Control of Plant Virus Diseases by Pathogen-Derived Resistance in Transgenic Plants

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Plant viruses have an enormous negative impact on agricultural crop production throughout the world, and, consequently, agronomists and plant pathologists have devoted considerable effort toward controlling virus diseases during this century. Prior to the advent of genetic engineering, traditional plant breeding methodology was sometimes successfully applied to develop resistance to viruses of agronomically important crops. In addition, standard techniques of plant pathology, including quarantine, eradication, crop rotation, and certified virus-free stock, have been important tools to control virus diseases, although each has disadvantages, such as expense, questionable effectiveness, and lack of reliability on a yearly basis.

The prospects for pathogen-mediated intervention in virus disease development were first realized in 1929 when H.H. McKinney demonstrated that tobacco could be protected from infection by a severe strain of TMV by prior inoculation with a milder strain of TMV (see refs. in Gadani et al., 1990). This type of protective measure, known as cross-protection, has since been employed throughout the world on several important crops, including tomato, papaya, and citrus (see refs. in Beachy et al., 1990; Gadani et al., 1990; Hull and Davies, 1992). However, this labor-intensive type of protection is expensive and it necessitates the use of an infectious virus as a control measure.

A problem of particular concern is that strains of viruses vary in their virulence on different crops and even within varieties of the same crop. A virus used to protect one crop could potentially cause serious diseases on alternate crops or varieties growing nearby. Therefore, it would be preferable to have a better understanding of cross-protection in order to employ a carefully regulated mechanism that could protect plants from the deleterious effects of virus infections and in order to derive strategies to prevent the cross-protecting virus from escaping to adjacent crops.

Over the past decade, the tools of molecular biology have permitted rapid advances in our understanding of plant viruses and their replicative strategies, yet we know little about the mechanistic aspects of cross-protection. Several theories have been advanced that provide models that can be tested. Arising from these hypotheses has been the generalized concept of pathogen-derived protection using transgenic plants. This idea, as elaborated by Sanford and Johnston (1985), proposes that the expression of certain genes of a pathogen, in this case a virus, in a host would disrupt the normal balance of viral components and thereby interfere with the virus life cycle. In the most successful instances, such disruptions would prevent the replication and/or movement of the virus beyond the initially infected cell. Even with less effective interference in the replication cycle, pathogen-derived resistance might modulate the disease symptoms and result in only a localized infection. In this review, we present some general features of the replication cycle of plant viruses and discuss several strategies for development of pathogen-derived resistance using transgenic plants.

Plant viruses differ considerably in the morphology of the virus particle and in the form of genetic material used to encode the virus genes. These various genomes include single- or double-stranded DNA, double-stranded RNA, or ssRNA in a message (plus)-sense or minus-sense format (Fig. 1). The general strategies underlying the expression of these genomes are diverse, but ultimately mRNAs are transcribed for translation of structural and nonstructural proteins that are required to fulfill the viral life cycle (Fig. 1). Despite differences in their replication strategies, all plant viruses have broadly similar steps in their life cycles: they must enter a host plant cell, generally by puncturing the cell wall, following abrasive mechanical damage, or via fungi, insects, mites, or nematodes that penetrate the plant cell wall during infection or feeding. The virus particle is then thought to swell or partially disassemble, which exposes the viral DNA or RNA to the cellular milieu (Verduin, 1992). If the virus possesses mRNA as genetic material, translation will begin to produce the virus-specific proteins required for replication (Fig. 1). DNA viruses generally enter the nucleus and utilize host enzymes to produce mRNAs suitable for translation.

A critical event in infection by most plus-sense RNA viruses is the production of replicase protein(s) that, in concert with

Abbreviations: CMV, cucumber mosaic virus; CPMP, coat protein-mediated protection; DI, defective interfering; PEBV, pea early-browning virus; PLRV, potato leafroll virus; PVX, potato virus X; PYY, potato virus Y; satRNAs, satellite RNAs; ss, single-stranded; TMV, tobacco mosaic virus; TSWV, tomato spotted wilt virus.
Figure 1. Schematic representation of a plant virus and its life cycle in a plant cell. The top part of the diagram depicts a representative genome of a simplified plant virus with the individual genes (boxed) and their functions. "X" may be any number of genes at any position with various functions such as an additional replicase factor, proteases, insect transmission factor, inclusion body protein, and/or a regulatory protein. The linear or circular viral genome is encapsidated in virus particles whose morphology is characteristic for the virus group. The viruses enter the plant cell either through mechanical wounds or upon transmission by a fungal or arthropod vector that penetrates the cell wall. Upon entry, the virus particles disassemble and, depending on the composition of the genome (shaded boxes), the replication cycle occurs in the nucleus or the cytoplasm. Some viruses also can have split genomes, and the ssRNA viruses have plus-, minus-, or ambisense RNA genomes. Certain events that are separated in the diagram are actually tightly linked as exemplified by the cotranslational disassembly shown to occur for some plus-sense RNA viruses. The functions delineated by the white boxes indicate processes that can occur in either the cytoplasm or in the nucleus, depending on the virus. Examples of transcriptional regulation at the promoter level are observed for geminiviruses (ssDNA) and caulimoviruses (double-stranded DNA). Both virus groups also employ posttranscriptional regulation including splicing, 5' to 3' processing, and perhaps nuclear export. Although many plant viruses produce subgenomic mRNAs for the translation of individual genes, many have adapted various translational strategies to allow translation of multiple cistrons from one single mRNA. Examples are ribosomal frame-shifting for dianthoviruses (ssRNA), suppression of termination for tobamoviruses (ssRNA), and reinitiation of translation for caulimoviruses. Potyviruses and comoviruses (ssRNA), as well as others, produce polyproteins that are very effectively processed into the individual proteins through viral-specific proteolysis. Viruses move from cell to cell in the symplast through plasmodesmata, either as complexes consisting of the viral genome and a specialized movement protein or as whole virus particles. The requirements of viral gene products and presumably the events involved in localized cell-to-cell movement vary with different viruses and are not yet clearly understood. Long-distance movement through the phloem is mechanistically distinct from localized movement and the requirement for involvement of virus gene products may vary with these two processes.

the cellular machinery, produce progeny by replicating the parental genome (Fig. 1). Most RNA viruses are thought to spread from cell to cell via the plasmodesmata as genomic RNA that is protected from degradation and assisted in movement by association with a movement protein or, for optimum long-distance movement, in conjunction with a functionally active coat protein (reviewed in Citovsky and Zambryski, 1991). Thus, each stage of the infection cycle (Fig. 1) has the potential of being perturbed, i.e., at uncoating, translation, replication, and/or movement. The goal of constructing genetically engineered plants resistant to virus infection is to express a portion of the viral genome, either with or without expression of an encoded protein, that will interfere with some particular aspect of the multiplication cycle.
Successful strategies based on pathogen-derived resistance include CPMP, expression of a coding region embedded in the replicase (for instance, the 54-kD protein of TMV), use of antisense RNAs that are the complement of the plus- or minus-sense template of the virus, or use of satRNAs that can presumably overwhelm the viral RNA replicase and thereby suppress specific events required for infection. For more detailed discussion and expanded references, consult several recent reviews (Beachy et al., 1990; Gadani et al., 1990; Dawson and Hilf, 1992; Hull and Davies, 1992; Register and Nelson, 1992).

**CPMP**

In 1986, Beachy and co-workers demonstrated that the expression of the coat protein gene of TMV in transgenic tobacco plants could provide a considerable level of protection against virus infection (reviewed by Beachy et al., 1990; Register and Nelson, 1992). Since then, CPMP in transgenic dicotyledonous plants has proven effective for more than 20 plant viruses (Hull and Davies, 1992). Recently, CPMP has also been extended to monocots, as demonstrated by expression of the coat protein of rice stripe virus in transgenic rice, where it provides protection against the homologous virus that is obligately vectored by viruliferous planthoppers (Hayakawa et al., 1992). These findings open new avenues for plant protection in the most important agricultural crops. In most instances, CPMP extends only to the virus or to related strains with substantially similar coat protein, but there are a few instances where the expression of the viral coat protein of one virus can provide at least some limited protection of transgenic plants against heterologous virus infections (Beachy et al., 1990; Gadani et al., 1990; Hull and Davies, 1992; Pang et al., 1992). In most cases, CPMP acts only against the virus, whereas inoculum consisting of naked virus RNA is frequently able to elicit infections. However, exceptions to this general rule exist, as with PVX, a plus-sense ssRNA virus where CPMP is effective against both DNA and viral inoculum (Braun and Hemenway, 1992). These different results suggest that multiple protective mechanisms may be involved in the cross-protection phenomenon.

For reasons that are not understood, the accumulation of large amounts of coat protein is not necessarily correlated with the most effective cross-protection. In some cases, high levels of protection are obtained in conjunction with very low accumulation of coat protein in transgenic plants. For instance, field protection to PVY, a plus-sense ssRNA virus, occurs in transgenic plants producing undetectable levels of PVY coat protein (Kaniewski et al., 1990, and refs. therein). This important finding might be applied to a number of crops because PVY and other potyviruses infect a broad range of plant species and cause yield reductions on economically important crops. CPMP has also been shown to greatly reduce the titer of PLRV in plants transformed with the PLRV coat protein gene. In this instance, transgenic plants accumulated PLRV coat protein transcripts, but the PLRV coat protein was not detected (Kawchuk et al., 1991). Lindbo and Dougherty (1992) have postulated that protection sometimes results from coat protein mRNA accumulation and is independent of a requirement for coat protein expression per se.

Their results with tobacco etch virus, a potyvirus, show that the use of nontranslated RNA complementary to the minus-sense (noncoding) replicative template can provide an efficient means of control of homologous virus infections. From these results, Lindbo and Dougherty (1992) propose that hybridization of the transgenic nontranslated coat protein RNA to the minus-sense template of the viral RNA inhibits the production of the plus-sense (infectious) progeny virus. However, not all the transgenic plants were resistant, indicating that variable levels of transcript expression in different cells and other unknown factors occurring during the production of transgenic plants may influence the effectiveness of protection.

Three research groups have demonstrated that transformed tobacco expressing significant, low, or undetectable levels of the nucleocapsid protein gene of TSWV, an ambisense ssRNA virus, are protected from TSWV infection (Pang et al., 1992, and refs. therein). This virus system also presents some interesting alternative possibilities for the mechanism of protection. The early reports showed that a wide range of accumulation of nucleocapsid protein was effective in protecting transgenic plants from TSWV infection. However, a recent report provides evidence that this protection is not "coat-protein" mediated in the traditional sense, but might be a transcript-mediated form of protection (de Haan et al., 1992).

In these experiments, plants expressed nucleocapsid transcripts that had both the start codon rendered ineffective and a frame-shift mutation near the beginning of the open reading frame. This strategy resulted in the production of nucleocapsid transcripts without the ability to translate the open reading frame in the transgenic tobacco plants. These plants had a level of protection that was similar to that of plants transformed with the intact nucleocapsid open reading frame (de Haan et al., 1992). Therefore, the nucleocapsid transcript appears to provide substantial protection against mechanical inoculation of TSWV and transmission of the virus by thrips that is independent of a requirement for a functional nucleocapsid protein. The authors make an interesting point that transcript-mediated protection may be preferable to CPMP because this precludes the need for accumulation of a foreign protein in crop plants. The mechanism of protection may be a form of antisense RNA (see below) capable of forming an RNA-RNA hybrid with the genomic RNA of the ambisense virus that alters the ratio of synthesized virus proteins. Alternatively, the expressed nucleocapsid mRNA may bind to and compete for virus- and/or host-associated replicase proteins (de Haan et al., 1992), or otherwise shift transcriptional or replicative events so that virus multiplication is reduced.

**REPLICASE-MEDIATED PROTECTION**

A recent development of considerable importance in pathogen-derived resistance has been the demonstration by Zai- tin and co-workers that expression of the 54-kD protein of TMV in transgenic plants offered higher levels of protection against a TMV infection than CPMP (Carr et al., 1992). The 54-kD protein is presumed to be derived from a subgenomic RNA of TMV that contains an open reading frame overlapping the carboxy-terminal portion of the 183-kD replicate
coding region, but this protein has not been detected either in infected plants or in plants transformed with this open reading frame (Carr et al., 1992). Experiments by Carr et al. (1992) show that the 54-kD protein, rather than the RNA transcript, is responsible for virtual immunity to TMV challenge in transgenic plants. The authors speculate that variations of the temporal expression and accumulation of the 54-kD protein in transgenic plants may disrupt the replication cycle of TMV. Similar experiments have been performed with PEBV, a bipartite plus-sense ssRNA virus (MacFarlane and Davies, 1992). As with TMV, expression of this putative 54-kD replicase-based protein of PEBV in transgenic plants provided protection against challenge by the homologous virus and two closely related strains of PEBV. Some of the plants were susceptible, and nucleotide sequence analyses of the transgene revealed the presence of mutations that prevented the translation of the PEBV 54-kD protein. Thus, although the 54-kD protein was not detected in protective transgenic plants, the open reading frame and the putative protein appeared to be essential for protection. MacFarlane and Davies (1992) detected two virus variants that overcame the replicase-based resistance in inoculated plants that were maintained for a prolonged period of time. This is not unexpected, since viruses are rapidly replicating entities. Thus, it is highly likely that strains able to circumvent resistance will evolve, particularly as such genes become widely used in crop protection, and it can be expected that multiple forms of protection strategies will be necessary to realize the maximum potential for virus-free transgenic crops when they are subjected to field conditions.

In analogous experiments, Braun and Hemenway (1992) expressed full-length and amino-terminal portions of the replicase gene of PVX in tobacco and found good resistance to subsequent PVX infection. In a comparison with plants expressing the coat protein gene of PVX, they observed that transgenic plants expressing the replicase derivatives provided more effective protection against virus infection than CPMP. As was the case with other replicase-based strategies, transcripts but not the predicted protein were detected in the transgenic plants, even though in vitro experiments indicated that the transcripts were translationally competent (Braun and Hemenway, 1992). A related study (Anderson et al., 1992) showed that a defective replicase protein of CMV, a plus-sense ssRNA virus with three components, protected transgenic plants from virus challenge. The defective protein may act as a dominant negative mutant that interacts with wild-type components of the replicase system to inactivate the complex and therefore interfere with the virus life cycle (Anderson et al., 1992). These recently developed replicase-based strategies offer new possibilities for protecting plants from the deleterious effects of virus infection, including yield reduction, and they will also increase our understanding of strategies utilized by viruses for replication in plant cells (Fig. 1).

cRNA OR ANTISENSE RNA STRATEGIES

The use of RNA complementary to part of the viral genome (antisense RNA) is another potential pathogen-derived resistance strategy that may have some utility for protecting plants from systemic virus infection (for a review, see Bejarano and Lichtenstein, 1992). In one case, expression of an RNA transcript complementary to a replication-associated portion of the viral genome of tomato golden mosaic virus, a ssDNA virus that replicates in the nucleus, resulted in a positive correlation between the accumulation of antisense RNA and reductions in symptom development of virus-inoculated plants (Day et al., 1991). In other experiments, transgenic potato plants expressing an RNA complementary to the coat protein gene of PLRV, a phloem-limited plus-sense ssRNA virus, also provided protection from virus infection comparable to that of transgenic plants expressing PLRV coat-protein (Kawchuk et al., 1991). Further research is needed to clarify the mechanism(s) of CPMP, and we may find that this form of protection is due, at least in part, to complementary (or antisense) RNA interactions with the virus genome.

The results of Lindbo and Dougherty (1992) that were discussed earlier certainly suggest that transcript accumulation rather than coat protein accumulation could result in plus "sense" RNA interference with replication of the minus-sense replicative intermediate. Thus, antisense RNA technology directed to the coding template may be useful for many viruses and could be particularly effective for those restricted to particular tissues, such as PLRV, which are phloem limited and dependent on aphids for transmission. Antisense RNA technology may also be applicable to viruses that replicate in the nucleus, such as the ssDNA-containing geminiviruses, where replication probably occurs in close proximity to the site where antisense RNA transcripts are produced. Perhaps the inability of antisense transcripts to be transported to cytoplasmic replication sites may partly explain why earlier studies with the coat protein-based antisense RNAs were unsuccessful against high-titer viruses such as CMV and PVX.

satRNAs

satRNAs are small RNAs that are not infectious by themselves and require helper viruses for their replication and encapsidation (reviewed in Palukaitis et al., 1992). In some cases, satRNAs enhance the severity of symptoms in conjunction with the helper virus infection, and in other cases the symptoms are ameliorated. The transgenic expression of satRNAs of a number of viruses has decreased virus symptoms and/or titers in a manner that appears to mimic a natural system (Palukaitis et al., 1992). A probable risk of this strategy is that in transgenic plants these satRNAs could mutate during their amplification and, in conjunction with a virus infection, exhibit a shift from an attenuating form to a virulent satRNA. Moreover, a virus or satRNA producing a mild reaction on one host plant could elicit severe symptoms on another host or in combination with a different strain of helper virus. However, practical experience with field tests in China have provided no evidence to support this hypothesis. In an 8-year study on tomato and pepper plants using mild or attenuating combinations of CMV and satRNA to mechanically inoculate plants, severe strains of satRNA in conjunction with CMV infections have not yet emerged (Tien and Wu, 1991).

From a practical plant breeding perspective, utilization of
more than one form of resistance should be expected to reduce the frequency of appearance of virus strains able to infect target crop plants that are released to growers for production over large acreages. An additional advantage of multiple forms of protection has been reported by Yie et al. (1992), who found elevated resistance to CMV in transgenic tobacco expressing both a CMV coat protein gene and a satRNA of CMV that attenuates virus symptom expression. This strategy appears to combine both the protective effects of CPMP and the interference of virus replication provided by the satRNA. In field trials, Yie et al. (1992) found that chimeric plants provided twice the protection of plants individually transformed with either CMV coat protein or satRNA. These findings suggest that it will be advantageous to utilize multiple sources of pathogen-derived resistance in plants when such sources are available, since multiple "artificial resistance" genes may lead to both enhanced and more durable forms of resistance.

**ALTERNATIVE STRATEGIES FOR PLANT PROTECTION**

In addition to the resistance strategies described above, we believe that considerable potential exists for development of new strategies for virus protection. For instance, DI RNAs, which are truncated forms of a wild-type virus, accumulate in concert with some natural virus infections to ameliorate viral symptoms and titer (Hillman et al., 1987). Expression of cloned forms of these DI s in transgenic plants could yield a potent form of pathogen-derived protection. Although native DI RNAs have been described only for a limited number of plant viruses, Marsh et al. (1991) serendipitously constructed cloned deletion mutants of RNA-2 of brome mosaic virus that effectively interfered with synthesis of native viral RNA in protoplasts. Co-workers in our lab (H. Zhou, D.K. Robert, A.O. Jackson, unpublished data) have also obtained similar results with barley stripe mosaic virus deletion mutants. Such synthetic DI RNAs should provide potent sources of resistance, although further studies are required before we can readily predict which regions of the genes can be deleted to yield DI molecules that effectively interfere with replication of their parental viruses.

It is also likely that plant-derived genes will provide information that can be used to engineer resistance to viruses. Many plant viruses induce severe hypersensitive resistance reactions when inoculated to plants. In some cases, viral proteins have been identified that can induce such a host response (reviewed in Dawson and Hilf, 1992), and several research groups are actively attempting to identify corresponding host-resistance genes. Understanding how these host and virus genes interact to produce resistance may provide alternative and generally applicable strategies for producing virus-resistant plants using cloned resistance genes that have been modified in various ways. Use of these genes could elicit a necrotic (hypersensitive) response in individually infected cells early in the virus life cycle and mimic natural resistance genes by prohibiting local or systemic movement of the virus.

Other emerging strategies with potential for control of virus infections include the use of small catalytic RNAs (ribozymes). These molecules, which occur naturally in some virus systems, have the potential to be engineered to disrupt virus replication (for discussion and refs. see Gadani et al., 1990; Hull and Davies, 1992). Virus-mediated activation of toxic genes in transgenic host plants, over-expression of viral movement proteins, use of dominant negative mutants, and expression of combinatorial antibodies to specific virus proteins are strategies currently being explored in several laboratories for protection of plants from virus infections. We are optimistic that in the near future we will have a much more sophisticated understanding of the mechanisms of virus replication and movement and of factors controlling host range. We hope to be able to utilize this knowledge in developing viable strategies that can expand our repertoire of methods available to protect plants against virus diseases.

**NOTE**

To maintain brevity, we have not provided a comprehensive reference list and have been unable to provide direct citations of many relevant papers.

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