthetic enzymes and the underlying molecular mechanisms are being uncovered that control floral scent production and emission. In some instances, the mechanism involved in the loss of fragrance by particular varieties or species is being studied.

To evaluate the possibility of producing monoterpenes in 'Eilat' carnation, transgenic plants expressing the linalool synthase gene isolated from *Clarkia breweri* were produced. GC-MS analysis revealed that leaves and flowers of the transgenic carnation emit linalool and its derivatives. However, this did not lead to detectable changes in flower scent for human olfaction (Lavy et al., 2002). Metabolic engineering of floral scent for ornamentals is further reviewed by Dudareva and Pichersky in this volume.

CONCLUSION

Horticulture plays a vital role in maintaining both environmental and human health. Commercially, horticulture production is large and complex. The complexity of the horticultural market and various obstacles has resulted in a significant lag of biotech products making an impact in the marketplace. We are confident that with horticulture's growing importance, the development of new value-added traits, continued refinements in the regulatory system, increased public acceptance, and the continued dedication of many talented scientists, any remaining obstacles to bring biotechnology-enhanced horticultural products to the tables, lawns and gardens of consumers throughout the world will be overcome.

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Ornamental Plant Transformation

Mark H. Brand

SUMMARY. Ornamental plant transformation has advanced considerably in the last decade. Now over 40 genera have been reported to be transformed. The primary methods of creating transgenic ornamental species have been *Agrobacterium tumefaciens*-mediated transformation and microprojectile bombardment. The vast majority of reports indicate the use of *Agrobacterium*-mediated transformation employing binary vectors and *vir* helper plasmids or supervirulence genes. Many reports are of transformation with the uidA reporter gene driven by the 35S cauliflower mosaic virus promoter, but recent efforts are now focusing on trait genes including disease resistance, flower color, flower longevity, floral scent and plant habit. Greater use of tissue specific and inducible promoters promises to enhance the functionality and usefulness of introduced trait genes. While technical challenges for production of transgenic ornamental plants still exist, the greatest challenges to realizing the potential benefits of transgenic ornamental plants are questionable public acceptance of transgenic plants and the prohibitive costs of generating environmental impact data needed to gain regulatory clearance.
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KEYWORDS. *Agrobacterium*-mediated transformation, microprojectile bombardment, transgenic, ornamental, gene gun, biolistics

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Transformation of ornamental plants has lagged behind transformation of important food and fiber crops, primarily due to economic reasons. With major agronomic crops there are relatively few species to focus on and large acreages of each crop, so the substantial economic costs of transgenic plant development can be justified. With ornamentals, there are numerous plant species grown, and no one species represents the same economic potential as corn or cotton, for example. A 1991 review of floricultural crop biotechnology (Woodson, 1991) indicated that only petunias were routinely transformed by *Agrobacterium*. Currently, over 40 ornamental genera have been transformed and several are routinely transformed (Table 1).

There are some compelling reasons to make transgenic ornamentals. Mol et al. (1995) discuss how traits such as flower color, plant size, plant architecture, flower vase life and fragrance are suitable targets for transgenic ornamental plant improvement. Table 1 shows that these traits are indeed those that researchers have focused on with ornamentals. In addition, considerable effort has been expended to create transgenic ornamental plants with enhanced disease resistance. The most commonly inserted trait gene in ornamentals, as with all plants, has been the uidA, GUS reporter gene.

Efficient tissue culture systems are a requirement for ornamental plant transformation. Again, because there are so many ornamental plant species, development of tissue culture systems for all species of interest has progressed more slowly than for major agronomic crops. Nonetheless, there now exist regeneration systems for a number of important ornamental species (Geneve et al., 1997). The most widely used approach for ornamental species has been either direct or indirect adventitious shoot formation (Zucker et al., 1998; Table 1). For some important ornamental species, such as rose, embryogenesis has been the regeneration pathway that was necessary (Xianquian et al., 2003; Marchant et al., 1998a). The most commonly used explants for ornamental plant transformation via adventitious shoot regeneration have been leaves or leaf pieces, or stem pieces (Table 1). These can be harvested from established in vitro shoot cultures or from greenhouse-grown plants. Use of greenhouse explants can only be applied to species where surface sterilization can be done with high success and little contamination. This method is generally not successful with recalcitrant species. Other commonly used explants for transformation include hypocotyls, protocorm-like bodies (orchids), nodules, bulb scales and petioles (Table 1).

In addition to tissues that are competent for regeneration, transformation systems need an efficient DNA delivery system, agents for selec-

TABLE 1. Selected reports of transgenic ornamental plants.

Scientific Name	Tissue Used	Transformation Method	Vector Used	Selection Marker	Promoter Used (trait)	Trait Gene	Reference
Aesculus glabra	embryogenic callus	*agrobacterium*-mediated	EHA 105	HPT	CaMV 35S	uidA	Trick and Finer, 1999
Agapanthus praecox ssp. *orientalis*	leaf derived embryogenic calli	*agrobacterium*-mediated	EHA 101; LBA 4404	HPT; NPTII	CaMV 35S	uidA	Suzuki et al., 2001
Alstroemeria (tetraploid)	embryogenic callus and proembryos	particle bombardment	1 µM gold	bar	maize ubi1	uidA; luc	Lin et al., 2000
Alstroemeria pelegrina var. *alba* × *A. magenta* hybrids	suspension cells from ovaries	*agrobacterium*-mediated	EHA 101; LBA 4404	HPT; NPTII	CaMV 35S	uidA	Akutsu et al., 2004
Anthurium andraeanum and A. 'UH1060'	root cuttings	*agrobacterium*-mediated	LBA 4404	NPTII	CaMV 35S	att	Chen et al., 1997
Begonia tuberhybrida	young leaf explants	*agrobacterium*-mediated	LBA 4404	NPTII	CaMV 35S	uidA, rol A, B, C	Kiyokawa et al., 1996
Begonia ×*hiemalis*	young leaf discs	*agrobacterium*-mediated	AGL0; LBA 4404	HPT; NPTII	CaMV 35S	uidA	Kishimoto et al., 2002
Betula pendula	leaves	*agrobacterium*-mediated	LBA 4404	NPTII	pin-2	uidA	Keinonen-Mettala et al., 1998
Betula pendula	leaves	*agrobacterium*-mediated	LBA 4404	NPTII	CaMV 35S	uidA	Lemmetyinen et al., 1998

TABLE 1 (continued)

Scientific Name	Tissue Used	Transformation Method	Vector Used	Selection Marker	Promoter Used (trait)	Trait Gene	Reference
Brassia, *Cattleya*, *Doritaenopsis*	protocorm-like bodies	particle bombardment	gold		CaMV 35S	bar	Knapp et al., 2000
Campanula carpatica	hypocotyls	*agrobacterium*-mediated	AGL0; LBA 4404	NPTII	CaMV 35S; fbp1	uidA; etr1-1	Sriskandarajah et al., 2004
Campanula glomerata	leaf explants	*agrobacterium*-mediated	EHA 105; LBA 4404	NPTII	CaMV 35S	uidA; ipt	Joung et al., 2001
Chrysanthemum ×morifolium	stem segments	*agrobacterium*-mediated	AGL0	NPTII	CaMV 35S; Lhca3.St.1	uidA	Annadana et al., 2001
Chrysanthemum ×morifolium	stem segments	*agrobacterium*-mediated	AGL0	NPTII	CaMV 35S; UEP1; chs-A; EPF2-5; CER6; PMC	uidA	Annadana et al., 2002
Chrysanthemum ×morifolium	thin cell layers	*agrobacterium*-mediated; agrolistics; particle bombardment; sonication	LBA 4404; gold	NPTII	CaMV 35S	uidA	Teixeira da Silva and Fukai, 2003
Chrysanthemum ×morifolium	leaf discs	*agrobacterium*-mediated	EHA 105	NPTII	CaMV 35S	phytochrome B1	Zheng et al., 2001
Chrysanthemum ×morifolium	leaf blade explants	*agrobacterium*-mediated	LBA 4404	NPTII	CaMV 35S	Lc	Boase et al., 1998

Chrysanthemum ×morifolium	stem segments	*agrobacterium*-mediated	C58; MP90	NPTII	CaMV 35S	RCC2	Takatsu et al. 1999
Cyclamen persicum	etiolated hypocotyls	*agrobacterium*-mediated	EHA 105; LBA 4404	NPTII	CaMV 35S	uidA	Boase et al., 2002
Cyclamen persicum	etiolated petioles	*agrobacterium*-mediated	AGL0; LBA 4404	HPT; NPTII	CaMV 35S	uidA	Aida et al., 1999
Cymbidium	protocorm-like bodies	particle bombardment	gold	NPTII	CaMV 35S	uidA	Yang et al., 1999
Datura meteloides	ex vitro leaves	*agrobacterium*-mediated	LBA 4404	NPTII	CaMV 35S	uidA	Curtis et al., 1999
Dendrobium	protocorm-like bodies	particle bombardment	gold; tungsten	NPTII	CaMV 35S; duplicate CaMV 35S	uidA	Nan and Kuehnle, 1995
Dendrobium	protocorm-like bodies	*agrobacterium*-mediated	AGL1; EHA 105	HPT	CaMV 35S	uidA	Men et al., 2003
Dendrobium	protocorm-like bodies	*agrobacterium*-mediated	LBA 4404	NPTII	CaMV 35S	DOH1 antisense	Yu et al., 2001
Dendrobium	callus; in vitro inflorescense tips	particle bombardment	gold		CaMV 35S; HBT; ubi1	GFP	Tee et al., 2003
Dianthus caryophyllus	ex vitro stem segments	*agrobacterium*-mediated with microprojectile prewounding	AGL0	NPTII	CaMV 35S	antisense f3h	Zucker et al., 2002

TABLE 1 (continued)

Scientific Name	Tissue Used	Transformation Method	Vector Used	Selection Marker	Promoter Used (trait)	Trait Gene	Reference
Dianthus caryophyllus	ex vitro stem segments	*agrobacterium*-mediated with microprojectile prewounding	AGL0	NPTII	CaMV 35S	*linalool* synthase	Lavy et al., 2002
Dianthus caryophyllus	leaf explants	*agrobacterium*-mediated	AGL0	NPTII	FBP1; CaMV 35S	etr1-1	Bovy et al., 1999
Dianthus caryophyllus	ex vitro stem segments	agrobacterium-mediated	AGL0	NPTII	CaMV 35S	rolC	Zucker et al., 2001
Dianthus caryophyllus	petal explants	*agrobacterium*-mediated	AGL0	NPTII	CaMV 35S	uidA	van Altvorst et al., 1996
Dianthus caryophyllus	ex vitro stem segments	*agrobacterium*-mediated with microprojectile prewounding	EHA 105; AGL0	NPTII	CaMV 35S; MAS	uidA	Zucker et al., 1999
Dianthus caryophyllus	?	*agrobacterium*-mediated		NPTII	CaMV 35S; MAS	antisense ACO	Savin et al., 1995
Dianthus caryophyllus	ex vitro stem segments	particle bombardment	gold	bar		uidA	Zucker et al., 1995
Dianthus caryophyllus	ex vitro stem segments	*agrobacterium*-mediated	AGL0	NPTII	NOS	uidA	Nontaswatsri et al., 2004
Echinacea purpurea	in vitro leaves, petiole, roots	*agrobacterium*-mediated	LBA 4404	NPTII	CaMV 35S	chs	Wang and To, 2004
Eschscholzia californica	in vitro cotyledons and embryogenesis	*agrobacterium*-mediated	GV3101	NPTII	CaMV 35S	uidA	Park and Facchini, 2000

Euphorbia	callus	particle bombardment	tungsten	NPTII; HPT	enhanced CaMV 35S; CaMV 35S;UBQ3;UBQ10	antimicrobial peptide genes	Smith et al., 2001
Eustoma grandiflorum	leaves	particle bombardment	tungsten	NPTII; HPT	enhanced CaMV 35S; CaMV 35S;UBQ3;UBQ10	antimicrobial peptide genes	Smith et al., 2001
Eustoma grandiflorum	in vitro leaf explants	*agrobacterium*-mediated	A281; EHA 105	NPTII		uidA; TSWV	Semeria et al., 1996
Eustoma grandiflorum	in vitro stem and root pieces	particle bombardment	1.1µM gold	bar	CaMV 35S	uidA	Takahashi et al., 1998
Eustoma grandiflorum	ex vitro leaf pieces	*agrobacterium*-mediated	A722	NPTII	CaMV 35S	antisense FLS	Nielsen et al., 2002
Eustoma grandiflorum	ex vitro leaf pieces	*agrobacterium*-mediated	A722	NPTII	CaMV 35S	uidA; antisense CHS	Ledger et al., 1997
Forsythia ×intermedia	internode pieces	*agrobacterium*-mediated	EHA 101	NPTII	CaMV 35S	uidA	Rosati et al., 1996
Forsythia ×intermedia	internode pieces	*agrobacterium*-mediated	EHA 101	NPTII	CaMV 35S	DFR	Rosati et al., 1997
Forsythia ×intermedia	internode pieces	*agrobacterium*-mediated	EHA 101	HPT	CaMV 35S	ANS	Rosati et al., 2003
Gentiana triflora × G. scabra	suspension cells	particle bombardment	gold	HPT	CaMV 35S	uidA	Hosokawa et al., 2000
Gerbera hybrida	in vitro petiole, leaf and shoot tips	*agrobacterium*-mediated	LBA 4404	NPTII		uidA	Nagaraju et al., 1998

TABLE 1 (continued)

Scientific Name	Tissue Used	Transformation Method	Vector Used	Selection Marker	Promoter Used (trait)	Trait Gene	Reference
Gerbera hybrida		*agrobacterium*-mediated			CaMV 35S	antisense CHS	Elomaa et al., 1996
Gladiolus 'Jenny Lee'	suspension cells	particle bombardment	1 µM gold	bar	CaMV 35S	uidA	Kamo et al., 2000b
Gladiolus 'Jenny Lee'	suspension cells	particle bombardment	1 µM gold	bar	CaMV 35S; duplicate CaMV 35S;Act1;UBQ3	uidA	Kamo et al., 2000a
Impatiens (New Guinea)	shoot tips	*agrobacterium*-mediated	GV101	NPTII	CaMV 35S	uidA	Chou, 2003
Impatiens wallerana	shoot tips	*agrobacterium*-mediated	GV101	NPTII	CaMV 35S	uidA	Chou, 2003
Iris germanica	suspension cells	*agrobacterium*-mediated	LBA 4404	HPT; NPTII	CaMV 35S	uidA	Jeknic et al., 1999
Kalmia latifolia	in vitro stem segments	*agrobacterium*-mediated	LBA 4404	NPTII		uidA	Pavingerova and Sediva, 1999
Lilium 'Acapulco'	filament callus	*agrobacterium*-mediated	EHA 101	HPT; NPTII	CaMV 35S	uidA	Hoshi et al., 2004
Lilium longiflorum	bulblet scale callus	particle bombardment	1 µM tungsten	bar	maize ubiquitin	uidA	Watad et al., 1998
Liquidambar styraciflua	nodule cultures	particle bombardment	gold	HPT	CaMV 35S	uidA	Kim et al., 1999
Liquidambar styraciflua	in vitro leaf explants	*agrobacterium*-mediated	A136	NPTII	CaMV 35S	uidA;anionic peroxidase	Sullivan and Lagrimini, 1993

Lobelia erinus	in vitro hypocotyl and root pieces	*agrobacterium*-mediated	GV3101	NPTII	CaMV 35S	uidA	Payne and Lloyd, 1998
Lobelia erinus	in vitro leaf or cotyledon discs	*agrobacterium*-mediated	EHA 105	HPT; NPTII	CaMV 35S	uidA	Tsugawa et al., 2004
Muscari aremeniacum	leaf derived embryogenic calli	*agrobacterium*-mediated	EHA 101; LBA 4404	HPT; NPTII	CaMV 35S	uidA	Suzuki and Nakano, 2002
Oncidium	protocorm-like bodies	*agrobacterium*-mediated	EHA 105; LBA 4404	HPT	CaMV 35S	uidA	Liau et al., 2003
Osteospermum ecklonis	in vitro leaf fragments	*agrobacterium*-mediated	GV3101	NPTII	CaMV 35S	rol genes	Giovannini et al., 1999
Pelargonium	suspension cultures	particle bombardment	tungsten	NPTII; HPT	enhanced CaMV 35S; CaMV 35S;UBQ3;UBQ10	antimicrobial peptide genes	Smith et al., 2001
Pelargonium 'Frensham'	petioles	*agrobacterium*-mediated	LBA 4404	NPTII	CaMV 35S	uidA	KrishnaRaj et al., 1997
Pelargonium 'Frensham'	petioles	*agrobacterium*-mediated	EHA 105	NPTII	modified CaMV 35S	Ace-AMP1	Bi et al., 1999
Pelargonium ×*domesticum*	leaf explants	*agrobacterium*-mediated	EHA 105; LBA 4404	NPTII	CaMV 35S	rolC	Boase et al., 2004
Petunia axillaris × (*P. axillaris* × *P. hybrida*)	leaf discs	*agrobacterium*-mediated	LBA 4404		CaMV 35S	CHR	Davies et al., 1998

TABLE 1 (continued)

Scientific Name	Tissue Used	Transformation Method	Vector Used	Selection Marker	Promoter Used (trait)	Trait Gene	Reference
Petunia axillaris × (*P. axillaris* × *P. hybrida*)	?	*agrobacterium*-mediated	LBA 4404	NPTII	CaMV 35S	rolC	Winefield et al., 1999
Petunia hybrida		*agrobacterium*-mediated	LBA 4404	NPTII	enhanced CaMV 35S	linalool synthase	Lucker et al., 2001
Petunia hybrida	?	*agrobacterium*-mediated	EHA 101	NPTII	CaMV 35S	F3'5'H	Mori et al., 2004
Petunia hybrida	leaf squares	*agrobacterium*-mediated	LBA 4404	HPT	CaMV 35S	boers	Shaw et al., 2002
Petunia hybrida	leaf pieces	*agrobacterium*-mediated	LBA 4404	NPTII	CaMV 35S	CHS	Napoli et al., 1990
Petunia hybrida	pollination	vacuum infiltration of pollen with *agrobacterium*	AGL0		CaMV 35S	bar; uidA	Tjokrokusumo et al., 2000
Petunia hybrida	leaf explants	*agrobacterium*-mediated	?	NPTII	SAG12	ipt	Clark et al., 2004
Petunia hybrida	leaves	particle bombardment	tungsten	NPTII; HPT	enhanced CaMV 35S; CaMV 35S;UBQ3;UBQ10	antimicrobial peptide genes	Smith et al., 2001
Phalaenopsis	protocorm-like bodies	*agrobacterium*-mediated	LBA 4404	HPT	CaMV 35S	uidA	Chai et al., 2002
Rhododendron (elepidote)	in vitro stem and leaf pieces	*agrobacterium*-mediated	LBA 4404	NPTII	CaMV 35S	uidA	Ueno et al., 1996

Rhododendron (elepidotes)	in vitro stem segments	*agrobacterium*-mediated	LBA 4404	NPTII		uidA	Pavingerova et al., 1997
Rhododendron catawbiense	in vitro leaves	particle bombardment	gold	NPTII	CaMV 35S	uidA	Knapp et al., 2001
Rhododendron simsii (evergreen azalea)	callus	*agrobacterium*-mediated	AGL0	NPTII	CaMV 35S	uidA	Mertens et al., 1997
Rosa hybrida	leaves and callus	*agrobacterium*-mediated	GV3101	NPTII	CaMV 35S	uidA	Xiangquian et al., 2002
Rosa hybrida	embryogenic callus	*agrobacterium*-mediated	GV3101	NPTII	CaMV 35S	Ace-AMP1	Xiangquian et al., 2003
Rosa hybrida	embryogenic callus	particle bombardment	gold	NPTII	CaMV 35S	RCH10	Marchant et al., 1998a
Rosa hybrida	in vitro stem slices	*agrobacterium*-mediated	GV3101	NPTII	CaMV 35S	uidA; rolA B C	van der Salm, et al., 1997
Rosa hybrida	embryogenic callus	*agrobacterium*-mediated	LBA 4404	NPTII	?	GFP	Kim et al., 2004
Rosa hybrida	embryogenic callus	particle bombardment	gold	NPTII	CaMV 35S	uidA	Marchant et al., 1998b
Saintpaulia ionantha	in vitro leaves and petioles	*agrobacterium*-mediated	EHA 105; A281	NPTII	CaMV 35S; MAS	uidA	Mercuri et al., 2000
Sedum erythrostichum	leaf segments	*agrobacterium*-mediated	GV3101	bar	CaMV 35S	uidA; NPTII	Yoon et al., 2002
Torenia fournieri	?	*agrobacterium*-mediated	?	NPTII	CaMV 35S	CHS; DFR	Aida et al., 2000
Verbena xhybrida	in vitro stem segments	*agrobacterium*-mediated	AGL0	NPTII	CaMV 35S	GFP	Tamura et al., 2003

TABLE 1 (continued)

Ace-AMP1 = allium antimicrobial protein 1
ACO = ACC oxidase
Act1 = rice actin
antisense CHS = chalcone synthase
att = atticin
boers = ethylene receptor from *Brassica*
CER6 = eceriferum promoter
CHR = chalcone reductase
chs = petunia chalcone synthase
chs-A = chalcone synthase promoter
EPF2-5 = zinc finger transcription factor
etr1-1 = insensitivity to ethylene
F3′,5′H = flavonoid 3′5′-hydroxylase from *Vinca major*
f3h = flavanone 3-hydroxylase
fbp1 = flower specific promoter
FBP1 = flower-specific petunia promoter
GFP = green fluorescent protein
HBT = 35S fused to maize C4PPDK
ipt = isopentenyl transferase
ipt = isopentenyl transferase
Lc = maize flavonoid regulation
Lhca3.St.1 = promoter from potato
MAS = mannopine synthase promoter
pin-2 = wound inducible
PMC = multicystatin
RCC2 = rice chitinase
RCH10 = rice chitinase
rolA B C = from *Agrobacterium rhizogenes*
SAG12 = senescens-associated transcriptional promoter
TSWV = nucleoprotein of tomato spotted wilt tospovirus
ubi1 = ubiquitin 1
UBQ10 = ubiquitin from *arabidopsis*
UBQ3 = *arabidopsis* ubiquitin
UBQ3 = ubiquitin from *arabidopsis*
UEP1 = ubiquitin extension protein promoter

tion of transgenic tissues, a reasonable transformation rate, genotype independent reproducibility, cost effectiveness and a tight time frame to avoid somaclonal variation (Hansen and Wright, 1999). The selection agent reported for the vast majority of ornamentals has been neomycin transferase II (*npt II*) (Table 1). Hygromycin phosphotransferase (*hpt*) and phosphinothricine-N-acetyl transferase (*bar*) have also been employed, but to a much lesser extent. The promoter used to drive trait gene expression in transgenic ornamentals has been almost exclusively the 35-S cauliflower mosaic virus promoter (35S CaMV) (Table 1). In the last few years there has been increased use of some additional promoters, such as ubiquitin (UBQ), actin (Act1) and a variety of tissue specific, species specific or inducible promoters.

The most used methods of DNA delivery for ornamental plants have been I-mediated transformation and microprojectile bombardment (Table 1). Alternative methods of DNA delivery, such as infiltration (whole plant transformation), silicon carbide fiber-mediated transformation, electroporation of cells and tissues, electrophoresis of embryos, microinjection, transformation via pollen-tube pathway and liposome-mediated transformation have seen little use with ornamentals (Rakoczy-Trojanowska, 2002; Zucker et al., 1998). Both *Agrobacterium*-mediated transformation and microprojectile bombardment are patented and those patents have implications for the potential commercialization of an ornamental species that has been improved or altered by either transgenic method. A patent with broad claims to transformation of dicots in general with a non-oncogenic *Agrobacterium* was issued to Washington University in 2000, but it has an initial priority date of 1983 (Barton et al., 2000). This patent probably applies to the general practice of using "disarmed" *Agrobacterium* lacking functional tumorigenic genes to produce transgenic ornamental plants. There are other patents that claim dicot transformation using binary vectors and co-integrated vectors that may also apply to creation of transgenic ornamental plants (*www.cambiaip.org/Whitepapers/Transgenic/AMT/books/whole.html*).

Several patents have also been granted to John Sanford and his coinventors for microprojectile bombardment, with the first one awarded in 1990 (Sanford et al., 1990). Again, these patents likely apply to improved transgenic ornamental plants produced via microprojectile bombardment. Researchers must be aware of the need to secure "freedom to operate" with either of these two popular transformation methods if they hope to commercialize a transgenic plant resulting from their efforts. In addition, most, if not all, genetic components (promoters, selectable markers and trait genes) are protected intellectual property

and arrangements will need to be made for access if eventual commercialization is anticipated.

There are several review articles that describe the process of plant transformation using *Agrobacterium tumefaciens* and microprojectile bombardment, as well as other methods of plant transformation (Walden and Wingender, 1995; Hansen and Wright, 1999; Veluthambi et al., 2003; Zucker et al., 1998; Rakoczy-Trojanowska, 2002). These reviews should be consulted for general information on plant transformation methods since ornamental plant transformation is principally similar to that of other groupings of plants.

AGROBACTERIUM-MEDIATED TRANSFORMATION

This transformation method takes advantage of the natural ability of the soil microorganism *Agrobacterium tumefaciens* to transfer DNA to plant cells. During transformation, a specific segment of the vector, the T-DNA, is transferred to the plant cell and is inserted into the plant's nuclear genome. The T-DNA region can be substituted with engineered DNA sequences coding for selectable markers and/or genes of interest. DNA transfer functions are mediated by a set of virulence genes located separately from the T-DNA region. Parts of the *vir* gene set sense the existence of wounded plant tissue and phenolic compounds and activate other *vir* genes. These *vir* genes replicate and process the DNA to be transferred and facilitate its movement to the plant cell nucleus.

Agrobacterium transformation has several advantages for plant transformation, including its simplicity and low cost, precise transfer and integration of DNA with defined ends, linked transfer of marker and trait genes, a high frequency of stable transformation with single-copy insertions, a low incidence of gene silencing and the ability to transfer large T-DNA pieces (Velethambi et al., 2003). The vast majority of ornamental plant transformations reported in the literature were made using *Agrobacterium* (approximately 80%), with a much smaller percentage made by microprojectile bombardment.

Agrobacterium tumefaciens strains used for ornamental plants are binary vector systems where the recombinant T-DNA and the *vir* region reside on separate plasmids. The most commonly used *Agrobacterium tumefaciens* for ornamental plant transformation has been LB4404, comprised of an octopine-type *vir* helper plasmid, a disarmed Ti plasmid and a binary vector plasmid carrying the trait gene(s) (Hoekema et al., 1983). The L, L-succinamopine-type EHA 101 and EHA 105 (Hood et

al., 1993), also with *vir* helper plasmids and "supervirulent" genes have also seen wide use in ornamental species. Other binary vectors seeing common use are AGLO and GV3101. While *Agrobacterium* has historically been considered a poor transformation method for monocot plants, the development of improved supervirulent vectors and *vir* helper plasmids has largely overcome this limitation. Table 1 shows that monocot ornamental genera including *Agapanthus*, *Alstroemeria*, *Anthurium*, *Dendrobium*, *Iris*, *Lilium*, *Muscari*, *Oncidium*, and *Phalenopsis* have been successfully transformed using *Agrobacterium tumefaciens*.

MICROPROJECTILE BOMBARDMENT

This transformation method is also known as biolistics, particle bombardment or particle acceleration. The equipment used for microprojectile bombardment is often referred to as a "gene gun." It gets this name because heavy, DNA-coated particles are "shot" into target plant tissues, directly through cell walls and membranes (Klein et al., 1987; Sanford, 1988). Gold or tungsten particles between 1 and 4 μM are surface coated with DNA by precipitation with spermidine. The particles are accelerated at target cells using compressed gas, electrical discharge or gun powder. The PDS 1000/He Particle Delivery System has been commonly used with ornamental species. It uses high-pressure helium, released by a rupture disk, and a partial vacuum to propel a macrocarrier coated with microcarrier particles toward target cells. A partial vacuum is needed to allow the microscopic microcarriers to travel straight to the target and maintain velocity. The macrocarrier is stopped by a screen and the microcarriers detach and continue their progress into target cells. The accelerated particles pass through the cell wall and cell membrane and land in various parts of the cell. The DNA dissociates from the microcarrier and moves into the nucleus where it is integrated into the genome of the plant (Yamashita et al., 1991).

The advantages of microprojectile bombardment are the ability to transform plants not infected by *Agrobacterium*, specialized vectors are not needed, multiple DNA fragments/plasmids can be simultaneously co-bombarded, elimination of false positive reporter gene expression seen with *Agrobacterium*, and transformation can be applied to plants where high efficiency regeneration systems are lacking and organelle transformation is a possibility (Veluthambi et al., 2003). On the other hand, biolistics requires specialized and expensive equipment, has a

low efficiency of transformation relative to *Agrobacterium* and needs adjustments to be made to the firing protocol for each plant species or tissue type (Sanford, 1988). Perhaps the biggest drawback to the gene gun is high copy number and rearrangement of transgenes, often leading to gene silencing or genomic rearrangements (Hansen and Wright, 1999; Veluthambi et al., 2003).

Despite the limitations of microprojectile bombardment, it has been used successfully to transform several ornamental monocot genera including *Alstroemeria*, *Gladiolus*, *Lilium*, and several orchid genera (Table 1). In addition, the dicot genera *Chrysanthemum*, *Dianthus*, *Euphorbia*, *Eustoma*, *Gentiana*, *Liquidambar*, *Pelargonium*, *Petunia*, *Rhododendron* and *Rosa* have been transformed by microprojectile bombardment (Table 1). Due to patent issues and potential future commercialization of transgenic plants, microprojectile bombardment was utilized instead of *Agrobacterium* for transformation of *Rhododendron* (Knapp et al., 2001). With *Dianthus*, microprojectile bombardment was used in combination with *Agrobacterium* to enhance transformation efficiency (Zucker et al., 2000; Lavy et al., 2002; Zucker et al., 1999).

Clearly, transformation of ornamental plants has progressed dramatically in the last decade, to the point where it is realistic to expect that almost any species can be transformed if sufficient time and resources are directed at the task. Still, most transformed ornamentals contain only single genes that are coupled to a selectable marker. As with other groups of plants, transferring multiple foreign genes, such as those for a biosynthetic pathway, remains a challenge. Two ways to achieve this are: (1) to introduce several genes in a single step, or (2) cross different transgenic plants containing individual genes. The second option is generally not possible with most ornamental species due to their high degree of heterozygosity. Crossing phenotypically superior single transgene plants would undoubtedly unravel combinations of desired ornamental traits that took years of traditional breeding to assemble in one genotype.

Initially, most ornamental plants were transformed with *uidA* driven by the CaMV 35S promoter (Table 1). More recently, trait genes for disease resistance, floral scent, flower longevity, flower color and plant habit have become the focus of transformation efforts, marking a maturation of ornamental plant transformation. In addition, tissue specific and inducible promoters are beginning to be employed to improve the function and usefulness of trait genes in ornamentals. One example of a transgenic ornamental crop that has been commercialized is various color-modified blue, lavender and purple carnations (*www.florigene.*

com). These carnations possess transgenic alterations in the delphinidin pigment and petal pH. These transgenic carnations are available in Australia and in North America as cut flowers, but North American cut flowers are grown in Ecuador and Columbia.

Although technical challenges exist for transgenic ornamentals, perhaps the bigger challenges are consumer acceptance of transgenic plants, intellectual property issues and government regulatory requirements. Public resistance to genetically modified plants is strong in Western Europe and North America (Zucker et al., 1998). Ornamental plants may actually face less public resistance than food and fiber crops because the focus will be on environmental aspects rather than human safety issues. In reality, the single most substantial hurdle that must be cleared to bring a new transgenic ornamental plant to market is the cost of developing the necessary environmental impact data required by regulatory agencies. This is especially true for perennial ornamental species that will be marketed as whole, live plants intended for outdoor growing and use. The development of the information needed to obtain regulatory approval is on the order of millions of dollars. For most ornamental crops, the market value of the entire crop simply does not justify such a substantial expenditure.

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Molecular Phylogeny-Assisted Breeding of Ornamentals

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SUMMARY. Phylogenetic information is useful for the exploitation of ornamental germplasm in introducing novel resources or selecting closely related species for the introgression of horticultural interesting traits. However, in traditional plant systematics, limited numbers of morpho-

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logical parameters have been evaluated as key characters, and hence, have often caused confusion in classification and induced many conflicts among taxonomists. On the other hand, recently developing molecular phylogeny provides more accurate and stable information for the relationship within each taxon level.

In this article, we show some examples to apply molecular phylogenetic data on exploitation of ornamental germplasm in introducing novel resources with genus *Rhododendron* (Ericaceae) and on selection of closely related species for the introgression of horticulturally interesting traits with genus *Dendrobium* (Orchidaceae). doi:10.1300/J411v17n01_03

KEYWORDS. Phylogeny, breeding, sequence, ITS, *matK*

INTRODUCTION

Regarding the reproductive or breeding system, most ornamental plant species are cross-pollinated or preferentially cross-pollinated (Horn, 2002). Interspecific hybridization plays a major role in developing new floriculture crops and cultivars. The success or failure of producing interspecific hybrids depends on the cross ability which is generally correlated to the genetic relationship or distance between parental species. Traditionally, the morphological or reproductive characters are utilized as taxonomic key characters, i.e., position, number and shape of floral organs, which have determined the genetic relationship within each taxon level such as family, genus and species.

Breeding selection has been mainly based on the analysis of these morphological and physiological parameters, because these parameters are relatively easy to obtain in their visible nature, but only a small number of traits can be evaluated for practical use and they are not stable within different age, different habitat or cultivation and varied by climate of each year. Morphological taxonomy or phylogeny have often caused confusion in classification and induced many conflicts among taxonomists due to this obscure information.

Recent progress of molecular approaches has revealed more accurate relationships in plant phylogeny. Instead of ambiguous morphological characters, molecular information is directly obtained from genetically inherited substance, DNA, and which is much more stable and neutral

with some exceptions. Besides a limited number of visible morphological traits, molecular data can be provided from an enormous number of genetic loci in 3 distinct genomes (nuclear, mitochondria, and chloroplast) within the plant cell. Their number is virtually unlimited; their only limitation being the genome size of the organism and the resources which can be devoted to the analyses. Their resolution can be high enough to detect single base pair differences between genomes (Debener, 2002). Thus the molecular data allows much more detailed analyses of the structure of plant genomes (Paterson et al., 1991) and hence, is more adaptable to distinguish each taxon precisely such as family, genus, subgenus, section, species, varieties and even population or individuals. The analysis of genetic diversity between populations, individuals, varieties and species has been a major task in modern plant breeding (Allard, 1988).

From these advantages, during the past two decades, study method of phylogeny and selection method of breeding have been increasingly complemented, and to some extent replaced by molecular analyses (Paterson et al., 1991; Gebhardt and Salamini, 1992; Mohan et al., 1997; Savolainen and Chase, 2003). The information about the genetic distance between ornamental plant genotypes or species obtained from molecular analyses may be applied in several ways. In the case of phylogenetic information about the relationships between different ornamental taxa, the data is used to infer the evolutionary history of the cultivated varieties. And this information is also useful for the exploitation of novel breeding material in that either hybrid ornamental species may be re-synthesized or in selecting closely related species for the introgression of horticulturally interesting traits (Debener, 2002).

In this article, we show some examples to apply molecular phylogenetic data on exploitation of ornamental germplasm in introducing novel resources with genus *Rhododendron* (Ericaceae) and on selection of closely related species for the introgression of horticultural interesting traits with genus *Dendrobium* (Orchidaceae).

METHODS OF MOLECULAR PHYLOGENY

In plant molecular phylogeny, the chloroplast genome has been extensivley analysed due to its large amount of copies and relatively slow evolution. Within chloroplast genome, two of the genes have been often sequenced for constructing evolutionary trees, *rbc*L (RuBisCO large subunit) and *mat*K (maturase K). Between them, the *mat*K is

known to evolve approximately 3 times faster than *rbc*L, and is therefore a powerful tool for phylogenetic reconstruction within angiosperm families and genera (Hilu and Liang, 1997).

On the other hand, sequence analysis of nuclear genome has been focused on the neutrally-evolved regions such as the internal transcribed spacer region (ITS) between ribosomal DNA genes.

Besides these sequence analyses, several molecular markers, such as restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP) and microsatellites (SSR) can be also very effective to study diversity, especially within species or the relationships between closely related species. General descriptions of different methods and their application to plant genetics are reviewed by Avise (1994), Staub et al. (1996), Karp et al. (1998) and Weising et al. (1998). The application of molecular information to ornamental plant breeding occurred later than in the major agricultural crops, thus reflecting the general delay in the adoption of advanced breeding strategies (Arus, 2000).

Selected studies on molecular phylogeny or genetic distances among ornamental plants are listed in Table 1.

APPLICATION OF MOLECULAR PHYLOGENY TO ORNAMENTAL PLANT BREEDING

Rhododendron and Menziesia (Ericaceae)

The genus *Rhododendron* of the heather family (Ericaceae) is well known for its attractive flowers as garden shrubs and pot plants. They have developed predominantly in East to Southeast Asia and comprise over 1,000 species (Chamberlain et al., 1996). This large genus has posed systematic problems in terms of infrageneric circumscription and ranks. Such unstable circumstances are caused by the great diversity of vegetative organs and the relatively uniform floral morphology.

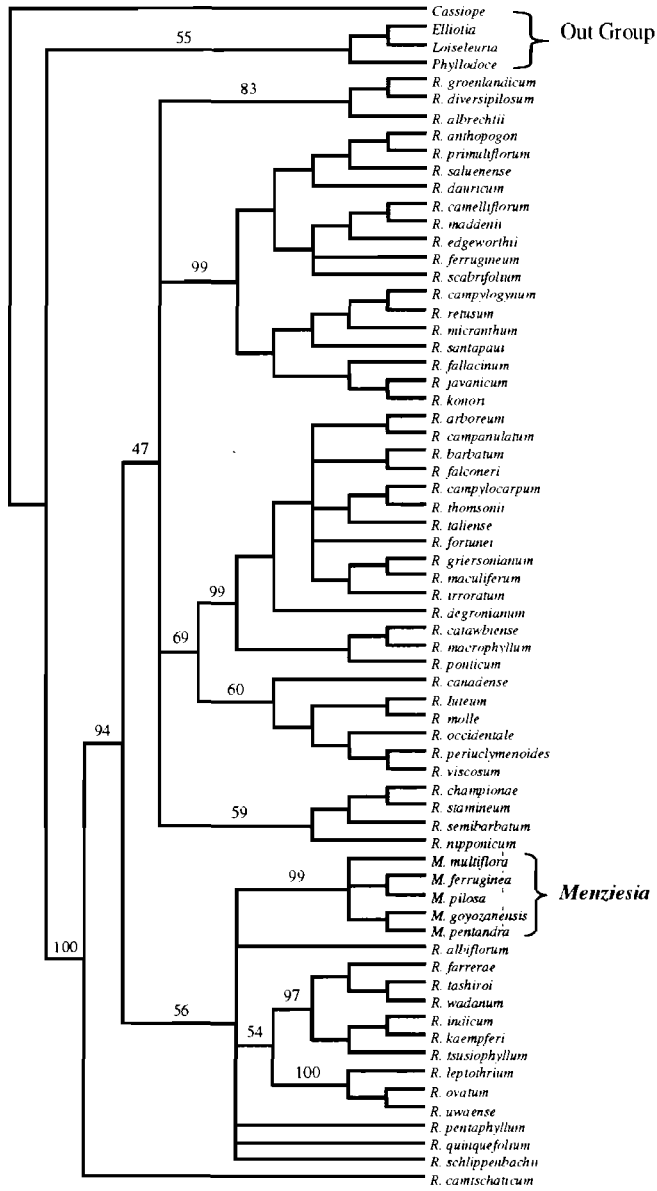
We analyzed *matK* with *trnK* intron sequences of chloroplast (cp) genome of the genus *Rhododendron* and its closely related genera (Kurashige et al., 1998, 2001), and elucidated a substantial part of the phylogenetic relationships (Figure 1). Recently, we also analyzed sequences of internal transcribed spacer region (ITS) of the 18S-26S nuclear (nr) ribosomal DNA (Kurashige et al., 2001).

TABLE 1. Selected studies on molecular phylogeny or genetic distances among ornamental plants.

Genus or Subfamily	Method	Reference
Aconitum	ITS sequence	Utelli et al., 2000
Anemone	RFLP	Hoot et al., 1994
Aquilegia	ITS sequence	Ro et al., 1997
Arachis	RAPD	Gimenes et al., 2000
Banksia	RAPD, trnL-F sequence	Maguire et al., 1997
Bidens-Coreopsis	ITS sequence	Kim et al., 1999
Cattleya	RAPD	Benner et al., 1995
Clivia	RAPD	Ran et al., 2001
Coelogyne	RFLP, matK, ITS sequence	Gravendeel et al., 2001
Cyclamen	ITS sequence	Anderberg et al., 2000
Cypripedioideae	ITS sequence	Cox et al., 1997
Dendranthema (*Chrysanthemum*)	DAF	Scott et al., 1996
Dendrobium	rbcL, ITS sequence	Yukawa et al., 2000
Diseae	ITS sequence	Douzery et al., 1999
Euphorbia (*Poinsettia*)	RFLP	Ling et al., 1997
Gentian	trnL sequence	Gielly et al., 1996
Geranium	DAF	Startman and Abbit, 1997
Hedera	ITS sequence	Vargas et al., 1999
Hemerocallis	AFLP	Tomkins et al., 2001
Lilium	ITS sequence	Dubouzet et al., 1999
	trnT-L-F, atpB-rbcL sequence	Nishikawa et al., 2002
Magnolia	matK, psbA-trnH, atpB-rbcL sequence	Azuma et al., 1999
Malva, Lavatera	ITS sequence	Ray, 1995
Nympaea	rbcL, matK, 18SrDNA sequence	Les et al., 1999
Orchididinae-Habenariinae	ITS sequence	Bateman et al., 2003
Orchis	ITS sequence	Aceto et al., 1999
Paeonia	psbA-trnH, trnL-F, matK, ITS sequence	Sang et al., 1997
Petunia	DAF	Cerny et al., 1996
Phlox	ITS sequence	Ferguson et al., 1999
Rhododendron	matK sequence	Kurashige et al., 2001
	AFLP	de Riek et al., 2001
Rhododendroideae	matK sequence	Kron, 1997
Rosa	RAPD	Ben-Mier and Vainstein, 1994
		Debner et al., 1996
		Jan et al., 1998
Sidalcea	ETS, ITS sequence	Andersen and Baldwin, 2003
Stanhopea	ITS sequence	Williams and Whitten, 1999
Trillium	matK sequence	Osaloo et al., 1999
Viola	RAPD	Ko et al., 1998

Total DNA was extracted from fresh leaf tissues following the methods of Kobayashi et al. (1998) or Yukawa et al. (2000). Sequences were determined by first PCR-amplifying either the cp *matK* gene with its flanking *trnK* introns or the nr ITS region from a total DNA. Single-stranded DNA for dideoxy sequencing was produced in a second round

FIGURE 1. Strict consensus of most parsimonious Fitch trees based on *matK* and *trnK* intron sequences for *Rhododendron* and *Menziesia*.



Numbers above internodes indicate bootstrap values from 1,000 replicates.

of amplification using the double-stranded product as a template. Both the forward and reverse strands were sequenced for all taxa.

All parsimony analyses were conducted with PAUP, Phylogenetic Analysis Using Parsimony, Version 3.1 (Swofford, 1993). The heuristic search option with 100 random replicates (Maddison, 1991) was used to perform Fitch parsimony analyses (Fitch, 1971). Branch lengths for trees were calculated by ACCTRAN optimization (Swofford and Maddison, 1987). For assessment of the relative robustness for clades found in each Fitch parsimony analysis, the bootstrap method (Felsenstein, 1985) was used.

All the results of these studies revealed the nested position of genus *Menziesia* in the genus *Rhododendron.*

Menziesia is a small genus in Ericaceae which includes 9 species composed of 2 North American and 7 Japanese species. The flower is small and bell-shaped, and is white, yellow, pink or brick red colors blossoming in spring-summer time. They are relatively dwarfed and have soft branches. They also have cold and shade tolerance. From the horticultural point of view, these traits are desirable new characters to improve Japanese azalea which is included in the subgenus Tsutsusi group. Interspecific pollinations within genus *Rhododendron* are usually only successful when both parents belong to the same subgenus; however, several inter-subgeneric hybrids were successfully obtained (Noguchi, 1932; Akabane, 1971). Therefore, it has a possibility to create new intergeneric hybrids between *Menziesia* and *Rhododendron*, according to the result of close relationship between them by molecular phylogenetic data. If *Menziesia* could be hybridized with *Rhododendron*, hybrid progenies will have novel characters such as large bell-shaped flowers with various colors and cold tolerant shrub.

For this purpose, we tried to make intergeneric hybrids between *Menziesia* and *Rhododendron* (Kita et al., 2004). In our understanding, this is the first approach to make intergeneric hybrids between these two genera.

We tried to make intergeneric hybrids by hand pollination between *Menziesia multiflora* and 7 *Rhododendron* species (Table 2). *Menziesia multiflora* is the most popular *Menziesia* species in Japan that is distributed widely and has attractive pendulous flowers. Some of the intergeneric combinations between *M. multiflora* and *Rhododendron* species were crossed reciprocally.

Capsule sets were observed 60 days after pollination. Capsules were collected 120 days afte pollination and sterilized in 1% sodium hypochlorite solution for 10 min followed by three washes in sterile distilled

TABLE 2. Frequency of capsule set and number of immature seeds per capsule in reciprocal crosses between *Menziesia multiflora* and *Rhododendron* species.

Cross combination (female × male)		Number of capsule set/ number of crosses (% of capsule set*)	Average number of immature seeds per capsule
M. multifrora × *Rhododendron* spp.			
M. multiflora	*R. kaempferi*	26/132 (19.7)	3.46
M. multiflora	*R. kiusiamun*	32/165 (19.4)	7.81
M. multiflora	*R. tashiroi*	60/79 (75.9)	34.55
M. multiflora	*R. wadanum*	5/22 (22.7)	0
M. multiflora	*R. pentaphyllum*	15/27 (55.6)	0
M. multiflora	*R. quinquefolium*	4/16 (25.0)	0
M. multiflora	*R. ovatum*	5/12 (41.7)	0
Rhododendron spp. × *M. multifrora*			
R. kaempferi	*M. multiflora*	0/23	-
R. kiusiamun	*M. multiflora*	18/26 (69.2)	10.44
R. tashiroi	*M. multiflora*	0/30	-
R. wadanum	*M. multiflora*	0/17	-
R. pentaphyllum	*M. multiflora*	0/20	-
R. quinquefolium	*M. multiflora*	0/10	-
R. ovatum	*M. multiflora*	0/12	-

* Number of capsule set/number of crosses × 100

water. When *Rhododendron* species were used as maternal parents for crossing, capsule set was observed only in a cross of *R. kiusianum* × *M. multiflora* (Table 2). On the contrary, in crosses when *M. multiflora* was used as a female parent, capsule sets were observed in all 7 cross combinations. Immature seeds were obtained in crosses of *R. kiusianum* × *M. multiflora*, *M. multiflora* × *R. kaempferi*, *M. multiflora* × *R. kiusianum* and *M. multiflora* × *R. tashiroi*. The greatest number of immature seeds per capsule was obtained in a cross of *M. multiflora* × *R. tashiroi*.

After sterilization, immature seeds were dissected from capsules, and then cultured on Anderson's rhododendron medium (Anderson, 1984) supplemented with 50 mg * l^{-1} gibberellic acid (GA_3), 30 g * l^{-1} sucrose, and solidified with 3 g * l^{-1} gellan gum after adjusting at pH 5.0. Obtained immature seeds could germinate in all cross-combinations. Germinated seedlings were then transferred to the same medium with 10 mg * l^{-1} N6-[2-isopentenyl] adenine (2ip), 30 g * l^{-1} sucrose and 3 g * l^{-1} gellan gum, pH 5.0 to induce multiple shoots. Germination ratio was calculated one month after cultivation on the medium. Vigorously grown seedlings were transferred to the same medium with 30 g * l^{-1} sucrose and 3 g * l^{-1} gellan gum, without phytohormone, pH 5.0 to

induce roots under the same condition. Rooted plants were potted in soil and acclimatized.

Leaf color of the seedlings was classified into 4 classes which were green, pale-green, sectional chimera variegated both green and albino leaf segments, and albino (Table 3). Most of the seedlings in any crosses showed pale-green or albino leaf colors. Green seedlings could be obtained only in crosses when *M. multiflora* was used as a maternal parent although the frequency of green seedlings was still low (Figure 2).

For intergeneric hybrid confirmation, PCR-RFLP analysis was conducted. Total DNA was extracted from fresh leaf tissues following the methods of Kobayashi et al. (1998). ITS region of nrDNA was amplified by polymerase chain reaction (PCR). The PCR products were digested by 0.1-0.5 units of appropriate restriction enzymes for each parental combination at 37°C for 2 hours. Banding patters of restricted fragments in ITS region were different between *M. multiflora* and *Rhododendron* spp. used in this study. The ITS region of *M. multiflora* was digested into two fragments (400 and 500-bp) by *Nsp*V restriction enzyme, whereas those of *Rhododendron* species were digested into 750-bp and 150-bp bands by *Afl* II enzyme. Therefore, hybrid seedlings possessed 4 bands (750, 500, 400 and 150-bp) after double digestion with *Nsp*V and *Afl* II enzymes (Figure 3). All of the investigated seedlings had both specific restricted fragments, confirming intergeneric hybrids between *Menziesia* and *Rhododendron* (Figure 4).

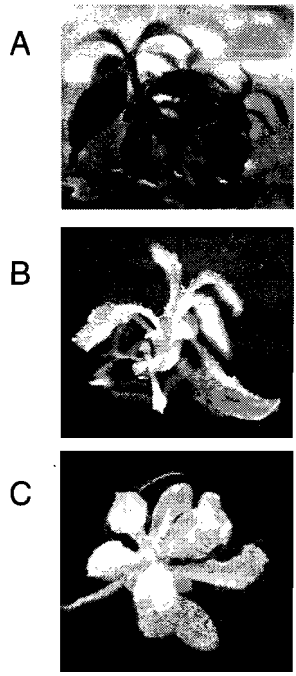
The success of intergeneric hybridization of *Menziesia* with *Rhododendron* reflects a close relationship between two genera, *Menziesia* and *Rhododendron*. This result indicates molecular phylogenetic data can be used as a guide for a breeding program.

TABLE 3. Germination rate of immature seeds and leaf colors of progenies by crossing between *Menziesia multiflora* and three *Rhododendron* species.

♀	♂	Number of immature seeds cultured	% of germination[z]	Number of seedlings (% of seedlings[x])			
				G[y]	PG	Sec	Albino
M. multiflora	*R. kaempferi*	90	34.4	7 (17.1)	19 (46.3)	2 (4.9)	13 (31.7)
M. multiflora	*R. kiusiamun*	250	47.2	10 (8.5)	8 (6.8)	0	100 (84.7)
M. multiflora	*R. tashiroi*	2073	33.6	96 (13.8)	77 (11.0)	107 (15.4)	417 (59.8)
R. kiusiamun	*M. multiflora*	188	17.6	0	9 (27.3)	0	24 (72.7)

(z): Number of germinated seeds/number of cultured seeds × 100
(y): Leaf colors are G; green, PG; pale-green, Sec; sectional chimera of green and white, and albino
(x): Number of seedlings in each color/total number of seedlings × 100

FIGURE 2. Hybrid seedlings between *Menziesia multiflora* × *Rhododendron tashiroi.*



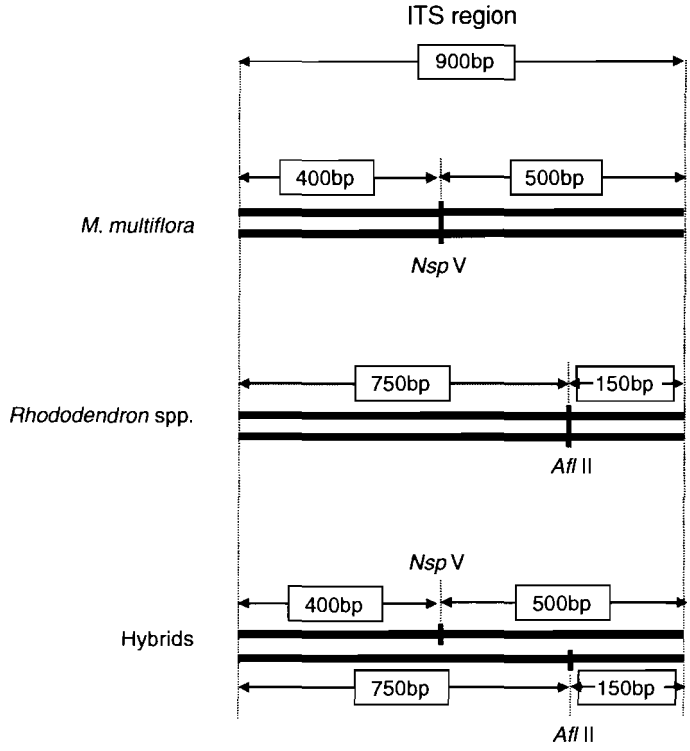
A: green; B: chimera, arrow indicates green segment; C: albino

Dendrobium (Orchidaceae)

We also applied this molecular strategy on *Dendrobium*, which is the largest genus in orchid family, by using cp *matK* with *trnK* introns and ITS region (Yukawa, 2000, 2001; Wongsawad et al., 2001, 2002). In these studies, more concrete relationships in subtribe Dendrobinae was established and relatedness or relationships among species were clarified (Figure 5).

For *Dendrobium*, we chose section *Callista* to improve their characters by crossing. Because most of species in section *Callista* are easily recognized by their attractive inflorescences, which are pendent like bunches of golden grapes from the dark green upright stems. Although section *Callista* bears such an attractive flower character and thus has a great potential for pot plants, its short vase life within a week prevents commercialization so far.

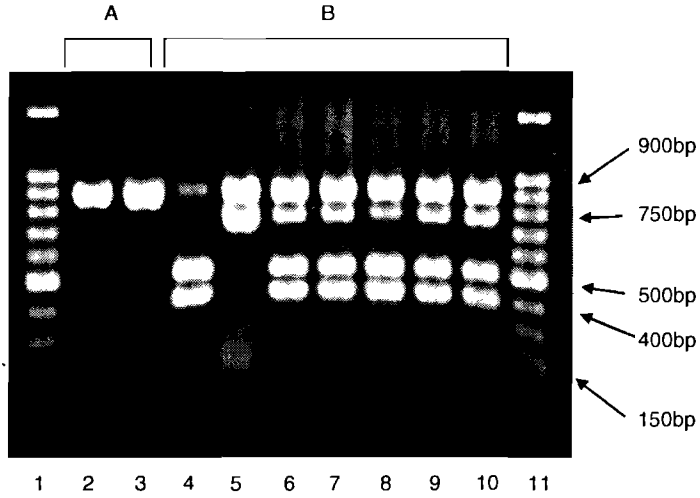
FIGURE 3. Restriction sites of ITS region of *M. multiflora*, *Rhododendron* spp. and their hybrids, *Menziesia multiflora*.



From our molecular results, we have tried to cross some species of section *Callista* with related sections such as *Pedilonum* and *Calyptrochillus* that have flowers with quite a long vase life

We crossed by hand pollination between section *Callista* and its related sections which have long-lasting flowers, according to the results of molecular phylogeny in *Dendrobium*. Seeds were collected from seed pods and sterilized in 1% sodium hypochlorite solution for 5 min followed by 5 washes in sterile distilled water. After sterilizing, seeds were germinated on the medium with 2 g * l^{-1} Hyponex 6.5-6-19 (Hyponex), 0.25 mg * l^{-1} $Ca(NO_3)_24H_2O$, 2 g * l^{-1} Bacto tryptone (Difco), 10 g * l^{-1} sucrose having pH 5.0 and solidified with 8 g * l^{-1} agar and cultured under fluorescent light with a 16 hour photoperiod with a light intensity of 50 µmol * m^{-2} * s^{-1} at 25°C. Then the seedlings

FIGURE 4. Profiles of PCR products (A) and restriction fragments of the products digested with *Afl II* and *Nsp V* (B). Lanes 2 and 4, *M. multiflora*; Lanes 3 and 5, *R. tashiroi*; Lanes 6-10, hybrid from *M. multiflora* × *R. tashiroi*; Lanes 6 and 7, green hybrid; Lane 8, pale-green hybrid; Lanes 9 and 10, albino hybrid; Lanes 1 and 11, 100-bp ladder.



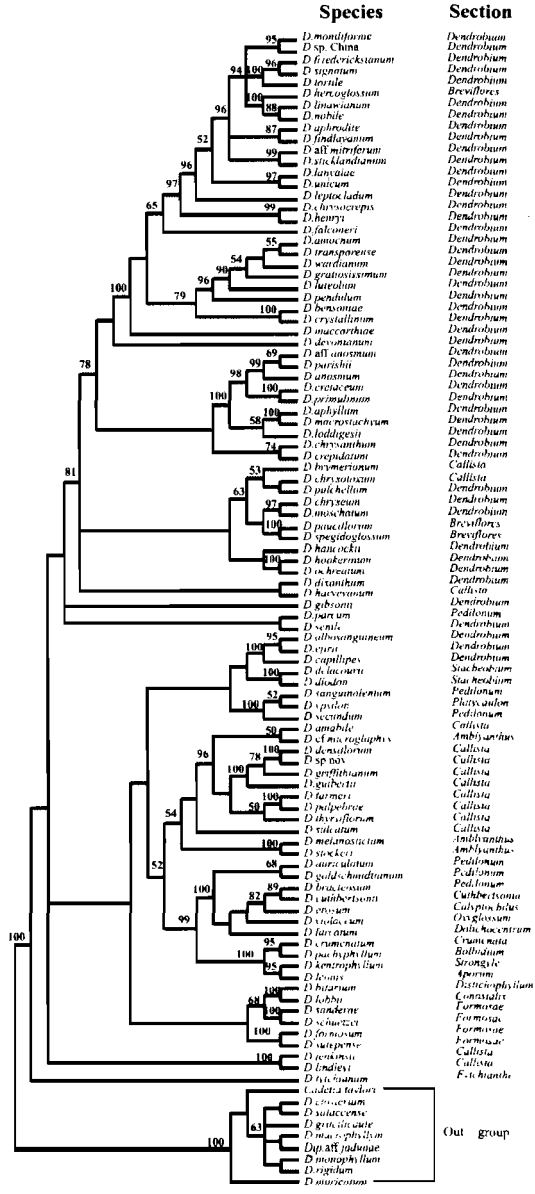
derived from intersectional crosses were transplanted in a flask on the modified Vacin and Went medium (Sagawa and Kunisaki, 1984; Sagawa, 1990) and cultured under the same condition.

We have obtained seedpods between section *Callista* and its related sections in molecular phylogeny (Table 4). Finally, we successfully got many seedlings from 4 intersectional crosses: *D. victoria-reginae* (sec. *Calyptrochilus*) × *D. palpebrae* (sec. *Callista*), *D. mohlianum* (sec. *Calyptrochilus*) × *D. lindleyi* (sec. *Callista*), *D. palpebrae* × *D. pseudoglomeratum* (sec. *Pedilonum*), and *D. kuhlii* (sec. *Pedilonum*) × *D. amabile* (sec. *Callista*). After transplanting to the pots, we are going to check their hybridity by PCR-RFLP analysis as mentioned earlier. All of the results indicate proof of hybridity. We have obtained many seedlings in several combinations and continue to culture at the moment.

CONCLUSION

In our results of sequence analysis for genus *Rhododendron* and its closely related genera, all the species of genus *Menziesia* were nested

FIGURE 5. Strict consensus of most parsimonious Fitch trees based on *matK* with *trnK* introns and ITS region sequences for *Dendrobium*.



Numbers above internodes indicate bootstrap values from 200 replicates.

TABLE 4. Cross combination between section *Callista* and other sections successfully bearing seedpods.

Female	Male
D. amabile (sec. *Callista*)	*D. pseudoglomeratum* (sec. *Pedilonum*)
D. amabile	*D. kuhlii* (sec. *Pedilonum*)
D. chrysotoxum (sec. *Callista*)	*D. victoria-reginae* (sec. *Calyptrochilus*)
D. palpebrae (sec. *Callista*)	*D. pseudoglomeratum*
D. palpebrae	*D. victoria-reginae*
D. lindleyi (sec. *Callista*)	*D. mohlianum* (sec. *Calyptrochirus*)
D. mohlianum	*D. lindleyi*
D. lawesii (sec. *Calyptrochillus*)	*D. guibertii* (sec. *Callista*)
D. pseudoglomeratum	*D. palpebrae*
D. pseudoglomeratum	*D. thyrsiflorum* (sec. *Callista*)
D. kuhlii	*D. amabile*
D. victoria-reginae	*D. palpebrae*

within *Rhododendron* species. Without this molecular information, we couldn't image making intergeneric hybrids between these two genera. Fortunately, we could get intergeneric hybrid seedlings by several combinations. Thus, molecular information allowed us to exploit the new germplasm for breeding of *Rhododendron*.

In the case of *Dendrobium*, section *Callista* has attractive flowers although the flower longevity is quite short. From the result of molecular analysis, we have tried to make new hybrids between section *Callista* and its related sections that have longer flowering time. This means the molecular information was useful to select the closely related species for the introgression of horticulturally interesting traits.

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Improvement of Cold Tolerance in Horticultural Crops by Genetic Engineering

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SUMMARY. Chilling and freezing temperatures often adversely affect the productivity and quality of horticultural plants. Attempts to enhance cold tolerance through traditional breeding have achieved limited success, mainly due to the complexity of the genetics associated with a plant's response to low temperatures. Recently, the improvement of cold tolerance by genetic engineering has been achieved in many species. Nevertheless, transgenic plants with greater tolerance have been reported in only a few horticultural examples. Their production has led to a new era in improving performance. In this review, we first present recent advances in understanding cold acclimation. We then describe a few cases in which improved cold tolerance has been achieved with transgenic plants. doi:10.1300/J411v17n01_04

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INTRODUCTION

Low temperature (LT) is a major limiting factor in the geographical distribution of horticultural plants, often adversely affecting crop development, growth, and productivity. This results in huge losses to economic yield around the world. Furthermore, many important species are often cultivated in areas where temperatures fall below the optimum required for their normal growth and development (McKersie and Leshem, 1994). For example, the growing season of annual plants is generally established by the length of the frost-free period, so that economic losses often occur in tropical and sub-tropical species due to unseasonable episodes of LT stress. The performance of perennial species can also be affected by cold conditions (Wisniewski and Bassett, 2003). These may include extremely low midwinter temperatures, frost that occurs early in the fall before plants have become fully acclimated to the cold, or frost in the spring when deacclimation, i.e., the loss of cold hardiness, has occurred. The result is damage to over-wintering buds, developing blossoms, or even vegetative tissues.

In addition to the direct effects of LT on plant survival and productivity, susceptibility to injury limits the areas in which woody ornamentals and perennial fruit crops can be planted. Therefore, LT-stress tolerance has been one of the primary considerations in deciding which horticultural species can be established in a particular geographical region. Recent advances in plant molecular biology have not only allowed a better understanding of the adaptive responses to LT but also have led to the development of new approaches for improving cold tolerance.

In this review, we first present the current understanding of both LT-induced injury and how some species adapt to cold temperatures. We then focus on various attempts to protect higher plants against LT stress through genetic engineering, with special emphasis being placed on a few cases in which transgenic horticultural crops with enhanced cold tolerance have been developed.

LOW-TEMPERATURE STRESS IN HORTICULTURAL CROPS

Chilling and freezing are the two major types of LT stresses in plants. Chilling stress occurs at temperatures either above 0°C or below 0°C in

the absence of ice formation. In contrast, freezing stress is associated with temperatures below 0°C and always involves the formation of ice crystals in the tissues.

Chilling Injury

Chilling injury occurs in many tropical and sub-tropical horticultural plants, such as banana, beans, cucumber, melon, tomato, and citrus. In temperate regions, these crops are commonly exposed to low, but non-freezing temperatures (0~15°C) during the growing season (Paull, 1990). The extent of chilling injury depends on the species, variety, part of the plant affected, and its developmental stage, as well as the severity and duration of the cold period (Saltveit and Morris, 1990). In chilling-sensitive plants, injury can occur at all stages of development, except for seeds that are dormant and dry (Saltveit and Morris, 1990). Seeds of some sensitive plants, such as soybeans, sweet corn, and cotton, are particularly vulnerable during the initial stages of imbibition, when low temperatures can result in seed death, decreased germination rates, increased decay, and the production of low-vigor or abnormal seedlings (Herner, 1990). If exposure to chilling occurs after a sensitive plant has become established, it may exhibit wilting and/or low leaf water potentials (Pardossi et al., 1992). These symptoms are caused primarily by the uncontrolled opening of the leaf stomata while the permeability of the roots to water is simultaneously reduced (McWilliam et al., 1982).

The reproductive stage, which includes the development of floral organs, flowering, fruiting, and seed formation, is most sensitive to LT (McKersie and Leshem, 1994). Although storage at low temperatures is the most effective means for maintaining fruit viability, the refrigeration of chilling-sensitive commodities, e.g., tomato, cucumber, or banana, often results in a loss of quality, as manifested by pitting, discoloration, abnormal ripening, and weakening of the tissues, thereby rendering the product susceptible to pathogenic decay (Wang, 1990).

Many different mechanisms have been explored to describe the process of chilling injury; the phase transition of membrane lipids has been suggested as the primary cause. Lyons (1973) has proposed that LT is first perceived by the acyl tails of the phospholipid molecules in cell membranes. This results in a membrane phase transition, i.e., the lateral phase separation of membrane lipids to form distinct domains of gel and liquid-crystalline phases. These changes are purportedly dependent on lipid composition, with a greater level of unsaturated lipids being corre-

lated with a lower number of phase transition events (Lyons and Raison, 1970). For example, an analysis of the molecular species of individual phospholipid classes has shown a negative relationship between chilling tolerance and the sum of the relative contents of the 16:0/16:0 and 16:0/16:1 fatty acids in the phosphatidylglycerol of the thylakoid membranes (Murata, 1983). Many efforts to alter the degree of unsaturation have resulted in modified chilling sensitivity (Murata et al., 1992; Kodama et al., 1994; Moon et al., 1995). Ultimately, changes in the membrane lipid phase transition prevent proper functioning of integral membrane proteins. This results in increased leakiness of the membranes, which then leads to a loss of compartmentation, decreased rates of mitochondrial oxidative activity, reduced energy supplies and utilization, lowered photosynthetic rates, disrupted metabolism, the accumulation of toxic substances, cell autolysis, and death (Wang, 1982).

The inhibition of photosynthesis is the first of the secondary events following membrane phase transition. This failure to maintain photosynthesis is associated with steps involved in regulating stomatal aperture (McKersie and Leshem, 1994). In chilling-tolerant species, the size of the stomatal opening is generally reduced at LT, in part because water conductivity through the root plasma membrane is decreased (McWilliam et al., 1982). For example, some tolerant plants can maintain steady water potentials by closing their stomata and preventing excessive transpirational water loss, whereas chilling-sensitive species often exhibit wide-open stomata at LT (Emaus et al., 1983). Cold-induced stomatal closure might also occur through the direct effects of temperature on the guard cells themselves (Honour et al., 1995). In addition, LT may affect the enzymes and ion channels responsible for the active maintenance of guard-cell osmotic potential (Ilan et al., 1995). Stomatal aperture is also influenced by plant growth regulators, such as abscisic acid (ABA). ABA binds to receptors on the outside of the stomatal guard-cell plasma membrane, and induces a signal transduction cascade involving increases in cytoplasmic calcium (Assmann and Shimazaki, 1999). This action eventually reduces guard-cell osmotic potential via loss of K^+ and Cl^-, causing the stomata to close.

When chilling-sensitive plants are exposed to LT under strong illumination, electron transport through Photosystem II (PS II) is inhibited. McKersie and Leshem (1994) have presented several hypotheses to explain the causes of such 'photoinhibition.' First, LTs promote excessive excitation of the photosystems by reducing the demand for chemical energy in processes such as CO_2 fixation. Consequently, over-excitation favors the transfer of energy from light to oxygen molecules, causing

photo-oxidative damage to the reaction center D1 protein. Second, the activity of enzymes that scavenge reactive oxygen species (ROS) decreases at LT (Richter et al., 1990); the accumulated ROS subsequently "escape" to other sites in the chloroplast or cytosol where they initiate degradation reactions. Third, turnover of the D1 protein in the reaction center is slow at LTs (Gong and Nilsen, 1989) and, therefore, its assembly into new PS II complexes is blocked. Finally, LTs inhibit the formation of zeaxanthin that quenches excitation energy and dissipates it as heat, further reducing the cells' ability to rid themselves of excess energy (Demming-Adams, 1990).

Another major contributing factor to chilling injury is oxidative stress. In addition to this chilling-induced photoinhibition in sensitive plants, membrane phase transition and dysfunction of other metabolic and repair processes, including a potential decrease in scavenger system capacities, can all contribute to the proliferation of ROS. For example, catalase activity can decrease in response to LTs in various crop plants (Fadzillah et al., 1996; Hsieh et al., 2002). This decline is likely caused by the failure of repair synthesis and suppressed translation (Feierabend et al., 1992), as well as reduced expression of the catalase gene (Hsieh et al., 2002).

Transgenic tomato plants overexpressing the antisense catalase gene show a 2- to 8-fold reduction in total catalase activity, and a 2-fold increase in levels of H_2O_2 in leaf extracts (Kerdnaimongkol and Woodson, 1999). These plants are more sensitive to exogenous applications of H_2O_2 and also become more susceptible to chilling stress. Suppression of catalase activity in these plants likely leads to a failure to scavenge the ROS that accumulate, resulting in increased sensitivity to oxidative stress. In contrast, transgenic tobacco, genetically engineered with chloroplastic *CuZn-SOD* from pea, retains higher rates of photosynthesis under intense light and LTs, compared with wild-type (WT) plants (Gupta et al., 1993). Moreover, overexpression of a mitochondrial manganese superoxide dismutase (*MnSOD*) and superoxide dismutase (*FeSOD*) in the chloroplasts of transformed maize enhances foliar tolerance to chilling and oxidative stress when leaf discs are incubated in the pro-oxidant herbicide methyl viologen (van Breusegem et al., 1999a, 1999b).

Freezing Injury

Freezing injury occurs when the external temperature drops below the freezing point of water (0°C). Some plants susceptible to chilling in-

jury can be killed by the first touch of frost, while many that are native to cold climates can survive extremely low temperatures without damage (Levitt, 1978). When plant tissues freeze, ice forms in their intercellular spaces, reducing water potential and leading to a loss of water from the cells. At −10°C, more than 90% of the osmotically active water will generally move out of the cells into those spaces (Thomashow, 1998). Therefore, freeze-induced dehydration has a number of damaging effects on cells, e.g., multiple forms of membrane lesions and the denaturation of proteins (Thomashow, 1998). Upon thawing, freezing-damaged protoplasts lose turgor and are unable to prevent cellular water and osmolites from leaking into their surroundings.

At the whole-plant level, symptoms of freezing injury may include desiccation or burning of foliage; water-soaked areas that progress to necrotic spots on leaves, stems, or fruit; and death of portions or the entire plant (Ingram et al., 2001). The various effects of such stress include freezing of citrus fruits; midwinter damage to deciduous crops; and early-frost injury to flowers, vegetables, and developing fruit; as well as limiting the potential number of perennial species that can be planted within a particular cold hardiness zone (Ashworth, 1986). Spring frost damage in reproductive organs usually causes internal and external morphological abnormalities that affect normal fruit development or even cause abscission. In several of the early-blooming members of Rosaceae (e.g., strawberry, cherry, peach, almond, and apricot), floral damage is the main form of freezing injury (Rodrigo, 2000). Varying levels of freezing tolerance are associated with different plant organs: in cabbage (*Brassica oleracea* L. var. *capitata*), the relative tolerance is petiole < upper pith (stem) < middle pith < lamina < lower pith (Manley and Hummel, 1996). It has been suggested that the cold hardiness mechanism in the foliar portions of cauliflower (*Brassica oleracea* L. var. *boytis*) is not expressed in the curd because freezing always proves fatal, as evidenced by florets having a flaccid and water-soaked appearance upon thawing (Fuller et al., 1989).

Plants have evolved two major means for surviving freezing stress. The first, supercooling of the cellular water, is an avoidance mechanism by which protoplasmic water can remain unfrozen to its homogeneous ice nucleation point of approximately −40°C, when the source of the nucleation agent is absent (Thomashow, 1998). Deep supercooling has been observed in the dormant flower buds and ray parenchyma cells of a number of deciduous fruit crops, including grape, blueberry, and several *Prunus* species (Ashworth, 1989). In some woody plants, anatomical and biochemical adaptations to their cell walls and vascular systems

are believed to limit the spread of ice-frozen tissues into supercooled areas (Fujikawa et al., 1997). For example, the degree of esterification of pectins is modified in tissues that experience deep supercooling. Such modifications may change the pore size of the cell walls and inhibit ice penetration into supercooled compartments (Wisniewski and Davis, 1995). Nevertheless, supercooled plant tissues can suffer irreversible damage once ice nucleation occurs.

The second, and most common, mechanism for survival is the development of tolerance to freezing temperatures. Cold acclimation is the process by which plants acquire tolerance through their exposure to low but non-freezing temperatures (Thomashow, 1998). Our current understanding of cold acclimation will be explained in more detail below.

Approaches and Their Limitations in Overcoming LT Problems

To overcome the problems associated with low-temperature stress and to improve production efficiency, more stress-tolerant crops must be developed. Traditional breeding strategies that have attempted to utilize the natural genetic variation within a species, interspecific or intergeneric hybridization, or induced mutation using tissue culture techniques, have met with only limited success (Flowers and Yeo, 1995). Those approaches are confounded by the complexity of stress-tolerance traits, low genetic variance in yield components under stress conditions, and a lack of efficient selection techniques (Cushman and Bohnert, 2000). Furthermore, quantitative trait loci (QTL) that are linked to tolerance at one stage of development can differ from those associated with tolerance at other stages (Foolad and Yin, 2000). Once identified, these useful QTLs not only require extensive breeding to restore the desirable traits but also prove time-consuming when removing the chromosomal segments that interfere with the recurrent parent genome. That is, when conventional breeding programs try to introduce such a single cold-tolerance trait into a high-yielding variety, many undesirable attributes (which often decrease crop values) are also transferred to the offspring. Therefore, an expensive and time-consuming process of backcrossing is required to develop a high-quality variety with one additional feature. Moreover, crossing is limited only to individuals of the same or closely related species.

In contrast to traditional breeding, genetic engineering with a small number of stress-tolerance genes appears to be a more attractive and rapid approach. First, it allows the introduction of isolated genes, i.e., single traits, into a crop without affecting other desirable attributes.

Second, genetic engineering empowers breeders to transfer genetic material between unrelated plant species or even genes from phylogenetically distant species, such as a virus, bacteria, or animals. Thus breeders can more precisely produce a plant variety with a single new trait.

Present engineering strategies rely on the transfer of one or a couple of genes that encode either biochemical pathways or endpoints of signaling pathways (Nelson et al., 1998). These gene products can protect, directly or indirectly, against LT stresses. Such strategies invariably are based on the existence of correlations between a specific stress-protective function and a consequence of that stress. Some of the approaches reported in the literature (Tables 1 and 2) include the overexpression of biosynthetic enzymes for protein kinases, transcription factors, and cold-regulated, oxidative stress-related, lipid-modifying, or compatible solute-synthesis genes.

MOLECULAR BIOLOGY OF COLD ACCLIMATION

Cold Acclimation

To survive winter, most species living in temperate regions undergo adaptive changes in the fall. Some plants acquire tolerance to freezing temperatures via prior exposure to low, nonfreezing temperatures, a process called cold acclimation (Hughes and Dunn, 1996). For example, non-acclimated rye is killed at about $-5°C$, but after being cold-acclimated, can survive freezing to about $-30°C$ (Thomashow, 1999). This acclimation process is correlated with a number of cellular and metabolic changes. Recent studies have started to define the molecular basis of these changes, which has led to the characterization of a large number of genes upregulated by LTs (reviewed by Thomashow, 1999; Shinozaki and Yamaguchi-Shinozaki, 2000; Browse and Xin, 2001; Zhu, 2001; Chinnusamy and Zhu, 2002; Shinozaki et al., 2003). Although the regulatory means for controlling LT responses are not fully understood, a better knowledge of cold-acclimation mechanisms would undoubtedly provide new strategies for improving freezing tolerance in horticultural plants.

Morphological, Anatomical, and Physiological/Biochemical Changes During Cold Acclimation

The process of cold acclimation is very complex, involving a number of morphological, physiological, and biochemical alterations. The most

TABLE 1. Summary of engineered chilling tolerance in plants

Gene	Origin	Transgenic species	References
Protein kinase			
OsCDPK7 (Ca-dependent protein kinase)	Rice	Rice	Saijo et al. (2000)
OsMAPK5 (Mitogen-activated protein kinase)	Rice	Rice	Xiong and Yang (2003)
AtDBF2 (Protein kinase)	*Arabidopsis*	Tobacco	Lee et al. (1999)
Transcription factors			
ICE1 (Inducer of CBF expression1)	*Arabidopsis*	*Arabidopsis*	Chinnusamy et al. (2003)
CBF1 (CRT/CRE binding factor)	*Arabidopsis*	Tomato	Hsieh et al. (2002) Lee et al. (2003)
SCOF-1 (Soybean cold-inducible factor1)	Soybean	Tobacco	Kim et al. (2001)
DREB1A (Dehydration-responsive element binding protein)	*Arabidopsis*	Tobacco	Kasuga et al. (2004)
Osmyb4 (Myeloblastosis binding factor)	Rice	*Arabidopsis*	Vannini et al. (2004)
OSISAP1 (Zinc-finger protein)	Rice	*Arabidopsis*	Mukhopadhyay et al. (2004)
Cold-regulated (COR) genes			
CuCOR19	Citrus	Tobacco	Hara et al. (2003)
RCI3 (Rare cold-inducible gene)	*Arabidopsis*	*Arabidopsis*	Llorente et al. (2002)

TABLE 1 (continued)

Gene	Origin	Transgenic species	References
Compatible solute synthesis genes			
COD/codA (Choline oxidase)	*Arthrobacter globiformis*	*Arabidopsis*	Alia et al. (1998); Hayashi et al. (1997)
	"	Rice	Sakamoto et al. (1998)
	"	Tomato	Park et al. (2003, 2004)
BADH (Betaine aldehyde dehydrogenase)	Barley	Rice	Kishitani et al. (2000)
bet a and bet b (Choline dehydrogenase and betaine aldehyde dehydrogenase)	*E. coli*	Tobacco	Holmstrom et al. (2000)
ApGSMT andApDMT (Glycine sarcosine methyltransferase and dimethyltransferase)	*Aphanothece halophytica*	*Arabidopsis*	Waditee et al. (2005)
TPSP (Trehalose-6-phosphate synthase/phosphatase)	*E. coli*	Rice	Garg et al. (2002); Jang et al. (2003)
GS2 (Chloroplastic glutamine synthetase)	Rice	Rice	Hoshida et al. (2000)
Lipid-modifying genes			
GPAT (Glycerol-3-phosphate acyltransferase)	*Arabidopsis*	Tobacco	Murata et al. (1992)
	"	Rice	Yokoi et al. (1998)
	Arabidopsis and Spinach	"	Ariizumi et al. (2002)
	Squash	Tobacco	Sakamoto et al. (2003)

Gene	Origin	Transgenic species	References
fad7 (Chloroplast ω-3 fatty acid desaturase)	*Arabidopsis*	Tobacco	Kodama et al. (1994)
des9 (Acyl-lipid Δ9 desaturase)	*Anacystis nidulans*	Tobacco	Ishizaki-Nishizawa et al. (1996)
	Synechococcus vulcanus	"	Orlova et al. (2003)
Oxidative stress-related genes			
Cu/Zn-SOD (Cu/Zn superoxide dismutase)	Pea	Tobacco	Gupta et al. (1993)
Mn-SOD (Mn-superoxide dismutase)	Tobacco	Maize	van Breusegem et al. (1999a)
Fe-SOD (Fe-superoxide dismutase)	Tobacco	Alfalfa	McKersie et al. (2000)
	Arabidopsis	Maize	van Breusegem et al. (1999b)
GST/GPX (Glutathione S-transferase/ peroxidase)	Tobacco	Tobacco	Roxas et al. (2000)
GPX (Glutathione peroxidase)	*Chlamydomonas*	Tobacco	Yoshimura et al. (2004)
CAT (Catalase)	Wheat	Rice	Matsumura et al. (2002)
Nt107 (Glutathione S-transferase)	Tobacco	Tobacco	Roxas et al. (1997)
APX (Ascorbate peroxidase)	Tobacco and Spinach	Tobacco	Yabuta et al. (2002)
	Pea	Cotton	Kornyeyev (2003)
ALR (Aldose/aldehyde reductase)	Alfalfa	Tobacco	Hegedüs et al. (2004)

TABLE 2. Summary of engineered freezing tolerance in plants

Gene	Origin	Transgenic species	References
Protein kinases			
AtNDPKs (NDP kinase)	*Arabidopsis*	*Arabidopsis*	Moon et al. (2003)
ANP1 (Mitogen-activated protein kinase kinase kinase)	*Arabidopsis*	Tobacco	Kovtun et al. (2000)
NPK1 (Mitogen-activated protein kinase kinase kinase)	*Tobacco*	Maize	Shou et al. (2004)
Transcription factors			
ICE1 (Inducer of CBF expression1)	*Arabidopsis*	*Arabidopsis*	Chinnusamy et al. (2003)
CBF1 (CRT/DRE binding factor)	*Arabidopsis*	*Arabidopsis*	Jaglo-Ottosen et al. (1998); Kasuga et al. (1999); Gilmour et al. (2000)
	"	Canola	Jaglo-Ottosen et al. (2001)
	"	Strawberry	Owens et al. (2002)
	Canola	Canola	Gusta et al. (2002)
	"	Flax	"
	Sweet cherry	*Arabidopsis*	Kitashiba et al. (2004)
SCOF-1 (Soybean cold-inducible factor1)	Soybean	Tobacco	Kim et al. (2001)
ABI3 (Seed-specific transcriptional activator)	*Arabidopsis*	*Arabidopsis*	Tamminen et al. (2001)
OsDREB1A (Dehydration-responsive element binding protein)	Rice	*Arabidopsis*	Dubouzet et al. (2003)

Gene	Origin	Transgenic species	References
ZmDREB1A	Maize	*Arabidopsis*	Qin et al. (2004)
Osmyb4 (*myeloblastosis binding factor*)	Rice	*Arabidopsis*	Vannini et al. (2004)
CBF4	*Arabidopsis*	*Arabidopsis*	Haake et al. (2002)
CaPF1 (*ERF/AP2 transcription factor*)	Hot pepper	*Arabidopsis*	Yi et al. (2004)
Cold-regulated (COR) genes			
COR15a (Cold-regulated gene)	*Arabidopsis*	*Arabidopsis*	Artus et al. (1996); Steponkus et al. (1998)
CuCOR19 (Dehydrin gene)	Citrus	Tobacco	Hara et al. (2003)
Wcs19 (Late embryogenesis abundant protein)	Wheat	*Arabidopsis*	Ndong et al. (2002)
RAB 18 and COR47	*Arabidopsis*	*Arabidopsis*	Puhakainen et al. (2004)
LTI29 and LTI30	"	"	"
Compatible solute-synthesis genes			
COD/codA (Choline oxidase)	*Arthrobacter globiformis*	*Arabidopsis*	Sakamoto et al. (2000)
	"	Tobacco	Konstantinova et al. (2002)
COD/cox	*Arthrobacter pascens*	*Arabidopsis*	Huang et al. (2000)
P5Cs (Pyrroline-5-carboxylate-synthetase)	*Vigna aconitifolia*	Tobacco	Konstantinova et al. (2002)
	Arabidopsis	Tobacco	Konstantinova et al. (2002)
AtProDH (Antisense proline dehydrogenase)	*Arabidopsis*	*Arabidopsis*	Nanjo et al. (1999)

TABLE 2 (continued)

Gene	Origin	Transgenic species	References
Lea-Gal (Antisense α-galactosidase)	Tomato	Petunia	Pennycooke et al. (2003)
SPS (Sucrose phosphate synthase)	*Arabidopsis*	*Arabidopsis*	Strand et al. (2003)
wft1/wft2 (Fructosyltransferase)	Wheat	Ryegrass	Hisano et al. (2004)
SacB (Levan sucrase)	*Bacillus subtillis*	Tobacco	Konstantinova et al. (2002)
Oxidative stress-related genes			
Mn-SOD (Mn-superoxide dismutase)	Tobacco	Alfalfa	McKersie et al. (1999)
	Wheat	Canola	Gusta et al. (2002)
Fe-SOD (Fe-superoxide dismutase)	Tobacco	Alfalfa	McKersie et al. (2000)

obvious changes are morphological, involving the partial or total loss of aerial organs and the formation of specialized organs, such as buds and tubers (Ferullo and Griffith, 2001). For example, the prostrate or rosette growth form is assumed to be a morphological consequence of development at LT, and can serve as a selection criterion for cold hardiness (Roberts, 1984). Anatomical analyses have also been used to find morphological "markers" for an effective breeding program. In the case of the potato, hardier species, such as *Solanum acaule* and *S. commersonii*, have smaller cells, a thicker palisade, and two to three times greater stomatal indices than do non-hardy species, such as *S. tuberosum* (Li and Palta, 1978).

The effect of cold temperatures on the ultrastructure of plant cells has been long studied (see review by Kratsch and Wise, 2000). The extent of the alterations in cell components is apparently related to the degree of chilling and the length of exposure. Studies have suggested that cold temperature-related changes involve a wide range of components. Garber

and Steponkus (1976) have reported the formation of a paracrystalline array of proteins in the thylakoid membranes of cold-acclimated spinach plants. In a comparison with particles in the membranes of non-acclimated thylakoids, they have also noted a decreased number of particles, representing the PS II complex, on the inner fracture face of acclimated thylakoid membranes and a homogenization of two sizes of particles.

Cold-hardening temperatures cause both univacuolated and multivacuolate mesophyll cells to form in acclimated plants, whereas only univacuolate cells exist in non-acclimated plants (Huner et al., 1983). The chloroplast ultrastructure in the former exhibits an increase in smaller granal stacks while the size of the photosynthetic unit remains the same. O'Neill et al. (1981) have found that the vesiculated smooth endoplasmic reticulum enlarges in cold-hardened leaf cells, suggesting that freezing tolerance is enhanced because more substrate is available for vesicle formation and subsequent extension of the plasma membrane. Consequently, this allows the plant to adapt to the reduction in membrane surface area that occurs during freeze-induced dehydration and later rehydration during thawing.

Cellular and metabolic changes during cold acclimation include a rise in the levels of sugars, soluble proteins, proline, and organic acids, as well as the appearance of new isoforms of proteins and an altered lipid membrane composition (Hughes and Dunn, 1996). In early fall, all perennial plants accumulate carbohydrate reserves in the form of starch or fructan, which is converted into soluble sugars when the cold period begins (Levitt, 1978). Their principal forms are oligosaccharides, such as sucrose, raffinose, and stachyose (Bachmann et al., 1994; Olien and Clark, 1995; Hill et al., 1996). The storage of soluble sugars is concomitant with an increase in the activities of several enzymes associated with carbohydrate metabolism, i.e., amylase, sucrose phosphate synthase, sucrose synthase, ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), and others (Hurry et al., 1994; Nielsen et al., 1997; Reimholz et al., 1997). In fact, this accumulation of free sugars seems to involve the enhancement of all photosynthetic pathways (Hurry et al., 1994). In addition, increased amylase and sucrose synthase activities are related to the appearance of new, cold-specific isozymes (Nielsen et al., 1997; Reimholz et al., 1997). A rise in the steady-state levels of mRNAs that encode sucrose phosphate synthase has also been observed in potato (Reimholz et al., 1997). This indicates that changes in carbohydrate metabolism, with respect to cold acclimation, are under transcriptional and/or post-transcriptional control.

Other compatible solutes, such as proline (Pro) and glycinebetaine (GB), are also accumulated in response to LTs. An increase in the content of those solutes under stress conditions has been found in a number of species; their levels have, in some cases, been positively correlated with freezing tolerance (Bohnert et al., 1995; Rhodes and Hanson, 1993). A causal relationship between Pro accumulation and tolerance to freezing stress has been demonstrated in *Arabidopsis thaliana* (Nanjo et al., 1999). Transgenic *Arabidopsis* plants with an antisense construct of proline dehydrogenase (*AtProDH*), which catalyzes Pro degradation, accumulate higher levels of Pro and show enhanced tolerance to freezing stress (Nanjo et al., 1999). In contrast, Xin and Browse (1998) have found that, under normal growing conditions, a freezing-tolerant *Arabidopsis* mutant, *eskimo1*, can accumulate a 30-fold higher level of Pro due to greater expression of the Δ1-pyroline-5-carboxylate synthetase (*P5Cs*) gene, which catalyzes Pro biosynthesis, compared with WT plants.

Glycine betaine accumulates naturally in distantly related plant species in response to various stresses (Rhodes and Hanson, 1993). In strawberry leaves, levels of GB can rise nearly two-fold after four weeks of cold-acclimation treatment, during which time their cold tolerance increases from −5.8 to −17°C (Rajashekar et al., 1999). Naidu et al. (1991) have reported that the concentration of GB can more than double (from 7.9 to 17.9 μmol/g dry weight) in cold-acclimated wheat seedlings in response to 5 d of cold stress (4°C). Likewise, GB in the winter type of barley accumulates to five times the basal level over three weeks at 5°C, but is only doubled in some spring types (Kishitani et al., 1994). In that barley research of near-isogenic lines of the same cultivar, accumulated levels of GB in leaves at LTs are well correlated with the percentage of green leaves that survive freezing injury (−5°C). Furthermore, the exogenous application of GB improves freezing tolerance in plants that are not natural accumulators (Harinasut et al., 1996; Chen et al., 2000; Makela et al., 2000; Sakamoto et al., 2000; Park et al., 2003). Studies *in vitro* have shown that GB is effective in stabilizing the structures of enzymes and proteins, as well as in protecting membrane properties (Papageorgiou and Murata, 1995).

Temperature and water availability affect both physical and biological properties of cell membranes. Therefore, it is assumed that plasma membranes undergo chemical alterations during cold acclimation so as to adjust to stress. During freezing, the formation and growth of extracellular ice crystals can cause lesions in the plasma membrane, resulting in the loss of osmotic responsiveness during subsequent thawing

(Steponkus, 1984). Cold-acclimated plants manifest not only a rise in the degree of lipid unsaturation, but also changes in lipid composition and the ratio of lipid to protein in membranes (Palta et al., 1993). Steponkus et al. (1988) have shown that increased levels of unsaturated species of phosphatidylcholine mimic the behavior of protoplasts from acclimated plants, and suffer less expansion-induced lysis during thawing. In *Arabidopsis thaliana*, the lipid composition of the plasma membrane changes significantly as plants acclimate (Uemura et al., 1995). The proportion of phospholipids increases from 46.8 to 57.1 mol% of the total lipids, with little change in the proportions of the phospholipid classes. Although the proportion of di-unsaturated species of phosphatidylcholine and phosphatidylethanolamine rises, mono-unsaturated species are still the predominant species. The proportion of cerebrosides decreases from 7.3 to 4.3 mol%, with only small changes in the proportions of the various molecular species. Finally, the proportion of free sterols decreases from 37.7 to 31.2 mol%, but with only small changes in the proportions of sterylglucosides and acylated sterylglucosides (Uemura et al., 1995).

Cold-Regulated (Cor) Genes

Biochemical and physiological changes that occur during cold acclimation are regulated by LTs through modifications in gene expression. Cold-regulated (*COR*) gene expression is critical to plants for imparting tolerance to both chilling (Gong et al., 2002; Hsieh et al., 2002) and freezing (Thomashow, 1999). Moreover, the expression of specific genes up-regulated by LT is highly correlated with the development of freezing tolerance (Thomashow, 1999).

Because tolerance is inducible, it has been commonly assumed that this induction involves the synthesis of novel peptides, which, by means of their enzymatic activity or structural properties, confer tolerance to the tissue. A number of *COR* genes have been characterized from different plant species (Hong et al., 1988; Houde et al., 1992; Lin and Thomashow, 1992; Monroy et al., 1993). For example, Lin and Thomashow (1992) have isolated four *COR* genes from *Arabidopsis thaliana*. Their Northern hybridization has indicated that the level of mRNA from each of these genes increases dramatically during the first 4 h of treatment at 5°C. The level then remains high for the duration of the acclimation period, and declines after the plants are transferred to warm temperatures. All four *COR* genes are ABA-responsive and accumulate if plants are sprayed at room temperature with that growth regulator. The peptides

from these genes are boiling-stable, in contrast to most peptides that are denatured and form a precipitate if an aqueous solution is boiled (Guy, 1990; Thomashow, 1999).

Crt/Dre Binding Factors (Cbfs)

Many *COR* genes have in their promoter regions one or several copies of the *CRT* (C-repeat)/*DRE* (Dehydration Responsive Element) *cis*-element, which has the core sequence CCGAC that is responsive to LT as well as dehydration (Yamaguchi-Shinozaki and Shinozaki, 1994; Stockinger et al., 1997). Stockinger et al. (1997) have isolated *CBF1* (*CRT*-binding factor 1), a cDNA clone for *CRT/DRE*-binding protein. It contains a DNA binding motif (AP2 domain) that is found in the *Arabidopsis APETALA2* (*AP2*) protein, where it functions in floral morphogenesis, as well as in the tobacco *EREBP1* family, where it is involved in ethylene-responsive gene expression (Jofuku et al., 1994; Ohme-Takagi and Shinshi, 1995). More recently, Liu et al. (1998) have used a yeast one-hybrid program to isolate five *CRT/DRE*, which they have named *DREB*s (*DRE*-binding proteins) and have classified into two groups–*DREB1* and *DREB2*. Although each group contains similar AP2 domains, it has low sequence similarity outside that domain. Three *DREB1* proteins are encoded by genes tandemly repeated in the order *DREB1B* (= *CBF1*), *DREB1A* (= *CBF3*), and *DREB1C* (= *CBF2*). Two *DREB2* proteins, *DREB2A* and *DREB2B*, also exist (Stockinger et al., 1997; Gilmour et al., 1998; Liu et al., 1998). Expression of *DREB1A* and its homologues, *DREB1B* and *DREB1C*, is induced by LT stress, whereas expression of *DREB2A* and *DREB2B* is induced by dehydration and salt stresses (Liu et al., 1998). Therefore, two independent *DREB* proteins, *DREB1* and *DREB2*, function as transcription factors in LT and dehydration signal transduction pathways, respectively, to activate *CRT/DRE cis*-elements.

Zarka et al. (2003) have recently found the transcription factors involved in *CBF2* expression, and have identified a 125-bp promoter segment containing two regions, designated *ICEr1* and *ICEr2* (induction of *CBF* expression region 1 or 2). By themselves, the regions are only weakly responsive to low temperatures, but in combination, impart a robust cold response.

Further analysis of the cold-sensing mechanism involved in *CBF* regulation has revealed that a cold shock is not required for bringing about the accumulation of *CBF* transcripts, but instead, the thermosensing circuitry is likely monitor absolute temperature and act like a

rheostat increasing output (i.e., the levels of *CBF* transcripts), with greater degrees of input (i.e., lower temperatures) (Zarka et al., 2003). Even though this cold-sensing mechanism becomes desensitized within a few hours of exposure to a given LT, e.g., 4°C, this desensitization does not preclude a robust response to a further decrease in temperature. Plants that have been adapted to 4°C for 14 d and that have low levels of CBF transcripts produce more transcript when transferred to 0°C or −5°C. In contrast, resensitizing to that temperature requires between 8 and 24 h of exposure to 22°C.

Inducer of Cbf Expression (Ice)

The *CBF/DREB1* genes are themselves induced by LTs. This induction is transient, preceding that of downstream genes with the *DRE/CRT cis*-element (Thomashow, 1999). Gilmour et al. (1998) have proposed that a transcriptional factor, present at warm temperatures, recognizes the *CBF* promoters and is activated in response to LTs by a signal transduction pathway that also exists at warm temperatures. Recently, Chinnusamy et al. (2003) also have identified a transcriptional activator, *ICE* (Inducer of *CBF* Expression), that can recognize the promoters of the *CBF* genes and induce their expression.

ICE1 encodes a MYC-like helix-loop-helix (bHLH) transcriptional activator (Chinnusamy et al., 2003). A dominant-negative *ice1* mutation blocks expression of *CBF3* and decreases the expression of many genes downstream of *CBF*s, such as *RD29A, COR15A* and *COR47*. This leads to a significant reduction in plant chilling and freezing tolerance. Interestingly, transgenic *Arabidopsis* lines that constitutively overexpress *ICE1* do not show *CBF3* expression at warm temperatures, but have a higher level of *CBF3* transcript at low temperatures, suggesting that cold-induced modification of the *ICE1* protein or a transcriptional cofactor is necessary for *ICE1* to activate the expression of *CBF*s (Chinnusamy et al., 2003).

Finally, in contrast to *CBF3* expression, the *ice1* mutation produces somewhat greater levels of cold-induced *CBF2* transcripts, indicating that the mechanisms for expression differ within the *CBF/DREB1* gene family (Chinnusamy et al., 2003).

Mutational Analysis of Freezing Tolerance in Arabidopsis thaliana

Many *Arabidopsis* mutants with increased or decreased freezing tolerance have been isolated and have proven very useful in identifying the

genes and proteins involved in cold acclimation. A classical mutagenesis approach has enabled the isolation of *sfr* (sensitivity to freezing) mutants that fail to gain tolerance after cold acclimation (Warren et al., 1996). Based on an analysis of these mutants for cold-induced gene expression, the *sfr6* mutants are deficient in the genes *kin1*, *cor15a*, and *cor78/rd29A*, all of which contain the *CRT/DRE* motif in their promoters (Knight et al., 1999). Further study with the *sfr6* mutant has found that the expression of cold-induced genes is activated post-transcriptionally by the interaction of *CBF1/DREB1* and *DREB2* with the *CRT/DRE* promoter element (Boyce et al., 2003). In contrast, the freezing sensitivity of cold-acclimated *sfr4* has shown the greatest deficit among the *sfr* mutants (Warren et al., 1996), due to its continued susceptibility to membrane lesions that are caused by lyotropic formation of the hexagonal II phase, and which are also associated with the low sugar content in this mutant's cells (Uemura et al., 2003).

A constitutively freezing-tolerant mutant, *eskimo1*, exhibits greater tolerance than WT plants in the absence of cold acclimation (Xin and Browse, 1998). These mutant plants accumulate a 30-fold greater level of Pro due to higher expression of the *P5Cs* gene compared with WT plants under normal growing conditions. However, the expression of several cold-regulated genes involved in freezing tolerance is not increased. This suggests that *ESKIMO1* may activate a different signal transduction pathway from the *CRT/DRE*-related pathway (Xin and Browse, 1998).

Ishitani et al. (1997) have isolated a large number of mutants with deregulated cold-responsive gene expression, including *cos* (constitutive expression of osmotically responsive genes), *los* (low expression of osmotically responsive genes), and *hos* (high expression of osmotically responsive genes). Xiong et al. (1999) have characterized an *Arabidopsis* mutant, *hos5*, which exhibits increased expression of the osmotic stress-responsive *rd29A* gene under osmotic stress but not when under cold stress. Moreover, this osmotic-stress hypersensitivity found in *hos5* does not affect the *aba* or *abi* mutants, suggesting that its sensitivity only in the *hos5* mutant is ABA-independent. The *hos1* and *hos2* mutants show enhanced induction of both *CBF*s and their downstream *cor* genes only under cold stress (Ishitani et al., 1998; Lee et al., 2001). Non-acclimated *hos1* and *hos2* mutants are less cold-tolerant than WT plants, whereas the expression of *CBF*s is maintained at a higher level for up to 24 h during this stress. *HOS1* and *HOS2* are, therefore, assumed to be negative regulators of cold signaling pathways by modulating the expression level of the *CRT/DRE* binding factors. *HOS1* encodes

a RING finger protein as a possible E3 ubiquitin ligase that is present in the cytoplasm at normal growth temperatures, but accumulates in the nucleus upon LT treatment. Therefore, Lee et al. (2001) have proposed that *HOS1* displays cold-regulated nucleo-cytoplasmic partitioning, which may play an important role in communicating cold-generated signals in the cytoplasm to the nucleus by targeting certain positive regulators of *CBF*s for ubiquitination and degradation.

The *los1* mutant, in which the *COR* genes are no longer induced by LT, enhances the expression of *CBF/DREB1*s but fails to develop freezing tolerance. This mutant, defective in the translation elongation factor 2 gene, blocks new protein synthesis specifically at low temperatures, indicating that cold-induced transcription of *CBF/DREB1*s is feedback-inhibited by either their gene products or the products of their downstream target gene (Guo et al., 2002). Another mutant, *los2*, is also destructed in the accumulation of cold-responsive gene transcripts. The *LOS2* gene encodes a bi-functional enolase involved in the glycolytic pathway. *LOS2* protein can bind to the promoter of *STZ/ZAT10*, a zinc finger transcriptional repressor. Induction of *STZ/ZAT10* is strongly increased and sustained in the *los2* mutant whereas its expression is rapid and transient in WT plants (Lee et al., 2002). The *los4* mutant plants are very sensitive to chilling stress, particularly in the dark; their chilling sensitivity is reversed by constitutive expression of the *CBF3* gene (Gong et al., 2002). The *LOS4* gene, considered the first positive regulator in the expression of *CBF*s, encodes a DEAD-box RNA helicase, suggesting that RNA metabolism is involved in cold acclimation.

Stockinger et al. (2001) have proposed that transcriptional activation of a *COR* gene by *Arabidopsis CBF1* might be mediated by homologs of the yeast histone actyltransferase (*HAT*) *GCN5* and the transcriptional adaptor proteins *Ada2*. In *Arabidopsis*, the *AtADA2* proteins interact with the *AtGCN5* protein. Moreover, both those proteins are found to interact with *CBF1*. Recently, Vlachonasios et al. (2003) have reported that isolating the *Arabidopsis* mutants, which are disrupted by T-DNA insertion of *ADA2* and *GCN5*, leads to induced expression of *CBF*s, as is seen in WT plants. However, subsequent transcription of the *COR* genes is reduced in both mutants. Non-acclimated *ada2b-1* mutant plants are more freezing-tolerant than the non-acclimated wild-types, suggesting that *ADA2b* may directly or indirectly repress a tolerance mechanism that does not require the expression of *CBF* or *COR* genes.

Another series of freezing-sensitive mutant plants, *frs1* (freezing sensitive 1), show a wilty phenotype and excessive water loss in both cold-acclimated and non-acclimated plants, but they recover after treat-

ment with exogenous ABA (Llorente et al., 2000). Complementation analysis has revealed that the *frs1* mutation is a new allele of the *ABA3* locus, and that gene expression in those mutants is altered in response to dehydration. Therefore, the freezing tolerance triggered by ABA-regulated proteins probably protects plants mainly from freezing-induced cellular dehydration.

The involvement of IP_3 (inositol 1,4,5-trisphosphate) has been demonstrated in the stress signal pathway (Xiong et al., 2001). A mutation, *fiery1* (*fry1*), shows enhanced expression of ABA- and stress-responsive genes when treated with ABA, cold, drought, or salt. *FRY1* encodes an enzyme with inositol polyphosphate 1-phosphatase that is involved in the catabolism of IP_3. In response to ABA and osmotic stress, there is a transient increase of inositol 1,4,5-trisphosphate (IP_3) in WT plants (Lee et al., 1996), whereas the *fry1* mutant accumulates a significantly higher level of IP_3. In contrast to the greater induction of cold-responsive genes, the *fry1* plants are defective in their cold acclimation and germination. Hence, *FRY1* is a negative regulator of cold-responsive gene expression through the modulation of IP_3 levels (Xiong et al., 2001).

GENETIC ENGINEERING FOR COLD TOLERANCE IN PLANTS

Genetic engineering, with either one or a small number of genes being introduced into a crop species, has achieved considerable progress toward improving tolerance to LT stresses, including chilling and freezing. Among the LT-induced genes already isolated, several major groups have been adopted for enhancing tolerance. These include protein kinases, transcription factors, cold-regulated genes, oxidative stress-related genes, lipid-modifying genes, and compatible solute-synthesis genes (Tables 1 and 2).

Protein Kinase

In early cold signaling, low temperatures are sensed via alterations in membrane fluidity or through cytoskeletal reorganization that affects the calcium channels (Knight and Knight, 2001). Under cold stress, these transient increases in cytosolic Ca^{2+} are mainly perceived by Ca^{2+} binding proteins, e.g., calmodulin and Ca^{2+}-dependent protein kinases (*CDPK*s) (Zielinski, 1998). Overexpression of a rice calcium-dependent

protein kinase (*OsCDPK7*) results in increased chilling- and osmotic-stress tolerances in rice (Saijo et al., 2000). Expression of *OsCDPK7* also induces some stress-responsive genes in response to high-salt or drought conditions, but not the cold, suggesting that the downstream pathways leading to such cold or salt/drought tolerance differ (Saijo et al., 2000).

In plants, various stresses, including the cold, stimulate the accumulation of reactive oxygen species (ROS), such as superoxide, hydrogen peroxide, and hydroxyl radicals (Hasegawa et al., 2000). These ROS serve as a signal that induces scavengers and other protective mechanisms, as well as the damaging agents that contribute to stress injury in plants (Prasad et al., 1994). Mitogen-activated protein kinase (*MAPK*)-signaling pathways are actively involved in transducing oxidative signaling (Ligterink and Hirt, 2001). Kovtun et al. (2000) have shown that the *Arabidopsis* mitogen-activated protein kinase kinase kinase (ANP1) can be induced specifically by H_2O_2. Furthermore, transgenic tobacco plants that express a constitutively active tobacco *ANP1* (*NPK1*) display elevated tolerance to multiple environmental stress conditions and repressed expression in auxin-inducible promoters (GH3). NDP kinase (*NDPK*) is believed to be a housekeeping enzyme that maintains the intracellular levels of all dNTPs except ATP (Moon et al., 2003). This enzyme is also associated with H_2O_2-mediated *MAPK* signaling in plants. Proteins from transgenic *Arabidopsis* plants that overexpress *Arabidopsis NDPK2* (*AtNDPK2*) show high levels of autophosphorylation and *NDPK* activity, and have fewer ROS than the wild-type. In contrast, mutants that lack *AtNDPK2* have higher levels of ROS than do WT plants. Constitutive expression of *AtNDPK2* in *Arabidopsis* plants confers enhanced tolerance to multiple environmental stresses that elicit ROS accumulation *in situ* (Moon et al., 2003).

Transcription Factors

Transgenic expression of the *CBF* genes leads to improved cold tolerance in many species (Tables 1 and 2). Constitutive expression of the *CBF1* or *CBF3* genes in transgenic *Arabidopsis* plants not only induces multiple *COR* genes without prior cold treatment, but also renders those plants more freezing-tolerant than the controls (Liu et al., 1998; Jaglo-Ottosen et al., 1998; Kasuga et al., 1999; Qin et al. 2004; Kitashiba et al. 2004). However, use of the strong constitutive 35S cauliflower mosaic virus (*CaMV*) promoter to drive expression of *CBF3* results in severe growth retardation under normal conditions. In comparison, the over-

expression of *CBF3*, as driven by the stress-inducible promoter such as *RD29A* or ABRC1, gives rise to only minimal effects on plant growth, with greater tolerance to stress (Kasuga et al., 1999, 2004; Lee et al., 2003). Jaglo-Ottosen et al. (2001) have reported that overexpression of the *Arabidopsis CBF1* genes in canola increases tolerance in both acclimatized and non-acclimatized plants. Furthermore, they have found that transcripts encoding *CBF*-like proteins also accumulate in response to LT in wheat and rye (which cold-acclimate), as well as in tomato, a freezing-sensitive species that does not cold-acclimate. Hence, components of the *CBF* cold-responsive pathway are believed to be highly conserved in flowering plants, and are not limited only to those that cold-acclimate. Furthermore, transgenic expression of *CBF1* in tomato plants induces a higher level of the *CATALASE1* (*CAT1*) gene, resulting in improved chilling tolerance with less H_2O_2 under either normal or LT conditions (Hsieh et al., 2002). Those research results have suggested that heterologous *CBF1* expression in transgenic tomato may induce several oxidative stress-responsive genes to protect plants from chilling stress. Finally, *Arabidopsis* plants that overexpress *CBF3* not only have elevated levels of *COR* proteins, but also higher Pro and total sugar contents (Gilmour et al., 2000). Increased levels of the latter two occur with cold acclimation in a wide variety of plants, and are thought to contribute to the enhancement of freezing tolerance, in part, by stabilizing membranes.

In addition to *CBF*s, analysis of the expression patterns of genes induced by both dehydration and LT have demonstrated broad variations in the timing of their induction and in their responsiveness to ABA (reviewed by Bray, 1997; Shinozaki and Yamaguchi-Shinozaki, 1997). The transient increase in ABA during cold stress as well as the enhancement of freezing tolerance by its exogenous application indicates that this growth regulator must play a critical role in cold acclimation (Thomashow, 1999). Analysis of the promoter regions of *cor15a*, *rd29a*, and *cor6.6* has revealed the presence of *ABRE*s (ABA-responsive elements), or PyACGTGGC (Baker et al., 1994; Yamaguchi-Shinozaki and Shinozaki, 1994; Wang et al., 1995). Gene expression through these *ABREs* is regulated in plants by the transacting protein that comprises the basic domain/leucine zipper (bZIP) structure (Aguan et al., 1993; Kusano et al., 1995; Lu et al., 1996; Choi et al., 2000). Kim et al. (2001) have cloned a novel cold-inducible zinc finger protein from soybean, *SCOF1*, containing two C_2H_2-type zinc fingers and a putative nuclear localization signal, KRKRSKR. *SCOF1* is weakly induced by ABA as well as LT. Its level of transcription is increased up to 3 d after

cold stress; in contrast, expression of *DREB1* decreases to a minimum level within 1 d (Liu et al., 1998). This temporal sequence in the expression pattern of *DREB1* and *SCOF1* indicates that the initial induction of *COR* gene expression by *DREB1* is synergistically increased by *SCOF1* during cold stress (Kim et al., 2001). Constitutive expression of *SCOF1* in *Arabidopsis* results in the expression of multiple *COR* genes (*cor15a*, *cor47*, and *rd29b*) and freezing tolerance. Although it does not directly bind to *ABRE* or *CRT/DRE* motifs, it does enhance the DNA binding activity of *SGBF1*, a bZIP transcription factor. Thus, *SCOF1* interacts with *SGBF1* to regulate *COR* gene expression through activation of *ABRE* in the ABA-dependent pathway of cold-stress signal transduction (Kim et al., 2001).

Microarray analysis has been used to monitor the complex changes in response to LT in the *Arabidopsis* transcriptome. Seki et al. (2001) identified 40 transcriptional factors that belong to AP2/EREB, zinc-finger, ERF, WRKY, bZIP and MYB families and Fowler and Thomashow (2002) have provided a direct evidence for the activity of at least 15 cold-regulated transcription factors not participating in the CBF cold-response pathway. *Arabidopsis* plants expressing the pepper ERF (ethylene-responsive factor)/AP2 affected expression of genes that contain either a GCC or a CRT/DRE box in their promoter region and displayed tolerance against freezing and pathogen stresses (Yi et al., 2004). In addition, the expression of *Osmyb4* or zinc-finger protein from rice in *Arabidopsis* transgenic plants also increases LT tolerance, suggesting that the existence of cross-talk between the LT stress and other stresses (Vannini et al., 2004; Mukhopadhyay et al., 2004).

cor Genes

LT induces the expression of many *cor* genes (reviewed by Thomashow, 1999; Shinozaki and Yamaguchi-Shinozaki, 2000; Browse and Xin, 2001; Zhu, 2001; Chinnusamy and Zhu, 2002; Shinozaki et al., 2003). In *Arabidopsis*, these include the *lti* (low temperature-induced), *kin* (cold-induced), *rd* (responsive to desiccation), and *erd* (early dehydration-inducible) genes. Their products help plants adapt to subsequent LT stress.

Overexpression of *cor15a*, which encodes a polypeptide, modestly increases the freezing tolerance of chloroplasts in non-acclimated *Arabidopsis* plants (Artus et al., 1996; Steponkus et al., 1998). This effect appears to result from the mature *cor15a*-encoded polypeptide, *cor15am*, decreasing the propensity of membranes to form deleterious hexagonal

II phase lipids upon freeze-induced dehydration (Artus et al., 1996; Steponkus et al., 1998). Furthermore, Hara et al. (2003) have suggested that a citrus *cor15a*, dehydrin (a Group-2 LEA protein), improves tolerance in transgenic tobacco plants by scavenging radicals to protect membrane systems. Transgenic *Arabidopsis* plants constitutively expressing multiple dehydrin genes including *RAB18* and *COR47* or *LTI29* and *LTI30* exhibited lower LT50 values and improved survival under freeing stress compared to the control plants (Puhakainen et al., 2004). They also found that the acidic dehydrin LTI29 was translocated from cytosol to the vicinity of the membranes during cold acclimation in transgenic plants. However, the overexpression of individual cold-induced genes has provided little improvement in tolerance at the whole-plant level (Artus et al., 1996; Kaye et al., 1998; Steponkus et al., 1998). Therefore, those individual components of freezing tolerance perhaps can work only within the context of a broader cold-acclimation response (Browse and Xin, 2001). Ndong et al. (2002) have reported that the wheat *wcs19* is a stromal protein belonging to a new class of organelle-targeted Group-3 *LEA* proteins. Its constitutive expression in *Arabidopsis* can protect only cold-acclimated leaves from freezing-induced damage.

Compatible Solute-Synthesis Genes

Compatible solutes, or osmoprotectants, are highly soluble compounds that are nontoxic at high concentrations and which are accumulated at elevated levels by many plant species in response to abiotic stresses (Rhodes and Hanson, 1993). These solutes include polyols and sugars, e.g., mannitol, sorbitol, and trehalose; amino acids, such as proline; and betaines and related compounds (Rhodes and Hanson, 1993; McNeil et al., 1999). With advances in plant molecular genetics, the roles of compatible solutes have been strengthened by the performance of transgenic plants that overexpress or express genes related to their biosynthesis under various stress conditions (reviewed by Chen and Murata, 2002).

Sugars

In the plant kingdom, most species do not seem to accumulate detectable amounts of trehalose, a non-reducing disaccharide of glucose, with the notable exception of the highly desiccation-tolerant "resurrection plants" (Wingler, 2002). Genetic transformation for enhanced accumu-

lation of trehalose in dicot species has resulted in undesirable pleiotropic effects, including stunted growth and altered metabolism under normal conditions (Goddijn et al., 1997; Romero et al., 1997). On the other hand, overexpression of *E. coli* trehalose-6-phosphate synthase/phosphatase (*ots A/B*) in rice is driven by either a constitutive promoter (Jang et al., 2003) or a stress-inducible or tissue-specific promoter (Garg et al., 2002). As a result, trehalose is accumulated in amounts of up to approximately 1 mg per gram fresh weight (FW), which contrasts with the negligible levels seen in non-transgenic controls. Transgenic lines produced by both groups (Garg et al., 2002; Jang et al., 2003) exhibit enhanced tolerance to various stresses without growth retardations or morphological alterations. In addition, increased trehalose levels are correlated with higher soluble carbohydrate contents and an elevated capacity for photosynthesis under stress and non-stress conditions. These observations are consistent with a suggested role in modulating sugar sensing and carbohydrate metabolism, rather than in osmoregulation (Garg et al., 2002).

In addition, *Arabidopsis* plants with increased expression of sucrose phosphate synthase (*SPS*) mitigate the inhibitory effect of cold stress on photosynthesis, and maintain the mobilization of carbohydrates from source leaves to sinks, leading to improved freezing tolerance (Strand et al., 2003). Antisense expression of a tomato *Lea-Gal* gene, which encodes α-galactosidase to catalyze the hydrolysis of raffinose, results in an increase in endogenous raffinose and enhanced tolerance by both non-acclimated and cold-acclimated plants (Pennycooke et al., 2003). Transgenic expression of genes, which are involved in fructan synthesis such as bacterial fructan polymerase (*SacB*) or wheat fructosyltransferase (*wft1* and *wft2*) increased freezing tolerance in tobacco and perennial ryegrass, respectively (Konstantinova et al., 2002; Hisano et al., 2004).

Proline (Pro)

In many plants, the stress-inducible accumulation of free proline (Pro) is caused by both the activation of Pro biosynthesis and the inactivation of Pro degradation (Kiyosue et al., 1996). Transgenic *Arabidopsis* plants with an antisense proline dehydrogenase (*AtProDH*), which catalyzes Pro degradation, accumulate higher levels of Pro (~ 0.6 mg/g FW) and show enhanced freezing tolerance, with a 33% survival rate and 59% ion leakage, versus a 100% mortality rate in WT plants (Nanjo

et al., 1999). In addition, Konstantinova et al. (2002) have introduced Δ1-pyroline-5-carboxylate synthetase, as derived from *Arabidopsis thaliana* and *Vigna aconitifolia* (Lines AtP5Cs and VacP5Cs), into tobacco plants. In that research, Pro contents have increased up to 8- and 15-fold, respectively, under cold stress. After the transgenic tobacco is exposed to freezing temperatures under field conditions, they show higher survival rates (50 to 90%), compared with the WT plants (0% survival).

Glycine Betaine (GB)

Glycine betaine (GB) is among the common betaines most widely distributed in higher plants. However, several taxonomically distant species, e.g., *Arabidopsis*, rice, and tobacco, are considered non-accumulators (Rhodes and Hanson, 1993), such that exogenous GB applications to these species actually improve their growth under various stresses (Harinasut et al., 1996; Chen et al., 2000; Makela et al., 2000; Sakamoto et al., 2000). Genes that encode the enzymes involved in GB biosynthesis have been cloned from higher plants, *E. coli*, and microorganisms (Rozwadowski et al., 1991; Deshnium et al., 1995; Rathinasabapathi et al., 1997; Nyyssola et al., 2000). Furthermore, genetic engineering of the GB biosynthetic pathway into non-accumulators has proven effective in enhancing stress tolerance (see review by McNeil et al., 1999; Sakamoto and Murata, 2000, 2002; Chen and Murata, 2002).

Rice plants have been transformed with the *codA* gene, which encodes choline oxidase (*COD*) that converts choline to GB, with or without the target sequence for chloroplasts (Sakamoto et al., 1998). When *COD* is designed to remain in the cytosol, GB levels are five times greater (~5 μmol/g FW) than when it is targeted to the chloroplasts. Under stress conditions, the content of GB in transgenic plants appears to be much lower (up to 5 μmol/g FW) than in GB accumulators (up to 40 μmol/g FW), regardless of the host plant species. For example, transgenic tobacco plants, engineered to produce GB, accumulate that compound in quantities of no more than 0.1 μmol/g FW (Holmstrom et al., 2000; Huang et al., 2000). On the other hands, transgenic utilization of two bacterial N-methyltransferase enzymes, such as glycine sarcosine methyltrasferase (*ApGSMT*) and dimethylglycine methyltransferase (*ApDMT*), in *Arabidopsis* plants accumulated much higher levels of GB than that of choline monooxigenase (*CMO*) transgenic *Arabidopsis* plants (Waditee et al., 2005).

In contrast, exogenous application of choline as a substrate to several species of transgenic plants can greatly increase GB levels (Nuccio et al., 1998; Huang et al., 2000). Nevertheless, modeling of the kinetics for labeled choline metabolites, after [^{14}C]-choline has been applied to transgenic tobacco that expresses a spinach gene for *CMO*, has demonstrated that choline import into the chloroplasts is a major constraint on the synthesis of GB in these organelles (McNeil et al., 2000). Hence, two major limiting factors have been hypothesized for the accumulation of GB in transgenic plants–substrate availability and the transport of choline across chloroplast membranes (Nuccio et al., 1998, 2000; Huang et al., 2000; McNeil et al., 2000). In particular, the availability of substrate can be increased by the engineering of phosphoethanolamine N-methyltransferase (*PEAMT*), a key enzyme in the choline-biosynthetic pathway (McNeil et al., 2001). When *PEAMT* is introduced into transgenic tobacco plants already engineered to produce GB, its overexpression enables plants to accumulate up to 50 times more free choline and 30 times more GB than those transformed with vector alone (McNeil et al., 2001).

Recently, transgenic plants expressing the *codA* gene show reduced oxidative damage under LT stress (Park et al., 2004). Despite having higher levels of H_2O_2 the transgenic lines were phenotypically normal and recorded higher activities of H_2O_2 scavenging enzymes under both normal and stress conditions (Park et al., 2004). It has suggested that H_2O_2 produced by *COD* activity in transgenic lines stimulates the expression of antioxidant enzymes to maintain safe levels of H_2O_2, resulting in enhanced tolerance to oxidative stress induced by LT stress.

Lipid-Modifying Genes

Because membranes are critical sites of LT-induced injury, the engineering of more stress-tolerant cell membranes has attracted much attention. Increased levels of unsaturated membrane lipids lead to higher fluidity, improved cold tolerance, and greater photosynthetic ability in transgenic plants (Tables 1 and 2). For example, tobacco plants have been transformed with acyl-ACP:glycerol-3-phosphate acyltransferase (*GPAT*), from both a chilling-sensitive squash plant and chilling-tolerant *Arabidopsis* (Murata et al., 1992). Levels of saturated phosphatidyl glycerol decline in the transgenic tobacco containing the *Arabidopsis* enzyme, in contrast to plants with the squash enzyme. Correspondingly, transformants with the *Arabidopsis* enzyme are more chilling-tolerant than those with the squash enzyme. The cDNAs for either *Arabidopsis*

GPAT or spinach *GPAT* have also been introduced into rice (Yokoi et al., 1998; Ariizumi et al., 2002). Under LT conditions, both the level of unsaturated fatty acids and the rate of net photosynthesis in the leaves of transgenic plants are much higher than in the controls.

A chloroplast ω-3 fatty acid desaturase has been shown to increase the amount of dienoic and trienoic fatty acids and, consequently, enhance resistance (Kodama et al., 1994). A broad-specificity Δ9-desaturase gene (*Des9*) from the cyanobacterium *Anacystis nidulans* or *Synechococcus vulcanus* has also been inserted into tobacco plants, resulting in enhanced chilling tolerance (Ishizaki-Nishizawa et al., 1996; Orlova et al., 2003). The enzyme introduces a *cis*-double bond in specific saturated fatty acids of various membrane lipids. Because lipid biosynthetic activities in higher plants are localized to the plastid and endoplasmic reticulum, the enzyme is fused to a transit peptide of the pea *Rubisco* small subunit in order to achieve correct targeting. As a result, the amount of affected Δ9-monosaturated fatty acids in the transgenics is increased up to 17-fold (Ishizaki-Nishizawa et al., 1996). Furthermore, when tobacco plants are exposed to 1°C for 11 d, those expressing *Des9* show no signs of chlorosis compared with the WT plants.

Oxidative Stress-Related Genes

Reactive oxygen species (ROS), including the superoxide (O_2^-), hydroxyl radicals (OH^-) and hydrogen peroxide (H_2O_2), cause oxidative damage to cells during several important metabolic processes, including the mitochondrial electron transport system and the chloroplast photosystems (Foyer et al., 1994). The amount of ROS increases when plants are exposed to various environmental stresses (reviewed by Foyer et al., 1994; Mittler, 2002). Under such stresses, PS I uses oxygen as an alternative electron acceptor. This oxygen reduction results in the production of superoxide and its dismutation product H_2O_2. Hydrogen peroxide and superoxide cooperate in the formation of highly toxic oxygen species, such as hydroxyl radicals (Wise, 1995). Furthermore, peroxidation of the membrane lipids or photoinhibition of PS I can be observed in chilling-sensitive plants (Gupta et al., 1993). These symptoms typically occur in plants suffering from oxidative stress. Therefore, this enhanced protection is also advantageous under LT conditions.

To scavenge ROS, plants have developed enzyme systems that include superoxide dismutase (*SOD*), ascorbate peroxidase (*APX*), catalase (*CAT*), monodehydroascorbate reductase (*MDAR*), dehydroascorbate

reductase (*DHAR*), and glutathione reductase (*GR*). Recent studies have demonstrated that plants produce ROS as signaling molecules to control their biological processes in response to abiotic stress (Neill et al., 2002; Vranová et al., 2002). In addition, researchers have attempted to develop transgenic plants with modified ROS-scavenging mechanisms as a means of increasing the functional capacity of such enzymes against LT-induced stress (Tables 1 and 2).

Manipulation of the expression of enzymes involved in ROS-scavenging systems has focused on *SOD, APX*, and *GR*, all isoenzymes that are mainly targeted into the cytosol, mitochondria, or plastids (Gupta et al., 1993; McKersie et al., 1993; Kornyeyev et al., 2003; Foyer et al., 1995; Yoshimura et al., 2004). For example, transgenic tobacco, which is genetically engineered with chloroplastic *CuZn-SOD*, shows improved resistance to intense light and LTs, even though the elevated activity of the native *APX* associated with higher *SOD* activity in these plants may be most critical to their recovery of photosynthesis (Gupta et al., 1993). Overexpression of *Mn-SOD* from tobacco also confers protection against freezing damage in alfalfa (McKersie et al., 1993) as well as chilling damage in cotton (Allen, 1995) and maize (van Breusegem et al., 1999a, 1999b). During field trials, transgenic alfalfa that potently expresses *Mn-SOD* and *Fe-SOD* in the chloroplasts has higher winter survival rates and herbage yield than do the control plants, with no detectable difference in the pattern of primary freezing injury, as shown by vital staining (McKersie et al., 1999, 2000). Transgenic overproduction of *APX* in cotton increases four-fold higher activity of *APX* than that of WT during exposure to 10°C and 500 μmol photons/m^2/s^1 (Kornyeyev et al., 2003). These plants did not exhibit as large of an increase in cellular H_2O_2 that was evident in WT shortly after the imposition of the chilling treatment, together with showing less PS I and PS II photoinhibition. Transgenic tobacco that expresses both Glutathione S-transferase (*GST*) and glutathione peroxidase (*GPX*) genes in the cytoplasm also shows improved tolerance to chilling temperatures and salt stress during germination and seedling development (Roxas et al., 1997, 2000). This improvement in growth appears to depend on increased oxidation of the glutathione pool, which is necessary for the effective scavenging of toxic H_2O_2 by *GPX* and for the maintenance of other antioxidants, such as ascorbate and tocopherols. Furthermore, transgenic tobacco plants overexpressing *Chlamydomonas* GPX in chloroplast seems to be more tolerant to several stresses, including chilling, salt and oxidative stress, than the cytosol-targeted lines (Yoshimura et al., 2004). This can be explained by the fact that the ma-

jor sites of ROS production in the plant cell are the organelles with highly oxidizing metabolic activities or with sustained electron flows: chloroplasts, mitochondria, and microbodies. In addition, transgenic tobacco plants overproducing alfalfa NADPH-dependent aldose/aldehyde reductase also show higher tolerance to LT photoinhibition and cadmium stress (Hegedüs et al., 2004).

GENETIC ENGINEERING FOR COLD TOLERANCE IN HORTICULTURAL CROPS

Even though many species have been transformed for enhanced tolerance to chilling (Table 1) and freezing (Table 2), the number of transgenic horticultural crops with such traits is relatively small.

The CbfI Transgenic Tomato

The *CRT/DRE* binding factor 1 (*CBF1*) induces expression of the *COR* genes, resulting in enhanced tolerance to chilling (Table 1) or freezing (Table 2) without cold acclimation. *CBF1* homologues have been isolated from various chilling-tolerant and -sensitive species, indicating that cold-regulated *CBF*-like genes are not limited only to plants that can be cold-acclimated (Jaglo-Ottosen et al., 2001). Constitutive expression of the *Arabidopsis CBF1* gene in tomato increases tolerance to chilling, but not to freezing stress (Hsieh et al., 2002). Moreover, using known *Arabidopsis COR* cDNAs, such as *COR47, COR15a*, and *KIN1*, as probes with *CBF1* transgenic tomatoes does not hybridize to any tomato transcripts, implying that alternative tomato protein(s) may be functioning as stress protectants (Hsieh et al., 2002).

Hsieh et al. (2002) have also found that the *CBF1* gene induces higher levels of the *CATALASE1* (*CAT1*) gene and catalase activity, resulting in improved oxidative tolerance with lower H_2O_2 content under either normal or LT conditions. In chilling-sensitive maize, *CAT3* gene expression and its enzymatic activities are increased during cold acclimation; the improvement of chilling tolerance conferred by acclimation is correlated with up-regulation of this gene (Prasad, 1997). Transgenic tomato plants that overexpress antisense *CAT1* are also more sensitive to oxidative stress and chilling injury (Kerdnaimongkol and Woodson, 1999). Hsieh et al. (2002) have proposed that a putative *CRT/DRE* binding site may exist on the maize *CAT3* and rice *CAT-A* promoter, based on DNA sequence analysis. Recently, Seki et al. (2001) have reported

that the *Arabidopsis CAT3* gene is induced by cold, drought, and the overexpression of *DREB1a* (= *CBF3*). Therefore, based on all these studies, Hsieh et al. (2002) have concluded that the enhancement of chilling tolerance in *CBF1* transgenic tomato may be due to the induction of the *CAT1* gene.

CBF1 transgenic tomato plants exhibit apparent dwarfism along with reductions in fruit set and number of seeds per fruit. Similar growth retardation has been observed in *Arabidopsis* transformed with the *DREB1a* construct (Kasuga et al., 1999). However, the application of GA_3 to transgenic tomato plants can reverse this phenomenon while not affecting their chilling tolerance. This suggests that overexpression of *CBF1* may interfere with GA biosynthesis in transgenics. Kasuga et al. (1999) have achieved enhanced tolerance without harming growth by replacing the *CAMV* 35S promoter with an inducible promoter in transgenic *Arabidopsis*. Their results demonstrate the potential benefit of using a stress-inducible promoter, such as *RD29A*, as a way to overcome the problem of dwarfism in *CBF1* transgenic plants.

The Cbf1 Transgenic Strawberry

Floral damage is a serious consequence of exposure to freezing temperatures, particularly in early-blooming species, such as strawberry, cherry, peach, almond, and apricot (Rodrigo, 2000). In strawberry plants, their early bloom and extreme mid-winter temperatures may increase the occurrence of injuries to flower and crown tissues, respectively. Therefore, Owens et al. (2002) have cloned orthologs of the *Arabidopsis CBF1* gene from strawberry (*Fragaria ×ananassa* Duchesne) and sour cherry (*Prunus cerasus* L.), and have studied the expression of *CBF1* in both species. They have also used *Agrobacterium*-mediated transformation in strawberry, with the *Arabidopsis CBF1* gene driven by a constitutive CaMV 35S promoter. This has led to enhanced freezing tolerance in transgenic leaf tissues, but not in the receptacle tissues, although the level of *CBF1* transcript is similar in both (Owens et al., 2002).

In *Arabidopsis* transformed with reporter genes driven by the *cor15a* and *cor78* promoters, Horvath et al. (1993) have noted that the reporter is not expressed in the ovaries following cold acclimation, despite reporter gene expression being detected in many vegetative and reproductive tissues. Moreover, the *Arabidopsis sfr6* mutant, which is deficient in freezing tolerance, shows no expression of the *COR* genes in re-

sponse to LTs, even though their expression of *CBF3* following cold exposure is normal (Knight et al., 1999). Altogether, these observations raise the possibility that either (1) different signal transduction pathways lead to the induction of *CBF* in strawberry floral tissues, or (2) cold acclimation can be induced by an alternative pathway or by a *CBF* paralog that has yet to be detected in such experiments (Owens et al., 2002).

The A-Gal Transgenic Petunia

Transcriptome-profiling experiments in *Arabidopsis* indicate that extensive changes in gene expression occur during cold acclimation, and that a substantial number of the genes that are up-regulated during that time are involved in metabolism under cold conditions (Fowler and Thomashow, 2002). In addition to *COR* gene expression, one notable biochemical/physiological alteration during cold acclimation is the accumulation of compatible solutes, such as soluble sugars (Guy, 1990). In *Arabidopsis*, transcripts and enzyme activity are strongly up-regulated for sugar biosynthesis at LTs (Strand et al., 2003), which suggests that such an accumulation may be related to carbon storage and a role as a cryoprotectant. Uemura et al. (2003) have studied the sugar-deficient mutant (*sfr4*), and have shown that this defect in its cells during cold acclimation is largely responsible for the impaired increase in freezing tolerance. However, supplementing those mutant plants with sucrose restores their tolerance to a level similar to that found with the wild-types after cold acclimation. Moreover, Castonguay and Nadeau (1998) and Taji et al. (2002) have reported that variations in freezing tolerance are more closely related to a plant's capacity to accumulate raffinose family oligosaccharides (RFOs) than to its ability to store sucrose.

In response to environmental conditions, the endogenous concentration of soluble sugars in higher plants is controlled through synthesis, degradation, and transport (Strand et al., 2003). RFOs, particularly raffinose, accumulate as a reaction to cold stress, with altered rates of raffinose biosynthesis (Taji et al., 2002). This biosynthesis is regulated by two enzymes, galactinol synthase (*GolS*) and raffinose synthase (*RafS*) (Pennycooke et al., 2003). Raffinose degradation proceeds by the action of α-galactosidase (α-*Gal*), which hydrolyzes Gal-containing oligosaccharides (Pennycooke et al., 2003). In addition, at least three putative *GolS* genes in *Arabidopsis* are members of the *CBF* regulon (Fowler and Thomashow, 2002), while one of them is induced

in response to LTs and the overexpression of *CBF3/DREB1a* (Taji et al., 2002).

The tomato *Lea-Gal* gene (α-galactosidase) has been introduced into petunia in the sense and antisense orientations (Pennycooke et al., 2003). The content of total soluble sugars in non-acclimated antisense lines is approximately three to five times higher than that in the non-acclimated WT and sense lines. More than 50% of the increase in total soluble sugars is due to the accumulations of raffinose in cold-acclimated antisense lines and sucrose in cold-acclimated WT and sense lines (Pennycooke et al., 2003).

The raffinose content of non-acclimated antisense plants is up to 22-fold higher than in the wild-types, and up to 53-fold greater after cold acclimation. Under non-acclimated conditions, all except one antisense line are significantly more freezing tolerance (up to a 2°C increase) than the WT plants. Freezing tolerance is about −5°C for cold-acclimated WT plants, whereas the cold-acclimated antisense lines range from −6 to −8°C. Thus, down-regulating α-Gal in petunia results in an elevated level of raffinose and increased tolerance at the whole-plant level for both non-acclimated and cold-acclimated plants. In contrast, overexpression of the α-Gal gene causes endogenous raffinose to decrease, along with the impairment of freezing tolerance. Moreover, differences in the maximum level of tolerance between the antisense and sense lines are more closely related to the capacity of plants to accumulate raffinose rather than sucrose (Pennycooke et al., 2003). These results, therefore, suggest that engineering for raffinose metabolism via transformation with the antisense construct of α-Gal provides an additional method for improving freezing tolerance.

The Coda Transgenic Tomato

Tomato plants, which normally do not accumulate glycine betaine (GB), are susceptible to chilling stress. We have transformed tomato (*Lycopersicon esculentum* Mill. cv. Moneymaker) with the *codA* gene, which encodes choline oxidase (*COD*), using the transit peptide sequence of a small subunit of the tobacco Rubisco gene to target the *COD* into chloroplasts (Park et al., 2003, 2004). These transgenic plants can accumulate up to 0.23 mmol of GB per gram FW in their leaves. After exposure to stress conditions, GB levels in their leaves are lower than those reported in *Arabidopsis* (0.8~1.2 mmol/g FW; Hayashi et al., 1997), *Brassica juncea* (0.64~0.82 mmol/g FW; Prasad et al., 2000), or

rice (1.1~5.3 mmol/g FW; Sakamoto et al., 1998), and are much lower than those in natural GB-accumulators such as spinach (30 to 40 μmol/g FW) (Rhodes and Hanson, 1993). Therefore, it may be necessary to generate plants with the capacity to synthesize higher levels of GB by enhancing the availability of choline, the GB precursor (McNeil et al., 2001).

In further physiological studies, seeds of homozygous transgenic lines have been found to germinate earlier, with and without cold treatment. When exposed to low temperatures, the dispersion rates of germination events increase in the wild-type, but not in the transgenic lines, compared with those under non-stress conditions. Within one week after the end of the chilling treatment, seeds of two transgenic lines show higher germination rates (93% and 83%) than those of WT seeds (53%). *In-vitro* growth of transgenic seedlings, under either a 16-h photoperiod or 24-h darkness, also have enhanced tolerance to LT stress. Although neither the WT nor the L1 and L2 transgenic plants grow at all in the light during chilling, upon transferring all the plant types to warm conditions, the transgenic seedlings (with a 100% survival rate) resume normal growth while the WT seedlings suffer extensive chilling injury, as manifested by necrotic lesions and moribund shoot tips. When chilled in darkness, transgenic seedlings continue to grow even at LT conditions, but the development of WT plants is completely inhibited. Upon their return to the greenhouse after 4 d of LT stress, five-week-old greenhouse-grown transgenic plants recover more quickly, with significantly less extensive chilling injury ($P < 0.01$). Furthermore, only about 20 to 25% of the total leaf area on the transgenic plants is damaged, compared with about 55% injury on the WT plants.

When WT and transgenic plants that contain 2~3 open flowers in the first inflorescence are exposed to LT, the former retain only about 82% of their flowers, but all flowers remain on the latter. Although this stress reduces the number of fruit in both types of plants, fruit set is higher ($P \leq 0.052$) in the transgenics. Consequently, they yield, on average, 30% more fruit after an episode of chilling stress. Therefore, these results indicate that the endogenous production of GB in transgenic tomato plants, as a consequence of *codA* expression, increases cold tolerance not only at the vegetative but also the reproductive stage, both of which are known to be most sensitive to environmental stresses.

CONCLUSIONS AND PERSPECTIVES

With the advances made in the molecular biology of plant cold acclimation, our understanding of injuries and LT tolerances has improved significantly in the past ten years. This has contributed to the impressive results gained in research to improve plant cold tolerance by genetic engineering. In addition to studies of "model plant systems," various genes related to the cold-defensive mechanism have been shown to confer tolerance in crop species, such as rice, maize, and canola, as well as a few horticultural plants, i.e., tomato, strawberry, and petunia. We expect that genetic engineering will continue to be used for generating additional horticultural species with enhanced tolerance.

To date, most of the successful examples of engineered tolerance have been achieved by transferring a single cold-tolerance gene, although the particular conferring mechanisms have greatly differed. Constructs with the sense orientation for overexpression or the antisense orientation to down-regulate gene expression have been successfully used. In the case of transformation with transcription factors, such as the *CBF* gene, this single transgene has been capable of inducing the expression of a battery of downstream *COR* genes. This approach appears to be the most effective way to confer a high level of tolerance. In addition, these cold-hardy transgenic plants very often show improved tolerance to salinity, oxidative stress, and drought as well. Nevertheless, when driven by a constitutive promoter, high expression for this type of gene often leads to undesirable side effects, such as lower transformation and regeneration frequencies, difficulty in obtaining transgenic plants with a high level of *CBF* transgene expression, severe growth retardation, and reduced seed and fruit formation. Fortunately, a stress-inducible promoter can be used to alleviate such problems (Kasuga et al., 1999). Alternatively, if one can identify and clone a small number of key *COR* genes involved in cold acclimation, a plant can be transformed with those genes, either singularly or in combination with a few such genes, to produce transgenic plants with improved tolerance but minimal detrimental effects.

Further improvement of cold tolerance via genetic engineering is still possible. First, a better understanding of the stress protection pathways in plants may allow us to selectively activate a minimum number of necessary genes at the right time and at the right place, as nature does. Second, better transformation technologies in horticultural species will allow us to improve tolerance in additional crops. Moreover, introducing different genes, which are individually effective in enhancing toler-

ance by various modes of action, into a single plant may result in the production of extremely stress-tolerant plants. However, it is also will be necessary to ensure the genetic manipulation that is achieved does not perturb growth or yield. Finally, the performance of transgenic plants should be verified in the lab as well as in the field; collaboration among different plant-science disciplines will lead to better evaluation of their practical utility.

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Engineering for Drought Tolerance in Horticultural and Ornamental Plants: Lessons from the Studies with Model Plants

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SUMMARY. Drought is one of the major factors limiting plant growth and productivity. Plant adaptation to drought is dependent on molecular networks for drought perception, signal transduction, expression of a subset of genes and production of metabolites that protect and maintain the structure of cellular components. In general, the drought response pathways can be classified into two categories: one is dependent on the stress hormone abscisic acid (ABA) and the other is ABA-independent. Many genes in these pathways have been identified, thereby providing guidance in choosing genes for engineering of drought tolerance. The review highlights the genes that mediate drought response and tolerance, and discusses lessons learned from engineering for drought tolerance in

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model plants, such as *Arabidopsis*, rice and tobacco. Because success of drought tolerance engineering is dependent on not only protein coding regions but also appropriate promoters, this article also reviews the promoters that are crucial for successful engineering of stress tolerance. doi:10.1300/J411v17n01_05

KEYWORDS. Ornamental plants, drought response, drought tolerance, abscisic acid, overexpressing genes

INTRODUCTION

Field-grown plants are constantly under unfavorable environmental conditions, including abiotic stresses such as drought, flooding, extreme temperatures, excessive salts, heavy metals, and high photon irradiation. Because of their immobility, plants have to make metabolic and structural adjustments to cope with stressful environmental conditions. These responses often lead to a wide variety of biochemical and physiological changes such as the accumulation of various organic compounds of low-molecular weight, collectively known as compatible solutes, osmolytes or osmoprotectants. Examples are sugars (e.g., trehalose), sugar alcohol (e.g., mannitol), amino acids (e.g., proline) and polyamines (Kasukabe et al., 2004). Structural proteins, such as, dehydrin, late-embryogenesis-abundant (LEA) and response-to-ABA (RAP) proteins, are also important for drought tolerance (for review, see Bajaj et al., 1999). The osmolytes and structural proteins directly protect plant cells against stresses. The other groups of gene products regulate gene expression and signal transduction in abiotic stress responses, as revealed in genetic (Ishitani et al., 1997), genomic (Seki et al., 2002; Hazen et al., 2005) and proteomic (Riccardi et al., 2004; Hajheidari et al., 2005) analyses. One of the drought response pathways is dependent on ABA; the other is not.

THE ABA-DEPENDENT DROUGHT RESPONSE PATHWAY

ABA is called a stress hormone. It is ubiquitous in lower and higher plants, and has also been found in algae (Hirsch et al., 1989), fungi

(Yamamoto et al., 2000) and even mammalian brain tissue (Le Page-Degivry et al., 1986). The response of plant cells to ABA involves a signal network containing receptors, secondary messengers, protein kinases and phosphatases, chromatin remodeling proteins, transcriptional regulators, RNA binding proteins, and protein degradation complexes, as summarized in several excellent reviews (Lovegrove and Hooley, 2000; Rock, 2000; Finkelstein and Rock, 2001; Schroeder et al., 2001; Ritchie et al., 2002; Hare et al., 2003; Himmelbach et al., 2003; Kuhn and Schroeder, 2003; Chinnusamy et al., 2004; Fan et al., 2004). ABA signaling networks are conserved among higher plant species; information derived from several plant species have been used to compile a network map of ABA signaling (Finkelstein and Rock, 2001; Himmelbach et al., 2003).

Receptors, G Proteins and Secondary Messengers

The site and nature of ABA perception were addressed in barley aleurone cells and guard cells of several plant species. Externally applied, but not microinjected, ABA could repress gibberellin (GA)-induced α-amylase expression in aleurone protoplasts, suggesting an extracellular perception of ABA (Gilroy and Jones, 1994). In contrast, introduction of ABA into the cytoplasm by microinjection (Schwartz et al., 1994) or a patch-clamp electrode (Allan et al., 1994) triggered or maintained stomatal closure, arguing for intracellular perceiving sites. One promising receptor candidate is ABAP1, which is located in membrane fractions of ABA-treated barley aleurone cells. Another candidate is GCR1, a putative G protein-coupled receptor identified in *Arabidopsis* (Pandey and Assmann, 2004).

In cereal aleurone cells, the activation of a plasma-membrane-bound ABA-inducible phospholipase D (PLD) is essential for ABA response (Ritchie and Gilroy, 2000). This process is GTP-dependent; addition of GTPγS transiently stimulates PLD in an ABA-independent manner, whereas treatment with GDPβS or pertussis toxin blocks the PLD activation by ABA. These data suggest the involvement of G-protein activity in the ABA response of barley (Ritchie and Gilroy, 2000). Monomeric G-proteins also regulate ABA responses (Lemichez et al., 2001; Yang, 2002). Rop10, a plasma membrane-associated small GTPase, appears to negatively regulate ABA responses in seed germination and seedling growth of *Arabidopsis* (Yang, 2002).

The primary intracellular messenger of ABA responses is Ca^{2+}, which also mediates the signaling of other hormones (Barkla et al., 1999). ABA activates the vacuolar H^+ATPase and regulates the influx of Ca^{2+} across the plasma membrane through ABA-activated channels (Schroeder and Hagiwara, 1990; Hamilton et al., 2000). In addition, the concentration of Ca^{2+} in cytosol ($[Ca^{2+}]_{cyt}$) is further modulated by other secondary messengers including inositol 1,4,5 triphosphate (InsP3), phosphatidic acid (PA), myo-inositol hexakisphosphate (InsP6), sphingosine-1-phosphate (S1P), hydrogen peroxide (H_2O_2), nitric oxide (NO), cyclic ADP ribose (cADPR), and cyclic GMP (cGMP) (Wu et al., 1997; Leckie et al., 1998; Himmelbach et al., 2003). The genes encoding this group of proteins are shown in Table 1.

Phosphatases and Kinases

Mutation studies suggest that several *Arabidopsis* protein phosphatases 2C, such as ABI1 and ABI2, function as negative regulators of

TABLE 1. Genes Involved in Early ABA Signaling Events and Production of Secondary Messengers

Gene[a]	Accession[b]	Gene Product	Reference
HvABAP1	AAF97846	ABA-binding protein	(Razem et al., 2004)
AtERA1	NP_198844	Farnesyl transferase, beta-subunit	(Cutler et al., 1996; Pei et al., 1998)
AtGCR1	AAN15633	G protein-coupled receptors	(Pandey and Assmann, 2004)
AtGPA1	AAA32805	Heterotrimeric GTP-binding (G) protein	(Wang et al., 2001)
At5PTase1	AAD10828	Inositol 5-phosphatase	(Burnette et al., 2003)
AtFRY1	AAC49263	Inositol polyphosphate-1-phosphatase	(Xiong et al., 2001a)
AtIP5PII	NP_849402	Ins(1,4,5)P_3 5-phosphatase	(Sanchez and Chua, 2001)
AtrbohD	NP_199602	NADPH oxidase catalytic subunit genes	(Kwak et al., 2003)
AtrbohF	NP_564821	NADPH oxidase catalytic subunit genes	(Kwak et al., 2003)
AtNIA1	CAA31786	Nitrate reductase	(Desikan et al., 2002)
AtNIA2	AAK56261	Nitrate reductase	(Desikan et al., 2002)
AtNOS1	AAU95423	Nitric oxide synthase	(Guo et al., 2003)
AtPLC1	BAA07547	Phospholipase C1	(Sanchez and Chua, 2001)
AtPLDa1	Q38882	Phospholipase D	(Zhang et al., 2004b)
AtROP2	Q38919	Rho-type small GTPase	(Li et al., 2001)
AtROP10	Q9SU67	Rop subfamily of Rho GTPases	(Zheng et al., 2002)
AtSYP61	AAK40222	SNARE superfamily of proteins, SNAP receptor	(Zhu et al., 2002)
AtSphK	BAB07787	Sphingosine kinase	(Coursol et al., 2003)

a: The first two letters of gene names refer to the organism from which the gene is cloned: *At, Arabidopsis thaliana*; *Cp, Craterostigma plantagineum*; *Hv, Hordeum vulgare*; *Lt, Larrea tridentata*; *Os, Oryza sativa*; *Pv, Phaseolus vulgaris*; *Ta, Triticum aestivum*; *Vf, Vicia faba*; *Zm, Zea mays*.
b: Genbank® accession numbers for the peptide sequences.

ABA signaling (Merlot et al., 2001; Himmelbach et al., 2003; Leonhardt et al., 2004). ABI2 and ABI1 physically interact with PKS3 (or its homologue CIPK3), a Ser/Thr protein kinase. This kinase is also associated with the calcineurin B-like Ca^{2+} binding protein, SCaBP5 (or its homologue CBL), forming a complex that negatively controls ABA sensitivity (Guo et al., 2002; Kim et al., 2003). Another calcium sensor (CBL9) functions as a negative regulator of ABA signaling and biosynthesis (Pandey et al., 2004). In contrast, the protein phosphatase 2A encoded by *RCN1*, functions as a positive regulator of ABA signaling (Kwak et al., 2002).

Protein kinases also can function as positive regulators of ABA signaling. Calcium-dependent protein kinases (CDPKs) contain a protein kinase domain and a carboxyl-terminal calmodulin-like structure that directly binds calcium (Cheng et al., 2002). Two *Arabidopsis* CDPKs (AtCPK10 and AtCPK30) activate an ABA-inducible barley promoter in the absence of the hormone (Cheng et al., 2002). ABA and H_2O_2 activate the *Arabidopsis* mitogen-activated protein kinase kinase kinase, ANP1, which initiates a phosphorylation cascade involving two mitogen-activated protein kinases (MAPK), AtMPK3 and AtMPK6 (Kovtun et al., 2000). Overexpression of *AtMAPK3* increases ABA sensitivity while inhibition of MAPK activity by inhibitor PD98059 decreases ABA sensitivity (Lu et al., 2002). Sucrose nonfermenting1-related protein kinases function as activators of ABA signaling in rice (Kobayashi et al., 2004) and wheat (Johnson et al., 2002). The genes encoding phosphatases and kinases involved in ABA signaling are shown in Table 2.

TABLE 2. Kinases and Phosphatases Involved in ABA Signaling

Gene	Accession	Gene Product	Reference
AtCDPK1	BAA04829	Calcium-dependent protein kinases	(Sheen, 1996)
AtCDPK1a	EAA19816	Calcium-dependent protein kinases	(Sheen, 1996)
AtPKS3	AAK26842	Protein kinase	(Guo et al., 2002)
AtCIPK3	NP_850095	Ser/Thr protein kinase	(Kim et al., 2003)
HvPKABA1	BAB61736	Ser/Thr-protein kinase	(Gómez-Cadenas et al., 1999)
AtSCaBP5	AAC26008	Ca(2+) binding protein	(Guo et al., 2002)
AtCBL9	AAL10301	Calcineurin B-like proteins	(Pandey et al., 2004)
AtRCN1	AAC49255	Protein phosphatase 2A	(Kwak et al., 2002)
AtABI1	NP_194338	Protein phosphatase 2C	(Leung et al., 1994; Wu et al., 2003)
AtABI2	O04719	Protein phosphatase 2C	(Finkelstein, 1993)
AtP2C-HA	AAG51849	Protein phosphatase 2C	(Leonhardt et al., 2004)
AtPP2CA	BAA07287	Protein phosphatases 2C	(Tahtiharju and Palva, 2001)

Transcriptional Regulation

Cis-Acting Elements

ABA response is dependent on promoter complexes called ABRCs (Shen and Ho, 1995; Shen et al., 1996; Shen and Ho, 1997, 1998; Shen et al., 2001; Shen et al., 2004). For two barley genes, each ABRC consists of an ACGT core containing element (ACGT box) and a coupling element (CE1 or CE3), forming two different ABRCs called ABRC1 and ABRC3. These two promoter complexes are different in the sequences of the coupling elements, the orientation constraints of the coupling elements and the distances between an ACGT-box and a CE (Shen et al., 2004). Extensive deletion and point mutation analyses suggest that the ACGT element requires the sequence 5′-ACGTGGC-3′ and the elements CE1 and CE3 require the sequences CCACC and GCGTGTC, respectively. It is suggested that the ACGT box and CE3 are functionally equivalent because the OsTRAB1/ABI5 binds to both the ACGT box and CE3 element *in vitro* (Hobo et al., 1999b). However, recent data indicate that the coupling between an ACGT-box and a CE or between two ACGT boxes is essential for a high level of ABA induction; two copies of CE3 are much less active (Shen et al., 2004). Furthermore, a partially purified nuclear extract from barley embryos has specific binding activity for the ACGT-box present in ABRC3. It recognizes the wild type version of the ABRC3 and two copies of the ACGT-box but possesses low affinity for two copies of the coupling element CE3, suggesting that it is likely a bZIP protein that is different from ABI5 binds to the CE3 element *in vivo* (Casaretto and Ho, 2003; Shen et al., 2004).

Another *cis*-acting element, CATGTG, has been identified in the promoter of the EARLY RESPONSE TO DEHYDRATION1 (ERD1) gene, which encodes a ATP binding subunit of the caseinolytic ATP-dependent protease (ClpA) homologous protein (Tran et al., 2004). The element is similar to the binding site of the MYC protein (Urao et al., 1996). However, it was recently found to be bound by a group of proteins that belongs to a large multigene family of plant-specific NAC transcription factors encoded by more than 100 members (Riechmann et al., 2000).

Trans-Acting Factors

Several transcription factors have been well documented to mediate ABA signaling. The ABI5-type bZIP proteins from *Arabidopsis*, sun-

flower, wheat, barley, and rice bind as dimers to the ACGT-box or CE3 to activate the promoters (reviewed in Finkelstein and Rock, 2001). ABI5 is up-regulated by ABA through an increase in the transcript level as well as the stability of the protein. AP2-type proteins from maize and barley, ZmABI4, HvDRF1, ZmDBF1 and ZmDBF2 (Narusaka et al., 2003), interact with CE1 or its related sequences (Himmelbach et al., 2003; Xue and Loveridge, 2004). AtMYC2 and AtMYB2 bind to MYC and MYB recognition sites, respectively, and function as activators of ABA signaling (Abe et al., 2003). Recently, ABA inducible NAC activator proteins also were found to interact with the MYC site (Fujita et al., 2004; Tran et al., 2004). The activities of ABI5 and its orthologues/homologues (Table 3) are modified by some kinases (Johnson et al., 2002; Kagaya et al., 2002; Lu et al., 2002), VP1 (Casaretto and Ho, 2003; Suzuki et al., 2003), FUS3, and LEC1 (Finkelstein and Rock, 2001). WRKY proteins can serve as activators of ABA signaling. Of the 81 published *OsWRKY* genes (Zhang et al., 2004c; Xie et al., 2005), at least two function as activators of ABA signaling in aleurone cells (Xie et al., 2005). A WRKY gene from a xerophytic evergreen C3 shrub, creosote bush (*Larrea tridentate*) that thrives in vase arid area also encodes an activator of the ABA signaling pathway (Zou et al., 2004).

The activity/assembly of the transcription complex for ABA signaling appears to be modulated by at least four classes of transcriptional repressors. The first class of repressors is bZIP proteins that negatively regulate ABA-induced gene expression by sequestering bZIP activators or competing with bZIP activators for binding to the ACGT-box. For example, two rice bZIP proteins (OsZIP-2a and OsZIP-2b) do not bind to ABRE by themselves. However, they heterodimerize via the leucine zipper with EmBP-1 (Table 3) and prevent it from binding to the ACGT-box (Nantel and Quatrano, 1996). In contrast, ROM2 binds to the ACGT-box but functions as a repressor (Chern et al., 1996). The second class of repressors is protein phosphatases. In addition to ABI1 and ABI2 protein phosphatases 2C described above, the C-terminal domain phosphatase-like protein, AtCPL3, also functions as a repressor of ABA signaling. AtCPL3 specifically down-regulates ABA-responsive gene expression possibly by contacting and dephosphorylating the carboxyl terminal domain (CTD) of the RNA polymerase II, thereby blocking transcription initiation (Koiwa et al., 2002). The third class of repressors is homeodomain proteins that bind to the cis-acting element, CAATTATTA; ATHB6 physically interacts with ABI1 and acts downstream of ABI-1 in mediating ABA signaling (Himmelbach et al.,

TABLE 3. Genes Involved in ABA-Controlled Transcriptional Regulation

Gene	Accession	Gene Product	Reference
AtABI4	AAF18736	AP2 domain protein	(Finkelstein et al., 1998)
ZmABI4	AAM95247	AP2 domain protein	(Niu et al., 2002)
AtABI3	NP_189108	B3 domain protein	(Ezcurra et al., 2000)
AtFUS3	AAC35246	B3 domain protein	(Gazzarrini et al., 2004)
OsVP1	BAA04066	B3 domain protein	(Hattori et al., 1995)
HvVP1	AAO06117	B3 domain protein	(Casaretto and Ho, 2003)
PvALF	T10864	B3 domain protein	(Bobb et al., 1997)
ZmVP1	CAA04889	B3 domain protein	(McCarty et al., 1991; Schultz et al., 1998)
AtMYC2	Q39204	bHLH	(Abe et al., 2003)
AtIMB1	AAO22056	bromodomain proteins	(Duque and Chua, 2003)
AtABF3	BAD43614	bZIP protein	(Choi et al., 2000; Kang et al., 2002)
AtABF4	AAF27182	bZIP protein	(Choi et al., 2000; Kang et al., 2002)
AtABI5	AAD21438	bZip protein	(Finkelstein and Lynch, 2000; Carles et al., 2002)
HvABI5	AAO06115	bZip protein	(Casaretto and Ho, 2003)
OsTRAB1	XP_482899	bZip protein	(Hobo et al., 1999a)
OsZip-1a	AAC49556	bZip protein	(Nantel and Quatrano, 1996)
OsZip-2a	AAC49557	bZip protein	(Nantel and Quatrano, 1996)
PvROM2	T10985	bZip protein	(Chern et al., 1996)
TaEmBP-1	P25032	bZip protein	(Guiltinan et al., 1990; Hill et al., 1996)
AtCPL1	NP_193898	C-terminal domain phosphatase-like	(Koiwa et al., 2002)
AtCPL3	NP_180912	C-terminal domain phosphatase-like	(Koiwa et al., 2002)
AtLEC1	NP_173616	HAP3 subunit of CCAAT protein	(Brocard-Gifford et al., 2003)
AtHB5	P46667	Homeodomain protein	(Johannesson et al., 2001; Johannesson et al., 2003)
AtHB6	AAD41726	Homeodomain protein	(Himmelbach et al., 2002)
AtMYB2	BAA03534	MYB protein	(Abe et al., 2003)
AtRD26	NP_849457	NAC protein	(Fujita et al., 2004)
HvWRKY38	AAS48544	WRKY protein, homologue of OsWRKY71	(Mare et al., 2004)
LtWRKY21	AY792618	WRKY protein, activator	(Zou et al., 2004)
OsWRKY24	DAA05089	WRKY protein, repressor	(Xie et al., 2005)
OsWRKY45	DAA05110	WRKY protein, repressor	(Xie et al., 2005)
OsWRKY72	BK005075	WRKY protein, activator	(Xie et al., 2005)
OsWRKY77	BK005080	WRKY protein, activator	(Xie et al., 2005)
AtMARD1	AAK92226	zinc-finger protein	(He and Gan, 2004)

2002). The fourth class of repressors is WRKY proteins (Xie et al., 2005). The genes encoding these regulators are listed in Table 3.

Posttranscriptional Regulation

ABA regulation is also exerted at the posttranscriptional level (Table 4). ABA induces the expression of several RNA binding proteins, including:

1. the maize glycine-rich protein MA16 that preferentially interacts with uridine and guanosine-rich RNA fragments (Freire and Pages, 1995),
2. AtABH1 and AtCBP20 that form a dimeric *Arabidopsis* mRNA cap-binding complex (Hugouvieux et al., 2002),
3 AtSAD1 that is similar to multifunctional Sm-like small nuclear ribonucleoproteins, and
4 the dsRNA binding protein HYL1, mutations in which lead to enhanced levels of ABI5 and MAPK (Lu and Fedoroff, 2000).

Except for MA16, whose function remains unknown, all these RNA binding proteins function as negative regulators of ABA signaling. Another RNA binding protein, AKIP1, is a substrate of the protein kinase AAPK. Phosphorylated AKIP1 interacts with the mRNA that encodes a dehydrin, a protein implicated in cell protection under stress conditions (Li et al., 2002).

Protein degradation is also part of ABA signaling (Hare et al., 2003). A nuclear-localized ABA regulated protein AFP, which physically interacts with ABI5 as shown by a yeast two-hybrid assay and co-immunoprecipitation, functions as a negative regulator of ABA signaling (Lopez-Molina et al., 2003). Proteasome inhibitor studies show that ABI5 stability is regulated by ABA through ubiquitin-related events. Both AFP and ABI5 are colocalized in nuclear bodies that also contain COP1, a RING-finger- and WD40-repeat-containing protein that functions as a key repressor of seedling de-etiolation (Ang et al., 1998). COP1 possesses autoubiquitination activity (E3) *in vitro* and can ubiquitinate, hence likely promote the degradation of, MYB-type transcription factors (Seo et al., 2003). Although COP1 has not been shown

TABLE 4. Genes Involved in ABA-Controlled Posttranscriptional Regulation

Gene	Accession	Gene Product	Reference
AtAFP	AAF67775	ABI five binding protein	(Lopez-Molina et al., 2003)
AtHYL1	AAG49890	Double-stranded RNA binding protein	(Lu and Fedoroff, 2000)
AtABH1	NP_565356	mRNA CAP binding protein	(Hugouvieux et al., 2001)
AtSAD1	AAK61592	U6-related Sm-like small ribonucleoprotein	(Xiong et al., 2001b)
AtPRL1	NP_193325	WD40 domain protein	(Nemeth et al., 1998)
AtOST1	CAC87047	Ser/Thr protein kinase	(Mustilli et al., 2002; Yoshida et al., 2002)
VfAAPK	AAF27340	Ser/Thr protein kinase	(Li et al., 2002)
VfAKIP1	AAM73765	RNA-binding protein	(Li et al., 2002)
CpCDT-1		Regulatory RNA or small peptide	(Furini et al., 1997)

to mediate ABA signaling, the mutation of another WD-40 protein, PRL1 (Table 4), results in oversensitivity to ABA (Nemeth et al., 1998), suggesting that PRL1 is a repressor of ABA signaling. Phosphorylation of the ABI5 stabilizes the protein probably by blocking its AFP-promoted degradation by the 26S proteasome (Lopez-Molina et al., 2003). These data suggest that AFP and PRL1 modulate ABA signaling by promoting degradation of transcriptional activators.

THE ABA-INDEPENDENT DROUGHT RESPONSE PATHWAY

The ABA-independent drought response pathway was first demonstrated by analyzing the *Arabidopsis RESPONSE TO DEHYDRATION29A* (*RD29A*) gene (Yamaguchi-Shinozaki and Shinozaki, 1994). In addition to ABRE, the promoter of *RD29A* contains a dehydration-responsive element (DRE), CCGA(C/G), which is identical to the C-repeat (Yamaguchi-Shinozaki and Shinozaki, 1994; Stockinger et al., 1997). This element is bound by transcription factors belonging to the ETHYLENE-RESPONSIVE ELEMENT BINDING FACTOR (ERF)/APETALA2 (AP2) family, called C-REPEAT-BINDING FACTOR (CBF) or DRE-BINDING FACTOR (DREB) (Stockinger et al., 1997; Liu et al., 1998).

LESSONS LEARNED FROM STUDIES WITH MODEL PLANTS

Overexpressing Genes Encoding Enzymes for Functional and Structural Metabolites Helps Improve Drought Tolerance

Enzymes for Detoxification

Drought induces the expression of genes related to oxidative stress. Overexpression of two groups of genes, encoding aldehyde dehydrogenase and superoxidase respectively, enhances plant tolerance to drought (Table 5). Aldehyde dehydrogenase catalyzes the oxidation of toxic aldehydes, which accumulate as a result of side reactions of reactive oxygen species with lipids and proteins. Overexpression of the *Arabidopsis ALDH3* gene resulted in enhanced tolerance to drought (Sunkar et al., 2003). Similarly, reduction of the cytotoxic methyl-

TABLE 5. Genes Used in Engineering for Drought Tolerance

Gene[a]	Accession[b]	Gene Product	Reference	Transgenic Species	Resistance to	Reference
Genes encoding detoxification enymes						
AtALDH3	AJ306961	aldehyde dehydrogenase	(Kirch et al., 2001)	*Arabidopsis*	drought	(Sunkar et al., 2003)
MsALR	AA660319	aldehyde dehydrogenase	(Oberschall et al., 2000)	tobacco	drought	(Oberschall et al., 2000)
NpMnSOD	X14482	superoxide dismutase	(Bowler et al., 1989)	alfalfa	drought	(McKersie et al., 1996)
Genes encoding enzymes for osmoprotectant production						
VaP5CS	M92276	[delta]-pyrroline-5-carboxylate synthetase	(Hu et al., 1992)	tobacco	drought	(Kishor et al., 1995)
ScTPS1	X68214	trehalose-6-phosphate synthetase	(Bell et al., 1992)	tobacco	drought	(Romero et al., 1997)
EcOtsA and EcOtsB	X69160	trehalose-6-phosphate synthetase	(Kaasen et al., 1994)	rice	drought and salinity	(Garg et al., 2002; Jang et al., 2003)
EcMtlD	X06794	mannitol-1-phosphatase dehydrogenase gene	(Davis et al., 1988)	wheat	drought and salinity	(Abebe et al., 2003)
McIMT1	M87340	myo-inositol O-methyltransferase	(Vernon and Bohnert, 1992)	tobaco	salinity and drought	(Sheveleva et al., 1997)
AsAdc	X56802	arginine decarboxylase (putrescine synthesis)	(Bell and Malmberg, 1990)	rice	drought	(Capell et al., 1998)
DsAdc	AJ251898	arginine decarboxylase	(Capell et al., 2004)	rice	drought	(Capell et al., 2004)
CfSPDS	E63760	spermidine synthase	(Kasukabe et al., 2004)	*Arabidopsis*	drought, salinity and freezing	(Kasukabe et al., 2004)
Genes encoding chaperone and structural proteins						
HvA1	X78205	LEA protein (group 3)	(Hong et al., 1988)	rice	drought and salinity	(Xu et al., 1996)
AtCALTPI	AF208832	basic lipid transfer protein	(Jung et al., 2005)	*Arabidopsis*	drought and salinity	(Jung et al., 2005)
Genes encoding transcription factors						
AtABF3	AF093546	ABRE-binding protein	(Choi et al., 2000)	rice	drought and salinity	(Oh et al., 2005)
AtNAC019	AY117224	NAC; binding to CATGTG	(Tran et al., 2004)	*Arabidopsis*	drought	(Tran et al., 2004)
AtNAC055	AB049070	NAC; binding to CATGTG	(Tran et al., 2004)	*Arabidopsis*	drought	(Tran et al., 2004)

TABLE 5 (continued)

Gene[a]	Accession[b]	Gene Product	Reference	Transgenic Species	Resistance to	Reference
AtNAC072	AY091428	NAC; binding to CATGTG	(Tran et al., 2004)	*Arabidopsis*	drought	(Tran et al., 2004)
AtCBF1	U77378	APATALA2/ERF	(Stockinger et al., 1997)	*Arabidopsis*	drought, salinity and freezing	(Jaglo-Ottosen et al., 1998)
AtCBF3/ AtDREB1a	AF074602, AB007787	APATALA2/ERF	(Gilmour et al., 1998; Liu et al., 1998)	*Arabidopsis*	drought, salinity and freezing	(Liu et al., 1998; Dubouzet et al., 2003)
				canola	drought and freezing	(Jaglo et al., 2001)
				rice	drought	(Oh et al., 2005)
				tobacco	drought	(Kasuga et al., 2004)
AtCBF4	AB015478	APATALA2/ERF	(Haake et al., 2002)	*Arabidopsis*	drought and cold	(Haake et al., 2002)
AtWIN1/ AtSHN	AAR20494	APATALA2/ERF	(Broun et al., 2004)	*Arabidopsis*	drought	(Aharoni et al., 2004)
LeERF1	AY044236	APATALA2/ERF	(Zhang et al., 2004a)	tobacco	drought	(Zhang et al., 2005)
AtSTZ	X95573	Zinc finger protein	(Lippuner et al., 1996)	*Arabidopsis*	drought	(Sakamoto et al., 2004)
AtCBP20	AF140219	20kDa subunit of the nuclear mRNA cap binding complex (nCBC)	(Kmieciak et al., 2002)	*Arabidopsis*	drought	(Papp et al., 2004)

a: The first two letters of gene names refer to the organism from which the gene is derived. *At*, *Arabidopsis thaliana* (*Arabidopsis*); *Ca*, *Capsicum annuum* (cayenne pepper); *Cf*, *Cucurbita ficifolia* (figleaf gourd); *Ds*, *Datura stramonium* (jimsonweed), *Ec*, *Escherichia coli*; *Gm*, *Glycine max* (soybean); *Le*, *Lycopersicon esculentum* (tomato); *Mc*, *Mesembryanthemum crystallinum* (common iceplant); *Ms*, *Medicago sativa* (alfalfa), *Np*, *Nicotiana plumbaginifolia* (Tex-Mex tobacco); *Sc*, *Saccharomyces cerevisiae* (yeast); and *Va*, *Vigna aconitifolia* (moth bean).
b: Genbank® accession numbers for the genes.

glyoxal by overexpressing a drought-inducible alfalfa (*Medicago sativa*) gene that encodes a NADPH-dependent aldose/aldehyde reductase protected transgenic tobacco against drought (Oberschall et al., 2000). Superoxide dismutase catalyzes the reduction of superoxide anions to hydrogen peroxide. Alfalfa plants overexpressing a manganese superoxide dismutase gene were more resistant to drought, as determined by chlorophyll fluorescence, electrolyte leakage, and regrowth from crowns (McKersie et al., 1996).

Amino Acids

The enzyme Δ1-pyrroline-5-carboxylate synthetase (P5CS) catalyzes the conversion of glutamate to Δ1-pyrroline-5-carboxylate, which is then reduced to proline. Overexpression of this gene resulted in accumulation of proline and enhanced root biomass in transgenic tobacco under drought-stress conditions (Kishor et al., 1995). Controlling the amount of proline produced in transgenic plants is important because too much proline is toxic to plant growth (Nanjo et al., 2003).

Sugars and Sugar Alcohols

Trehalose is a non-reducing disaccharide that was thought to be present only in some bacteria, fungi, insect and desiccation-tolerant plants (Eastmond et al., 2002). However, recent data indicate that trehalose-6-phosphate synthase 1 (TPS1), which catalyses the first step in trehalose synthesis, is present in *Arabidopsis* (Gomez et al., 2005) and essential for its embryo maturation (Avonce et al., 2005). Nevertheless, overexpression of the yeast TPS1 gene led to improved drought tolerance in tobacco (Romero et al., 1997). Overexpressing trehalose biosynthetic genes (*otsA* and *otsB*, as a polycistron) in rice resulted in accumulation of trehalose at levels 3-10 times that of the controls, higher soluble carbohydrate levels and an elevated capacity for photosynthesis, and enhanced tolerance to drought (Garg et al., 2002; Jang et al., 2003).

Mannitol also accumulates upon drought to alleviate the stress. The *MtlD* gene from *Escherichia coli* encodes a mannitol-1-phosphate dehydrogenase that catalyzes the reversible conversion of fructose-6-phosphate to mannitol-1-phosphate, which is converted to mannitol via nonspecific phosphatases in transgenic plants. Transgenic wheat plants expressing *MtlD* were more tolerant to drought and salt stress (Abebe et al., 2003). However, the amount of mannitol accumulated was too small to protect against stress through osmotic adjustment. Therefore, improved growth performance of mannitol-accumulating calli and mature leaves is more likely due to other stress-protective functions of mannitol, such as scavenger of reactive oxygen species (Shen et al., 1997a). Transgenic tobacco plants overexpressing a myo-inositol O-methyltransferase gene from the common iceplant (*Mesembryanthemum crystallinum*) accumulated (up to 600 mM) of methylated inositol (D-ononitol). These transgenic plants were hence resistant to drought and salt stress (Sheveleva et al., 1997).

Polyamines

Polyamines, such as putrescine (diamine), spermidine (triamine) and spermine (tetraamine), are a group of nitrogenous compounds, which accumulate in plants under abiotic stresses, including drought and salt. Overexpression of an oat or jimsonweed (*Datura stramonium*) arginine decarboxylase gene in rice under the control of the maize ubiquitin promoter (*Ubi-1*) produced much higher levels of putrescine, spermidine and spermine that protected the plants from drought (Capell et al., 1998; Capell et al., 2004). However, constitutive overexpression of this gene severely affected developmental patterns (Capell et al., 1998). *Arabidopsis* plants overexpressing *CfSPDS*, a spermidine synthase gene from figleaf gourd (*Cucurbita ficifolia*), exhibited a significant increase in spermidine synthase activity and spermidine content in leaves together with enhanced tolerance to various stresses including drought, chilling, freezing, salinity, hyperosmosis, and paraquat toxicity. Under chilling stress, the transgenic plants also displayed a remarkable increase in arginine decarboxylase activity and conjugated spermidine contents in leaves compared to the wild type (Kasukabe et al., 2004).

Overexpressing Genes Encoding Chaperones and Structural Proteins Can Lead to Enhanced Drought Tolerance

Drought can cause denaturation of many proteins, rendering them nonfunctional. Late embryogenesis proteins (LEA) could protect cells against stress by controlling the proper folding and conformation of structural proteins and enzymes (Dure III et al., 1989; Soulages et al., 2003). Overexpression of *HVA1*, which encodes a group 3 LEA protein in barley, conferred dehydration tolerance to transgenic rice plants (Xu et al., 1996). Overexpression of the cayenne pepper (*Capsicum annuum*) *CALTPI* gene (encoding a basic lipid transfer protein) also helped improve stress tolerance. Under the control of the cauliflower mosaic virus (CaMV) 35S promoter, the *CALTPI* transgene conferred salt and drought tolerance to transgenic *Arabidopsis* plants (Jung et al., 2005).

Regulating the Expression of Genes Encoding Transcription Factors and RNA-Binding Proteins Is a Favored Strategy for Drought Tolerance Engineering

Mounting lines of evidence suggest that overexpression of some genes encoding transcription factors for the ABA-dependent and ABA-

independent signaling pathways is an effective approach of engineering drought tolerance (Table 5). Overexpression of the *Arabidopsis ABF3* gene, a component of the ABA-dependent pathway, increased tolerance of the transgenic rice plants to drought (Oh et al., 2005). *Arabidopsis CBF4* gene was up-regulated by drought, but not by low temperature. Overexpression of *CBF4* in *Arabidopsis* plants resulted in the activation of downstream genes whose promoters contain DRE/C-repeat elements, and enhanced tolerance to drought (Haake et al., 2002). Similarly, transgenic plants overexpressing any of the three NAC genes, which encode proteins binding to the MYC site, showed enhanced expression of several stress-inducible genes and significantly increased drought tolerance (Tran et al., 2004).

For the ABA-independent pathway, overexpression of the *Arabidopsis CBF1*, *DREB1A/CBF3* and *SHINE* (*SHN*) genes, which encode AP2/ERF transcription factors binding to the DRE/C-repeat, improved drought tolerance in *Arabidopsis* (Jaglo-Ottosen et al., 1998; Liu et al., 1998; Dubouzet et al., 2003; Aharoni et al., 2004), canola (Jaglo et al., 2001), rice (Oh et al., 2005), and tobacco (Kasuga et al., 2004). *SHN* overexpressors displayed significant drought tolerance and recovery (Aharoni et al., 2004). Similarly, TERF1, a tomato ERF/AP2 protein, activated GCC box- or DRE-driven reporter gene expression in transient expression assays, subsequently increasing the tolerance to drought and the osmoticum, PEG6000, in tobacco (Zhang et al., 2005).

Drought tolerance also could be achieved by overexpressing other genes. The *Arabidopsis* SALT TOLERANCE ZINC FINGER (STZ) and *ARABIDOPSIS* ZINC FINGER2 (AZF2) proteins contain two canonical Cys-2/His-2-type zinc-finger motifs (Lippuner et al., 1996). Transgenic *Arabidopsis* overexpressing these genes showed growth retardation and tolerance to drought. It was suggested that AZF2 and STZ function as transcriptional repressors to increase stress tolerance following growth retardation (Sakamoto et al., 2004). The *Cap Binding Protein20* (*CBP20*) gene encodes the 20kDa subunit of the nuclear mRNA cap binding complex. Plants overexpressing this gene showed enhanced drought tolerance (Papp et al., 2004).

Obviously, enhanced tolerance to drought could be achieved with overexpressing appropriate genes encoding transcription factors. But what is the mode of action? A transcription factor controls the expression of a battery of genes in one or more branches of a signaling network. Overexpression of this key regulator could mobilize genes, which encode chaperons, structural proteins, and enzymes for osmoprotectant synthesis, to cope with a stress. Indeed, the drought tolerant plants

overexpressing *DREB1A/CBF1* under the control of the constitutive *CaMV 35S* promoter or the stress-inducible *RD29A* promoter, activated the expression of many stress-tolerance genes such as *RD29A*, *Kin 1*, *Cor6.6*, *Cor15a*, *RD17* and *P5CS* (Kasuga et al., 1999). CBF3-expressing plants had elevated levels of proline and total soluble sugars, including sucrose, raffinose, glucose, and fructose. Plants overexpressing *CBF3* also had elevated *P5CS* transcript (Gilmour et al., 2000). Of course, overexpression of transcription factor genes could lead to structural changes that help plants tolerate drought. For example, plants overexpressing SHN, which is the same as *WIN1* (Broun et al., 2004), have increased cuticle permeability, altered leaf and petal epidermal cell structure, trichome number, and branching as well as reduced stomatal density (Aharoni et al., 2004).

Promoters Are Crucial for Drought Tolerance Engineering

With a few exceptions (Jang et al., 2003; Oh et al., 2005), overexpression of genes described above under the control of a constitutive promoter (*CaMV 35S* or *Ubi-1*) induced strong expression of the target genes in transgenic plants, even under unstressed condition, and caused dwarfed phenotypes in the transgenic plants. Constitutive overproduction of molecules, such as trehalose (Romero et al., 1997) or polyamines (Capell et al., 1998), causes abnormalities in plants grown under normal conditions. The expression of *DREB1A* driven by the *CaMV 35S* promoter resulted in severe growth retardation under normal growing conditions (Kasuga et al., 1999).

Using ABA or stress-inducible promoters can minimize the negative effects on the growth of transgenic plants. For instance, expression of *DREB1A* under the control of the stress inducible *RD29A* promoter had minimal effects on plant growth while providing a greater tolerance to stress conditions (Kasuga et al., 1999; Kasuga et al., 2004). Also, expression of a gene is not always the higher the better. Some gene products, such as chaperons, are needed in a large quantity, hence a very strong promoter is needed. In contrast, genes for polyamine biosynthesis might better to be driven by an ABA/drought-inducible promoter of moderate strength. Therefore, a collection of ABA/stress inducible promoters that are able to confer different levels of expression is as crucial as the protein-coding cDNA clones in engineering plants for drought tolerance.

Molecular switches with different levels of ABA induction and transcription strength have been constructed based on the analysis of two

ABA responsive promoters in barley (Table 6). One copy of the 49-bp *HVA22* ABRC1 is able to confer more than 30-fold ABA induction and additional copies of ABRC1 added to the reporter construct led to even higher level of ABA induction. The 68-bp ABRC3 of *HVA1* gene was even stronger than the *HVA22* ABRC1; one copy of this fragment led to 20-fold induction with the absolute level of GUS activity twice as high as that obtained with the *HVA22* ABRC1. Moreover, the presence of two coupling elements further enhanced the expression of the construct when they interacted with the ACGT-box either from *HVA22* or *HVA1*. When tested in transgeneic rice, the induction of GUS activity conferred by one copy of ABRC1 varied from 3- to 8-fold with different treatments or in different rice tissues. Transgenic rice plants harboring four copies of ABRC1 showed 50% to 200% higher absolute GUS activity than those with one copy of ABRC1 under the control or ABA treatment conditions (Su et al., 1998).

Tissue specific expression of a transgene is also an important consideration. For example, osmolytes accumulated in leaf tissue would not be beneficial to root tissue under osmotic stress. Some transcription factor genes must be expressed in certain tissues/organs to correctly exert their regulatory roles. Thus, there is a need to identify promoters that are not

TABLE 6. ABA Responsive Molecular Switches

Switch Type	Copy No.	Activity	Fold
A3-CE1	1	100%	38X
(ABRC1)	2	162%	55X
	3	272%	87X
	4	517%	130X
CE3-A2 (66-bp)	1	220%	20X
CE3-A2 (22-bp)	1	30%	9X
(ABRC3)	2	75%	20X
	3	150%	25X
CE3-A3-CE1	1	423%	40X
CE3-A2-CE1	1	256%	53X

HVA22 complex consists of an ACGT-box (A3) and a distal CE1. The normalized GUS activity from the ABA-treated sample of the single copy ABRC1 construct is taken as 100% throughout this table. "Fold" stands for fold induction calculated as described (Shen et al., 1996). The ABA response complex in *HVA1* promoter consists of an ACGT-box (A2) and the proximal CE3. The ternary ABA response complexes consist of two coupling elements and an ACGT-box. Adapted from Shen et al., 1996.

only ABA/stress-inducible but also tissue- and/or developmental-stage-specific.

CONCLUSIONS

Tremendous progress has been made in engineering drought tolerance in the past decades, as demonstrated with genes encoding various types of proteins described above. The following issues need to be considered in engineering drought tolerant plants.

(1) Choice of genes: in addition to the genes listed in Table 5, those listed in Tables 1 through 4 are also good candidates. However, response to drought is multigenic, thus difficult to control and engineer. In addition, field-grown plants are subjected to multiple stresses. The response of *Arabidopsis* plants to a combination of drought and heat stress was found to be distinct from that of plants subjected to drought or heat stress alone. As many as 454 transcripts were specifically expressed in plants under a combination of drought and heat stress. Also, proline that accumulated in plants subjected to drought did not accumulate in plants during a combination of drought and heat stress. Instead, the major osmoprotectants under this combined stress condition are sucrose and other sugars such as maltose and glucose (Rizhsky et al., 2004). Therefore, one approach is to identify genes that are induced by a combination of stresses. As an example, a *WRKY* gene was induced by a combination of drought and heat (Rizhsky et al., 2002). Of course, overexpression of stress-induced genes doesn't always lead to enhanced stress tolerance. For instance, *DREB2* is induced by dehydration, but overexpression of this gene in transgenic *Arabidopsis* plants failed to improve their stress tolerance (Liu et al., 1998). Therefore, it might be necessary to test genes in model plants, combine multiple genes that have been demonstrated to be effective in protecting model plants against stresses, and apply them to plants with longer generation time periods.

(2) Choice of promoters and other genetic elements: to minimize the probability of gene silencing in transgenic plants, each gene needs to be flanked by a different ABA/stress-inducible promoter and terminator. Terminators contain *cis*-acting elements that are crucial for tissue specificity (Dietrich et al., 1989; Rastinejad and Blau, 1993; Rastinejad et al., 1993) and expression levels (Ingelbrecht et al., 1989). Matrix attachment regions (MARs)/scaffold attachment regions (SARs) help reduce homology-dependent gene silencing in certain cases (Allen et al., 2005;

Halweg et al., 2005; Xue et al., 2005). Therefore, inclusion of these types of elements could help improve gene expression level, especially in later generations of transgenic plants.

(3) Target of genes for organelles: it is ideal to target transgenes from prokaryotes to chloroplasts because codon usage and translation apparatus are similar between prokaryotes and chloroplasts. This approach has been demonstrated to be successful in alleviating the toxic effect of glycine betaine (Sakamoto and Murata, 1998) or mannitol (Shen et al., 1997b). In addition, this approach can reduce the risk of spreading transgenes via plant pollens. An alternative approach is to modify prokaryotic genes based on the codon usage of a target plant.

(4) Sizes of gene constructs: transformation efficiency decreases as the size of transgene(s) gets larger. Bidirectional promoters, which can direct the expression of two genes, can be used to minimize the size of constructs (Xie et al., 2001). In addition, several genes can be linked together as a polycistronic unit, to be driven by a single promoter (Kaasen et al., 1994). Inclusion of a linker between two coding sequences may help enhance the stability of fusion proteins (Robinson and Sauer, 1998).

(5) Selection and test of transgenic plants: selection of plants that harbor only one copy of a transgene can help reduce the possibility of gene silencing. In addition, the expression level of a transgene may vary in different transgenic lines due to the position effect. Therefore, several lines, preferably those with high levels of expression, need to be evaluated. Also, because expression levels of transgenes may drop in later generations, it is necessary to check the expression levels of transgenes in those lines of plants before an experiment is carried out in a large scale. It is hoped that the invaluable information derived from the model plants described in this review will increase the probability of success for applications of drought-related genes and promoters in horticultural and ornamental plants.

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Transgenic Approaches to Disease Resistance in Ornamental Crops

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SUMMARY. Viral, bacterial, and fungal diseases of ornamental plants cause major losses in productivity and quality. Chemical methods are available for control of fungal diseases, and to a lesser extent for bacterial diseases, but there are no economically effective chemical controls for viral diseases except to control vector species. Host plant resistance is an effective means of controlling plant diseases, and minimizing the necessity for the application of pesticides; however, there are many ornamentals in which no natural disease resistance is available. It is pos-

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sible to introduce resistance derived from other species, or even from the pathogen itself, by genetic engineering. This allows the introduction of specific, or in some instances broad spectrum, disease resistance into plant genotypes that have been selected for desirable horticultural characters; in contrast, introduction of natural resistance by traditional breeding may take many cycles of breeding to combine disease resistance with desirable ornamental quality. This article briefly reviews existing work on transformation systems for ornamentals, and discusses the various approaches to introducing resistance to viral, bacterial, and fungal diseases, and to nematode infestations. These include pathogen-related proteins, R genes, and general pathogen resistance; anti-microbial peptides; expression of anti-pathogen antibodies; viral sequences; ribozymes; antiviral peptides; ribonucleases; and ribosome-inactivating proteins. Examples are given of application of these approaches to disease resistance in other types of crop and model plant systems, and actual or potential application to disease resistance in ornamentals. Future prospects for obtaining plants with multiple pest and disease resistances are discussed. doi:10.1300/J411v17n01_06 *[Article copies available for a fee from The Haworth Document Delivery Service: 1-800-HAWORTH. E-mail address: <docdelivery@haworthpress.com> Website: <http://www.HaworthPress.com>.]*

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INTRODUCTION

Grower cash receipts for U.S. floriculture and environmental horticulture crops, as estimated by USDA's Economic Research Service (USDA-ERS, 2004), reached $14.3 billion in 2003, of which $5.6 billion represented floral crops. Grower cash receipts for all floriculture crops (cut flowers, cut greens, flowering and foliage potted plants, and bedding and garden plants) have increased 4-6 percent per year since 1991. The nursery and greenhouse industry comprise one of the fastest growing segments of US agriculture. Two-thirds of the value of U.S. floriculture production in 2003 consisted of bedding and garden plants and potted flowering plants, while woody landscape plant producers accounted for over 50% of the greenhouse and nursery crop value.

The Importance of Diseases and Disease Resistance in Ornamental Crops

More than perhaps any other sector of agriculture and horticulture, the visual quality of the ornamental product at the retail level is critical. This is especially true for cut flowers and potted plants. Visible symptoms of disease therefore have a major impact on quality, in addition to any effect on productivity.

It is generally accepted that viral crop diseases rank second only to those caused by fungi in terms of economic importance (Hadidi et al., 1998; Matthews, 1998). However, unlike fungal diseases where chemical methods aimed at prevention of infection have been quite successful, control of viral and bacterial diseases has been much more problematic. Direct and indirect effects of viral and bacterial infections include: reduction in growth, reduction in vigor, costs of attempting to maintain crop health, and, of significance to the ornamental industry, reduction in quality and/or market value (Hadidi et al., 1998).

While other chemical control methods are also available, the industry standby for control of many pests and diseases has been pre-plant fumigation with methyl bromide. This soil sterilant has been especially important in control of soil-borne diseases and nematodes, but will be withdrawn from use in most countries effective in 2005. Although other chemicals can replace some of the uses of methyl bromide, alternative methods for control of nematodes and soil-borne diseases are needed for sustainable production of all types of crops, including ornamentals. Species of *Fusarium*, *Phytophthora*, *Pythium*, *Rhizoctonia*, and *Sclerotinia* are the most common soil-borne fungal pathogens in ornamental production.

Plant diseases cause significant losses in the production and quality of ornamental crops, and are very difficult to control; moreover, new diseases occur as different crops are introduced or grown in new areas (Chase et al., 1995; Larson, 1992; Powell and Lindquist, 1992). At the final "production" stage of growing and distributing ornamental plants, losses due to viral and bacterial infections can range from 10 to 100%, depending upon the virus- or bacteria-host combination. Many crops are susceptible to multiple viruses, each of which may cause serious economic losses, and infected plant material may not be acceptable for export (Loebenstein et al., 1995). Typically several (or many) different crops are grown in the same facility. At least 125 different viruses have been identified that infect and cause disease in ornamental plants (Cohen, 1995).

Control of viral and bacterial diseases of floral crops usually focuses on use of "clean stock," i.e., propagation materials that have been indexed and shown to be free of known pathogens. However, the use of virus-free propagation material is not in itself adequate, as many viruses can also be transmitted by an insect vector, such as aphids, whiteflies or thrips. In addition, several important viruses of ornamentals affect multiple crop genera, making it important that all crops grown in the facility are virus-free in order to prevent transmission between different crops. Current control measures for viruses and bacteria in floral crops rely on early detection and removal of infected planting material from production areas as well as preventative measures to control the insect vectors (Matthews, 1998; Powell and Lindquist, 1992). Screening of greenhouses, isolation of virus- and bacterial-tested "clean" propagation stock from production areas, eliminating weeds and non-production reservoir plants from greenhouses, monitoring of insect populations and judicious use of pesticides are all needed for control.

The use of resistant varieties is the most commonly-used strategy for control of disease in many crop species. Conventional breeding strategies require the identification of sources of disease resistance genes, a difficult task in ornamentals given the diversity of floral and nursery crop species that are susceptible to such a large and diverse group of pathogens. Neither wild nor improved germplasm with desirable disease resistance is available for many genera of ornamental plants. Also, the overriding importance of appearance and general horticultural traits, the large number of cultivars which are produced per crop, and the rapid turnover in cultivars, have made breeding for disease resistance extremely difficult in floral crops. Even where effective disease resistance can be identified in related germplasm, introgression of a single gene into horticulturally desirable plant lines may require multiple back-cross generations, and may result in reduced expression of the resistance compared to the source material. New tools and genes have been developed for use in the genetic engineering of plants to introduce effective resistance to plant diseases and to understand the mechanisms of resistance. This approach should allow increases in both productivity and quality of ornamental plants in an environmentally friendly manner, thereby reducing the use of and reliance on chemical control of pests and diseases.

Progress and Unique Issues in the Transformation of Ornamental Plants

Recent developments in biotechnology have provided new opportunities to solve practical horticultural problems. The development of

technologies for gene identification and gene transfer into plants has provided the opportunity for genetically engineering disease resistance into horticulturally desirable cultivars without altering critical quality traits (Daub et al., 1996; Hadidi et al., 1998; Hull, 2002). There are essentially three sources of transgenes for protecting plants against viruses (or fungal and bacterial pathogens): natural resistance genes; genes derived from viral sequences (pathogen-derived resistance); and genes from various other sources (Hull, 2002).

Plants have their own networks of defense against plant pathogens that include a vast array of proteins and other organic molecules produced prior to infection or during pathogen attack. Not all pathogens can attack all plants and a single plant is not susceptible to all plant pathogenic fungi, viruses, bacteria or nematodes. Recombinant DNA technology allows the enhancement of inherent plant responses against a pathogen by either using single dominant resistance genes not normally present in the susceptible plant (Keen, 1999) or by choosing plant genes that intensify or trigger the expressions of existing defense mechanisms (Campbell et al., 2002; Rommens and Kishore, 2000). What is useful in one plant/pathogen system may be transferred to another, increasing the recipient plant's ability to defend itself from a previously uncontrollable pathogen. Many new strategies show promise, some limitations, and exciting opportunities to develop new tools for combating plant pathogens.

Transformation of ornamental plants has lagged somewhat behind efforts in the major field crops largely because of the sheer variety of genera that are utilized as ornamentals, and because fewer groups have worked on the regeneration systems that are necessary for an efficient transformation system to be developed for a particular crop. Nonetheless, we are now at a point where transformation systems have been demonstrated for a significant number of ornamentals (Davies et al., 2003; Deroles et al., 1997, 2002; Griesbach, 1994; Hutchinson et al., 1992; Robinson and Firoozabady, 1993; Schuerman and Dandekar, 1993, Zuker, 1998), and constructs other than reporter genes are being introduced into important ornamentals (e.g., Deroles et al., 2002). In many crops, transformation or regeneration systems are problematic in that they are cultivar-dependent; these problems are beginning to be overcome as different methods are employed, allowing much less cultivar restriction in regeneration and transformation of, for example, roses (Castillon and Kamo, 2002), gladiolus (Kamo et al., 1995a,b, 1997), carnation and chrysanthemum (reviewed in Deroles et al., 2002).

A small number of transgenic ornamentals are available in the marketplace, with the most notable being the series of seven carnation lines offered by Florigene, which are currently offered for sale in Australia, Canada, and the USA (http://www.florigene.com.au). The cultivars 'Moondust' and 'Moonshadow' were the first to be offered for sale, with novel mauve and purple flower color derived by insertion of the 3′,5′-hydroxylase gene using an *Agrobacterium*-mediated transformation method based on stem explants (C. Lu, cited in Deroles et al., 2002). Additional lines vary in the intensity of flower color from pastel lavender to a dark blue-purple (http://www.florigene.com.au). White-flowered transgenic chrysanthemum were produced from the pink-flowered cultivar 'Moneymaker' following introduction of an additional copy of the chalcone synthase gene (Robinson and Firoozabady, 1993). The loss of pigmentation was interpreted to be a result of co-suppression (Napoli et al., 1990; Courtney-Gutterson, 1994) which is now thought to be a manifestation of RNA silencing. However, as there are many non-transgenic white-flowered varieties of chrysanthemum available, it is doubtful whether the white form of 'Moneymaker' would have any commercial value.

Most of the transgenic ornamental plants have been developed with quality-enhancing genes such as flower color rather than disease resistance genes because of the perception that it will be more profitable to introduce consumer-valued traits. Rose, the number one cutflower on the Dutch market and popular world-wide, has recently been a target of transformation studies for ornamental plants since a transformation system is now available for this previously difficult-to-transform plant. The development of a somatic embryogenesis system from embryogenic callus of rose has resulted in transgenic rose plants.

Level of transgene expression is often critical as most studies have shown a correlation between level of transgene expression and resistance. Most studies have relied on using the CaMV 35S promoter for dicots and the maize ubiquitin 1 promoter for monocots to obtain a high level of transgene expression. Two studies demonstrated that a pathogen-inducible promoter also works for resistance (Logemann et al., 1992; Coutos-Thevenot et al., 2001). Part of the delay in testing ornamental and woody plants for disease resistance is that an efficient transformation system must first be developed along with the isolation and characterization of effective promoters that can be applied to these systems.

Many studies have been done using antifungal genes in transgenic plants, but much work remains if high levels of resistance are to be

achieved. The work on floral and nursery plants has only just begun. Perennial ornamentals pose a challenge that most field crops do not, in that transgene expression must be stable over multiple seasons and repeated cycles of dormancy in the same plant. Kamo et al. (2000 a,b) have demonstrated that GUS expression from several promoters was not adversely affected following multiple cycles of dormancy in the floral monocot gladiolus, but these results may not hold for all transformed plants. Perennial and vegetatively propagated plants also may suffer disease problems that are propagated along with the crop. This is especially true for virus diseases, but there are also fungal and bacterial diseases that primarily enter the crop as infected propagation materials. Results using model transformation systems such as tobacco, *Arabidopsis*, and crops of agronomic importance have shown that activity of the antifungal genes differs for each plant species, making it important to test these same antifungal genes in floral and nursery plants for evaluation of their activity. Woody nursery plants are infected with fungal pathogens that cause cankers (*Nectria*, *Cytospora*, *Botryosphaeria*), anthracnose (*Discula*), wilt (*Verticillium*, *Fusarium*), powdery mildew (*Sphaerotheca*) and leaf spot (*Diplocarpon*, *Entomosporium*, *Marssonina*, *Alternaria*, and *Septoria*). The antifungal genes available have not yet been tested against many of the fungi of importance in ornamentals.

As technologies for regeneration and gene transfer in plants improve and broaden across diverse genera, the prospect of using transgenic approaches to develop disease tolerant ornamental crops becomes more promising. Transgenic approaches to breeding woody ornamentals are especially attractive considering the space required to grow out and evaluate germplasm to identify disease resistance traits, and the long generation time of some woody plants, which makes introgression of traits via backcrossing difficult. As with any transgenic approach to plant improvement, the costs, potential benefits, and risks of using genetic engineering to create disease resistant ornamental plants must be carefully considered. Several features unique to either woody plants or ornamental plants are relevant in assessing these factors. Unlike many agronomic crops, woody and perennial ornamental crops remain in the field for years or decades. Thus, the expression of the transgene must be durable in all seasons that the plant could come under attack from the pathogen, and must also be stable enough to withstand multiple cycles of plant dormancy. Scorza et al. (2001) have shown that resistance to plum pox potyvirus conferred by post-translational gene silencing in transgenic plum was stable over multiple seasons. The

long-lived nature of these perennial ornamentals also poses unique problems for environmental risk assessment, since the plant can serve as a reservoir, either for an escaping transgene or for a plant pest that has overcome the engineered resistance. On the other hand, few ornamental plants are cultivated in a typical monoculture, so the selective pressure on pathogens to overcome the transgenic resistance is not as strong as it would be in monocultured agronomic crops. Because the fruit and seed of many ornamental plants serve as food for wildlife, the aspects of ingestion and seed dispersal by wildlife must also be considered. However, unlike most agronomic and other horticultural crops, FDA issues regarding human ingestion should not be an issue with ornamental crops. Issues regarding gene recombination in seed production are minimized in the many ornamental crops that are vegetatively propagated.

In our group we are interested in introducing disease resistance to a variety of ornamentals. We have demonstrated effective resistance against *Bean yellow mosaic virus* (BYMV) in the model host *Nicotiana benthamiana* (Kamo and Hammond, 1995a,b), and have transformed Gladiolus with similar constructs (Kamo et al., 1997). We have also demonstrated resistance to *Cucumber mosaic virus* (CMV) in *N. benthamiana* by expression of anti-CMV single-chain ScFv antibodies (Hsu et al., 2004), and expressed antibodies against BYMV in *N. benthamiana* (Maroon and Jordan, 1998, 1999). We have transformed Gladiolus with anti-CMV antibodies, CMV coat protein (CP), and a defective CMV replicase gene (Kamo, Jordan, and Hsu, unpublished), and are working towards transformation of Gladiolus for fungal resistance (Kamo et al., 1997; and Kamo, unpublished) and *Ornithogalum* for resistance to *Ornithogalum mosaic virus* (OrMV) and related potyviruses (De Villiers et al., 2000; Hammond, unpublished). We currently have regeneration protocols for *Prunus* (flowering cherry, Cheong and Pooler, 2003) and *Cercis* (redbud, Cheong and Pooler, 2004), and are developing transformation methods for these genera. We hope to introduce genes for disease resistance, specifically fungal resistance in *Cercis* and viral and/or bacterial resistance in *Prunus*. Transgenic approaches to disease resistance in these crops is especially appealing in light of the fact that natural sources of resistance are difficult to identify (*Cercis*) or quarantine issues limit the amount of germplasm available for importation for breeding (*Prunus*).

REVIEW OF MECHANISMS OF PATHOGEN RESISTANCE

Pathogen-Related (PR) Proteins, R Genes, and General Pathogen Resistance

Plants are armed with many different defense mechanisms against microbial invasion. Among these is the induced resistance which depends on the activation of defense mechanisms by invading microbes triggering the synthesis of pathogen-related (PR) proteins.

One major form of induced resistance is the elicitation of a hypersensitive reaction, the most effective response through which plants resist fungal, bacterial, viral and nematode infection. The reaction is typically induced in plants by incompatible or necrogenic pathogens and is characterized by the rapid death of plant cells at the site of penetration or infection, leading to restriction of movement of the pathogen, thus preventing the pathogen from spreading to other parts of the plant. In other cases, the response to the pathogen may also render tissue distal to infection sites or necrotic areas able to defend against subsequent challenge infection by the same or different pathogens. This phenomenon is known as systemic acquired resistance. A third form of induced resistance is caused by the colonization of roots by certain non-pathogenic microbes, mainly plant-growth promoting rhizobacteria. Once the resistance is induced, the plants exhibit resistance to challenge inoculation of foliar pathogens. This type of resistance is referred to as induced systemic resistance.

The response of a plant to invasion by a particular pathogen is highly specific (Flor, 1947) and is governed by the gene-for-gene hypothesis (Flor, 1971). For a plant to mount a resistance response, it must carry a resistance "R" gene and there must be a corresponding avirulence "avr" gene in the pathogen. The result of this gene-for-gene interaction triggers a cascade of signal transaction pathways.

Pathogen-related (PR) proteins are plant proteins whose synthesis is induced in pathological or related situations by pathogens. Early studies on *Tobacco mosaic virus* (TMV-) infected Samsun NN and Xanthi-nc tobacco revealed that PR proteins had relative mobility (Mr) in the range of 15kDa to 30 kDa, isoelectric points (pI) between 4.0 and 6.0, and were protease resistant and localized predominately in the intercellular spaces of hypersensitive tissues (Van Loon et al., 1987). PR proteins with high pI values, medium molecular weight, and intracellular localization also have been reported (Kauffmann et al., 1987; Legrand et al., 1987; Melchers et al., 1994). Protoplasts of Samsun NN

tobacco, when inoculated with TMV, release into the medium an inhibitor of virus replication that is sensitive to proteolytic enzymes (Gera et al., 1990). The inhibitor protein has been isolated from the intercellular fluids of induced tissues or cells as well as from extracts of inoculated protoplasts. It has an estimated Mr of about 23 kDa (Gera et al., 1990).

Fourteen families of PR proteins have been classified (Huang, 2001). The biological function of PR proteins has yet to be fully elucidated. Some PR proteins have been identified as chitinases, β-1,3-glucanases, chitosanases, thaumatin-like proteins, proteinases and proteinase inhibitors. PR proteins are important in disease resistance in plants. They may lyse cell walls of invading pathogens (with chitinase and/or β-1,3-glucanase), liberate elicitors of defense reactions (with β-1,3-glucanase), hydrolyze peptide phytotoxins produced by pathogen (with proteinase) and inactivate the proteases secreted by the pathogens during the infection process (with proteinase inhibitor).

PR proteins play a direct role in inhibiting plant pathogens, but only a handful of them have been characterized by their biological function and cloned. Significant information has been accumulated in support of the gene-for-gene interaction in recent years. This knowledge has come from the application of molecular techniques to the study of plant-pathogen interactions. To date, the majority of R genes and their corresponding avr genes that have been isolated and characterized involve plant-bacterium and plant-fungus interactions (Mindrinos et al., 1994; Grant et al., 1995; Song et al., 1995; Salmeron et al., 1996; Martin et al., 1993; Parker et al., 1994; Lawrence et al., 1995; Jones et al., 1994). Identification of genes involved in plant-virus interactions has been limited, however (e.g., Whitham et al., 1994).

Antifungal and Antibacterial Peptides

Many organisms express peptides that have activity against various bacterial and/or fungal pathogens. These antimicrobial peptides form part of the defense arsenal of many plants and insects, as well as higher animals. Several different families of these peptides have been identified, many of which are broadly categorized as cationic antimicrobial peptides (e.g., Hancock et al., 1995; Osusky et al., 2000, and references therein). This group includes the cecropins, which are found in the hemolymph of many invertebrates, and accumulate in response to injury or infection (Boman and Hultmark, 1987). Another group of peptides, the magainins, are excreted from specialized glands in the skin of

amphibians (Bevins and Zasloff, 1990). A third group, the defensins, have members that are found in insects (Hoffmann and Hétru, 1992), mammals (Lehrer et al., 1993; Ganz and Lehrer, 1994), and plants (Broekhart et al., 1995). A fourth type of antimicrobial peptide is represented by the potato peptides snakin-1 (Segura et al., 1999) and snakin-2 (Berrocal-Lobo et al., 2002).

Both cecropins and magainins are short, highly basic, and essentially linear peptides (typically 20-40 residues) that form amphipathic helices that can form ion channels by integration into microbial membranes (Duclohier, 1994). The affected cells leak electrolytes and die if the loss is not reversed. The structure of defensins is more complex; defensins are Cys-rich peptides that have cystine-stabilized three-dimensional structure, with many peptides having anti-parallel β-sheets. There are differences in the length, number of cystine bonds, and folding patterns (Boman, 1995). Many of the plant defensins are found in seeds, and show some conservation; 14 representative members from seven different plant families are 45-54 residues long, are stabilized by at least two disulfide bridges, and have eight conserved Cys, two Gly, one Glu, and one aromatic (Trp, Tyr, or Phe) residues (reviewed in Broekhart et al., 1995). The insect defensins have 34-43 residues connected by three disulfide bridges, and are secreted into the hemolymph from the insect fat body following pathogen induction, as are the cecropins (Hoffmann and Hétru, 1992). The mammalian defensins have 29-34 residues, are stabilized by three disulfide bridges, and are produced in various specialized cells of the gut, airways, and circulatory system (Lehrer et al., 1993; Ganz and Lehrer, 1994). Snakin-1, a representative of another class of antimicrobial peptides, is highly basic, has 63 residues (of which 12 are Cys) with a short central hydrophobic domain flanked by highly polar termini, and has some motifs in common with hemotoxic, disintegrin-like snake venoms (Segura et al., 1999). Additional linear Gly-/His-rich (Park et al., 2000) and macrocyclic Cys-knot (Tam et al., 1999) antimicrobial peptides have also been reported. Over 800 peptides with anti-bacterial activity are known, generally ranging from 15-45 residues (Boman, 2003). Other molecules such as thionins, glucanases, chitinases, and other PR proteins are also known to have various effects in plant defense; a plant cystatin (an inhibitor of cystein proteases) was recently shown to have antifungal activity (Pernas et al., 1999).

Multiple native and synthetic antimicrobial peptides have been expressed in various species of transgenic plants. In some instances more

than one peptide has been expressed in the same plant, as each peptide has a different spectrum of activity. For example, two broad groups of plant defensins can be distinguished based on their morphogenic effects on fungal hyphae. The "morphogenic" defensins from the Brassicaceae and Saxifragaceae cause a reduction in hyphal elongation, and an increase in hyphal branching while the "non-morphogenic" defensins from the Asteraceae, Fabaceae, and Hippocastanaceae slow hyphal extension with little effect on hyphal morphology; there are also differences in their antifungal spectrum (Broekhart et al., 1995). In general, the plant defensins have much more activity against fungi than against bacteria, but the potato defensin Pth-St1 has activity against *Ralstonia (= Pseudomonas) solanacearum* and *Clavibacter michiganensis* (Moreno et al., 1994). A third subclass of plant defensins, the "α-amylase inhibitor" type, inhibits insect and human α-amylase, thus conferring some resistance to herbivory rather than against pathogens (see Broekhart et al., 1995). Snakin-1 has activity against several bacterial pathogens (including *C. michiganensis*), causes aggregation of both gram-positive and gram-negative bacteria, and is also active against fungal pathogens including *Botrytis cinerea* (Segura et al., 1999).

In non-transgenic plants, anti-microbial peptides are expressed either constitutively in particular organs such as floral parts or seeds that are particularly susceptible to insect or pathogen attack, or in a wound-inducible manner (e.g., Broekhart et al., 1995; Segura et al., 1999; Berrocal-Lobo et al., 2002). It has also been shown that some anti-microbial peptides are induced by various stress stimuli, including infection by viruses, bacteria, and fungi, or treatment with salicylic acid or jasmonic acid (Linthorst, 1991).

A large number of both native and synthetic peptides has been tested against varying spectra of fungal and bacterial plant pathogens, with differential activity often observed. In some cases, activity against fungal pathogens was shown to be greater than against bacteria (e.g., Segura et al., 1999; Berrocal-Lobo et al., 2002; Saitoh et al., 2001; Ali and Reddy, 2000). Hexapeptide combinatorial libraries have been examined for antimicrobial activity (Blondelle et al., 1995; Reed et al., 1997; López-Garcia et al., 2000), and a hexapeptide and derivative pentapeptide with activity against phytopathogenic fungi was identified (Reed et al., 1997). However, sequence-related derivatives of another effective anti-fungal hexapeptide were found to lack appreciable activity (López-Garcia et al., 2000). Cavallarin et al. (1998) demonstrated that variants of cecropin A differed in spectrum and degree of antifungal activity, with *Phytophthora infestans* being particularly susceptible to

an 11-residue N-terminal domain with amphipathic a-helix structure. Pernas et al. (1999) showed that chestnut cystatin inhibited the growth of three pathogenic fungal species, but not growth of a saprophytic fungus, *Trichoderma viride*. Marcus et al. (1997) have isolated a gene for a macadamia nut antimicrobial peptide, MiAMP1, that inhibits several plant pathogenic fungi, oomycetes, and gram-positive bacteria *in vitro*, and lacks apparent plant or mammalian toxicity.

Several native and synthetic peptides have been expressed in various transgenic plants, including cecropin derivatives (e.g., Jaynes et al., 1993; Nordeen et al., 1992; Allefs et al., 1995; Osusky et al., 2000). Different peptides also have differential stability against proteolytic degradation in various plant species; Owens and Heutte (1997) showed that MB39, a structural analogue of cecropin B, had a significantly longer half-life in leaf intercellular fluid in nine out of ten crop species examined, while retaining similar anti-fungal and anti-bacterial activity. Efficiency of translation may also vary between species, depending upon codon usage, and substitution of preferred codons may strongly influence expression levels (e.g., Perlak et al., 1991). There may also be significant differences between genotypes of a particular plant species in response to a particular peptide; a cecropin-mellitin chimeric peptide conferred powerful resistance against several bacterial and fungal pathogens in potato cv. Desiree, but induced an undesirable lesion-mimic phenotype in cv. Russet Burbank, suggesting that its application may be cultivar specific (Osusky et al., 2000). A lesion-mimic phenotype was also observed in transgenic potato expressing bacterio-opsin, which activates defense responses typical of systemic acquired resistance; the plants showed enhanced resistance to *P. infestans* isolate US1 (A1 mating type), but not to isolate US8 (A2 mating type) or *Erwinia carotovera* (Abad et al., 1997). Such reactions may also cause unpredictable results in breeding with transformed plants.

Varying results have been reported with different peptides, host plants, and pathogens. Jaynes et al. (1993) reported enhanced resistance to bacterial wilt of tobacco caused by *R. solanacearum* in plants expressing cecropin B. Allefs et al. (1995) found that cecropin B had negligible effect on resistance of transgenic potato to *E. carotovera*, whereas horseshoe crab peptide tachyplesin I conferred some resistance to *Erwinia* (Allefs et al., 1996). Osusky et al. (2000) observed resistance to the fungi *Phytophthora cactorum* and *Fusarium solani*, and to bacterial rot caused by *E. carotovera*, in potato expressing a chimeric cecropin-melittin peptide. The tubers retained resistance to spoilage for over a year at 4°C, suggesting that the peptide will also protect against

post-harvest pathogens. Furthermore, the peptides elicited no adverse reaction in mice and their enteric microflora when fed raw potato tuber, which is an important safety consideration (Osusky et al., 2000). The insect antibacterial peptide sarcotoxin IA was expressed in tobacco from a modified PR1a promoter, which is activated by salicylic acid or by necrotic lesion formation following pathogen attack; resistance to *E. carotovera* and *Pseudomonas syringae* was dependent on the expression level of sarcotoxin IA, and plants expressing higher levels of the peptide were also resistant to the fungal pathogens *Rhizoctonia solani* and *Pythium aphanidermatum* (Mitsuhara et al., 2000). Constitutive over-expression of *Arabidopsis* thionin Thi2.1 in the susceptible *Arabidopsis* ecotype Columbia enhanced resistance against *Fusarium oxysporum* f.sp. *matthiolae* (Epple et al., 1997), with reduced loss of chlorophyll, reduced fungal growth, and significantly increased hyphal abnormalities.

Individual peptides, chimeric peptides, or dual peptides separated by a proteolytic cleavage site have also been expressed from plant viral vectors to assess their activity against a variety of pathogens, and in a number of host species. This is a potent method for determining the spectrum of antimicrobial activity and assessing any adverse effects on different hosts faster than is possible by transformation and regeneration of multiple species. Saitoh et al. (2001) expressed the defensin WT1 from *Wasabi japonica* from a *Potato virus X* (PVX) vector. The expressed level of WT1 was insufficient to enhance resistance of PVX(WT1)-infected *Nicotiana benthamiana* against *Botrytis cinerea*, although WT1 purified from such plants strongly inhibited *B. cinerea* and *Magnaporthe grisea*, and less effectively the bacterium *Pseudomonas cichorii*, in *in vitro* assays (Saitoh et al., 2001). The same group has also expressed another *W. japonica* antimicrobial protein, WjAMP-1, from a PVX vector (Kiba et al., 2003). Expression of WjAMP-1 results in processing to a hevein-like domain with antifungal activity, and a C-terminal domain (equivalent to the C-terminus of hevein) which has both antifungal and antibacterial activity (Kiba et al., 2003). WjAMP-1 purified from wasabi leaves inhibited *Alternaria alternate*, *B. cinerea*, *F. solani*, and *M. grisea* in a dose-dependent manner, while a C-terminal His-tagged WjAMP-1 produced in *N. benthamiana* inhibited not only the fungi, but also three *Pseudomonas* species, *Agrobacterium tumefaciens*, and *E. coli*; the His-tag-containing recombinant protein was less potent than the native protein (Kiba et al., 2003). Zhao and Hammond (2000) used a PVX vector to express a translational fusion of snakin-1 and potato defensin pseudothionin 1 (Pth1) separated by the

Foot and mouth disease virus (FMDV) protease 2A, such that the FMDV protease self-cleaves the molecule co- or post-translationally to yield separate snakin-1 and Pth1 peptides. Snakin-1 is active against several fungal and bacterial pathogens, while Pth1 has a different antimicrobial spectrum and activity, and both synergistic and additive effects have been observed when the two peptides are combined (Moreno et al., 1994; Segura et al., 1999). Activity against *C. michiganesis* was observed in plants infected with the PVX vector expressing the snakin-1/Pth1 combination (R.W. Hammond, personal communication).

Reports of transgenic ornamentals expressing antimicrobial peptides include snapdragon transformed with cecropin (Kuenhle, in Robinson and Firoozabady, 1993), and anthurium transformed with attacin (Chen and Kuenhle, 1996). Geranium has been transformed with an antimicrobial protein (Ace-AMP) from onion for resistance to *B. cinerea* (Bi et al., 1999), and with cecropin against *Xanthomonas campestris* (Renou et al., 2000). Petunia has been transformed for fungal resistance (Esposito et al., 2000), and rose callus cultures have been transformed with cecropin B for resistance to bacteria to extend vase life (Derks et al., 1995). Rose transformed with Ace-AMP1 showed enhanced resistance to powdery mildew caused by *Sphaerotheca pannosa* (Li et al., 2003). Oncidium orchids expressing the antimicrobial sweet pepper ferredoxin-like protein (pflp) showed enhanced resistance to soft rot caused by *Erwinia carotovera* (Liau et al., 2003).

Antibody-Mediated Pathogen Resistance

A recent scientific breakthrough has presented another possibility for controlling plant diseases through the use of transgenic plants that produce antibodies to specific plant pathogens (During et al., 1990). Complete antibodies or antibody fragments have been expressed in plants by transient expression using viral vectors, agroinfiltration, or biolistics, or after stable integration of a transgene directly into the plant genome (Schillberg et al., 2001). Functional antibodies expressed in plants, sometimes called 'plantibodies,' can be used either to inhibit plant physiological functions or to establish pathogen resistance (De Jaeger et al., 2000). Plant expression of antibodies that bind antigens essential for the infection process or pathogenesis could block infection entirely or ameliorate the symptoms of infection through a reduction in the effective titer of the target antigen. For example, the effective "neutralization" of one or more viral proteins (e.g., viral proteases, replicases, movement proteins) should interfere with the efficiency of infection, vi-

rus assembly, movement of virus within the host, symptomatology, aphid transmission, and/or virus replication and thereby form the basis of a mechanism for conferring disease tolerance or resistance to the engineered host plant. This antibody-mediated resistance in plants is an alternative approach to the pathogen-derived resistance described above, or could be combined with other approaches.

Antibodies for Viral Resistance: There are several reports of transgenic plants expressing full-size antibody or antibody fragments against the coat proteins of several different plant viruses (Fecker et al., 1997; Tavladoraki et al., 1993; Voss et al., 1995; Zimmerman et al., 1998). These plants generally showed, via a delay in symptom development, a partial protection against the virus in the early stages of infection, suggesting a possible role of the antibodies in plant protection. The antibody molecules may bind to the nucleoproteins to prevent uncoating in the early stage of infection, or bind to the nucleoprotein molecules to prevent assembly of virions in the later stages of virus replication or movement within the plant. The recent development of transgenic model host plants (*N. benthamiana*) expressing antibodies against BYMV and other potyviruses (Maroon and Jordan, 1998, 1999, and unpublished), and CMV (Hsu et al., 2004) and of ornamental host gladiolus expressing antibodies against CMV CP, which show various degrees of resistance to local and systemic virus infection (Hsu and Kamo, unpublished), illustrate the potential of this technology as a means of controlling these wide-host range viruses in ornamental plants.

Antibodies produced against non-structural viral proteins required for viral replication processes or movement in the plant should be better sources of resistance in transformed plants than antibodies against structural proteins. Unfortunately, there are only a few reports of even the initial development of the recombinant antibodies to viral non-structural proteins. Hust et al. (2002) have developed a recombinant antibody against *Plum pox virus* viral protease, a protein which acts as the major protease in the cleavage of the potyviral polyprotein into functional proteins. This antibody can detect 19 different potyviruses, but has yet to be expressed in plants. A recombinant antibody directed against an epitope of the *Tomato spotted wilt virus* (TSWV) G1 glycoprotein conserved among a large number of tospoviruses has been expressed in plants (Franconi et al., 1999). However, these plants were not reported to have been challenged-inoculated with virus to determine the extent this potentially broad-spectrum antibody-mediated resistance against TSWV, a virus of major economic importance to the ornamental industry.

In the reports to date, the expression of antibodies or antibody fragments in plants for pathogen resistance has been marginally effective, usually only leading to a delay in disease development (Stoger et al., 2002; Ziegler and Torrance, 2002). However, these studies have demonstrated that an antibody-based approach can create pathogen resistant crop plants, particularly against plant viruses. The success of this approach will be improved when viral proteins other than the coat protein are used as the target. Antibodies directed against evolutionarily conserved functional domains, in such proteins as viral protease, movement protein and replicase, should provide more potent, broad spectrum resistance against viral pathogens (Schillberg et al., 2001). Pyramiding resistance by simultaneously expressing several plantibodies with different target specificities will increase the likelihood of developing long-lasting, broad-spectrum resistance. The successful application of an antibody-based resistance to other pathogens, such as bacteria, fungi, nematodes and insects is promising and may well become another strategy for the molecular breeding of pathogen resistant crops and plant lines.

Antibodies for Fungal Resistance: Antibody-mediated fungal resistance in plants was demonstrated only recently. Monoclonal antibodies that inhibit fungal growth have been identified (Hiatt et al., 2001). Peschen et al. (2004) show that fusion proteins, consisting of any one of three antifungal peptides fused to a single chain antibody reactive with a *Fusarium* cell wall antigen, conferred high levels of protection in transgenic *Arabidopsis* against *Fusarium oxysporum* f.sp. *matthiolae*. Either the fungus-specific antibody or any one of the antifungal peptides supplied alone, or the antibody and any one of the peptides as mixtures, inhibited an inferior response compared to the fusion protein as *in vitro* inhibitors of fungal growth, indicating the necessity of the physical association in the fusion protein for maximal protection (Peschen et al., 2004).

Antibodies for Bacterial Resistance: It has also been recently demonstrated that an antibody-mediated resistance strategy may also be applicable to bacterial diseases. Le Gall et al. (1998) developed transgenic tobacco expressing stolbur phytoplasma-specific antibodies that were resistant to infection when top-grafted onto tobacco plants heavily infected with the stolbur phytoplasma. Transgenic maize plants expressing antibodies against the corn stunt spiroplasma *Spiroplasma kunkelii* were developed by Chen and Chen (1998); however, these plants showed no distinct resistance to infection under greenhouse conditions.

Antibodies for Nematode Resistance: Nematicidal plants can be engineered with antibodies against secretory feeding proteins (Stiekema

et al., 1997; Schillberg et al, 2001; Schots et al., 1992). Antibodies directed against *Meloidogyne incognita* root knot nematode secretions involved in the plant nematode infection process have been produced and expressed in transgenic tobacco, with limited success (Rosso et al., 1996; Baum et al., 1996). It has been hypothesized that these antibodies would be more effective if they were expressed in the plant cytosol where feeding occurs, rather than the apoplast.

TRANSGENIC PLANTS IN VIRUS CONTROL

The concept of pathogen-derived resistance (originally suggested by Sanford and Johnston, 1985) has stimulated research on obtaining virus resistance through genetic engineering. Pathogen-derived resistance is mediated either by the protein encoded by the transgene (protein-mediated) or by the transcript produced from the transgene (RNA-mediated). Extensive research with genes from viruses and other sources has documented the efficacy of viral sense or antisense genes (e.g., coat protein, replicase, satellite RNAs, defective interfering RNAs) in protecting plants against virus infection following transfer and expression of these genes in plants (reviewed in Goldbach et al., 2003; Hadidi et al., 1998; Hull 2002; Ziegler and Torrance, 2002).

Several lines of research indicate that the best approach for this pathogen-derived "virus-induced" resistance is one mediated by an RNA-based post-transcriptional gene silencing (PTGS) mechanism. This plant defense system, one aspect of RNA silencing, results in degradation of mRNA produced both by the transgene and the virus (Hammond et al., 1999; Lu et al., 2003; Vance and Vaucheret, 2001; Wassenegger, 2002). In general, protein-mediated resistance provides moderate protection against a broad range of related viruses while RNA-mediated resistance offers high levels of protection only against closely related strains of a virus (Dawson, 1996; Goldbach et al., 2003; Lu et al., 2003).

Using various coat protein (CP) sense, CP antisense, or replicase sense viral genes, several groups are working to introduce virus resistance into various ornamentals, including chrysanthemum (Sherman et al., 1998; Yepes et al., 1999), gladiolus (Hammond and Kamo, 1995a, 1995b; Kamo et al., 2000a, 2000b; Kamo, Hsu, and Jordan, unpublished), lily (Langeveld et al., 1997), and various orchids (Deroles et al., 2002).

Examples of virus resistance that are particularly relevant to ornamentals include a number of viruses with wide host ranges that include several ornamentals, such as *Arabis mosaic virus* (a nepovirus, ArMV), CMV (a cucumovirus), *Chrysanthemum virus B* (a carlavirus, CVB), TMV and other tobamoviruses, as well as tospoviruses (including TSWV) and many potyviruses. Spielmann et al. (2000) reported a delay in infection, and some escape from infection, in *N. benthamiana* expressing ArMV CP. Multiple constructs have conferred resistance to CMV in tobacco, tomato, and cucurbits expressing CMV CP or replicase genes (e.g., Gonsalves et al., 1992; Anderson et al., 1992), dsRNA (Kalantidis et al., 2002), or satellite RNA (Harrison et al., 1997). Hsu et al. (2004) have shown that anti-CMV single-chain antibodies (ScFv) confer significant resistance in *N. benthamiana*. Chrysanthemum has been transformed with different forms of the CVB CP gene, but resistance data are not yet available (Mitiouchkina et al., 2004). Resistance to tobamoviruses has been reported in a variety of transformed plants, including expression in tobacco of truncated replicase (Golemboski et al., 1990), defective movement protein (Cooper et al., 1995), CP (Powell-Abel et al., 1986), and anti-TMV ScFV (Zimmermann et al., 1998). Resistance to tospoviruses has been conferred by various N-protein constructs (e.g., Pang et al., 1993), and an anti-viral peptide (Rudolph et al., 2003). Jan et al. (2000) showed that a composite tospovirus (TSWV partal N-gene)-potyvirus (*Turnip mosaic virus*; TuMV) CP construct resulted in PTGS and resistance against both TSWV and TuMV. Resistance against *Tomato yellow leaf curl virus* (TYLCV–an ssDNA geminivirus) has been obtained in tomato from expression of the CP gene (Kunik et al., 1994) and in *N. benthamiana* from a truncated replicase protein (Noris et al., 1996); TYLCV also infects the ornamental lisianthus, for which transformation protocols are available (see Deroles et al., 2002).

There are several ornamentals that have already been transformed with viral or anti-viral genes in order to obtain virus resistance, although not all published studies include resistance data. Chrysanthemum has been transformed with a number of different types of construct, with varying results. Yepes et al. (1995) used biolistic transformation of leaf or stem explants to obtain a total of 82 transgenic lines from four cultivars. The construct used was the TSWV N-gene, and stem explants regenerated more efficiently than leaf pieces. In a subsequent paper, Yepes et al. (1999) compared the biolistic method to *Agrobacterium*-mediated transformation, and utilized the N-genes of TSWV, *Impatiens necrotic spot virus* (INSV), and *Groundnut ringspot virus* (GRSV). In

neither case was resistance analyzed, and the plant lines were eventually destroyed without resistance being assessed (D. Gonsalves, personal communication to J. Hammond). Sherman et al. (1998) used *Agrobacterium* to transform chrysanthemum cv. 'Polaris' with either a full-length (N^+), a translationally-truncated (Nt), or an antisense ($N-$) copy of the N-gene of a dahlia isolate of TSWV; one Nt and two $N-$ lines were fully resistant to challenge by viruliferous thrips carrying a virulent chrysanthemum isolate of TSWV. Several N+ and other lines were infected but showed significantly reduced symptoms of TSWV compared to non-transgenic controls (Sherman et al., 1998). Chrysanthemum have also been transformed for effective resistance to *Chrysanthemum stunt viroid* (CSVd) with the dsRNA-specific nuclease Pac1 (Ishida et al., 2002), and with various CP constructs of CVB, although no resistance data are available (Mitiouchkina et al., 2004).

Resistance to mechanical inoculation with TSWV has also been demonstrated in four cultivars of *Gerbera* (Korbin et al., 2002), and in *Osteospermum* (Allavena et al., 2000). *Osteospermum* has also been transformed with several constructs derived from *Lettuce mosaic virus* (potyvirus), but no resistance assays were reported (Mörbel et al., 2002). Borth et al. (2004) have characterized partial resistance in transgenic *Dendrobium* orchids transformed with the *Cymbidium mosaic virus* (CymMV) CP or mutated movement protein gene. Kamo et al. (1997, and unpublished) have transformed gladiolus with BYMV CP and antisense RNA constructs, and anti-CMV ScFv antibodies that were each effective in *N. benthamiana* (Hammond and Kamo, 1995a,b; Hsu et al., 2004), as well as CMV CP and defective replicase constructs (Kamo, Hsu, and Jordan, unpublished). The BYMV CP and antisense gladiolus lines showed delayed virus accumulation, but not effective resistance (Kamo et al., submitted), while the other lines are undergoing analysis. Lily has been transformed with a defective CMV replicase gene (Lipsky et al., 2002), and De Villiers et al. (2000) attempted transformation of *Ornithogalum* with the OrMV CP gene, but no information on resistance in either crop is available. Similarly, Berthomé et al (2000) report transforming geranium (*Pelargonium* ×*hortorum*) with the *Pelargonium flower break virus* CP gene, or rat 2′,5′-oligoadenylate synthetase (2-5A), or yeast Pac1 dsRNA-specific RNase, but without resistance assessment.

Defective Interfering RNA and DNA: Defective interfering RNAs (DI-RNAs) or DNAs (DI-DNAs) are deletion mutations of the viral genome that are able to replicate in a parasitic fashion, utilizing the

replicase complex of an active infection; DI-RNAs and DI-DNAs are not able to replicate on their own. In most cases DI-RNA reduces the replication level of the parental virus, resulting in reduced symptom expression, although at least one DI-RNA intensifies symptom expression (Li et al., 1989). DI-DNAs have similar competitive effects on DNA viruses (Stanley et al., 1990). Kollar et al. (1993) showed that a *Cymbidium ringspot virus* (CymRSV) DI-RNA protected transgenic *N. benthamiana* againt CymRSV infection, while Stanley et al. (1990) demonstrated protection against geminivirus infection from a DI-DNA. Stanley et al. (1997) have also shown the presence of a naturally-occurring DI-DNA in *Ageratum yellow vein virus* (geminivirus) infections of *Ageratum conyzoides*. Rubio et al. (1999) developed a DI-RNA from *Tomato bushy stunt virus* (tombusvirus, TBSV) that conferred broad-spectrum protection against related tombusviruses in *N. benthamiana*; the DI-RNA was expressed at low levels in healthy transgenic plants, but was amplified to very high levels following TBSV infection, and resulting in plant recovery.

Ribozymes: Ribozymes (RNA molecules that autocatalytically cleave sequences complementary to their binding site) have potential to confer resistance against viruses if expression levels and activity are sufficient. Some viroids and viral satellite RNAs self-process from multimeric replicative forms by ribozyme activity, and some success has been achieved in protecting transgenic plants against specific pathogens. Yang et al. (1997) showed that a 'hammerhead' ribozyme protected transgenic potato against *Potato spindle tuber viroid* (PSTVd), whereas an inactive mutant ribozyme did not; this finding was significant, as de Feyter et al. (1996) had shown that a ribozyme against TMV functioned as an antisense RNA rather than as a ribozyme (probably by inducing PTGS). One line of transgenic melon expressing a ribozyme against *Watermelon mosaic virus 2* (potyvirus, WMV2) was found to have immunity to WMV2 (Huttner et al., 2001).

Anti-Viral Peptides: Another anti-viral approach that may have considerable applicability to ornamentals is the expression of dominant interfering peptides ('aptamers') that interact with essential viral proteins. The first example of this approach is the expression in *N. benthamiana* of a 29 amino acid peptide selected for interference with multiple tospovirus N-proteins (Rudolph et al., 2003). The transgenic plants were highly resistant to TSWV, GRSV, and *Chrysanthemum stem necrosis virus*, while a lower level of resistance was observed with *Tomato chlorotic spot virus*, and delayed disease development with INSV (Rudolph et al., 2003).

Broad Spectrum Resistance to Viruses

Ribonucleases: It may be possible to introduce broad spectrum resistance against RNA viruses and viroids, based on the expression of ribonucleases specific for double-stranded RNA (dsRNA). DsRNA is a feature of the replication of RNA viruses and viroids, but is not normally found in healthy plant cells. Two approaches that have been tested are the expression of the yeast dsRNA-specific RNase Pac1, and the mammalian interferon-induced 2′,5′-oligoadenylate synthetase (2-5A)/RNase L system. Transgenic potato expressing Pac1 were resistant to *Potato virus Y* (PVY) and PSTVd, and chrysanthemum were resistant to CSVd, while tobacco expressing both Pac1 and 2-5A/RNase L were resistant to TMV, CMV, and PVY (Ishida et al., 2002). In contrast, Ogawa et al. (1996) observed that plants expressing 2-5A and RNAse L were extremely resistant to CMV, but that PVY-inoculated plants all died within 20 days after inoculation despite restriction of PVY infection to the inoculated leaf.

Ribosome-Inactivating Proteins: A number of ribosome-inactivating proteins (RIPs) have been described from a variety of plant species, and several have been expressed in transgenic plants to examine virus resistance. Perhaps the best known of these is the pokeweed antiviral protein (PAP) from pokeweed (*Phytolacca americana*), which was discovered by virtue of its ability to inhibit transmission of various plant viruses (Duggar and Armstrong, 1925). Some 50 years later it was determined that the purified protein was a potent inhibitor of protein synthesis (Irvin, 1975). PAP is the most potent antiviral protein among all plant RIPs tested by Stevens (1981). The activity of PAP, and its similarity to other RIPs such as trichosanthin, ricin, luffin, momorcharin, have been reviewed by Tumer et al. (1999); PAP is a type I RIP, and depurinates both eukaryotic and prokaryotic ribosomes, whereas type II RIPs such as ricin affect primarily eukaryotic ribosomes.

Transgenic expression of PAP at high levels in tobacco caused a stunted and mottled phenotype, and plants were sterile, but plants expressing lower levels had a normal appearance and were fertile (Lodge et al., 1993). Transgenic plants expressing wild-type PAP or a two amino acid mutant form, PAP-V, were resistant to infection by various viruses, whether transmitted mechanically or by aphids; the transgenic PAP was enriched in the intercellular fluid of transgenic plants, as it is in pokeweed (Lodge et al., 1993). Transgenically expressed PAP therefore has the potential to inhibit infection by multiple viruses. Some other RIPs, including trichosanthin and dianthin, have since been shown

to confer virus resistance in transgenic plants (Lam et al., 1996; Hong et al., 1996); however, others such as barley RIP and ricin, which are not active on tobacco ribosomes, also lack antiviral effects (Taylor et al., 1994).

PAP mutants that retain the active site, but which have a C-terminal deletion, retain antiviral activity, but do not depurinate ribosomes (Tumer et al., 1997), showing that the antiviral activity could be separated from the toxicity of the wild-type PAP. PAP and various non-toxic mutants also confer fungal resistance in transgenic tobacco (Zoubenko et al., 1997). The combination of a barley endosperm RIP with a barley class-II chitinase (which has activity against fungal cell walls) increased resistance in transgenic tobacco to *Rhizoctonia* over the RIP alone (Jach et al., 1995).

Compared to conventional breeding for virus resistance, genetic engineering provides a quicker and more precise technology to obtain plants that are resistant to viruses; however, most transgenic virus-resistant plants are still under laboratory development. The few commercially grown virus-resistant crops include papaya expressing *Papaya ringspot virus* coat protein (Ferreira et al., 2002) and multiple virus resistant cucurbits (Fuchs et al., 1997).

TRANSGENIC PLANTS IN FUNGAL RESISTANCE

All antifungal genes currently used to develop transgenic ornamental plants have already been used for transgenic agronomic crops that are plagued with the same genera of fungi, *Fusarium*, *Rhizoctonia*, and *Botrytis*. These include RIPs, plant chitinases, plant glucanases, thaumatins, thionins, AMPs, stilbene synthase, lysozyme, and fungal chitinases. There is one report showing that an R gene that confers resistance to powdery mildew, *Erysiphe orontii*, *E. cichoracearum*, and *Ooidium lycopersici* in *Arabidopsis* also conferred resistance *to E. orontii* and *O. lycopersici* in transgenic *Nicotina tabacum*, and to *E. cichoracearum* in transgenic *N. benthamiana* plants (Xiao et al., 2003).

Several classes of PR proteins have been cloned and tested for antifungal activity. Class I chitinases are PR3 proteins and have been found to lyse hyphal tips of fungi, especially when combined with a glucanase or RIP. The three classes of chitinases are based upon a structural analysis of the chitinase gene (Huynh et al., 1992). Class I chitinases are basic isoforms with an amino-terminal cysteine-rich domain and a highly conserved catalytic domain. Class II enzymes have a

catalytic domain similar to that of the Class I enzymes but Class II lacks the cysteine-rich domain. Class III chitinases are acidic isoforms that lack homology with the Class I and II chitinases. A rice chitinase under the control of the CaMV 35 S promoter has been used to enhance resistance to *Botrytis cinerea* and *Rhizoctonia solani* in transgenic rice plants (Tabei et al., 1998; Datta et al., 2001) and powdery mildew, *Uncinula necator*, in grapevine (Yamamoto et al., 2000). There was only slight resistance to anthracnose, *Elisinoe ampelina*, in grapevine (Yamamoto et al., 2000). Resistance to powdery mildew, *Erysiphe graminis*, was induced in wheat plants transformed with the barley seed class II chitinase under the control of the maize ubiquitin 1 promoter (Bliffeld et al., 1999). Chitinase genes have been isolated from several fungi, *Rhizopus oligosporus*, *Trichoderma harzianum*, and *Saccharomyces cerevisiae*, and all have shown antifungal activity in plants (Terakawa et al., 1997; Lorito et al., 1998; Carstens et al., 2003). The *chi*1 gene from *Saccharomyces* was used to transform tobacco plants, and the transgenic plants showed suppressed symptoms when infected with *Sclerotinia sclerotiorum* and *Botrytis cinerea* (Terakawa et al., 1997).

Transgenic tobacco and potato plants containing the endochitinase gene, ThEn-42, cloned from *Trichoderma* showed a high level of tolerance or were completely resistant to *Alternaria alternata*, *A. solani*, *Botrytis cinerea*, and *Rhizoctonia solani* (Lorito et al., 1998). From 5 to 10% of the transgenic tobacco plant lines were completely resistant to *A. alternata*. The tobacco lines were also highly resistant and some lines were almost completely resistant to *Botrytis*. The majority (65-80%) of transgenic plants survived infection with *Rhizoctonia* whereas most of the nontransformed plants died. *CTS1-2* coding for the chitinase from *Saccharomyces* showed antifungal activity against *Botrytis* in transgenic tobacco plants (Carstens et al., 2003). Germination and hyphal growth of *Botrytis cinerea* were inhibited by up to 70% by leaf extracts from the transgenic plants. A chitinase gene, *chiA*, has also been cloned from the bacterium *Serratia marscescens*, and tolerance to *Rhizoctonia solani* by transgenic tobacco plants was shown in the field (Howie et al., 1994). Resistance to powdery mildew, *Erysiphe graminis*, and rust was induced when transgenic wheat plants expressed the antifungal protein, ag-AFP isolated from *Aspergillus giganteus*, in combination with a barley class II chitinase (Oldach et al., 2001). In contrast, wheat plants transformed with a barley type I RIP did not confer resistance to the same fungus, while in a detached leaf infection, the F1 progeny lines showed a decrease in susceptibility (50-70%) when challenged with *Bo-*

trytis (Oldach et al., 2001). These studies show that some fungal genes may be more effective than plant genes in controlling fungi.

Thaumatin proteins are class V PR proteins (PR 5). Expression of a thaumatin-like protein has been shown to enhance resistance to *Rhizoctonia solani* in transgenic rice plants and to delay development of *Fusarium graminearum* symptoms in transgenic wheat plants (Datta et al., 1999; Chen et al., 1999).

RIPS have conferred resistance in both transgenic tobacco and rice plants to *Rhizoctonia solani*. Logemann et al. (1992) subcloned the barley RIP under the control of the wound and pathogen inducible *wun1* promoter, and three R_0 tobacco plants containing this construct were resistant to *Rhizoctonia*. Some of the transgenic tobacco plants grew as well in soil that had been inoculated with *Rhizoctonia* as control plants grown in non-inoculated soil. The R_1 plants showed expression of the RIP transgene. The RIP pokeweed antiviral protein II, PAPII, that is relatively less toxic to plant cells than other RIPs, was used to transform tobacco (Wang et al., 1998). Two out of eight transgenic tobacco lines that were isolated were found to be resistant to TMV and PVX, as well as *Rhizoctonia solani*. Only 30-40% of the transgenic tobacco plants died as compared to 90% mortality of control plants when grown in soil infected with *Rhizoctonia solani*.

Unlike dicots, there has been difficulty regenerating monocot plants that express the barley RIP when the RIP is located in the cytoplasm. Transgenic wheat plants expressing the barley RIP could not be regenerated (Bliffeld et al., 1999), and transgenic rice plants with RIP contained only truncated copies of RIP or did not express RIP. Bieri et al. (2000) used a DNA construct of the barley RIP containing a signal peptide that caused RIP to be correctly transported to the apoplast, and transgenic wheat plants that expressed RIP were isolated. Unfortunately, antifungal activity could not be demonstrated by these transgenic wheat plants. More recently a modified maize RIP under control of the rice *rbcs* promoter combined with the rice basic chitinase gene, RCH10, under the control of the actin, *Act1*, promoter was used to transform rice (Kim et al., 2003). These transgenic rice plants showed expression of both the RIP and chitinase genes; the plants were resistant to *Rhizoctonia solani* but not two other fungi, *Bipolaris* and *Magnaporthe*. A positive correlation between transgene expression and resistance was shown.

Fungal disease resistance resulting from transgenic expression of phytoalexins has also been demonstrated. Tobacco, barley, wheat, and grapevine rootstock plants transformed with a stilbene synthase gene,

Vst I, isolated from grapevine resulted in enhanced resistance to *Botrytis cinerea* (Hain et al., 1993; Leckband and Lorz, 1998; Coutos-Thevenot et al., 2001). Transgenic grapevine rootstock plants showed an accumulation of the phytoalexin, resveratrol, at levels 5-100 times that of the control plants when the *Vst*I gene was under control of the pathogen-inducible PR10 promoter from alfalfa (Coutos-Thevenot et al., 2001). Following inoculation of the grapevine leaves with *Botrytis cinerea*, all leaves of the control plants became infected whereas the best transgenic clones showed only 40% infection and very slow mycelial growth in infected plants.

Resistance against *Fusarium oxysporum* resulted from overexpression of a thionin in *Arabidopsis* (Epple et al., 1997). Complete resistance against *Erwinia carotovora*, *Phytophthora cactorum*, and *Fusarium solani* was achieved in potato using a modified cecropin peptide (Osusky et al., 2000). The amino terminus was modified to reduce the toxicity of the cecropin peptide, and its α-helical nature was maintained. All control plants died after challenge with the pathogen, and all transgenic plants survived without evidence of infection. The main problem with this cecropin peptide was that the phenotype of one cultivar of potato was unaffected, but another cultivar had curly leaves with smaller and branched tubers resulting from expression of the peptide. Another synthetic antimicrobial peptide, D4E1, under the control of the CaMV 35S promoter, conferred resistance to *Aspergillus flavus*, *Verticillium dahliae*, and *Colletotrichum destructivum* in transgenic tobacco (Cary et al., 2000).

Combinations of more than one antifungal gene are more effective than a single gene for resistance. The combinations tried have been random, and successful antifungal resistance does not yet appear to rely on a specific combination of genes for resistance against a particular fungal pathogen. Jach et al. (1995) tested single resistance genes, either a barley class II chitinase, a β-1,3-glucanase, or a type I RIP, and compared a single gene in transgenic tobacco plants for resistance conferred to *Rhizoctonia* to combinations of the same genes (chitinase combined with glucanase or chitinase combined with RIP). There was more protection from *Rhizoctonia* infection using the antifungal gene combinations rather than single genes. The protection against *Rhizoctonia* was shown to result from a synergistic interaction rather than additive interaction between the antifungal genes. A transgenic wheat plant containing both a chitinase and β-1,3-glucanase showed delayed *Fusarium* symptoms in the greenhouse but no tolerance in the field (Anand et al., 2003).

All studies describing antifungal activity in transgenic plants were tested for antifungal activity using *in vitro* or greenhouse testing. Results from the *in vitro* tests indicate resistance as measured quantitatively, and only a few of the transgenic plants have shown complete resistance. The real test of antifungal resistance is in the field, as resistance demonstrated in greenhouse tests does not necessarily carry over to the field (Anand et al., 2003). Anand et al. (2003) demonstrated that transgenic wheat expressing either a thaumatin-like protein or both a chitinase and β-1,3-glucanase showed delayed symptoms in the greenhouse when infected with *Fusarium graminearum*; however, these same plants were no different from nontransformed plants in the field. There are few studies demonstrating field-level resistance of an antifungal gene (Grison et al., 1996; Gao et al., 2000). The first field study to demonstrate fungal tolerance resulting from a chitinase gene involved transgenic tobacco expressing the *chiA* gene from *Serratia marcescens* (Howie et al., 1994). Tobacco plants were challenged with *Rhizoctonia solani*, and several plant lines were tolerant in most, but not all of the field trials. *Brassica napus*, oilseed rape, plants transformed with a tomato chitinase gene under the control of the CaMV 35S promoter showed tolerance in two field trails to three fungi, *Cylindrosporium concentricum, Phoma lingam*, and *Sclerotinia sclerotiorum* (Grison et al., 1996). Transgenic potato plants transformed with the alfalfa antifungal peptide defensin isolated from seeds of *Medicago sativa* were resistant to *Verticillium dahliae* in the field (Gao et al., 2000).

Two groups have transformed roses for blackspot and *Diplocarpon rosae* resistance. A basic chitinase gene (RCH10) isolated from rice and under the control of the CaMV 35S promoter was introduced into embryogenic callus of a floribunda rose cultivar 'Glad Tidings' using the gene gun (Marchant et al., 1998). Leaf disks from twenty transgenic plants lines grown in the greenhouse were inoculated with a conidial suspension of *Diplocarpon rosae*, and the resulting lesion diameter measured. A reduction in disease was seen as 65% of the transformed plants had lesions that were smaller than that of the control. The extent of resistance correlated with expression level of the chitinase that ranged from 4-15 times that of nontransformed plants. Roses have been transformed using *Agrobacterium tumefaciens* with different combinations of antifungal genes: a Class II chitinase, a Class II β-1,3-glucanase, a Type I ribosome inhibiting protein (RIP), or T-4 lysozyme (Dohm et al., 2001, 2002). All genes were under the control of the

CaMV 35 S promoter. The majority of transgenic plants expressed the transgene, and 80 plant lines were analyzed for blackspot resistance. Expression of the cytosolic proteins, chitinase, glucanase, and lysozyme, did not result in blackspot resistance. The susceptibility to blackspot was reduced by 40% in transgenic plants that secreted the RIP into the extracellular space when plants were challenged by inoculation with *Diplocarpon* conidia in the greenhouse.

Roses have been transformed with the antimicrobial protein gene, Ace-AMP1, under the control of the CaMV 35 S promoter with a duplicate enhancer region for resistance to powdery mildew, *Sphaerotheca pannosa* var. *rosae* (Li et al., 2003). Ace-AMP1 was isolated from onion seeds and selected for transformation studies because of its fungistatic activity in vitro against a wide variety of fungi and because unlike other AMPS, its antimicrobial activity is not affected by cations at physiological pH (Cammue et al., 1995). Seven transgenic rose lines were selected and challenged with powdery mildew using both detached leaflets and plants in the greenhouse. Six of the seven transgenic plant lines were more resistant to powdery mildew than the control, and surprisingly there was no correlation between level of Ace-AMP1 gene expression and resistance. A genetic analysis involving ten genotypes and eight races of powdery mildew has recently identified the first resistance gene against powdery mildew of rose, *Rpp1*, that appears to be a single dominant gene (Linde and Debener, 2003).

Other ornamental plants that have been transformed for fungal resistance include scented geraniums, bentgrass, chrysanthemums, *Petunia hybrida*, carnation, and African violets. Ace-AMP1 under the control of the CaMV 35S promoter with duplicated enhancer has been used to transform scented geraniums for *Botrytis cinerea* resistance (Bi et al., 1999). Seven transformed plants were obtained, and three were selected based upon transgene expression. The most resistant plant line of geranium showed a 50% reduction in sporulation *in vitro* as compared to the control. The more Ace-AMP1 protein that was produced correlated with more resistance to *Botrytis* sporulation. Bentgrass, *Agrostis* spp., transformed with the *bar* gene under the control of the maize ubiquitin 1 promoter were resistant to the herbicide glufosinate that inhibits glutamine synthetase (Wang et al., 2003). Transgenic plants of bentgrass sprayed with glufosinate were more resistant to *Rhizoctonia solani* and *Sclerotinia homoeocarpa* than transgenic plants that had not been sprayed with the herbicide. Application of glufosinate to the transgenic bentgrasses rather than expression of the *bar* gene caused a reduction in fungal symptoms. Transgenic chrysanthemum, *Dendranthema grandi-*

florum, plants that expressed the rice chitinase gene, RCC2, showed resistance to *Botrytis cinerea* (Takatsu et al., 1999). Over 3000 explants were used for *Agrobacterium*-mediated transformation, and only 16 lines analyzed by PCR were found to contain the chitinase gene. Eleven of the lines showed expression of the chitinase gene, and these were used for inoculation *in vitro* with *B. cinerea* conidia. A qualitative assessment was made of the symptoms, and three plant lines showed slight symptoms as compared to the control plants.

The reports on transformation of *Petunia hybrida*, African violet, and carnation with antifungal genes are brief and require further documentation before conclusions can be made as to the outcome of these antifungal genes in conferring fungus resistance. *Petunia hybrida* was transformed with the endochitinase from *Trichoderma harzianum* alone or in combination with osmotin for *Botrytis cinerea* resistance (Esposito et al., 2000). Carnations containing various combinations of the osmotin, PR-1 or chitinase genes were developed for *Fusarium oxysporum* resistance (Zuker et al., 2001). African violets were transformed with glucanase and chitinase genes for *Fusarium oxysporum* and *Pythium* resistance (Ram and Mohandas, 2003).

TRANSGENIC PLANTS FOR BACTERIAL RESISTANCE

Ornamental crops are subject to a number of economically important bacterial diseases, most commonly caused by *Erwinia*, *Pseudomonas*, *Ralstonia*, and *Xanthomonas* spp. (Powell and Lindquist, 1992). Plant bacteria normally enter plants through wounds and natural openings, and then spread and multiply intercellularly inside plant tissues or in xylem vessels. Since most bacteria can multiply rapidly under favorable conditions, and currently no effective and non-phytotoxic bactericides are available, bacterial diseases are very difficult to control and often result in significant economic losses in the production and quality of ornamental crops when an outbreak occurs.

Among the most destructive bacterial diseases of ornamentals is bacterial blight in geranium and anthurium caused by *Xanthomonas campestris* (Kuehnle et al., 1995). The prevalence of the pathogen had almost wiped out geranium production before the 1960s, and the outbreak of the pathogen in the early 1980s had resulted in about $4 million decline in Hawaii's anthurium production from 1986 to 1993 (Kuehnle et al., 1995). Even today, despite advances in integrated control measures, this disease remains a problem. Another well-known bacterial disease of

ornamentals is bacterial wilt of geranium caused by *Ralstonia solanacearum.* According to APHIS figures, the year 2003 introduction of the exotic biotype of *R. solanacearum*, race 3 biovar 2, on imported geranium and its subsequent outbreak in geranium in 127 nurseries in 27 states have resulted in the destruction of over 2 million geranium and other plants at an estimated $5 million loss to the geranium industry.

Once disease symptoms are observed, the damage has already been done and the disease is very difficult to control; therefore, management of bacterial diseases in ornamentals depends mainly on pathogen exclusion and prevention, including strict sanitation and the use of pathogen-free propagating materials. The use of genetically engineered crops offers another novel, cost-effective and environmentally sound strategy for control of bacterial diseases. Very limited effort, however, has been devoted to the development of transgenic crops resistant to bacterial diseases, as compared to fungal and viral diseases, and the research has mostly been done in tobacco and potato plants. Only a few attempts have been made to improve resistance of ornamental crops to bacterial diseases (Kuehnle et al., 1993, 1995; Renou et al., 2000).

The first engineered bacterial resistance was developed by Anzai et al. in 1989 in tobacco against *Pseudomonas syringae* pv *tabaci.* Since then, many transgenic approaches have been developed that confer either partial or "complete" resistance to plant pathogenic *Erwinia*, *Pseudomonas*, *Ralstonia*, or *Xanthomonas* spp. (Mourgues et al., 1998). The genes used in those transgenic approaches are isolated from diverse organisms including arthropods, mammals, bacteria, fungi and plants. In general, 3 sources of genes have been explored:

1. genes coding for antimicrobial peptides or proteins;
2. genes coding for enzymes that either detoxify or desensitize plants to bacterial toxins; and
3. genes that enhance natural disease resistance in plants (During, 1996; Herrera-Estrella and Simpson, 1995; Kuehnle et al., 1995; Loffler and Florack, 1997; Mourgues et al., 1998).

Genes Coding for Antimicrobial Peptides or Proteins Isolated from Anthropods, Bacteriophage, Human and Plants: The antibacterial peptides and proteins used so far for genetic engineering of bacterial resistance include cecropins and attacins from the giant silkmoth, lysozymes from T4 bacterial phage and human, tachyplesin from horseshoe crab, lactoferrin from human and bovine sources, and hordothionin from barley.

Cecropins are lytic peptides isolated from the giant silkmoth *Hyalophora cecropia*. They are a family of small basic proteins (~4 kDa) effective against both Gram-positive and Gram-negative bacteria by affecting the permeability of the inner and outer bacterial membranes (Jaynes et al., 1987). Based on their high lytic activity against plant pathogenic bacteria, Jaynes et al. (1987) proposed the idea of introducing antibacterial protein genes from insects to enhance plant resistance. By expressing a modified cecropin Shiva-1 gene under the control of the potato proteinase inhibitor II promoter in tobacco, Jaynes et al. (1993) reported a delayed bacterial wilt symptoms and reduced disease severity and mortality after inoculation with *R. solanacearum* by a stem inoculation assay, although no statistical analysis of the disease assay was described. Such enhanced disease resistance, however, was not observed in the same transgenic tobacco plants inoculated by a wounded root assay (Jaynes et al., 1993). Since *R. solanacearum* is a soil pathogen and normally enters plants through wounds or natural openings in roots, the biological significance of the enhanced disease resistance in transgenic tobacco against *R. solanacearum* remains questionable. In contrast to the results obtained by Jaynes et al. (1993), no resistance to *R. solanacearum* and the wildfire pathogen *P. syringae* pv. *tabaci* has been observed under greenhouse and field conditions in transgenic tobacco, or to the soft rot pathogen *Erwinia carotovora* in transgenic potato, expressing an unmodified cecropin B gene (Allefs et al., 1995; Belknap, 1993; Florack et al., 1995). The negative results were attributed to the rapid degradation of the peptide by endogenous proteases (Florack et al., 1995).

Attacins are also lytic peptides isolated from the giant silk moth that are about 20kDa in size, with their target probably being the outer bacterial memberane (Engstrom et al., 1984). The introduction of the attacin E gene into apple and pear plants has resulted in significant reduction of symptoms caused by the fire blight pathogen *Erwinia amylovora* in several transgenic lines (Norelli et al., 1993; Reynoird et al., 1999).

Lysozymes are enzymes with a specific hydrolytic activity against the bacterial cell-wall peptidoglycan. Transgenic potato plants expressing T4 bacteriophage lysozyme had a higher level of resistance to *Erwinia carotovora atroseptica*, the causal agent of soft rot and black leg, than the untransformed control (During et al., 1993). Expressing a human lysozyme in tobacco plants resulted in a slightly increased resistance to *P. syringae* pv. *tabaci* (Nakajima et al., 1994).

Tachyplesin I is a 2.3 kDa antimicrobial peptide isolated from horseshoe crabs *Tachypleus tridentatus* (Allefs et al., 1996). The expression

of this peptide under the control of a CaMV 35S promoter in potato resulted in less rot caused by *Erwinia* spp. under aerobic conditions and only slightly less rot under anaerobic conditions than control tubers (Allefs et al., 1996).

Lactoferrin is a member of a family of iron-binding glycoproteins that possess both antibacterial and antiviral activities. When a human lactoferrin gene was expressed in tobacco, the transgenic plants produced a smaller protein with much higher antibacterial activity (Mitra and Zhang, 1994). As a result, transgenic tobacco displayed a much-delayed onset of disease symptoms caused by *R. solanacearum* (Zhang et al., 1998). When a bovine lactoferrin gene was expressed in pear, most of the transgenic clones showed an increase in fire blight resistance against *E. amylovora* in *in vitro* and greenhouse tests (Malnoy et al., 2003).

The effect of utilizing type I thionins, small antimicrobial proteins isolated from wheat and barley, for transgenic resistance against bacterial diseases is unclear. Carmona et al. (1993) reported that constitutive expression of the barley hordothionin, but not the wheat purothionin, in tobacco resulted in a significant decrease in the growth of *P. syringae* pv. *syringae* and a reduced percentage of necrotic lesions. On the contrary, similar studies by Florack et al. (1994) did not demonstrate any effect on resistance to bacterial pathogens in transgenic tobacco, probably due to the lack of secretion of the hordothionin into the intercellular spaces where the bacteria reside.

More recently, a synthetic gene *msrA1* encoding a cecropin A-melittin (the major component of bee venom) cationic peptide chimera was expressed in potato in an attempt to develop a broad-spectrum resistance to plant pathogens (Osusky et al., 2000). When challenged with different plant pathogens under stringent conditions, transgenic 'Desiree' potato plants displayed a high level of resistance to both bacterial (*E. carotovora*) and fungal (*Phytophthora cactorum* and *Fusarium solani*) pathogens (Osusky et al., 2000).

Since antimicrobial proteins are effective against a range of plant pathogens, engineering such proteins in ornamental crops may confer resistance against either multiple bacterial pathogens or both bacterial and fungal pathogens. So far, however, there are only two preliminary reports of transgenic ornamentals with some degree of resistance to bacterial diseases through the expression of antimicrobial proteins. Kuehnle et al. (1993) reported that transgenic anthurium plants expressing an attacin gene under the control of a double CaMV 35S promoter showed some tolerance to *X. campestris* pv. *dieffenbachiae* strain D150, the

most virulent anthurium blight strain known. More recently, Renou et al. (2000) expressed a chimeric cecropin gene in pelargoniums and claimed that three out of the six transgenic clones of pelargonium showed a reduced symptom caused by the blight pathogen *Xanthomonas campestris* pv. *pelargonii*. In neither case, however, was any statistical analysis was performed for the disease assay, no translation and subcellular location of the peptide were determined, and no experiments were conducted to determine if the observed partial disease resistance will hold up under field conditions.

Genes Coding for Enzymes That Detoxify or Are Insensitive to Bacterial Toxins: Many plant pathogenic bacteria produce toxins that induce disease symptoms and render the host plants more susceptible to invasion. Genetic engineering aimed at resistance to bacterial toxins has resulted in the only complete resistance (defined as lack of disease symptom development) to bacterial diseases (Anzai et al., 1989; De La Fuente-Martinez et al., 1992; Mourgues et al., 1998; Zhang et al., 1999). The tobacco wildfire pathogen *P. syringae* pv. *tabaci* produces an enzyme that inactivates its own toxin, tabtoxin. Complete resistance to *P. syringae* pv. *tabaci* was obtained by expressing the tabtoxin detoxification enzyme in tobacco (Anzai et al., 1989). Different from *P. syringae* pv. *tabaci*, the bean blight pathogen *P. syringae* pv. *phaseolicola* produces an enzyme insensitive to its own toxin, phaseolotoxin. Transgenic bean plants expressing the phaseolotoxin insensitive enzyme did not develop any chlorotic disease symptoms in inoculated leaves, whereas all untransformed control plants developed severe symptoms (De La Fuente-Martinez et al., 1992). Similarly, almost no disease was observed by Zhang et al. (1999) in transgenic sugarcane plants expressing an albicidin detoxifying gene *albD* cloned from another bacterium possessing biocontrol abilities against *Xanthomonas albilineans*, the xylem-invading causal agent of sugarcane leaf scald disease. All these results have demonstrated that genetic engineering by expression of a toxin-resistance protein can confer resistance to both disease symptom development and multiplication of a plant pathogenic bacterium in its host (Zhang et al., 1999).

P. syringae pv. *syringae* produces a plant toxin, syringomycin. This bacterium has a wide host range including both woody and herbaceous ornamentals and is responsible for a number of economically important diseases of ornamental crops. Identifying an enzyme, either from the pathogen itself or from an antagonistic microorganism, which detoxifies or is insensitive to syringomycin may led to the development of

transgenic ornamentals with high levels of resistance against this pathogen.

Genes That Enhance Natural Disease Resistance in Plants; the Hypersensitive Response: As stated previously, the hypersensitive response (HR) is one of the most important defense mechanisms in plants. It occurs only in incompatible plant-pathogen interactions and is characterized by rapid and localized cell death surrounding the infection site. This HR lesion is believed to inhibit further spread of the pathogen and to generate a signal that activates host defense mechanism and induces long-lasting systemic resistance to a broad spectrum of plant pathogens (Ross, 1961). Programmed cell death in plants is believed to be activated during the HR response (Mittler et al., 1995). Different transgenic systems have been developed that mimic the activation of programmed cell death in higher plants and may be used for broad-range resistance against plant pathogens (Mourgues et al., 1998). In tobacco, a lesion-mimic phenotype was engineered by expressing a *Halobacterium halobium* gene encoding a light-driven bacterio-opsin (bO) proton pump (Mittler et al., 1995). The transgenic plants contained a high systemic level of salicyclic acid and displayed an enhanced disease resistance to two viruses and to the bacterium *P. syringae* pv. *tabaci* (Mittler et al., 1995). In contrast, transgenic potato expressing the *bO* gene produced an increased level of salicylic acid and were resistant to a fungal pathogen but not to *E. carotovora,* suggesting that the engineered resistance may be limited to certain plant-pathogen systems (Abad et al., 1997).

One of the earliest events that occurs during plant-pathogen recognition is the production of active oxygen species including H_2O_2 (Mehdy, 1994). The accumulation of such oxygen species is believed to play an important role in plant defense (Mehdy, 1994). Wu et al. (1995) reported that a fungal gene which encodes glucose oxidase was expressed in potato, and generates H_2O_2 when glucose is oxidized. Transgenic potato plants displayed strong resistance to *E. carotovora* under both aerobic and anaerobic conditions, probably mediated by an elevated level of H_2O_2 (Wu et al., 1995). The transgenic plants also exhibited enhanced resistance to the late blight pathogen *Phytophthora infestans*, suggesting that expression of an active oxygen species-generating enzyme in transgenic plants confers broad range disease resistance in plants (Wu et al., 1995).

In the last decade, several disease resistance (R) genes have been isolated from plants, including the *Pto* and *Prf* genes of tomato that confer resistance to *avrPto*-containing *P. syringae* pv. *tomato*. Oldroyd and

Staskawicz (1998) demonstrated that resistance to a broad spectrum of pathogens (bacteria and viruses) can be achieved by the overexpression of *Prf*, a component of the *Pto* resistance pathway, probably by activating the *Pto* and *Fen* pathways in a pathogen-independent manner and leading to the activation of systemic acquired resistance.

Since plant resistance is often determined by one or a few resistance genes, such gene(s) would be the best source for engineering disease resistance in ornamentals. Results obtained by Thilmony et al. (1995) suggested that R genes isolated from one species, tomato, can function in a related but sexually incompatible plant species, tobacco. It remains to be determined, however, whether R genes can function in a more diverse, sexually incompatible species. Since it is known that *avr* genes are under frequent mutation, durability of the transgenic approach utilizing R genes also needs to be determined (Mourgues et al., 1998).

Results from some of the genetic approaches discussed above are encouraging and have the potential to be applied to ornamentals to improve their resistance against the same or similar bacterial pathogens or against a broad spectrum of plant pathogens. Successful application of these genetic approaches in ornamentals, however, will rely largely on the availability of transformation protocols for ornamental crops, and the availability of suitable genes that confer resistance to plant pathogens of interest (Loffler and Florack, 1997). Successful application will also depend on the correct temporal and spatial regulation of gene expression, so that not only sufficient quantities of biologically active gene products can be produced, but also that these products will be secreted at the right time and correct sub-cellular location to combat the invading bacterial pathogens.

TRANSGENIC PLANTS IN NEMATODE CONTROL

Plant parasitic nematodes are responsible for up to $100 billion in worldwide crop loss annually (Sasser and Freckmann, 1987), with damage and loss to ornamental plants occurring primarily to field-grown woody ornamentals. The most significant damage to herbaceous ornamentals is caused by relatively few genera (Chase et al., 1995; Powell and Lindquist, 1992). Most of the economic losses of field-grown ornamental crops are due to damage by the root-knot nematode (*Meloidogyne* spp.) (Benson, 2001), although the cyst nematode, the lesion nematode (*Pratylenchus vulnus*), ring nematode (*Criconemella xenoplax*), stunt nematode (*Tylenchorhynchus claytoni*), dagger nematode (*Xiphinema*

diversicaudatum), and foliar nematodes (*Aphelenchoides* spp.) can also be problematic in some genera (Benson, 2001). Control of nematodes has historically focused on field treatments such as chemical fumigation, soil solarization, crop rotation, flooding, and fallowing; cultural practices such as mulching; biological control; the use of disease-free planting stock; and the use of disease resistant host plants (Benson and Dunn, 2001). Nematode control can be difficult since most infestations go unnoticed until symptoms are severe and cultural controls ineffective. Long-term environmental concerns over several nematicidal fumigants have forced growers to rely on more sustainable control methods, including host plant resistance.

Ornamental plants exhibit varying levels of resistance to nematode species, depending on genus, species, and cultivar (Benson and Barker, 1982). For example, some hollies (*Ilex* spp.) are susceptible to root knot nematode but tolerant to stunt and lesion nematodes. Because screening and subsequent breeding of woody ornamental plants requires a substantial investment of time and space, genetic engineering for resistance is especially attractive in this group of plants. Although transgenic resistance to nematodes has been achieved only in herbaceous crops to date, several strategies appear to be promising for both herbaceous and woody ornamental plants as well. These include transformation with genes aimed directly at the nematode to deter feeding, migration, or to inhibit growth and reproduction; or genes that can inhibit the formation of the specialized giant or feeding cells that form in the plant root at the site of nematode infection and are essential for nematode feeding (Lilley et al., 1999; Atkinson et al., 2003; Grundler, 1996). Two essential elements of an effective control strategy incorporate genes that encode an anti-nematode effector protein, peptide, or interfering RNA and plant promoters that direct a specific pattern of expression of that anti-nematode effector (reviewed in Atkinson et al., 2003). Specific examples of anti-nematode effectors include: naturally occurring plant resistance genes, toxins (such as *Bacillus thuringiensis* endotoxins), protease (and other enzyme) inhibitors, and lectins. The characteristics of a plant promoter required to drive an effective nematode resistance depends upon the type, specificity and expected site of expression of the intended effector. Expression of a protein or peptide that is a nematode-specific toxin might be driven by a promoter that is root specific or even constitutive. The expression of a general cytotoxic protein, however, might be more exacting (i.e., produced only at nematode feeding sites or during root invasion) if the aim is to prevent or abate the formation of nematode-induced feeding structures. Albeit still in its infancy,

the use of biotechnology, as a part of an integrated pest management strategy to provide nematode control, does offer a potential solution with benefits to the producer, the consumer and the environment.

Genetic engineering with genes that have a direct effect on the nematode itself has been shown to be effective primarily with the use of protease inhibitors to prevent protein metabolism by the nematode. Transgenic potatoes expressing the cowpea trypsin inhibitor (Atkinson, 1993) were one of the first demonstrations of the effectiveness of this approach against the cyst nematode, *Globodera pallida*. Subsequent work on cysteine protease inhibitors in tomato (Urwin et al., 1995), *Arabidopsis* (Urwin et al., 1997, 2000), rice (Vain et al., 1998), and potato (Urwin et al., 2001) has resulted in plants with reduced populations of, and damage, by nematodes. Recent reports indicate that combining natural partial host plant resistance with partial transgenic resistance conferred by a cysteine protease inhibitor may result in full resistance (Atkinson et al., 2003).

Other transgenic approaches that have been effective against the nematode itself include engineering with a lectin gene in potato (Burrows et al., 1998), or cloned R-genes from naturally-occurring resistant plants (e.g., Cai et al., 1997; Milligan et al., 1998). As work continues to identify plant genes expressed in response to nematode feeding (e.g., Wilson et al., 1994; Favery et al., 1998), it should be possible to direct the expression of various nematicidal genes using inducible or tissue-specific promoters.

Another approach to transgenic control of nematodes in ornamental plants is to alter or ablate the specialized giant or feeding cells essential for feeding for some nematode species (Ohl et al., 1997; Opperman and Conkling, 1998). Identification and cloning of genes specific to these cells (Goddjin et al., 1993; Bird and Wilson, 1994; Opperman et al., 1994) could result in the identification of cell-specific promoters that could then be used in conjunction with cell ablation technology (e.g., barnase; Mariani et al., 1990). Alternatively, inhibition of feeding site development can be accomplished using transgenic antisense constructs to a nematode responsive element. Such an approach has been effective in tobacco (Opperman et al., 1994; Opperman and Conkling, 1996).

Although transgenic approaches to nematode resistance in ornamental plants appears promising, the possible environmental effects of this transgenic technology must be considered in light of the fact that woody ornamental plants may stay in the ground for years (in nurseries) or decades (in landscapes), thus having the potential to have long-term ef-

fects on non-target soil organisms. A model for a sequential approach to this risk assessment has been proposed by Cowgill and Atkinson (2003) for cysteine protease inhibitors in transgenic potato, which resulted in the preliminary conclusion that the presence of the transgene had little effect on one non-target herbivorous insect. However, it is important to consider other fauna, including above-ground organisms (e.g., pollinators and parasitoids) and below-ground organisms (e.g., other nematodes, collembola, and earthworms) (Groot and Dicke, 2002).

CONCLUSION AND FUTURE PROSPECTS

Results obtained to date suggest that there are multiple approaches to obtaining increased resistance to diseases in transgenic plants. Effective resistance conferred by transgenes has significant potential to increase crop productivity and quality, and at the same time to allow growers to lessen their reliance upon agrochemicals to control pests and diseases.

The greatest success to date has been obtained against virus infections, with a variety of strategies yielding significant levels of resistance. Resistance caused by expression of viral sequences, antiviral antibodies, ribozymes, antiviral peptides, or dsRNA-specific nucleases is likely to be quite durable, as such resistance mechanisms are not known to have previously imposed selection pressure in virus evolution; evolution to escape resistance based on RNA silencing and sequence homology would require multiple mutations and multiple cycles of replication in plants that restrict the level of replication (Hammond et al., 1999). The durability of resistance against an individual pathogen would be further increased by combining (pyramiding) different resistance mechanisms against the same pathogen; where feasible pyramiding transgenic resistance with host resistance genes will increase both the effectiveness and durability of disease resistance (Hammond et al., 1999). Effective virus resistance against one virus will also protect against the synergism that sometimes occurs in mixed infections, provided that the transgene is not responsible for the synergism (Hammond et al., 1999).

Combining resistance against different diseases is also possible by transgenic approaches, as multiple genes can be introduced into a horticulturally-desirable genotype on a single construct. Introduction of multiple transgenes against different viruses has been demonstrated (e.g., Fuchs et al., 1997). The use of multiple transgene constructs appears to be a much more efficient means of introducing multiple re-

sistance genes into a well-adapted genotype than combination by conventional breeding and multiple cycles of selection. Linkage of the desired resistance genes is established by design, making further breeding simpler, and obviating any necessity to break possible linkage between resistance genes and potentially undesirable characters by conventional breeding. Pyramiding of resistance genes against all of the major viral, fungal, and bacterial diseases of a particular crop would benefit crop productivity and quality.

In some cropping systems it may be valuable to transform rootstocks, rather than the cultivars that are grafted as scions. Rootstock transformation has the potential to control soil-borne diseases (some fungal and bacterial diseases; nematodes; and viruses with nematode or fungal vectors) and provide protection to multiple cultivars grafted as scions. RNA silencing has also been shown to be graft-transmissible in some instances (e.g., Palauqui et al., 1997), and thus resistance in the rootstock has the potential to confer resistance in a non-transgenic scion. Crops where rootstock resistance might be applicable to multiple non-transgenic scion cultivars include roses and ornamental *Prunus*, among others.

It will be important for scientists developing transgenic plants for disease resistance to conduct thorough trials to evaluate resistance, and to include field trials in which growers can see the effectiveness of transgenic resistance in comparison to existing genetic resistance (or lack of resistance). Unless growers are convinced of the economic and ecological benefits of utilizing transgenic resistance approaches, it is unlikely that disease-resistant transgenic ornamentals will be widely grown or reach consumer acceptance, despite the promising results reported from research to date. Cooperation between scientists and growers will be necessary in order to realize the potential for combining effective resistance to multiple pathogens into horticulturally desirable cultivars of a wide range of ornamentals.

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Genetic Engineering Approach to Enhance Adventitious Root Formation of Hardwood Cuttings

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SUMMARY. Vegetative propagation by cuttings is critical to the horticultural industry, particularly the ornamental and fruit sections. This article briefly reviews the traditional approaches to improve root formation, specifically with hardwood cuttings. The focus of this review has

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been placed on the nontraditional strategies, including infection with *Agrobacterium rhizogenes* and genetic engineering with *rol* genes, or *rol*B gene alone from *A. rhizogenes* and *iaa*M gene from auxin biosynthesis genes (*iaa*M, *iaaH*) from *A. tumefaciens*. Recent results with these new strategies have shown great potential in enhancing adventitious root formation in difficult-to-root woody species. The future perspective in understanding adventitious rooting mechanisms and new strategies for improving rooting are also discussed. doi:10.1300/J411v17n01_07 *[Article copies available for a fee from The Haworth Document Delivery Service: 1-800-HAWORTH. E-mail address: <docdelivery@haworthpress.com> Website: <http://www.HaworthPress.com>*

KEYWORDS. Adventitious rooting, *Agrobacterium rhizogenes*, *A. tumefaciens*, genetic transformation, hardwood cutting, *iaa*M gene, *rol*B gene

INTRODUCTION

In horticulture, vegetative propagation by stem cuttings is the most efficient and economical method to produce a large quantity of clonal plants of proven genetic quality, particularly fruit trees, rootstocks and ornamental plants, while at the same time maintaining the superior genetic makeup of the plants. Although softwood, semi-hardwood or microcuttings can be used, propagation by hardwood cuttings offers numerous advantages over other cutting propagation methods in propagating woody plants. Collection of cuttings from fall to spring offers flexibility; rooting induction can be carried out in the greenhouse or simpler field facilities; and water, chemical, energy, and labor inputs are much less than propagation by softwood cuttings. The propagated plants are generally stronger and thus transplant easier and survive better.

A considerable amount of research effort has been devoted to understanding the genetic control and environmental interactions which determine adventitious root formation from hardwood cuttings (for reviews, see Davis et al., 1989; Davis and Haissig, 1994). A large body of literature has also been generated on inducing adventitious rooting for various hard-to-root species, but techniques that are effective across a wide range of recalicitrant taxa remain to be developed (for examples, Zaczek et al., 1997; Griffin et al., 1999). Among numerous factors that affect adventitious root initiation, plant growth hormones, particularly auxin,

have been shown to be a key player (Blakeley, 1994; Harbage and Stimart, 1996; Blazkova et al., 1997; Chao et al., 2001). Auxins are used nearly universally to stimulate root induction in both tissue culture and in conventional propagation. However, these treatments are frequently ineffective for hardwood cuttings (Macdonald, 1986; Dirr, 1998; Griffin et al., 1999), may be a consequence of changes in endogenous auxin content, especially free indole acetic acid (IAA) (Le, 1985; Alvarez et al., 1989), auxin metabolism (Battacharya et al., 1975; Pythoud and Buchala, 1989), or target cell sensitivity to exogenously applied auxin (James, 1983a, b) during stem maturation. However, all of these explanations may be interrelated, because the level of free IAA can be regulated in several ways, including by degradation, transport, synthesis, and inactivation through conjugation (altered auxin metabolism).

The physiological and nutritional conditions of stock plants and environmental factors can also influence the competence of cuttings for root formation (e.g., Blanzich, 1989; Davis, 1989; Griffin et al., 1999). Various techniques such as etiolation, pruning, hedging, and grafting mature trees onto juvenile rootstocks have been employed to improve rooting in some woody plants (e.g., Griffin et al., 1999; Zaczek et al., 1997). However, the positive effects observed may be mediated by changes in auxin content or perception (sensitivity).

One of the reasons why it is difficult to pinpoint the factors responsible for the lower auxin responsiveness of hardwood stem cuttings relates to a lack of suitable experimental material. The ideal comparison would be between two plants that have a distinct phenotypical contrast in root initiation response but have little or no difference in genotype; and these plant materials can be an inbred line that roots easily and a single mutation form that does not root (Hackett and Murray, 1994). Such materials are largely available in *Arabidopsis* or other plants in the form of mutants. However, very few woody plants have been found to exhibit the mutant form in rooting and most woody plants are genetically highly heterozygous (Dai et al., 2004). Lund et al. (1996) studied mutant tobacco (*rac*) which does not root and found that the *rac* mutant tobacco undergoes cell division but does not initiate adventitious roots in response to exogenous auxin. Their further research showed that the mutant plant blocks auxin activation of the *HRGPnT3* promoter but not of *iaa4/5* or *gh3* indicating that the activation of specific auxin signal transduction pathways was compromised in the mutant (Lund et al., 1997). Recently, Ludwig-Müller (2003) studied rooting potential in relation with peroxidase activity in one easy-to-root and one difficult-to-root species of the ornamental plant *Grevillea*. The research showed

much higher total peroxidase activity at the time point of adventitious root formation in the hard-to-root species *G. petrophioides* than in the easy-to-root *G. rondea*. However, since these are two different species and in vitro shoot segments were used, it is hard to conclude whether the high peroxidase activity actually caused rooting difficulty.

ROOTING INDUCTION BY INFECTION OF AGROBACTERIUM RHIZOGENES

When manipulating the rooting capability with traditional techniques of application of exogenous hormones, the uptake, transport, and compartmentation of IAA are difficult to control. As a consequence, assumptions have to be made to interpret the data obtained, and the exact mechanism of rooting induction is difficult to identify. Biotechnological approaches may provide powerful tools to facilitate understanding of root formation and development, and may offer economically feasible methods for propagating difficult-to-root woody species. One of the techniques is to induce root formation by infecting stem cuttings with *Agrobacterium rhizogenes*. *A. rhizogenes* is a common soil-borne bacterium that has been shown to cause secondary root proliferation, or "hairy roots" at the site of infection (Riker, 1930). But use of this organism to intentionally induce rooting in woody plants has been relatively recent. Strobel and Nachmias (1985) first demonstrated that *A. rhizogenes* mutant strain MT232 enhanced rooting in almond bare root seedlings. This technique has been extended to several fruit trees and forest species, including olive cuttings (Strobel et al., 1988), difficult-to-root apple microcuttings (Patena et al., 1988), chestnut cuttings which otherwise would be impossible to root (Rinallo and Mariotti, 1993), *Eucalyptus* species (Aron, 1992), coniferous species *Pinus montocola*, *P. Bankiana*, and *Larx laricina* (McAfee et al., 1993), willow (Hauth and Beiderbeck, 1992), *Alnus* species (Savka et al., 1992), *Rosa hybrida* (Salm et al., 1996), and *Populus* (Tzfira et al., 1998, 1999).

The mechanism underlying this rooting induction is related to *Agrobacterium* biology, and is attributed to transfer of part of the DNA from its root-inducing (Ri) plasmid to the plant nuclear genome (White et al., 1985). The induction of the "hairy roots" phenotype is largely due to the *rol*B gene in the Ri plasmid, although other genes, such as *rol*A and *rol*C, may also contribute (Cardarelli et al., 1987a, b; Spena et al., 1987; Vilaine et al., 1987; Schmulling et al., 1988; Capone et al., 1989). The symptoms observed following *A. rhizogenes* infection are suggestive of

auxin effects resulting mainly from an increase in cellular sensitivity to auxin, or from an altered auxin metabolism, rather than actual auxin production. The sensitivity of the tips of Ri-induced roots to exogenous auxin was 100-1000 times higher than that of regular roots (Shen et al., 1988, 1990; Walden et al., 1993). Estruch et al. (1991) have shown that the *rol*B-encoded protein hydrolyzes the indole glucosides and releases IAA from its glucoside conjugates. This could be the first of a cascade of events leading to an alteration of plant physiological and developmental processes. The RolB protein has been located on the membrane (Filippini et al., 1996) and is involved in auxin perception (Maurel et al., 1994).

ROOTING INDUCTION BY TRANSFER OF ISOLATED ROL GENES

Understanding of the mechanism of root induction with *A. rhizogenes* infection has resulted in another method of promoting rooting, i.e., transformation of plants with isolated *rol*ABC genes, or only the *rol*B gene. Successful root induction by transfer of *rol* genes was first demonstrated in several herbaceous plants, including cucumber, tobacco, potato, lettuce, etc. (Tepfer, 1984, Cardarelli et al., 1987a; Vilaine et al., 1987; Capone et al., 1989; Curtis, 1996). Röder et al. (1994) showed that the *rol*B gene under control of the tetracycline inducible promoter significantly increased the cellular sensitivity to applied auxin and rooting induction in tobacco plants. Rugini et al. (1991) reported that *rol*ABC transformed kiwi (*Actinidia chinesis*) showed typical "hairy roots" phenotypes in vitro. Leaf discs and leafy microcuttings from the transgenic plants showed 100% rooting in hormone free medium, while controls showed only 30-40% rooting (Rugini et al., 1991). Rooting induction following *rolABC* transformation has also been reported in *Populus* (Tzfira et al., 1998, 1999).

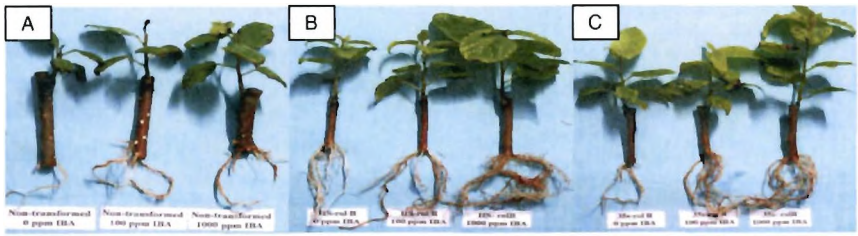
The usefulness of the *rolB* gene for rooting induction in fruit trees has been evaluated extensively in the lab of Dr. Margareta Welander using dwarfing apple rootstocks. In the in vitro rooting test, transgenic clones of M26 rootstock rooted at frequencies of 83-100% on the hormone free rooting medium, while only 1% of the control rooted (Welander et al., 1998; Welander and Zhu, 2000). Furthermore, the number of roots produced was also increased from 3.5 to 9 (Zhu et al., 2001). Although root length and root morphology did not differ between the transgenic clones and the untransformed control plants, some of the transgenic plants

showed a significant reduced node number and internode length compared with the control plants (Zhu et al., 2001). Similar work has also been done with apple rootstock M.9:29 (Welander and Zhu, 2000), and Jork 9 (Sedira et al., 2001). Because rooting of hardwood cuttings was not tested, the question as to whether this gene can overcome the rooting difficulties associated with hardwood cuttings remains unanswered.

Since the increases in auxin sensitivity conferred by the RolB protein are not restricted to the process of adventitious root induction, expression of the *rolB* gene under the control of a constitutive promoter, such as the CaMV 35S promoter or its own promoter, may adversely influence overall plant growth and development (Zhu et al., 2001). One solution may be to express the *rol*B gene under the control of an inducible promoter. To test this possibility, Dai et al. (2004) transformed a hybrid aspen (*Populus canescens* × *P. grandidentata)* with a *rolB-GUS* fusion protein under the control of a heat shock promoter. Aspen trees root easily with microcuttings and greenwood cuttings, but are very difficult to root with hardwood cuttings, therefore, offering excellent opportunity to study the mechanism of adventitious rooting. The fusion of the *rol*B gene with the *uid*A (GUS) gene downstream allows easy monitoring of gene expression.

Unlike previously reported results (Rugini et al., 1991; Welander et al., 1998; Sedira et al., 2001; Zhu et al., 2001), Dai et al. (2004) tested rooting in hardwood cuttings. Six weeks after the hardwood cuttings were quick-dipped in IBA solution (0, 0.49, or 4.9 μM), cuttings from the *rol*B transformants exhibited significantly higher rooting percentages than those from non-transformed plants. Among the *rolB* transformants, the heat shock-*rol*B-transformed plants gave significantly higher rooting rates than the CaMV 35S-*rol*B transformed plants (Dai et al., 2004). Individual transgenic clones within each gene construct also showed significant differences with a range of 12.5% to 88.9% for 35S-*rol*B-transformed plants, and a range of 20% to 100% for heat shock-*rol*B-transformed plants. Heat treatment (42°C for 2 h prior to IBA treatment) significantly increased the rooting rate when treated cuttings were exposed to 0.49 or 4.9 μM IBA. High concentrations of IBA did not result in significant difference in rooting frequency, but yielded more roots (Figure 1). This research demonstrated that transfer of the *rol*B gene could improve the rooting of hardwood cuttings of aspen trees, and that the heat shock promoter is superior to the 35S promoter in this context (Dai et al., 2004). The *rol*B product appears to respond to not only endogenous auxin, with rooting frequencies being improved even without auxin application, but also to exogenous auxin

FIGURE 1. Adventitious roots produced from hardwood cuttings of control (A), heat shock *rol*B transformed plants (B) and CaMV-*rol*B transformed plants (C) after a quick-dip in indole-butyric acid solution (from left to right) at 0, 0.49, and 4.9 μM.



because of the positive correlation between the amount of induced roots and the concentration of exogenous auxin.

The research by Dai et al. (2004) created a gain-of-function woody plant that has contrasting phenotypes in rooting ability with hardwood cuttings (Hackett and Murray, 1994). These plants can serve as the equivalent of the mutant form of the wild type which exhibits low rooting with hardwood cuttings. This "mutant" form, coupled with the easy-to-monitor *rol*B-GUS fusion gene under a constitutive CaMV 35S and an inducible heat shock promoter should greatly facilitate dissection of the rooting initiation process and help us to understand the mechanism of rooting induction in hardwood cuttings. Further research will be needed to measure the free and conjugated auxin contents and correlate it with the rooting frequency and the rooting quantity in order to elucidate the actual rooting mechanism. Also, although this research showed that the hardwood cuttings of juvenile trees can be rooted, it is unknown whether hardwood cuttings of mature trees can be rooted.

ROOTING INDUCTION BY TRANSFER OF AUXIN SYNTHASE GENES

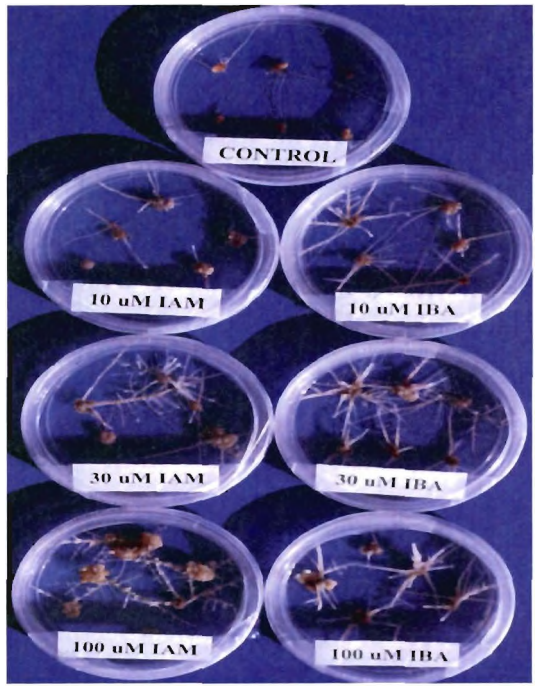
The auxin synthase genes, *iaa*M and *iaa*H, from *A. tumefaciens* have also been transferred into plants to induce root formation. The gene *iaa*M encodes tryptophan monooxygenase which converts tryptophan to indoleacetamide (IAM); the second gene, *iaa*H, encodes indoleacetamide hydrolase which converts IAM to IAA (Schroder et al., 1984; Thomashow et al., 1984, 1986). Both genes have been separately trans-

ferred to petunia, and the *iaa*M gene alone was shown to be sufficient for root induction (Klee et al., 1987). The iaaM transformants produced a 10-fold excess of IAA, and root primordia were observed in the stems of these plants, but the primordia did not develop into normal roots. These results strongly suggest that the high level of auxin is required for root initiation, but continued high auxin levels are inhibitory to root development (Klee et al., 1987). The transgenic petunia with *iaa*H gene showed normal phenotypes without causing root primordial formation, but the plants acquired an ability to utilize low concentrations of NAM (naphthalene-3-acetamide) as an auxin and formed roots, while the non-transformed control plants did not. When both *iaa*M and *iaa*H genes were transferred to tobacco, the transgenic plants also increased free auxin and conjugated IAA (Sitbon et al., 1992), and the adventitious root formation was prolific along the stem of transgenic plants (Sitbon et al., 1992). These data strongly suggest that the internal IAA level is a major factor in controlling root formation. These genes, however, have not been tested for root induction in woody plants.

The *iaaM* and *iaaH* genes have previously been used for aspen transformation; however, the goals of the research were to alter growth and wood formation, not to improve the adventitious root formation (Tuominen et al., 1995, 2000). More recently, Cheng's lab transformed an aspen hybrid with the *iaa*M gene under the CaMV 35S promoter and three inducible promoters: a heat shock promoter, wounding inducible promoter, and the auxin inducible GH3 promoter (Bosela and Cheng, unpublished). Preliminary research established that exogenous IAM was effective for root induction in aspen *in vitro*. Using leaf explants approximately 10-30 higher concentrations of IAM were required to induce the same degree of rooting as was observed for the IAA and IBA treatements. However, using callus explants the efficacy of IAM appeared superior to that of IAM and approximately equivalent to that of IBA (Figure 2). In addition, the 35S-*iaaM* transformants exhibited several phenotypes indicative of auxin overproduction including spontaneous root formation from callus and lower frequencies of adventitious shoot regeneration, leaf hyponasty (primarily *in vitro*), anomalous leaf venation patterns and reductions in leaf size, and increased levels of apical dominance following *ex vitro* rooting (Figure 3). However, the shoot lines regenerated did not produce spontaneous adventitious roots and stem cuttings from the transformants have not been tested for improved rooting ability to date.

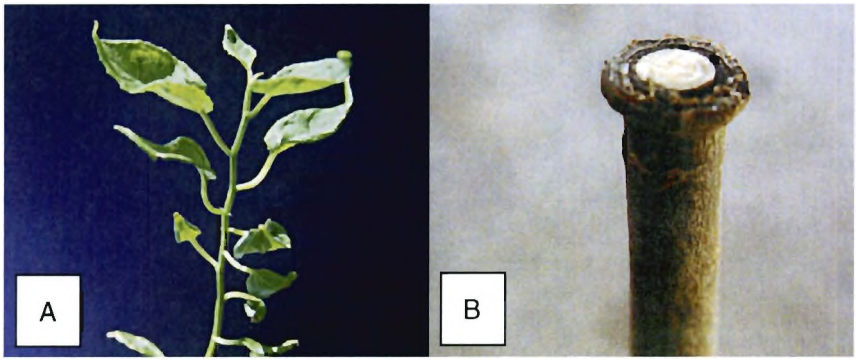
Full scale rooting tests with the transformants containing inducible promoters are also wanting, but promising results have been obtained in

FIGURE. 2. The effects of IBA and IAM on root induction from hybrid aspen (*P. canescens* × *P. grandidentata*) callus explants. The callus explants were cultured for 3-4 wks in the dark on MS medium with IBA or IAM at the concentrations indicated.



preliminary tests. In one experiment with semi-hardwood cuttings, control plants rooted at 52%, while a mean rooting frequency of 84% was obtained for the HS-iaaM-transgenic plants with some lines rooting at 100% (Cheng and Osburn, unpublished). In a second experiment employing hardwood cuttings from the wounding inducible-*iaaM* transformants, physical wounding of the cuttings by slicing with a razor blade nearly doubled the rooting frequency (26 to 48%), but subsequent treatment of the cuttings with salicylic acid, a chemical that has been shown to inhibit promoter activiation, blocked the increase in rooting frequency, with only 21% of the cuttings rooting. However, the rooting frequencies of the controls were not significantly affected by either treatment (Bosela and Cheng, unpublished). Collectively, these results suggests that the *iaa*M gene may also prove effective for adventitious

FIGURE 3. Aberrent phenotypes indicative of auxin overproduction in hybrid aspen transformed with a *CaMV35S-iaaM* construct. Several of the transformants exhibited spontaneous leaf hyponasty in vitro or upon ex vitro rooting (A). Following transfer to the grenhouse many of the transformants exhibited severe apical dominance. Several failed to regrow following decapitation. The transformant shown (B) also produced callus from margin of the pruning cut indicative of abnormal (transgene-mediated) auxin biosynthesis.



root induction in woody cuttings. Most importantly, all of the transgenics that employed inducible promoters were morphologically indistinguishable from control plants, indicating that using an inducible promoter driving the *iaa*M gene is a feasible strategy to induce root formation without causing morphological abnormalities that have been shown in annual plants under a constitutive promoter control.

PERSPECTIVES

Propagation by hardwood cuttings is the most economical means of vegetative propagation for producing large quantities of woody plants without altering the genetic constitution. Previous research in academia and private nurseries using traditional techniques has facilitated the hardwood cutting propagation of several woody plants. Unfortunately, these traditional strategies are not effective in many other species, making propagation of those plants more expensive and resource-consumptive. Recent research in genetic engineering with *rol*B and *iaa*M genes has shown great potential to improve the rooting of difficult-to-root species. Transgenic aspen with an improved rooting capability can serve as

a form of the gain-of-function mutant of the non-transgenic aspen. By further analyzing the *rol*B and *iaa*M transgenic plants at the physiological, anatomical and molecular level, scientists can better understand the adventitious rooting process, and pinpoint some critical control elements. Recent availability of the whole genome sequence of a woody plant, *Populus*, will no doubt assist in further understanding the mechanisms of adventitious root formation, consequently offering additional strategies for improving adventitious rooting of hardwood cuttings of difficult-to-root species.

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Flowering

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SUMMARY. The transition from vegetative growth to flowering in higher plants is controlled by environmental conditions and developmental regulation. The complexity of this transition is created by an intricate network of signalling pathways that monitor the developmental state of the plant as well as environmental conditions (light, temperature, and photoperiod). *Arabidopsis thaliana* (Brassicaceae family) is an excellent model system in which to approach this complexity. Currently four genetic pathways have been identified: the light-dependent, the autonomous, the vernalization and the gibberellin pathway, all integrated by the function of regulatory genes at the integration pathway. The integrated signal of the floral induction is transmitted to the floral meristem identity genes and floral morphogenesis is performed. The promotion of flowering, by both increasing the number of flowers and advancing time of flowering, as well as creating novelty in the flower structure, are major desirable traits in ornamental plant breeding. The possibility of using molecular techniques to transfer genes in major ornamentals has greatly

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increased the resources available to plant breeders. Moreover, native genes can be over expressed or suppressed. The recent explanations of the mechanisms of flowering in *Arabidopsis* have led new sets of genes to be available. These include genes which affect flowering and flower architecture. In a few cases these genes can change the flowering time of species unrelated to the plant from which they were isolated. doi:10.1300/J411v17n01_08

KEYWORDS. *Arabidopsis thaliana*, flowering-time genes, meristem-identity genes, organ-identity genes, *rol* genes

INTRODUCTION

The transition from vegetative growth to flowering in higher plants is controlled by environmental conditions and developmental regulation. Flowering was the first plant photoperiodic response to be discovered. The first good experimental evidence was presented during the early years of the twentieth century. Tournois (1912) attempted to explain why hemp flowers vigorously if planted early in the spring but remains in a vegetative state if planted in late spring or summer. Garner and Allard (1920) demonstrated that in soybean plants there is a seasonal timing mechanism that regulates the first open blossom. The authors developed terms necessary to describe the photoperiodic response of flowering such as short-day and long-day plants. In 1936 Cajlachjan, working on floral initiation, coined the term *florigen* for the unidentified hypothetical flowering hormone thought to be present in photo induced leaves and plants (Devlin and Witham, 1983).

In the last 15 years, knowledge of the molecular and genetic mechanisms that underline floral induction, floral pattering and floral organ identity has been exploded. Elucidation of basic mechanisms has derived mainly from work in three dicot species: *Antirrhinum majus*, *Arabidopsis thaliana* and *Petunia hybrida.* Although Antirrhinum and Petunia have contributed fundamental breakthroughs to our understanding of flower development, it is from *Arabidopsis* that the most detailed and comprehensive picture of the molecular mechanisms underlying flower development has been obtained (Bernier, 1988; Yanofsky, 1995; Koornneef et al., 1998). For *Arabidopsis*, its convenient genetics and

small genome allowed genes to be cloned based on their map position. For *Anthirrinum* (snapdragon), active transposable elements had already been cloned, and this coupled with a large series of characterized flower mutants was the basis of cloning genes with unstable transposon-induced mutants. At present the full complexity of the flowering network can only be approached in *Arabidopsis* where the necessary tools are available, and extensive efforts are being made to describe related pathways (Araki, 2001; Muradov et al., 2002; Komeda, 2004).

MOLECULAR BASIS OF FLOWERING

The transition from vegetative to reproductive phase, the floral transition, is the most dramatic phase change in plant development (Figure 1). The complexity of this transition is created by an intricate network of signalling pathways that monitor the developmental state of the plant as well as environmental conditions (light, temperature, photoperiod). *Arabidopsis thaliana* (Brassicaceae family) is an excellent model system in which to approach this complexity, because it responds to many of the environmental conditions that control flowering in other species: all ecotypes are long-day plants (LD) that flower under long rather than short days, and are annual plants. Intensive genetic analysis of flowering-time mutants (late and early flowering) has identified most of the genes that in *Arabidopsis* regulate flowering. Currently four genetic pathways have been identified: the light-dependent, the autonomous, the vernalization and the gibberellin pathway, all integrated by the function of regulatory genes at the integration pathway (Muradov et al., 2002). The integrated signal of the floral induction is transmitted to the floral meristem identity genes and floral morphogenesis is performed (Jack, 2004). This convergence of pathways on a common set of genes may enable the integration of different responses, so that the plant can produce a coordinated flowering response under conditions in which multiple environmental and developmental parameters are changing simultaneously (Boss et al., 2004). Also, genetic analysis of *Arabidopsis* varieties, showing natural variation in flowering time, has demonstrated how the activity of these pathways can be altered in nature and how balancing the effects of different environmental stimuli on flowering time is important in plants adapting to growth in different geographical locations (Muradov et al., 2002).

FLOWER MORPHOLOGY IS SPECIFIED BY FLORAL ORGAN IDENTITY GENES

The beautiful diversity in flower architecture is due to changes in four organ types: sepals, petals, stamens and carpels (Figure 2). The flower organogenetic process is under genetic control and mutants that

FIGURE 1

FIGURE 2

disrupt the normal processes are well known (Meyerowitz et al., 1989). It was argued that the normal function of such homeotic mutant genes was to define the identity of the organs. From the early 1980s, new technology allowed the responsible genes to be cloned, their molecular nature to be deduced and their expression patterns to be mapped (Smyth, 2005).

The first homeotic gene to be cloned was the *DEFICIENS* gene of Anthirrinum (Sommer et al., 1990), soon followed by the *AGAMOUS* gene of *Arabidopsis* (Yanofsky et al., 1990). In each case, the genes encoded transcription factors related to several already known in humans and yeast and together were called the MADS family. Recent evidence suggests that MADS proteins function together in complexes larger than a dimer. The "quartet" model postulates that tetramers of MADS proteins specify floral organ identity (Theissen, 2001). The organ identity genes belong to the MADS family with the exception of the A class gene *APETALA2*. *AP2* encodes a putative transcription factor that is a member of a small plant-specific gene family; its function is regulated posttrascriptionally by a specific microRNA miR172 by inhibition of translation (Chen, 2004).

The action of organ identity genes was initially summarized in the ABC model (Coen and Meyerowitz, 1991). The A class genes (*AP1* and *AP2*) specify the identity of sepals and petals that develop in whorls 1 and 2; moreover, they repress C class activity in whorls 1 and 2. The B class genes *AP3* and *PISTILLATA* (*PI*) are required to specify the identity of petals in whorl 2 (A + B petals) and stamens in whorl 3 McGonigle et al., 1996). The C class gene *AGAMOUS* (*AG*) is necessary to specify the identity of whorl 3 stamens (B + C stamens) and whorl 4 carpels. The second major function of C class is to repress A class activity in whorls 3 and 4. Orthologues of ABC genes have been isolated from *Antirrhinum* (Ma and dePamphilis, 2000), petunia (van der Krol and Chua, 1993) and gerbera (Yu et al., 1999), and homeotic mutants of *Primula* are currently being studied (Webster and Gilmartin, 2003). Recent studies have refined the ABC model and extended it to many other species (Espinosa-Soto et al., 2004). The discovery of another set of MADS genes, *SEPALLATA1*, *2* and *3* (Pelaz et al., 2000), redundantly involved in defining the petal, stamen and carpel domain of the flower primordium, had led to a revision of the ABC model. The *SEP* genes are referred to as E class genes. The "ABCE" model (Jack, 2004) postulates that sepals are specified by A activity alone (tetramer of MADS proteins AP1-AP1), petals by A + B + E (tetramer of MADS proteins AP1-SEP-AP3-PI), stamens by B + C + E (tetramer of MADS

proteins AP3-PI-AG-SEP) and carpels by C + E (tetramer of MADS proteins AG-AG-SEP-SEP). There is another set of genes, initially characterized in petunia that was previously named D class genes (Colombo et al., 1995). The two genes *FBP7* and *FBP11* function to specify placenta and ovule identity in petunia; the *Arabidopsis* ortholog genes *STK*, *SHP1* and *SHP2* (Pinyopich et al., 2003) are considered D class genes too, because they specify ovule identity in this species.

The failure of floral organs to develop with the correct identity in A, B, C, D and E class mutants demonstrates that the ABCDE genes are necessary to specify floral organ identity (Jack, 2004). Genes required for the establishment of floral-organ polarity and floral symmetry have been studied in *Antirrhinum* (Zik and Irish, 2003).

FLOWER MERISTEM IDENTITY GENES

Genes involved in the determination of floral meristems were first identified from mutants in which flowers were replaced by shoots with inflorescence-like properties. The *FLORICAULA* gene from *Anthirrhinum* (Coen et al., 1990) and *LEAFY*, its ortholog from *Arabidopsis* (Weigel et al., 1992), encode transcriptor factors that impose a floral identity on primordial that arise from the flank of shoot apical meristems, after their floral induction. The interactions between *LFY* and three MADS-box transcription factors, *AP1*, *CAL* and *FUL* repress *TERMINAL FLOWER 1* (*TFL1*), which maintains the apical meristem (growing point at tip of stem) of the inflorescence (arrangement of flowers on a floral stem) in an indeterminate state, with an unlimited growth (Boss et al., 2004). Constitutive expression of the *LFY* transgene accelerates the time to flowering. In these plants *AP1* expression was enhanced in floral primordial and also detected in leaf primordial (Liljegren et al., 1999). Thus, *LFY* induces the expression of *AP1* (Wagner et al., 1999).

Orthologues of the meristem identity genes *LFY*, *AP1* and *TLF1* (*FLO*, *SQUA* and *CEN*), as well as their co-regulator *UNUSUAL FLORAL ORGANS* (*UFO*), have been isolated from *Antirrhinum*. During early stages of flower development, both *LFY* and *AP1* are expressed throughout the floral meristem (Gustafson-Brown et al., 1994). *LFY* stimulates floral organ identity genes by functioning together with the coactivator stem cell-promoting gene *WUSHEL* (*WUS*) to activate the C class gene *AG* and together with *UFO* to activate the B class gene *AP3*. *AP1* to-

gether with *UFO* has a positive regulatory effect on B class gene *AP3* (Jack, 2004).

PATHWAYS CONTROLLING FLOWERING TIME IN ARABIDOPSIS

In *Arabidopsis*, four different pathways (photoperiodic, vernalization, autonomous and gibberellin) control flowering time and they are integrated between them at the point of the integration pathway. The gene *FLOWERING LOCUS C* (*FLC*) encodes a MADS box transcription factor, expressed predominantly in shoot and root apices, that acts to quantitatively repress flowering through repression of the floral pathways integrators *FT*, *LFY* and *SOC1*. Several flowering pathways converge to regulate the level of *FLC* expression. The floral repressor *FLC* plays a central role in vernalization requirement and response and it is negatively regulated by the autonomous pathway. Moreover, *FLC* regulates the level of expression of the gene *SOC1*, which was isolated as a suppressor of *CO* in the photoperiod pathways . In a recent paper (Sung et al., 2003) it is proposed that the *EMBRYONIC FLOWER* (*EMF*) genes may function independent of a regular flowering pathway (described below) and are developmental repressors that allow plants to stay at a vegetative state. A new mechanism based on chromatin regulation (Wagner, 2003) is proposed for the EMF-mediated floral repression (Moon et al., 2003). The gene *EARLY BOLTING IN SHORT DAYS* (*EBS*) is part of a transcriptional repressor complex that modulates chromatin structure and it is required to repress *FT* expression and the initiation of flowering in short days (Piñeiro et al., 2003).

The Photoperiod Pathway

One of the most important controlling flowering time is the photoperiod. Molecular-genetic approaches have identified genes required for the day length response in *Arabidopsis*. Some of these genes encode regulatory proteins specifically involved in the regulation of flowering, while others encode components of light signal transduction pathways or are involved in circadian clock function (Reeves and Coupland, 2000). Red light is accepted by the phytochrome proteins, which are encoded by *PHYA* to *PHYE* genes. Blue light receptors are named cryptochrome proteins and are encoded by the *CRY1* and *CRY2* genes (Levy and Dean, 1998). Under red light *PHYB* functions by repression of the

CO function (Putteril et al., 1995). Under blue light *CRY2* inhibits *PHYB* and induces flowering (Lin, 2002). *CRY1* cooperates with *CRY2* to repress the function of *CO* and *GI* genes. The genes *LHY*, *CCA1*, *ELF3* and *TOC1* process the physical signal which is transmitted to the *GIGANTEA* (*GI*) gene and the resultant signal activates the *CO* gene. The *GI* protein is expressed with circadian rhythmicity. The co mutants are late flowering under LD (Koorneef et al., 1991). The *CO* gene has homology with to the Zn-finger domain proteins of transcriptional factor (Putteril et al., 1995). The quantity of the *CO* message was proportional to the earliness of flowering in transgenic plants and seems to be rate limiting for flowering. *CO* promotes flowering by activating the expression of *FT* and *SOC1* (Samach et al., 2000).

The Vernalization Pathway

Some plants need exposure to low temperature for several weeks to flower. The genetic control of vernalization was elucidated by crossing winter annual varieties that require vernalization with summer annual varieties that do not (Michaels and Amansino, 2000). Several genes have been cloned. The *FRIGIDA* (*FRI*) gene is a positive regulator of the *FLC* repressor for flowering. The strong up-regulation of *FLC* by *FRI* creates a requirement for vernalization. The *Arabidopsis* early flowering ecotypes, such as Columbia Landsberg erecta, have mutations in the *FRI* gene (Johanson et al., 2000). The molecular analysis of *FRI/FLC* suggests that vernalization acts by reducing *FLC* expression in response to extended exposure to cold. There are two genes identified in the process of vernalization, *VERNALIZATION* (*VRN*) 1 and *VRN2* (Gendall et al., 2001) with a repressible role on gene *FLC*; *VRN2* codes for a protein with homology to Polycomb group (PcG) proteins and it is postulated that *VRN2* may function to keep the FLC-chromatin state for down-regulation. Another study has identified the *VERNALIZATION INDEPENDENCE* genes (*VIP1* to *VIP7*). The *VIP4* was cloned and encodes a PcG protein (Zhang and van Nocker, 2002). The gene *HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES 1* (*HOS1*) appears to regulate the response to cold; it encodes a RING finger protein that may serve as a ubiquitin ligase and be a negative regulator for cold signalling (Muradov et al., 2002).

The Autonomous Pathway

The autonomous pathway was identified via a group of mutants that are late flowering under all photoperiods and are highly responsive to

vernalization (Muradov et al., 2002). They all contain higher levels of *FLC* mRNA than do wild type plants or late-flowering mutants. Therefore, in wild-type plants this pathway can be considered to negatively regulate *FLC* expression. Several of the genes within this pathway have been cloned. *FCA*, *LUMINIDEPENDENS* (*LD*) and *FPA* are maximal expressed at the apex of the plant and inflorescences and are low expressed in mature leaves and roots. The way in which their respective proteins regulate *FLC* expression is not known yet. Another gene, *EARLY IN SHORT DAYS 4* (*ESD4*), is closely associated with the regulation of *FLC* in the autonomous pathway (Reeves et al., 2002).

The Gibberellin Pathway

Gibberellins (GAs) are plant growth regulators important in many aspects of plant growth and in *Arabidopsis* they promote flowering (Wilson et al., 1992). Several mutations affecting GA biosynthesis have been identified and the mutant genes cloned. GAs promote flowering by increasing the transcriptional activity of the floral meristem identity gene *LEAFY* (Blazquez et al., 1998). With exposure to LDs increased *GA* content in the leaf with its subsequent transport to the shoot apex could represent one component in the regulation of the vegetative to floral transition at the apex of the LD plant *Lolium temulentum* (King and Evans, 2003). On the other end, GAs inhibit flowering in grapevine, champagne cultivar Pinot Meunier (Boss and Thomas, 2002).

The Integration Pathway

Studies in *Arabidopsis* have led to the identification of components within individual signaling pathways that affect flowering, and to their positioning within molecular hierarchies. Furthermore, distinct signaling pathways are known to converge on the activation of the same flowering-time genes. This convergence of pathways on a common set of genes may enable the integration of different responses, so that the plant can produce a coordinated flowering response under conditions in which multiple environmental parameters are changing simultaneously (Mouradov et al., 2002). The four genetic pathways all converge to induce the flower meristem genes. *LFY* regulates the transcription of *AP1*, *AP3* and *AG* and gives the floral identity to the meristem. Thus, *LFY* is the switch of floral development but is not the floral evocation. Another gene, FT, is the important switch of the floral evocation (Kardailsky et al., 1999; Kobayashi et al., 1999). When *FT* gene was constitutively ex-

pressed, transgenic plants flowered very early irrespective of photoperiod. *FT* has high homology to *TFL1* and has six members in the *Arabidopsis* genome. The protein encoded by the *FT* gene seems to be homologous to the phosphatidylethanolamine-binding protein (PE-BP) and belongs to the same group of *TLF1* and *CEN* genes (Bradley et al., 1997). *FT* is expressed maximum at the onset of floral induction, it is positively regulated by *LD* condition and it requires the *CO* gene for positive regulation in LD condition. The *CO* gene directly interacts with the *FT* gene (Samach et al., 2000). *FT* appears to be regulated also by *FLC*, a key of the autonomous and vernalization pathway. The expression of *FT* and *SOC1* is controlled positively not only by light pathway, by also by the autonomous pathway acting through *FLC* negatively. The signal of the vernalization increases *SOC1* expression presumably via reduction of *FLC* levels and *SOC1* can be up-regulated by a gibberellin pathway as well. *SOC1* and *FT* act as the convergence of all the pathways.

APPLICABILITY OF FLOWERING GENES IN FLORICULTURE

The promotion of flowering, by both increasing the number of flowers and advancing time of flowering, as well as creating novelty in the flower structure are major desirable traits in ornamental plant breeding. With classical breeding, the available gene pool for new traits is limited to the genetic background of the parents. In addition, the high heterozygosity in many valuable floricultural crops, such as rose, chrysanthemum and carnation, and the limited knowledge of their genetic background, hamper advances in breeding (Vainstein, 2002). Moreover, many varieties of ornamental plants are sterile. Biotechnological techniques, such as genetic engineering, are a useful addition to classical breeding for the introduction of novel traits into plants and the production of new varieties. Increases in both the efficiency of transformation procedures and the regeneration of transgenic plants have enabled the improvement of ornamental varieties by molecular breeding (Tanaka et al., 2005).

The possibility of using molecular techniques to transfer genes, not only within and between plant species, but also from other kingdoms (from bacteria and virus), has greatly increased the resources available to plant breeders. Moreover, native genes can be overexpressed or suppressed. Recent insights into the mechanisms of flowering in *Arabid-*

opsis have led new sets of genes to be available (Table 1). These include genes which affect flowering and flower architecture. In a few cases these genes can change the flowering time of species unrelated to the plant from which they were isolated. The flowering-time gene *CONSTANS* (*CO*), which has been cloned from *A. thaliana* Landsberg erecta (Putteril et al., 1993), was transferred in the genome of the ornamental species *Osteospermum ecklonis* in order to alter the onset of flowering. The *CO* cDNA, kindly provided by Dr. George Coupland from John Innes Centre, Norwich, UK was cloned in the expression vector pGREEN under the control of the constitutive promoter 35S. Genetic transformation of leaf tissue was performed using *Agrobacterium tumefaciens* strain AGL1. *CO* constitutive expression was detected in leaf tissue of a checked transgenic clone. In this work we showed that the early flowering phenotype reported over-expressing *CO* gene in *Arabidopsis* (Putterill et al., 1995) was not observed in *35SCO Osteospermum* plants cultured in *in vivo* conditions (Giovannini et al., 2002). Most probably in this species the transition from vegetative to reproductive phase is under the control of a different genetic pathway (e.g., vernalisation pathway). The precocious flowering of transgenic plants ectopically expressing the floral meristem-identity genes suggested a potential for application in biotechnology. The constitutive expression of the gene *LFY* promoted flower initiation in hybrid aspen (Coupland, 1995) and citrus (Peña et al., 2001), shortening tree juvenile phase; furthermore, *AP1* was as efficient as *LFY* in the initiation of flowers in citrus and did not produce any severe developmental abnormality, whereas *AP1* did not seem to have any effect in hybrid aspen (Weigel and Nilsson, 1995) and in *O. ecklonis* (Giovannini, unpublished results). The expression of genes transferred across genera is not always predictable and requires considerable trial and error to arrive at a cost-effective conclusion. Moreover, ornamentals have a rather small market for each species in comparison to major food crops. Orthologous genes involved in the flowering transition have not been isolated yet in ornamental species.

The Effect of rol Genes on Flowering

One of the most effective approaches to manipulate plant growth and development is by genetic modification of hormonal balance. The *rol* genes of the Ri plasmid of *Agrobacterium rhizogenes* are bacterial genes that modify plant hormone biosynthesis and/or perception and when they are expressed in plants alter several of the plant's developmental processes and affect their architecture (Gaudin et al., 1994). Al-

TABLE 1. Representative genes of the different classes that participate to flower induction and development in *Arabidopsis thaliana*.

Gene Class	Abbreviation	Gene name	Gene family	References
Organ identity	*AP2*	*APETALA2*	Transcription factor	Chen, 2004
	AP3	*APETALA3*	MADS box	Bowman et al., 1993
	PI	*PISTILLATA*	MADS box	Bowman et al., 1993
	AG	*AGAMOUS*	MADS box	Yanofsky et al., 1990
	SUP	*SUPERMAN*	MADS box	Bowman et al., 1993
Shoot meristem identity	*TFL1*	*TERMINAL FLOWER1*	Similar to animal Raf1 kinase inhibitors	Bradley et al., 1996
Floral meristem identity	*LFY*	*LEAFY*	MYB transcription factor	Weigel et al., 1992
	EMF2	*EMBRYONIC FLOWER2*	PcG	Sung et al., 2003
	CAL	*CAULIFLOWER*	MADS box	Bowman et al., 1993
	FUL	*FRUITFULL*	MADS box	Gu et al., 1998
	AP1	*APETALA 1*	MADS box	Bowman et al., 1993
	UFO	*UNUSUALFLORALORGANS*	F-box	Simon et al., 1994
	WUS	*WUSCHEL*	Homeodomain transcription factor	Mayer et al., 1998
Flowering time	*CO*	*CONSTANS*	Transcription factor	Putteril et al., 1995
	FRI	*FRIGIDA*		Johanson et al., 2000
	VIP	*VERNALIZATION INDEPENDENCE*	PcG	Zhang H., and S. van Nocker, 2002
Itegrator	*FLC*	*FLOWERING LOCUS C*	MADS box	Michaels and Amansino, 1999
	SOC1	*SUPPRESSOR OF OVEREXPRESSION OF CONSTANS*	MADS box	Samach et al., 2000
	FT	*FLOWERING LOCUS T*	Similar to animal Raf1 kinase inhibitors	Kardailsky et al., 1999

though specific differences are found between species and between transgenic lines in general, these plants show a dwarfed phenotype, due to shorter and more numerous internodes, reduced apical dominance with increased lateral branching, smaller, wrinkled leaves, and increased rooting potential, with shorter and more branched roots; flowering is either delayed or inhibited and the number of flowers sometimes decreases or is stimulated; flowers are usually of the same size, although they may be smaller, sometimes altered in shape and fertility is reduced or even lost, although surprisingly, in some cases it is retained (Tepfer, 1984; Christey et al., 2001). Therefore, *rol* genes can be used not only to modify the morphology of a plant, but also to influence the process of flowering.

Among the *rol* genes, termed *rol*A, B, C and D, *rolC* has been the most widely studied because its effects are the most advantageous in terms of improving ornamental and horticultural traits. In addition to the dwarfness and the increase in lateral shoots that lead to a bushy phenotype, rolC-plants display more, smaller flowers, and advanced flowering; surprisingly, these plants may have better rooting capacity and they show almost no undesirable traits (Casanova et al., 2005). Transgenic *Osteospermum* plants with the *rolC* gene, driven by a constitutive promoter, exhibited flowering earliness and an increased number of flowers per plant in comparison to control plants, in open field tests; the flower head diameter was significantly reduced in *35SrolC* and *rolABC* plants (Giovannini et al., 1999). The bacterial gene *rolD* accelerates induction of the flowering process and enhances flower formation in tobacco (Mauro et al., 1996), and offers promising applications for the promotion of flowering. Although the biochemical functions of *rol* genes remain poorly understood, they are useful tools for improving ornamental flowers, as their expression in transgenic plants yields many beneficial traits. The capacity to produce more flowers or even to change flower shape, as revealed with some species, is a desirable modification for any ornamental plant.

FUTURE TREND

The first transgenic flower was reported in 1987 by Meyer et al., and since then an increasing number of ornamental species has been transformed successfully. However, whereas several transgenic crops have already been introduced commercially, among ornamental flowers only carnations with a range of violet colors, including cv. Moondust and cv.

Moonshadow, produced by Florigene (Australia), are currently on the market (Tanaka et al., 2005).

In the case of floricultural crops, molecular regulation of flowering and floral traits via the application of foreign genes could contribute to the improvement of ornamental plants (Morandini and Salamini, 2003). Consequently, increased knowledge about the functions of the genes involved in flowering would greatly benefit their application in both floriculture and horticulture. The efficient function of multiple genes is currently a common technical limitation (Tanaka et al., 2005).

In the near future plant biotechnology and plant breeding will continue to work alongside to improve horticultural and floricultural plants.

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Plant Shape: Molecular Aspects of Leaf Morphogenesis

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SUMMARY. Plant morphogenesis is controlled by the integration of endogenous genetic programs and responses to exogenous signals. The leaf is a good subject for studying plant morphogenesis, the diversity of which is reflected in leaf shape. Early control of leaf shape relies on controlling leaf initiation at the shoot apical meristem (SAM), the rates and planes of cell division, and the polarity-dependent differentiation of leaf cells. Final leaf form involves coordination of the rates of division, enlargement, and differentiation of leaf cells. In addition, recent genetic studies have revealed a different mechanism that plays an important role in regulating leaf shape: the control of spatial and temporal expression by microRNA and programmed cell death. This brief review focuses on the genetic regulation of leaf shape, from the perspective of the spatial and temporal balance between cell division, cell

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enlargement, and cell differentiation, with special emphasis on our own studies. doi:10.1300/J411v17n01_09

KEYWORDS. Cell division, cell elongation, leaf morphogenesis, microRNA, polarity, programmed cell death

INTRODUCTION

The leaf is a major component of the shoot; it is the organ that is the key to a full understanding of plant morphogenesis and plant biodiversity. Leaves are the primary photosynthetic organs, and their shapes are adapted to the natural environment for efficient photosynthesis. The early control of leaf shape relies on controlling leaf initiation at the shoot apical meristem (SAM), changes in the rates and planes of cell division, and polarity-dependent differentiation of leaf cells (Steeves and Sussex, 1989). Subsequent shaping of leaves in later development also involves biophysical forces. Therefore, the process of leaf expansion, namely, the correlation between cell division and elongation, plays an important role in establishing leaf morphology (Ashby, 1948; Arkebuer and Norman, 1995). The rates of cell division and elongation at each stage contribute to the final shape of a leaf (Steeves and Sussex, 1989; Tsukaya, 2002, 2003) and play important roles throughout leaf development. Nonetheless, the mechanisms that control these basic aspects of leaf development are poorly understood, because the pattern of growth within the lamina is surprisingly complex (Steeves and Sussex, 1989). Several recent molecular genetic studies, including studies of cell proliferation, cell differentiation, and the cell elongation pattern in leaves, have helped us to understand the complexity of leaf shape.

This review focuses on the genetic regulation of leaf shape, from the perspective of the spatial and temporal balance between cell division and cell enlargement, with special emphasis on our own studies. In addition, we also focus on recent genetic studies of microRNA and programmed cell death, which play an important role in regulating leaf shape in different manners. Genes that control leaf shape during plant development are summarized in Table 1. Please see other recent reviews that cover early events in leaf initiation and primary morphogenesis (e.g., Dale, 1988; Tsukaya, 1995; Smith and Hake, 1992;

TABLE 1. Genes that control leaf shape.

Genes	Classification	Function on leaf shape or phenotypes of leaves	References
Class-1 KNOX			
KNOTTED1(*KN1*)	*KNOX* gene in maize	dominant gain-of-function mutation: knotted and altered leaves loss-of-function mutation: extra vegetative leaves	Smith et al., 1992 Kerstetter et al., 1997
SHOOT MERISTEMLESS (STM)[a]	*KNOX* gene in *Arabidopsis*	loss-of-function mutation: shoot meristemless	Long et al., 1996
KNAT1,2,6[a]	*KNOX* gene in *Arabidopsis*	35S-*KNAT1* plants: lobed leaves	Lincoln et al., 1994; Chuck et al., 1996; Serikawa et al., 1996
Rough Sheath1 (*RS1*)	*KNOX* gene in maize	dominant gain-of-function mutation: sheath-like tissue at the base of the blade	Becraft and Freeling, 1994
NTH15	*KNOX* gene in tobacco	35S-*NTH15* plants: dwarf phenotype with abnormal leaf morphology	Tamaoki et al., 1997
OSH1	*KNOX* gene in rice	35S-*OSH1* plants: knotted and altered leaves	Matsuoka et al., 1993
LeT6	*KNOX* gene in tomato	35S-*LeT6* plants: indeterminate features in compound leaf	Janssen et al., 1998
POTH1	*KNOX* gene in potato	35S-*POTH1* plants: dwarf phenotype with short and altered leaves	Rosin et al., 2003
PHANTASTICA (*PHAN*)[a]	MYB gene of *Antirrhinum*	loss-of-function mutation: narrow and altered leaves	Waites et al., 1998
Rough Sheath2 (*RS2*)	MYB gene of maize	loss-of-function mutation: dwarfism, leaf twisting, and altered leaves	Schneeberger et al., 1998
ASYMMETRIC LEAVES1 (*AS1*)[a]	MYB gene of *Arabidopsis*	loss-of-function mutation: lobed leaves with short petioles	Tsukaya and Uchimiya, 1997; Byrne et al., 2000
AS2[a]	leucine-zipper motif protein	loss-of-function mutation: lobed leaves	Semiarti et al., 2001
BLADE ON PETIOLE (*BOP*)[a]	Not identified yet	loss-of-function mutation: lobed leaves and blades on petioles	Ha et al., 2003
Dorsoventral polarity			
ARGONAUTE1 (*AGO1*)[a]	PIWI and PAZ domain	loss-of-function mutation: narrow and pointed leaves	Bohmert et al., 1998
PHABULOSA (*PHB*)[a]	homeodomain-leucine zipper	dominant gain-of-function mutation: trumpet-shaped leaf, adaxialized leaf	McConnell et al., 2001
PHAVOLUTA (*PHV*)[a]	homeodomain-leucine zipper	dominant gain-of-function mutation: altered and adaxialized leaf	McConnell et al., 2001
REVOUTA (*REV*)[a]	homeodomain-leucine zipper	dominant gain-of-function mutation: outgrowth of leaves	Talbert et al., 1995
LATERAL ORGAN BOUNDARIES (*LOB*)[a]	LOB domain protein	35S-*LOB* plants: round and curled leaf blades and short petioles	Shuai et al., 2002
YAB1/FIL[a]	zinc finger, HMG box-like domain	35S-*FIL* plants: altered and abaxialized leaves	Sawa et al., 1999
KAN1,2,3[a]	GARP domain	35S-*KAN1* plants: narrow and abaxialized leaves	Kerstetter et al., 2001
DROOPING LEAF (*DL*)	YABBY family gene in rice	loss-of-function mutation: drooping leaf *ACTIN:DL* plants: curled and cylinder-like leaves	Yamaguchi et al., 2004

TABLE 1 (continued)

Genes	Classification	Function on leaf shape or phenotypes of leaves	References
Cell division			
cdc2aAt[a]	Cdc2 kinase	dominant negative mutation: normal shaped leaves with reduced cell number	Hemerly et al., 1995
CycD2[a]	D-type cyclin	35S-*CycD2;At*-tobacco plants: rapid elongation of leaves	Cockcroft et al., 2000
CycD3[a]	D-type cyclin	35S-*CycD3;1* plants: small leaves	Dewitte et al., 2003
ICK1/KRP[a]	cyclin-dependent kinase inhibitor	35S-*ICK1* plants: serrated and small leaves	Wang et al., 2000
AINTEGUMENTA (*ANT*)[a]	*APETALA2*-like family	loss-of-function mutation: narrow and pointed leaves 35S-*ANT* plants: larger leaves	Mizukami and Fischer, 2000
CURLY LEAF (*CLF*)[a]	polycomb-group gene	loss-of-function mutation: narrow and small leaves	Kim et al., 1998a; Goodrich et al., 1997
Pointed first leaf2 (*PFL2*)[a]	ribosomal protein S13-homolog	loss-of-function mutation: narrow and pointed leaves	Ito et al., 2000
ROTUNDIFOLIA4 (*ROT4*)[a]	novel small peptide	dominant mutation: round leaf blades and short petioles	Narita et al., 2004
NbDEK	Phytocalpain of tobacco	loss-of-function mutation: narrow and destorted leaves	Ahn et al., 2004
Polar cell elongation			
ANGUSTIFOLIA (*AN*)[a]	transcriptional co-repressor (CtBP)	loss-of-function mutation: narrow leaves with altered trichomes	Kim et al., 2002
ROTUNDIFOLIA3 (*ROT3*)[a]	cytochrome P450 (CYP90C1)	loss-of-function mutation: round leaf blades and short petioles	Kim et al., 1998b, 1999
MicroRNA			
AGO1[a]	miR165/MiR166	potential targets of miRNA are *PHB/PHV/REV*	Emery et al., 2003; Kidner and Martienssen, 2004
JAW[a]	miR159	dominant gain-of-function mutation: serrated leaves with curvature Potential targets of miRNA are *TCP2/TCP4*	Palatnik et al., 2003

[a]Genes which were isolated from *Arabidopsis thaliana*.

Dengler, 1999; Goliber et al., 1999; Sinha, 1999; Dengler and Tsukaya, 2001; Eshed et al., 2001; Kessler and Sinha, 2004).

GENES THAT CONTROL LEAF SHAPE DURING EARLY DEVELOPMENT: DORSOVENTRAL POLARITY

The size and shape of organisms and the organs within them are generally conserved within a species, suggesting genetic control over these parameters. Mutations at many loci lead to altered shape and size, suggesting complex regulation of the overall morphology of an organism. Recent studies on the genetic control of leaf morphogenesis allowed us to identify genes that affect leaf shape.

Leaves are defined as indeterminate organs, with genes that are involved in establishing leaf dorsoventrality. Lateral organs contain two primary axes of polarity: a proximal-distal axis and an adaxial-abaxial axis. These polarities are established relatively early during leaf development, and are defined relative to the SAM (Sussex, 1955). A number of mutations that alter leaf polarity have been described (Table 1).

Mutations in *PHANTASTICA* (*PHAN*) of *Antirrhinum*, which encodes MYB transcript factor (Waites et al., 1998), lead to a loss of dorsoventrality in leaves, resulting in narrow, altered leaves. Mutations in *ASYMMETRIC LEAVES1* (*AS1*) of *Arabidopsis*, which encodes a *PHAN* ortholog, result in the formation of lobed leaves that curl downward and display vascular pattern defects (Tsukaya and Uchimiya, 1997; Byrne et al., 2000; Ori et al., 2000; Semiarti et al., 2001). Mutations in *AS2*, which encodes a leucine-zipper motif protein, lead to shape defects that are similar to those that result from the accumulation of *as1* and ectopic *KNOX* RNA in leaves (Semiarti et al., 2001). *AS1* and *AS2* are necessary for repressing the class-I *KNOX* genes *KNAT1*, *KNAT2*, and *KNAT6* in the leaf primordium (Byrne et al., 2000; Ori et al., 2000; Semiarti et al., 2001).

Genes that regulate adaxial-abaxial polarity in *Arabidopsis* are *PHABULOSA* (*PHB*), *PHAVOLUTA* (*PHV*), and *REVOLUTA* (*REV*), which encode class-III homeodomain/Leu zipper transcription factors (McConnell et al., 2001; Otsuga et al., 2001; Bowman et al., 2002). Semi-dominant gain-of-function mutations in *PHB* and *PHV* result in the formation of adaxialized leaves (McConnell et al., 2001). In addition, members of the *YABBY* and *KANADI* gene families regulate adaxial-abaxial polarity by specifying abaxial cell fate (Sawa et al.,

1999; Siegfried et al., 1999; Eshed et al., 2001; Kerstetter et al., 2001; Bowman et al., 2002; Emery et al., 2003).

Recently, several studies of microRNA (miRNA) have suggested that miRNA controls leaf polarity by repressing the *PHB/PHA/REV* and *ARGONAUTE1* genes (Table 1; Emery et al., 2003; Juarez et al., 2004; Kidner and Martienssen, 2004). In addition, a dominant mutation in the *JAW* locus, *jaw-D*, which produces a different miRNA that guides messenger RNA (mRNA) cleavage of several *TCP* genes, results in serrated leaves with curvature (Palatnik et al., 2003), implying that miRNA-mediated control of leaf morphogenesis has important roles in the variation in leaf form.

SPATIAL AND TEMPORAL PATTERNS OF CELL DIVISION AND CELL ENLARGEMENT

Cell elongation in leaves continues throughout leaf expansion, while cell division can stop before the leaf is mature (Dale, 1976). Our previous genetic and histological study of the *curly leaf* (*clf*) mutation illustrates a good way to examine the balance between cell division and cell enlargement in leaf expansion (Kim et al., 1998a). The leaves of the *clf* mutant are narrow, short, and curl upwards, while the average length of the hypocotyl and primary root of the *clf* mutant do not differ significantly from those of the wild type (Goodrich et al., 1997; Kim et al., 1998a). The width and length of leaf blades of the *clf-25* mutant are significantly smaller than those of the wild type at the same stage; a reduction in the size and number of leaf cells results in the narrow short leaves (Kim et al., 1998a). During leaf cell elongation, two phases were noted. While cells were dividing, the size of cells in the *clf-25* mutant did not differ from those of the wild type. After completion of the cell-division phase (4 d after the appearance of the leaf primordium) in the observed area of the leaf blade, expansion continued in both the wild type and the *clf-25* mutant. The rate of cell elongation at this stage was lower in the *clf-25* mutant than in the wild type (Kim et al., 1998a). These data show that the spatial and temporal balance between cell proliferation and cell enlargement in leaves can affect leaf morphology. The proper balance of leaf cell proliferation and enlargement in wild-type plants, both spatially and temporally, was revealed in a recent study of *Arabidopsis* using transgenic plants harboring a G2/M-specific marker gene, *Cyc1At* promoter-*GUS* (Donnelly et al., 1999). GUS-positive cells in the leaves of transgenic plants were distributed uniformly at the early stage of leaf

primordia. Four days after the appearance of the leaf primordium, GUS activity in the leaf blade was still high, but its activity was reduced to a limited area of the petiole, basal portion of the mid-vein, and the margin of the leaf blade (Donnelly et al., 1999). At 8 days, the distribution of GUS-positive cells began to show a longitudinal gradient along the leaf blade, with most in the basal part of the leaf blade. At 12 days GUS-positive cells were limited to the basal third of the leaf blade (Donnelly et al., 1999). By contrast, after cell division decreased, palisade cell enlargement continued until the leaf was fully expanded (Donnelly et al., 1999). These results support the cell size control of cycling model proposed by Francis (1998), in which a minimum size is required for commitment to cell division in a tissue-specific manner.

CELL DIVISION IN LEAF MORPHOGENESIS

Whether the factors controlling cell division can affect leaf morphogenesis directly is controversial. However, close cooperation between cell proliferation and cell differentiation is thought to be an important factor affecting leaf morphogenesis.

We can divide the genes that control cell division into two classes: those for the cell cycle machinery itself, such as plant cyclin-dependent kinases (CDKs) and the cyclin family, and those that control the cell cycle machinery or cell proliferation directly (Table 1). CDKs and cyclins control cell cycle timing and the cell division pattern and frequency to generate the final plant architecture (Hemerly et al., 1999; Mironov et al., 1999). Inhibition of cell division in tobacco using the *cdc2aDominant negative* allele resulted in leaves with normal shape, but smaller overall size, which contained fewer, but larger cells (Hemerly et al., 1995); this suggests that altering the rate of cell division in these leaves did not affect the overall leaf shape. In contrast to the *cdc2a dominant negative* allele of tomato, *2S2-cdc2a.N147 transgenic Arabidopsis*, which has constructs containing the mutant *cdc2a.N147* under the control of the *At2S2* promoter (Conceição and Krebbers, 1994), has a distorted phylotactic pattern, and altered cotyledons and leaf shape (Hemerly et al., 2000). These experimental data suggest that altered leaf shape is caused by a reduction in cell number in the SAM.

In tobacco, overexpression of the plant D-type cyclin CycD2 increases the rates of cell division and leaf initiation, and accelerates plant growth without affecting morphology (Cockcroft et al., 2000). By contrast, *CycD3;1* overexpression in *Arabidopsis* increases CycD3;1-asso-

ciated kinase activity and reduces the proportion of cells in G1-phase of the cell cycle, leading to striking alterations in development. The cotyledons of plants overexpressing *CycD3;1* are larger than those of wild-type plants, but contain smaller cells. Leaf shape in overexpressing plants is altered radically, with a failure to develop the distinct spongy and palisade mesophyll layers (Dewitte et al., 2003). Recently, a study of virus-induced gene silencing (VIGS) of *NbDEK*, which encodes the calpain of *N. benthamiana*, a calcium-dependent cysteine protease, supported the idea that *CycD3;1* over expression regulates leaf shape (Ahn et al., 2004). VIGS of *NbDEK* resulted in narrow, distorted leaves via the control of *CycD3* expression (Ahn et al., 2004). Moreover, several molecular genetic studies of the cell proliferation inhibitors, such as *ICK1* (cyclin-dependent kinase inhibitor) and *KRP2* (kip-related protein) support the idea that cell division affects leaf morphogenesis directly (Riou-Khamlichi et al., 1999; Wang et al., 2000; De Veylder et al., 2001). Leaves of transgenic plants overexpressing *ICK1* were smaller and serrated (Wang et al., 2000). CDK activity in 35S-*ICK1* plants was reduced, which altered the length/width ratio and shape of leaves (Wang et al., 2000). Interestingly, the leaf cells in 35S-*ICK1* plants were two or three times larger, while the number of leaf cells was significantly reduced (Wang et al., 2000). This suggests that cell division affects leaf shape. This idea is supported by a study of transgenic plants that ectopically express *Arabidopsis CycB1* (*Cyc1At*), a mitotic cyclin gene, via the *cdc2* promoter (Doerner et al., 1996).

A study of transgenic plants that overexpress *CycD3*, a G1 cyclin, indicated that cell-cycle regulators affect leaf shape directly (Riou-Khamlichi et al., 1999). In addition, *AINTEGUMENTA* (*ANT*), which encodes a transcription factor with an AP2 domain in *Arabidopsis*, regulates the number of cells incorporated into developing leaves (Mizukami and Fischer, 2000). The *ant* mutant has reduced proliferation, leading to smaller leaves with fewer, but larger-than-normal, cells, like *cdc2a dominant negative* plants. Overexpression of *ANT* produces enlarged organs with superficially normal morphology and normal-sized cells. Therefore, *ANT* appears to prolong the period during which cells maintain division competence, without disrupting pattern controls. Our previous study of the *pointed first leaf2* (*pfl2*) mutant, which has an insertion mutation in the *AtRPS13A* gene encoding a cytoplasmic ribosomal protein S13-homolog resulting in narrow and pointed leaves with fewer but larger cells (Ito et al., 2000), also supported the hypothesis that cell proliferation affects leaf shape.

By contrast, a recent study based on overxpression of *rotundifolia4* (*rot4-1D*), a novel small peptide, suggested that cell proliferation could control leaf length in a polar manner (Narita et al., 2004). The *rot4-1D* mutant had short, rounded leaf blades and short petioles, similar to the *rot3* mutant. Interestingly, in contrast to dominant negative *cdc2a*, *ant* mutants, which have fewer, but enlarged, cells in leaves, *rot4-1D* has fewer cells in leaf blades, with no difference in cell size (Narita et al., 2004). These data imply that ROT4 controls polarity-dependent cell proliferation during wild-type leaf morphogenesis.

POLAR ELONGATION OF LEAF CELLS IN LEAF MORPHOGENESIS

As discussed above, variation in the leaf index can be attributed to two different histologic phenomena in leaf blades: changes in cell proliferation and changes in cell expansion. As our study of the leaves of the *clf* mutant showed, the process of cell enlargement can be divided into two distinct phases (Kim et al., 1998a). The earlier phase of cell enlargement has a tight relationship with the process of cell proliferation, as described above. The later phase of cell enlargement involves a polar-dependent process of leaf cell elongation. A previous genetic study of the *angustifolia* (*an*) and *rotundifolia3* (*rot3*) mutations revealed that genetic regulation of the polar elongation of cells controls the two-dimensional growth of the leaf blade (Table 1; Tsukaya, 1995; Tsuge et al., 1996).

The *AN* gene regulates the width of leaf cells in a polarity-dependent manner and functions independently of *ROT3* (Tsuge et al., 1996). The *an* mutant has comparatively narrow leaves of normal length, i.e., a defect in cell elongation in the leaf-width direction, while cells elongate further than wild-type cells in the leaf-thickness direction (Tsuge et al., 1996); the total number of leaf cells is little different from that in the wild type (Tsuge et al., 1996). A cytological analysis of cortical microtubules (MTs), which are important in regulating the polar elongation of cells, in the *an* mutant revealed an abnormal arrangement of cortical MTs in leaf cells (Kim et al., 2002). This suggests that the *AN* gene regulates the polarity of cell growth by controlling the arrangement of cortical MTs. The AN protein interacted with the ZWICHEL protein, a kinesin-like protein that might interact with tubulin, in a yeast two-hybrid system (Folkers et al., 2002), supporting our idea that it controls the polarity of cell growth by controlling the arrangement of cortical MTs.

The *AN* gene has been identified by positional cloning (Kim et al., 2002) and found to encode a member of the C-terminal binding protein (CtBP) family, the members of which act as transcriptional co-repressors in animals (Turner and Crossley, 2001). The *AN* gene was the first member of the CtBP family to be found in plants. The AN protein can also self-associate in the yeast two-hybrid system (Kim et al., 2002). Furthermore, microarray analysis has suggested that the AN gene regulates the expression of certain genes, e.g., the gene involved in cell wall formation, *MERI5*, a member of the xyloglucan endotransglucosylase/hydrolase family (Kim et al., 2002).

Unlike *an* mutants, *rot3* mutants have short leaves and petioles with normal leaf width, suggesting that *ROT3* controls leaf length specifically. The *rot3* mutant has a defect in the polar elongation of leaf cells in the leaf-length direction, but leaf cell proliferation is normal (Tsuge et al., 1996). The *ROT3* gene was cloned using a T-DNA tagging strategy (Kim et al., 1998b). A molecular genetic study of the *ROT3* gene suggested that it encodes a novel cytochrome P450, CYP90C1 (Kim et al., 1998b),.which has high homology to the cytochrome P450s involved in brassinosteroid (BR) biosynthesis. A study of transgenic plants overexpressing the *ROT3* gene revealed that it regulates polar elongation in leaf cells, but does not affect leaf cell numbers throughout leaf expansion (Kim et al., 1999). In addition, the leaves of transgenic plants were found to be longer than those of wild-type plants, but the same width (Kim et al., 1999). Therefore, the *ROT3* gene might specifically regulate leaf elongation. Recently, we found that an exogenous supply of BR restored the phenotype of short leaves of the rot3 mutant (Kim et al., 2005). In addition, an exogenous supply of a high concentration of brassinolide (BL) reduces the transcript levels of the *ROT3* gene (Kim et al., 2005), suggesting that *ROT3* is involved in BR biosynthesis.

BRs are general plant growth factors that regulate both the division and elongation of cells in all plant organs (Szekeres et al., 1996; Azpiroz et al., 1998; Nakaya et al., 2002). The *CPD/CYP90A1* and *DWARF4/CYP90B1* knockout mutants, which have high homology to the *ROT3* gene and are involved in brassinosteroid biosynthesis, show severe dwarfism, with a non-polar defect in leaf expansion that can be restored by the exogenous supply of BR (Szekeres et al., 1996; Azpiroz et al., 1998). The *rot3* mutant differs from the BL-related mutants in the cited studies, as it has no defects in the proliferation of cells in the leaf lamina, stem elongation, or skotomorphogenesis (Tsuge et al., 1996; Kim et al., 1998b). Therefore, all members of the CYP90 gene family, except

ROT3, are involved in the general regulation of the proliferation and enlargement of plant cells. The *ROT3* gene appears to have evolved from a family of genes that are general regulators of plant growth.

REMODELLING LEAF SHAPE BY PROGRAMMED CELL DEATH

Programmed cell death (PCD), or apoptosis, is a genetically encoded, active process that results in the death of individual cells, tissues, or whole organs. PCD is a major driving force in the achievement of animal form and development, and in the maintenance of cell homeostasis (Ganan et al., 1998). In plants, PCD is necessary for the differentiation of specialized cell types, such as tracheary elements; the deletion of tissues with temporary functions, such as the embryonic suspensor and aleurone cells; and organ or shoot morphogenesis, such as the formation of certain leaf lobes and perforations, and the elimination of stamen primordia cells in unisexual flowers (Greenberg, 1996; Beers, 1997; Pennell and Lamb, 1997; Kuriyama and Fukuda, 2002). Although the primary signals that initiate the PCD pathway have not been identified in any of these cases, they are presumably internal, and respond to positional and temporal cues that are elaborated during plant development.

During leaf development, PCD affects leaf form in some *Monstera* species (Kaplan, 1984). The appearance of the leaves of the Swiss cheese plant, *Monstera deliciosa*, results from light-regulated tissue death in the leaf primordia, where specific patches within developing lamina die and are not replaced, forming a deeply lobed leaf (Kaplan, 1984). Incidentally, the lace plant, an *Aponogeton* species, uses PCD during leaf development in a quite different way (Sergueff, 1907). A recent study of the lace plant indicated that during leaf remodeling, PCD is progressive (Gunawardena et al., 2004). Perforations caused by synchronous cell death result in a lattice pattern between the intersections of the longitudinal and numerous transverse veins of the leaves; finally, the PCD zone spreads peripherally, with two to three more cell layers forming a gradient of progression through the PCD process, resulting in the mature net-like leaves (Gunawardena et al., 2004). Therefore, the exquisite leaf patterns of the lace plant are formed through a combination of cell death and subsequent growth of the lamina. While this type of cell death in plants appears similar to that involved in pattern forma-

tion in animals, essentially nothing is known about the developmental cues, signaling pathways, or execution of cell death in these unique cases of developmental PCD. Further molecular approaches using Swiss cheese and lace plants should help to elucidate the remodeling mechanism in leaf morphogenesis via PCD.

CONCLUSIONS

Leaf initiation and the subsequent characteristics of organ formation are the result of an orchestration of events that begins in the SAM and takes place throughout early leaf development. The genes that regulate the acquisition of stem-cell fate and meristem maintenance can profoundly affect leaf shape and size when expressed ectopically in the leaf, perhaps reflecting the dependence of leaf shape on the timing of cellular differentiation. The significance of the effects of these ectopic perturbations in model organisms seems obscure. However, enough data are emerging from comparative developmental studies to suggest that the same mechanisms are utilized in nature to vary leaf form. In addition, differential synthesis, transport, and perception of hormonal signals can modulate shape characteristics. These events must be coordinated at the level of cell division and expansion to lead to the final organ form.

The mechanism joining cell division, cell elongation, and leaf morphogenesis is still controversial. There are major unresolved questions about perturbations in the course of cell division and cell enlargement, including how the patterns of cell division are controlled with such precision and how cells are regulated spatially and temporally during organ morphogenesis. The answers to these questions have eluded researchers for decades. Several of the studies introduced in this review have contributed partial answers to some of these questions. The advent of new techniques in genetic and molecular analyses will help us to unfold these mysteries. Recent studies have revealed that PCD is one of the factors that control leaf morphogenesis, via remodeling of leaf shape.

The genes controlling leaf shape that have been isolated and characterized to date offer considerable promise for the improvement of crops and horticultural plants. It should be possible to engineer plants using these genes to design horticultural novelties.

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Improvement of Horticultural and Ornamental Crops Through Transgenic Manipulation of the Phytochrome Family of Plant Photoreceptors

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SUMMARY. Consumer demands drive continuous developments in the production of horticultural and ornamental crops. In addition to improvements in quality and nutritional content, crop producers must supply an increased variety of products to a year-round market. The availability of many horticultural and ornamental crop products is dependent on the timing of reproductive development. The time at which many plants initiate sexual or vegetative reproduction is governed by a number of interacting environmental factors such as daylength, light quality and temperature. Artificial manipulation of the growing environment is therefore frequently used to ensure production meets retailers' marketing programs, a strategy which can often result in high energy costs. An alternative approach involves the manipulation of genes encoding proteins responsible for perceiving and transducing environmental stimuli,

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in particular, the genes encoding the phytochrome family of plant photoreceptors. Alterations in the expression of genes encoding phytochromes can modulate not only the timing of reproductive development, but also plant architecture. Such approaches can therefore be used to modulate a variety of phenotypic traits such as height, lateral branching and harvest yield, while enabling growers to tailor crop reproduction to their marketing needs. In this review, we will discuss examples of crop improvement using transgenic manipulation of phytochrome expression, along with benefits and disadvantages of such approaches. doi:10.1300/J411v17n01_10

KEYWORDS. Phytochrome, photomorphogenesis, transgenic, plant architecture, reproductive development

THE PHYTOCHROME FAMILY OF PLANT PHOTORECEPTORS

In addition to providing an energy source for photosynthesis, light signals convey information to plants about the surrounding environment. Through the operation of specialised photoreceptors, plants are able to monitor the quantity, quality, periodicity and direction of incident light. The information derived from light signals is used to modulate multiple developmental processes from seed germination and seedling establishment through to mature plant architecture and reproductive development. Interaction of light signals with the endogenous circadian oscillator provides plants with a means to monitor daylength (photoperiod) and consequently anticipate seasonal changes. The ability to adapt vegetative and reproductive strategies to a changing light environment confers considerable selective advantage to plants growing in natural communities. Three principle families of signal-transducing photoreceptors have been identified and characterised in higher plant tissues. These are the red/far red (R/FR) absorbing phytochromes (Kendrick and Kronenberg, 1994) and the blue/UV-A absorbing cryptochromes and phototropins (Cashmore et al., 1999; Briggs and Huala, 1999).

The phytochromes are homodimeric chromoproteins. Each subunit consists of a polypeptide of approximately 1,200 amino acids covalently attached to a light-absorbing tetrapyrrole chromophore, phytochromo-

bilin, via a thioether linkage to a conserved cysteine residue (Lagarias and Rapport, 1980). A light-induced isomersiation of the chromophore, coupled with differential interactions between the chromophore and polypeptide moieties, enable the phytochromes to exist in two spectrally distinct, stable forms. Synthesised in the inactive Pr form (absorbing maximally in the R region of the spectrum at about 660 nm), phytochrome activity is acquired upon phototransformation to the Pfr isomer (absorbing maximally in the FR region of the spectrum at about 730 nm). Upon absorption of a photon of light, Pfr is transformed back into Pr. The reversible photochromicity of phytochrome, together with the considerable overlap in the absorption spectra of Pr and Pfr, means that phytochromes exist as an equilibrium mixture of the two forms under almost all irradiation conditions.

Monocotyledonous angiosperms contain three major phytochromes, A, B and C, the apoproteins of which are encoded by the genes *PHYA*, *PHYB* and *PHYC* (Mathews and Sharrock, 1997). More recent duplication events have resulted in the existence of additional phytochrome species in dicotyledonous plants. In the model species *Arabidopsis thaliana*, five discrete apophytochrome-encoding genes, *PHYA-PHYE* have been isolated and sequenced (Mathews and Sharrock, 1997). Phytochromes A, B, C and E share only 46-53% sequence identity while *PHYD* encodes an apoprotein which shares 80% sequence similarity with the product of the *PHYB* gene (Clack et al., 1994). An initial gene duplication event is believed to have separated *PHYA* and *PHYC* from *PHYB/D/E*. The subsequent separations of *PHYA* from *PHYC* and *PHYE* from *PHYB/D* have resulted in three sub-families of *PHY* genes, *A/C*, *B/D* and *E* (Smith, 2000).

THE ROLE OF PHYTOCHROMES IN PLANT DEVELOPMENT

The roles of individual phytochromes in mediating plant development are complex and have been largely inferred from studies of loss-of-function mutants in *Arabidopsis*. In addition to possessing some unique photoregulatory functions, different members of the phytochrome family adopt redundant, synergistic and in some cases, mutually antagonistic actions.

In terms of photoreceptor protein abundance, phytochrome A (phyA) is the predominant phytochrome species in etiolated seedlings. Phytochrome A is susceptible to rapid proteolytic degradation upon conver-

sion to its Pfr form (Quail, 1994). This unique property is related to the ability of phytochrome A to function as a FR light sensor in etiolated seedlings (e.g., Hennig et al., 2000). Phytochrome A operates via two distinct modes of action for the responses of etiolated seedlings to FR (Smith and Whitelam, 1990). In the FR High Irradiance Response (FR-HIR), prolonged irradiation with continuous FR is required, and the extent of the HIR is dependent upon the fluence rate of FR (see Smith and Whitelam, 1990). In contrast, very low fluence responses (VLFR) are initiated by fluences of light as low as 10^{-9} mol m^{-2} and are fully saturated by very low concentrations of Pfr. Because of this, the VLFR response mode does not display prototypical R/FR reversibility (see Smith and Whitelam, 1990). Of course, seedlings growing in the natural environment would never be exposed to prolonged FR. Nevertheless, there is evidence that FR-HIR is of fundamental importance in seedling establishment under natural canopy shade conditions. It has been shown that for *Arabidopsis* seedlings growing under dense vegetational shade, a FR-rich light environment, *phyA* mutants display severely impaired de-etiolation and significant numbers fail to become established and die (Yanovsky et al., 1995).

Relative FR-enrichment of the local light environment occurs in areas of dense vegetation and results from the selective absorption of photosynthetically active R wavelengths by chlorophyllous tissue. The parallel reflection of FR wavelengths creates a local reduction in red to far red ratio (R:FR), providing plants with a unique and unambiguous signal that competitors are nearby (Smith and Whitelam, 1997). In high R:FR conditions, phytochromes B, D and E act redundantly together to constrain elongation growth and repress flowering. Reductions in the R:FR ratio of incident light shift the photoequilibrium of phytochromes B, D and E towards the inactive Pr form, so reducing the inhibition of elongation and flowering. The resulting elongation and early flowering responses are termed the shade avoidance syndrome (Robson et al., 1993; Devlin et al., 1999; Franklin et al., 2003b). Rapid elongation following the perceived threat of vegetational shading enables plants to overtop potential competitors and raise their leaves towards the light, responses often accompanied by a reduction in lateral branching. The parallel acceleration of flowering enables plants to rapidly set seed in unfavourable conditions and represents a crucial competitive strategy in rapidly growing populations (Ballaré et al., 1990; Smith and Whitelam, 1997). The shade avoidance response has significant agronomical implications. The channelling of resources towards rapid elongation growth can result in reduced plant biomass, lower seed production and an in-

creased susceptibility to lodging, making shade avoidance an important determinant in the planting density of many crops (Ballaré et al., 1990; Schmitt et al., 1995; Robson et al., 1996). These problems are most apparent in monocultures, where plants of similar architecture are unable to compete effectively for light (Robson and Smith, 1997). The action of phyA in FR-rich environments enhances plant survival by preventing excessive elongation growth which could ultimately prove lethal (Yanovsky et al., 1995; Salter et al., 2003). In addition to its unique role as a FR sensor, phyA functions throughout *Arabidopsis* development to promote flowering in long days (Johnson et al., 1994; Neff and Chory, 1998), modulate mature leaf architecture (Franklin et al., 2003a,b) and redundantly suppress internode elongation with phyB and phyE, thus maintaining a compact rosette phenotype (Devlin et al., 1998).

Phytochromes B, D and E represent the most recently evolved members of the phytochrome family and form a distinct subgroup (Mathews and Sharrock, 1997). The role of these phytochromes in mediating responses to low R:FR has led to speculation that shade avoidance may have provided the selective pressure for their evolution (Devlin et al., 1998). In addition to their crucial role in regulating elongation and flowering responses to low R:FR, phyB, D and E also act redundantly to mediate multiple physiological responses throughout the lifecycle of *Arabidopsis*. Such roles include the R/FR reversible promotion of seed germination (Hennig et al., 2002), the regulation of cotyledon expansion (Franklin et al., 2003b), functional interaction with the B photoreceptor cryptochrome 1 (cry1) (Hennig et al., 1999), and regulation of expression of the transcription factor ATHB-2 (Franklin et al., 2003b). Expression of the ATHB-2 gene is predominantly controlled by changes in R:FR ratio (Carabelli et al., 1993,1996) and the ATHB-2 protein is believed to play a role in regulating cotyledon expansion, growth of the vascular system and lateral root formation (Steindler et al., 1999).

The recent identification of *Arabidopsis* mutants at the *PHYC* locus has revealed roles for this phytochrome in the R-mediated inhibition of hypocotyl elongation, possibly through modulation of phyB function (Franklin et al., 2003a; Monte et al., 2003). A role for phyC, similar to that of phyA, was also revealed in the modulation of leaf architecture (Franklin et al., 2003a; Monte et al., 2003). Despite the relatively close phylogenetic relationship between phyA and phyC, no identifiable role for phyC was identified in FR sensing (Franklin et al., 2003a; Monte et al., 2003). Mutant combinations deficient in PHYC displayed elongated hypocotyls in blue light (B), an effect most pronounced at low fluence rates (Franklin et al., 2003a). Under these conditions, the B photoreceptor

cryptochrome 2 (cry2) predominates (Lin et al., 1998), suggesting a possible functional interaction between phyC and cry2 in this response.

Overall, the multifunctional and diverse roles of different phytochrome species in mediating plant development has made genetic manipulation of their expression a target for the improvement of horticultural and ornamental crops. Alterations in phytochrome gene expression provide a means for modulating a variety of desirable phenotypic traits such as seed germination, plant size, plant architecture and harvest yield. Furthermore, the intricate co-ordination of light signals and the central circadian oscillator in co-ordinating the timing of reproduction and other events makes manipulation of phytochrome gene expression a powerful tool for the developmental modulation of several commercially grown crops.

MODULATION OF PLANT ARCHITECTURE THROUGH GENETIC MANIPULATION OF PHYTOCHROME GENE EXPRESSION

Modulation of plant height is a major objective in the production of both horticultural and ornamental crops. For many greenhouse crops height control of is often essential for optimising the efficient handling and rapid establishment of plants as well as for the aesthetic enhancement of ornamental floriculture. The control of excessive elongation growth is also essential for the prevention of lodging in many species while reduction of plant height can confer significant economic advantage to growers of horticultural crops through reallocation of assimilates to the harvest unit.

There are a number of methods for regulating plant height in horticultural crops. For many years the use of chemical growth retardants has been a standard practice in the production of glasshouse crops. In particular, inhibitors of the synthesis of the plant hormone gibberellin have been widely used (Rademacher, 1991). However, increased consumer concerns over possible health risks and environmental impacts have led to the search for cost-effective alternatives to chemical height control. A number of alternative methods have been described, including temperature management regimes, the use of mechanical conditioning, the manipulation of light quality within the greenhouse and gene manipulation. Modifying the difference between day and night temperatures, or DIF (differential between day/night temperature) such that the night temperature is higher than that of the day temperature (negative DIF) is

an effective method of reducing plant height (Myster and Moe, 1995). However, this approach involves high energy costs and can be detrimental to plant viability at certain times of year.

It has been recognized that alteration of light quality, specifically the R:FR ratio, provides a very effective means of altering plant height and other aspects of plant development. In the greenhouse, R:FR ratio manipulation can be achieved by either the provision of supplementary lighting or by the use of spectrally selective filters. The use of $CuSO_4$ solutions, to selectively attenuate FR wavelengths, has provided ample evidence of the potential benefits of controlling R:FR ratio and the consequent shade avoidance responses displayed by plants. For example, removal of FR with $CuSO_4$ has been shown to lead to dramatic reductions in plant height in several plant species, including chrysanthemum, tomato and lettuce (Mortensen and Stromme, 1987; McMahon and Kelly, 1995; Reddy et al., 1996). Similarly, $CuSO_4$ filters fitted around the individual internodes of field-grown soybean plants have been shown to effectively reduce elongation shade avoidance responses, although this is clearly not suggested as a practical proposition for commercial growers (Ballaré et al., 1990). The manipulation of R:FR ratio through the use of $CuSO_4$ filters affects aspects of plant growth and development other than plant height. For instance, chlorophyll content and flowering time are also altered. This is not surprising given that these responses are well established components of the shade avoidance syndrome. Although liquid $CuSO_4$ filters have been shown to be very effective, the difficulties associated with materials handling, high construction costs and the hazardous nature of $CuSO_4$ have meant that the use of such filters is of limited value to commercial growers. Consequently, there has been intense interest in the development of spectrally selective greenhouse films. At the present time, a suitable plastic material with the ability to selectively remove FR is not commercially available, although some experimental materials have been evaluated (see Rajapaske et al., 1999).

The manipulation of photoreceptor genes provides an alternative means of exploiting the power of light quality signals for the modification of plant height. Specifically, the modification of ornamental and horticultural crops through genetic manipulation of phytochrome levels potentially presents an energy-efficient, year-round alternative to current horticultural practices. The constitutive expression of *PHY* genes in laboratory grown plants has been well documented to result in exaggerated light-mediated responses. For example, ectopic expression of oat *PHYA* in tobacco plants leads to substantial dwarfing, decreased apical

dominance, increased pigmentation and delayed leaf senescence (Cherry et al., 1991; Jordan et al., 1995). When grown in low R:FR ratio conditions, transgenic plants displayed a suppression of the normal shade avoidance elongation response through persistence of a normally transient FR-HIR (McCormac et al., 1991, 1992). A dwarf phenotype was also observed in transgenic tomato and potato plants constitutively expressing heterologous PHYA gene sequences (Boylan and Quail, 1989; Heyer et al., 1995; Yanovsky et al., 1998). Manipulation of the expression of other PHY genes can also modify plant height. Transgenic expression of PHYB in *Arabidopsis* (Wagner et al., 1991; McCormac et al., 1993) and tobacco (Halliday et al., 1997) resulted in an increased sensitivity to R and reduced extension growth at all R:FR ratios.

Horticultural examples of plant architecture management through genetic manipulation of *PHY* gene expression are limited. Constitutive expression of an oat *PHYA* gene in tomato produced plants with decreased stature and increased pigmentation in both leaves and fruits (Boylan and Quail, 1989). Enhanced pigmentation is associated with elevated levels of lycopene and vitamin C, in addition to increased fruit firmness. The production of lycopene in tomato fruit is regulated by phytochromes, independently of ethylene production (Alba et al., 2000). Studies linking increased lycopene consumption to reduced rates of prostate cancer in adult males (Giovannucci et al., 1995) indicate potential health benefits from the production of tomato fruits with altered *PHY* levels. By comparison, analysis of tomato mutants containing loss-of-function mutations at the *PHYA* locus (*fri*^{1}) revealed fruit with improved paste qualities. Mutant fruits contained fewer seeds that their wild type (WT) counterparts and displayed an increased proportion of soluble solids and improved paste viscosity (Alba et al., 1999). These advantages were, however, offset by reduced yields in both the glasshouse and the field. The same study revealed tomato plants mutated at the *PHYB1* locus (*tri*^{3}) to produce significantly more fruits per plant when grown under glasshouse conditions. The properties and size of fruits were similar to WT, suggesting *PHYB1* mutations may improve yield without adverse effects on fruit quality (Alba et al., 1999).

The constitutive expression of *Arabidopsis PHYB* has been extensively examined in potato plants. In addition to reduced plant height and decreased apical dominance, significant differences were observed in leaf morphology and photosynthetic performance when grown under glasshouse conditions (Thiele et al., 1999). The smaller, thicker leaves of transgenic plants contained increased pigment levels and decelerated chlorophyll degradation. The increased photosynthetic performance

and longer lifespan of transgenic plants resulted in greater biomass production and ultimately increased tuber yields (Thiele et al., 1999). Field studies have since revealed ectopic expression of *Arabidopsis PHYB* in potato to increase crop photosynthesis and tuber yield at high planting densities (Boccalandro et al., 2003).

The ectopic expression of a heterologous *PHY* gene has also been shown to modify the growth and development of cherry. In particular, transgenic expression of rice *PHYA* has been found to modify plant growth responses to proximity-dependent FR signals and, in some cases, to increase tolerance to the threat of vegetational shading (Muleo et al., 2003).

In commercial apple production it is frequently desirable to have dwarf rootstocks since the properties of the rootstock can influence the growth characteristics of the scion. However, it is often the case that dwarf rootstocks are more difficult to root by cuttings compared with more vigorous ones. For this reason, there has been interest in the development of techniques to introduce dwarfing characteristics that do not affect rooting, into elite rootstocks. The ectopic expression of the *Arabidopsis PHYB* gene has been shown to effectively lead to dwarfing of the apple rootstock M26 with no adverse effects on rooting ability (Hoelfors et al., 2000).

Consumer demand for increased variety, uniform morphology and aesthetic perfection make ornamental plants ideal targets for genetic manipulation. Expression of the tobacco *PHYB1* gene in chrysanthemum was used to produce plants with shorter stature, reduced branch angles and higher chlorophyll content (Zheng et al., 2001). Despite pleiotropic effects on plant architecture, expression of *PHYB* had little effect on flower formation or development (Zheng et al., 2001).

MODULATION OF REPRODUCTIVE DEVELOPMENT THROUGH GENETIC MANIPULATION OF PHYTOCHROME GENE EXPRESSION

Sensitivity to the timing of light and darkness, termed photoperiodism, enables plants to adapt to seasonal changes in their surroundings (Thomas and Vince-Prue, 1997). In photoperiodically sensitive species, the onset of sexual or vegetative reproduction is governed by the relationship between the day-length received and a threshold or critical day-length. The timing of reproduction often only occurs when days are sufficiently short (short-day plants; SDP) or long (long-day plants;

LDP). Plants insensitive to photoperiod are termed day neutral. Daylength measurement involves the integration of temporal information, provided by the circadian oscillator with light/dark discrimination provided by specific photoreceptors. Whether or not a SDP flowers in light/dark cycles is dependent primarily upon the length of the dark rather than the light period (Thomas, 1991). A light treatment (night-break) given during the perceived "dark-period" can prevent flowering in SDP (Borthwick et al., 1952). Action spectra for night-break promotion of flowering in LDP have shown maximum effectiveness of R and reversibility by FR (Downs, 1956). In the LDP *Arabidopsis*, flowering is accelerated under long day conditions. Here, the presence of light is perceived through either phyA or cry2 (Yanovsky and Kay, 2002).

The tuberization of potato plants is photoperiodically-regulated, with induction in many species occurring under short daylengths. Induction of tuberization in *Solanum tuberosum* L. subsp. *andigena* has been shown to involve phyB in the production of a graft-transmissible inhibitor (Jackson et al., 1998). Plants with reduced levels of PHYB, as a result of antisense inhibition, lost photoperiodic sensitivity and were able to tuberize in both long and short-day conditions (Jackson et al., 1996). The timing of tuberization in short day potato species is delayed by growth in longer photoperiods. Reduction of phyA levels through antisense inhibition was shown to reduce this delay, with transgenic plants tuberizing significantly earlier than wild type controls (Yanovsky et al., 2000). The bulbing process in many Allium species is also regulated by photoperiod (Lancaster et al., 1996), presenting an opportunity for potential future manipulation.

Over-expression of *PHY* genes has been used successfully to modulate flowering time in the ornamental crop *Aster* (Asteraceae) (Wallerstein et al., 2002). *Aster* is a long-short day plant. The transition from rosette to inflorescence requires long days with a short-day requirement for subsequent floral initiation. Overexpression of either oat *PHYA* or *Arabidopsis PHYB* reduced the daylength requirement for inflorescence development in *Aster*. While over-expression of *PHYA* shortened the critical daylength requirement, over-expression of *PHYB* shortened the length of night-break needed to induce inflorescence development. When grown under commercial conditions (autumn through spring), over-expression of either *PHYA* or *PHYB* substantially increased the yield of flowering shoots (Wallerstein et al., 2002).

A potentially adverse effect of *PHY* overexpression was discovered in transgenic hybrid aspen (*Populus tremula* ×*tremuloides*), transformed

with oat *PHYA* (Olsen et al., 1997). Perception of daylength in temperate-zone tree species enables apical growth cessation and cold acclimation prior to the onset of low autumn temperatures. In addition to dwarfing stem growth, constitutive expression of *PHYA* reduced the critical daylength for elongation growth from 15 h to below 6 h. The resulting delay in growth cessation prevented leaf abscission and the development of cold hardiness in transgenic plants. *PHYA*-overexpressing lines subsequently displayed increased stem tissue damage when subjected to freezing temperatures.

FUTURE PERSPECTIVES

The genetic manipulation of *PHY* gene expression has clear potential benefits for the improvement of horticultural and ornamental crops whilst avoiding some of the disadvantages of current practices used to manipulate plant architecture and flowering time. The overexpression of *PHY* genes to modify crops does, however, present potential problems. The majority of transgenic approaches use constructs driven by constitutive viral promoters. Such promoters drive expression in virtually all tissues and are not subject to conditioning by endogenous factors. Without spatial and temporal control over promoter activation, the introduced *PHY* gene is expressed ectopically at unnaturally high levels, throughout the plant's lifecycle. Phytochromes perform multifunctional and overlapping roles throughout plant development. Transgenic expression of a single *PHY* gene can therefore result in pleiotropic phenotypes, often altering both plant architecture and the timing of reproductive development. The manipulation of *PHY* gene expression to modify a single crop trait can therefore often result in unfavourable effects on other plant processes.

A potential solution to this problem involves the targeted expression of transgenic constructs through the use of tissue-specific promoters. Directed overexpression of *PHYA* in tobacco has been shown to effectively "mask" stems and leaves to localised low R:FR signals (Rousseaux et al., 1997). Such results suggest the possibility of producing transgenic crop plants in which specific tissues are "blinded" to perceived signals of canopy density, without deleteriously altering the photomorphogenic competence of other plant organs. Further refinements of this technology could include the use of temporal and developmentally specific promoters to direct the expression of transgenic *PHY* constructs. In this way, plant growth could be specifically tailored to eliminate unwanted

photomorphogenic responses, without impairing the light-foraging ability of the whole crop. Such technological advances would provide a cost-effective method of manipulating plant development whilst enhancing plant survival in densely planted canopies. The benefits of such approaches are evident and further research in this area should confer significant future advantage to both the growers and consumers of horticultural and ornamental crops.

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Problems and Challenges of Invasive Ornamental Plants and Molecular Tools to Control Their Spread

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SUMMARY. Invasive plants, one of the most devastating ecological problems in the 21st century, cause an estimated $35 billion loss per year to the economy in the United States alone. More than 50% of all invasive plant species and 85% of invasive woody plant species were introduced originally for ornamental and landscape use. Because many non-native ornamentals are commercially important and widely utilized for various purposes, completely banning their use and prohibiting their import are unpractical solutions. On the other hand, currently used methods to control the spread of non-native plants are ineffective, expensive, or environmentally problematic. Recent advances in plant molecular biology and plant genetic transformation may enable us to create sterile cultivars of these non-native ornamental crops of high commercial value. The use of sterile cultivars should reduce or eliminate the undesirable spread of some non-native invasive plants into natural areas. doi:10.1300/J411v17n01_11

KEYWORDS. Invasive ornamental plants, green industry, sterile cultivars, seedless fruits, transgenic plants

INTRODUCTION

Invasive plants are ranked second, behind habitat loss, as the greatest threat to biological diversity and ecosystem function in the United States (Brumback, 1998; Pimentel et al., 1999). Invasive plants silently and constantly encroach into parks, preserves, wildlife refuges, and urban areas. In the United States, invasive plants have taken over 100 million acres of land and are increasing at a rate of 10% annually (Pimentel et al., 2000). Economic and environmental damages caused by invasive plants in the United States amount to approximately $35 billion annually (Hall, 2000; Pimentel et al., 1999). This figure does not include non-monetary losses to ecosystem functions, human health effects, habitat loss for native species, and reductions in biodiversity.

Ornamental horticulture is an important industry of U.S. agriculture. Agricultural production has shifted from traditional food to ornamental horticulture over large areas of the United States. The green industry, however, has been a significant contributor to the spread of invasive plants. More than 5,000 species of non-native ornamental plants have escaped cultivation and have become naturalized in minimally managed

habitats (Morse et al., 1995). According to Reichard and Campbell (1996), more than 50% of all invasive plants were introduced for horticultural or ornamental purposes. The majority of invasive non-native woody plants were introduced intentionally as ornamental plants. Reichard and Campbell (1996) documented that 85% of the 235 invasive woody plants in the United States were originally introduced as ornamental plants while an additional 14% were introduced as agricultural plants. Furthermore, Hall (2000) found that of the 454 plants listed in Campbell's list of "plants that hog the garden" (Campbell, 1999), 292 were still being sold in nurseries. New invasive plant species are identified nationwide virtually every year. The repeated sale and widespread introduction of invasive ornamental plants have boosted the chance of colonization and establishment of these plants in natural areas (Enserink, 1999). Invasive plants used in ornamental horticulture play an important role in changing the look, feel, and function of natural areas in many parts of the United States.

IMPACTS OF INVASIVE ORNAMENTAL PLANTS

Gardens, arboretums, federal or local governmental agencies, commercial nurseries, and the general gardening public have long been conducting extensive plant exploration and introductions. The collected plants were used for scientific research and for the development of new commercially valuable ornamental plants (Orlean, 1998; Kilnkenbourg, 1999). Also, the use of many invasive plants has been promoted by governmental agencies for soil erosion control. For example, kudzu (*Pueraria lobata*) was first introduced as a ground cover and was later promoted for soil erosion control by the U.S. Soil Conservation Service (SCS). Scotch broom (*Cytisus scoparius*) was originally introduced as an ornamental and later promoted by the SCS for erosion control. Scotch broom is still used as a highway planting in western states and has now spread to over 500,000 acres in the state of California alone, where it displaces native flora and fauna (Office of Technology Assessment, OTA, 1993).

The use of exotic ornamental plants has resulted in the introduction of many aggressive invaders. The underlying reason that many exotic ornamental plants are invasive is that they possess positive traits that are highly desirable to gardeners, landscapers, and the nursery industry: they reproduce easily, establish rapidly and grow quickly under adverse and diverse environmental conditions. For example, these plants often

produce large quantities of fruits and seeds, and their seeds are easily distributed to remote areas with the help of wind, water, or wildlife. Examples of these invasive ornamentals are oriental bittersweet (*Celastrus orbiculatus*), privet (*Ligustrum* spp.) and Japanese barberry (*Berberis thunbergii*). Some invasive ornamental plants spread vegetatively via underground rhizomes, stems that root easily once they touch the ground, or adventitious rooting of plant stem fragments. These traits are also desirable for gardeners because plants with these advantageous characteristics will quickly fill in empty spaces in the backyard landscape or water garden. English ivy (*Hedera helix*) and water hyacinth (*Eichornia crassipes*) are two familiar examples. Rapid growth is another characteristic of invasive plants in general. Gardeners and landscapers often look for plants that can quickly fill patches in a garden or rapidly cover the ground to out-compete weeds. From a nursery perspective, plants that establish quickly and grow fast provide rapid turnover at production facilities with minimal investment, which reduces production costs and provides a financial boost to the bottom line of a business. Invasive ornamental plants are also generally adaptive to adverse and diverse environmental conditions, such as soil type, water requirements, and nutrient levels and they are often devoid of severe disease and insect infestations. These traits are attractive to nursery growers and consumers because they require minimal maintenance and need fewer fertilizers and pesticides. With such favorable traits, profit margins for plants that are invasive or potentially invasive are often much higher than for native noninvasive plants. Table 1 shows sales figures for some of the invasive or potentially invasive ornamental plants (USDA, 1998), indicating that many of these invasive ornamental plants are significant cash crops for the green industry.

Although ornamental plants are intended for use in gardens and other managed landscape areas, invasive ornamentals often escape from cultivated settings and invade natural areas, where they colonize, establish and reproduce at a rapid pace. Even though there is no report that quantifies direct monetary losses caused by the incursion of invasive ornamental plants in natural areas, the economical impacts for a limited number of invasive plants have been estimated. For example, purple loosestrife (*Lythrum salicaria*) costs $45 million annually in forage losses and control costs (Hall, 2000; Pimentel et al., 1999). Purple loosestrife is spreading at a rate of 285,000 acres per year. In addition, ornamental invasive plants have caused considerable non-monetary damage to ecosystems, contributing to a loss of ecological ecosystem function and biological diversity that cannot be easily determined. The

TABLE 1. Sales of Some Invasive Ornamental Plants in the United States (1998)

Plant	Operations	Total number of plants (× 1,000)	Total sales ($ × 1,000)
Ground covers *	1,343	48,859	83,576
Euonymus	1,125	7,715	41,085
Spireas	1,190	5,852	34,948
Callery pear	1,097	1,553	30,088
Norway maple	840	918	22,334
Privet	832	2,655	17,351
Climbing clematis	931	3,698	14,096
Weigelas	702	1,348	9,725
Other vines**	761	4,193	25,497

* Including some of the most invasive plants, such as English ivy.
** Including some of the most invasive plants, such as Japanese honeysuckle vines.

spread of purple loosestrife has altered the fundamental structure of emergent wetlands, caused biomass reductions for 44 indigenous plants, and reduced native wildlife that depended on those indigenous plants (Hall, 2000; Pimentel et al., 1999). Unfortunately, this plant is still sold in nurseries and garden centers. In the United States, privet (*Ligustrum japonica*) has invaded 2.4 million acres in the South (Meier, 1999), Scotch broom (*Cytisus scoparius*) has invaded over 1 million acres in Washington and Oregon alone, and spotted knapweed (*Centaurea maculosa*) now covers 7.2 million acres. Kudzu has taken over 10 million acres of natural habitat in the Southeast.

Invasive plants, once established, can effectively compete with native plants for resources. Typical examples include English ivy and kudzu. Both species reproduce quickly through vegetative propagation, and grow and establish rapidly. They quickly overtake backyards, abandoned or disturbed fields, road right-of-ways, vacant buildings, woodlands, and forest edges. The dense growth and green, waxy leaves of English ivy and kudzu form a thick canopy that prevents sunlight from reaching native herbs and other vegetative seedlings. Vines can climb and envelop branches and entire trees, restricting sunlight and eventually killing the smothered vegetation. The term, "ivy desert," has been used to describe such a situation (Westbrooks, 1998; Reichard and White, 2001). A similar "kudzu desert," attributed to the spread of

kudzu, is also evident everywhere throughout the southeastern United States. Kudzu is often referred to as the "vine-that-ate-the South."

Japanese barberry, a beautiful but highly invasive ornamental shrub originally imported from Japan, is spreading throughout the northeast region of the United States, and is likely one of the most destructive invasive plants in the area. Japanese barberry leafs out in early spring before native shrubs and other understory species begin to grow. The plant can reach 2 to 6 feet in height and it forms thick stands that exclude nearly all native vegetation. The loss of native plants, a consequence of the spread of invasive non-native plants, is often followed by the disappearance of many endangered species. For example, giant reed (Arundo donax), an escaped garden grass and a serious threat to parks and other public areas in southern California, is destroying habitat for the least-Bill's vireo, an endangered bird (Raver, 1999). Baby's breath (*Gypsophila paniculata*) destroys habitat for a threatened thistle (Randall, 1996; Raver, 1999). Melaleuca tree (*Melaleuca quinquenervia*) has invaded the Everglades so densely that it has crowded out many species of native wildlife (Randall, 1996).

Some invasive plants modify the ecosystem by changing soil water and salt content, therefore altering the hydrology and salinity of an area. Salt-cedar trees (*Tamarix* spp.), which have invaded the American southwest, decrease water reserves by increasing evapotranspiration, thereby modifying desert riparian areas (Walker and Smith, 1997). The iceplant (*Mesembryanthemum crystallinum*) in coastal California takes up salt from soils and deposits it on the surface, making these ecosystems uninhabitable for indigenous plants. Yellow iris (*Iris pseudacorus*) was instrumental in changing a Potomac River marsh to a mesic forest that favored ash (*Fraxinus*) and willows (*Salix*) by creating a raised seedbed with its rhizomes (Hall, 2000). Some invasive plants can also hybridize with closely related native plants to create invaders that are even more aggressive. Bradford pear generally produces few seeds. However, if it is hybridized with other pear species, the highly viable seeds that are produced become extremely competitive in natural areas, as frequently observed in the southeastern United States.

CURRENT CONTROL METHODS, THEIR UTILITIES AND LIMITATIONS

The control and eradication of invasive ornamental plants are technically difficult, often complicated, and costly. Current management

techniques to deal with the spread of invasive plants include mechanical or physical removal, the application of herbicides, or biological control. These methods, however, may be ineffective or inapplicable for invasive ornamental plants. Mechanical removal, such as hand pulling, hoeing, tilling, mowing, shredding, root pulling (power grubbing), chaining, and bulldozing have been used to eradicate some invasive plants. For widespread invasive ornamental plant species such as Japanese barberry and multiflora rose (*Rosa multiflora*), however, these methods are often too expensive or impractical to implement. While chemical control is the primary method of weed control in most grazing rangelands in the United States, herbicides often cause pollution and may damage non-target species. Herbicides may be non-selective and ineffective in the control of monocot and some dicot ornamentals such as butterfly bush (*Buddleia davidii*), which can re-establish quickly from its base (University of Florida IFAS Extension, 2004).

Biological control utilizing beneficial insects or microbial pathogens has been evaluated extensively and is successfully being used to control some invasive plants. Unfortunately, biological control is not currently available for most invasive plants and some biological control agents may not be applicable to invasive ornamental plants due to their non-selectivity. For instance, a native virus that attacks multiflora rose has been proposed as a potential biological control agent. Multiflora rose is invasive across most of the temperate United States. It was originally introduced as an ornamental and as a 'living fence' to keep grazing cattle in pastures and away from farmhouses. Multiflora rose grows aggressively and produces a large number of seeds that are dispersed by birds as they feed on the rosehips. This invasive shrub produces dense thickets that exclude many native plants (National Park Service, 2004). The native virus, however, has not been used to control the spread of multiflora rose because it also infects other roses of commercial value and could cause significant damage to the rose industry. The virus had also been found to infect other economically valuable plants that are related to rose, such as apples and some types of berries (Szafoni, 1990).

In addition to the above-mentioned control methods, replacement plant lists for invasive or potentially invasive ornamental plants are also being compiled. The question remains as to whether these alternative species are truly desirable substitutes for invasive ornamental plants. Consumers look for plants that establish quickly, withstand biotic and abiotic stresses and grow without much care. Unfortunately, these characteristics are the same traits that make plants invasive. Thus, alternative or replacement plants that do not possess these characteristics are

often not as attractive as their invasive counterparts to consumers and the green industry.

REGULATIONS AND CONCERNS OF THE GREEN INDUSTRY

Due to the economical and ecological damages caused by the spread of non-native invasive ornamentals, some ornamental plants have been banned in several U.S. states. Many more species may be banned in the future. A number of plants currently under consideration for a ban are cause for considerable economic concern to the green industry throughout the country. For example, during the 2004 legislative session, the Connecticut Legislature's Environment Committee called for the immediate bans of a large number of invasive or potentially invasive ornamental plants. A ban of this type would significantly affect the state's green industry (Table 2). The Environment Committee introduced two bills that would have banned Norway maple, Japanese barberry, winged euonymus (*Euonymus alatus*), and multiflora rose. Japanese barberry and winged euonymus, for example, are very popular shrubs in landscape plantings in Connecticut and across the nation, accounting for $15-20 million in annual sales in Connecticut alone in 2002. Both species produce large numbers of seeds that are easily spread by birds. The proposed Connecticut bill also called for a $100 fine per plant for violation of the ban. The Connecticut green industry expressed its serious concerns over these bills and the economical consequence of banning these ornamental crops (Davis, 2004). Following continued discussions between the Environment Committee and the newly formed Connecticut Invasive Plants Council, a compromise bill was reached in 2004 to ban 81 non-native invasive or potentially invasive plants, with the $100 fine per plant retained. The ban, however, excludes 15 ornamental plants of economic concern to the green industry, including Japanese barberry, winged euonymus, and Norway maple. These 15 ornamental species may be considered for possible banning in future legislative sessions.

Florida is also considering a ban on a large number of plant species of ornamental interest that are produced mainly within the state for distribution to other parts of the country. Species of particular economic interest to the Florida nursery industry that are currently under consideration for a ban include coralberry (*Ardisia crenata*), asparagus fern (*Asparagus densiflorus*), camphor tree (*Cinnamomum camphora*), taro

TABLE 2. Invasive or Potentially Invasive Plants That May Be Banned in Connecticut and Their Impact to the Connecticut Green Industry (The data are conservative estimates of sales figures of invasive or potentially invasive plants provided by Connecticut Green Industry members in 2004)

Plant	Wholesale value ($)	Retail value ($)
Amur maple (*Acer ginnala*)	280,000	560,000
Black locust (*Robinia pseudoacacia*)	24,000	48,000
California privet (*Ligustrum ovalifolium*)	42,000	84,000
Eulalia (*Miscanthus sinensis*)	842,000	1,684,000
European privet (*Ligustrum vulgare*)	4,000	8,000
Glossy buckhorn (*Frangula alnus*)	5,000	10,000
Japanese barberry (*Berberis thunbergii*)	2,450,000	4,900,000
Porcelain berry (*Ampelopsis brevipedunculata*)	3,000	6,000
Norway maple (*Acer platinoides*)	450,000	900,000
Rugose rose (*Rosa rugosa*)	190,000	380,000
Water hyacinth (*Eichhornia crassipes*)	40,000	80,000
Winged euonymus (*Euonymus alatus*)	2,245,000	4,490,000

(*Colocasia esculenta*), Surinam cherry (*Eugenia uniflora*), Chinese banyan tree (*Ficus microcarpa*), common lantana (*Lantana camara*), Chinese privet (*Ligustrum sinense*), Japanese honeysuckle (*Lonicera japonica*), heavenly bamboo (*Nandina domestica*), strawberry guava (*Psidium cattleianum*), half-flower (*Scaevola sericea*), and umbrella tree (*Schefflera actinophylla*) (Jolly, 2004). If the ban is enacted, the loss of these plants to the trade could represent a significant reduction in income to the green industry both locally and nationally.

Although the most effective solution to address the problems caused by invasive ornamental plants would be to ban the use of all invasive or potentially invasive plants and to prohibit the import of new plants that are either invasive or potentially invasive, it may not be the best solution, at least for some invasive plant species. Many invasive and potentially invasive ornamental plants that have been introduced are used to supplement the market with new and unusual landscape plants or innovative food, medicinal, energy, or fiber crops. Simply prohibiting new imports and completely banning the use of all invasive and potentially invasive plants would be politically, socially, and economically problematic. Nursery growers, landscape designers, and others who are as-

sociated with the green industry have become increasingly concerned that some highly valuable ornamental crops are or will be classified as invasive, which may in turn lead to a ban on these plants, prohibiting their importation, movement, use, or sale. The American Nursery and Landscape Association (ANLA) that lobbies on behalf of the green industry has been opposed to "regulatory overkill" and "extremism" when dealing with invasive or potentially invasive ornamental plants.

One possible solution to this problem is to neutralize the invasive characteristics of economically important non-native plant species before they are planted in the landscape. Previously, traditional breeding methods have been used to select or breed sterile cultivars of invasive ornamental plants. Vetiver grass (*Vetiveria zizanioides*) has been extensively used because of its ability to stop soil erosion, increase soil fertility, increase forage production, purify water, and decrease water pollution, but it has also been found to be invasive in Australia, South Africa, Thailand, Hawaii, and the southern United States. To reduce its invasiveness, a sterile cultivar that sets no viable seed, registered as 'Monto vetiver' was developed. The cultivar underwent 7 years of testing and evaluation before its release was approved. 'Monto vetiver' grass has been used for soil and water conservation (Queensland Government Natural Resources and Mines, 2004). However, as was determined for purple loosestrife, several "sterile" cultivars that have been marketed for years have since been proven fertile. Anderson and Ascher (1993) determined fertility levels of a number of "sterile" cultivars of purple loosestrife and showed that these plants are not sterile. Although most of these cultivars are self-incompatible, they can produce large amounts of seed when used as a male or female parent in crosses (Ellis, 1998; The Nebline, 2000). Because a very limited number of sterile cultivars have been developed for invasive ornamental plants to date and because traditional breeding methods to produce sterile cultivars are technically difficult or time-consuming, new technologies such as plant gene transfer technology should be used to create true sterile cultivars for economically important invasive ornamentals.

BIOTECH TOOLS TO NEUTRALIZE THE INVASIVENESS OF NON-NATIVE ORNAMENTALS

Plant gene transfer technology has been shown to be a powerful means to modify growth and developmental processes of higher plants, including the production of sterile plants. There are a number of molec-

ular tools (fusion genes) that can be used to develop sterile cultivars of invasive ornamental plants. The following is a summarization of a selection of the tools that may be useful to neutralize the invasiveness of exotic ornamental crops.

Male and Female Sterility

Male sterility is defined as the inability of the male to fertilize the ovum, and female sterility is defined as the inability of the female organ to conceive. In both cases, the sterility can be caused by inadequacy in structure or function of the genital organs. To create male and female sterility, male and female organs of plants have been used as targeted sites. In recent years, several types of male and female sterility technologies have been developed. One group of male or female sterility technologies is based on targeted expression of cytotoxin genes in anther, pollen, stigma, or ovary. Cytotoxin genes used for creating sterility include ribonucleases (De Block and Debrouwer 1993; Denis et al., 1993; Goldman et al., 1994; Mariani et al., 1990; Zhan et al., 1996), diptheria toxin A chain (DTA, ADP-ribosyl-transferase) (Kandasamy et al., 1993; Thorsness et al., 1991), and the ribosome inactivating protein (RIP) (Cho et al., 2001). In the case of the ribonuclease toxin, for instance, Mariani et al. (1990) used a tobacco tapetum-specific gene promoter (TA29) to control the expression of the barnase gene cloned from a soil bacterium, *Bacillus amyloliquefaciens* in tobacco and oilseed plants and induced male sterility. Expression of the barnase gene specifically destroyed the tapetal cells and successfully prevented pollen formation, thereby producing male-sterile plants. Because the expression of the barnase gene was restricted to tapetum tissues, the vegetative growth and floral development of the transgenic plants were normal.

Similarly, female sterility has been achieved using the barnase gene. Goldman et al. (1994) used a stigmatic secretory zone specific gene promoter sequence, the 5′ untranslated region of the STIG1 gene cloned from tobacco plants, to drive the expression of the barnase genes. The pistils of transgenic plants that expressed the STIG1-barnase fusion gene underwent normal development but lacked the stigmatic secretory zone and therefore became female sterile. Pollen grains of the transgenic plants could germinate on the ablated stigmatic surface although they failed to penetrate the transmitting tissue of the style, suggesting that pollen was normal. Using a developing seed specific gene promoter, the FBP7 gene promoter, to control the expression of the barnase gene in tobacco plants, Colombo and his colleagues (1997) were able to generate

transgenic plants that produced no ovules or seeds. The promoter of the FBP7 gene cloned from petunia plants was specifically active in the coat of developing seeds and is completely silent in the gametophytically derived tissues. Because normal seeds were produced if wildtype plants were pollinated with transgenic plants, the pollen of the FBP7-barnase plants was fertile.

A second group of male or female sterility technologies is the use of fusion genes that can lead to alterations in specific metabolic pathways, endogenous plant hormone concentrations, or hormonal signaling pathways. For example, inhibition of pyruvate dehydrogenase production in mitochondria resulted in male sterility (Yui et al., 2003). Tissue-specific repression of an extracellular invertase, Nin88, caused male sterility (Goetz et al., 2001). Disruption of flavonoid biosynthesis through manipulation of chalcone synthase gene expression could lead to male sterility (van Tunen et al., 1988; van der Meer et al., 1992). Overexpression of an endo-beta-1,3-glucanase gene caused male sterility (Worrall et al., 1992; Tsuchiya et al., 1995). On the other hand, expression of a bacterial enoyl-CoA hydratase/lyase enzyme, which led to re-routing of the phenylpropanoid pathway, also caused male sterility (Mayer et al., 2001; Hernould et al., 1998).

Plant hormones play an important role in the reproductive processes of higher plants. An inhibition of expression of an ethylene-forming enzyme, l-aminocyclopropane-l carboxylate oxidase, in a pistil has been shown to be effective to disrupt ovule development and therefore caused female-sterility (De Martinis and Mariani, 1999). Reduction in jasmonic acid concentration in anthers led to male sterility due to defects in anther and pollen development (Park et al., 2002; Sanders et al., 2000). Expression of the rolC gene, cloned from *Agrobacterium rhizogenes*, resulted in male sterility and reduced female fertility, presumably due to changes in hormone concentration (Schmulling et al., 1993). Huang et al. (2003) showed that tissue-specific overexpression of the CKX1 gene involved in oxidative cytokinin degradation in transgenic maize (*Zea mays*) resulted in male-sterile plants. Similarly, expression of the gai gene involved in GA signal transduction in anthers and pollen of tobacco and *Arabidopsis* resulted in the abortion of these respective tissues (Huang et al., 2003). Male infertility or abnormal male organ development as a result of the expression of the CKX1 or gai genes both could be restored by applications of exogenous cytokinins.

A third group of technologies to create male or female sterility is to alter the development of specific tissues or organs of the plant reproductive system via ectopic expression of homeotic genes. Genetic and mo-

lecular studies demonstrate that homeotic genes act alone and together to specify the fate of floral organ primordia in higher plants. Changes in reproductive organ development can be achieved by manipulation of the expression of homeotic genes (Lohmann and Weigel, 2002, 2004). For example, ectopic expression of the *Brassica napus* AGAMOUS gene, which regulates the development of stamens and pistils, leads to the conversion of stamens into carpel-like structures in transgenic tobacco plants (Mandel et al., 1992). Expression of the AGAMOUS homolog, TAG1, resulted in the replacement of stamens with petaloid organs and the conversion of pistils to non- reproductive organs (Pnueli et al., 1994). In both cases, the transgenic plants were male sterile.

Of the three major technologies to produce male or female sterile plants discussed above, the first one, the reproductive organ- or tissue-specific expression of a cytotoxin gene such as the barnase gene appears to be simplest, most effective, and widely applicable technology for creating sterile ornamental cultivars. The application of the other two approaches to a diverse collection of invasive plant species, however, could be difficult. One reason is that the response of different plant species to changes in concentrations of metabolites or hormones can vary dramatically, and such variations can exacerbate further under certain environment. The homeotic conversion of one type of organ to another can dramatically affect flower morphology. For some ornamentals, such alterations could be undesirable. Another advantage of the use of a cytotoxin gene such as the barnase gene is that Mariani et al. (1992) have demonstrated that the expression of the TA29 promoter-barstar (a potent inhibitor of barnase also cloned from *Bacillus amyloliquefaciens*) fusion gene can restore male fertility of plants that express the TA29 promoter-barnase gene. The utility of the barstar gene is that it can be used to eliminate the cytotoxic effect of barnase in non-targeted tissues or organs. Also, the barstar gene can be used to restore fertility of barnase-mediated sterility conditionally so that multiplication of seeds from the sterile plants becomes possible.

Seed Sterility

Seed sterility is defined as the inability to germinate. "Terminator" seed technology (Oliver et al., 1998) is one well-known example of seed sterility. Scientists at the Delta and Pine Land Company linked a late embryogenesis specific gene promoter to a lethal gene, but the gene is interrupted by a blocking sequence so that the lethal gene is not active. The blocking sequence is bounded by the loxP sequences of the loxP/

Cre DNA recombination system from bacteriophage P1 (Austin et al., 1981). The loxP sequence is recognized by the Cre recombinase protein, and deletion of the intervening DNA occurs if the two loxP sequences are in the same orientation (Sauer, 1992). The interrupted lethal gene is introduced into a parental line. The Cre gene is under the control of a chemically induced gene promoter such as the tetracycline inducible gene promoter and introduced into anther parental line. Both of these lines can produce viable seed if self-pollinated. If both transgenic lines are hybridized, the progeny will contain both the interrupted lethal gene and the chemically inducible Cre gene. The Cre gene is expressed upon chemical treatment of the seed, and, as a consequence the blocking sequence within the two loxP sites will be removed, thereby directly connecting the lethal gene to the late-embryogenesis active gene promoter. As a result, when this promoter becomes active during maturation of the second-generation seed, the expression of the lethal gene specifically in seed makes the seed incapable of germination. A similar technology described by Kuvshinov et al. (2001) was termed "recoverable block of function" (RBF). A lethal gene, the barnase gene, was driven by a germination-specific promoter and the barstar (an inhibitor of barnase) gene was placed under the control of a heat-shock promoter. Under natural conditions, the barstar gene does not act because the heat-shock promoter is not induced and the seeds therefore cannot germinate. When needed, seed fertility can be restored through a temperature shock.

Parthenocarpy

Fruit development in higher plants normally requires pollination, fertilization, and seed development that stimulate cell division of specific floral tissues. In some cases, parthenocarpic (seedless) fruit development can proceed without pollination, fertilization, or seed development. Traditionally, parthenocarpic fruits are produced from mutants, triploid plants, or flowers treated with exogenous growth regulators (Varoquaux et al., 2002). However, because of technical difficulties associated with these methods, none of them has been widely applicable in ornamental plants. For instance, development of triploids of some species can be complicated due to the presence of an interploid block that prevents the normal development of a triploid embryo (Ranney, 2000). Also, the application of growth regulators specifically to floral organs of large numbers of plants is laborious and unpractical in commercial settings.

Gene transfer technology has offered a powerful tool to produce seedless fruits. Parthenocarpy was first reported in auxin-overproducing transgenic petunia (Klee et al., 1987) and then in tobacco plants (Guilfoyle et al., 1993). With ovary- or fruit-specific gene promoters to direct expression of the iaaM gene or *rolB* gene, seedless fruits were produced from eggplant, tomato and watermelon plants (Acciarri et al., 2002; Carmi et al., 2003; Li et al., 2000; Rotino et al., 1997). In our case, we used two types of gene promoters to drive the expression of the auxin biosynthetic gene, iaaM, cloned from *Agrobacterium tumefaciens* (Klee et al., 1987), the auxin inducible, ovary specific GH3 promoter cloned from soybean (Hagen et al., 1991) and the ovary specific AGL5 promoter (Sieburth and Meyerowitz, 1997). Using these two fusion genes, we produced transgenic *Arabidopsis*, tobacco, tomato, and watermelon plants (Li, 1998). All plants that expressed the GH3 promoter-iaaM or AGL5 promoter-iaaM gene were capable of producing seedless fruits. Also, because overproduction of auxin in the transgenic plants was restricted to the ovary and developing fruit, no obvious side effects were evident. The seedless tomato and watermelon fruits produced from the transgenic plants contained similar levels of acids and sugars, vitamins, and other nutrients relative to the seeded fruits produced from control plants. Furthermore, the seedless tomatoes and watermelons were normal in size or were significantly larger than those produced from the wildtype control plants grown under identical conditions, although fruit productivity per plant was not significantly altered.

Non-hormonal genes have also been shown to be capable of producing seedless fruits. For example, Yao et al. (2001) have shown that the loss of function mutation in the MdPI MADS-box transcription factor, a homolog of *Arabidopsis* mutant pistillata, confers parthenocarpic fruit development in apple plants. Ampomah-Dwamena et al. (2002) reported that repression of a tomato MADS box gene, TM29, using either co-suppression or antisense techniques, resulted in infertile stamens and ovaries and subsequently led to the production of parthenocarpic fruits. However, these transgenic tomato plants also produced aberrant flowers with morphogenetic alterations in the organs of the inner three whorls. Ito and Meyerowitz (2000) reported that overexpression of the cytochrome P450 gene could lead to production of parthenocarpic fruits. Wildtype *Arabidopsis* fruits developed to normal size only if the ovules were fertilized. When expression of the cytochrome P450 gene was hyper-activated, fruits grew without fertilization and reached nearly normal size. When wild-type pollen was used to pollinate the cytochrome P450 overexpressing plants, the pollinated fruits became more

than 10% longer and 40% wider than wild-type fruits but they produced very few seeds.

Advantages and Disadvantages of Transgenic Approaches to Neutralize the Invasiveness of Non-Native Ornamental Plants

Although there are a number of molecular tools that can be used to create male sterility, female/seed sterility, or parthenocarpy, any single one alone may not be effective enough to neutralize the invasiveness of an invasive ornamental plant. For instance, a male-sterile Japanese barberry plant can still produce seeds if pollinated by a closely related plant growing within a reasonably short distance. One possible solution to create plants that are completely sterile is to use both male- and female-sterile genes together in a single plant. Furthermore, many ornamentals such as Japanese barberry produce showy fruits that are of high ornamental value, but male sterility and female sterility will lead to the production of no fruits or undersized fruits which are undesirable characteristic to consumers. To circumvent that problem, we believe that a combined use of male-sterility, female-sterility, and parthenocarpic technologies may lead to development of 'super-sterile' cultivars that are both male and female sterile but also bear normal-sized fruits (seedless). We have recently constructed such a gene cassette that contains a male- and female-sterile gene and a parthenocarpy gene, named "super-sterile' gene cassette. We are currently testing the effectiveness of this gene cassette in several invasive ornamental plant species.

Like other methods to control the spread of invasive plants, there are also disadvantages associated with the gene transfer approach. One disadvantage is that delivery of sterile and parthenocarpy genes into many of the ornamental plants, such as Japanese barberry, winged euonymus, Russian olive (*Eleaegnus angustifolia*), and Norway maple, can be technically difficult because no genetic transformation methods have yet been developed for these species. Very recently, however, we have successfully used *Agrobacterium* to transform winged euonymus (Chen et al., 2006). Second, although ornamental plants are not used for food and introduced genes (transgenes) would be well contained because of their sterile nature, some consumers may still be reluctant to purchase these plants because they are "genetically modified." Third, sterile technologies can be effective in eliminating or reducing the invasiveness of these modified plants but they have little utility in preventing the further spread of unmodified plants that have already escaped from the managed landscape to natural areas. Finally, the sterile technologies as dis-

cussed here are not applicable for some invasive plants such as kudzu and English ivy whose invasiveness is dependent on rapid vegetative propagation.

On the other hand, plant gene transfer-mediated neutralization of invasiveness also offers several distinct advantages. First, male- and female-sterility genes or parthenocarpy genes could be broadly applicable to many species whose invasiveness is mediated through sexual reproduction. If a sterility gene or a parthenocarpy gene works well in Japanese barberry, it should also work well in winged euonymus and Norway maple. Second, the targeted expression of a cytotoxin gene in pollen or ovules would produce little or no effect on the morphology of floral organs, although it would lead to male or female sterility. Aesthetics of floral organs of ornamental plants is a critical consideration for consumers. Third, production of sterile or seedless cultivars via gene transfer techniques can be a faster process and therefore more cost effective in comparison to traditional methods. In addition, sterile flowers may senesce more slowly, as demonstrated in Easter lilies, carnations and roses (Daniell, 2002), a desirable trait for many ornamentals. Transgene escape has been a subject of public concern, but for "super-sterile" cultivars the combination of male sterility, female sterility and parthenocarpy traits in a single plant makes the escape of transgenes extremely unlikely. Finally, although early prediction has been proposed for new introductions of non-native plants, that process normally takes years and the prediction may not be reliable under some circumstance. The plant gene transfer-mediated production of "super-sterile" cultivars may allow new introductions of highly valuable non-native plants without worrying about a possibility of these plants to become invasive.

PERSPECTIVES

A large number of popular non-native ornamental plants are invasive or potentially invasive. These plants have already caused and will continue to cause significant damage to our ecosystem. However, completely prohibiting new imports and totally banning the use of all invasive and potentially invasive ornamental plants are socially, politically, and economically unfeasible. Current methods to control the spread of invasive ornamental plants may be ineffective, technically difficult, and expensive. Development of sterile forms of economically important ornamental crops can offer an excellent alternative to address the invasive problem of exotic ornamentals whose invasiveness is de-

pendent on sexual reproduction. Sterile plants can be grown and used for landscaping or ornamental purposes while virtually eliminating any possibility that these plants could sexually reproduce and become invasive. Also, a large number of molecular tools can be used for gene transfer-mediated production of sterile cultivars of economically important ornamentals that currently exist on the market or are likely to be introduced.

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Metabolic Engineering of Flower Color in Ornamental Plants: A Novel Route to a More Colorful World

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SUMMARY. Advances in the knowledge of plant pigment pathways at genetic, biochemical and molecular levels, and the establishment of genetic transformation methods for an increasing number of plant species have paved the way to genetic engineering of flower and plant color for ornamental purposes. From trial-and-error approaches based on the available few genes, the trend is now to comprehensively study the target species, genotypes and pigment pathways to precisely address specific breeding goals. This review examines the state of the art in this field, from the pioneering stages to fundamental and applied research on flavonoids, which is being extended to carotenoids and is also likely to involve other pigments in the near future. doi:10.1300/J411v18n01_01 *[Article copies available for a fee from The Haworth Document Delivery Service: 1-800-HAWORTH. E-mail address: <docdelivery@haworthpress.com> Website: <http://www.HaworthPress.com>*

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INTRODUCTION

Ornamental plants paint the world around us with a plethora of flower and leaf colors. From the domestication or import of wild species to the increasingly sophisticated breeding strategies, man has pursued his quest to improve flowering plants. Flower color, size, abundance and recurrent flowering are major traits in ornamental plant breeding because of their immediate visual impact. Flower color is the trait with the greatest genetic engineering prospects, since genetics, biochemistry and molecular bases of plant pigments are now well understood. Genetic transformation tools will likely help to increase the palette of colors in ornamental species in coming years. Major plant pigments are flavonoids and carotenoids. However, in some plants, flower color may also be highly influenced by other compounds such as betalains, chlorophylls, and indigo-related pigments. Betalains are present in only a restricted number of taxa and their pathway still has to be characterized and studied from the molecular standpoint. As for other "minor" pigments, chlorophylls play a major role in photosynthesis and are also responsible for rare cases of green flowers. Indigo-related compounds are important dyes in the textile industry, but have not yet found application in the flower biotechnology industry. Finally, pigments from the above-mentioned classes present exclusively in fungi, algae and bacteria could also be produced in plants by heterologous expression of non-plant genes.

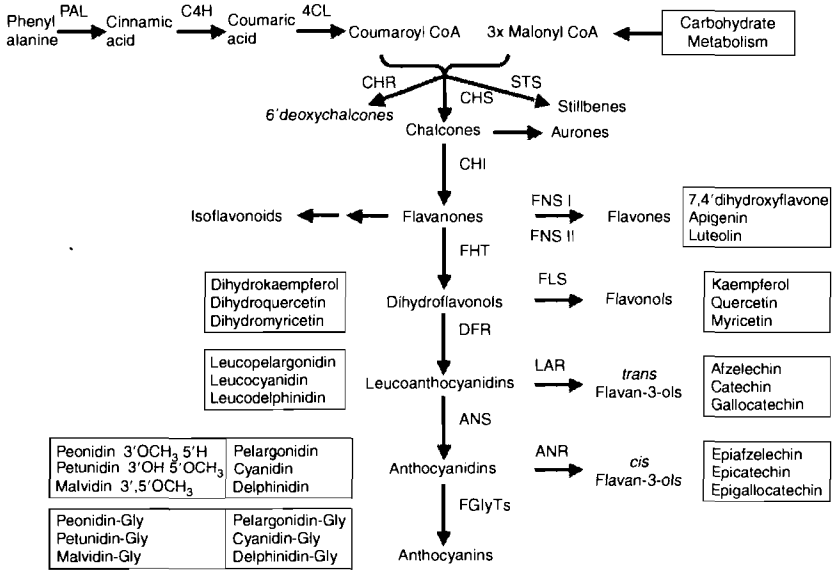
Genes which affect major ornamental traits such as those governing the genesis of flower whorls, meristem fate in the transition from the vegetative to the reproductive state, flower symmetry, cell shape, etc. are beyond the scope of this article. Instead, this review will mainly focus on flavonoids and carotenoids which have been studied intensively for ornamental and biotechnology purposes. For these two classes of compounds, the major molecular and biochemical features of their biosynthesis will be described, and achievements and future prospects highlighted. The reader will be referred to the main reviews in the field.

FLAVONOIDS

Flavonoids are water-soluble secondary metabolites, typically synthesized in epidermal cells of various organs to protect plants against

UV radiation and as a response to biotic and other abiotic challenges. The flavonoid pathway is one of the best-studied plant secondary pathways from the molecular, biochemical and genetic standpoint (Forkmann, 1993; Holton and Cornish, 1995; Springob et al., 2003). Nevertheless, recent findings of new structural genes (Tanner et al., 2004; Xie et al., 2003) and the lack of knowledge about some crucial steps such as proanthocyanidin formation will, certainly, in the near future, stimulate further research and generate results that could be exploited for applied ornamental purposes. Enzymatic reactions of the flavonoid pathway (Figure 1) mostly occur in the cytoplasm, and end products are accumulated in the vacuole, where they undergo a series of final modifications via acylation, complexation and polymerization. Each flavonoid class is characterized by a specific chemical structure of the aglycone molecule (Figure 2), which undergoes substitutions to give several end-products within each class. The first enzyme of the pathway, chalcone synthase (CHS), condensates p-coumaroyl-CoA with three malonyl-CoA molecules to form chalcones. Subsequent steps leading to anthocyanin biosynthesis involve chalcone isomerase (CHI), flavanone 3-hydroxylase (FHT), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS) and flavonoid glycosyltransferases (FGlyTs). For FglyTs, glycosylation at the C3 (carbon 3) position on the flavonoid molecule reflects the usual situation for most species, even though flavonoids with various glycosylation patterns at different positions have been characterized, due to the action of different enzymes encoded by members of the large plant glycosyltransferase gene family (Vogt and Jones, 2000). A number of structural genes encode enzymes involved at branch points of the pathway (Figure 1). Flavone synthases (FNS I and FNS II) and flavonol synthase (FLS) lead to the synthesis of flavones and flavonols, respectively. Leucoanthocyanidin reductase (LAR) and anthocyanidin reductase (ANR) enzymes are involved in the synthesis of flavan-3-ols, which are monomers that form high molecular weight proanthocyanidins (PAs). The condensing enzyme giving rise to PA biosynthesis is currently one of the few missing steps of flavonoid biosynthesis that remains to be characterized. Finally, flavonoid 3'hydroxylase (F3'H) and flavonoid 3' 5'hydroxylase (F3'5'H) modifying enzymes control the hydroxylation pattern of the B ring, a key feature determining the final color of the anthocyanin molecule (Figure 3). It is also worth mentioning that some plant species or families possess specific flavonoid enzymes. Leguminous plants are able to produce isoflavonoids and derived phytoalexins, whose synthesis is initiated by chalcone reductase (CHR), isoflavone synthase (IFS) and isoflavone reductase (IFR) enzymes.

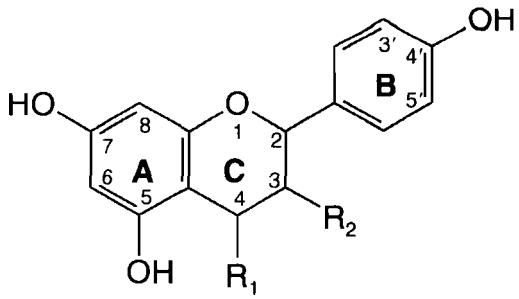
FIGURE 1. Schematic representation of the flavonoid pathway. For each class of flavonoid molecules, the three aglycones showing different hydroxylation pattern in the β ring are noted. Abbreviations: 4CL, 4-coumarate:CoA ligase; ANR, anthocyanidin reductase; ANS, anthocyanidin synthase; C4H, cinnamate-4-hydroxylase; CHI, chalcone isomerase; CHR, chalcone reductase; CHS, chalcone synthase; DFR, dihydroflavonol 4-reductase; FGlyTs, flavonoid glycosyltransferases; FHT, flavanone 3-hydroxylase; FLS, flavonol synthase; FNS, flavone synthase; LAR, leucoanthocyanidin reductase; PAL, phenylalanine ammonia lyase; STS, stilbene synthase.



Grape and a few other plants possess stilbene synthase (STS), a polyketide synthase that is quite similar to CHS, which leads to resveratrol and other antioxidant stilbenes (Figure 1).

Many flavonoid compounds confer pigmentation, namely anthocyanins, 3-deoxyanthocyanins (or phlobaphenes), chalcones, aurones, flavones and flavonols. Among these, the anthocyanins represent the most widespread class of flavonoid pigments, giving rise to a wide array of colors in most plants, while 3-deoxyanthocyanins are mainly present in monocot grasses. The final color appearance mostly depends on the substitution pattern of the B ring of the anthocyanin pigment molecule (Figure 3), to give pink, orange (pelargonidin), red (cyanidin), blue (delphinidin) and purple/magenta (peonidin, petunidin and malvidin).

FIGURE 2. Chemical structure and nomenclature of the main flavonoid aglycone molecules.

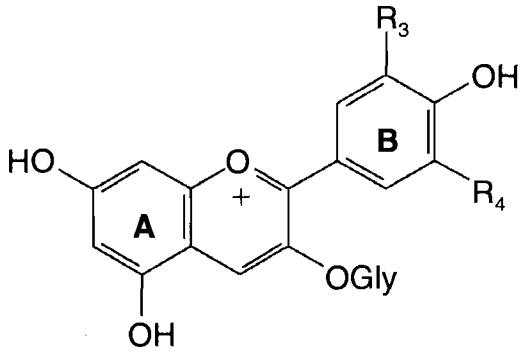


Flavonoid class	R_1	R_2	Double bond site(s)
Flavanones	O	H_2	-
Flavan-4-ols	OH	H_2	-
Flavones	O	H	2-3
Dihydroflavonols	O	OH	-
Flavonols	O	OH	2-3
Leucoanthocyanidins	OH	OH	-
Flavan-3-ols	H_2	OH	-
Anthocyanidins	H	OH	1-2, 3-4
Anthocyanins	H	O-Gly[a]	1-2, 3-4

[a] various glycosylating sugar residues

Other flavonoid classes as flavones and flavonols complete the palette, providing yellow colors and acting as co-pigments, thus modulating the hue and brightness of anthocyanin pigmentation as well as the appearance and "depth" of white color. Interestingly, the mere presence of the anthocyanin pigment does not ensure the display of the corresponding color, since the pigmentation has also been shown to be strongly dependent on vacuolar pH and the interaction between anthocyanins, co-pigments and metal ions (Forkmann, 1993) and, more recently, on the glycosylation position (Fukuchi-Mizutani et al., 2003). This holds particularly true for delphinidin-derived pigments, where the transgenic approaches to obtain new products in major ornamental species lacking blue flowers such as roses has generated limited marketable results to date. A notable exception is represented by "blue" carnation varieties successfully obtained through biotechnology, which were released in

FIGURE 3. Chemical structure and nomenclature of the six anthocyanin aglycones.



Anthocyanin (3-Gly)	R_3	R_4
Pelargonidin	H	H
Cyanidin	OH	H
Delphinidin	OH	OH
Peonidin	OCH_3	H
Petunidin	OCH_3	OH
Malvidin	OCH_3	OCH_3

the late 1990s and marketed in Australia, Japan, USA and Europe (Mol et al., 1999; Tanaka, Tsuda, and Kusumi, 1998).

GENETIC ENGINEERING OF FLAVONOID PIGMENTS

The wealth of genetic and biochemical information about the flavonoid pathway has set the stage for genetic engineering. Modification of flower pigmentation has been achieved since the 1980s as one of the first successes of applied plant biotechnology, either through heterologous transgene expression or silencing of endogenous genes (e.g., Courtney-Gutterson et al., 1994; Meyer et al., 1987). Since then, progress in cloning and knowledge of structural and regulatory genes of the flavonoid pathway have prompted flower biotechnologists to use different genes and strategies to change the color of flowering ornamental plants. From model species such as petunia, snapdragon and chrysanthemum, the list of engineered plants has been expanding at a fast pace (Forkmann and Martens, 2001). The list is still expanding, also due to

the increasing availability of gene sequences and the development of transformation protocols for new plant species. Table 1 summarizes some key results for a number of key genes.

To avoid "trial and error" approaches, the biochemical diversity of flavonoid composition of target organs (usually petals or tepals) within species should first be studied, while testing the *in vivo* effects of specific inhibitors of many enzymes (mono- and dioxygenases) of the pathway on the floral phenotype, and conducting enzymatic studies on different steps of the flavonoid pathway in target plants. The impact of such preliminary biochemical studies for designing rational genetic engineering strategies to modify flavonoid biosynthesis in plant tissues was recently highlighted (Martens et al., 2003). For instance, enzymatic characterization, recently enhanced by the successful use of bacterial or eukaryotic heterologous expression systems (Martens, Teeri, and Forkman, 2002; Vogt and Jones, 2000), revealed striking substrate specificities for DFR and FGlyT enzymes. These observations may provide a basis for some future projects aimed at engineering flower color.

Another important issue concerns the expression of the transgene in the target tissue. The cauliflower mosaic virus (CaMV) 35S gene promoter has been extensively used because of its ability to drive strong transgene expression in flower organs. Nevertheless, its constitutive expression in most plant organs and tissues is a major drawback for using it for "chirurgical" engineering, which could be achieved using flower-specific promoters. Also, intellectual property issues related to the CaMV 35S promoter have to be taken into account.

Once the target species has been characterized and the transgenes isolated, a number of strategies could be adopted to achieve the final goal.

Down-regulation of flavonoid genes. Repression of the flavonoid pathway at a single enzyme step resulted in the impairment of pigment synthesis or rerouting towards the synthesis of new compounds in branches upstream from the down-regulated gene/enzyme. Down-regulation of gene expression has often been achieved by gene silencing, either through the expression of antisense RNA or homologous sense RNA (co-suppression) or, more recently, using RNA interference constructs. CHS genes are typical targets of this strategy, resulting in inhibition of the whole flavonoid pathway. White flowers were first successfully obtained with antisense CHS constructs in petunia and tobacco (van der Krol et al., 1988). Antisense strategies and co-suppression against CHS and DFR genes were later extended to important market species such as chrysanthemum (Courtney-Gutterson et al.,

TABLE 1. Genetic engineering of flower or plant color. For each gene, some examples of biotechnology-based modifications of pigment pathways are reported. For carotenoid pathway, examples of natural mutants with modified floral phenotype are also reported. For abbreviations, see text and figure legends.

Pathway Gene	Target plant	Construct (source)	Transgene effect	Reference
Flavonoid CHS	Petunia, Tobacco Chrisanthemum	Antisense CHS (*Petunia*) Antisense CHS (*Chrisanthemum*)	Loss of pigmentation, patterning Loss of pigmentation	van der Krol et al., 1988 Courtney Gutterson et al., 1994
STS	Tobacco	Sense STS	Reduced pigmentation, male sterility	Fischer, Budde, and Hain et al., 1997
CHR	Petunia Tobacco	Sense CHR (*Medicago*) Sense CHR (*Pueraria*)	Novel 6′-deoxychalcones Depigmentation, novel 5-deoxyflavonoids	Davies et al., 1998 Joung et al., 2003
FHT	Carnation	Antisense FHT (*Dianthus*)	Reduced pigmentation, increased scent	Zuker et al., 2002
DFR	Petunia (white) *Torenia* (blue)	Sense DFR (maize) Antisense DFR (*Torenia*)	Novel orange flowers (pelargonidin) Bluer flowers, increased flavones	Meyer et al., 1987 Aida et al., 2000
ANS	Forsythia	Sense ANS (*Matthiola*) + Sense DFR (*Antirrhinum*)	Bronze color (*de novo* cyanidin over carotenoids)	Rosati et al., 2003
FGlyTs RT	*Eustoma* Petunia	Sense FGluT (*Antirrhinum*) Antisense RT (*Petunia*)	New anthocyanin and acylation patterns Different anthocyanin pattern	Markham, 1996 Brugliera et al., 1994
FLS	Petunia, Tobacco Petunia	Antisense FLS (*Petunia*) Sense FLS	Different pigmentation, decreased flavonols Increased anthocyanins, decreased flavonols	Holton, Brugliera, and Tanaka, 1993 Tanaka, Tsuda, and Kusumi ,1998
FNS II	*Torenia* (purple)	Sense FNS II (*Torenia*)	Paler flowers (decreased flavones)	Ueyama et al., 2002
F3′5′H	Carnation (white) Tobacco Tobacco, Petunia	Sense F3′5′H + DFR Sense F3′5′H (*Campanula*) Sense F3′5′H (*Eustoma*, *Petunia*)	Violet flowers (delphinidin) Purple flowers (delphinidin) *Different pigmentations (delphinidin)*	Holton, 1996 Okinaka et al., 2003 *Shimada et al., 1999*
Regulatory genes	Tobacco Tobacco Petunia	Sense Myc-like (*Perilla*) Sense Myb-like (*Fragaria*) Sense Myc-like (maize *Lc*)	Increased pigmentation Loss of anthocyanins and flavonols Increased leaf and flower pigmentation	Gong et al., 1999 Aharoni et al., 2001 Bradley et al., 1998
Carotenoid PSY1	Tomato	Antisense 35S::PSY (tomato)	Loss of pigmentation in flowers and fruits	Bird et al., 1991
CCS	*N. benthamiana*	Sense CCS (pepper)	Orange patterned leaves	Kumagai et al., 1998
CrtO	Tobacco	Sense *CrtO* (*Hematococcus*)	Red nectaries	Mann et al., 2000
CRTISO	Tomato mutants	*tangerine* mutants	Light-pigmented flowers, orange-red fruits	Isaacson et al., 2003
B	Tomato Tomato mutants	Antisense B *Beta* and *Old Gold* mutants	Patterned orange flowers	Ronen et al., 2000
Other cases GFP	*Osteospermum* and *Eustoma*	Sense GFP	Fluorescent flowers	Mercuri et al., 2001

1994), gerbera, rose and other flowering plants (reviewed in Davies and Schwinn, 1997). Mostly, these approaches have led to the creation of white flowers. Nevertheless, a wide array of hues–from white to wild-type color–has been obtained in the transformed clones due to the modulation of transgene expression caused by post-translational RNA degradation, insertion position effects, methylation, etc. (Jorgensen et al., 1996; Metzlaff et al., 1996). These antisense (and some sense) approaches have surprisingly produced transformed plants with varying spatial pigmentation patterns. Symmetric and erratic pigmentation patterns of antisense CHS transformants were reported in *Petunia* (van der Krol et al., 1988) and *Eustoma* (Deroles et al., 1995), but not in species that naturally have no patterning, which would suggest that this phenomenon is likely restricted to species showing flower developmental instability.

More recent gene down-regulation approaches highlighted the importance of co-pigment molecules in changing the visual perception of pigments. Suppressing FLS gene expression in *Petunia* decreased the size of the flavonol pool, and displayed red flowers since cyanidin appears more intensely red in the absence of flavonols (Tanaka, Tsuda, and Kusumi, 1998). Inhibition of DFR in *Torenia* boosted the flavone content, which made wild-type violet flower color more intensely blue (Aida et al., 2000). Co-suppression of FNSII in *Torenia* reduced the amount of flavones and increased that of flavanones, generating transgenic flowers paler than wild-type ones (Ueyama et al., 2002).

A surprising transgene effect was observed when down-regulating FHT genes in carnation. The expected loss of pelargonidin pigmentation was accompanied by increased floral scent in transgenic lines, due to an increase in methyl benzoate emission. Flavonoid and volatile compound pathways share cinnamic acid as common precursor, so a possible rerouting of metabolites was hypothesized (Zuker et al., 2002).

Glycosylation is the final modification which improves the stability and water solubility of flavonoid molecules prior to their import into the vacuole, but also determines the type and extent of successive modifications such as methylation, acylation, complexation and further glycosylation. Therefore, FGlyTs play a crucial role on flower color. For instance, *Petunia* plants transformed with an antisense construct against rhamnosyl transferase (RT) showed a reduction in malvidin and an increase in petunidin and delphinidin levels, probably due to impairment of flavonoid methyltransferase action on anthocyanin molecules (Brugliera et al., 1994). An increasing number of FGlyT genes are being identified as members of a large plant glycosyltransferase superfamily, and func-

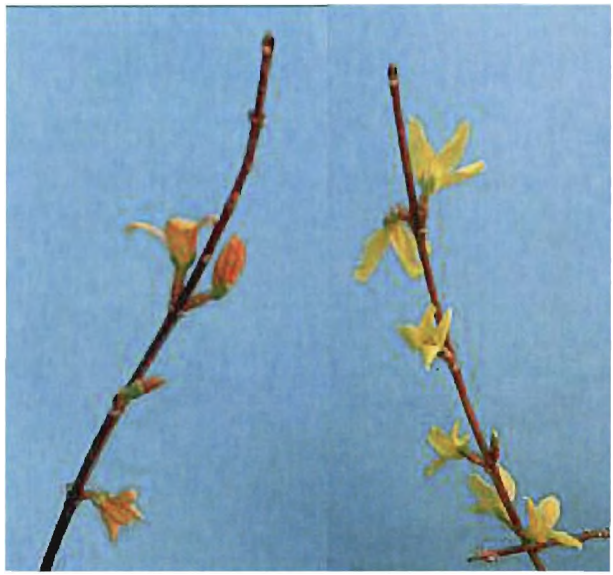
tional assessment of their properties (e.g., substrate specificity, see Table 1) should facilitate their use for genetic engineering of flower color.

Finally, transformation with transcription factor genes can also down-regulate the expression of genes in the flavonoid pathway, as shown by Aharoni et al. (2001), who reported the impairment of anthocyanin biosynthesis in tobacco flowers using a strawberry Myb-like transcription factor.

Overexpression of flavonoid genes. This strategy aims at increasing the synthesis of existing compounds or enabling the synthesis of new flavonoids by boosting or rerouting the substrate flow towards end products of the pathway. Overexpression of structural genes has been widely used to accumulate higher levels of anthocyanins but only a few examples will be presented here. In fact, the first case of genetic engineering of flower color was the creation of novel orange-flowered petunias obtained by introducing a maize DFR gene in a line with white flowers (Meyer et al., 1987). *Petunia* DFR is not able to convert dihydrokaempferol into leucopelargonidin, so the introduced DFR complemented the lack of activity on 4′ hydroxylated compounds and enabled the synthesis of orange pelargonidin pigments in transgenic flowers, leading to the creation of a floricultural novelty.

As an example of minor ornamental species and flavonoid gene stacking, the authors of the present article produced forsythia plants with a novel flower color. In this popular early-flowering ornamental shrub, anthocyanins accumulate in sepals but not in petals or anthers. The invariably yellow petal color results from the accumulation of carotenoids, while flavonol glycosides are the main constituents of the flavonoid fraction in this organ. Molecular and biochemical preliminary studies revealed that transcriptional block of the ANS gene and to a lesser extent of the DFR gene were responsible for the lack of anthocyanin synthesis in petals (Rosati et al., 1997; Rosati et al., 1999). Therefore, flower color was modified by subsequently transforming forsythia with a DFR gene of *Antirrhinum* and an ANS gene of *Matthiola* (Rosati et al., 2003). As expected, the double transformants displayed a novel bronze-orange petal color due to *de novo* synthesis of cyanidin anthocyanins over the wild-type yellow carotenoid background (Figure 4). Single transformants with either gene had an unchanged floral phenotype and pigmentation of vegetative organs (stems, petioles, leaf veins) was visible, but it was far more intense in double transformants. Both double and single transformants also had a modified flavan-3-ol accumulation pattern, in agreement with the most re-

FIGURE 4. Phenotype of forsythia cv. 'Spring Glory' wt (right) and DFR + ANS double transformants (left) at full bloom.



cent characterization of specific steps leading to PA (condensed tannin) biosynthesis, another biologically important class of antioxidant and pigment molecules (Xie et al., 2003).

Overexpression of genes encoding transcription factors of the flavonoid pathway can also lead to the activation of specific sets of genes (Table 1). Studies on tobacco by Gong et al. (1999) and petunia by Bradley et al. (1998) showed that it is possible to increase flower pigmentation by up-regulating anthocyanin pathway genes through the constitutive expression of *Myc*-like activators from dicot and monocot species, respectively. Strong pigmentation of leaf and vegetative organs was also observed in petunia transformed with the maize *Lc* construct. These recent studies and others published previously (reviewed by Holton and Cornish, 1995) demonstrate the interchangeability of transcription factors in up- and down-regulating the whole flavonoid pathway, even in phylogenetically distant species.

Overexpression of foreign genes to introduce new functions in target plants. Progress in the knowledge of genes/enzymes governing taxon-specific branches of the flavonoid pathway has led to the introduction

of novel functions in engineered plants. A typical example is given by the introduction of the F3′5′H activity in species lacking blue flowers–first of all, rose–for *de novo* production or increase of delphinidin pigments. Genetic engineering of different Solanaceae plants with heterologous and homologous F3′5′H enzymes gave rise to various pigmentation levels in tobacco and petunia flowers (Table 1). Florigene Ltd and Suntory Ltd developed a series of "blue"-flowered transgenic carnation varieties that are presently marketed in Japan, USA and Australia. These new varieties were obtained by concomitant transformation with *Petunia* DFR and F3′5′H genes, which enabled *de novo* synthesis of delphinidin (Holton, 1996). Nevertheless, vacuolar pH conditions and co-pigmentation effects have so far hampered the development of true blue carnations, as well as that of delphinidin-containing roses and chrysanthemums (Forkmann and Martens, 2001). Further investment is thus needed to achieve the "Holy Grail" of flower breeding.

Engineering of model Solanaceae plants with legume CHR genes, resulting in rerouting of the flavonoid pathway towards novel deoxyflavonoid compounds, and the use of STS genes are further examples of "functional gain." In a white-flowered petunia line, the introduction of a CHR gene from *Medicago* led to the development of yellow flowers due to the production of 6′ deoxychalcones and the concomitant reduction of flavonols (Davies et al., 1998). In tobacco, transformation with a CHR gene from *Pueraria* decreased anthocyanin levels but not total flavonoid content in flowers, indicating substantial homeostasis in total flavonoid metabolite flow (Joung et al., 2003). As for STS, repression of anthocyanin biosynthesis in tobacco by transformation with a sense STS gene produced flowers with reduced pigmentation but also male sterility (Fischer, Budde, and Hain, 1997). This important trait could therefore be manipulated in Solanaceae and other taxa for which pollen vitality depends on the presence of flavonols.

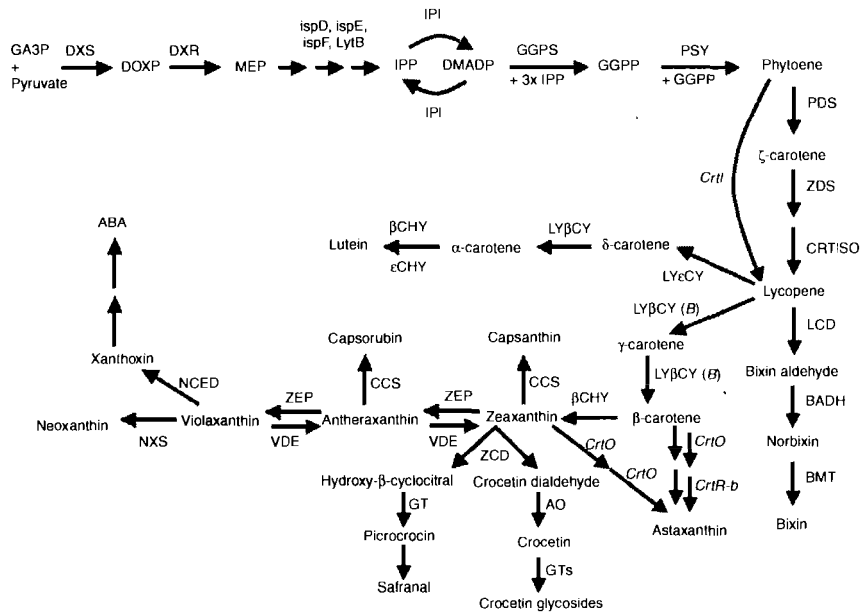
Recently isolated genes (e.g.,, anthocyanidin reductase, flavone synthase, leucoanthocyanidin reductase and various glycosyl transferases) and expressed sequence tag (EST) sequencing programs from species accumulating specific flavonoids (e.g., aurones, isoflavonoids) are new tools that could be implemented to possibly reroute the flavonoid pathway towards the synthesis of novel pigment compounds, thus enlarging the color palette or facilitating the introduction of new features (pathogen and/or pest resistance or pharmaceutical/nutritive value of different crop plants).

CAROTENOIDS

Carotenoids are a large family of C40 compounds derived from successive condensations of C5 isopentenyl pyrophosphate (IPP; Figure 5), which is also required for the synthesis of important molecules such as cytokinins, gibberellins, chlorophylls, terpenoids, sterols, and quinones (Cunningham and Gantt, 1998; Hirschberg, 2001). Carotenoid pigments give yellow, orange, pink and red colours to flowers and fruits, and they are also present in fungi, bacteria and algae. In plants, carotenoids play fundamental roles, contributing to light energy transduction and photoprotection in the photosynthetic apparatus. In addition, they carry out other biologically important functions as signaling molecules in plant-microorganism interactions, as an important source of pro-vitamin A and antioxidant compounds in human and animal diets, and, most relevant to this article, as a source of pigmentation for flowers, fruits and seeds (Cunningham and Gantt, 1998).

In plants, carotenoid biosynthesis was shown to be the result of tight regulation of gene expression, promoted by developmental and environmental cues and controlled by rate-limiting steps (Corona et al., 1996; Cunningham and Gantt, 1998; Giuliano, Bartley, and Scolnik, 1993; Matthews, Luo, and Wurtzel, 2003). Carotenoid biosynthesis starts with the production of colorless phytoene by phytoene synthase (PSY). Subsequent desaturations of the chromophore region by phytoene desaturase (PDS) and zeta-carotene desaturase (ZDS) enzymes, and isomerization by carotenoid isomerase (CRTISO) lead to the synthesis of lycopene, the major red tomato pigment (Figure 5). Note that bacteria have genes coding for multifunctional enzymes of this part of the pathway, exhibiting both desaturase and isomerase properties, which produce lycopene directly from phytoene (Hirschberg, 2001). These bacterial genes have also been used in plants and proved to be perfectly functional, for instance, in the carotenoid engineering of Golden Rice (Ye et al., 2000). Cyclization of lycopene by lycopene β- and ε-cyclases (LyβCY and LyεCY) constitutes a branching point in the carotenoid pathway (Figure 5). α-Carotene is converted into lutein by carotene hydroxylases (εCHY and βCHY), while β-carotene is transformed by βCHY into zeaxanthin, which enters the xanthophyll cycle via the alternating activity of zeaxanthin epoxidase (ZEP) and violaxanthin deepoxidase (VDE). Carotenes and xanthophylls provide orange and yellow pigmentation in flowers and leaves, respectively. Xanthophylls with two β rings are also precursors of absiscic acid (ABA) which regulate

FIGURE 5. Schematic representation of isoprenoid and carotenoid pathways. Specific steps leading to the biosynthesis of bixin from lycopene in *Bixa orellana* (Bouvier, Dogbo, and Camara., 2003), astaxanthin in bacteria and algae (Mann et al., 2000), and crocetin, picrocrocin and safranal in *Crocus sativus* (Bouvier et al., 2003) are also shown. Product abbreviations: ABA, abscisic acid; DMADP, dimethylallyl diphosphate; DOXP, 1-deoxy-D-xylulose 5-phosphate; DPME, 4-diphosphocytidyl-2C-methyl-D-erythritol; GA3P, glyceraldehyde 3-phosphate; GGPP, geranylgeranyl diphosphate; IPP, isopentenyl diphosphate; MEP, 2-C-methyl-D-erythritol. Gene/enzyme abbreviations: AO, aldehyde oxydoreductase; *B*, chromoplast-specific lycopene β-cyclase gene; BADH, bixin aldehyde dehydrogenase; BMT, norbixin methyltransferase; CCS, *capsanthin-capsorubin synthase*; βCHY, *carotene β-ring hydroxylase*; εCHY, carotene ε-ring hydroxylase; *CrtI*, bacterial phytoene desaturase gene; CRTISO, carotenoid isomerase; *CrtO*, bacterial β-carotene ketolase gene; *CrtR-b*, bacterial β-carotene hydroxylase gene; DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; DXS, 1-deoxy-D-xylulose 5-phosphate synthase; GGPS, GGPP synthase; GT, UDPG-glucosyl transferase; ispD, DPME synthase; ispE, DPME kinase; ispF, MEP 2,4-cyclodiphosphate synthase; IPI, IPP isomerase; LCD, lycopene cleavage dioxygenase; LYβCY, lycopene β-cyclase; LYεCY, lycopene ε-cyclase; LytB, (*E*)-4-hydroxy-3-methyl-but-2-enyl diphosphate reductase; NCED, 9-cis epoxycarotenoid dioxygenase; NXS, neoxanthin synthase; PDS, phytoene desaturase; PSY, phytoene synthase; ZDS, ζ-carotene desaturase; VDE, violaxanthin deepoxidase; ZEP, zeaxanthin epoxidase.



seed dormancy and organ abscission. It is noteworthy that specific carotenoids confer unique pigmentation, fragrance and taste to flowers and fruits in some species. For example, the color of red pepper varieties is due to the action of the capsanthin-capsorubin synthase (CCS) enzyme, a κ cyclase homologous to β- and ε-cyclases and displaying partial β-cyclase activity (Figure 5). In pepper chromoplasts, CCS converts antheraxanthin and violaxanthin into capsanthin and capsorubin, respectively (Bouvier et al., 1994), which accumulate in specific lipoprotein fibrils containing the major carotenoid-associated fibrillin protein (Deruère et al., 1994). In *Crocus* flowers, three apocarotenoids, i.e., crocetin, picrocrocin and safranal, are key molecules, respectively, responsible for the orange-yellow color, bitter taste and aroma of saffron through the cleavage of zeaxanthin by the zeaxanthin cleavage dioxygenase (ZDC) enzyme (Bouvier et al., 2003). In a subsequent elegant study on *Bixa orellana*, a tropical tree from South America, Bouvier, Dogbo, and Camara (2003) reconstructed the three enzymatic steps starting from the cleavage of lycopene and leading to the synthesis of bixin (annatto), which is the second most important dye used in the food and cosmetic industries worldwide. Several carotenoids present only in bacteria and algae as a result of the impressive combinatorial chemistry of their carotenoid pathway (e.g., Albrecht et al., 2000; Mann et al., 2000) could also be synthesized in transgenic plants (Mann et al., 2000).

Ornamental pigmentation by carotenoids was first developed to attract pollinators and animals for seed dispersal, but its potential has not yet been fully explored by plant breeders. Indeed, no reports of genetic engineering of carotenoids to specifically modify flower or plant color have been reported. However, an analysis of the literature revealed a number of studies which, if properly pursued and exploited, might give rise to genetic manipulation of carotenoid for ornamental purposes. Strategies for modifying the carotenoid pool in flowers of a given plant species are essentially the same as those used for flavonoids. However, given the role of carotenoids in photosynthesis, it is essential to use organ- or organelle-specific promoters to restrict transgene expression to target organs. In this respect, to gain further insight into the probable transgene effects, the effects of inhibitors of specific steps of the carotenoid pathway (e.g., Norflurazon for phytoene desaturase, CPTA for lycopene cyclases) or the analysis of publicly available tomato germplasm and other mutant collections (e.g., http://zamir.sgn.cornell.edu/mutants/) should also be explored to overcome the need for

empirical studies. The following examples will clearly explain the cautionary issues mentioned above.

GENETIC ENGINEERING OF CAROTENOID PIGMENTS

Genetic engineering research has been more focused on flavonoids than carotenoids for potential ornamental applications because of the more recent progress in gene cloning and the limited presence of carotenoid pigments in flowers of major ornamental species. Since the advent of Golden Rice (Ye et al., 2000), a major breakthrough in biotechnology geared towards nutritional enhancement, the momentum of research on the development of carotenoid-engineered products has increased dramatically.

Tomato is maybe the most widely studied species with respect to carotenoid biosynthesis. Evident ripening-associated modifications in its fruit phenotype and the development of mutant collections have made it a model species and a possible starting point for studying potential ornamental applications of molecular engineering of the carotenoid pathway.

In the first work of interest for flower color breeding, Bird et al. (1991) produced transgenic tomatoes with an antisense pTOM5 (coding for a PSY gene) construct under control of the CaMV 35S promoter. The plants had yellow fruits and paler yellow flowers due to the inhibition of the whole pathway. Interestingly, the total carotenoid content was not affected in leaves, and this was independent of the constitutive expression driven by the CaMV 35S promoter. In fact, plants have two PSY genes: *psy1*, corresponding to the pTOM5 clone, mostly expressed in fruits and flowers, and *psy2*, mostly expressed in leaves. Overexpression of pTOM5 *psy1* thus only altered the carotenoid composition in chromoplast-containing organs. In *Petunia*, transformation with *psy*, *lycy* and *chy* constructs driven by the CaMV 35S promoter produced minor changes in petal color due to a very slight increase/modification of existing carotenoid levels/patterns (Davies et al., 2003). Other carotenoid gene constructs altered the leaf carotenoid composition, thus highlighting the deleterious effects of the CaMV 35S promoter in driving constitutive transgene expression, and the difficulty of modifying flower carotenoid color in a species with limited chromoplast development in petals of flowers such as *Petunia*.

Studies on species accumulating particular carotenoid compounds have provided further tools for ornamental biotechnologists. The expression of a pepper CCS gene in tobacco leaves transfected with a viral expression vector led to high capsanthin accumulation, with a concomitant reduction in β-ring xanthophylls (Kumagai et al., 1998). Leaves showed a patterned orange phenotype, associated with major modification in plastid structure. This and similar approaches could be used for genetic engineering of foliage ornamental plants. Cloning of *Crocus* ZDC has paved the way to engineering of flower and food fragrance through enzymatic cleavage of zeaxanthin. As also envisaged by the authors of the original paper (Bouvier, Dogbo, and Camara, 2003), the expression of the three *Bixa* enzymes for annatto synthesis in engineered tomato fruits could lead to bixin production. These studies, together with studies on 9-*cis* epoxycarotenoid dioxygenase (NCED) enzymes involved in ABA biosynthesis (e.g., Tan et al., 2003), have highlighted the biological importance and biotechnological applications of plant carotenoid cleavage dioxygenases.

The use of the chromoplast-specific promoter of tomato *Pds* (Corona et al., 1995) succeeded in targeting expression in chromoplast-containing organs without altering the leaf carotenoid content in various examples. In tobacco flowers, the nectary is the only tissue containing chromoplasts. Mann et al. (2000) introduced the *CrtO* gene from the algae *Haematococcus*, which encodes a beta-carotene ketolase. The nectary color of transgenic flowers was changed from yellow to red due to accumulation of the high-value pigment astaxanthin. Although of limited ornamental value, this revealed the feasibility of expressing non-plant genes for flower color engineering. Metabolic engineering of lycopene, beta-carotene and xanthophyll content was reported in tomato fruits for nutritional enhancement (Dharmapuri et al., 2002; Römer et al., 2000; Rosati et al., 2000). Introduction of a bacterial gene or plant genes under the control of either CaMV 35S or tomato *Pds* promoters did not modify the flower color of transgenic lines in any of the above-mentioned studies. This is likely due to concurrent factors, such as the use of sense constructs that actually "push" the metabolite flow towards already existing violaxanthin, the activity of both lycopene β- and ε-cyclases in flowers and the presence of alternative lycopene β-cyclases in tomato. In an attempt to modify flower color, one of the authors of the present review transformed tomato and forsythia with a construct containing the *Capsicum* CCS gene under control of the tomato *Pds* promoter (C. Rosati, unpublished). The strategy was aimed at rerouting carotenoid biosynthesis in flowers from β-β xanthophylls to-

wards capsanthin and/or capsorubin in order to produce solid red flowers. The failure in altering the wild-type yellow flower phenotype was probably due to the lack of fibrillin and other carotenoid-associated proteins typical of pepper chromoplasts or transgene co-suppression because of the high sequence similarity between CCS and homologous lycopene β-cyclase genes.

Studies on natural and induced tomato mutants generated information on possible carotenoid engineering strategies in flowers. *Old-gold* and *old-gold crimson* mutants have tawny orange flowers and deep red fruits that lack β-carotene. This is the result of a mutation at the *B* locus, coding for an alternative lycopene β-cyclase, which leads to the accumulation of up to 12% of lycopene in *old-gold* flowers. *Tangerine* mutants have pale yellow flowers due to the absence of CRTISO activity (Isaacson et al., 2002; Figure 5). This reduces the conversion of *cis* forms into the corresponding enzymatically-modifiable *trans* isomers, down-regulating β-β xanthophyll formation in flowers. Note that the CRTISO step is difficult to regulate at the molecular level since spontaneous carotenoid isomerization also occurs in the presence of light. A study of *Delta* mutants, deficient in lycopene ε-cyclase activity, is another example. This mutation does not produce any modifications in the carotenoid profile in flowers, most probably because the major carotenoid pigment is violaxanthin while lutein accounts only for only 7% of all flower carotenoids (Ronen et al., 1999).

Finally, the above-mentioned studies are being integrated in a study of carotenoid biosynthesis in flower species (e.g., Moehs et al., 2001; Zhu et al., 2002, 2003) and especially the recent breakthroughs in knowledge on plant isoprenoid biosynthesis (Adam et al., 2002; Lichtentaler, 1999), which provides precursors for carotenoid biosynthesis. Moreover, more in-depth knowledge on genes coding for carotenoid-associated proteins and transcription factors of the carotenoid pathway will help in elucidating the most critical steps of carotenoid biosynthesis (Vishnevetsky et al., 1999). This ever increasing body of knowledge will eventually pave the way to engineering of flower and leaf color in ornamental plant species.

MINOR PIGMENTS AND OTHER APPROACHES USED TO MODIFY FLOWER COLOR

Other pigments include betalains, chlorophylls, indigo-related compounds, and melanin. However, despite the existence of some patents

for biotechnological applications of the few cloned genes involved in their synthesis, no reports on genetic engineering of such pigments have been published to date.

The engineering of flower color with green fluorescent proteins (GFP) is worth mentioning (Mercuri et al., 2001). This "new" approach produced fluorescent colors, through enzymatic reactions of the transgenic protein, not via the synthesis of specific pigments. Flowers of *Osteospermum* (syn. *Dimorphoteca*) and *Eustoma* GFP transgenic plants fluoresce when irradiated with UV or blue light. However, the authors indicated that there could be possible limiting factors to this approach, such as the masking effect of GFP fluorescence by the red autofluorescence of chlorophyll, as well as absorbance or reflectance effects by flavonoids and anthocyanins accumulated in flowers. Even if these products will probably never be marketed, they represent an elegant and original way to produce ornamental novelties.

CONCLUSIONS

Since the first report on metabolic engineering of the flavonoid pathway (Meyer et al., 1987), several strategies have been successfully applied to modify flower color through molecular breeding. Despite this, only a few transgenic floricultural crops have been marketed to date. This could be partially explained by the moratorium on genetic modified organisms (GMOs) in Europe. It is likely that when the GMO embargo is lifted, these biotechnology-developed ornamental products will spread and reach a wider public because of their non-edible nature. However, another reason may be the fact that most new ornamental varieties generated so far through metabolic engineering have only limited commercial value. Indeed, for blue flowers, several strategies based on flavonoid pigments did not generate the desired "true blue" phenotype, mainly because the final color obtained depends on many other factors such as vacuolar pH, co-pigmentation, anthocyanin modifications (acylation, glycosylation, methylation), and interactions with metal ions. Current knowledge regarding the contribution of endogenous and environmental factors to flower color modulation is still scarce even though recent progress has been made (Fukuchi-Mizutani et al., 2003; Gonnet, 2003; Yamaguchi et al., 2001). These encouraging results, together with data obtained through from various EST sequencing programs on species accumulating specific flavonoids and carotenoids and recently-isolated genes will likely provide new tools to redirect both pathways towards the synthesis of novel flower pigmentation patterns.

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Metabolic Engineering of Floral Scent of Ornamentals

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SUMMARY. Flowers of many plant species emit floral scent to attract pollinators. Such scents are often complex mixtures of various small aliphatic molecules, mostly terpenoid, phenylpropanoid, and benzenoid compounds. However, a large number of commercial flower varieties have lost their scent during the traditional breeding process. This lack of scent has been recognized as one of the major problems in the floriculture industry. Recent discoveries of genes encoding enzymes catalyzing the synthesis of scent compounds, and advances in our understanding of the regulation of scent biosynthetic genes, now make it possible to engineer plants with increased scent or with newly introduced aromas. Past approaches for metabolic engineering of floral scent have used various strategies, including the introduction of new scent genes and the block-

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age of existing non-scent pathways to divert metabolites into scent production. However, these experiments have only been partially successful. Problems have included the lack of specificity in targeting the expression of the introduced gene to flowers and unexpected biochemical reactions that metabolized the new scent compounds into non-volatile byproducts. Future attempts at scent genetic engineering will need to utilize specific floral gene promoters and be based on a clearer understanding of metabolic fluxes. doi:10.1300/J411v18n01_02

KEYWORDS. Floral scent, metabolic engineering, secondary metabolites, scent genes, ornamentals

BIOLOGICAL AND ECONOMICAL IMPORTANCE OF FLORAL SCENT

Floral scent is typically a complex mixture of low molecular weight, mostly lypophilic compounds emitted by flowers into the atmosphere. The relative abundances and interactions between these compounds give the flower its unique, characteristic fragrance. In nature, structure, color, and scent are critical factors in attracting pollinators to flowers. The same features determine consumers' attraction to cut flowers, potted flowering plants, and flowering herbaceous plants, which play an important role in human life from an esthetic point of view. Humans, like insects, strongly associate scent with specific flowers, e.g., rose or jasmine. While there is certainly a wide variation in human taste, most people prefer the scents of bee-pollinated, and especially moth-pollinated, flowers, which they often describe as "sweet-smelling." Humans' attraction to scent is not only determined by the composition of the fragrance but also by the overall level of the volatiles (Burdock, 1995).

While we do not usually consider floral scent as a necessity, nonetheless the presence of the floral scents we love contribute to our decision to cultivate and propagate specific plant species. Unfortunately, a large number of commercial flower varieties have lost their scent during the selection and breeding processes, which have traditionally focused on maximizing post-harvest shelf-life, shipping characteristics, and visual esthetic values (i.e., color, shape) of flowers (Zuker et al., 1998). One problem is that floral scent has rarely been a target trait in commercial

breeding programs, so such a trait is often lost by chance during the selection of segregating progeny for other traits. Moreover, traditional breeding of ornamentals has been unintentionally selected against scent due to the negative correlation between longevity and fragrance (Vainstein et al., 2001). The lack of a distinctive scent in many modern floricultural varieties, cut flowers in particular, has been recognized as one of the major problems in the floriculture industry. However, man's admiration of flower fragrance could potentially make volatile substances a high-impact commercial commodity.

Scent production in the floriculture industry is facing two major challenges: to create flowers with improved scent quality and/or newly introduced aromas that consumers can enjoy and to maintain the fragrant bouquet during the postharvest period. Genetic engineering could possibly contribute toward realizing both of these goals, and by doing so will increase the value of ornamentals. Floral scent engineering could also have a strong economic impact on the yield of many agricultural crops that rely on insect pollinators, including most fruit trees (Free, 1970; McGregor, 1976), berries (Currie et al., 1992), nuts, oilseeds, and vegetables (McGregor, 1976; DeGrandi-Hoffman, 1987). Novel composition of floral scents will have an increased interest from the perfume industry, which is constantly looking for new aromas. Production of biologically active volatile compounds in flowers could also be used in aroma therapy, pending proof of their efficacy. In addition, flowers could become factories for the manufacture of a variety of new natural flavors for the food industry.

Genetic engineering of floral scent not only has practical implications but also touches the fundamental questions about the biosynthesis and regulation of secondary metabolites. For example, it could help uncover the effect of gene addition or modification on steady-state levels of metabolites and fluxes in the pathways. This information could be generalized to rationally design experiments to manipulate fluxes of other plant metabolic networks. Moreover, the genetic manipulation of floral scent will provide a foundation for studying the role of individual volatiles in pollinator attraction and may allow us in the future to fill important gaps in our understanding of plant-insect interactions, since in addition to pollinator attraction some volatile compounds found in floral scent may function as repellents in direct defense against microbes and animals or as attractants of natural predators of the attacking herbivores thus indirectly protecting the plant.

Existing knowledge clearly indicates that engineering plants to synthesize and emit more volatiles from their flowers is now possible.

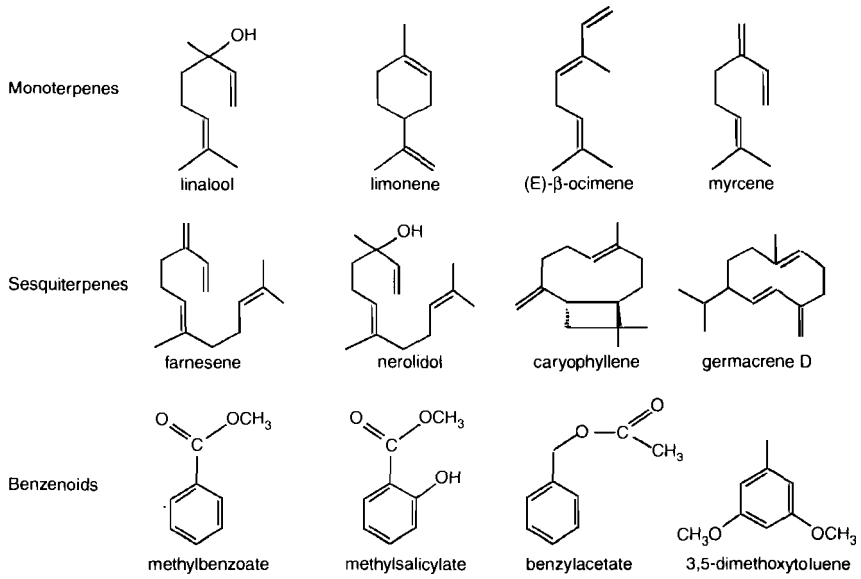
While for crop plants, engineering floral scent is only the first step in obtaining a useful product (optimizing it for insect interactions is the next, more complicated step), enhancing or creating *de novo* scent in ornamentals and cut flowers is a much more manageable undertaking with immediate commercial payoff. It also has the added advantage that it will create a product that the consumers can directly benefit from, yet will engender little public apprehension since the product will not be internally consumed. Thus, using genetic engineering to create fragrant flowers that consumers can enjoy is likely to improve the public's appreciation of the technology and to increase its acceptability in general.

BIOCHEMISTRY OF FLORAL SCENT

The chemical composition of floral scent has been extensively studied for over one hundred years due to the commercial value of floral volatiles to the perfume industry. To date, a total of more than 700 floral scent compounds have been described from more than 60 plant families (Figure 1). Floral fragrances belong to a broad category of secondary metabolites and are dominated by terpenoid (monoterpenes and sesquiterpenes), phenylpropanoid, and benzenoid compounds (Croteau and Karp, 1991; Knudsen and Tollsten, 1993; Knudsen et al., 1993). Fatty acid derivatives and a range of other chemicals, especially those containing nitrogen or sulfur, are also sometimes present. Each of these classes, in turn, can be further modified to contain different functional groups, e.g., alcohols, aldehydes, ketones, acids, ethers, and esters (Knudsen et al., 1993).

Terpenoid compounds, such as the volatile monoterpenes linalool, limonene, myrcene, and *trans*-β-ocimene, and sesquiterpenes, such as farnesene, nerolidol and caryophyllene, are common constituents of floral scents. Monoterpenes and sesquiterpenes have a common biosynthetic origin and are synthesized through the condensation of the universal five-carbon precursors, isopentenyl diphosphate (IPP) and its allylic isomer dimethylallyl diphosphate (DMAPP). Monoterpenes are derived exclusively from geranyl diphosphate (GPP, C_{10}), which is the result of a head-to-tail condensation of IPP and DMAPP catalyzed by GPP synthase (GPPS, EC 2.5.1.1) (Poulter and Rilling, 1981; Ogura and Koyama, 1998). The sesquiterpenes are derived from farnesyl diphosphate (FPP, C_{15}), which is originated through the sequential

FIGURE 1. Representative compounds of floral scent.



head-to-tail addition of two IPP units to DMAPP. The biosynthesis of IPP and DMAPP in higher plants can proceed via two parallel independent pathways located in two different subcellular compartments. In the cytosol IPP could be synthesized from the classical mevalonic acid (MVA) pathway which starts from the condensation of acetyl-CoA (Qureshi and Porter, 1981; Newman and Chappell, 1999), whereas in plastids IPP is formed from pyruvate and hosphoglyceraldehyde via the methyl-erythritol-phosphate (MEP) pathway (Eisenreich et al., 1998; Lichtenthaler, 1999; Rohmer, 1999). The cytosolic pool of IPP serves as a precursor of FPP and, ultimately, sesquiterpenes, while the plastidial pool of IPP provides the precursors of GPP and, ultimately, monoterpenes. Recently, evidence has been provided that the plastidic MEP pathway can supply IPP (which is interconvertible with DMAPP) to the cytosol in *Arabidopsis thaliana* (Laule et al., 2003).

Phenylpropanoids constitute a large class of secondary metabolites in plants, however only those that are reduced at the C9 position (to either the aldehyde, alcohol, or alkane/alkene) and/or which contain alkyl additions to the hydroxyl groups of the benzyl ring or to the carboxyl group (i.e., ethers and esters) are volatile. In addition, many benzenoid

compounds, which lack the three-carbon chain and originate from *trans*-cinnamic acid as a side branch of the general phenylpropanoid pathway, are also volatile. All phenylpropanoids/benzenoids are derived from phenylalanine via a complex series of branched pathways.

The first committed step in the biosynthesis of benzenoid compounds is catalyzed by the well known and widely distributed enzyme L-phenylalanine ammonia-lyase (PAL; EC 4.3.1.5), just as in the biosynthesis of other phenylpropanoids. PAL catalyzes the deamination of L-phenylalanine (Phe) to produce *trans*-cinnamic acid. Formation of benzenoid compounds from cinnamic acid requires the shortening of the side chain by a C_2 unit which might occur via the CoA-dependent β-oxidative pathway with formation of four CoA-ester intermediates, via the CoA-independent non-β-oxidative pathway with benzaldehyde as a key intermediate, or via a combination of these pathways (Boatright et al., 2004). While some early steps in the biosynthetic pathways to benzenoid volatile compounds are yet to be elucidated, common modifications of downstream products such as hydroxylation, acetylation, and methylation reactions have been described.

Other fragrance compounds, such as short-chain alcohols and aldehydes, are formed by metabolic conversion or degradation of phospholipids and fatty acids through the concerted action of lipoxygenases, hydroperoxide lyases, isomerases, and dehydrogenases. In addition, a variety of nitrogen-containing and sulfur-containing floral volatiles are produced by various pathways.

PRESENT AVAILABILITY OF SCENT BIOSYNTHETIC GENES

In the last six years, investigations into floral scent in a number of laboratories have resulted in the characterization of many genes encoding enzymes responsible for the synthesis of scent compounds (Table 1). Some of these genes were isolated from floral tissues whereas others were isolated from vegetative tissues and are responsible for the biosynthesis of volatile compounds which are also found in floral scents. This list includes genes for the biosynthesis of monoterpenoid compounds such as *(S)*-linalool synthase from *Clarkia breweri* and *Arabidopsis thaliana* (Dudareva et al., 1996; Chen et al., 2003b), (R)-linalool synthase from bergamot mint (*Mentha citrate*) and annual wormwood (*Artemisia annua*) (Crowell et al., 2002; Jia et al., 1999), myrcene

synthase from snapdragon (*Antirrhinum majus*) and grand fir (*Abies grandis*) (Dudareva et al., 2003; Bohlmann et al., 1997), *(E)*-β-ocimene synthase from snapdragon (*Antirrhinum majus*) and *Arabidopsis thaliana* (Dudareva et al., 2003; Faldt et al., 2003a), myrcene/*(E)*-β-ocimene synthase from *Arabidopsis thaliana* (Bohlmann et al., 2000), (+)-3-carene synthase from salvia (*Salvia stenophylla*) and Norway spruce (*Picea abies*) (Hoelscher et al., 2003; Faldt et al., 2003b), (−)-limonene synthase from spearmint (*Mentha spicata*), grand fir (*Abies grandis*), and *Perilla frutescens* (Colby et al., 1993; Bohlmann et al., 1997; Yuba et al., 1996), (+)-limonene synthase from lemon (*Citrus limon*) (Lucker et al., 2002), (+)-α-pinene synthase and (−)-α-pinene synthase from loblolly pine (*Pinus taeda*) (Phillips et al., 2003), (−)-β-pinene synthase from annual wormwood (*Artemisia annua*), lemon (*Citrus limon*) and grand fir (*Abies grandis*) (Lu et al., 2002; Lucker et al., 2002; Bohlmann et al., 1997), 1,8-cineole synthase from sage (*Salvia officinalis*) (Wise et al., 1998), γ-terpinene from lemon (*Citrus limon*) (Lucker et al., 2002), and geraniol synthase from sweet basil (*Ocimum basilicum*) (Iijima et al., 2004). Genes responsible for the biosynthesis of sesquiterpenes include caryophyllene synthase from *Arabidopsis thaliana* and annual wormwood (*Artemisia annua*) (Chen et al., 2003b; Cai et al., 2002), germacrene A synthase from goldenrod (*Solidago canadensis*) and chicory (*Cichorium intybus*) (Prosser et al., 2002; Bouwmeester et al., 2002), germacrene D synthase from roses (*Rosa hybrida*) (Guterman et al., 2002), terpene synthase 1 from maize (*Zea mays*), (Schnee et al., 2002), (*E*)-β-farnesene synthase from peppermint (*Mentha ×piperita*) and yuzu (*Citrus junos*) (Crock et al., 1997; Maruyama et al., 2001), and (*E, E*)-α-farnesene synthase from apple (*Malus domestica*) (Pechous and Whitaker, 2004). In addition, genes for terpene-modifying enzymes such as rose (*Rosa hybrida*) geraniol/citronellol acetyl transferase (Shalit et al., 2003) and mint limonene hydroxylases (Lupien et al., 1999) have also been identified.

Several genes responsible for the biosynthesis of benzenoid/phenylpropanoid compounds have also been recently isolated, such as *S*-adenosyl-L-methionine (SAM):(iso) eugenol O-methyl transferase from *Clarkia breweri* (Wang et al., 1997), eugenol methyltransferase and chavicol methyltransferase from sweet basil (*Ocimum basilicum*) (Gang et al., 2002), *S*-adenosyl-L-methionine:salicylic acid carboxyl methyl transferase from *Clarkia breweri*, snapdragon (*Antirrhinum majus*), and Madagascar jasmine (*Stephanotis floribunda*) (Ross et al., 1999; Negre et al., 2002; Pott et al., 2002), *S*-adenosyl-L-methionine:benzoic

TABLE 1. Current isolated and characterized genes responsible for the biosynthesis of volatile compounds

Gene	Main Product	Species	References
(*S*)-linalool synthase	(*S*)-linalool (ND)	*Clarkia breweri*	Dudareva et al., 1996
	(*S*)-linalool (100%)*	*Arabidopsis thaliana*	Chen et al., 2003b
(*R*)-linalool synthase	(*R*)-linalool (100%)	bergamot mint (*Mentha citrata*)	Crowell et al., 2002
	(R)-linalool (100%)	annual wormwood (*Artemisia annua*)	Jia et al., 1999
myrcene synthase	myrcene (100%)	snapdragon (*Antirrhinum majus*)	Dudareva et al., 2003
	myrcene (100%)	grand fir (*Abies grandis*)	Bohlmann et al., 1997
(*E*)-β-ocimene synthase	(*E*)-β-ocimene (96.6%)	snapdragon (*Antirrhinum majus*)	Dudareva et al., 2003
	(*E*)-β-ocimene (94%)	*Arabidopsis thaliana*	Faldt et al., 2003a
myrcene/(*E*)-β-ocimene synthase	myrcene (56%), (*E*)-β-ocimene (20%)	*Arabidopsis thaliana*	Bohlmann et al., 2000
(+)-3-carene synthase	(+)-3-carene (73%)	salvia (*Salvia stenophylla*)	Hoelscher et al., 2003
	(+)-3-carene (78%)	Norway spruce (*Picea abies*)	Faldt et al., 2003b
(−)-limonene synthase	(−)-limonene (94%)	spearmint (*Mentha spicata*)	Colby et al., 1993
	(−)-limonene (< 100%)	grand fir (*Abies grandis*)	Bohlmann et al., 1997
	(−)-limonene (< 100%)	*Perilla frutescens*	Yuba et al., 1996
(+) limonene synthase	(+)-limonene (99.15%)	lemon (*Citrus limon*)	Lucker et al., 2002
(+)-α-pinene synthase	(+)-α-pinene (97%)	loblolly pine (*Pinus taeda*)	Phillips et al., 2003
(−)-α-pinene synthase	(−)-α-pinene (79%)	loblolly pine (*Pinus taeda*)	Phillips et al., 2003
(−)-β-pinene synthase	(−)-β-pinene (94%) and α-pinene (6%)	annual wormwood (*Artemisia annua*)	Lu et al., 2002
	(−)-β-pinene (81.4%)	lemon (*Citrus limon*)	Lucker et al., 2002
	(−)-α-pinene (42%) and (−)-β-pinene (58%)	grand fir (*Abies grandis*)	Bohlmann et al., 1997
1,8-cineole synthase	1,8-cineole (79%)	sage (*Salvia officinalis*)	Wise et al., 1998
γ-terpinene	γ-terpinene (71.4%)	lemon (*Citrus limon*)	Lucker et al., 2002
geraniol synthase	geraniol (100%)	sweet basil (*Ocimum basilicum*)	Iijima et al., 2004
caryophyllene synthase	(−)-(*E*)-β-caryophyllene and α-humulene (ND)	*Arabidopsis thaliana*	Chen et al., 2003b
	β-caryophyllene (97%)	annual wormwood (*Artemisia annua*)	Cai et al., 2002
(+) germacrene A synthase	(+)-germacrene A (98%)	goldenrod (*Solidago canadensis*)	Prosser et al., 2002
	(+)-germacrene A (100%)	chicory (*Cichorium intybus*)	Bouwmeester et al., 2002
germacrene D synthase	germacrene D (100%)	roses (*Rosa hybrida*)	Guterman et al., 2002
terpene synthase 1	(*E*)-nerolidol (29%), (*E*)-β-farnesene (26%), (*E,E*)-farnesol (45%)	maize (*Zea mays*)	Schnee et al., 2002
(E)-β-farnesene synthase	(*E*)-β-farnesene (85%)	peppermint (*Mentha ×piperita*)	Crock et al., 1997
	(E)-β-farnesene (ND)	yuzu (*Citrus junos*)	Maruyama et al., 2001

Gene	Main Product	Species	References
(*E*,*E*)-α-farnesene synthase	(*E*,*E*)-α-farnesene (100%)	apple (*Malus domestica*)	Pechous and Whitaker, 2004
geraniol/citronellol acetyl transferase	geranyl acetate, citronellyl acetate	roses (*Rosa hybrida*)	Shalit et al., 2003
(−)-limonene-3-hydroxylase	(−)-*trans*-isopiperitenol	peppermint (*Mentha ×piperita*)	Lupien et al., 1999
(−)-limonene-6-hydroxylase	(−)-*trans*-carveol	spearmint (*Mentha spicata*)	Lupien et al., 1999
S-adenosyl-L-methionine (SAM):(iso) eugenol O-methyl transferase	methyleguenol, *iso*-methyleugenol	*Clarkia breweri*	Wang et al., 1997
*E*ugenol O-methyl transferase	methyleguenol	Sweet basil (*Ocimum basilicum*)	Gang et al., 2002
Chavicol O-methyl transferase	methylchavicol	Sweet basil (*Ocimum basilicum*)	Gang et al., 2002
S-adenosyl-L-methionine: salicylic acid carboxyl methyl transferase	methylsalicylate, methylbenzoate	*Clarkia breweri*	Ross et al., 1999
	methylsalicylate, methylbenzoate	snapdragon (*Antirrhinum majus*)	Negre et al., 2002
	methylsalicylate, methylbenzoate	Madagascar jasmine (*Stephanotis floribunda*)	Pott et al., 2002
S-adenosyl-L-methionine:benzoic acid carboxyl methyl transferase	methylbenzoate	snapdragon (*Antirrhinum majus*)	Dudareva et al., 2000
S-adenosyl-L-methionine:benzoic acid/ salicylic acid carboxyl methyl transferase	methylsalicylate, methylbenzoate	petunia (*Petunia hybrida*)	Negre et al., 2003
	methylsalicylate, methylbenzoate	*Arabidopsis thaliana*	Chen et al., 2003a
	methylsalicylate, methylbenzoate	tobacco (*Nicotiana suaveolens*)	Pott et al., 2004
orcinol O-methyl transferase	dimethoxytoluene	roses (*Rosa hybrida*)	Lavid et al., 2002
S-adenosyl-L-methionine: jasmonic acid carboxyl methyl transferase	methyljasmonate	*Arabidopsis thaliana*	Seo et al., 2001
acetyl-coenzyme A:benzyl alcohol acetyltransferase	benzylacetate	*Clarkia breweri*	Dudareva et al., 1998
benzoyl-coenzyme A:benzyl alcohol benzoyl transferase	benzylbenzoate	*Clarkia breweri*	D'Auria et al., 2002
benzoyl-coenzyme A:benzyl alcohol/ phenylethanol benzoyl transferase	benzylbenzoate, phenylethylbenzoate	petuinia (*Petunia hybrida*)	Boatright et al., 2004
acetyl-coenzyme A:*cis*-3-hexen-1-ol acetyltransferase	*cis*-3-hexen-1-yl acetate	*Arabidopsis thaliana*	D'Auria et al., 2002
alcohol acyltransferase	hexyl acetate, octyl acetate	strawberry (*Fragaria*)	Aharoni et al., 2000

* Numbers in paranthesis show the percent of the total product
ND–not determined

acid carboxyl methyl transferase from snapdragon (*Antirrhinum majus*) (Dudareva et al., 2000), *S*-adenosyl-L-methionine:benzoic acid/salicylic acid carboxyl methyl transferase from petunia (*Petunia hybrida*), *Arabidopsis thaliana*, and tobacco (*Nicotiana suaveolens*) (Negre et al., 2003; Chen et al., 2003a; Pott et al., 2004), acetyl-coenzyme A:benzyl alcohol acetyltransferase from *Clarkia breweri* (Dudareva et al., 1998), benzoyl-coenzyme A:benzyl alcohol benzoyl transferase from *Clarkia breweri* (D'Auria et al., 2002), benzoyl-coenzyme A:benzyl alcohol/phenylethanol benzoyl transferase from *Petunia hybrida* (Boatright et al., 2004), acetyl-coenzyme A:*cis*-3-hexen-1-ol acetyltransferase from *Arabidopsis thaliana* (D'Auria et al., 2002), alcohol acyltransferase from strawberry (*Fragaria*) (Aharoni et al., 2000), and orcinol O-methyl transferase from rose (*Rosa hybrida*) (Lavid et al., 2002). In addition, the *S*-adenosyl-L-methionine: jasmonic acid carboxyl methyl transferase responsible for the formation of the lipoxygenase derivative methyl jasmonate was recently isolated and characterized from *Arabidopsis thaliana* (Seo et al., 2001).

Many enzymes in secondary metabolism can act on several similar substrates. For example, the SAMT enzymes of several species have been shown to methylate not just salicylic acid but also benzoic acid (Ross et al., 1999; Negre et al., 2002, 2003; Chen et al., 2003; Pott et al., 2004). Which compound is methylated may depend on the concentration of the substrate, benzoic acid or salicylic acid, available in the cell (Negre et al., 2003; Pott et al., 2004). Similarly, acyl transferases can use different substrates (D'Auria et al., 2002; Boatright et al., 2004). This phenomenon may facilitate floral scent engineering, as a wide spectrum of scent compounds may be obtained with a fewer number of genes. Moreover, many genes in secondary metabolism have the unusual ability to produce more than one product. Examples include many terpene synthases (Bohlmann et al., 2000; Schnee et al., 2002) (Table 1). Therefore, the introduction of a single gene to a plant could likely lead to the formation of several new volatile compounds.

For introduced genes to exert their effect on scent production, the availability of substrates in the same cell, and in the same compartment within the cell, where the enzymes catalyzing the reaction is destined to, must be insured. Indeed, recent investigations of floral scent emission in snapdragon clearly show that the concentration of substrates in the cell plays an important role in the regulation of biosynthesis of floral volatiles (Dudareva et al., 2000; Kolosova et al., 2001a). Unfortunately, at present only limited information on cellular and subcellular localiza-

tion of enzymes and substrates, as well as flux rates, is available concerning most of the pathways leading to the synthesis of floral volatiles.

REGULATION OF FLORAL SCENT PRODUCTION

The isolation of genes responsible for the formation of floral scent volatiles has facilitated the investigation of the regulation of scent biosynthesis. When examined, it has been shown that the flowers synthesize scent compounds *de novo* in the tissues from which these volatiles are emitted. The emission levels, corresponding enzyme activities, and the expression of genes encoding scent biosynthetic enzymes are all temporally and spatially regulated during flower development. Typically, expression of scent genes is relatively uniform with highest levels in petals. *In situ* hybridization and immunolocalization have demonstrated that the biosynthesis of volatile compounds occurs almost exclusively in the epidermal cells of floral organs, from which they can easily escape into the atmosphere after being synthesized (Dudareva et al., 1996; Dudareva and Pichersky, 2000; Kolosova et al., 2001b). Transcriptional regulation of expression of these genes at the site of emission and the level of supplied substrates for the reactions were found to be the major factors controlling scent production, and, indirectly, scent emission during flower development. When regulation of rhythmic emission of methylbenzoate was investigated, it was found that the level of substrate (benzoic acid) also plays a major role in the regulation of circadian emission of methylbenzoate in diurnally (snapdragon) and nocturnally (*Petunia* cv. *Mitchell* and *Nicotiana suaveolens*) emitting flowers (Kolosova et al., 2001a).

GENERAL APPROACHES AND PITFALLS IN METABOLIC ENGINEERING OF FLORAL SCENTS

Bioengineering of floral scents could entail either the modification of existing pathways and/or the introduction of new enzymes to produce novel compounds not normally found in the host plant. The modification of endogenous pathways can be achieved via the engineering of single steps or multiple points in a pathway to increase or decrease metabolic flux to the target compound, through the blocking of competitive pathway(s), or through the introduction of a new step that redirects the metabolic flux toward a particularly desirable molecule. However, the

introduction of novel genes or the enhancement of activities of pre-existing genes may not be successful in modulating floral scent. A lack of substrate availability and the metabolism of newly synthesized compounds, including their conjugation and storage can significantly limit volatile emission. The biosynthesis and accumulation of these compounds in some cell types where they cannot be emitted can potentially cause lethality since many of the scent compounds are cytotoxic and are likely to cause cell death. The redirection of the metabolic flux toward a desirable compound may also have a deleterious effect on the plant as a result of depletion in the available levels of the general precursors required for normal plant development. To avoid such undesirable effects and to achieve emission, it may be necessary to use promoters that target expression of these introduced genes to specific cell and tissue types in the flower during plant growth and development. To date, however, experiments designed to engineer floral scent have not yet used flower-specific promoters, and should therefore be considered as "first generation" experiments. These experiments are described below.

METABOLIC ATTEMPTS TO MODIFY FLORAL SCENT USING NONFLOWER-SPECIFIC PROMOTERS

In the last three years several attempts have already been made to bring about the synthesis of new floral scent components in flowers. The linalool synthase (LIS) from *Clarkia breweri* (Dudareva et al., 1996), which converts GPP to (3*S*)-linalool, a widespread monoterpene emitted by flowers of many species and by plants after herbivore attack, was the most frequently used gene in these attempts. The LIS gene was introduced under the control of the cauliflower mosaic virus (CaMV) 35S constitutive promoter into *Petunia hybrida* W115 (Lucker et al., 2001) and carnation (*Dianthus caryophyllus*) (Lavy et al., 2002), both lacking this monoterpene. Transgenic petunia plants expressing the heterologous gene produced linalool but the entire product was converted into the non-volatile compound linaloyl β-D-glycoside by the action of endogenous glucosyltransferase. Consequently, the newly introduced linalool had no effect on the olfactory properties of the flowers or vegetative parts of the transformants. Moreover, a comparative analysis of the LIS gene expression and monoterpene production in different floral tissues as well as in roots, stems, and leaves revealed that the differences between organs in the amount of the synthesized linalool or

its glycoside depend more on the availability of the substrate GPP in the tissue than on the expression of the LIS gene (Lucker et al., 2001).

In transgenic carnations, biosynthesis of linalool in flowers and leaves was also achieved; however, a significant fraction of the produced linalool was further oxidized to *cis*- and *trans*-linalool oxide by an endogeneous enzyme. Transgenic flower extracts only contained detectable levels of *trans*-linalool oxide, suggesting that the transgenic tissue did not accumulate linalool, rather it was rapidly emitted instead. Although linalool and linalool oxide constituted almost 10% of the total volatiles emitted from the transgenic carnation flowers, the overall levels of linalool and the other scent compounds were so low that most humans failed to detect a change in floral aroma in smell tests (Lavy et al., 2002).

Although this experiment was not done to change the levels of floral volatiles, it is useful to note here that the *Lis* gene was introduced into tomato (*Lycopersicon esculentum* Mill.) under the control of the tomato late-ripening-specific *E8* promoter to improve the flavor of the fruits (Lewinsohn et al., 2001). In this case, the transgenic tomato fruits accumulated > 50-fold more linalool than wild-type plants without altering the biosynthesis of other non-volatile terpenoids such as tocopherols, lycopene, β-carotene, and lutein. Although some of the linalool was further oxidized to the volatile 8-hydroxylinalool, the concentrations of linalool and 8-hydroxylinalool in the ripe fruit of transgenic plants were sufficient for olfactory detection (Lewinsohn et al., 2001).

The pioneering experiments discussed above showed that volatile compounds can be synthesized in heterologous systems via metabolic engineering. However, these studies also revealed additional unexpected problems that can be encountered in the genetic engineering of flower fragrance: modification of the scent compound into a non-volatile form, e.g., glycosylation, masking by other volatiles, or the emitted amount being insufficient for olfactory detection by humans.

Recently, a complex metabolic engineering experiment to alter the monoterpene volatile profile of *Nicotiana tabacum* was reported (Lucker et al., 2004b). In this experiment, the three transgenic tobacco lines, each carrying a different monoterpene synthase (γ-terpinene cyclase, (+)-limonene cyclase, and (−)-β-pinene cyclase, see above), were sequentially crossed to obtain a line containing all three monoterpene synthases. This transgenic tobacco line emitted β-pinene, limonene, γ-terpinene, and a number of side products from their leaves and flowers, in addition to the regular terpenoids also emitted by the parental

line. The total level of the monoterpenes was greatly increased (10- to 25-fold) leading to drastic changes in the leaf and flower fragrance profiles, and this level of emission was sufficient for detection by the human nose (El Tamer et al., 2003). Modifications such as glycosylation or hydroxylations described for petunia, tomato and carnations transformed with linalool synthase (Lucker et al., 2001; Lewinsohn et al., 2001; Lavy et al., 2002) were not detected in these transgenic tobacco plants, in part because the monoterpene produced did not contain a hydroxyl group amenable to glycosylation. The introduced monoterpene synthases compete for the same substrate, GPP, and the magnitude of monoterpene emission could be expected to depend on the relative expression and protein levels of monoterpene synthases, their maximal velocities, and substrate affinities for GPP. In leaves the levels of compounds were close to that predicted based on the K_m values of the enzymes for GPP; however, in flowers the emission levels of the main products of the introduced monoterpene synthases were comparable and did not affect the endogenous linalool production, suggesting that the GPP pool did not limit monoterpene production (Lucker et al., 2004b).

Monoterpene emission in the transgenic tobacco line expressing all three monoterepne synthases was further modified by introducing the mint gene for limonene-3-hydroxylase, which catalyzes the hydroxylation of (+)-limonene with the formation of (+)-*trans*-isopiperitenol, under the control of the 35S promoter (Lucker et al., 2004a). The resulting (+)-*trans*-isopiperitenol, an uncommon compound in the plant kingdom, was emitted as a major component of the volatile spectrum of the plants possessing all four integrated transgenes. An interesting observation is that while monoterpene synthases are localized in the plastids, the limonene-3-hydroxylase enzyme is an endoplasmic reticulum-localized protein in mint and was likely localized to the ER in the transgenic tobacco as well, although this was not directly determined (Lucker et al., 2004a). These results suggest that the manipulation of metabolic pathways involving multiple cellular compartments can be implemented by multiple gene transfer, thereby facilitating the production of desirable molecules in transgenic plants.

ATTEMPTS TO MODIFY FLORAL SCENT VIA THE REDIRECTION OF METABOLIC FLUX

An alternative molecular genetic approach for the olfactory enhancement of flower fragrance by increasing the flux through the target path-

way was recently demonstrated in carnations (Zuker et al., 2002). The antisense suppression of the flavanone 3-hydroxylase (*f3h*), encoding a key enzyme in the anthocyanin pathway, led to the loss of the original orange/reddish color of flowers and to increased methylbenzoate production thereby altering the flower scent that could be detected by humans (humans typically have a keener perception of benzenoids than of terpenoids). Since both anthocyanins and methylbenzoate originate from the phenylpropanoid pathway, these results suggest that the enhanced scent production in transgenic anti-*f3h* flowers is due to redirecting the metabolic flux toward benzoic acid, which is the precursor of methylbenzoate.

While the redistribution of the metabolic flux in the phenylpropanoid pathway did not have a deleterious effect on the plant, the redirection of the metabolic flux toward monoterpene production may lead to deprivation of essential compounds formed from common intermediates, for example, thiamin and pyridoxol, both of which are derived from 1-deoxy-D-xylulose-5-phosphate, as well as diterpenes (e.g., gibberellins as phytohormones), tetraterpenes (e.g., caratenoids as photosynthetic pigments), and several polyterpenes (e.g., phytol side chain of chlorophyll). An example of such unintended consequences includes an attempt to increase carotenoid formation in transgenic tomato plants by diverting the metabolic flux to this branch of the terpenoid pathway which resulted in a decrease of gibberellin production and the formation of dwarf plants (Fray et al., 1995).

METABOLIC ENGINEERING VIA THE UPREGULATION OF PATHWAY FLUX

To date, the metabolic engineering of floral scent has mainly concentrated on the introduction of genes responsible for the final steps of the formation of volatile compounds. Although this approach was successful in some cases (Lucker et al., 2004a, 2004b), the level of end-product accumulation can be limited by substrate availability. There are clear indications in the examples described above (Lucker et al., 2001, 2004a) that the supply of GPP could be a limiting factor in the biosynthesis of monoterpenes from the MEP pathway. GPP is synthesized from DMAPP and IPP and the improvement of the flux to these universal isoprenoid precursors will require the identification of the slow steps in the MEP pathway and the overexpression of the genes responsible for these steps. When DXP synthase, which catalyzes the first step in the MEP

pathways, the condensation of pyruvate and glyceroaldehyde-3-phosphate to form 1-deoxyxylulose-5-phosphate (DXP), was overexpressed in *Arabidopsis* transgenic plants, elevated levels of carotenoids and greater amounts of chlorophylls and tocopherols were observed (Estevez et al., 2001). Unfortunately, the levels of terpenoids emitted from the flowers of these transgenic Arabidopsis were not analyzed in these experiments. For the second step, conversion of DXP to MEP, overexpression of the corresponding enzyme in peppermint led to an almost 50% increase in the monoterpene fraction in this herb (Mahmoud and Croteau, 2001). Thus, these examples show that the increase of the flux through the basic terpenoid pathway could raise the levels of many desired end products, such as monoterpenes of floral scent.

Transcription factors with their ability to control both multiple pathway steps and cellular processes that are necessary for metabolite accumulation can also offer an efficient strategy to manipulate the flux through the metabolic pathways. However, to date, little is known about transcription factors that directly control production and emission of volatile compounds in plants.

FUTURE DIRECTIONS IN THE MODIFICATION OF FLORAL SCENT BY GENETIC ENGINEERING

The availability of genes responsible for the formation of floral volatile compounds is no longer a limiting factor in the metabolic engineering of floral scent. Flower-specific promoters that can help to minimize the unwanted side effects of transgenic manipulations are also available (e.g., Cseke et al., 1998). In addition, successful transformations have been developed for several cut flowers, including the most commercially important roses, chrysanthemums, carnations, and gerbera, although for most varieties it is still an "art form" (summarized in an excellent review by Zuker et al., 1998). Thus, the key limitation to improving the success rate of metabolic engineering of floral scent at the present time is our insufficient understanding of metabolic pathways. It is therefore not surprising that, to date, the various attempts at metabolic engineering of floral scent have mainly concentrated on the introduction of genes responsible for the final steps of the formation of volatile compounds. The identification and upregulation of earlier, rate-limiting steps of the pathways leading to the biosynthesis of substrates to affect an increase in the levels of desired end products should be the next step.

The generation of metabolic flux models of the metabolic pathways will also provide the information for rational metabolic engineering.

Overall, it is clear that genetic manipulation of floral scent is possible, but will require a more rational design based on a correct choice of species, prior knowledge of pathways including their cellular and subcellular localization, empirical testing, and correct use of promoters. Similar approaches can be used to engineer crop plants with introduced herbivore-induced volatiles, thereby enhancing our ability to exert biological control in such agroecosystems.

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Auxin, Cytokinin and Abscisic Acid: Biosynthetic and Catabolic Genes and Their Potential Applications in Ornamental Crops

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SUMMARY. Auxin, cytokinin, and abscisic acid (ABA) are important plant hormones that regulate many growth and developmental processes. In recent years, a number of genes involved in the metabolic and signaling pathways for auxin, cytokinin and ABA have been cloned and characterized. With organ- and tissue-specific or conditionally active gene promoters, it has become possible to manipulate concentrations of plant hormones *in planta* to create commercially desirable traits. Seedless fruit production and the extension of shelf life of green produce and ornamental plants are two successful examples of manipulating concentrations of these hormones *in planta*. In this review, we will focus our discussion on the effects of the over- or under-expression of genes involved in the biosynthetic and catabolic pathways of these hormones with an emphasis on their potential applications in ornamental crops.
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KEYWORDS. Auxin, cytokinin, abscisic acid, biosynthetic and catabolic genes, applications, ornamental crops

INTRODUCTION

The plant hormones auxin, cytokinin, and abscisic acid (ABA) control a variety of important processes during the plant life cycle. Each year, large quantities of synthetic hormones or growth regulators with auxin, cytokinin or ABA activity have been used in horticultural and nursery crops in the U.S. (Norcini et al., 1996). However, exogenous applications of these chemicals are expensive, often carry the risk of environmental contamination and worker exposure, and sometimes produce undesirable side-effects. Recent advances in the areas of molecular cloning and plant transformation have made it possible to manipulate concentrations of and tissue sensitivity to these hormones *in planta*.

AUXIN

Auxin is a group of plant growth regulators, with the most common example being indoleacetic acid (IAA). Auxin plays a fundamental role

in cell division and elongation and regulates diverse aspects of plant growth and development such as cambial activities, plant stature, shoot and root architecture and initiation, shoot strength, fruit set and size, fruit ripening, and senescence. Commercially, synthetic auxins such as indolebutyric acid (IBA) and naphthalenacetic acid (NAA), have been used to promote root growth and development, to promote uniform flowering, and to set fruit and prevent premature fruit drop while anti-auxins or auxin transport inhibitors have been used to reduce apical dominance (Weaver, 1972). Synthetic auxins 2, 4-D and 2, 4, 5-T have also been used as herbicides. Broad-leaved weeds like dandelions are much more susceptible to auxin type herbicides than narrow-leaved plants like grass and cereal crops.

In higher plants, auxin in the form of IAA is synthesized both from tryptophan (Trp) using Trp-dependent pathways and from an indolic Trp precursor via Trp-independent pathways. Genes encoding enzymes that are responsible for the rate-limiting steps of auxin biosynthesis can be used to regulate auxin level in plants, but none of these two pathways is fully elucidated (Woodward and Bartel, 2005). Plants can produce IAA by oxidation of indole-3-butyric acid (IBA) or by hydrolysing IAA conjugates, but these processes can also be plant species-specific. IAA is often linked to amino acids, sugars or peptides as a way of inactivation in plants. Although regulation of auxin concentration in plants could be achieved with alteration of either oxidation or conjugation of IAA, pathways for auxin inactivation in higher plants may vary from species to species, which makes this approach less attractive. Alternatively, manipulation of auxin transport may regulate auxin concentration in plants. For instance, the findings by Friml et al. (2002) have suggested that tissue- and organ-specific manipulation of the distribution of PIN protein or other auxin transporter proteins may lead to alterations in auxin levels in target tissues or organs.

Although many genes that are invovled in biosynthesis, inactivation, transport and signaling pathways of auxin have been cloned from higher plants, the understanding of the auxin metabolic pathways is imcomplete and much information on genetic and biochemical regulation is still missing. On the other hand, when expressed in plants, the *Agrobacterium* tryptophan monooxygenase gene (*iaaM*) alone or in combination with the *Agrobacterium* indoleacetamide hydrolase gene (*iaaH*) can lead to increase in auxin concentration. Alternatively, the expression of the *Pseudomonas syringae* iaaL gene can result in conjugation of IAA and therefore a reduction in auxin concentration in the plant cell (see review by Klee and Lanahan, 1995). The iaaM and iaaH proteins catalyze the

conversion of the amino acid tryptophan to IAA in plants (Klee et al., 1987). Tobacco, petunia and *Arabidopsis* plants constitutively expressing the *iaaM* gene alone also displayed increased apical dominance and reduced stem growth (Romano et al., 1995; Guilfoyle et al., 1993), indicating that the iaaM gene itself is sufficient to cause over-production of auxin in plants. The iaaM overexpressing plants often produce a large number of adventitious roots from intact leaves (Klee et al., 1987; Guilfoyle et al., 1993), a phenomenon that is not normally observed in wild-type plants treated with auxin exogenously. This finding suggests that overproduction of auxin *in planta* may be more effective than exogenous application of auxin in promoting root initiation. Rooting is a key and often-difficult step in asexual propagation of many horticultural and forest crops. Expression of the *iaaM* gene and other auxin related genes, if under the control of appropriate gene promoters, could enhance formation of adventitious roots of cuttings of hard-to-root ornamental and horticultural crops.

The most commercially valuable trait observed in auxin overproducing transgenic plants up to this point is the production of seedless fruits, pathenocarpy (Varoquaux et al., 2002). Production of seedless fruits from vegetable and fruit tree crops is projected to be one of the most important technologies in the agricultural industry over the next many years (Ortiz, 1998). Traditionally, seedless fruits are produced from mutants, triploid plants or flowers treated with exogenous growth regulators. Triploid plants and seedless fruit mutants are difficult to breed. The use of synthetic growth regulators to produce seedless fruits often causes environmental and health concerns, and is also ineffective in some fruit species because of insufficient uptake and translocation of the applied hormones to the target tissues. Parthenocarpy was initially observed in auxin-overproducing *iaaM* petunia (Klee et al., 1987) and tobacco plants (Guilfoyle et al., 1993). However because of the use of a constituative promoter to drive production of the *iaaM* gene (Klee et al., 1987; Guilfoyle et al., 1993), these transgenic plants exhibited auxin symptoms such as leaf epinacity, reduced stem growth and adventitious root formation from leaves and stems.

Later, with ovary specific gene promoters to direct expression of the *iaaM* gene, seedless fruits have been produced in eggplant (Rotino et al., 1997; Spena, 1998), tomato (Barg and Salts, 1996; Li, 1997; Pandolfini et al., 2002), strawberry and raspberry (Mezzetti et al., 2004) and watermelon (Y. Li Lab, unpublished data). In all these cases, because overproduction of auxin was restricted to the ovary and developing fruit, no obvious deleterious pleiotropic effects were evident. In addition to sat-

isfying the market demand and consumer preference toward seedless produce (e.g., grapes and watermelons), the transgenic seedless fruit technologies also often resulted in increased fruit productivity partially because of improved fruit set. For instance, Spena and colleagues (Mezzetti et al., 2004) demonstrated that transgenic seedless strawberry and raspberry plants showed a significant increase in fruit number, size and fruit yield. In three Rosaceae species tested, *Fragaria vesca*, *Fragaria* ×*ananassa* and *Rubus idaeus*, the expression of the *DefH9-iaaM* gene plants resulted in an increased number of flowers per inflorescence and an increased number of inflorescences per plant. Also, the weight and size of individual transgenic fruits were increased, approximately 180% in cultivated strawberry, 140% in wild strawberry, and 100% in raspberry. A potential application of the *iaaM* gene-mediated seedless fruit technology in ornamental horticulture is to eliminate seed production of invasive ornamental plants.

Poor pollination is a major cause of inadequate fruit set and undersized fruits in many vegetables and fruit trees, including greenhouse- and field-grown tomatoes. Diseases and environmental stresses such as low and high temperatures, low light intensity, and drought cause poor pollination and reduction of fruit yield. Production of fruits independent of pollination may therefore reduce or eliminate yield reduction problems associated with poor pollination. In the case of the transgenic seedless tomato, the size, weight, acid and sugar contents of fruits were significantly higher than in seeded fruits from wild-type plants (Barg and Salts, 1996; Li, 1997; Pandolfini et al., 2002). Also, a significant increase in total solids content was observed in the transgenic seedless tomato fruits, which could benefit the tomato processing industry. Tomato fruits contain 95% water and the remaining 5% consists of pulp, seeds, and soluble products (mostly sugars, organic acids, and flavor compounds). Most of the water is removed during processing of tomato products such as ketchup, paste and sauce. To a large degree, the price of these tomato products is determined by the cost of removing water. It is estimated that a 25% increase in total solids would save the US tomato industry at least $75 million a year (Chrispeels and Sadava, 1994).

Dwarfing and increased number of branches and flowers, important traits for many ornamental crops, can be achived by down-regulation in auxin level in plants using auxin transport inhibitors or anti-auxins (Weaver, 1972). It has also been previously shown that expression of the *Pseudomonas syringae* iaaL, which encodes an indoleacetic acid (IAA)-lysine synthase, is capable of reducing active auxin concentra-

tions in plants (Romano et al., 1991). The iaaL enzyme converts IAA to IAA-lysine, a biologically inactive form of auxin. IAA-lysine conjugates are not synthesized in plants normally and it appears that the conjugation is not reversible. With a strong constitutive and globally active *35S CaMV* promoter to control the expression of the *iaaL* gene, the IAA concentration in transgenic tobacco plants was 19-fold lower than in wild-type plants (Romano et al., 1991). Phenotypically, tobacco plants expressing the *iaaL* gene exhibited reduced apical dominance or increased branching, but also had underdeveloped root and vascular systems. Recently, we produced transgenic tobacco, petunia and chrysanthemum plants that expressed the *iaaL* gene under the control of a gene promoter that is more active in above-ground organs (McAvoy et al., 2003 and Y. Li lab, unpublished data). Some of the resulting transgenic plants were compact, with reduced stem height growth and more branches, and the leaves were darker green in color. However, many transgenic lines also had an underdeveloped root system, suggesting that there is a need to eliminate the *iaaL* gene's undesirable effect on root growth and development.

CYTOKININS

Cytokinins are a group of adenine derivatives that affect multiple aspects of plant growth and development, including cell division, vascular development, sink/source relationships, apical dominance, and leaf senescence (Binns, 1994; Mok and Mok, 2001). Exogenously applied cytokinins stimulate cell division, reduce apical dominance and delay senescence. Cytokinins have been used to maintain vigor in many vegetable and floriculture plants through improvement of the shelf life, prevention of post-harvest leaf deterioration and decrease in low temperature induced leaf yellowing (Ludford, 1995; Weaver, 1972). Several synthetic cytokinins have been registered for use on carnations and roses to increase lateral bud formation and branching, but large-scale commercial applications of cytokinins on horticultural crops have not been possible because of inefficient absorption of the hormone by plants and the high costs associated with exogenous applications (Gianfagna, 1995).

A pathway for cytokinin biosynthesis and metabolism is emerging from molecular and biochemical studies, particularly highlighted with the cloning of several genes encoding enzymes involved in cytokinin biosynthetic or metabolic pathways, such as *ipt* (Takei et al., 2001), which catalyzes the first committed step in cytokinin biosynthesis;

cytokinin oxidase (Houba-Hérin et al., 1999; Morris et al., 1999), which cleaves the N6 side chain from cytokinins; and several enzymes that catalyze the conjugation of sugar moieties to cytokinins (Martin et al., 1999a, 1999b, 2001). A model for cytokinin signalling has has been shown to be similar to prokaryotic two-component response pathways (Lohrmann and Harter, 2002). Cytokinin receptors (CRE1, AHK2, and AHK3) have been shown to act through other two-component elements, including His phosphotransfer proteins (AHPs) and the type-B ARRs, involved in the up-regulation of cytokinin inducible genes (Hwang and Sheen, 2001; Sakai et al., 2001). Although a large number of plant genes involved in cytokinin bisynthesis and catoblism have been cloned and characterized, the *ipt* (isopentenyl transferase) gene cloned from *A. tumefaciens* has been most frequenctly used to manipulate cytokinin levels in plants. IPT catalyzes the rate-limiting step for *de novo* cytokinin biosynthesis (McGaw and Burch, 1995), i.e., the addition of isopentenyl pyrophosphate to the N6 of 5′-AMP to form isopentenyl AMP (Chen, 1997). Isopentenyl AMP is the precursor of all other cytokinins, of which the three most commonly detected and physiologically active forms in plants are isopentenyl adenine, zeatin, and dihydrozeatin. Expression of the *ipt* gene under the control of a wide range of constitutive, tissue-specific or inducible gene promoters have demonstrated potential utilities of manipulating cytokinin concentrations *in planta.*

Phenotypes observed in cytokinin overproducing transgenic plants include increases in the number of lateral shoots, shorter internodes, formation of adventitious shoots directly from unwounded leaves and roots, altered source-sink relations and delayed senescence. For example, Estruch et al. (1991) reported that tobacco plants that were somatic mosaics for the expression of a *35S CaMV* promoter-*ipt* gene frequently produced adventitious shoots on veins at the unwounded leaf tip. With an auxin-regulated *SAUR* promoter-*ipt* fusion gene expressed in tobacco plants, Li et al. (1992) observed adventitious shoots developed on the unwounded leaf petioles and veins where the *SAUR* promoter is highly active. The SAUR promoter is specifically active in elongating regions of shoots and roots with strong expression in leaf and stem vascular tissues (Li et al., 1991). We also observed initiation of shoots from unwounded roots of *35S CaMV* promoter-*ipt* tobacco plants (Y. Li Lab, unpublished data). Because direct shoot formation from unwounded tobacco leaves and roots has not previously been reported with exogenously applied cytokinins, these results suggest that cytokinins overproduced *in planta* can be more effective than those exogenously applied.

Because cytokinin is a key hormone for shoot initation, the expression of the *ipt* gene has been used as a selectable marker gene in plant transformation. Selectable marker genes, conferring resistance to antibiotics or herbicides have often been used for selction of transgenic cells or plants during the production of transgenic plants (Yoder et al., 1994). However, there are concerns that antibiotic- or herbicide-resistance marker genes could spread to wild type plant relatives and pose problems to human health and the ecosystem (Dale et al., 2002). To circumvent these problems, several alternative selection systems including the use of the *ipt* gene as a positive marker have been developed for the selection of transgenic plants (see review by Zuo et al., 2002). Because overexpression of *ipt* continuously will negatively affect root initiation and produce bushy shoots, Ebinuma et al. (1997) placed 35S CAMV promoter-*ipt* fusion gene and the Ac/Ds transposable system in the same vector. The 35S promtoer-*ipt* DNA segment could then be excized from and re-inserted into the host genome. In rare cases, the excized DNA failed to re-integrate into the host genome, resulting in the *35-ipt* transgene being removed. The efficiency of this method, however, is very low. An improved version of the method was to use an inducible R/RS DNA recombination system to excise the *35S-ipt* gene once transgenic shoots were produced (Sugita et al., 2000).

Alternatively, an inducible expression system can be used to to control the expression of the *ipt* gene (Kunkel et al. 1999) to circumvent the problem of a constititutive expression. Transgenic shoots could be regenerated in a cytokinin-free medium if *ipt* expression is induced but the plants then would have no *ipt* expression once the inducer was removed. The *ipt* gene has been used effectively as a positive selectable marker for transformation of many plant species including tobacco, tomato, muskmelon and sweet pepper and the use of the ipt gene has also been demonstrated to improve transformation efficiency (Endo et al., 2001, 2002; Kunkel et al., 1999; Mihálka et al., 2003). Also, we have recently demonstrated that the *kn1* gene (Smith et al., 1992) could also be as effective as the ipt gene to replace antibiotic or herbicide resistance genes for plant transformation and to enhance plant transformation efficiency (Luo et al., 2006). Although the function of the *kn1* gene is not well understood, it has been shown that transgenic plants overexpressing *kn1* exhibit morphological alterations, including changes in leaf shape, loss of apical dominance, and the production of ectopic meristems on leaves, which are similar to the characteristics of *ipt*-overexpressing plants (Estruch et al., 1991; Li et al., 1992; Sinha et al., 1993; Lincoln et al., 1994; Hareven et al., 1996; Tamaoki et al., 1997).

The anti-sensence effect of exoegenously applied cytokinins has been confirmed with the overproduciton of cytokinin in planta using the *ipt* gene (Smart et al., 1991; Li et al., 1992; Gan and Amasino, 1995). For example, excised leaves of cytokinin overproducing transgenic tobacco plants showed a significantly prolonged retention of chlorophyll and delayed senescence when incubated for a prolonged period in a dark moist chamber at room temperature (Li et al., 1992). There were no signs of senescence in these detached leaves after 14 weeks of incubation. Even after 6 months of incubation, the leaves still remained green, healthy and turgid. While the antisenscence effects resulting from the expression of the *ipt* gene was largely demonstrated in dicot plant species previously, more recently a senescence-enhanced cysteine protease gene promoter cloned from maize was used to control the expression of the *ipt* gene in a monocot plant, ryegrass (Li et al., 2004). Transgenic ryegrass lines that express the *ipt* gene displayed a stay-green phenotype although some transgenic lines developed spontaneous lesions.

The most promising and useful *ipt* gene construct for commericilization so far is probably the *PSAG12-ipt* fusion gene that was developed by Gan and Amasino (1995). The *PSAG12* gene promoter is a senescence-specific promoter that is active only in senescencing organs. Transgenic tobacco plants *PSGA12-ipt* did not exhibit the developmental abnormalities usually associated with constitutive *ipt* expression (Gan and Amasino, 1995). Also, the leaves of the transgenic tobacco plants exhibited a prolonged photosynthetically active life span. Because the *PSAG12-ipt* gene was activated only at the onset of senescence in the lower mature leaves of tobacco, the use of the *PSAG12-ipt* gene resulted in cytokinin biosynthesis in these leaves, which inhibited leaf senescence and, consequently, attenuated activity of the *PSAG12-ipt* gene, preventing cytokinin overproduction.

When the *PSAG12-ipt* gene was introduced into lettuce plants, developmental and postharvest leaf senescence in mature heads were significantly delayed (McCabe et al., 2001). There were no significant differences in head diameter or fresh weight of leaves and roots compared to wild-type plants. At the stages of bolting and preflowering, relative to the wild-type controls, there was little decrease in chlorophyll, total protein, and Rubisco content in transgenic leaves. However, the transgenic *PSAG12-ipt* lettuce plants showed a 4-8 week delay in flowering, and premature senescence of their upper leaves, a somewhat surprising phenotype.

In another study, Schroeder et al. (2001) reported that ornamental *Nicotiana alata* plants expressing the *SAG12-ipt* gene exhibited 2- to

4-times fewer senesced leaves, significantly longer *in situ* flower life and greater shoot dry weight. The transgenic plants were shorter and produced more branches. On the other hand, the transgenic plants had 32% to 50% fewer flowers per branch. Chang et al. (2003) demonstrated that floral senescence of *SAG12-ipt* transgenic petunia plants was delayed 6 to 10 d relative to wild-type flowers, and the increase in endogenous ethylene production in flowers after pollination was delayed in ipt expressing flowers. Furthemore, flowers from ipt transgenic plants were less sensitive to exogenously applied ethylene and required longer treatment times to induce endogenous ethylene production, corolla senescence, and up-regulation of the senescence-related Cys protease phcp1.

It has been reported that transgenic plants that overproduced cytokinin were resistant to biotic and abiotic stresses. Smigocki et al. (1993) reported that expression of a wound inducible proteinase inhibitor II promoter-*ipt* gene in tobacco plants led to resistance to the tobacco hornworm and inhibited normal development of green peach aphid nymphs. The senescence-specific *SAG12* promoter-*ipt* gene has also been used to create flood tolerant plants. Flooding often causes premature senescence, which leads to cessation of growth and reduced yield in many crops. Zhang et al. (2000) determined the effects of cytokinin on flood tolerance using transgenic *Arabidopsis thaliana* plants that expressed the senescence-specific *SAG12-ipt* gene. Transgenic plants exhibited increased tolerance to waterlogging and complete submergence. More recently, Huynh et al. (2005) conducted a detailed study on *SAG12* promoter-*ipt* transgenic *Arabidopsis* plants. Two forms of flooding stress, total submergence and root waterlogging, were applied to the transgenic and wild-type plants. Expression of the *PSAG12-ipt* gene was associated with several phenotypic adaptations, including chlorophyll retention and increased biomass and carbohydrate content relative to wildtype plants. The transgenic plants exhibited improved recovery after waterlogging or submergence treatment. Their work established the relationship between flood tolerance and cytokinin accumulation in the *SAG12-ipt* plants.

To prevent leaf senescence of young transplants or shoot cuttings during storage under dark and cold conditions, we have recently placed the ipt gene under the control of a cold-inducible promoter, *cor15a,* from *Arabidopsis thaliana* and introduced it into petunia and chrysanthemum (Khodakovskaya et al., 2005). Transgenic petunia and chrysanthemum plants, as well as excised transgenic leaves remained green and healthy during prolonged dark storage (4 weeks at 25 degrees C) af-

ter an initial exposure to a brief cold-induction period (4 degrees C for 72 h) while excised leaves and intact plants of non-transformants senesced under the identical treatment. Molecular analysis demonstrated a marked increase in *ipt* gene expression in intact transgenic plants as well as in isolated transgenic leaves exposed to a short cold-induction treatment prior to dark storage. These changes correlated with elevated concentrations of cytokinins in transgenic leaves after cold treatment. Furthermore, the *cor15a-ipt* transgenic plants showed a normal phenotype when grown at 25°C. Hu et al. (2005) have also demonstrated that transgenic turfgrass expressing ipt driven by a maize ubiquitin promoter had significant increases in tillering ability, chlorophyll a and b contents, and tolerance to low temperature stress.

Desirable phenotypes have also been observed in the down-regulation of cytokinin. Success in the molecular cloning of the cytokinin oxidase genes from maize and *Arabidopsis* makes it possible to reduce concentrations of endogenous cytokinins in transgenic plants (Houba-Herin et al., 1999; Werner et al., 2001). Cytokinin oxidase catalyzes the degradation of cytokinins bearing unsaturated isoprenoid side chains, resulting in the overall reduced concentrations of active cytokinins (Armstrong, 1994). Because reducing the endogenous cytokinin concentration in plants has previously not been possible, cytokinin oxidase genes provide an exciting possibility to explore the commercial potential of artificially lowering the cytokinin content in plants. Werner et al. (2001) found that overexpression of cytokinin oxidase genes in tobacco plants led to significant reductions in the concentrations of various cytokinins. The transgenic plants developed stunted shoots with smaller apical meristems. The plastochrone was prolonged, and leaf cell production was only 3-4% of that found in wild type plants. In contrast, root meristems of the transgenic plants were enlarged and root elongation was enhanced, also the transgenic plants developed more branched roots.

Werner et al. (2003) produced a detailed analysis of phenotypes of transgenic *Arabidopsis* plants that individually expressed six different members of the cytokinin oxidase/dehydrogenase (AtCKX) gene family. Their results are consistent with the hypothesis that cytokinins have central, but opposite, regulatory functions in root and shoot meristems and indicate that a fine-tuned control of cytokinin catabolism plays an important role in ensuring the proper regulation of cytokinins function. Yang et al. (2003) cloned a novel cytokinin oxidase gene, *DSCKX1* from Dendrobium Sonia. In transgenic orchid plants overexpressing DSCKX1, the elevated level of cytokinin oxidase activity was accom-

panied by a reduction of cytokinin content. These plants exhibited reduced shoot growth and produced numerous long roots, and their calli also showed decreased capability of shoot formation. Conversly, antisense transgenic plants showed rapid proliferation of shoots and inhibition of root growth combined with higher endogenous cytokinin content than wild-type plants. Presumably, manipulation (over- or under-expression) of cytokinin oxidase gene expression in plants could lead to promotion of shoot, lateral and adventitious root formation in horticultural crops.

ABSCISIC ACID

Abscisic acid (ABA), a "stress" hormone, promotes seed dormancy and enhances tolerance of plants to environmental stresses such as drought and low temperatures. ABA is synthesized by cleavage of violaxanthin or neoxanthin and the cleavage of these xanthophylls is the rate-limiting step of stress induced ABA biosynthesis (Walton and Li, 1995). The gene encoding the xanthophylls cleavage enzyme has been cloned from several species (Schwartz et al., 1997). Increases in ABA content in plants have been achieved using two different strategies. One strategy to enhance ABA production is to overexpress 9-cis-epoxycarotenoid dioxygenase genes, engineered for either constitutive or inducible expression in transgenic plants. The constitutive expression of 9-cis-epoxycarotenoid dioxygenase genes results in an increase in ABA. These plants showed activation of both drought- and ABA-inducible genes, a reduction in leaf transpiration rate (Iuchi et al., 2001), marked increases in tolerance to drought stress (Iuchi et al., 2001; Qin and Zeevaart, 2002), and increased seed dormancy (Thompson et al., 2000). In contrast, antisense suppression and disruption of the *AtNCED3* gene gave a drought-sensitive phenotype. Alternatively, Frey et al. (1999) have shown that overexpression of a zeaxanthin epoxidase gene cloned from *Nicotiana plumbaginifolia* in both sense and antisense orientation leads to alterations in ABA content. The seeds from overexpressing lines had increased ABA content and subsequently delayed germination, whereas those from plants expressing antisense ABA2 had a reduced ABA content and germinated rapidly.

Strauss et al. (2001) have shown that expression of a single-chain variable-fragment antibody against ABA in potato plants caused reductions in free ABA content and reduced growth. Transgenic plants produced smaller leaves than untransformed plants. Leaf stomatal conductivity of

transgenic plants was increased due to larger stomatal pores. When expression of the antibody gene was restricted to seeds by using a seed-specific gene promoter from *Vicia faba*, the resulting transgenic tobacco plants were phenotypically similar to wild-type plants but the embryo developed green cotyledons containing chloroplasts, accumulated photosynthetic pigments and produced less seed storage protein and oil bodies (Phillips et al., 1997). The transgenic seeds germinated precociously if removed from seed capsules during development but were incapable of germination after drying. These studies demonstrate that it is possible to use transgenic technology to manipulate ABA levels to improve performance, such as stress tolerance and seed germination in horticultural crops.

CONCLUDING REMARKS

In comparison to exogenous applications of plant growth regulators, transgenic manipulation of either hormone concentration or sensitivity offers several advantages:

a. cost-effectiveness because once desirable transgenes are inserted into target plants, no additional manipulations are needed;
b. relatively low risk to human health and the environment compared to the exogenous application of synthetic growth regulators;
c. minimal side-effects when the transgene is expressed only in target organs and at specific developmental stages; and
d. increased effectiveness.

Although we now have a large number of genes available for manipulating hormone concentration and sensitivity, relatively little has been accomplished in using these genes to improve horticultural crops. One reason is that we do not have a wide range of gene promoters to control the expression of genes that can alter hormone concentration or sensitivity in an organ- and developmental stage-specific manner.

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Molecular Biology of the Metabolism and Signal Transduction of Gibberellins, and Possible Applications to Crop Improvement

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SUMMARY. The hormones gibberellins (GAs) have profound biological effects on several aspects of plant growth. The genes encoding enzymes of GA metabolism and proteins of GA signaling have been cloned, and their functions well characterized. Notably, progress has been made in our understanding of how GAs are synthesized and decom-

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posed in response to various environmental cues. The homeostasis of endogenous levels of GAs is maintained at the transcriptional level of the genes by feed-back and feed-forward regulatory mechanisms through the signaling pathway. Mutations of each gene result in dwarf or succulent growth phenotypes. Some mutations cause agriculturally important phenotypes, such as 'green revolution' semi-dwarf in cereal crops. This indicates that the genes involved in the metabolism and signaling of GAs have the potential to improve economically important crops through the manipulation of their functions in transgenic plants.
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KEYWORDS. Gibberellins (GAs), GA biosynthesis genes, GA signaling factors, growth regulation, molecular breeding

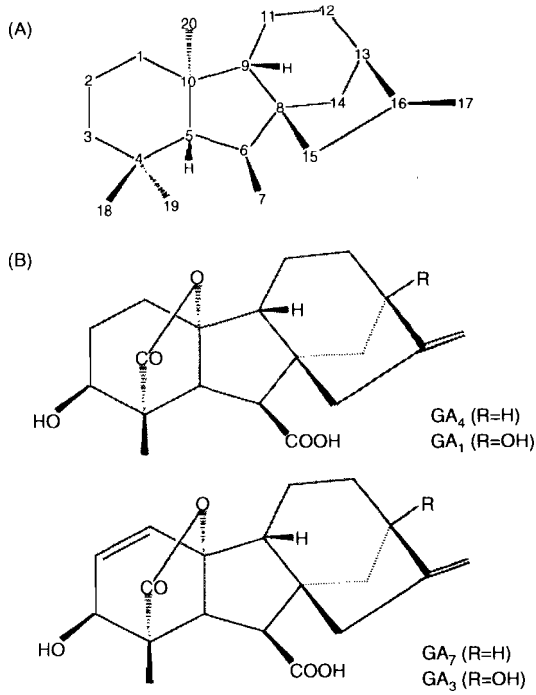
INTRODUCTION

Plants grow under the control of various environmental cues, including light, temperature and water in their niche. These stimulations are transmitted through a signal transduction pathway into the cell, and provoke proper physiological and biochemical responses. Plant hormones involved in these processes play an important role in plant development. Thus, studies on their metabolism and signaling processes will provide profound insight into how plants grow.

Gibberellins (GAs) are hormones that control plant development, including seed germination, stem elongation, flowering induction, stamen development, and stimulation of fruit growth with auxin. GAs are diterpenoids which have a basic *ent*-gibberellane skeleton, and 125 different homologues have been reported in higher plants and GA-producing fungi (Rademacher, 2000). However, only a few GAs are biologically active, e.g., GA_1, GA_4, GA_3, and GA_7 (Figure 1). Recent advances in research using GA-deficient and GA-insensitive mutants have provided information about the genes encoding the biosynthetic enzymes and the signaling factors (Hedden and Kamiya, 1997; Richards et al., 2001). This knowledge will open the way to growth manipulation of economically important crops.

In this article, recent findings about the enzymes and genes for GA biosynthesis and the current understanding of GA signaling will be reviewed. We also discuss the possibility of controlling crop growth through the genetic modification of GA metabolism and signaling.

FIGURE 1. Structures of a basic *ent*-gibberellane skeleton (A) and physiologically active GAs (B).



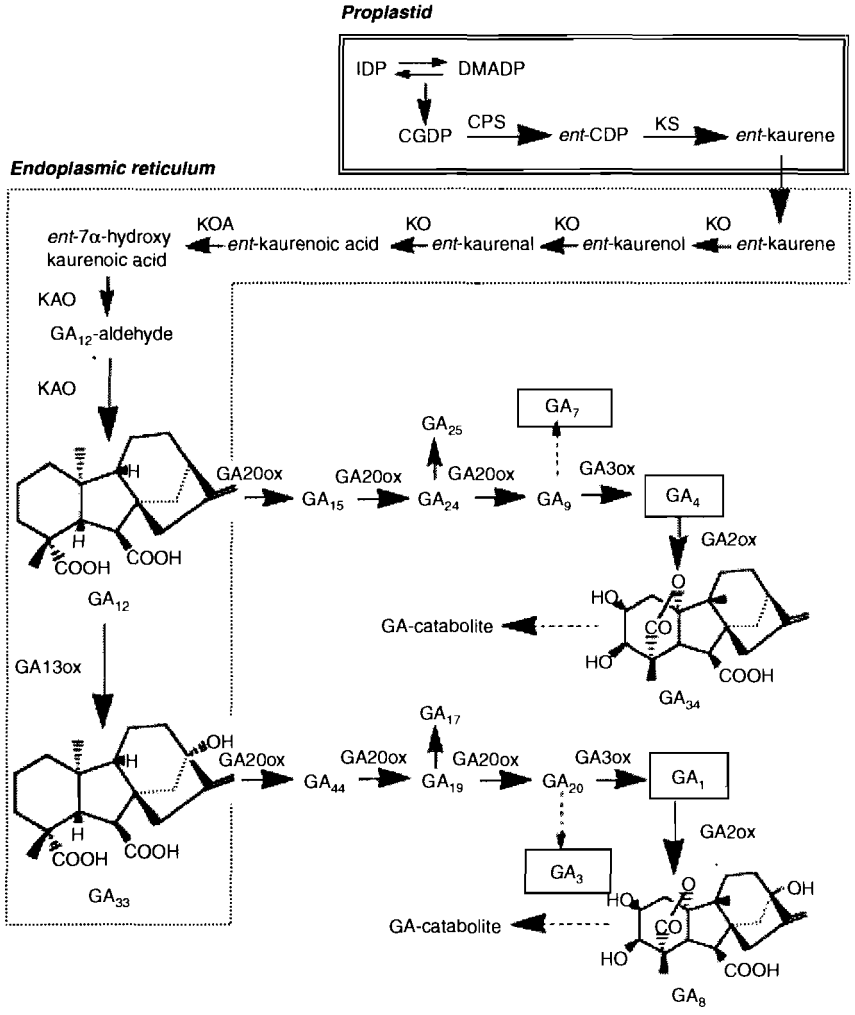
ENZYMES AND GENES OF GA BIOSYNTHESIS

Three stages in the biosynthesis of GAs according to the nature of enzymes and their localization in the cell have been reported (Rademacher, 2000) (Figure 2). The first stage is from geranylgeranyl diphosphate (GGDP) to *ent*-kaurene catalyzed by terpene cylases in the proplastid. The second stage is from *ent*-kaurene to GA_{12} catalyzed by monoxygenases associated with the endoplasmic reticulum. The final stage is the most important to synthesize or catabolyze biologically active GAs in the cytosol in which dioxygenases are involved.

Stage 1

Recent findings indicate that both mevalonate and methylerythritol phosphate pathways are involved in supplying dimethylallyl diphos-

FIGURE 2. The biosynthetic and catabolic pathway of gibberellin (GA) in higher plants. Boxes indicate biologically active GAs. Abbreviations of enzyme names are: *ent*-copalyl diphosphate synthase (CPS), *ent*-kaurene synthase (KS), *ent*-kurene oxidase (KO), *ent*-kaurenoic acid oxidase (KAO), GA 13-hydroxylase (GA13ox), GA 20-oxidase (GA20ox), GA 3β-hydroxylase (GA3ox), and GA 2β-hydroxylase (GA2ox). Based on Hedden and Phillips (2000).



phate (DMADP) and isopentenyl diphospahte (IDP), the substrates for the condensation reaction to synthesize GGDP (Kasahara et al., 2002). *ent*-Kaurene is synthesized from GGDP by a two-step cyclization reaction: from GGDP to *ent*-copalyl diphosphate (*ent*-CDP) catalyzed by copalyl diphosphate synthase (CPS) and from *ent*-CDP to *ent*-kaurene by *ent*-kaurene synthase (KS).

cDNAs of *CPS* have been cloned in several plant materials, such as *Arabidopsis* (*Arabidopsis thaliana* (L.) Heynh.) (Sun and Kamiya, 1994), pumpkin (*Cucurbita moschata*) (Smith et al., 1998), maize (*Zea mays*. L.) (Bensen et al., 1995) and pea (*Pisum sativum* L.) (Ait-Ali et al., 1997). The genes encoding CPS have also been found in rice (*Oryza sativa* L.) DNA databases (Sakamoto et al., 2004). KS was purified from immature seeds of pumpkin (Saito et al., 1995). This was followed by cDNA cloning of the gene based on partial amino acid sequence information on the enzyme (Yamaguchi et al., 1996). The gene was also cloned in *Arabidopsis* (Yamaguchi, Sun, et al., 1998) and found in the rice genome in multiple copies (Sakamoto et al., 2004).

High levels of activity by CPS and KS are detected in proplasitids from actively growing shoots of pea and wheat (*Triticum aestivum* L.) (Aach et al., 1995), suggesting that sufficient sources for GA biosynthesis are required in the tissue. Indeed, the enzymatic activity declined in mature tissue (Aach et al., 1997). This is partially consistent with expression patterns of the genes. *CPS* mRNA is abundant in very young tissue in pumpkin, but decreased in amount in accordance with plant growth (Smith et al., 1998). However, the level of *KS* mRNA is constant. The same is also true in *Arabidopsis*; the promoter activity of *GA1* encoding CPS is strong in rapidly growing tissue as well as vascular tissue of leaves, which are supposed to be a site of the synthesis of GAs for transport to other organs (Silverstone, Chang et al., 1997). On the other hand, *GA2*, a gene for KS, is expressed at relatively high levels in various tissues of *Arabidopsis* (Yamaguchi, Sun et al., 1998). Since GGDP is utilized as a common precursor for several large families of terpenoid products (Chappell, 1995), regulation of the conversion of GGDP into *ent*-kaurene is a critical step for the determination of the GA biosynthetic rate. Thus, it is likely in *Arabidopsis* that *GA1* is controlled by a very weak promoter, indicating that this gene is strictly regulated and may function as a gatekeeper to control the flow of metabolites into the GA biosynthetic pathway (Silverstone, Chang et al., 1997). Although developmental regulation of *CPS* and *KS* gene expression was reported, very little information is available on their environmental regulation. In lisianthus (*Eustoma grandiflorum* Shinn), a cut flower plant requiring

vernalization for stem elongation, *CPS* mRNA was accumulated in stem tissue during low temperature treatment (Mino et al., 2003). The result suggests that rosette plants exposed to cold temperatures prepare a source for GA biosynthesis and the condition helps stem elongation through the prompt synthesis of bioactive GA after vernalization.

Stage 2

ent-Kurenere oxidase (KO) is a multifunctional enzyme which catalyzes three consecutive reactions leading from *ent*-kaurene to *ent*-kaurenoic acid. The enzyme requires O_2 and NADPH and involves cytochrome P450 for activity (Murphy and West, 1969). A dwarf pea *lh-2* mutant could oxidize the radiolabeled intermediate *ent*-kaurenoic acid but not *ent*-kaurene, *ent*-kaurenol, or *ent*-kaurenal, whereas the wild-type plant was able to metabolize all four substrates (Swain et al., 1997). The GA-deficient *ga3* mutant of *Arabidopsis* showed a growth response to the application of *ent*-kaurenoic acid but not *ent*-kaurene (Helliwell et al., 1998). The results suggested that *LH-2* and *GA3* encode KO and a single enzyme catalyzes these three reactions. Direct evidence that KO catalyzes the three steps from *ent*-kaurene to *ent*-kaurenoic acid was obtained with *GA3* of *Arabidopsis*, a gene encoding cytochrome P450 monooxygeneas of the subfamily CYP701A (Helliwell et al., 1999). The extract from *GA3*-expressing yeast (*Saccharomyces cerevisiae*) cells incubated with *ent*-kaurenol contained *ent*-kaurenoic acid, while that of nontransformed yeast did not. The genes for KO were also isolated in pea (Davidson et al., 2004) and were found in the rice genome (Sakamoto et al., 2004). Northern-blot analysis shows that the pea *KO* gene (*PsKO1*) was expressed in all tissues examined, including stems, roots and seeds and *OsKO2* of rice was also broadly expressed in shoot apices, leaf sheaths, leaf blades, elongating stems, and immature panicles. However, there was a tendency in *Arabidopsis* for the expression patterns of *GA1* and *GA3* to be closely related (Helliwell et al., 1998), suggesting that genes encoding enzymes catalyzing successive steps in the GA biosynthetic pathway are under the control of a common mechanism. It was reported in *Thlaspi arvense*, a species requiring vernalization for stem elongation, that the activity of KO in the shoot tip increased after vernalization (Hazebroek et al., 1993), indicating that the genes encoding KO and/or the enzymes' activities are controlled by low temperature.

The last steps of stage 2 are controlled by *ent*-kaurenoic acid oxidase (KAO), which catalyzes the three steps from *ent*-kaurenoic acid to GA_{12} via *ent*-7α-hydroxy-kaurenoic acid and GA_{12}-aldehyde. The genes of KAO were reported in *Arabidopsis* and barley (Helliwell et al., 2001), pea (Davidson et al., 2003) and rice (Sakamoto et al., 2004). A yeast cell transformed with the cDNAs encoding the CYP88A subfamily of cytochrome P450 enzymes (KAO) of *Arabidopsis*, barley and pea showed all three reaction steps.

Stage 3

Most reactions of stage 3 proceed in the cytosol. The soluble dioxygenases, the enzymes primary involved in the stage, require 2-oxoglutarate as a co-substrate and Fe^{II} and ascorbate as co-factors. However, some initial reactions of the stage are still catalyzed by a monooxygenase bound to the endomembrane. In vegetative tissues of certain species, e.g., maize, rice, pea and lisianthus, GA_{53} is generated from GA_{12} by GA 13-hydoroxylase, followed by a subsequent oxidation reaction in the early 13-hydroxylation pathway. Maize *D3*, the gene encoding an enzyme with early 13-hydroxylase activity, was isolated via transposon tagging (Winkler and Helentjaris, 1995). On the other hand, in *Arabidopsis* and cucumber (*Cucumis sativus* L.), non-13-hydroxylation is a major metabolic pathway.

Successive oxidation steps from GA_{12} to GA_9 in the non-13-hydroxylation pathway or GA_{53} to GA_{20} in the early 13-hydroxylation pathway are catalyzed by GA 20-oxidase (GA20ox). A cDNA clone encoding GA20ox was first isolated from developing cotyledons of pumpkin (Lange et al., 1994). However, the recombinant protein catalyzed the three-step conversions of GA_{12} to GA_{25} and of GA_{53} to GA_{17}, in addition to the formation of C19-GAs, GA_1, GA_9, and GA_{20}. A cDNA of more common enzymes which convert 10-methyl GAs to the corresponding C19 lactones was isolated from *Arabidopsis* (Phillips et al., 1995; Xu et al., 1995). cDNA clones of *GA20ox* were also obtained from pea (Martin et al., 1996), bean (*Phaseolus vulgaris* L.) (Garcia-Martinez et al., 1997), spinach (*Spinacia oleracea* L.) (Wu et al., 1996), watermelon (*Citrullus lanatus*) (Kang et al., 1999), rice (Spielmeyer et al., 2002), apple (Kusaba et al., 2001) and hybrid citrus (*Citrus sinensis* × *Poncitrus trifoliata*) (Vidal et al., 2003). It is interesting that the *GA20ox* gene corresponds to the *Sd-1* locus of rice, and a deletion

within the coding region of this locus provides semidwarf "green revolution" *sd-1* mutant rice (Spielmeyer et al., 2002).

GA_9 and GA_{20} are transformed into the physiologically active GAs, GA_4 and GA_1, respectively, by the action of GA3β-hydroxylase (GA3ox). However, cell-free extracts of wild cucumber (*Marah macrocarpus*) and apple (*Malus domestica*) seeds are able to convert GA_9 and GA_{20} to another type of bioactive GA, GA_7 and GA_3, respectively (Albone et al., 1990), though it might be a side reaction of GA3ox activity (Hedden and Phillips, 2000). The cDNA clone of *GA3ox* was isolated first from *Araibidopsis* (Chiang et al., 1995), and subsequently from pea (Lester et al., 1997; Martin et al., 1997), tobacco (*Nicotiana tabacum* L.) (Itoh et al., 1999), pumpkin (Frisse et al., 2003) and hybrid aspen (*Populus tremula* × *P. tremuloides*) (Israelsson et al., 2004). It was reported that the *Le* gene of pea originally described by Mendel encodes GA3ox, and malfunction of this gene leads to a dwarf *le* phenotype (Lester et al., 1997; Martin et al., 1997).

The third enzyme of stage 3 is GA2β-hydroxylase (GA2ox) which reduces the biological activity of GA via hydroxylation at position 2β, suggesting that the enzyme is important for the turnover of bioactive GAs. Further steps in the catabolism of 2β-hydroxy GAs are catalyzed by a different enzyme system (Hedden and Kamiya, 1997). The genes encoding GA2ox were cloned from pea (Martin et al., 1999), runner bean (*Phaseolus coccineus*) (Thomas et al., 1999), rice (Sakamoto et al., 2001), *Arabidopsis* (Schomburg et al., 2003) and hybrid poplar (Busov et al., 2003). Increased expression of *AtGA2ox* of *Arabidopsis* in tobacco plants significantly reduced GA levels and induced a dwarf phenotype mimicking the GA-deficient mutant (Schomburg et al., 2003).

REGULATION OF GA METABOLISM

The levels of GAs in tissues and/or cells are tightly regulated, so that plants are able to grow properly in response to environmental stimuli. Developmental stage, light, temperature and endogenous levels of hormones can modify the metabolism of GA via regulation of each step in the biosynthetic and catabolic pathway. Furthermore, some genes encoding enzymes involved in the metabolism of GA, especially dioxygenases, compose multigene families in plants, and the expression of genes at each locus may be responsive to respective environmental signals. Thus the overall metabolic features of GAs are not always simple.

Development

As described earlier (see section, *stage 1* in *Enzymes and Genes of GA Biosynthesis*), the transcriptional level of *GA1* (CPS) of *Arabidopsis* was elevated in actively growing tissue and vascular tissue of expanded leaves. Since CPS tightly regulates the amount of *ent*-CDP in cells, the results that the primary step in GA metabolism after vernalization in *Thalaspi arvense* L. takes place in the shoot tip (Hazebroek et al., 1993) may be consistent with the nature of this enzyme. *GA1* of *Arabidopsis* and *An1* of maize CPS are single copy genes (Sun and Kamiya, 1994; Bensen et al., 1995), while at least two copies were found in pumpkin (Smith et al., 1998).

The two genes encoding KAO in *Arabidopsis* are expressed in all parts of the plant, with higher levels in inflorescences, inflorescence stems and siliques, the tissues in which larger amounts of bioactive GAs are required (Helliwell et al., 2001). In pea, however, *PsKAO2* is expressed only in developing seeds, while *PsKAO1* is expressed in vegetative tissue (Davidson et al., 2003). Thus, though mutation in the *PsKAO1* gene results in the extreme dwarf *na* phenotype, the mutant is able to synthesize bioactive GAs during seed development and set seeds thanks to the normal function of *PsKAO2*.

The genes for GA20ox and GA3ox are generally expressed at a higher rate in growing vegetative tissue and developing fruit tissue. On the basis of transcript levels determined by northern blot analysis, Garcia-Martines et al. (1997) reported that the pea *GA20ox* gene was highly expressed in young leaves, fully expanded internodes, very young seeds and expanding pods. The tobacco *GA3ox* gene was expressed at the site where bioactive GAs function, i.e., rib meristem, elongation zones of shoot apices, developing anthers and root tips (Itho et al., 1999). In *Arabidopsis*, the *GA4* (GA3ox) gene was expressed in developing siliques, geminating seeds and seedlings, but transcription of the *GA4H* (*GA4* homolog) gene was detected only in geminating seeds and young seedlings (Yamaguchi, Smith et al., 1998).

In situ hybridization analysis indicated that rice *GA2ox* mRNA was localized at the base of leaf primordia and young leaves (Sakamoto et al., 2001). Levels of the transcripts decreased quickly after transition from the vegetative to reproductive phase, suggesting that levels of bioactive GAs in shoot apical meristem are under the control of expression levels of *GA2ox*.

Light

A rosette plant of spinach induces stem elongation under long-day (LD) conditions and the process is mediated by elevation of endogenous levels of GAs. Consistent with this, more *GA20ox* mRNA was accumulated in LD than short-day conditions (Wu et al., 1996). Although the expression of *GA20ox* in petioles and young leaves was strongly up-regulated in LD conditions, that of *GA3ox* and *GA2ox* was not. This indicates that levels of physiologically active GAs in spinach are primarily controlled by *GA20ox* (Lee and Zeevaart, 2002). Transgenic antisense potato (*Solanum tuberosum*) plants with reduced levels of phytochrome B (PHYB) accumulated more *GA20ox* mRNA than wild-type plants, suggesting the involvement of PHYB in the control of GA biosynthesis (Jackson et al., 2000). Seed germination in *Arabidopsis* is regulated by light through PHYB, and expression of both the *GA4* and *GA4H* (GA3ox) genes is up-regulated by red light which induces germination, but down-regulated by far-red light which suppresses germination (Yamaguchi, Smith, et al., 1998). A similar response of *GA3ox* gene expression to red light was also found in lettuce (*Lactuca sativa* L.) seeds in which germination is regulated by the phytochrome. However, expression of the gene encoding GA20ox was unchanged or down-regulated by red light according to the respective locus (Toyomasu, 1998). The red light down-regulated expression of *GAox2* in germinating lettuce seeds, suggests that high endogenous levels of GAs are required for germination (Nakaminami et al., 2003).

For more details on the role of light in the regulation of GA biosynthesis, refer to the review of Garica-Martinez and Gil (2001).

Temperature

Exposure of imbibed seeds of *Arabidopsis* to low temperature promotes germination. Treatment with cold temperature, vernalization, is also required for many winter annuals and biennials for stem extension from a vegetative rosette. The involvement of GAs in the process was reported. However, few examples of how genes for the biosynthesis of GAs are implicated in the process have been obtained. In *Arabidopsis*, however, it was reported recently that several genes for GA biosynthesis are up-regulated in response to low temperature (Yamaguchi et al., 2004). Analysis by DNA microarray indicated that some transcripts encoding GA20ox and GA3ox accumulated at a much higher rate in the seeds at 4°C than 22°C, whereas the expression of GA2ox was down-

regulated at 4°C relative to that at 22°C. Furthermore, levels of bioactive GAs were increased in the seed at 4°C. Up-regulation of genes for GA biosynthesis, *CPS*, *GA20ox* and *GA3ox*, during vernalization treatment was reported in rosette plants of lisianthus (Mino et al., 2003). On the other hand, in hybrid citrus, high temperature induced expression of the *GA20ox* gene and an increase in the amount of GA_1 in the shoot (Vidal et al., 2003). These results indicate that the perception of the temperature required for plant growth leads to a harmonious expression of the genes for GA biosynthesis.

Hormones

Endogenous levels of GA were regulated by GA itself via negative feedback regulation of the expression levels of both GA20ox and GA3ox and feedforward regulation of the level of GA2ox. Both *GA20ox* and *GA3ox* of *Arabidopsis* were up-regulated in the mutant deficient in GA-biosynthesis (Chiang et al., 1995; Xu et al., 1995). The message of the *GA20* gene decreased on application of GA_3 in *Arabidopsis* (Phillips et al., 1995) and pea (Martin et al., 1996). In lithianthus, the accumulation of both *GA20ox* and *GA3ox* transcripts was reduced by GA_3 (Oka et al., 2001). Conversely, the expression of GA2ox was up-regulated in *Arabidopsis* (Thomas et al., 1999) and in rice (Sakai et al., 2003) on the application of a bioactive GA. However, some genes are not subject to feedback regulation. Yamaguchi, Smith et al. (1998) reported that *GA4H* (GA3ox) in *Arabidopsis* was predominantly expressed during seed germination and was not down-regulated on application of GA_4. It is conceivable that *GA4H* developed this specific function to circumvent down-regulation of GA biosynthesis and maintain sufficiently bioactive GAs for seed germination. Feedback and feedforward regulation have been reported in the genes encoding dioxygenases, but not found so far in CPS, KS or monooxygenases.

Indole-3-acetic acid (IAA) regulates endogenous levels of bioactive GAs through regulation of the transcription of *GA3ox* and *GA2ox* in pea (Ross et al., 2000). Decapitation of the pea shoot decreased *GA3ox* mRNA levels, but application of IAA to the plants increased amounts of the transcript. On the other hand, *GA2ox1* transcript levels were increased by decapitation and reduced by the application of IAA. It was reported recently that nuclear DELLA protein (GRAS family protein; see section, *Arabidopsis* in *Molecular Genetics of GA Signaling*), a growth repressor destabilized by GA, is involved in cross-talk regulation between IAA and GAs (Fu and Harberd, 2003). In beech (*Fagus*

sylvatica L.) seeds, ethylene suppressed the expression of *GA20ox*, and the regulation of the gene by GA and ethylene plays an important role in the transition from seed dormancy to germination in this species (Calvo et al., 2004).

MOLECULAR GENETICS OF GA SIGNALING

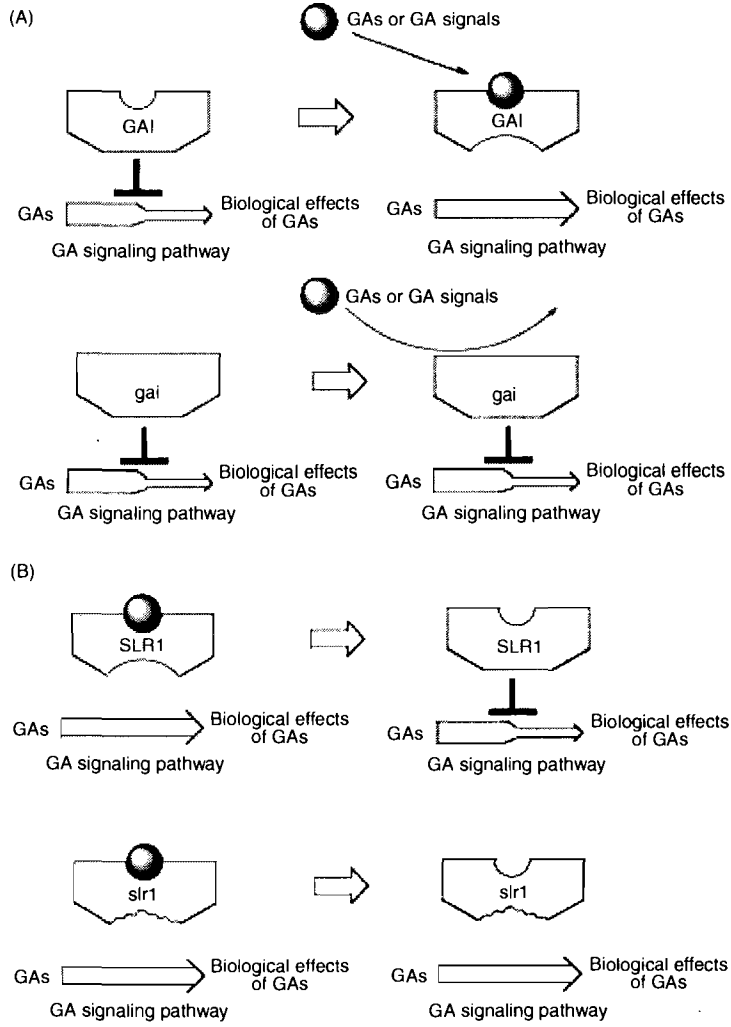
There are several reports describing the functions of the genes for GA signaling in plant species. Although the most extensive study was performed in *Arabidopsis*, information for other species, e.g., rice, wheat, barley and maize, has also become available.

Arabidopsis

Mutations in the genes encoding the GA signaling pathway induce a phenotype similar to GA-deficient dwarf or elongating shoots treated with an overdose of GA. As such a mutant, *Arabidopsis gai* (*ga insensitive*) showed a typical dwarf phenotype with dark green leaves, but the plant height could not be restored by application of bioactive GAs (Koornneef et al., 1985). Since feedback regulation is perturbed, the mutant has elevated levels of *GA4* (GA20ox) transcripts and endogenous GAs (Peng et al., 1997; Cowling et al., 1998). *GAI* encodes a protein of 532 amino-acid residues (Peng et al., 1997), classified as belonging to the GRAS (for *GAI*, *RGA* and *SCARECROW* of *Arabidopsis* genes) family of proteins (Pysh et al., 1999). High-level expression of *GAI* under the control of the CaMV 35S promoter or maize *Ubiquitn* promoter in rice induced the dwarf phenotype and increased GA20ox transcript levels (Fu et al., 2001). Altered ectopic expression of *GAI* in responses to GA in transgenic tobacco were also reported (Hynes et al., 2003). These results suggest that GAI functions as a negative regulator of GA signaling. GAI has a DELLA domain at the N-terminal which does not appear in other GRAS family proteins: an in-frame deletion of a 17-amino-acid residue segment in the domain results in a gain-of-function mutation in this locus (Peng et al., 1997). This means that the DELLA domain may function as a GA signal receiver. The GAI that receives GA signals normally releases suppression of the GA signaling pathway, whereas *gai* without the receiver continues to suppress and this eventually leads to a GA-deficient phenotype (Figure 3-A).

The second gene is *rga* (*repressor of ga1-3*) which was selected as a suppressor of the GA biosynthetic mutant *ga1-3* (a dwarf mutation with

FIGURE 3. Functions of regulators in the GA signaling pathway. Wild-type and mutant proteins function in the presence or absence of biologically active GAs or GA signaling in *Arabidopsis* GAI (A) and rice SLR1 (B).



reduced endogenous GA levels due to a defect of CPS) (Silverstone, Mak et al., 1997). RGA, a 587-amino acid protein, also has a DELLA domain at the N-terminal and the signal sequence to recruit the protein to the nucleus. Several deletions and point mutation creating a stop codon in the open reading frame of the loci resulted in *rga* being a loss-of-function mutation (Silverstone et al., 1998). Interestingly, *gai-t6*, another type of *gai* recessive mutation, alone has little effect to restore the dwarf phenotype of *ga1-3* to the wild type, but together with *rga-24*, has a synergistic effect to rescue it completely (Dill and Sun, 2001). This suggests that *RGA* plays a more dominant role than *GAI* in the GA-signaling pathway.

RGA protein in the nucleus is rapidly degraded after application of GA as witnessed by the reduction in green fluorescent protein-RGA fusion protein in the nucleus and immunoblot analysis using anti-RGA anti-body (Silverstone et al., 2001). *Arabidopsis SLEEPY1* (*SLY1*) encodes an F-box protein that is indispensable for maintaining normal GA signaling processes. The recessive mutation of the gene results in a GA-insensitive dwarf phenotype and the accumulation of high levels of RGA. Recently, it was reported that SLY1 protein directly interacts with RGA and GAI via the C-terminal GRAS domain and forms the SCF^{SLY1} E3 ligase complex for ubiquitination and subsequent degradation by the 26S proteasome (Dill et al., 2004; Fu et al., 2004). Destabilization of repressor proteins for GA signaling is tightly regulated by not only GAs but also a more complex genetic system.

The third gene is *RGL2* (*RGA-like2*) encoding a protein with a DELLA domain at the N-terminal and functions as a negative regulator of GA signaling of seed germination (Lee et al., 2002). The amount of *RGL2* transcript decreased during the germination of wild-type seeds, but remained at high levels in non-germinating *ga1-3* mutant seeds. This suggests that expression of *RGL2* is controlled by GA at the transcriptional level. Thus, GA regulates stem elongation via *GAI* and *RGA*, and seed germination via *RGL2*. A recent genetic analysis indicated that reduced elongation growth of petals and stamens in GA-deficient mutant *Arabidopsis* is controlled by the joint functions of *RGA* and *RGL2* (Cheng et al., 2004). However, there are up to 30 candidate GRAS ORFs in the *Arabidopsis* genome (Lee et al., 2002). This means that GA signaling through GRAS family proteins might be controlled by a more complex mechanism.

SPINDLY (SPY) is another type of negative regulator of responses of GA (Jacobsen and Olszewski, 1993; Jacobsen et al., 1996). The protein has ten tetratricopeptide repeat motifs which function as a pro-

tein-protein interaction domain and the C-terminal catalytic domain of cytosolic *O*-GlcNAc transferases, both of which play an important role in GA signaling (Thornton et al., 1999; Izhaki et al., 2001; Tseng et al., 2001). Using a barley transient assay system, Robertson et al. (1998) found that the expression of *HvSPY*, an ortholog of *SPY*, abolished the GA_3-induced activity of the α-amylase promoter that is controlled by GA. In lithianthus, expression of *EgSPY* was increased in the stem during vernalization, but not in non-vernalized plants (Mino et al., 2003). Since expression of the genes for GA biosynthesis was up-regulated during vernalizaiton, the enhanced induction of *EgSPY* might induce conditions in which adventitious stem elongation would be inhibited during vernalizaiton.

Finally, *GAMYB-like* genes, orthologs of GAMYB, a transacting factor for barley α-amylase promoter, are involved in GA signaling in growth and flowering responses in *Arabidopsis* (Gocal et al., 2001).

Rice

The shoots of the recessive *slender* (*slr1-1*) mutant elongate as if saturated with GAs, and the phenotype is not able to be suppressed by an inhibitor of GA biosynthesis. The results indicate that the product of the *SLR1* gene acts as a negative regulator and the loss-of-function of the gene does not suppress GA signaling, eventually resulting in a GA overdose phenotype even if low endogenous levels of bioactive GAs are maintained (Figure 3-B). Indeed, *SLR1* has significant homology to *GAI* and *RGA* of *Arabidopsis* (Ikeda et al., 2001). Like GRA of *Arabidopsis*, SLR1 was degraded by GID2 (gibberellin insensitive dwarf2), a rice F-box protein, through interaction between the SCF^{GID} complex and the phosphorylated SLR1, followed by ubiquitin-mediated degradation of the complex (Gomi et al., 2004). Proteolysis of SLR1 results in a more active GA signaling in the cells.

OsWRKY71 is another type of negative regulator as a transcriptional repressor of GA signaling in aleurone cells (Zhang et al., 2004). The protein is also degraded following exogenous GA treatment.

Wheat, Maize and Barley

Reduced height-1 (*Rht-1*), the so-called wheat 'Green revolution' gene, and maize *Dwarf-8* (*D8*) are orthologues of the *Arabidopsis GAI* (Peng et al., 1999; Hedden, 2003). These genes encode proteins that re-

semble nuclear transcription factors with a DELLA domain at the N-terminal and participate in GA signaling. A mutant gene, *rht-1*, of wheat brought about a semi-dwarf phenotype with lodging resistance and high grain yield. The *SLENDER1* (*SLN1*) locus of barley produces SLN1 (DELLA protein) which is necessary for repression of GAMYB (Chandler et al., 2002; Gubler et al., 2002).

APPROACH TO GENETIC MODIFICATION OF GA METABOLISM AND SIGNALING

Improving agricultural and horticultural traits, those of which affect seedling establishment in the field, growth rate, plant height, and flowering habit, is a major aim of plant breeding. Notably, semi-dwarf has several benefits in terms of growth not only for lodging resistance but also for high grain yield of cereal crops, and also contributes to increases in operating efficiency in orcharding, and decreases in the work rate of roadside trees. Reducing the endogenous levels of GAs is required to fulfill these breeding programs. On the other hand, elevated endogenous GA levels will be attributions for some other crops. The production of seedless grapes requires the application of GA to the flower cluster both during and after flowering. The treatment with GA of developing fruits of apple and persimmon (*Diospyros kaki* Thunb) will improve their quality. Some floricultural plants such as lisianthus need low-temperature or GA application to the vegetative rosette for year-round cut flower production. If we control the endogenous levels of GAs at the proper site and proper time, labor costs will be dramatically reduced. To this end, promoters of the genes exclusively expressed at flowering time, during fruit development and in the vegetative rosette as well as the genes for GA biosynthesis or decomposing genes are required. Recent advancements in plant molecular biology and transgenic techniques will help us to manipulate plant growth in terms of molecular breeding.

High-Level Expression of the Genes

It is expected that overexpression of genes involved in the biosynthesis of GA produced the phenotypes obtained with high-dose bioactive GAs. However, this is not true for all the genes involved in GA biosynthesis and plant species employed. Transgenic *Arabidopsis* plants that overexpress either *AtCPS*, *AtKS* or both have greatly in-

creased levels of the early intermediates *ent*-kaurene and *ent*-kaurenoic acid, but a lesser increase of later metabolites, and no GA overdose morphology was observed in the overexpression lines (Fleet et al., 2003). This suggests that the flux through the GA biosynthesis pathway is mainly controlled downstream of *ent*-kaurene format.

The GA20ox from developing pumpkin seeds produces physiological inactive GAs; it catalyzes the conversion of GA_{19} and GA_{24} to GA_{17} and GA_{25}, respectively, as major reaction products (cf. section, *Stage 3* in *Enzymes and Genes of GA Biosynthesis* and Figure 2). Overexpression of the gene in transgenic lettuce (*Lactuca sativa*) resulted in higher levels of GA_{17} and GA_{25} and lower levels of biologically active GA_1 and GA_4 compared with non-transgenic control plants. More importantly, the transgenic lettuce showed reduced growth (Niki et al., 2001). A transgenic semi-dwarf woody nightshade (*Solamum dulcamara* L.) was also obtained by overexpression of pumpkin *GA20ox* (Curtis et al., 2000). In contrast, overexpression of *Arabidopsis* GA20ox in transgenic *Arabidopsis*, which does not have diverting activity in the biosynthetic pathway to tricarboxylic acids (GA_{17} and GA_{25}), increased in bioactive GA and produced GA-overproduction phenotype (Huang et al., 1998). Similar results, that overexpression of *GA20ox* increased levels of GA and accelerated growth, were reported in transgenic potato (*Solanum tuberosum* L.) (Carrera et al., 2000) and transgenic tobacco (Vidal et al., 2001). In tree breeding programs, increased growth rates and stem volumes and shortening of rotation times are important. Overexpression of *GA20ox* led to a 20-fold increase in bioactive GA levels and increased growth and taller trees in hybrid aspen (Eriksson et al., 2000). However, overexpression of *GA3ox* in hybrid aspen had relatively minor effects on GA_1 and GA_4 homeostasis and caused no major changes in morphology (Israelsson et al., 2004). These results suggest that GA 20-oxidation, relative to GA 3-hydroxylation, acts as a limiting step in GA biosynthesis controlling shoot elongation.

Overexpression of *GA2ox* induced dwarf phenotypes in *Arabidopsis*, hybrid popular and rice, though the lines were obtained from activation-tagging mutant lines in the first two cases (Schomburg et al., 2003; Busov et al., 2003; Sakamoto et al., 2003). In transgenic rice in which expression of *GA2ox* is constitutively up-regulated by the actin promoter, seeds could not be produced. However, when the transgene was controlled by the promoter of the rice GA3ox gene, the transgenic plant showed normal grain development as well as a semi-dwarf phenotype (Sakamoto et al., 2003). Thus, it is very important to select the promoter,

when *GA2ox* is employed as a tool for reduction of endogenous GA levels.

Based on the suppressor function in the GA signaling pathway, an overdose of GAI/RGA proteins in cells produces the dwarf phenotype and suppresses a variety of GA responses (Peng et al., 1999; Fu et al., 2001). More importantly, since the function of the orthologs is conserved in GA signaling throughout the plant kingdom, the gene from one species is able to substitute for that of another species (Peng et al., 1999). Thus, the gene of one species (e.g., *Arabidopsis*) has the potential to create a range of dwarfed plants in various plant materials.

Low-Level Expression of the Genes

Down-regulation of the expression of the genes by the anti-sense technique is effective to control endogenous GA levels, and eventually, plant phenotype. Anti-sense copies of the *GA20ox* cDNA reduced levels of GA_{20} and GA_1 in transgenic potato plants, and the plants had a short internode and tuberized earlier than control plants (Carrera et al., 2000).

Recently, a more efficient siRNA technique has been developed to induce the sequence-specific inhibition of gene function (Fire, 1999; Sharp, 2001). The reverse genetic approach using the siRNA is more efficient to induce loss-of-function or reduction-of-function mutants than the anti-sense technique. For example, the four genes involved in the flower development of *Arabidopsis* (*AGAMOUS, CLAVATA3, APETALA1* and *PERIANTHIA*) suppressed by siRNA induced each recessive mutant phenotype more efficiently than the anti-sense constructs (Chuang and Meyerowitz, 2000). Thus, siRNA may be a robust tool for down-regulation of the genes encoding enzymes and proteins of GA metabolism and signaling.

CONCLUDING REMARKS

In the discovery and study of the genes controlling GA metabolism and GA signaling, pioneering work has provided fundamental insight into how GAs function in plants. An overview of the genes involved in the biosynthesis of GAs and in GA signaling has been obtained in *Arabidopsis*, and the number of orthologs has increased in many other plant species thanks to the sequence similarity of amino acids. The next step will be to learn more about the GA signaling mechanism, especially the receptor of GA, enhancer sequences of the 5′-upstream region

of GA-responsive gene and their trans-acting factors which specifically up- or down-regulate the transcription. We will need to broaden our understanding of economically important crops. We anticipate in the near future that cereals, and horticultural and floricultural crops will be improved in terms of their morphological and reproductive traits by molecular breeding based on GA metabolism and signaling.

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Ethylene: Current Status and Future Directions of Using Transgenic Techniques to Improve Flower Longevity of Ornamental Crops

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SUMMARY. Ethylene is a simple gaseous hormone that is involved in many aspects of plant growth and development. Two physiological responses that are commonly controlled by ethylene, fruit ripening and flower senescence, have been given the most significant attention in terms of crop improvement using the tools of modern plant molecular biology and biotechnology. Fortunately, the wealth of research on ethylene biosynthesis and signaling pathway has been providing tools for the

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genetic control of ethylene effects. Today, manipulation of ethylene biosynthesis and signaling is one of the most promising approaches to improve crops through genetic engineering. In this review we will discuss current status and future directions of transgenic techniques to control ethylene effects with a primary focus on flower senescence. doi:10.1300/J411v18n01_05

KEYWORDS. Ethylene synthesis, ethylene signaling, flower senescence, genetic engineering

INTRODUCTION

The main purpose of flowers and fruits is to produce seeds for dispersing offspring, and many plants have evolved petals to attract pollinators for successful pollination. Once the flower is successfully pollinated and fertilized, petals have fulfilled their biological role. As a consequence, plants have developed many mechanisms to shed or senesce petals in order to decrease the costs associated with maintaining organs with such high metabolic costs. After pollination, flowers from many different plant species show visual changes in many different ways, often mediated by the plant hormone ethylene (van Doorn, 1997). For example, some flowers close their corolla and others change color or form. In addition to visual changes, floral scent emission has also been shown to change after pollination in petunia and snapdragon (Negre et al., 2003). However, the most dynamic changes following pollination may be petal senescence or abscission. In some species such as carnation, orchids and petunia, petals wilt within a day or two after pollination. In other species such as *Pelargonium* and *Digitalis*, petals abscise quickly in response to pollination (Stead, 1992; van Doorn and Stead, 1997; Clark et al., 1997). Flowers that show petal senescence and abscission are usually sensitive to ethylene and ethylene treatment can mimic pollination (van Doorn, 1997, 2002). Although pollination is a main trigger of flower senescence, petals on all flowers eventually senesce or abscise, whether pollination occurs or not. In ethylene-sensitive flowers such as carnation and petunia, ethylene synthesis occurs at low levels without pollination and petal senescence occurs at a certain

point after anthesis that appears to be somewhat dependent on environmental factors.

Commercial value of flowers is immediately decreased upon the first signs of flower senescence. Therefore, improvement of flower longevity has been one of the most important subjects in the ornamental plant industry. Since it is known that ethylene plays a critical role in flower senescence, much effort has been focused on ways to diminish the effect of ethylene and to improve flower longevity especially for cut flowers and potted plants. Several methods of chemical control have been utilized to inhibit both ethylene biosynthesis and perception. Chemicals such as aminoethoxyacetic acid (AOA) and aminoethoxyvinylglycine (AVG) significantly reduce ethylene production and delay petal senescence and abscission of many species (Abeles et al., 1992; Baker et al., 1977; Wang et al., 1977; Wang and Baker, 1979). However, the concern of potential toxic effects and lack of control of effects due to ethylene generated from outside sources halted their commercial use (Abeles et al., 1992). After Veen et al. (1978) discovered that STS ($AgSO_4$, silver thiosulfate) had an effective anti-ethylene activity and prevented flower senescence in cut carnations, STS has become widely used for blocking ethylene effects in the ornamental industry for several decades. STS is thought to bind ethylene receptors and thus inhibit perception of ethylene. Flowers treated with STS confer ethylene insensitivity and petal senescence and abscission are effectively prevented (Abeles et al., 1992; Mor et al., 1984; Veen et al., 1978; Reid et al., 1980). This activity as an ethylene action inhibitor is desirable for commercial use in ornamentals because it is effective in preventing effects of exogenous ethylene that may occur during storage and transportation. Unfortunately, STS is a heavy metal pollutant, and in recent years commercial use has become more restricted because of environmental concerns. Thus, much effort has been made to develop non-toxic alternatives to STS. For example, 1-methylcyclopropene (1-MCP) is a more recently developed chemical found to block ethylene perception (Serek et al., 1994) and has been commercialized and used effectively in many ornamentals (reviewed by Sisler and Serek, 2003).

In addition to the development of new chemicals, genetic engineering is a promising approach to improve flower longevity. The distinct role of ethylene in flower senescence and an accessibility to genes involved in ethylene biosynthesis and signaling have allowed researchers to control ethylene effects through genetic engineering. In this review, we will summarize the current status of transgenic techniques to im-

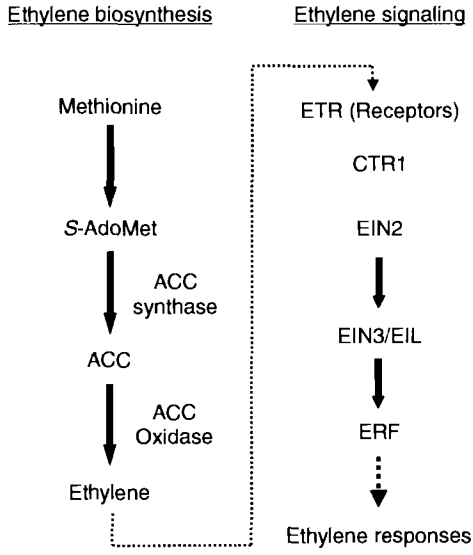
prove flower longevity and discuss issues associated with genetic control of ethylene effects.

OUTLINE OF ETHYLENE BIOSYNTHESIS AND SIGNALING PATHWAY

Ethylene Biosynthesis

The ethylene biosynthetic pathway has been established and genes for key enzymes have also been isolated (reviewed by Yang and Hoffman, 1984; Kende, 1993; Wang et al., 2002). Ethylene is synthesized from *S*-adenosyl-L-methionine (*S*-AdoMet) through a simple pathway in plants (Figure 1). In the pathway, *S*-AdoMet is converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase (ACS). During the formation of ACC, 5′-methylthioadenosine (MTA) is released from *S*-AdoMet and recycled to L-methionine through the Yang cycle to maintain methionine pools in plant tissue. ACC is then oxidized by ACC oxidase to form ethylene. The conversion of *S*-AdoMet to ACC by ACC synthase is generally thought to be the rate-limiting step in the

FIGURE 1. Outline of the ethylene biosynthesis and signaling pathway.



ethylene biosynthesis (Kende, 1993), but other experiments have also indicated that there can be regulation of ethylene synthesis at the level of ACC oxidase (Barry et al., 1996; Nakatsuka et al., 1998).

The genes for ACC synthase and ACC oxidase have been isolated from a number of different species. It has been demonstrated that both enzymes are encoded by multi-gene families and their members are spatially and temporally regulated by various endogenous cues and environmental stimuli (Fluhr and Matoo, 1996; Johnson and Ecker, 1998; Jones and Woodson, 1999a; Kende, 1993; Nakatsuka et al., 1998). In addition, biochemical evidence supports the possibility of post-translational regulation of ACS activity (reviewed by Wang et al., 2002). In climacteric fruits, which are defined by the concomitant increase in respiration and ethylene synthesis during ripening, pattern of ethylene synthesis changes dramatically. Immature fruits produce basal levels of ethylene and exogenous ethylene treatment does not stimulate further synthesis (System 1). In contrast, ethylene synthesis shifts to an autocatalytic mode in ripening fruit (System 2) (reviewed by Lelievre et al., 1997). Ethylene-sensitive flowers also show a similar pattern of ethylene synthesis during flower senescence (Woodson, 1994). The difference between the two systems can be explained by the differential expression of ACC synthase. In tomato, eight members of *ACS* gene family have been identified and their expression is differentially regulated (Barry et al., 2000; Nakatsuka et al., 1998). Barry et al. (2000) showed that System-1 ethylene is regulated by the expression of *LEACS1A* and *LEACS6*, whereas System-2 ethylene synthesis is initiated and maintained by ethylene-dependent induction of *LEACS2*. Climacteric fruits and flowers become more sensitive to ethylene as they mature (Jones, 2002; Liu et al., 1985; Woodson and Lawton, 1988). Exogenous ethylene treatment induces autocatalytic ethylene synthesis in mature fruits and flowers but not in immature fruits and flowers (Liu et al., 1985; Woodson and Lawton, 1988). These indicate that an increase in ethylene sensitivity is required for this shift. Unfortunately, the mechanism by which ethylene sensitivity is regulated is still unclear.

Ethylene Signal Transduction

Analysis of ethylene insensitive mutants in *Arabidopsis* has revealed components involved in ethylene signaling (Figure 1; reviewed by Kieber, 1997; Wang et al., 2002; Guo and Ecker, 2004). Ethylene is perceived by a family of membrane-localized receptors. ETR1, which encodes a histidine kinase with homology to bacterial two-component

regulators, was the first protein to be identified as an ethylene receptor (Chang et al., 1993). Multiple mutant alleles of *ETR1* have been identified and all result in dominant ethylene insensitivity. A total of five ethylene receptor genes have been cloned from *Arabidopsis*: ETR1, ETR2, ERS1, ERS2, and EIN4 (Hua et al., 1995; Hua et al., 1998; Sakai et al., 1998). Analyses of loss-of-function mutations in multiple receptors have shown that the receptors are negative regulators of ethylene responses (Hua and Meyerowitz, 1998). The receptors are believed to act through CTR1, which is homologous to the Raf family of serine/threonine kinases. Genetic evidence indicates that CTR1 is a negative regulator, as loss-of-function mutants show constitutive activation of ethylene signaling (Kieber et al., 1993; Huang et al., 2003). The next identified component is EIN2. EIN2 is a single copy gene and is the only gene known in which loss of function mutations result in complete loss of ethylene responsiveness (Roman et al., 1995; Chen and Bleecker, 1995). It has homology to Nramp metal transporters and is likely to be an integral membrane protein. Toward the end of the signaling pathway are the gene families for EIN3 and ERF1. EIN3 is a novel nucear-localized protein that belongs to a multigene family (Chao et al., 1997). Null mutations in *ein3* cause partial ethylene insensitivity, very likely because of functional redundancy of the family members. It has been shown that EIN3 binds to the promoter of ERF1 (Solano et al., 1998). ERF1 is a member of a large family of transcription factors. Overexpression of ERF1 in transgenic plants leads to a partial constitutive ethylene response, indicating that ERF1 is a positive transcription factor (Solano et al., 1998). Recently, it has been shown that EIN3 protein levels rapidly increase in response to ethylene and EIN3 protein quickly degrades in the absence of ethylene (Guo and Ecker, 2003). Furthermore, Yanagisawa et al. (2003) showed that ethylene enhanced the stability of EIN3. These results indicate that regulation of stability of EIN3 protein is the key step in the response to ethylene.

In recent years, several groups have isolated genes for ethylene signal components in several ornamental spices. For example, genes for ethylene receptors have been reported in *Pelargonium* (Dervinis et al., 2000), rose (Muller et al., 2000), carnation (Shibuya et al., 2002) and *Delphinium* (Kuroda et al., 2003). An *EIN2* homolog from petunia (Shibuya et al., 2004), and *EIN3* homologs from carnation (Waki et al., 2001; Iordachescu and Verlinden, 2003), petunia (Ciardi et al., 2003) and rose (Muller et al., 2003) have also been isolated. Analysis on these genes indicates that the ethylene signaling pathway is conserved in a variety of plant species.

Role of Ethylene in Flower Senescence

The involvement of ethylene in flower senescence of many plant species is clear because treatment of chemicals that inhibit ethylene biosynthesis or perception delays senescence. The knowledge of how ethylene controls flower senescence would help to develop a strategy for extending flower longevity through genetic engineering. Although the whole mechanism of ethylene-regulated senescence is far from being thoroughly understood, extensive research in orchids and carnation has revealed the role of ethylene as an inter-organ signal coordinating flower senescence.

Bui and O'Neill (1998) proposed a model for flower senescence induced by pollination in orchids based on detailed gene expression analysis for ethylene biosynthesis enzymes. After pollination, pollen-borne signals, including auxin, induce the expression of an *ACS* gene in the stigma, leading to ACC synthesis then ethylene production. This ethylene stimulates the autocatalytic production of ethylene in the stigma. ACC or ethylene itself is translocated from the stigma to the labellum and perianth, and the ethylene induces *ACS* and *ACO* mRNA accumulation, leading to the autocatalytic production of ethylene in the labellum. This ethylene is responsible for hyponasty and senescence of the labellum. In the perianth ACC is translocated from the stigma, ovary, or labellum and converted to ethylene, which triggers senescence. In the ovary pollen-borne auxin and unknown pollination factor(s) induce *ACS* gene expression, resulting in increased ACC synthesis. Based on the low level of ACC oxidase and ethylene production in the ovary, a limited amount of ACC is likely to be converted to ethylene, and this organ may also be a source of ACC for translocation to the perianth.

In carnation, ethylene has been also shown to be a primary signal for pollination-induced petal senescence (Jones and Woodson, 1997, 1999b). Pollination induces autocatalytic ethylene synthesis in the stigma/style and this stylar ethylene (and/or ACC) induces ethylene synthesis in the ovary. The ethylene produced by the styles or ovary acts as a diffusible signal and is perceived by the petals, leading to an increase in *ACS* and *ACO* expression, and subsequent autocatalytic ethylene production and rapid petal senescence. It has been suggested that the ethylene produced in the gynoecium (styles and ovaries) also triggers the onset of petal senescence in carnation during natural senescence. When petals were detached before the autocatalytic ethylene production had started, they exhibited a suppressed ethylene production and a prolonged petal life (Ten Have and Woltering, 1997). Furthermore, the careful removal of

gynoecium from carnation flowers inhibited the autocatalytic ethylene production in petals and delayed petal senescence (Shibuya et al., 2000). Subsequential regulation of ethylene responsible gene expression through flower organs also supports the role of ethylene as a mobile signal after pollination in petunia. *PhBSMT* genes, which are involved in flower scent production, are down-regulated by ethylene. The decrease of these transcripts occurred subsequently in the stigma/style, ovary and petal after pollination in wild-type petunia pants but not in ethylene-insensitive transgenic plants (Underwood et al., in 2005).

In addition to ethylene synthesis, it is known that ethylene sensitivity is regulated during flower senescence. For example, autocatalytic ethylene production is induced by ethylene treatment in mature carnation petals while the treatment does not induce in immature petals (Woodson and Lawton, 1988). Jones (2002) treated carnation floral organs at several developing stages with exogenous ethylene, and showed that these organs including styles, ovaries and petals became more sensitive to ethylene as flowers matured. In carnation, three members of carnation ethylene receptor gene family have been isolated (Shibuya et al., 2002). mRNA levels of two of these receptors decreased in the petal during natural senescence. It has been shown that ethylene receptors act as negative regulators of ethylene responses in *Arabidopsis* (Hua and Meyerowitz, 1998) and reduced expression of a receptor gene results in increased ethylene sensitivity in tomato (Tieman et al., 2000). The decrease in mRNA levels of ethylene receptors in carnation petals could potentially lead to increased sensitivity. However, it is not known whether the decrease in these genes is a result of a decrease in transcription or whether it is due to mRNA degradation. Recent research has indicated that regulation of abundance of EIN3 protein is a rate-limiting step in the ethylene signaling pathway in *Arabidopsis* (Guo and Ecker, 2003). To reveal the mechanism by which ethylene sensitivity is regulated during flower senescence, further analysis on signaling components at the protein level will be required.

Although the precise mechanism of flower senescence is still unknown, the critical role of ethylene in controlling flower senescence is clear. Since several genes that are responsible for ethylene biosynthesis and signaling in flowers are now available, it is possible to produce transgenic plants with prolonged flower longevity by manipulating expression of these genes.

CURRENT STATUS OF CONTROLLING ETHYLENE EFFECTS THROUGH GENETIC ENGINEERING

There are clear commercial advantages of controlling ethylene effects since ethylene plays a crucial role in fruit ripening and flower senescence in many important crops. To control ethylene effects, there are two basic approaches: one is suppression of ethylene biosynthesis and the other is suppression of ethylene perception or signaling. Several transgenic techniques have been usually attempted in *Arabidopsis* or tomato first and then applied to several other plant species (reviewed by Stearns and Glick, 2003). In ornamental crops, application of genetic engineering on ethylene has been reported only in a few species to date, including carnation, petunia and torenia (Table 1). However, the strategies developed in these species could apply to other ornamental crops.

SUPPRESSION OF ETHYLENE BIOSYNTHESIS

Two approaches have been shown to successfully reduce ethylene production through genetic modification in transgenic plants. One strategy is blocking the ethylene biosynthesis pathway by suppressing expression of genes that encode ACC synthase or ACC oxidase. Hamilton et al. (1990) expressed *pTOM13* (later identified as an ACC oxidase isoform by Hamilton et al., 1991) gene in the antisense orientation in tomato. In the resulting transgenic plants the level of *ACO* mRNA was significantly decreased. As a result of reduction of ACO expression, ethylene production in ripening fruit was decreased up to 87% compared to control and fruit ripening was significantly delayed. After this finding, this antisense strategy utilizing the *ACO* gene was applied to other species to reduce ethylene production, including carnation (Savin et al., 1995), cantaloupe (Auyb et al., 1996), broccoli (Henzi et al., 1999) and torenia (Aida et al., 1998). In transgenic carnation expressing an antisense carnation *ACO* gene driven by a constitutive promoter, ethylene production was reduced to less than 10% of non-transgenic control plants (Savin et al., 1995). These plants showed a significant increase in vase life up to nine days compared to non-transgenic plants where the vase life was around five days. It is also known that expression of a gene in sense orientation also co-suppresses the expression of the homologous target gene in plants. Aida et al. (1998) produced transgenic torenia expressing a torenia *ACO* gene in both sense and antisense orientations. Both types of transgenic plants showed a decreased ethyl-

TABLE 1. Examples of ornamental plants with suppressed ethylene biosynthesis and signaling.

Plant species	Gene construct	Reference
Suppression of ethylene biosynthesis		
Carnation (*Dianthus caryophyllus*)	Antisense *ACO* MAC promoter	Savin et al., 1995
Carnation (*Dianthus caryophyllus*)	Sense *ACO* 35S promoter	Kosugi et al., 2002
Torenia (*Torenia fournieri*)	Sense/Antisense *ACO* 35S promoter	Aida et al., 1998
Suppression of ethylene signaling		
Carnation (*Dianthus caryophyllus*)	*etr1-1* (*Arabidopsis*) 35S/FBP1 promoter	Bovy et al., 1999
Petunia (*Petunia hybrida*)	*etr1-1* (*Arabidopsis*) 35S promoter	Wilkinson et al., 1997
Petunia (*Petunia hybrida*)	*ers* (Brassica) 35S promoter	Shaw et al., 2002
Petunia (*Petunia hybrida*)	Sense/RNAi *EIN2* (*Petunia*) 35S promoter	Shibuya et al., 2004
Petunia (*Petunia hybrida*)	Sense *EIL2* (*Petunia*) 35S promoter	Ciardi et al., 2003

ene production and increased flower longevity with a greater increase in longevity of the co-suppression ACO plants than with the antisense plants. Co-suppression of the *ACO* gene also increased flower longevity in carnation by four days compared to controls (Figure 2; Kosugi et al., 2002).

ACS is a gene encoding ACC synthase that is thought to be the rate-limiting enzyme in ethylene biosynthesis. Oeller et al. (1991) showed that the antisense expression of the *ACS* gene reduces 99.5% of ethylene production in tomato plants. Control fruits kept in air began to produce ethylene 48 to 50 days after pollination, then fully ripen by 60 days, while the transgenic fruits did not show the ethylene and respira-

FIGURE 2. A transgenic carnation line expressing an *ACO* co-suppression construct under the control of the CaMV35S promoter. Flowers were removed at the full-opening stage from a non-transgenic (left) and the transgenic (right) plant and placed in water for eight days. Pictures were provided by Y. Kosugi.

tory burst, and fruits never turned red and soft (Oeller et al., 1991). Although expression of sense or antisense *ACS* gene has successfully reduced ethylene production in some species, this strategy has not been applied to many horticultural or ornamental crops. This could be attributed to the several characteristics of *ACS* genes: ACS is encoded by a multi-gene family, and its expression is regulated in a tissue- and stage-specific manner and is differently regulated by several stimuli including ethylene itself. These characteristics of *ACS* genes seem to make a transgenic strategy difficult, thus *ACO* genes have been often used to suppress ethylene biosynthesis.

A second approach used to reduce ethylene production is through engineering the degradation of its biochemical precursors using genes not in the direct pathway of ethylene synthesis. Klee et al. (1991) introduced a gene for ACC deaminase isolated from a soil-borne bacterium, *Pseudomonas* sp. strain 6G5, into tomato plants. Some soil bacteria use ACC as a sole nitrogen source through metabolizing ACC to α-ketobutyric acid by ACC deaminase. In the resulting transgenic tomato plants,

ethylene production was reduced corresponding to ACC deaminase expression levels. The level of ethylene was reduced by 90% in leaves and fruits, and fruit ripening was significantly delayed. However, this strategy has not applied for ornamental plats to date.

Manipulation of the ethylene biosynthetic pathway is an efficient way to reduce ethylene production thus to delay fruit ripening and flower senescence. An important feature of these transgenic plants is that they still have the ability to respond to ethylene. The transgenic tomato fruits expressing an antisense *ACO* gene or an ACC deaminase gene ripen normally when treated with exogenous ethylene (Hamilton et al., 1990; Klee et al., 1991). Petal senescence of carnation with reduced *ACO* expression is induced by ethylene treatment although it does not induce ethylene synthesis in these transgenic plants (Kosugi et al., 2000). This ability to perceive and respond to exogenous ethylene could be desirable for transgenic crops such as tomato, banana and melon. These crops are required to ripen before consumption, allowing producers to control the timing of ripening by exogenous ethylene treatment. However, this normal ethylene sensitivity appears to be a disadvantage for ornamental crops such as carnation, because flower senescence may be induced by exogenous ethylene that is produced by surrounding non-transgenic flowers during storage and transportation.

SUPPRESSION OF ETHYLENE SIGNALING

As was the case with chemical approaches to reducing ethylene sensitivity, it has been shown that manipulation of genes involved in ethylene perception or signal transduction is an efficient way to control the effects of ethylene in horticultural and ornamental crops. Wilkinson et al. (1997) produced transgenic tomato and petunia expressing a dominant mutant *Arabidopsis* ethylene receptor, *etr1-1*, under the control of the constitutive Cauliflower Mosaic Virus (CaMV) 35S promoter. *etr1-1* has a mutation in the amino terminal sensor domain resulting in the failure of ethylene binding, thus leading to decreased ethylene sensitivity (Chang et al., 1993; Schaller and Bleeker, 1995). Transgenic tomato plants expressing *etr1-1* showed ethylene-insensitive phenotypes including delayed fruit ripening and delayed flower abscission. In the petunia plants with the *etr1-1* transgene, it has been shown that flower senescence is significantly delayed compared to non-transgenic plants. Corollas of pollinated flowers remained turgid and structurally intact for at least eight days after pollination, while non-transgenic flowers

wilted by three days. Furthermore, exogenous ethylene treatment did not induce petal senescence of transgenic flowers (Wilkinson et al., 1997). This strategy is particularly desirable for the purpose of controlling ethylene effects through genetic engineering because these findings suggested that introducing a single gene could confer ethylene insensitivity in a variety of heterologus plant species. For example, Bovy et al. (1999) showed that carnation transformed with the *Arabidopsis etr1-1* gene have a delayed flower senescence phenotype. Vase life of non-transgenic carnation flowers was eight days in average, while the *etr1-1* transgenic flowers lasted up to 24 days (three-fold compared to control). This increased flower longevity as a result of inhibition at the level of ethylene perception in these transgenic plants was greater compared to transgenic plants with inhibited ethylene biosynthesis (Bovy et al., 1999). Manipulation of ethylene signaling components, in addition to the ethylene receptor, has also been shown to be a viable method to confer ethylene insensitivity. Transgenic petunia plants with reduced expression of the *EIN2* gene by co-suppression or RNAi (RNA interference) displayed a significant delay in flower senescence equivalent to the *etr1-1* petunia (Shibuya et al., 2004). Flower longevity of these transgenic plants was increased up to five-fold after pollination and ethylene treatment compared to non-transgenic control (Figure 3). Suppression of *EIN3* expression also reduced ethylene sensitivity in transgenic petunia. In petunia, three members of the *EIN3* gene family (*PhEIL1*, *PhEIL2* and *PhEIL3*) have been isolated (Ciardi et al., 2002). Petunia plants transformed with the sense *PhEIL2* exhibited delayed flower senescence (Figure 3). However, flower longevity was not increased in these transgenic plants as effectively as in *etr1-1* or EIN2 transgenic plants. It has been shown that antisense tomato plants with reduced expression of a single *EIN3* gene do not show a significant change in ethylene responsiveness, but reduced expression of multiple *EIN3* genes is required to reduce ethylene sensitivity likely due to functional redundancy (Tieman et al., 2001). In the *PhEIN2* co-suppression lines, mRNA level of *PhEIL3*, which shows a high homology to *PhEIL2*, was also decreased (unpublished). However, it might need to suppress mRNA expression of all members of *EIN3* gene family to greatly increase flower longevity.

Together, suppression of ethylene perception or signaling has been shown to successfully prevent ethylene effects. In particular, expression of a mutant ethylene receptor gene such as the *Arabidopsis etr1-1* will be an effective approach to manipulate ethylene responses because the

FIGURE 3. Transgenic petunia flowers for ethylene signaling components. Petunia plants (*Petunia ×hybrida* cv. Mitchell Diploid) were transformed with Arabidopsis *etr1-1* over-expression (etr), *PhEIN2* RNAi (EIN2-r) and *PhEIL2* co-suppression (EIN3-s) constructs under the control of the CaMV35S promoter. Flowers were removed from non-transgenic plants (WT) and transgenic plants on the day before anthesis, treated with ethylene for 18hr and then incubated for three days.

same gene construct can be used to transform a variety of heterologus plant species.

ISSUES OF ETHYLENE-INSENSITIVE TRANSGENIC PLANTS AND FUTURE DIRECTIONS

Genetic engineering provides powerful techniques to control the effects of ethylene in many ways. However, transgenic plants conferring constitutive ethylene insensitivity showed physiological side effects that would limit their commercial use. For example, ethylene-insensitive petunia plants produced with the *35S::etr1-1* construct showed a significant reduction in adventitious root formation (Clark et al., 1999). This inhibition of adventitious root formation was observed in transgenic petunia with the *EIN2* RNAi construct (Shibuya et al., 2004). This characteristic would be a severe problem in ornamental crops propagated through vegetative cuttings. The *etr1-1* petunia plants have also been shown to have a decrease in pollen viability, seed weight and seed germination (Clevenger et al., 2004). Furthermore, the *etr1-1* and EIN2

transgenic petunia lines that showed the lowest levels of ethylene sensitivity exhibited a high percentage of premature death in the greenhouse, which is likely due to reduced tolerance against abiotic stresses (Shibuya et al., 2004). Over-expression of the *etr1-1* in tobacco also increased susceptibility to fungal pathogens infecting roots (Knoester et al., 1998). In these transgenic plants, a constitutive promoter such as the CaMV 35S promoter was used to express transgenes, resulting in changes in the ethylene response even in untargeted tissues. Since ethylene is involved in many aspects other than flower senescence or fruit ripening, it is not surprising that constitutive ethylene insensitivity causes negative side effects in transgenic plants.

Clearly the key to circumvent these undesirable side effects will be a temporal and spatial control of transgene expression. For that purpose, a promoter that expresses a transgene only in the target tissue at the correct time will be required. Several genes have been reported to have flower-specific expression. They include floral organ identity genes, and genes involved in pigment or sent production in flowers. Petunia *FBP1* (floral binding protein 1), for example, is a floral organ identity gene and is expressed exclusively in petals and stamens (Angenent et al., 1992). Bovy et al. (1999) used the petunia *FBP1* promoter to drive the *Arabidopsis etr1-1* gene in carnation and obtained transgenic plants with delayed flower senescence. They observed a reduction in the loss of transformants in tissue culture and the greenhouse in the *FBP1::etr1-1* lines compared to transgenic lines with *35S::etr1-1*. Unfortunately, an extensive investigation of horticultural performance characteristics was not reported in this work. Transgenic petunia plants expressing the *etr1-1* gene under the control of a promoter isolated from *APETALA3* (*AP3*), a floral organ identity gene of *Arabidopsis*, have also been produced. This promoter expressed a reporter gene (*GUS*) specifically in flower tissues in petunia, as is the case in *Arabidopsis*. However, these *AP3::etr1-1* transgenic plants did not show a significant delay in flower senescence (unpublished). Strong GUS expression was detected in young flower buds of the *AP3::GUS* transgenic petunia but the signals weakened as flowers developed. This would suggest that expression of the *etr1-1* gene in later stages of flower development will likely be required to delay flower senescence. These results confirm that the temporal control of trangene expression is important as well as the spatial control. Gan and Amasino (1995) isolated a highly senseceneспecific promoter, P_{SAG-12}, from the *SAG12* gene, which is one of the senescence associated genes of *Arabidopsis*. Expression of this gene is up-regulated in an age-specific manner and is minimally regulated by

environmental factors. Interestingly, transgenic petunia plants expressing *ipt*, a cytokinin biosynthesis gene, displayed delayed flower senescence and had decreased sensitivity to ethylene (Chang et al., 2003). This promoter may be effective in driving ethylene related genes such as *etr1-1* and *EIN2*, diminishing ethylene effects without negative side effects in non-senescing tissues.

There may be a simple alternative approach to obtain transgenic plants with decreased ethylene sensitivity and delayed floral senescence without undesirable phenotypes. Shibuya et al. (2004) produced approximately 150 transgenic lines containing a constitutive CaMV35S promoter to drive co-suppression of *EIN2* and an RNAi construct for *EIN2* in petunia. Some lines showed a significant delay in flower senescence but also exhibited inhibition of adventitious root formation and premature death as mentioned above. A few lines showed a significant delay in flower senescence, but did not exhibit premature death and also produced a significant number of adventitious roots on vegetative propagules. In the latter lines, the suppression of *EIN2* expression was not uniform in tissues even though transgene expression was driven by the constitutive CaMV 35S promoter, most likely due to a positional effect. This lack of uniform suppression of *EIN2* mRNA expression reults in different levels of ethylene sensitivity in different tissues. These findings suggest that through selection of appropriate transgenic events, it may be possible to identify plants that have the desired levels of ethylene insensitivity in target tissues while avoiding deleterious, unwanted effects of reduced ethylene sensitivity.

CONCLUSIONS

Ethylene plays a major role in controlling flower senescence in many commercially important crops. Manipulation of ethylene biosynthesis and signaling using transgenic techniques is an effective way to increase flower longevity. However, alternation of ethylene biosynthesis or sensitivity in untargeted tissues or stages causes physiological side effects that would limit their commercial use. Effective spatial and temporal promoters for expression of transgenes and screening of transgenic lines that have desired phenotypes without negative side effects would resolve the problem. Once the whole mechanism of flower senescence is thoroughly understood, the knowledge would provide a new strategy to improve flower longevity.

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The Economics of Horticultural Biotechnology

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SUMMARY. Technological change has driven economic progress in agriculture and will continue to play a crucial role in the 21st century. The latest wave of technological change in agriculture is based on the ability to specifically modify crop genetics through recombinant DNA techniques. Biotech crop varieties have been adopted on a wide scale in some agronomic crops, but horticultural crops face hurdles for commercialization. Market barriers are blocking the production of biotech horticultural crops that have already been developed. High costs for research, development and regulatory approval combined with the small acreages planted and the diversity of varieties limit the potential for profitable applications of biotechnology for many fruits and vegetables, tree fruits and nuts, and nursery and landscape crops. Like most technological changes in agriculture, the introduction of modern biotechnology has

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met with spirited political opposition from some quarters. Such opposition could discourage adoption of biotech products that are wanted by some consumers, can be profitable for growers and could reduce the environmental impacts of crop production. doi:10.1300/J411v18n01_06

KEYWORDS. Horticulture, transgenic, biotechnology, regulation, technological change, intellectual property rights

INTRODUCTION

Agriculture has been an important engine of economic development in the United States. The mainspring of economic progress in agriculture has been productivity improvements driven by technological change that is fueled by research and development (R&D). Since World War II, agricultural productivity has more than doubled in the United States, as in many other countries. These gains can be attributed to new biological, mechanical and chemical technologies, including improved genetic materials, machines, pesticides, fertilizers and agronomic knowledge. The current wave of technological progress, especially biotechnology, promises continued expansion of the tools available to farmers to maintain productivity and profitability.

Biotechnology has transformed the production systems of major field crops such as soybeans, corn, cotton and canola. Since the first large-scale introduction in 1996, the global area planted to transgenic crops grew to 167 million acres in just seven years (James, C., 2004). Over sixty percent of those acres were in the United States. In 2004, biotech varieties represented 85 percent of the soybeans, 76 percent of the cotton and 45 percent of the corn acreage grown in the United States National Agricultural Statistics Survey (NASS).

To date, the most successful biotech crop varieties have emphasized "input traits" associated with crop production, such as genetic resistance to herbicides and to insects, rather than "output traits" related to product quality. High adoption rates suggest that biotechnology has offered significant economic benefits to farmers. Such benefits come from increased yields, lower risks, reduced use of chemical pesticides, savings in management, labor and capital equipment, and gains from re-

duced tillage and other modified production practices (Kalaitzandonakes, 2003).

Economic studies confirm that the farmer benefits from biotech crops have been large. Gianessi et al. (2002) conducted 40 detailed U.S. case studies of biotech cultivars to examine their actual or potential impacts on pest management. They estimated that in 2001, eight biotech cultivars adopted by U.S. growers provided a net value of $1.5 billion to growers, reflecting increased crop values and cost savings. They further estimated that the 32 other case-study cultivars, which are in the development pipeline but not yet commercialized, would have generated an additional $1 billion in benefits if they had been adopted, bringing the total potential benefit in 2001 to $2.5 billion. Of this annual total, the lion's share was for herbicide-tolerant crops ($1.5 billion per year), followed by insect-resistant crops ($370 million per year). These estimates do not represent the total economic impact because the geographic analysis was limited in scope, and they do not include any benefits to the seed and biotech firms that produced the technology. Similarly large benefits have been confirmed in other studies (e.g., Alston et al., 2003; Falk-Zepeda et al., 2000; Marra et al., 2003; Trigo and Cap, 2003).

In addition to direct economic benefits, research also suggests that biotechnologies have yielded a number of health and environmental benefits. For instance, insect-resistant crops have been found to reduce the number and volume of synthetic insecticide applications, thereby reducing farm-worker exposure and negative impacts on non-target species (e.g., Gianessi et al., 2002; Traxler et al., 2003). Likewise, some herbicide-tolerant crops have been shown to lead to significant reductions in potential negative environmental impacts from herbicide use (Nelson and Bullock, 2003). Herbicide-tolerant crops have also been found to facilitate the adoption of minimum tillage practices which, in turn, lead to reduced soil erosion and improved water quality (Kalaitzandonakes and Suntornpithug, 2003). Farmers attach significant value to these various nonmarket benefits (e.g., Alston et al., 2003; Phaneuf et al., 2004) but they may be of even greater value to others, including farm neighbors, farm workers, and others far away.

While biotechnology has been adopted rapidly in certain field crops, it has had limited commercial success in horticultural crops. Even though the first biotech crop to reach the market was the Flavr Savr™ tomato, and sweet corn, potato, squash and papaya varieties engineered to resist insects and viruses have been approved for commercial use and marketed, papaya is the only horticultural crop for which transgenic varieties have achieved a significant market share (about 70 percent of the

Hawaiian crop shipped to the U.S. mainland is transgenic). A number of technical, regulatory, and market factors combine to create hurdles for the utilization of biotechnology in horticultural crops. In this paper we discuss the influence of such factors on horticultural biotechnology investment and commercialization and offer an assessment of the future of horticultural biotechnology. We also discuss how public policy could facilitate the use of biotechnology in horticultural crops.

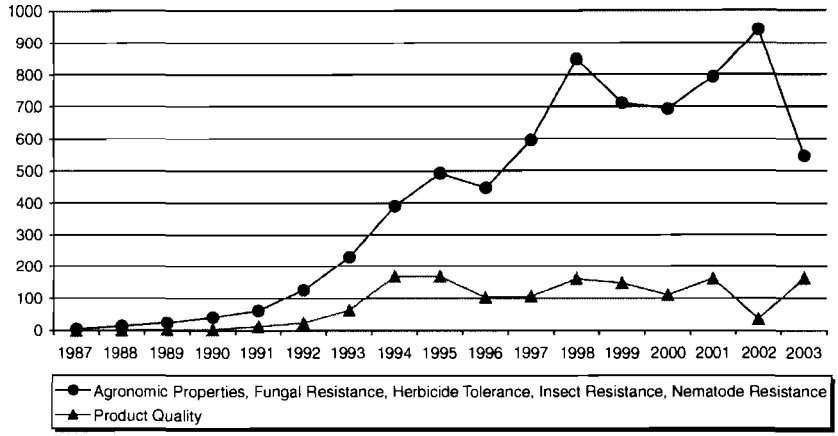
KEY INFLUENCES ON THE DEVELOPMENT OF CROP BIOTECHNOLOGIES

Technical and market factors explain the early focus of biotechnology R&D on herbicide-tolerant and insect-resistant field crops. Technologically, these first-generation agronomic traits were rather straightforward, as they required expression of only a single gene coding for one enzyme or toxin protein. Such single-gene events caused discrete and dramatic changes in phenotypes, and accordingly, they were relatively easy to further select and breed into commercial cultivars (McElroy, 1999). The $45 billion global crop protection industry provided a ready target market for these agronomic traits, and their rapid adoption has confirmed that the demand for alternatives to current pest control methods was strong.

Second generation enhanced quality traits are technically more complex. Quality traits are generally associated with many genes or gene complexes acting in concert (Mazur, 1999). To systematically advance such second generation crop biotechnologies, scientists have had to learn how to coordinate expression of several genes and target them to the proper tissues. For example, the development of rice grains with enhanced beta-carotene (vitamin A precursor) required the transfer of three genes and the targeting of their expression to the endosperm tissue of the seed (Ye et al., 2000). Similarly, engineering canola seeds to accumulate higher levels of beneficial omega-3 fatty acids required the transfer of three genes and their expression during seed development (Ursin, 2003). As a result of this greater technical difficulty, quality traits have lagged behind the development of agronomic traits, which account for the vast majority of biotech traits that have been field tested to date (Figure 1).

Other technical factors, beyond biotechnology, also contributed to the early application of first generation agronomic traits to major field crops rather than horticultural crops. Over the years, much greater re-

FIGURE 1. Field Trials by Phenotypic Category: Agronomic vs. Product Quality



Source: USDA APHIS

search attention and resources have been directed to the major field crops, so their genetics and physiology are better understood than for most horticultural crops. Experimentation with perennials such as grapes, nuts and fruit trees is also more difficult and more expensive because the experimental unit is larger and requires longer periods of time for evaluation, as some species require several years of growth before fruiting begins. Similarly, the diversity of propagation and marketing mechanisms also presents challenges, since many horticultural crops are vegetatively propagated from cuttings or grafting rather than by seed, or are perennial, bringing different issues for transformation, containment, and stewardship.

In addition to technical factors, market potential also drove the early focus of crop biotechnology R&D on agronomic traits for large acreage, pesticide-intensive crops such as corn, soybeans, cotton and canola. It is important to note that identifying, inserting and validating any genetic modification, as well as guiding a modified crop through the regulatory process is a lengthy process that typically lasts 7-10 years. R&D and regulatory costs are essentially the same across crops and traits. Such fixed costs must therefore be incurred by the innovator whether the technology could be adopted on one or a million acres, but the benefits are directly proportional to the number of acres on which the variety is adopted. This is why biotech companies have focused on developing

technologies for those traits that offer the largest value potential among the most widely planted field crops, especially feed-grain and fiber crops.

Consider, for instance, the economic opportunities for various first generation traits in the United States. The horticultural industry encompasses a diversity of fruit and vegetable crops, as well as many nonfood species, such as ornamentals, flowers and recreational turf grass. Table 1 illustrates that collectively horticultural crops compare well with major field crops in terms of total value–producing output roughly equal to that of row crops. In contrast, the total area planted to horticultural crops is small relative to field crops. For example, each year the U.S. corn acreage alone is almost seven times the acreage of all fruits and vegetables combined. As such, even the vegetable crops with the largest gross revenues, such as lettuce or tomatoes, are minor crops compared to major field crops in the eyes of biotechnology developers.

The comparatively minor consumption of agrochemicals by horticultural crops has similarly been a limiting factor. Table 2 shows the use of pesticides for major field and horticultural crops in 2003. Corn, soybeans, and cotton dominate in herbicide use, indicating the significant pressures for effective weed control in these crops. Similarly, corn and cotton dominate insecticide use. The large acreage and many pest pressures meant that field crops represented a significant economic opportunity for improved weed and insect control, and that is exactly where most R&D attention for herbicide tolerance and insect resistance was first directed. In contrast, most horticultural crops offer insufficient potential either because of small total acreage or significant fragmentation and diversity in pest control needs among varieties or regions where the crop is grown–a key issue for the larger acreage tree fruits (e.g., apples) and grapes. This difficulty in attracting commercial research and development of chemical pest controls for small-market crops has long been recognized and led to the establishment of the USDA IR-4 program, which supports research required to gain registration or special limited-use permits for agrichemicals for those crops (Holm and Kunkel, 2004). The small market potential for many horticultural crops is exacerbated by the abundance of niche varieties within crop species. While locally adapted varieties are also important for commodity field crops, horticultural markets are much more highly segmented by factors such as location, season, and consumer preferences, which drive the choice of variety. Diseases vary by location, so the types of resistant varieties required also vary. Diverse growing conditions and seasons require multiple adapted varieties to assure availability in the market every day

TABLE 1. Market Size of Various Crops in the US, 2003

	Value ($1000)	Area (1000 Ac)	World (1000 Ac)
Fruit and Tree nuts			
Almonds	1,419,432	550	4,282
Apples	1,729,949	396	12,995
Apricots	34,729	18	985
Avocados	390,868	67	940
Grapes, all types	2,534,264	951	18,570
Grapefruit	258,006	129	624
Kiwi	18,097	5	143
Oranges	1,611,812	792	9,056
Peaches	466,681	146	3,510
Strawberries	1,329,779	48	523
Total	12,876,732	4,059	139,298
Vegetable and Melons			
Artichokes	73,987	8	296
Asparagus	160,892	69	3,109
Bell Peppers	491,459	56	2,086
Carrots	632,469	95	2,548
Cauliflower	267,867	44	2,131
Garlic	147,745	37	2,820
Lettuce	2,089,461	312	2,455
Melons	812,228	275	11,695
Onions	958,032	166	7,437
Potatoes	2,679,902	1,273	46,675
Tomatoes	1,782,007	120	10,647
Dried Peas & Beans	497,392	2,010	83,097
Total	110,754,335	5,331	336,36
Field Crops			
Corn	24,803,566	78,736	352,432
Rice	1,485,031	3,022	379,200
Wheat	7,954,899	61,700	515,650
Soybeans	17,465,394	73,404	206,728
Cotton	3,777,132	13,479	79,455
Total	74,977,924	398,644	3,100,671*

Sources: USDA NASS 2004, FAOSTAT
*includes all oil crops, fiber crops, coarse grains

of the year. Introducing a trait into a horticultural species likely requires its introduction into multiple varieties to achieve market success.

In addition to the upfront R&D costs, the technology developer must also obtain regulatory approvals for a trait in a particular crop. It is difficult to obtain precise information on costs of regulatory approval

TABLE 2. Agrichemical Use and Application for Selected Crops in the United States–2003

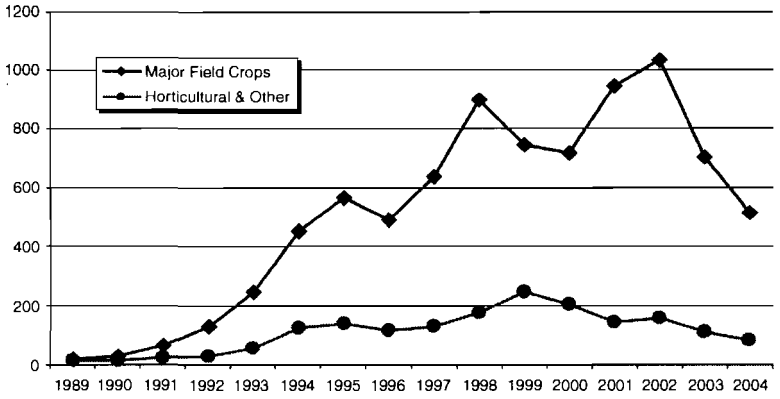
	Planted Acres	Herbicide		Insecticide		Fungicide		Other	
	1000 acres	*pct*	*1000 lbs*	*pct*	*1000 lbs*	*pct*	*1000 lbs*	*Pct*	*1000 lbs*
Field Crops									
Corn	72,770	95	149,136	29	7,465	0	232	0	
Barley	4,850	93	3,249	3	41	7	31	1	31
Fall Potatoes	1,024	91	1,577	84	1,571	91	6,538	47	75,386
Cotton	12,795	98	25,542	64	13,632	7	348	66	15,715
Soybean (2002)	71,670	99	86,742	6	1,077	0	108	0	
Spring Wheat (2002)	12,650	91	6,778	0		6	68	0	
Horticultural Crops									
Carrots	85	79	91	24	39	62	136	26	4,328
Lettuce	181	68	245	95	504	74	485	6	118
Watermelons	125	47	51	57	71	78	665	13	1,585
Tomatoes	97	58	45	85	252	86	1,995	52	10,192
Apples	338	52	472	95	9,100	85	4,423	65	166
Grapes	1,053	65	1,207	60	3,053	84	41,148	19	580

Source: USDA, NASS

for biotech crops and chemical pesticides, but according to available estimates the total cost of R&D–from "discovery" to commercial release of a single new pesticide or herbicide product–is $60-80 million, and regulatory approval alone can cost $6-8 million. Biotech varieties also face unique regulatory requirements that increase the cost of commercialization markedly relative to traditionally bred varieties (Redenbaugh and McHughen, 2004). A new technology must therefore generate enough revenue for the developer over its lifetime to cover these costs, and for some horticultural crops the total acreage is simply not sufficient.

Given the large fixed costs associated with developing and commercializing biotech crops, innovators have rationalized their R&D pipelines in recent years with almost 85 percent of all field trials being comprised of the eight dominant field crops (Figure 2). A majority of the remaining field trials are accounted for by crops such as tobacco and canola, leaving horticultural crops with relatively little, and shrinking attention. For example in 2003, corn varieties were tested with 356 field trials, while among the horticultural crops, creeping bentgrass and tomatoes represented the largest numbers of trials at 13 each and Kentucky bluegrass, papayas and petunias each had five field trials (USDA-APHIS).

FIGURE 2. Percent of U.S. Field Trials in Major Field and Horticulural Crops 1989-2003



Source: USDA APHIS
Major Crops Include: Corn, Cotton, Soybean, Wheat, Alfalfa, Potato, Beet, Rice, Tobacco, Canola, Barley

DEMAND FOR HORTICULTURAL BIOTECHNOLOGY

Demand factors have also influenced the development and commercialization of biotech traits in the horticultural industry. For some horticultural crops the per-acre use of agricultural chemicals can be even higher than in the field crops (Table 2). Market demands for blemish-free produce result in high crop protection costs for horticultural crops. The input traits that are valuable in field crops would therefore be equally desired by producers of horticultural crops. This was confirmed by the rapid initial adoption of insect- and virus-resistant potatoes, which greatly reduced insecticide applications. However, the refusal of large buyers, such as McDonalds and Simplot, to accept biotech potatoes eliminated markets and stalled adoption. In other cases, such as herbicide-tolerant lettuce, the industry itself blocked commercialization out of fear that their products would be targeted in the market place. Indeed, despite the high ranking of pesticide residues on foods in surveys of consumer concerns, little or no attempt has been made to market biotech horticultural products as having been produced with reduced pesticide use (James, 2004).

Sluggish demand for biotech traits targeting enhancements in processing characteristics has also been observed in the horticultural industry. Tomatoes engineered to have higher viscosity and therefore to be

more economical to process resulted in price reductions in canned tomato products that were initially welcomed by consumers in the United Kingdom (UK). However, as the controversy around biotech foods grew louder in UK and elsewhere in Europe in the late 1990s, large food retailers and food manufacturers voluntarily removed biotech foods from their shelves (Kalaitzandonakes and Bijman, 2003).

The examples above highlight the growing role of food processors and retailers in the adoption of horticultural biotechnology. Indeed, the relevance of the concerns of upstream channel participants is steadily increasing in parallel with their growing influence on the supply chain, through consolidation and market coordination. Perhaps most noticeably, consolidating supermarket chains have been taking over the market for fruits and vegetables, especially fresh produce, changing the way these products are marketed. Because fresh produce is perishable and subject to fluctuations in availability, quality and price, it presents special problems for supermarket managers compared with packaged goods. Supply-chain management, and the increasing use of contracts that specify production parameters, as well as product characteristics and price, is replacing spot markets for many fresh products. A desire for standardized products, regardless of where they are sourced around the world, could limit the development and adoption of products targeting smaller market segments, unless retailers perceive benefits and provide shelf space for diversified products. It therefore appears likely that consumer-oriented traits such as nutritional or flavor enhancement, extended freshness or other improved quality traits will be necessary in order to drive the introduction of biotech products into the horticultural marketplace.

Global trade is also a key influence on the demand for horticultural biotechnology. The seasonality and perishability of many horticultural crops make international trade necessary to maintain a constant supply of produce for consumers. For instance, although the United States is a net importer of fresh produce, it also exports a significant portion of its horticultural production volume (Cook, 2004). In 2001, U.S. fruit and vegetable production, concentrated mostly in the southern and Pacific coast states, totaled $25.7 billion. During 1999-2001, the United States (at $2.0 billion per year) ranked second in world fresh fruit exports. About 42 percent of US fresh fruits exports were directed to its NAFTA partners and 40 percent to East Asia (Japan, South Korea, Taiwan, China, and Hong Kong). Other markets of significance include the EU and countries in the Association of South-East Asian Nations. Over the

same period, the United States (at $1.2 billion per year), was similarly a top world exporter and importer of fresh vegetables.

The global sourcing of horticultural produce adds another dimension of complexity in the adoption of horticultural biotechnology. Differing regulatory policies across the globe have created an uncertain and complicated environment for the trade of biotech produce. The EU and Japan have instituted strict legislation governing the use and import of biotech crops, while the United States, Canada and others have adopted a policy of substantial equivalence. In this environment, approval of each biotech cultivar is required in each importing country, possibly with each country having different testing or labeling requirements. Segregating or channeling products for different markets is possible, but requires extensive (and expensive) changes in current production and distribution systems. Diverse national policies for labeling of biotech foods add further complexity to the marketing of horticultural biotechnology.

THE FUTURE OF HORTICULTURAL BIOTECHNOLOGY AND THE ROLE OF GOVERNMENT POLICY

Horticultural crops would benefit from the herbicide tolerance and insect resistance traits that have been successful in field crops, and would stand to benefit even more from development of genetic resistances to viral, fungal and bacterial diseases (Giannesi et al., 2002; Clark et al., 2004). The needs are arguably greater in the horticultural crops, since the options for chemical controls for weeds, insects and diseases are more limited due to the restricted markets they represent. In addition, as many perennial crops are vegetatively propagated, highly heterozygous and slow to reach sexual maturity, the ability to introduce a single advantageous trait into an elite cultivar without the necessity for extensive backcrossing would be extremely useful. In many cases, modifications could be made in rootstocks while leaving the scions that produce the marketable product essentially unchanged (Driver et al., 2004). Genetic enhancements that increase production yields, mitigate spoilage losses, improve ease of harvesting and reduce costs of processing would also be of significant value. Longer lasting flowers and novel ornamentals now in development would be attractive to the floriculture and landscape industries. Perennial crops including fruits and nuts would benefit from technologies that accelerate maturity of the plants by shortening the non-productive establishment period. Control of flowering to re-

duce the tendency for alternate bearing in some crops would help even out fluctuations in production. However, while proof of concept research has demonstrated the possibility for all these advances, this investment will go unrealized if the social, regulatory and economic environment maintains hurdles to their commercialization. The role that biotechnology will play in the horticultural industry, therefore, depends on coherent public policy reforms and on a receptive marketplace. Some key areas where public policy reforms could assist the development and commercialization of horticultural biotechnologies are highlighted below.

Intellectual Property Rights Policy

Without durable intellectual property rights the rate of innovation will slow, reflecting both underinvestment in research and under-adoption of research outcomes. This occurs as private investors cannot secure sufficient returns to their investments in certain types of research (such as developing new traits and crop varieties); as a result, their incentives to invest are reduced. On the other hand, when the rights to research results are protected, such as by patents or trade secrets, innovators can restrict access to the technology and charge monopoly prices. Intellectual property rights (IPRs) are a double-edged sword: to the extent that they provide a greater incentive for investing in research they could also result in lower adoption rates.

Governments have addressed the incentive problems in agricultural research in several ways. Federal and state governments (as well as industry) have funded agricultural research at public institutions such as the U.S. Department of Agriculture (USDA) and state agricultural experiment stations associated with land-grant universities. This approach allows an increase in total research without the problems associated with monopoly pricing of inventions. However, even though the investment has paid handsome dividends, it is increasingly difficult to sustain the past levels of funding for public agricultural R&D, in the face of general budget problems and declining political support for public science funding, including agricultural science (Alston, 2004).

Governments have also acted to strengthen IPRs applied to plants and animals as well as mechanical technologies. Changes in IPRs, especially in the 1980s, were crucial for the agricultural biotechnology development that followed. Partly as a reflection of enhanced IPRs, private-sector funding of agricultural research in the United States has been growing faster than public-sector funding and now exceeds it

(Pardey and Beintema, 2001). The balance in agricultural R&D between the private and public sectors varies among types of research. For instance, until recently the private sector emphasized agricultural R&D pertaining to mechanical and chemical technologies, especially pesticides, while the public sector was more important in other areas such as improving crop varieties. Private involvement was dominant in crop variety research in hybrid corn, sunflowers and vegetables, where the returns were well protected by the lower genetic quality of saved F2 seed from hybrid cultivars, trade secrets and other legal rights. Enhanced IPRs, combined with new scientific possibilities associated with modern biotechnology have stimulated private research investments in a time of waning support for public research. These factors together have resulted in a shift in the private-public balance in research to improve crop varieties. Accordingly, new attention must be paid to old questions about whether the private investment in crop research will be sufficient, whether the allocation of those resources (say, among crops) will be optimal, whether the results will be adopted rapidly and widely, and what role the government should play. For instance, the government could reform property-rights institutions to increase efficiency and reduce R&D costs. IPRs apply to research processes as well as products, and limited access to enabling technology or simply the high cost of identifying all of the relevant parties and negotiating with them, may be retarding some lines of research–a type of technological gridlock (Binenbaum et al., 2003; Graff et al., 2004). Nottenburg et al. (2002) suggest a government role in improving access to enabling technologies.

Public Involvement in Biotech

It may also be appropriate to increase support for public horticultural biotechnology research in some cases where there is a compelling public interest. This may be the case, for example, where a devastating disease threatens a horticultural industry and a biotech-based solution is the most viable option for developing resistant varieties. Another example might be the development of nutritionally enhanced food products. However, public institutions generally do not have access to the full range of enabling technologies and trait genes, nor the resources to satisfy the regulatory and stewardship requirements that are needed to develop a commercial biotech variety, making public-private partnerships an attractive avenue for development (Rausser and Ameden, 2004).

New licensing structures for enabling technologies developed in universities and public research institutions may be particularly helpful for

small-revenue crops (as well as for developing country applications) (Graff et al., 2004; Delmer et al., 2003). The Public-Sector Intellectual Property Resource for Agriculture (PIPRA) represents a significant development in this area (Delmer, 2004; www.pipra.org).

Regulation

Regulation and monitoring are needed to ensure that novel traits are assessed for both food and environmental safety prior to commercialization. However, such prudent precautions should not be so restrictive as to present insurmountable barriers to the commercialization of horticultural products that could provide significant benefits to producers and consumers as well as to the environment. Accordingly, the government could revise its regulations to increase efficiency and reduce costs for regulatory approvals. Instead of requiring a completely separate approval for each genetic transformation "event," it may be feasible to approve classes of technologies with more modest specific requirements for individual varieties. If a particular biotech trait was approved in a given species, it could be transformed directly into other elite cultivars of the same species without requiring a new regulatory package. Currently, a given approved insertion event must be transferred to other cultivars by backcrossing, a slow process that obviates much of the value of the biotech approach. In cases where backcrossing is impossible or impractical, as with long-lived or vegetatively propagated perennials, independent transformation of cultivars is the only option.

The issues faced by those attempting to obtain regulatory approval for biotech minor crops are virtually identical to those faced for small acreage chemical registrations. For this reason, the U.S. Department of Agriculture's IR-4 program that assists in the registration of agricultural chemicals for specialty crops could be broadened to support the registration of biotech varieties (Holm and Kunkel, 2004). It is logical that this program, or one like it, could be applied to assist in bringing biotech crops to market, particularly since many of them could ease the pressures on the program to maintain chemical registrations by engineering disease or insect resistance into the plants.

CONCLUDING COMMENTS

While the adoption of biotech field crops is spreading around the globe, biotech horticultural products are struggling to emerge into the marketplace. There is no shortage of targets and applications, many of

which have already been demonstrated or even marketed on a limited basis. However, it will be difficult for additional biotech traits that provide primarily grower benefits (input traits) to break into the horticultural market. Possible exceptions to this could be situations like that with papaya in Hawaii, where a disease or pest is so devastating that the entire industry is threatened and the only available solution is a biotech approach (Gonsalves, 2004). A similar situation could occur in California if the insect-vectored Pierce's disease spreads to its major grape-growing regions (CDFA, 2004).

While emergent needs might create opportunities for introduction of input traits in the production of certain horticultural crops, it is generally accepted that future horticultural biotech products will need to appeal to consumers to create demand and provide economic benefits to the processor and distributor segments of the supply chain as well to the producers. Nutritionally improved horticultural products that appeal to consumers could meet this need; however, most targets for nutritional improvement require metabolic engineering of multiple genes, which will require additional research to achieve. Testing requirements to obtain regulatory approval for nutritionally enhanced products will likely also be higher than for current products that do not substantially alter composition. Nonetheless, such consumer-oriented products may be required to open horticultural markets to biotech before benefits from input traits can be realized.

Horticultural crops represent gross market value roughly equivalent to that of the field crops, but are produced on less than 5 percent of the acreage. Thus, the potential for economic returns through sales of seeds or propagation materials is more limited for horticultural crops. Development costs are further increased and potential returns per cultivar are decreased by the need for multiple varieties adapted to diverse locations in order to provide season-long products. When these determinants of market potential are combined with high transaction costs for accessing IPR and for obtaining regulatory approvals, they paint a picture of an economic environment that is not favorable for private R&D investment in new horticultural biotech products. Public-sector pooling of enabling technologies and governmental support for programs to assist in meeting regulatory testing requirements, analogous to those supporting the registration of agrichemicals for minor crops, could lower these hurdles. Public institutions are continuing research in horticultural biotechnology, but will likely require private partnerships for commercialization of biotech cultivars. The lack of harmony in international regulations for sale and labeling of biotech food products could continue to restrict exports.

There are, however, some positive developments that could improve the environment for horticultural biotechnology. Public institutions and foundations are collaborating through PIPRA and other organizations to lower the intellectual property barriers for international agriculture and specialty crops (Delmer et al., 2003). The expanding adoption of biotech field crops is stimulating the establishment of uniform regulatory and biosafety protocols around the world, and the European Union is slowly beginning to relax its moratorium on approvals of biotech crops. Nutritionally enhanced "foods for health" are being developed in field crops, and, if accepted by consumers, these products could open the door for acceptance of similar products in horticultural commodities. A few ornamental biotech products are in the market, and additional ones may face lower hurdles for acceptance since they are not consumed as food. Thus, although the timeline for a significant economic impact of biotechnology on horticulture will likely be pushed back from earlier predictions, continued research, coupled with sensible public policies, will create products desired by consumers and an economic environment in which they can be supplied.

It is important to note here that our discussion in this paper has had a distinct U.S. perspective. This is, in part, due to the fact that until now the United States has led the world in the development and adoption of biotech products in agriculture. U.S. dominance may be expected to continue for some time to come in the development of biotech products, especially for field crops. In the case of horticulture, however, there are prospects for significant developments to occur outside the United States–China may take the lead in horticultural biotechnology. The Chinese economy, including its agriculture, is experiencing rapid growth and change. China has been rapidly expanding its horticultural industry and has a large and successful agricultural research system with rising strength in plant biotechnology. While China faces many of the same fundamental issues as in the United States, its large domestic market and rapidly expanding horticultural sector, combined with receptive attitudes to the development and adoption of biotech products, provide a comparatively favorable setting for horticultural biotech products. In an article titled, provocatively, "China aggressively pursuing horticulture and plant biotechnology," Huang and Rozelle discuss these various forces, and others, as they may condition China's role in leading the development of horticultural biotechnology products. Scientific and commercial progress in that country could result in spillover benefits to other countries (Huang and Rozelle, 2004) and could change the global environment for horticultural biotechnology.

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Challenges to Commercial Use of Transgenic Plants

Richard Meilan

SUMMARY. There is a well established and rigorous framework to regulate interstate movement and environmental release of transgenic plants. Three federal agencies, the U.S. Department of Agriculture, the Food and Drug Administration, and the Environmental Protection Agency, are involved in the regulatory process. To date, more than 10,000 field trials have been conducted and approximately 60 different crop-species combinations have been deployed commercially in the U.S. This includes a wide range of crops that have been genetically engineered for a variety of traits. The purpose of this article is to describe the process by which one obtains permission to release transgenic plants for both experimental field trials and for commercial purposes. doi:10.1300/J411v18n01_07

KEYWORDS. Bioconfinement, flowering control, genetic engineering, regulatory affairs, stewardship

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REGULATORY OVERSIGHT

The United States Department of Agriculture (USDA), the Environmental Protection Agency (EPA), and the Food and Drug Administration (FDA) are the three Federal agencies responsible for regulating the testing, movement, and commercial deployment of transgenic plants and their products. Oversight for these agencies was outlined by the Office of Science and Technology Policy in 1986 through the Coordinated Framework for Regulation of Biotechnology. The USDA, via the Animal and Plant Health Inspection Service, Biotechnology Regulatory Services (APHIS, BRS), has the authority to regulate interstate movement and environmental release of all transgenic plants by virtue of the Plant Protection Act. The EPA, through the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), regulates the planting and food and feed use of transgenic plants into which genetic material has been inserted that imparts a pesticidal property (plant-incorporated protectant, PIP). In addition to registering the use of a PIP, the EPA also establishes tolerances, or exemptions from the requirement of a tolerance, through the Federal Food, Drug, and Cosmetic Act (FFDCA). The EPA is also responsible for registering the agrochemicals used on engineered crops. Finally, the FDA is granted authority under the FFDCA to regulate transgenic food and feed crops or products from transgenic crops that may come in contact with food. A description of their monitoring protocol is provided in their Statement of Policy: Foods Derived from New Plant Varieties (1992). Thus, depending on the trait for which a crop has been engineered, more than one Federal agency may be involved in its regulation. More details on the biotechnology regulatory process and its history can be found on the Agricultural Biotechnology page at the APHIS website (http://www.aphis.usda.gov/brs/index.html). Below is a brief description of the requirements for each Federal agency involved in regulating transgenic plants. Due to space constraints and their overarching authority, the role played by APHIS is emphasized.

Regulatory Oversight by APHIS

Currently, two mechanisms are available for the importation, interstate movement, and release (field-testing) of genetically engineered (GE) plants. Initially (between June 1986 and April 1993), transgenic materials could be moved or tested only under a "permit." The permit application review process was lengthy (minimum of 180 days) because an Environmental Assessment (EA) was needed for each application. In

May 1997, APHIS introduced its "notification" system for a wide range of plant species, which streamlined the process (i.e., a 10-day review for interstate movement notifications and a 30-day review for notifications requesting environmental release) for organisms and traits with which APHIS had familiarity in managing risk. Transgenic plants requiring more careful scrutiny are still subject to the permitting process (e.g., those plants containing genes: (1) derived from a human pathogen, (2) encoding the production of a pharmaceutical, or (3) whose function is unknown). During their review, applications (for permits and notifications) are forwarded to the appropriate regulatory authority within the affected state(s), which then approves the request, denies it (with justification), or imposes requirements more stringent than those specified by APHIS.

Detailed instructions for filing a notification can be found on the following page of the APHIS website: http://www.aphis.usda.gov/brs/usergd.html. Briefly, a notification must contain the following information:

- *Responsible party* (the applicant, who is legally liable for noncompliance);
- *Duration of introduction* (notifications are usually valid for only 12 months, though extensions can be requested; longer periods are allowed, but annual status updates are required);
- *Recipient organisms* (scientific names of species transformed; limited to a single species);
- *List of regulated articles* (including line designation(s); description of gene inserted; expected phenotype; designation of genetic construct(s) used to transform plant(s); and genetic components that went into assembling construct(s), such as promoters, coding sequences, terminators, and selectable markers);
- *Mode of transformation* (e.g., *Agrobacterium tumefaciens*);
- *Introduction* (where the transgenic material will originate, as well as its final destination); and
- *Certification* [statement in which the responsible party certifies that the regulated article will be handled in accordance with the relevant Performance Standards (see below) and regulations set forth in the coordinated framework (7 CFR 340.3)].

If permission to move or release transgenic materials is granted, a letter of acknowledgement is sent to the applicant. In that letter, APHIS

specifies the responsible party must comply with a list of Performance Standards, which include the following sections:

- *Shipping and maintenance at destination* (to prevent loss or environmental release of the regulated material);
- *Identification scheme* (to prevent accidental mixing of regulated and non-regulated materials);
- *Termination procedures* (including steps that will be taken to "devitalize" the plant material);
- *Monitoring* (to ensure devitalization was successful and to prevent regulated material from persisting in the environment); and
- *Training* (to ensure that these standards are clearly communicated and adhered to by all employees who have access to the regulated material).

Before transgenic plants can be grown for commercial purposes, a petition for non-regulated status is submitted to APHIS. This is done only after extensive evaluation that typically involves several years of field tests. Instructions for submitting a petition can be found at the APHIS website (http://www.aphis.usda.gov/brs/petguide.html). These include requirements for both a complete molecular characterization of the transgenic plants and data on potential environmental impacts. If the petition is approved, the transgenic plant is no longer regulated (i.e., unrestricted distribution and planting is granted (although APHIS could impose restrictions, if needed). Since the Coordinated Framework was established in 1986, more than 10,000 field trials have been conducted in the U.S.; APHIS has granted non-regulated status to approximately 60 different crop-species combinations (a complete listing can be found on the APHIS website). To date, no detrimental effects have been reported. Although 14 tree species have been field-tested, with the exception of papaya, non-regulated status has not yet been granted for any of them.

Function of EPA

The EPA is involved in regulating GE plants when they have been engineered to contain a PIP that imparts resistance to a specific pest. That evaluation considers the extent to which the introduced PIP is toxic to the target pest as well as the potential for unintended exposures through the expression of PIPs in GE plants. The EPA also assesses the potential for targeted pest populations to acquire resistance to the PIP as

a result of its widespread use. All petitions involving plants containing a Bt (*Bacillus thuringiensis*) gene must include a resistance management plan that details steps that will be taken to minimize the risk of insects becoming resistant. EPA's role in regulating GE plants also encompasses: (1) establishing tolerance, or exemptions from the requirement of a tolerance, for the PIP; (2) registering herbicides to which crops are engineered to be tolerant, if the product was not previously registered for use with that species; and (3) assessing the risks associated with new exposures to herbicide residues.

Role of FDA

The FDA regulates human food and animal feed derived from all plant varieties, and holds products obtained from GE plants to the same rigorous safety standards required of all other foods. These efforts can include collecting data on allergenicity, digestibility, and toxicology of the protein encoded by the transgene. Information on safety and regulatory issues related to GE plants can be viewed at the FDA website (http://www.cfsan.fda.gov/~lrd/biotechm.html).

Other Considerations

While many other countries are developing their own regulations, U.S. agencies are, to the extent possible, trying to harmonize their regulatory framework with rules being promulgated elsewhere. The existing U.S. structure is also being revised. To aid in the formulation of new regulations for herbaceous perennials, APHIS hosted a public hearing in Baltimore, MD, during January 2003, at which they solicited input from experts in various related fields, representatives from industry and non-governmental organizations (NGOs), and the general public. A similar meeting was held in Washington, DC, in June 2003 to obtain input regarding regulations for woody perennials. In January 2004, APHIS announced its intention to prepare an Environmental Impact Statement (EIS) in which the agency will evaluate its biotechnology regulations and consider several possible changes (http://www.usda.gov/Newsroom/0033.04.html). These modifications are likely to include the development of a multi-tiered system to replace the current permit and notification system, along with enhancements to the deregulation process to provide for greater flexibility in approving products as well as long-term monitoring. Any changes to the regulations are expected to

be science- and risk-based, but they are also likely to be influenced by consumer issues, trade considerations, and politics.

Finally, while working toward commercialization of engineered products, potential petitioners should be mindful that:

- Early consultation with the appropriate regulatory agencies can save a lot of time and needless actions and delays.
- A petition for non-regulated status can include more than one independent line (transformation event).
- Species-gene combinations are evaluated on a case-by-case basis.
- With the appropriate permission, it is possible to use safety data submitted by previous petitioners.
- Gene flow is not considered a risk *a priori*; it must be evaluated to determine whether a perceived risk is genuine.
- Under rare circumstances, it may be possible to commercialize a product derived from a transgenic plant without deregulation by APHIS. However, the developer must still abide by permit and notification requirements and other regulations specified under 7 CFR, section 340. In addition, if the inserted gene encodes a PIP or the plant has any potential food or feed use, the EPA and FDA still remain involved. In general, this alternative approach is unwise and leaves the developer open to significant liability and financial risk.
- Pharmaceutical-producing plants might always be regulated in some way (i.e., they may never be fully deregulated by USDA).

TRANSGENE CONFINEMENT

The National Research Council recently released a report entitled: "Biological Confinement of Genetically Engineered Organisms" (http://www.nap.edu). The committee that drafted the report was asked to address the following questions:

- What is the status of various bioconfinement strategies?
- What methods are available and how feasible, effective, and costly are they?
- How can bioconfinement failures be mitigated?
- What methods are available for detecting failures?
- What are the probable ecological consequences of large-scale bioconfinement?

- What new information is needed for addressing any of the above questions?

The report's major conclusions are listed below.

- The effectiveness of a confinement method will vary depending on the organism, environment, and the scale over which it is applied.
- To evaluate efficacy, the genetically engineered organism (GEO) should be compared with its progenitor before release. For trees, it may be necessary to begin field tests before such comparisons are possible.
- Various confinement methods should be tested separately and in combination, in a variety of appropriate environments, and in representative organisms.
- The need for bioconfinement should be considered early in the development of a GEO.
- Non-food crops should be utilized for genes encoding products that need to be kept out of the food supply.
- An integrated confinement system should be based on risk.
- The stringency of confinement should reflect the consequences of GEO escape.
- It is unlikely that any single confinement method will be 100% effective.
- The use of redundant methods will increase the likelihood of accomplishing the desired confinement level.
- Government regulators should consider the potential effects a U.S. confinement failure could have on other nations, and vice versa.
- International cooperation should be sought for managing GEO confinement.
- Social and ethical values should be considered when assessing the stringency needed for confinement.
- Transparency and public participation are needed to develop and implement the most appropriate bioconfinement approach.

The need for bioconfinement in several major crop classes is discussed below. Because the public comment period for a petition to deregulate herbicide-tolerant creeping bentgrass (*Agrostis stolonifera*) has recently ended, and because there is considerable familiarity with this case, that species will serve as a model for describing work done with an herbaceous perennial. Likewise, considering that poplar (*Populus*)

has been used in more transgenic research than any other tree species, it will be highlighted in the section on woody perennials.

Herbaceous Annuals

In general, herbaceous annual crops are heavily domesticated and have virtually no wild relatives with which they are inter-fertile. In addition, the products harvested (e.g., seed, fruit, pollen) are generally derived from the flowers. Thus, there is neither a need nor a desire to prevent flowering in these species.

Herbaceous Perennials

Other than cotton, which is intentionally grown as an annual, no transgenic herbaceous perennial crops have yet been commercialized. However, a number of transgenic perennial grasses are being developed at both public and private institutions. These grasses have been engineered for tolerances to herbicides, drought, disease, and cold; enhanced quality (e.g., digestibility, nutritional content); reduced stature; and lignin content. Traits are currently being investigated for creeping bentgrass, bluegrass and fescues, as well as in alfalfa, another herbaceous perennial. As of this writing, petitions requesting the deregulation of two products, glyphosate-tolerant creeping bentgrass and glyphosate-tolerant alfalfa, have been submitted to the USDA. The *CP4 EPSPS* gene, which confers tolerance to glyphosate (the active ingredient in the herbicide Roundup®), has been inserted and expressed in those plants. This gene is identical to that utilized in other crops, such as canola, corn, cotton, and soybean, which have been planted on tens of millions of acres since the commercialization of Roundup Ready® soy in 1995.

Creeping Bentgrass

Research on glyphosate-tolerant creeping bentgrass (GTB) has been conducted since the mid-1990s. This product offers the opportunity to apply glyphosate directly on crops grown either for their seed or as sod and turf, without causing altered performance and growth, or environmental adaptations. Weeds, such as annual bluegrass and rough stalk bluegrass, are extremely difficult to manage within an existing sward of bentgrass. Although mechanical or physical control measures exist, they can be very destructive, especially on a golf course where aesthet-

ics and continuous play are desirable. Furthermore, selective herbicides for such weedy grasses are not available.

In seed-production operations, great care is taken to establish pure stands of bentgrass by first applying pre-emergent herbicides. However, on golf courses weed presence is usually tolerated, so that both unwanted and desired species must be contended with simultaneously, often requiring greater use of pesticides, water, labor, etc. However, if growers were able to control those weeds during the initial production phase, seed purity would increase and golf course superintendents would need to manage only for the creeping bentgrass. Removing the annual bluegrass or rough stalk bluegrass would also eliminate the diseases and insects to which those species are specifically susceptible, which in turn, would significantly reduce reliance on pesticides and potential chemical exposure by farm workers, golfers, area residents, local wildlife, and the environment in general.

Because GTB is not engineered to produce its own pesticides, the USDA is the principal regulatory authority for its oversight. Therefore, prior to commercialization, its developers must petition that agency to deregulate the product so it can be freely transported and planted. In addition, application must be made to the EPA to extend the current registration of specific glyphosate formulations to include their application on GTB. The FDA must also be consulted because creeping bentgrass straw is used as an animal feed.

APHIS has acquired substantial expertise and knowledge about a number of widely planted crops, including cotton, corn, canola, and soybean. However, the specific data required from a petitioner seeking deregulation are based on the plant species transformed and its expressed trait(s). For example, the inserted DNA must be fully characterized and the biology and life history of the plant clearly documented. This information has been agreed upon by USDA-APHIS, the EPA, the Canadian Food Inspection Agency (CFIA), and Health Canada, in a series of meetings beginning in 1998. Such criteria are listed at the USDA website and should be consulted by applicants when determining the studies needed for achieving regulatory clearance for a transgenic crop. By tailoring their requirements to the specific plant and trait under consideration, the agencies can perform a thorough and appropriate EA.

Environmental Assessment

To assess the environmental and non-target safety of GTB, transgenic and non-transgenic creeping bentgrasses have been examined at all

stages of their life cycles to determine if their potential for becoming a plant pest has been affected in any way, as is done with annual plants. These studies compared seed and vegetative establishment, plant growth, flowering, pollen viability and longevity, fecundity, seed germination, seedling vigor, insect resistance, disease susceptibility, and various botanical characteristics. University researchers, along with scientists from The Scotts Company and Monsanto, performed more than 90 individual experiments between 1999 and 2003 at 65 field locations. Those sites represent the northern (or cool), southern (or warm), and transition-zone climates to which turf grasses are adapted. The experimental environments comprise both managed and unmanaged ecosystems, with extreme variations in light, moisture, soil type, nutrition, competition, and temperature. Studies were also conducted to assess the possibility for intra- and inter-specific and inter-generic out-crossing, and the relative fitness of putative hybrids. Finally, herbicide trials were performed to determine the efficacy of glyphosate alternatives for controlling both GTB and hybrids.

Based on the results from these studies, the petitioners have been determined that GTB and its progeny are substantially equivalent to non-transgenic creeping bentgrass.

Stewardship

Seed Production

As part of their usual risk assessment, the USDA, FDA, and EPA do not require a comprehensive stewardship plan. However, stewardship has become an important component of transgenic crop management and should be considered by the developer well in advance of commercialization. Such a plan should address, to the extent possible, that the transgenic plant: (1) remains in the country(s) for which regulatory approval has been obtained, (2) is kept in the environment where first planted, and (3) is used as intended. Thus, while gathering data to support the deregulation of GTB, it has been important to consider the appropriate stewardship practices for all users of the product (i.e., seed and sod producers and golf course managers).

To meet the current demand for creeping bentgrass seed used on golf courses in the U.S., approximately 7,000 acres are farmed annually; about 98% of this acreage is in the Willamette Valley of Oregon. However, to satisfy the anticipated demand by sod farms and golf courses for GTB seed, considerably less acreage, perhaps no more than 3,000 acres,

probably would be needed. This amount of seed could be grown by less than two dozen contract growers, each of whom could be sufficiently isolated geographically (if required) to prevent out-crossing to or from conventional seed farms.

The Scotts Company and Monsanto have worked with growers in Jefferson County, Oregon, and the Oregon Department of Agriculture (ODA) to establish an 11,000-acre Control Area within which only bentgrasses developed through biotechnology would be grown. This Area was approved by the ODA after two public hearings in June 2002, and is located approximately 100 miles east of the Willamette Valley. These two farming regions are also separated by the Cascade Mountain Range. Because the considerable distance and physical barriers will block gene flow to conventional bentgrass fields in Oregon, the risk is managed to help avoid unintentionally exporting GTB to countries in which import approval has not been obtained.

Golf Courses and Sod Production

Creeping bentgrass is a low-stature, fine-textured, soft, dense, carpet-like turf grass that tolerates low mowing. Therefore, frequent watering, optimum fertilization, and disease and soil management practices are needed to prevent competition from other grass or broad-leafed weed species. Even under optimal nutrition and watering regimes, this crop is susceptible to a wide range of diseases, including pink snow mold, brown patch, and dollar spot. Because of its intense cultural requirements, creeping bentgrass is not suitable for planting by homeowners. It is instead used for putting greens, tees and fairways, lawn bowling greens, grass tennis courts, and other specialized applications. Therefore, The Scotts Company and Monsanto intend to market GTB exclusively for the golf-course market through seed and sod production, rather than for residential, industrial, or other recreational applications.

To grow GTB, both sod farms and golf courses will obtain licenses that specify good stewardship management practices. For example, managers on sod farms maintain their growing turf at the same height as its final intended use, between 1/8 to 3/4 inches. Under those conditions, creeping bentgrass is unlikely to flower, pollinate, or set mature seed (Lush, 1988). Therefore, appropriate stewardship will be focused on: (1) implementing precautionary measures that minimize potential seed and stolon scatter or movement via equipment to other courses or farms; (2) maintaining GTB turf at heights that preclude pollination, flowering, and development of mature seed heads; (3) devitalizing stolons and

aerification cores that could enable unintentional vegetative propagation; and (4) controlling GTB volunteers through herbicide applications and monitoring, either after a golf course is renovated or abandoned or when a sod farm has been harvested. These practices should significantly reduce the potential for undesired establishment, reproductive growth, and gene flow to wild relatives. Moreover, mechanical measures and a number of herbicides other than glyphosate are available for removing unintended GTB growth and eliminating environmental risk. However, just as a pesticide applicator takes precautions to ensure an herbicide does not drift to non-target plants, responsible stewardship will mean maintaining transgenic turf grass only where it is intended.

In summary, the regulatory assessment of herbaceous perennials is similar to that of annuals. While collecting research data, it is important to consider the unique characteristics of the transformed plant and the expressed trait. Communication with the appropriate regulatory agencies and academic and industry stakeholders is strongly recommended as early as possible in the product-development process so that the pertinent environmental, non-target, human safety, and stewardship concerns are addressed. Doing so will facilitate the review process and potentially reduce the time spent gaining approval. Websites for U.S. regulators as well as the countries intended for export or environmental release should be consulted for further information; these sites are updated as regulatory requirements change.

Woody Perennials

Although numerous transgenic crops are currently being grown for commercial purposes (see: http://www.aphis.usda.gov/brs/), papaya represents the only commercial deployment of a transgenic woody perennial. Its release resulted from a special effort to save an entire industry from the ravages of ubiquitous ringspot virus in Hawaii (Gonsalves, 1998). This unique case has involved virtually no environmental risk because papaya originally had been introduced to Hawaii (meaning there are no inter-fertile wild relatives) and because the Pacific Ocean is an effective physical barrier to transgene escape.

As of now, all other transgenic tree species in the U.S are being grown only for research purposes, for three reasons: (1) existing regulations were written with the perspective of agronomic row crops, which are highly domesticated and have few, if any, wild relatives; (2) these plant systems have ecological issues that are different from annual row crops; and (3) biotechnological techniques have been slower to develop

for trees (mainly the latter). Even though APHIS has never stated that transgene escape will not be allowed, it has made clear that efforts must be taken to mitigate the risk of transgene spread from those species, at least during the early stages of development. Several strategies can be employed to achieve transgene confinement.

With the current state of technology, it is impossible to guarantee absolute sterility for any species. However, from a purely scientific or even a risk-reduction perspective, complete sterility may not always be needed before transgenic trees can or should be grown commercially. This is especially true for riparian species, such as poplar, that can be grown under fertigation in xeric environments, such as east of the Cascade Mountains in the Pacific Northwest. Native poplars are largely absent from this landscape. Given their reproductive isolation and the half-life of pollen under very dry conditions, there may be no justification for preventing the establishment of even fully fertile transgenic trees in that region. Triploid genotypes of poplar are available that have greatly reduced innate fertility; transgenics produced in these clones might safely be grown in areas where no inter-fertile wild relatives are present.

The need for sterility depends on the trait being exploited; the environment within which the transgenics will be grown; the particular species; and various social, political, and ethical considerations. Each case must be analyzed individually. In some situations, the introduced gene imparts a competitive disadvantage; transgenic trees growing outside an intensively managed plantation would be unlikely to survive. Nevertheless, because of the uncertainty, many research groups around the world are exploring techniques to genetically engineer flowering control. For example, one common procedure is to ablate (eliminate) cells by expressing a deleterious gene in a tissue-specific fashion (Mariani et al., 1990). A second engineering method employs dominant negative mutations (DNMs). DNM genes encode mutant proteins that suppress the activity of co-existing wild-type proteins (Espeseth et al., 1993). A third technique involves gene silencing. Recent studies in a variety of eukaryotic organisms have shown that double-stranded RNA (dsRNA) is an inducer of homology-dependent gene silencing; the use of dsRNA to induce silencing is termed RNA interference (RNAi) (Hannon, 2002).

Aside from transgene confinement, reproductive sterility may be engineered for other reasons. First, rapid (juvenile) rates of growth can be maintained. During the early phase of its life, a plant utilizes all of its photosynthate for vegetative growth; after it undergoes the transition to maturity, some carbohydrate is metabolized for reproductive structures.

This diversion of energy causes a reduction in growth rates, particularly in trees (Eis et al., 1965; Tappeiner, 1969). Therefore, the earlier the flowering process is interrupted, the greater the potential benefit.

One major handicap for scientists working in the area of flowering control in trees is the long juvenile period. Understanding the genes involved in the regulation of floral development will not only expedite sterility research, but also potentially speed the progress to be gained through conventional breeding, by accelerating the onset of flowering. Moreover, delayed flowering is itself a possible confinement strategy, especially when trees are grown under short-rotation intensive culture. Thus, a great deal of effort is being made to identify genes that regulate the transition from vegetative to reproductive phase in a tree's life cycle.

Addressing the Concerns of NGOs

Several NGOs are opposed to the commercial deployment of genetically engineered plants, including trees. One of their primary concerns is that the spread of particular transgenes might increase the modified plants' competitiveness and, therefore, their invasiveness. Another issue is that farmers might lose their ability to use existing pest-control measures. For example, organic fruit and vegetable growers can now exogenously apply purified Bt toxin (which, in fact, is produced commercially using genetic engineering tools), and still maintain their "organic" rating. It is feared that through widespread use of Bt toxins, insect populations will develop resistance to that management tool, rendering it ineffective. Finally, there are people who are philosophically opposed to the idea of genetic engineering (e.g., it's "wrong" or "unnatural").

Any developments that arise from genetic engineering must be viewed in the proper context (in contrast to current practices). For example, companies now plant a single genotype (i.e., clone) of hybrid poplar on thousands of contiguous acres of land. Because those non-transgenic trees have been vegetatively propagated, all of their cells, including their pollen, contain exactly the same DNA. When this synchronous population starts flowering, it produces a monotypic pollen supply. The resulting pollen cloud can be carried considerable distances by the wind and can ultimately affect the genetic diversity of inter-fertile wild trees. Engineering flowering control alone (not coupled with any other trait) would prevent this gene flow from occurring. Under circumstances where it is important to protect sensitive genetic stocks (not just for

trees), engineered flowering control alone may be desirable. A second example pertains to herbicide usage. Some growers currently use environmentally detrimental compounds to control competing vegetation. To obviate this need, we have already engineered trees to tolerate an herbicide that is not volatile, carcinogenic, teratogenic, or mutagenic. In addition, this less injurious compound is soil-inactivated and biodegradable. Having such herbicide-tolerant trees not only can encourage the use of a more "environmentally friendly" product, but also reduce the overall reliance on agrochemicals.

Many public concerns surround the ecological ramifications of complete sterility. For example, insects or mammals may rely on pollen or seed as a food source. However, this argument is not so applicable to a species such as poplar, which is wind-pollinated and does not produce nectar or support a large number of insect or vertebrate pollinators. Furthermore, poplar seeds are very small, virtually devoid of endosperm, and have a short life span. Thus, they are not considered to provide a significant source of food for wildlife. However, for species whose pollen is a valuable food supply, it may be necessary to engineer those plants to produce pollen that, although nutritionally unaltered, is infertile. The intention is not to supplant wild trees with genetically engineered sterile versions; the goal is to plant trees that have been domesticated through genetic engineering into intensively managed plantations, on previously disturbed sites, to satisfy society's increasing demand for renewable resources. This paradigm will help protect native and old-growth forest from being harvested.

On the other hand, trees that never produce any pollen could be beneficial under certain circumstances. Planting pollen-less stock in some urban settings would reduce the pollen loads from species that are highly allergenic to a large proportion of the human population [e.g., *Cryptomeria japonica* and birch (*Betula*)]. It may also be desirable, from the perspective of a homeowner, to engineer street trees that are incapable of producing nuisance reproductive structures [e.g., sweetgum (*Liquidambar styraciflua*) and *Gingko biloba*]. This conundrum highlights the need to consider each crop-trait combination on a case-by-case basis.

To answer one criticism stated earlier about transgenic crops, several steps can be taken to minimize the risk of insects becoming resistant to Bt toxins. As mentioned previously, the EPA requires that petitions involving plants containing a Bt gene must incorporate a resistance management plan, which details the process to be followed in reducing the possibility of such a resistance developing to the PIP. These plans often

include the establishment of refugia, which are adjacent areas on which only non-transgenic plants of the same species are grown. Because insects cannot become resistant unless they are exposed to the toxin; the greater the exposure, the greater the chance of resistance development. Therefore, insects feeding on non-transgenic plants will remain susceptible to the toxin and mate with those that may become resistant by feeding on transgenic plants, thus maintaining a susceptible population.

Another strategy is to engineer plants with two Bt toxin genes, each of which being effective against the target insect via a different mechanism. The likelihood that insects will become resistant to both toxins is the product of the individual probabilities of them becoming resistant to each toxin. Furthermore, the non-target insects are much more likely to be exposed to exogenously applied Bt toxin (as is practiced by organic growers) than to a toxin produced inside plant cells (i.e., the insect actually has to eat some of the transgenic plant in order to become exposed). Again, we must put this issue in the proper context; the alternative to a PIP is the use of broad-spectrum insecticides that kill all insects, both targeted and beneficial.

Finally, there are great risks associated with doing nothing. With the recent increase in international trade agreements but a simultaneous lack of funding for Federal agencies charged with enforcing import laws, the potential for environmental catastrophes has risen tremendously. Given this, circumstances can occur under which it would be desirable to spread a transgene into the wild in order to contain a newly introduced environmental threat. For example, the Emerald Ash Borer (EAB, *Agrilus planipennis* Fairmaire) is an aggressive exotic pest recently arrived from Asia that is attacking and killing all the North American ash (*Fraxinus* spp.) trees it invades. First identified in Michigan in 2002, the EAB has since been detected in 13 counties in Michigan, two counties in Ohio, and one in Maryland (from transported nursery stock), as well as in Windsor, Ontario (Haack et al., 2002). To prevent the spread of the EAB, these areas are under quarantine to restrict the transportation of ash trees, branches, nursery stock, logs, and firewood. This pest is fatal to all trees it attacks, no means are available for its control, and evidence suggests that, unchecked, the EAB will spread throughout North America. Currently, in efforts to prevent its continued migration, when an infestation is found, all ash trees within a half-mile radius are fallen and incinerated. However, if nothing else is done to eradicate this highly destructive pest, American ash species may go the way of American chestnut and elm. Genetic engineering may be the only effective tool for managing the threat of EAB.

THE NEED FOR FIELD TRIALS

Transgenic plants should be field-tested for several reasons. The first is proof of concept–it must be shown that the inserted gene is having the expected effect. *Agrobacterium tumefaciens* is widely used to insert genes into plant cells, but these insertions occur at random locations within the genome. The DNA surrounding the site of insertion influences the efficiency with which the transgene is expressed (i.e., "positional effects"). In addition, transgenes can be differentially expressed under various conditions (e.g., Callahan et al., 2000). Thus, it is prerequisite to screen independent lines (plants derived from cells that have undergone separate gene insertion events) to verify that the transgene is being expressed at commercially useful levels. It may also be necessary to conduct field trials to determine if there is sufficient value so that end-users are willing to pay a biotechnology premium (licensing fee) for use of a genetically modified plant. Finally, long-term field studies are needed to detect somaclonal variation and assess the stability of transgene expression (Meilan et al., 2002, 2004).

INTELLECTUAL PROPERTY CONSIDERATIONS

Other than directly isolating and cloning a gene of interest, it is virtually impossible to obtain genetic material for plant transformation without signing a Material Transfer Agreement (MTA). Standard agreements are now widely used, and usually specify that biological material (constructs, bacteria, plants) containing the genetic material covered by the agreement cannot be shared with a third party without the expressed written permission of the provider. University researchers who use transgenic plants only for research purposes may be able to obtain an exemption from paying license fees. However, even those scientists are well advised to keep intellectual property (IP) issues in mind when configuring their transformation vectors, especially if there is any chance that the resulting transgenic plants could enter a commercial stream.

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Risk, Trust, and Consumer Acceptance of Plant Biotechnology: Implications for Genetically Modified Ornamental Plants

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SUMMARY. This discussion summarizes current knowledge about rewards, risks, and reality surrounding the public's perception of genetically modified (GM) plants and foods. Differences in perception and acceptance of GM products between European and US publics are briefly described. The discussion includes recommendations for effective communication with the public. Pitfalls are identified that restrict credible discourse about the risks and benefits of GM ornamental plants and biotechnological products.

The U.S. gardening public is increasingly informed about technologies that influence their hobby interests, including plant biotechnology. Results of a preliminary and on-going survey of Tennessee Master Gardener Volunteers are presented that reveal consumer concerns similar to ones already voiced in the U.S. about GM foods. Discussion concludes

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by integrating these considerations, within the context of ongoing debate about GM foods, to provide implications about the success of novel GM ornamental plant introductions in the near future. doi:10.1300/J411v18n01_08

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INTRODUCTION

To any real extent, contemporary discourse has failed to consider public perception and concern about genetically engineered (GE) ornamental plants in dialogue about acceptable plant biotechnology. Aesthetic applications like ornamental plants have largely been overshadowed by the current debate surrounding genetically modified (GM) foods. Compared with this GM commodity, proponents of plant biotechnology expect little public opposition to genetically engineered ornamental plants. But almost all GM products have raised ethical and moral issues among public and environmental interest groups. Perception and acceptance of plant biotechnology, in general, has varied across geographic borders and along demographic lines.

Today, the greatest acceptance of GM foods occurs in the U.S. and Canada (Cantley et al., 1999; Hoban, 1999; Lusk and Sullivan, 2002; Rousu et al., 2003; Gaskell, et al., 1999, 2004; Lapan and Moschini, 2004; Noussair et al., 2004). But even in the U.S., despite regulatory approval and the fact that no adverse human health effects have been linked with GM foods (Braun, 2002; Stewart, 2004), the public expresses significant concerns. Vocal opponents are twice as numerous as proponents (Gaskell et al., 1999; US-FDA, 2000; Lapan and Moschini, 2001).

In 1991, Lacy and colleagues reported that the public was becoming more aware about biotechnology in agriculture. Yet by 2000, genetically modified (GM) foods still had not entered public awareness in U.S. mainstream society. In fact, U.S. focus group participants did not express concerns of safety about GM products, but rather "outrage" that GM foods had entered the U.S. food supply without their awareness (US-FDA, 2000). In Europe, repeated Eurobarometer surveys document the erosion of public support for biotechnology and GM foods

(Braun, 2002). Growing public awareness and concern centers on environmental impacts, food safety, socio-economic effects and moral issues about biotechnology (Hoban et al., 1992; Boulter, 1997; Uzogara, 2000; Beckwith et al., 2003; Gaskell et al., 2004).

Our lack of understanding about consumer perception and acceptance for GM ornamental plants represents a critical knowledge gap. Until consumer research is conducted to evaluate consumer perception of ornamental genetically modified organisms (GMOs), we must draw parallels and make inferences from literature that has explored public perceptions of safety, risk, and acceptance about GM foods. This existing research helps frame specific questions and concerns about GM ornamental plants. What does our history with GM foods suggest about the likely success of GM ornamental commodity introductions? If we keep the public ignorant about regulatory procedures and scientific oversight, do we expect the public to be any less concerned about the health of their children playing on GM turf or climbing in GM trees? Is there a function for genetically engineered ornamental plants that will promote greater acceptance of biotechnology and GM foods? What responsibility and means do scientists have to integrate this technology with the economic interests of the Green Industry, a growing segment of the U.S. agricultural market? Finally, what outcome should we expect if introductory efforts of GM ornamental plants fail?

A FOUNDATION FOR UNDERSTANDING: PUBLICLY DEBATED PROS AND CONS OF BIOTECHNOLOGY

World population growth projections are severe: estimates exceed 7.6 billion people by 2020 (UN, 1996). To meet anticipated food demands of 2025, average cereal crop yields will need to be 80 percent higher than the average cereal yield in 1990 (Borlaug, 1997). To meet this demand, biotechnology, including plant genetic engineering, is expected to be instrumental. Norman Borlaug, the Father of the Green Revolution, joined proponents of contemporary biotechnology to advocate the synthesis of biotechnological tools with conventional plant breeding. The Consultative Group on International Agricultural Research (CGIAR) supports Borlaug's assertion of critical worldwide need, stating that "every instrument of agricultural transformation should be mobilized in [efforts] to feed the hungry, help the poor, and protect the environment" (Serageldin, 2000).

Beyond crop yield, GM advocates cite other benefits including improved post-harvest stability, higher crop productivity, decreased reliance on chemical pesticides, reduced pesticide costs and environmental pollution, new crop varieties, and more nutritious foods to relieve dietary deficiencies in developing countries (Uzogara, 2000; Huffman, 2003; Gaskell, et al., 2004; Stewart, 2004). Proponents of plant genetic engineering acknowledge that GMOs have more predictable gene expression than plants produced through conventional breeding methods (Sharma et al., 2002; Stewart, 2004). In turn, agricultural biotechnology advocates frequently promote outreach to describe the similarities of biotechnological processes with conventional plant breeding. Yet, 25 percent of a polled cross-section of U.S. citizens felt it was morally wrong to change plants using even classical breeding techniques (OTA, 1987). Still, among the public majority who widely regard plant breeding as safe, products of conventional plant hybridizations are acceptable because crosses are generally constrained by natural reproductive barriers and biological sexual compatibility (Sharma et al., 2002).

Opponents of plant biotechnology, including international environmental non-governmental organizations, argue beyond increases in yield and production efficiency. Concerns they express extend from environmental and genetic drift to the creation of new viruses and toxins (see review, Uzogara, 2000). Other studies suggest that opposition to GM products stems from irrational fears and trade protectionism (Uzogara, 2000). Specifically for GM foods, fears include potential toxicity and allergenicity, altered nutritional quality, and transfer of antibiotic resistance from GM products (Uzogara, 2000). Critics also cite shifts in power relationships and economic dependencies among world citizens, change in practical and cultural relationships to land and nature, increased agricultural commercialization, and patent and intellectual restriction of genetic knowledge that gives unequal trade advantages to agribusiness. Meanwhile, traditional forms of farming could be displaced in developing and industrialized nations. Widespread adoption of GM crops is also argued to promote a loss of plant genetic diversity (Schibeci et al., 1997; Uzogara, 2000; Huffman, 2003; Murray, 2003; Wu, 2004).

Ethical issues are also publicly stated concerns about GMOs (Boulter, 1997; Beckwith et al., 2003). Among Public Perception of Agricultural Biotechnologies in Europe (PABE) project participants, for example, biotechnology processes evoked ethical concerns that nature has been pushed beyond its limits (Marris, 2001). Uzogara (2000) describes plant biotechnology as "creating species" in which plant metabolism is tai-

lored to achieve a desired objective. Statements like this seem to reinforce the viewpoint of GMO opponents who cite objections to scientific and industrial intent to "play God," risking environmental stability by disrupting the delicate balance of nature (Woodard and Underwood, 1997).

For consumers who have a familiarity and awareness of genetic engineering, it may be too late for outreach efforts to alter their views. Strength of prior attitudes about technology limits the ability of new information to affect acceptance, even when coming from credible sources. In a study of consumer perceptions about GM beer and yoghurt, prior attitudes accounted for 90 to 95 percent of perceived benefits, and 86 to 90 percent of perceived risks (Frewer et al., 1999).

Still, public perception is complex and dynamic: viewpoints shift as products become more familiar. As public knowledge about new technologies increases, positions can be reinforced as opinions are found that support a given view (Rogers, 1983). Consumers also update personal assessments of risk when presented with new information (Smith et al., 1990), adjusting perception to reflect new media attention and new product introductions (Boulter, 1997; Smith et al., 1990; Hoban and Kendall, 1993). Unsurprisingly, public understanding and interpretation of risk about genetic engineering and biotechnology will be critical to widespread acceptance and use of GMOs.

UNDERSTANDING PUBLIC PERCEPTION OF RISKS AND BENEFITS FROM GMOS

Efforts to understand consumer willingness to accept risk and risk-averse behavior are confounded by how risk is defined by researchers and perceived by the public. For example, college students were less concerned about "natural" risks including drought, floods, and earthquakes than human-generated risks like biotechnology (McDaniels et al., 1995). Discussion about risk is further challenged by the need to accurately interpret consumer *perception* of risk versus scientific assessments of *actuarial risk*, *technical risk*, or *sound-science*-based risk assessments, particularly when associated with GM product introductions (Sandman, 1987; Hoban et al., 1992; Boulter, 1997; Gaskell et al., 2004).

In general, the word *risk* as applied in the scientific sense of a probability of negative consequence, does not factor into public concern about GMOs. In regions where opposition to GMOs is high and the

topic figures prominently, people instead talk about moral difficulties, ethical issues, *dread* fears, and unknowable dangers (Slovic et al., 1985; Gaskell et al., 1999, 2004).

In Service to the "Public Good"...?

Claims of benefit from GM crops include higher productivity, lower pesticide costs for producers, less environmental pollution from pesticides, and new crop varieties that will alleviate famine in developing countries (Gaskell et al., 2004). Yet, the public does not appear to agree the benefits that are promoted, particularly by industry, are the same incentives that stimulate commercialization of GM products (Gaskell et al., 2004). Nor do farmers (Gillespie and Buttell, 1989) and citizens in developing countries expect to receive the same benefits that agribusiness will accrue from the availability and acceptance of GMOs (Boulter, 1997; Gaskell et al., 2004). In fact, farmers producing some GM crops (e.g., *Bt* corn) potentially incur net losses when pest pressures are too low for yield improvements to cover fixed technology fees (Carpenter, 2001). Technology fees for *Bt* corn alone return about $128 million to agribusiness and its shareholders, while the public only gains about $1.90 per person per year in reduced corn costs (Wu, 2004). Wu (2004) also modeled environmental and health benefits associated with GM-*Bt* corn, in terms of reduced pesticide use and mycotoxin production, finding only modest public benefit at about $0.02 per *Bt* corn acre.

What Influences Public Acceptance of GMOs More, Perception of Risk or Lack of Evident Benefit?

Awareness about biotechnology is increasing in the U.S. About 40 percent of a randomly sampled pool of consumers correctly believed that "foods produced through biotechnology" were already available in grocery stores (Hoban, 1999). Still, despite almost 15 years of commercialization, genetically engineered foods widely remain in the "unknown" category of risks perceived by the U.S. public (Peterson, 2000).

Marris (2001) identified the need to clarify myths surrounding public understanding and acceptance about GMOs. The myths, which were defined as factors "so evident" that they seem to need no further substantiation, included the need to educate the public about science to influence GM acceptance, and the belief that product benefits are the primary consideration driving public acceptance of GM foods.

Moon and Balasubramanian (2004) suggest that public attitudes about agrobiotechnology are more influenced by perception of risks than perceived benefits. In fact, the lack of any readily apparent GM product benefits may constitute the greatest challenge to consumer acceptance, regardless of the perceived level of risk (Gaskell et al., 2004; Moon and Balasubramanian, 2004). Lusk (2003) suggests that price premiums for GM foods may be earned if potential health benefits are promoted to consumers. Others have argued that acceptance of biotechnology, particularly outside the U.S., is probably influenced by multiple factors acting in concert (Slovic et al., 2002; Rowe, 2004). For example, how desirable the benefit or outcome of an action is expected to be influences the perception of risk associated with that action. An action expected to produce a desired benefit will be viewed as less risky than an action yielding an unattractive or uninteresting outcome (Slovic et al., 2002).

Still, acceptance of GM foods differs if consumers are classified as "skeptical" or "relaxed" about new technologies, or if they perceive a trade-off in both benefits and risks. In the latest Eurobarometer survey, 60% of respondents who were skeptical about technology believed that GM foods provided no benefits and carried risk. As GM food benefits were perceived to increase by respondents, the possible "risk to future generations" became a more important consideration for accepting GM foods (Gaskell et al., 2004).

If Consumers Perceive Risks from GMOs, Do They Subsequently Reject GM Foods?

Despite scientific evidence supporting the safety of GM crop production and food consumption, agribusiness and the Green Industry face considerable market risk if consumers reject GM products (Wu, 2004). In the case of food for infants, for example, no industry has yet been willing to integrate GM crop commodities into baby food products (Uzogara, 2000).

In 2002, with famine threatening the health of more than 14 million people, government leaders in the sub-Saharan African nations of Zambia, Zimbabwe and Mozambique rejected U.S. food donations that contained GM corn (Cauvin, 2002). A widespread premise held that decisions to reject food relief were based on fears of environmental and health risks. Instead, these views were confounded by concerns of Third World leaders that national commodity exports would subsequently be rejected in European markets and that European Union (EU) donor or-

ganizations would refuse to provide further financial aid (Cauvin, 2002; Wu, 2004). The conflicting viewpoints are partially explained because corn exports to the EU represented a small proportion of U.S. production: about 5 percent annually in the 1990s (Wu, 2004).

In France, 79% of respondents agreed or mostly agreed that "GMOs should simply be banned" and 89% were opposed to the presence of GMOs in human food products (Noussair et al., 2004). Similar results were demonstrated in the UK (Moon and Balasubramanian, 2004). Yet, a French willingness-to-pay survey challenged expectations that consumers would really reject GM products. Only 35 percent of polled participants were unwilling, in practice, to purchase food products (biscuits) made with a non-specified type of GMO. About 65 percent of the respondents were either indifferent to GMOs, willing to purchase inexpensive GM products or valued the presence of GMOs in their products. Within the respondent group, nearly 25 percent were unwilling to pay less for their biscuit choice, even after they were told it contained GMOs (Noussair et al., 2004).

Consumer Acceptance of GM Foods: Content and Quantity Matters

The proportion of GMO content in a food product has been shown to influence consumer acceptance. Biscuits with ingredients stated to contain either less than 1.0 percent or 0.1 percent GMOs were more preferred than GMO biscuits with an unspecified amount of genetically modified material (Noussair et al., 2004). In a contrasting U.S. willingness-to-pay study, bids were not significantly different when GM products contained from 1 to 5 percent (the highest presented proportion) GMOs (Rousu et al., 2003). Acting on this principle, Korea, Thailand, Japan, and Brazil have independently established acceptable levels of GMO food "impurity" that range from 3 to 5 percent above which foods must carry a GM label (Rousu et al., 2003).

In the U.S. and Canada, acceptance for GM foods is generally greater than in Europe and Asia (Cantley et al., 1999; Hoban, 1999; Lusk and Sullivan, 2002; Rousu et al., 2003; Gaskell et al., 1999, 2004; Lapan and Moschini, 2004; Noussair et al., 2004). In an auction-bid study, students at Kansas State University were presented a bag of genetically modified corn chips and allowed to bid for the chance to exchange the food for GM-free chips. Only 30% of the students paid a price premium for the GM-free chips, averaging 7 cents per ounce. Only 20% were willing to pay at least $0.20 per ounce. Yet, it is relevant to note that

individual actions differ when hypothetical situations (e.g., independently-reported willingness-to-pay) are compared with a real commitment to purchase (Neill et al., 1994; List and Shogren, 1998).

Certain Demographic Variables Help Predict Consumer Acceptance of GMOs

In a sociological context, it is relevant that the same generation that is currently entering retirement and embracing gardening as a hobby (ANLA, 2000) also grew up watching the genre of radiated, mutant monster movies that followed World War II. These low-budget films were prevalent Saturday matinee features between 1945 and 1965. These movies exacerbated public fear of atomic radiation (Boyer, 1985) and helped establish many stereotypical images by which the public views science and scientists (Haynes, 1994; Boulter, 1997).

While many demographic measures are imperfect predictors of public stance on GMOs and biotechnology, others are more reliable (Lusk and Sullivan, 2002). Demographically women, middle-to-older age groups, and people with limited education are most likely to oppose biotechnology. Women voice greater opposition than men to genetically engineered foods, specifically citing concern about environmental effects and food safety concerns (Burton et al., 1991; Hoban et al., 1992; Florkowski et al., 1999). GMO acceptance generally increases with increased income (Heiman et al., 2000). The influence of education is less clear, with greater acceptance (Heiman et al., 2000; Bak, 2001) or less acceptance (Martin and Tait, 1992; Gaskell et al., 1998; Marris, 2001) observed as levels of education increase. Moreover, self-acknowledged familiarity with GM technology may confound interpretation of survey results. In the Midwestern U.S., participants who claimed to be aware of GM technology were most likely to reject GM foods. This suggests that their initial sources of information presented primarily negative perspectives (Huffman, 2003).

In survey results from 1987, the Office of Technology Assessment also reported that people who described themselves as "very religious" were likely to oppose genetic engineering. Increased awareness about morally contentious science and issues has also been linked with diminished support for the subject (Evans and Durant, 1995). Yet, moral objections raised by the public involve broad-ranging beliefs, concerns, and fears that exceed strictly religious values (Lacy et al., 1991).

Cost of Mandatory Labeling for Food GM Content

U.S. consumers paid little notice when GM commodities were integrated into their food supply. Whether successful integration is credited to general public acceptance, lack of awareness, or ambivalence the result was less expensive product introductions for agribusiness producers. The cost of segregating and labeling GM corn in the U.S., for example, has been estimated at $416 million (Wu, 2004). In today's market, projected expenses increased the cost of corn by 12 percent and soybeans by 11 percent (Lin et al., 2000). Mandatory labeling would also place impractical burdens on U.S. food processing, from grain elevator to market shelf (Lin et al., 2000; Moon and Balasubramanian, 2004). If mandatory labeling was required to identify and disclose GM commodity contents, costs modeled for U.S. producers are expected to generate consumer backlash in commercial markets. This is particularly true if more expensive GM products fail to demonstrate appreciable benefits to consumers (Lapan and Moschini, 2004; Wu, 2004). In fact, evidence suggests that non-GM products may be perceived as superior and thus command a price premium in the marketplace (Lusk et al., 2001; Huffman, 2003; Tegene et al., 2003).

STRUGGLING TO EARN AND MAINTAIN THE PUBLIC TRUST

GM foods were introduced during the 1990s using a "push" approach. This strategy made sure that GM seed supplies for farmers were adequate and provided a quick tactical gain, rather than a "pull" strategy that would have established consumer demand by promoting the benefits of agro-biotechnology (Moon and Balasubramanian, 2004). As a result, consumers accepted GM commodity products in the marketplace, bringing them home without conscious choice (US-FDA, 2000). Despite regulatory approval, some consumers continue to reject GM foods. When expressions turn to outrage, consumers begin to perceive risk than may actually exist (Grobe and Douthitt, 1995). In practice, the adverse influence of negative attributes about GM foods outweighs the favorable effect of positive ones (Moon and Balasubramanian, 2004). Hadden (1989) explains that outrage factors particularly affect risk perception when exposure is involuntary, the product is unfamiliar, or products involve issues of ethical or moral significance. Each factor is evident in the case of GM foods (Boulter, 1997; Beckwith et al., 2003).

The Age of Decline in Social Trust

Cultural, historical, and socioeconomic parameters have been used to explore the credibility that consumers give to academic, governmental, and industrial messengers (Becker, 1996; Cantley et al., 1999; Gaskell et al., 1999; Lusk and Sullivan, 2002; Huffman, 2003). As the pace of technology and awareness of world events quicken, trust increasingly functions as a substitute for knowledge (Luhmann, 1979). Yet, the concept of "social trust," in which people are willing to rely on experts and institutions to manage risk exposure, must be cautiously defined before it can explain public acceptance of a technology (Frewer et al., 2003). In part, the pace of new technology, which brings dangers that are not recognized at the time products are introduced, explains hesitancy of the public to trust (Boulter, 1997). More than ever, citizen action groups aggressively question institutionalized decision-making, revealing that the public is unwilling to extend social trust to decisions that impact their lifestyles (Laird, 1989; Huffman, 2003; Tegene et al., 2003). As new technologies emerge, the public expects government and industry leaders to demonstrate that regulatory procedures are more than sufficient to keep them safe (Boulter, 1997; Glickman, 1999).

In contemporary society, proponents of biotechnology must contend with public erosion of confidence in governmental authority. Perception about technology-related risk in general, and agrobiotechnology in particular, reflects a lack of civic trust in governmental ability to effectively manage risks stemming from that technology (Slovic et al., 1993; Hallman, 1996; Slovic, 1997; Cantley et al., 1999; Beckwith et al., 2003). This viewpoint is supported by growing awareness that consumers have not been given the opportunity of choice to make important decisions affecting their health and lifestyles (Hoban et al., 1992; Hoban and Kendall, 1998; Arntzen et al., 2003; Beckwith et al., 2003; Miles and Frewer, 2003; Moon and Balasubramanian, 2004).

The Origin of the Message Can Determine the Credibility of the Messenger

Trust is a complex concept linked with the messenger to perceptions of accuracy, knowledge and concern with public welfare (Frewer et al., 2003). The source of information, or affiliation of the messenger, influences the public's trust in the message. Regardless of the messenger, opinions and promotions that claim product benefit without presenting unbiased discussion of risks are received with skepticism and distrust

(Marris, 2001; Beckwith et al., 2003). Paradoxically, the most trusted examples in industry established credibility by providing frank assessments of risks and benefits that seem counter to their economic interests (Eagly et al., 1978; Boulter, 1997).

When presented an environmental group's (Greenpeace International) perspective, polled consumers from two high-income Midwestern U.S. cities became more likely to reject a GM labeled food for a plain-labeled product. However, when independent third-party contributors provided information about GMOs (scientifically-based statements taken from scientists, religious leaders, and academics), consumer acceptance exactly offset the negative impact of the environmental group perspective (Tegene et al., 2003; Huffman, 2004).

Public acceptance of information sources also varies by region and country (Cantley et al., 1999; Gaskell et al., 1999; Huffman, 2004). Within the EU, consumers were presented a list of sources and asked which were most likely to "tell the truth about genetically modified crops grown in fields." Environmental, consumer, and farming organizations topped the list, while national public entities and industries were least believed (Gaskell et al., 1999). In part, EU reluctance to believe government and industry pronouncements has been endorsed by public experience with bovine spongiform encephalopathy (BSE) (Gaskell et al., 1999). In the U.S., some surveys reveal a high level of support and public trust for agencies that regulate GM products: 90% and 84% for the USDA and FDA, respectively (Gaskell et al., 1999). Conversely, New Jersey residents indicated lower support for U.S. government and the biotech industry, more for environmental groups and local farmers, and most faith in the credibility of university scientists (Hallman, 1996).

Scientists, in particular, are held to a high public standard for credibility and trust (Hallman, 1996; Boulter, 1997; Hails and Kinderlerer, 2003). In turn, scientists are obligated by society to present scientific facts and likely developments accurately and without bias (Boulter, 1997; Wellcome Trust, 2001; Hails and Kinderlerer, 2003).

Within the U.S. Green Industry, the American Society of Landscape Architects (ASLA) has specifically addressed diminished confidence in regulatory authorities. In 2001, the ASLA issued a press release stating concern about GM technology, disbelief that the USDA review process is sufficient to assess public and environmental risk, and calling for a moratorium on experimental field trials until the process is reformed and an appropriate review panel is assembled (Cervelli, 2000; Argust, 2001).

For GM ornamental plant introductions to succeed, Green Industry professional associations will have to support the products of biotechnology. Landscape architects, in particular, are critical contributors to plant promotion by specifying the plants used in landscape designs and as a consequence, which plants are grown by nurseries (Garber and Bondari, 1992). Yet, landscape architects are not well informed about new plant introductions. Thus, plant marketing and promotional efforts seldom benefit from their input (Day and Garber, 1993).

The U.S. GMO Debate and the Message That Reinvigorated It

In the 1980s, scientific debate between molecular geneticists and ecologists gained a public audience in the U.S. Attention sparked awareness and action by political, industry, and environmental groups, which led to regulatory procedures currently employed for GM experimental field trials, product approval, and release (Braun, 2002; Sharma et al., 2002). Compared with ongoing dialogue in Europe, the U.S. public debate that led to established regulatory provisions for handling GMOs was brief. By the end of the 1980s the issue had disappeared from collective public memory (Gaskell et al., 1999). Public debate resurfaced in 1999 when a scientific note about GM corn pollen exposure and monarch butterfly mortality was published by Cornell University researchers.

Research Repercussions: Monarch Ascendant, Science Descendant?

Perception of risk by the public or by legislators as well as pressure on legislators from public and environmental action groups can directly influence scientific progress, causing policies and resources to be redirected (Glickman, 1999; Peterson, 2000). In 1999, Losey and colleagues reported observations that pollen from transformed N4640-*Bt* corn that expressed *Bacillus thuringiensis* (*Bt*) Cry protein, a lepidopteran-specific endotoxin, affected developmental delays and mortality when fed to Monarch butterfly (*Danaus plexippus* L.) larvae in no-choice laboratory assays (Losey et al., 1999). This research note triggered immediate and critical scientific responses (e.g., Hodgson, 1999). Not all of the subsequent scientific debate or media coverage was positive (Wu, 2004).

In response to public and political pressure a coordinated, multi-state research effort was launched to investigate ecological, temporal, and

plant physiological parameters regarding *Bt* corn and monarch butterflies. The risk assessment by the consortium concluded that protein expression levels differ and are regulated by the transformation event (Stanley-Horn et al., 2001) and that toxicity from pollen consumption was higher in certain transformations (e.g., Event 176 corn). The consortium agreed with regulators that products of GE should be screened for protein expression before environmental releases are approved. When ecological, physiological, and toxicological models were explicitly applied to monarch butterfly larvae in field and laboratory assessments, however, the risks of monarch exposure and mortality from *Bt* corn were found to be negligible (Hellmich et al., 2001; Oberhauser et al., 2001; Pleasants et al., 2001; Sears et al., 2001; Stanley-Horn et al., 2001; Zangerl et al., 2001).

In the case of monarch butterflies and *Bt*-corn, the high-profile consortium research effort was extensive. While initially contentious, it could be reasonably argued that public debate and political pressure expedited critical research to address scientific knowledge gaps. Still, the results of these studies reached far fewer members of the public than those who heard or read the original media reports (Wu, 2004). Despite extensive research that demonstrated negligible environmental concerns, consumer groups continue to cite monarch butterfly death from contact with *Bt* corn as a reason to limit GM product adoption (Klingeman, *see* preliminary survey data, *below*). Even university student and peer groups, who were familiar with the Losey (1999) report, were less familiar with the results of the ecological and environmental research that followed (Beckwith et al., 2003). Such misunderstanding clearly illustrates the challenge posed to scientists. Results of experimental research should be better advocated with scientists openly discussing the outcome of research efforts as well as failures and limitations of the process (Cantley et al., 1999; Uzogara, 2000).

SCIENCE'S UNDERSTANDING OF THE PUBLIC VERSUS PUBLIC UNDERSTANDING OF SCIENCE: CONFLICTING VIEWPOINTS AND THE "DEFICIT MODEL"

When scientists are polled, they identify the public with concepts loosely framed to describe an "anti-science, post-modern culture." Scientists report feeling that public support of and knowledge about science is generally in decline (Holton, 1993; Wellcome Trust, 2001). Advocates for this view suggest that public resistance to biotechnology,

including GM foods, is exacerbated by scientific illiteracy (e.g., Wyse and Krivi, 1978; OTA, 1987; Scholderer and Balderjohn, 1999). Certainly, much has been said about the lack of the basic biological knowledge of the public (Hoban, 1998; Lusk and Sullivan, 2002). For example, when presented as true or false the statement "Ordinary corn does not contain genes but genetically modified corn does," only 33 percent of the public correctly answered false (Hoban, 1998). In the U.S., the public has not given a lot of thought to genetic engineering in general (Hallman, 1996). Even when prompted, more than a quarter of surveyed New Jersey residents could not describe an image or concept evoked by the phrase "genetic engineering" (Table 1).

Ironically, lack of awareness about GM technology appears, in some cases, to lead to increased consumer acceptance (Lusk and Sullivan, 2002). Conversely, studies have also described a positive association between public confidence in science and higher level of education (Heiman et al., 2000; Bak, 2001). In direct consequence, the *deficit model* has been formulated to explain this relationship and is frequently cited with the need to promote scientific literacy.

TABLE 1. When prompted, New Jersey resident consumers ranged widely in the first thoughts and image responses given about the phrase "genetic engineering." Results suggest many U.S. consumers have not given much thought to genetic engineering (Adapted from Hallman, 1996).

What do you think of when you hear the words "genetic engineering"?	Responses among NJ Residents	Generalized Valuation
Don't Know	26.1%	NA
Science or Technology	11.8%	+
Test-Tube Babies, Embryos, Cloning	9.7%	–
Plants, Animals, or People (in general)	8.2%	0
Negative or Frightened	7.5%	–
Monster or Mutant allegories	7.1%	–
DNA or Chromosomes	5.8%	+/–
Medicine	4.1%	+
God or Creation issues	4.0%	–
Progress	3.9%	+
Cross-Breeding	3.5%	+
Neutral	2.6%	0
Nazi or Hitler imagery	2.3%	–
Artificial or Tampering	1.8%	–
Other	1.6%	NA

The deficit model asserts that educational and outreach efforts are essential to fill the public gap in scientific understanding (Marris, 2001). In turn, greater scientific literacy is expected to instill appreciation and win greater support for science (Bak, 2001). Yet, the sufficiency of the deficit model to explain consumer acceptance of GM has been questioned (e.g., Hails and Kinderlerer, 2003). Closer inspection suggests that greater scientific literacy enhances support for science. However, exposure to higher education does not guarantee support when the subject is controversial, like biotechnology. Recipients of college degrees and postgraduate majors contribute weakly to public attitudes about science: most likely because many advanced degrees do not include scientific expertise (Bak, 2001). In fact, evidence from Europe suggests that greater knowledge about GMOs increases public skepticism and polarization (Martin and Tait, 1992; Gaskell et al., 1998; Marris, 2001). In turn, a *dialog and debate* model has been proposed and applied in the UK in 2003 to a public forum and feedback with scientists, regulators, and governmental authorities (Turney, 2002; Hails and Kinderlerer, 2003).

STRATEGIES FOR EFFECTIVE PUBLIC OUTREACH

A comparison was made of issues presented through biotechnology literature published between 1987 (year of the first approved field-test of an engineered organism) and 1994 in opinion surveys, the popular press, and technical/regulatory articles. This exercise established different trends in issues emphasized by scientific- and public-oriented publications. Notably, technical and regulatory articles were less likely than public-oriented opinion polls and popular press articles to include ethical, safety, or value-based issues (Hagedorn and Allender-Hagedorn, 1997). Various public interest groups have responded to this communication gap, voicing their perception that scientific neglect for ethical and social issues about biotechnology is equivalent to contempt among scientists for public expression of concern and dissent (Wynne, 1995; Hagedorn and Allender-Hagedorn, 1997; Cantley et al., 1999). Hagedorn and Allender-Hagedorn repeat Hoyle's (1995) precautionary observation that "public approval of biotechnology is a mile wide–and an inch deep. It could change overnight."

If the public determines that it has been denied adequate opportunity to participate in biotechnology decisions, it may reject novel market introductions and breakthrough technologies (Hoban et al., 1992). His-

torical precedents including opposition to nuclear power generation (Gardner, 1982; Jasper, 1992) and fluoridation of water supplies (Martin, 1989) have led to rapid shifts in public perception and consequent backlash on government regulators. As already is evident in parts of Europe, the application of biotechnology for GM products can be publicly constrained and if so, benefits will not be realized (Fleising, 1991; Cantley et al., 1999; Glickman, 1999; Hails and Kinderlerer, 2003).

Communicating Science to the Public

To date, scientists have not developed an effective education program for the public that promotes the positive attributes of agrobiotechnology. In the U.S. and abroad, this communication gap has increased negative civic attitudes about GM products, including foods (Frewer et al., 2003; Moon and Balasubramanian, 2004). Contemporary advocates for better outreach methods argue that the public does not need to be *educated* about the scientific process to appreciate outcome of GM product development (Wellcome Trust, 2001; Hails and Kinderlerer, 2003). In fact, efforts to demystify the *process* of GM development are predicted to limit public acceptance of the GM product. Rather, outreach should emphasize benefits of the *products* of biotechnology (Tiedje, 1989).

The public needs to be provided sufficient information and background to make their own informed decisions about biotechnology (Boulter, 1997; Hoban and Kendall, 1998). Outreach is incomplete if it fails to address the economic, environmental, and ethical costs, as well as practical limitations of the GM product and its production (Boulter, 1997; Cantley et al., 1999; Uzogara, 2000; Moon and Balasubramanian, 2004; Rowe, 2004). Frewer and colleagues further caution that relying on the persuasive effort of scientific experts may not sufficiently address public concern. By misaligning the message and messenger, the public will reject the information as irrelevant or unbelievable (Frewer et al., 2003).

The Influence of News and the Media

The way that statements of risk are presented influences, in predictable ways, the likelihood that new products or practices are accepted. Appropriate discussion of outcomes from new technology can shift existing attitudes. Scientists cite confusion in popular press and media coverage as a principal barrier to accurately conveying information

about science to the public (Wellcome Trust, 2001). But the media cannot be wholly charged with polarizing the debate surrounding biotechnology.

Gaskell and others (2004) point out that media reports on other contemporary technologies do describe risks. Reports about mobile phone use include coverage about brain damage in children and associated traffic accidents. Yet, consumers willingly assume the risks associated with cell phone use because the technology is familiar and the benefits are immediately apparent. In other words, consumers are willing to accept riskier positions that offer beneficial trade-offs than less risky positions having potentially un-recoverable outcomes (Tversky and Kahneman, 1991).

Images and stories presented by the media do not necessarily direct individual behavior. Rather, persistent images from media outlets establish a framework for public expectation and reflect behaviors and opinions that their peers apparently value (Nelkin and Lindee, 1995).

When news is expected to have dramatic or wide-reaching impact, even among top-ranked sources, media coverage cannot be relied on to accurately report salient facts (e.g., Molitor, 1993). Slattery and others (2001) confirmed that a marked shift occurred between 1968 and 1996 toward sensational news reporting, particularly in local public broadcasts. In the case of *Bt* corn, for example, research results were far less dramatic for media outlets than the original story. In turn, the original report endures in public memory (Beckwith et al., 2003; Klingeman, preliminary survey data) because the results were not featured as widely.

To attract constructive media attention, evidence for GM benefit and procedures for sound environmental integration must be delivered in accurate and compelling ways. Educational materials about GMOs and biotechnology will also have to be creatively applied to maintain the interest of consumer audiences. For example, Avise (2004) summarizes 60 current biotechnological applications and experimental projects using straightforward, non-technical descriptions. He concludes each summary with an easy-to-understand "Boonmeter" device that categorizes his personal opinion of these efforts as "boondoggle," "hyperbole," "hopeful," or "boon" to humanity.

Websites that discuss the virtues and perils of genetic engineering are already overabundant. It is difficult to determine which sources credibly attribute their claims. But trusted, reliable Internet resources provide valuable resources and outreach tools. An example is "Transgenic Crops: An Introduction and Resource Guide," hosted by Colorado State University. The regularly updated webpage showcases emerging "Hot

Topics" and addresses issues of general public interest and concern. The format is engaging and relatively easy to understand. The site also includes slide sets, articles, lesson plans, and related links for teachers, journalists, nutritionists, and Extension agents (Byrne et al., 2004).

Recommendations to Scientists Working with the Public

We limit the eventual adoption of GM ornamental products by relegating research about consumer perception and risk analysis to the marketing departments of private industry. This is true, in no small part, because public trust is dependent upon the source and perceived intent, or position, of the messenger. Scientists in particular can meaningfully contribute to the debate because they are trusted by the public and expected to provide unbiased expertise.

Boulter (1997) and Braun (2002) take the view that scientists have a social obligation to more actively advocate their discoveries to a curious public. Braun (2002) further asserts that scientists should venture beyond their laboratories and professional journals to actively lobby with politicians and opinion leaders. But active advocacy of scientific discovery is counterproductive if the public interprets an overly optimistic message or thinks that the messenger is biased for personal gain or portrays benefits that exceed realistic expectations (Boulter, 1997; Beckwith et al., 2003). Mixed messages also occur when scientists, the expert authorities, openly disagree about controversial science (Boulter, 1997).

Boulter (1997) also recommends that scientists be aware of the ways that their profession is stereotyped by historical and contemporary fiction (Table 2). These characterizations pervade public perception of science and influence the message that is inferred from the scientific messenger. Scientific writers and spokespeople are best able to allay consumer concerns if they can circumvent stereotypes that are largely negative (Boulter, 1997; Wellcome Trust, 2001).

CAN GM ORNAMENTAL PLANTS BRIDGE THE GAP BETWEEN SCIENCE AND SOCIETY?

The U.S. nursery and floriculture industry represents a smaller portion of the economy than the $34 billion fresh produce industry, about $14.3 billion annually (USDA-ERS, 2004). But many of the same trends that influence the competitive development and introduction of new GM products act on garden and landscape plant consumers (Table 3). Fresh produce and plant selection by consumers are both driven by

TABLE 2. Stereotypical portraits of fictional scientists (adapted from Haynes, 1994; Boulter, 1997). To generate public interest in science and sustain adequate research funding, scientists need to present their work in socially relevant ways. Scientific advocates must balance appeal to a wider general culture with clichéd characterizations that will negate the credibility of the messenger.

Characterization	Stereotypical Attributes
Medieval alchemist	Secretive, obsessive. Pursues (unsuccessfully) an inherently evil objective. Overreaches God's divine prerogative by creating new life.
Foolish specialist out-of-touch with reality	Scientifically ineffectual, socially isolated, a moral failure.
Unfeeling, impersonal scientist	Respected, powerful, very successful (an expert authority), emotionally detached. Personal relationships traded as an expected sacrifice to science.
Helpless scientist	Scientific discoveries take over and become uncontrollably monstrous.
Scientific hero	Intellectual brilliance. Saves mankind from disasters.
Scientific idealist	Pursues ideals of scientific utopia.

packaging and promotion (Orton and Romig, 1990; Stegelin, 2001; Barton et al., 2002; Stanley, 2002). When plants are heavily promoted, poor performance in the landscape or garden, particularly in the absence of plant guarantees, limits repeat purchase behavior. In fact, about 10 percent of more than 500 plant consumers who acknowledged dissatisfaction with recent plant purchases reported that they abandoned gardening to pursue other recreational activities (Dennis and Behe, 2004). For this reason, the Green Industry should avoid dubious plant introductions with unconfirmed merits.

Arguably, Calgene Corporation's 1994 commercial launch of the MacGregor® (Flavr Savr®) tomato met that standard. Flavr Savr tomatoes were intended to extend vine-ripening time and post-harvest shelf life, but they were expensive and consumers did not appreciate the "Flavr®." The product was withdrawn after failing to rally market interest and maintain economic viability (Avise, 2004).

More Tests for the "Boonmeter"?

Some opponents view ornamental plant biotechnology with disdain and perhaps not without cause. For example, recent proposals to geneti-

TABLE 3. Several trends have influenced the retail food trade, as well as research and development of new fresh produce products, including GE foods. These same trends are also factors influencing consumer ornamental plant purchases and demand for novel plant products (adapted from Orton and Romig, 1990).

Product characteristic or trend	Effects sales of market produce and retail foods	Influences ornamental plant sales
Availability	+	+
Product branding	+	+
Consistency	+	+
Convenience	+	+
Health & lifestyle issues	+	+
Impulse purchases	+	+
Packaging	+	+
Taste	+	NA
Freshness	+	NA
Quality	+	+
Rate of new product introductions	+	+
Variety	+	+

cally modify apple trees and produce individualized live-tree memorials for dead relatives received UK government funding but met with scientific roadblocks, widespread skepticism, and ethical outrage. The UK National Endowment for Science Technology and the Arts (NESTA) extended $70,000 in initial support to two graduates of London's Royal College of Art and founders of the venture firm Biopresence™ to develop the first individualized tree memento. Personalized trees are expected to cost about $40,000. In response to expressed concerns, NESTA has decided to withhold its support pending consensus approval from the Advisory Committee on Releases to the Environment (ACRE), the Department for Environment, Food, and Rural Affairs, and English Nature. Consent will be contingent on modified plans to insert a "silent" coded segment of each human DNA sequence that would not change the gene length or the protein for which it codes (ABC Science Online, 2004). Given survey-stated, public aversion to attempts to insert animal and human genes into plants, it is difficult to expect widespread acceptance of these keepsakes (Hoban, 1993; Hallman, 1996; Hoban and Kendall,

1998; Lusk and Sullivan, 2002; Beckwith et al., 2003; Klingeman, preliminary survey data).

From Interest, to Familiarity, Then Desire: GM Ornamental Plants Can Grow Public Acceptance of Biotechnology

As the debate about biotechnology and GM food continues, the commercialization of GM ornamental commodities has quietly begun. Growths of the Green Industry and gardening as a hobby have created expectations of cornered markets and successful new commercial ventures. Academic and industrial teams are actively pursuing genetically engineered solutions to horticultural conundrums. Perhaps most notable among these is the almost Grail-like quest for a *true* blue rose. Murray (2003) concludes that applications of biotechnology to achieve ornamental color variants, like blue flowering roses, are "apparently benign, but a massive waste of money compared with simpler options." Yet, genetically modified ornamental plants are positioned to play a critical role bringing positive public attention to biotechnology's products.

To accomplish the objective of publicly accepted GMOs, gene technology in ornamentals should be applied to produce plants that satisfy a critical demand. For example, consumers are willing to pay a price premium for powdery mildew-resistant flowering dogwoods (Gardner et al., 2003; Klingeman et al., 2004). Genetic engineering to produce powdery mildew- and anthracnose-free flowering dogwoods, black spot-free roses, or lace bug-resistant azaleas would command market recognition. In turn, clearly demonstrated benefits, like improvements in plant health and environmental vigor, are expected to influence societal opinion leaders and help to overcome consumer concerns about biotechnology. Plants that meet consumer demand will earn recognition both through commercial trade and in the landscape (for more, see reviews by Harriman et al. and Alston et al., this volume).

Current Commercialization Efforts and the Market for GM Ornamental Plants

Since 1989, several commercial ventures, including Florigene and Keygene (The Netherlands), Calgene Pacific (Australia), Kirin, Sapporo, and Suntory (Japan), and DNA Plant Technology (USA), have genetically modified ornamental plants to create novel products primarily for the florist industry. Of these companies, Florigene, Ltd. was the first to commercialize its research.

Established as a joint venture in 1989, Florigene partnered with chrysanthemum breeder Fides to genetically engineer 'Moneymaker', a pink carnation cultivar. A chimeric chalcone synthase (CHS) gene isolated from chrysanthemum (*Dendrathenum grandiflora*) was introduced into 'Moneymaker' leaf disks using *Agrobacterium tumefaciens* as the transforming agent. By blocking the chalcone synthase pathway that regulates floral pigmentation, pigment synthesis was suppressed in chrysanthemum petals. Transformed plants were vegetatively propagated for field trials and yielded relatively stable expression of white or pale pink floral colors (Courtney-Gutterson et al., 1993, 1994). Subsequent testing produced 'Flori-ant', a white GE carnation cultivar that was used to test the Dutch patent process for a transformed flower. 'Flori-ant' has been patented, but because there are many white carnations in the trade, 'Flori-ant' carnations have not been actively marketed.

'Florigene Moondust' and 'Florigene Moonshadow' carnations (Figure 1) are the first commercially available, genetically engineered ornamental plants and were created using Florigene's patented *Blue Gene Technology*. 'Florigene Moondust' carnation was patented and released in 1996, followed by 'Florigene Moonshadow' in 1997. Since 2001, *Blue Gene Technology* has expanded Florigene's GM carnation product line. Moon Series carnations, which are grown in Ecuador, offer blue colored flowers ranging from pale lavender to nearly black. Initial price offerings, at about $10 for a potted plant (Avise, 2004), reflect the close competition of the floral market.

Florigene has also developed *Long Vase Life* (LVL) technology in carnations, in which the ACS gene, responsible for ethylene production, is suppressed. LVL carnations have not been commercialized. NovaFlora, Inc., a Philadelphia-based biotechnology firm, owns commercial rights to a gene marketed as *Flowering Time* technology that induces early floral induction by altering normal plant day-length requirements for flowering. NovaFlora also holds commercial rights to a gene that regulates apical meristem growth and the structure of inflorescences (the *Plant Architecture Gene*), as well as one promoted as the *Dwarfing Gene*, that controls height of ornamental plants and grasses.

To date, availability of GE ornamental plants has been limited to the floriculture industry. Other green industry groups, including turfgrass research and development firms, are seeking field test approvals or deregulation of landscape and recreation-related GM plants (Shea, 2004). Surveys indicate strong consumer support for GM turf, especially if the grass reduces maintenance needs. Just under 50 percent of New Jersey residents "strongly approved" the use of genetic engineering to produce

FIGURE 1. 'Florigene Moondust' and 'Florigene Moonshadow' carnations are the first widely available genetically engineered ornamental plants in commercial trade. 'Florigene Moondust' was patented and released in 1996, followed by 'Florigene Moonshadow' (pictured below) in 1997. Florigene's patented *Blue Gene Technology* produced the Moon Series of carnations, which present carnations in the blue range of colors from pale lavender to nearly black. Florigene, Ltd. has also developed *Long Vase Life* (LVL) technology in carnations, in which the ACS gene, responsible for ethylene production, is suppressed. LVL carnations have not been commercialized. To date, availability of GE ornamental plants has been limited to the cut flower industry (*Photo courtesy of J. Mason, Florigene, Ltd. with permission*).

"grass you don't mow" with about 25 percent more respondents "approving mildly" (Hallman, 1996; Harriman et al., this volume). Still, public interest groups and researchers express concern about gene flow from GM turfs (e.g., Pollack, 2004). Gene flow from creeping bentgrass (*Agrostis stolonifera* L.), engineered to express a *CP4 EPSPS* glyphosate-resistance gene, was widespread among seedling progeny of receptive sentinel and resident grass species located about 2 km downwind of the western Oregon experimental study zone. The *CP4 EPSPS* marker was also detected in seedling progeny of sentinel grasses up to 21 km away from the zone and 14 km from the zone in resident grasses (Watrud et al., 2004).

Preliminary Implications for GM Ornamental Plants from a Tennessee Master Gardener Volunteer Pilot Study

The Master Gardener program was established in 1972 in the State of Washington to provide a corps of volunteers that could assist Extension agents with consumer home horticulture questions (Bobbitt, 1997). Today, a generalized profile for a Master Gardener Volunteer would most likely describe a married (> 80 percent) woman (> 75 percent), more than 50 years old (> 55 percent) who has lived at her suburban (> 50 percent) household for more than 10 years (> 40 percent). Her annual household income likely exceeds $50,000 (> 50 percent) and she has had at least some college education (> 50 percent) (Rohs and Westerfield, 1996; Rohs et al., 2002).

In spring 2004, a four-page survey was sent to about 1,800 Master Gardener Volunteers across Tennessee. The survey solicited general opinions about plant biotechnology, as well as perceptions about science that more specifically addressed ornamental plant genetic engineering. Data, which are still being entered and analyzed, reveal that TN Master Gardener Volunteers express similar concerns to those voiced by U.S. consumers about GM foods. Respondents provided unprompted statements of hopes and concerns about GM ornamental plants. While very few Master Gardeners indicated desire for specific plant types, like a "true red iris and a true blue daylily" and a "[plant that would] emit vibrations to kill all dandelions, bindweed, and Johnsongrass," several general themes were apparent (Klingeman, preliminary survey data).

Master Gardeners described hopes and concerns related to unknown long-term consequences of releasing GM ornamental plants in the environment, including pollen drift and invasive plant spread. To a certain degree, respondents equated GM ornamentals with GM foods, express-

ing concerns about human health and the possibility that genes would be spread to fruits and vegetables in their home gardens. Dread fears (e.g., Slovic et al., 1985) were expressed in descriptions of kudzu-like monsters and "frankenbushes." Master Gardeners consistently voiced the hope that "heirloom" varieties and native plant species would not be lost. Respondents questioned the rationale of scientists and ability of regulators and legislators to control their experiments. Finally, they expressed various concerns that nature is delicately balanced, that Man could disrupt its equilibrium, and that genetic modifications were unnatural and an "affront to God." Advocates of GM ornamental plants expressed hope that plants would be affordable and available soon (Klingeman, preliminary survey data). These themes are consistent with concerns expressed about biotechnology and GM foods in other U.S. studies (Boulter, 1997; Uzogara, 2000; Beckwith et al., 2003; Moon and Balasubramanian, 2004).

CONCLUSIONS AND FUTURE RESEARCH

Despite the continuing worldwide debate and self-stated civic concern, a majority proportion of consumers seem willing to accept GM foods provided that they are sufficiently inexpensive and pose no apparent risks–even if the benefits of GMOs are unclear. Ultimately, consumers will judge emerging technologies, including plant biotechnology, evaluate their merits, and act to determine the success or failure of new products (Stenholm and Waggoner, 1992; Hoban and Kendall, 1998). It is important to evaluate and understand consumer reaction to biotechnological products, before significant investments for research and development are made (Hoban and Kendall, 1998).

During the last 15 years, export value of nursery and floriculture commodities has remained relatively stable with appraisals ranging between $204 and $305 million and averaging about $255 million a year (USDA-ERS, 2004). It will remain to be seen, however, how EU restrictions on future GM ornamental products might impact exportation value of this segment of the U.S. economy. Regardless of its potential commodity value, public perception, if negative, will predispose GM ornamental plants to failure in the market place (Glickman, 1999; Sharma et al., 2002).

Tennessee's Master Gardener Volunteers strongly favored plant labeling to identify ornamental plants that have been genetically engi-

neered. If public attitudes about labeling GM ornamental plants are consistent with the public's desire for labeled GM foods then Green Industry leaders can take proactive action and voluntarily adopt a labeling protocol for GM ornamentals, as has been suggested for GM foods (Uzogara, 2000; Lapan and Moschini, 2004; Beckwith et al., 2003; Moon and Balasubramanian, 2004).

Labeling efforts should not compound public misperception and confusion by using technical jargon. For example, while the word "biotechnology" was recognized by 57% of more than 2000 New Zealand adults, only about 9% felt they could explain the term (Schibeci et al., 1997). Phrases like "gene-technology," as adopted by the Australian Science and Technology Council, may be more acceptable to the public than "genetic engineering." The public has construed these phrases with independent (perhaps unregulated) individuals who, by biased action, satisfy the interests of governmental or corporate entities (Schibeci et al., 1997).

Concerns about unknown environmental consequences are expected to drive consumer acceptance and market success of GM ornamental plant introductions. Experience with public and consumer studies suggests that acceptance of GM ornamental plants would be best promoted by transforming familiar ornamental plants to make them pest- and disease-resistant. Subsequent outreach about newly developed GM ornamental plants should emphasize advantages of GM ornamental plants for integrated pest management systems. The concern that women express about the safety of GMOs is also significant because, within the U.S. gardening public, about 70 percent of consumers purchasing ornamental plant materials are women (ANLA, 2000). Efforts to promote the benefits and safety surrounding GM ornamental plants should specifically be addressed to the concerns of this market demographic.

Benefits of plant biotechnology should be emphasized in realistic and practical terms that can be equated with forms of agriculture that are already familiar. Discussion about risks and potential challenges, including ethical and social issues, should not be omitted and should be balanced against the anticipated benefits of biotechnology (Boulter, 1997). By openly discussing public fears about GM technology, we simultaneously address consumer concerns about the morality and ethical implications of biotechnology (Peterson, 2000).

Once the public loses trust it is not readily, if ever, regained (Slovik, 1997; Cantley et al., 1999). The costs of lost trust are incurred in politi-

cal fallout to regulatory institutions, industry economic vulnerability, and escalated media attention (Frewer et al., 2003). The extent to which U.S. residents currently distrust the U.S. government and biotechnology industries has not been adequately assessed. This knowledge gap has been cited as a critical obstacle to honest discourse about the benefits of this new technology (Hallman, 1996; Beckwith et al., 2003).

To allay public concern regarding safety and oversight of GM field trials, the processes of evaluating proposals to release GMOs, as well as conclusions about the safety of GM products should be made apparent within the public domain (Cantley et al., 1999; Gavaghan, 1999; Uzogara, 2000; Sharma et al., 2002). Specifically, both the scientific peer review process and regulatory channels by which GM field trial requests are screened should be better conveyed to the public and media (Wellcome Trust, 2001; Hails and Kinderlerer, 2003). Greater visibility of the regulatory process and responsible agencies will reassure consumers that there is functioning oversight of genetic engineering and that discrete penalties are meted out to deter renegade scientists. In turn, this will allay dread fears the public has expressed about biotechnology (Slovic et al., 1985; Boulter, 1997; Beckwith et al., 2003), including GM ornamentals (Klingeman, preliminary survey data).

Public confidence in the safety of governmental regulatory oversight for GM technology could be jeopardized if Federal agriculture budgets linked with biotechnology programs are cut. Public trust in the impartiality of U.S. academic institutions may also be compromised if budget shortfalls for U.S. higher education continue and research funds are instead supplied by the biotechnology industry. Moreover, contemporary academic pursuit of plant patent and process-patents may erode public confidence in the unbiased value of the academic message (e.g., Beckwith et al., 2003).

Results from the Tennessee pilot study of Master Gardener Volunteers will provide valuable insight and strategies for outreach about GM ornamental plants. But opinions and perceptions expressed by this special interest public group are not expected to represent general public concern. There is a need to better understand issues that are important to end-users of GM research efforts. Otherwise, if private industry is relied on to willingly share their consumer marketing and risk analysis reports, chances are limited that GM ornamental products will be advocated appropriately and introduced successfully.

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Index

