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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