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Engineering Resistance and Disease Management in Ornamental Crops

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ABSTRACT

Viral diseases of ornamental plants cause major losses in productivity and quality. Host pl1ant resistance offers an effective means of controlling plant diseases caused by viruses. It minimizes the necessity for the application of pesticides. However, there are many ornamentals in which no natural disease resistance is available. Genetic engineering allows the introduction of specific, in some instances broad spectrum, disease resistance derived from other species, or even from the pathogen itself into plant genotypes that have been selected for desirable horticultural properties.

Key words: Agricultural biotechnology; Floriculture; Genetic enhacement; Molecular breeding; Virus resistance.

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 an average annual increase of 4-6 percent 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 Disease and Disease Resistance in Ornamental Crops

More than 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 caused by viruses, therefore, have a major impact on quality. Unlike diseases incited by other pathogens where chemical methods aimed at prevention of infection have been quite successful, control of viral diseases has been much more problematic. Direct and indirect effects of viral and 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).

Many crops are susceptible to multiple viruses, each of which may cause serious economic losses. In addition, infected plant material may not be acceptable for export (Loebenstein *et al*., 1995). In practice, several 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 diseases of floral crops usually focuses on use of clean 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 strategies of controlling viral diseases in floral crops include early detection and removal of infected 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-tested clean propagation stock from production areas, elimination of weeds and non-production reservoir plants from greenhouses and surrounding areas, monitoring of insect populations and judicious use of pesticides are all needed for control.

The use of resistant varieties is by far the most recommended approach for control of disease in many crop species. Conventional breeding strategies require the identification of sources of disease resistance genes. This is a difficult task in ornamentals due to the diversity of floral and nursery crop species that are susceptible to such a large diverse group of pathogens. 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 requires multiple back-crossings which 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 that vector the virus.

Progress and Uunique Issues in the Ttansformation of Ornamental Plants

Advances in biotechnology have provided new opportunities to solve practical horticultural problems. The development of technologies for gene identification and gene transfer in 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: natural resistance genes, genes derived from viral sequences (pathogen-derived resistance), and genes from various other sources (Hull, 2002).

Transformation of ornamental plants has lagged behind efforts in the major field crops. This is further hampered by that fewer groups have worked on the regeneration systems that are necessary for an efficient transformation for a particular crop. In many crops, transformation or regeneration systems are cultivar-dependent. 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) allowing much less cultivar restriction in regeneration and transformation (Castillon and Kamo; 2002, Deroles *et al*.; 2002, Kamo, *et al*., 1995a, b, 1997).

As technologies for regeneration and gene transfer in plants improve and broaden across diverse genera, transgenic approaches to develop disease tolerant ornamental crops become more promising. Transgenic approaches for breeding woody ornamentals are attractive. Less space is required to grow out and evaluate germplasm in identifying disease resistance traits. Also, long generation time of backcrossing of woody plants can be eliminated. The costs, potential benefits, and risks of using genetic engineering to create disease resistant ornamental plants must be carefully evaluated. 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. The long-lived nature of these perennial ornamentals also poses unique problems for environmental risk assessment. The plant could 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. 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.

TRANSGENIC APPROACHES

Pathogen-derived resistance

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 (coat protein, replicase, satellite RNAs, defective interfering RNAs) in protecting plants against virus infection following transfer and expression of these genes in plants (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).

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* infections of *Ageratum conyzoides*. Rubio *et al*. (1999) developed a DI-RNA from *Tomato bushy stunt virus* (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 are RNA molecules that autocatalytically cleave sequences complementary to their binding site. Ribozymes 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. One line of transgenic melon expressing a ribozyme against *Watermelon*

mosaic virus 2 (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 *Tomato spotted wilt virus* (TSWV), *Groundnut ringspot virus* (GRSV), and *Chrysanthemum stem necrosis virus*, while a lower level of resistance was observed with *Tomato chlorotic spot virus*, and delayed disease development with *Impatiens necrotin spot virus* (INSV) (Rudolph *et al*., 2003).

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.

Ribosome-inactivating proteins

A number of ribosome-inactivating proteins have been described from a variety of plant species, and several have been expressed in transgenic plants (Stevens, 1981). Their activities have been reviewed by Tumer *et al*. (1999).

Antibodies for Viral Resistance

A recent scientific breakthrough that has presented another possibility for controlling plant diseases is the use of transgenic plants that produce antibodies to specific plant pathogens (During *et al*., 1990). Complete antibodies or single-chain variable fragment (scFv) antibodies 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). Neutralization of one or more viral proteins (viral proteases, replicases, movement proteins) should interfere with viral infection, virus assembly, movement of virus within the host, symptom expression, aphid transmission, and/or virus replication. Resistance by simultaneously expressing several antibodies with different target specificities will increase the likelihood of developing long-lasting, broad-spectrum resistance.

Transgenic Plants in Virus Control

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* (ArMV), *Cucumber mosaic virus* (CMV), *Chrysanthemum virus B* (CVB), *Tobaco mosaic tobamovirus* (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 (Gonsalves *et al*., 1992; Anderson *et al*., 1992), dsRNA (Kalantidis *et al*., 2002), or satellite RNA (Harrison *et al*., 1997). Chrysanthemum has been transformed with different forms of the CVB CP gene (Mitiouchkina *et al*., 2006). 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 (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.

There are 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. 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, INSV, and GRSV. 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). 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*, but no resistance assays were reported (Mörbel *et al*., 2002). Borth *et al*. (2006) have

characterized partial resistance in transgenic *Dendrobium* orchids transformed with the *Cymbidium mosaic virus* CP or mutated movement protein gene. Kamo *et al*. (1997, and unpublished) have transformed gladiolus with *Bean yellow mosaic virus* (BYMV) CP and antisense RNA constructs, that were each effective in *N. benthamiana* (Hammond and Kamo, 1995a,b) 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 *Ornithogalum mosaic virus* 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.

Transgenic plants expressing full-size antibody or antibody fragments against the coat proteins have been reported for several different plant viruses (Fecker *et al*., 1997; Tavladoraki *et al*., 1993; Voss *et al*., 1995; Zimmerman *et al*., 1998). High levels of resistance against TSWV were obtained in transgenic *N. benthamiana* expressing scFv antibodies targeting the TSWV N-gene product (Prins *et al*., 2005). The recent development of transgenic *N. benthamiana* and gladiolus expressing scFv antibodies against CMV (Hsu *et al*., 2006; Hsu and Kamo, *unpublished*) and *N. tabacum* expressing antibodies against TSWV NP protein showing various degrees of resistance to viral infection illustrates the potential of this technology as a means of controlling these wide-host range viruses in ornamental plants (Xu *et al*., 2006). A recombinant antibody directed against an epitope of the TSWV G1 glycoprotein

conserved among a large number of tospoviruses has been expressed in plants (Franconi *et al*., 1999). Antibodies directed against evolutionarily conserved functional domains, such as viral protease, movement protein and replicase should provide more potent, broad spectrum resistance against viruses (Schillberg *et al*., 2001).

CONCLUDING COMMENTS

Results obtained to date suggest that there are multiple approaches to achieve resistance to viral diseases in transgenic plants. Resistance attained by expression of viral sequences, antiviral antibodies, ribozymes, antiviral peptides, or dsRNA-specific nucleases is likely to be durable. Such resistance mechanisms are not known to have previously imposed selection pressure in virus evolution. The durability of resistance against an individual pathogen in a single plant would be further increased by additive effect of combining different resistance mechanisms against the same pathogen.

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 (Fuchs *et al*., 1997). The use of multiple transgene constructs appears to be a much more efficient means of introducing multiple resistance genes into a well-adapted genotype than combination by conventional breeding and multiple cycles of selection.

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.

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). Ten years after genetically enhanced varieties of major crops have become commercially available; adaptation of these varieties by U.S. farmers has become widespread (USDA-ERS, 2006). As we move forward in modern agriculture, the difficulty lies not so much in the world of science but rather in commercialization and adaptation of genetic engineering by the public. The ultimate success of agricultural biotechnology will depend on our ability to identify and measure its potential benefits and risks as well as their contribution.

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