# Differences in Protection Phenotypes in Tobacco Plants Expressing Coat Protein Genes from Peanut Stripe Potyvirus With or Without an Engineered ATG

## Brandt G. Cassidy and Richard S. Nelson

Samuel Roberts Noble Foundation, P.O. Box 2180, Ardmore, OK 73402 U. S. A.

Transgenic *Nicotiana benthamiana* plants expressing the full-length coat protein (CP) gene from the potyvirus, peanut stripe virus (PStV), with or without an engineered ATG initiation codon or containing an engineered ATG initiation codon with amino terminal deletions of 16 or 106 amino acids, were tested for resistance to virus infection. Two plant lines, both with engineered ATGs (ATG plus), did not accumulate virus in systemic leaves or show symptoms when mechanically inoculated. All other plant lines that were ATG plus (full length or deletions) exhibited a delay in the appearance of systemic symptoms and the symptoms were milder. Following the initial systemic spread of the virus, the younger systemic tissue appeared to escape infection, leading to a recovery of the plant. Western blot analysis revealed that virus-encoded CP was not detectable in the systemic leaf tissues from the plants which had recovered. The steady-state levels of both protein and mRNA from the genomic copy of the PStV CP gene were down-regulated in these upper systemic symptomless leaves. Upper systemic, symptomless leaves on recovered plants were resistant to infection by PStV upon reinoculation but susceptible to infection by tobacco etch virus. Protection or recovery phenotypes were not observed when transgenic plants, immune to PStV infection, were inoculated with other potyviruses or a tobamovirus. After challenge with PStV, plant lines expressing the CP messenger RNA without an engineered ATG (ATG minus) displayed an altered symptom phenotype compared with those on non-transgenic or ATG plus transgenic plants. Symptoms were delayed in appearance or did not appear on ATG minus plants and no recovery from the viral infection was seen even after an extended period of time on those plants exhibiting the delay phenotype. The possibility that an engineered ATG is necessary to obtain the recovery phenotype is discussed.

*Additional keywords*: coat protein-mediated resistance, co-suppression, pathogen-derived resistance.

Peanut stripe virus (PStV) is a member of the genus *Potyvirus* of the plant viruses. These viruses are composed of a single molecule of positive-sense ssRNA of approximately 10,000 nucleotides encapsidated by approximately 2,000 molecules of the coat protein (CP). The RNA genome is translated as a single polypeptide and processed by proteolysis into at least eight functional proteins (Riechmann et al. 1992). PStV has been detected in most of the regions of the world where peanuts (*Arachis hypogaea*) are grown and is a serious problem in Southeast Asia, India, and China, where yields from peanuts and other legumes are severely affected (Adalla and Natural 1988; Mishra et al. 1993). PStV has been observed in the United States (Demski et al. 1984); however, a rigorous seed testing program has prevented the spread of this seed-transmitted virus.

Efforts to identify a peanut variety with resistance to PStV have been unsuccessful, as none of the 10,000 *A. hypogaea* cultivars tested has shown resistance to PStV (Zeyong 1988; D. V. R. Reddy, personal communication). The tools of plant genetic transformation have now made it possible to engineer resistance to PStV as an alternative to other protection methods. Pathogen-derived resistance to plant viruses was first demonstrated by Powell Abel et al. (1986) who introduced the CP coding region of tobacco mosaic virus (TMV) into *N. tabacum* cv. Xanthi nn and obtained plants resistant to TMV infection. Since that time many examples of coat protein-mediated resistance (CP-MR) have been reported (reviewed in Hull and Davies 1992; Lindbo et al. 1993a; Reimann-Philipp and Beachy 1993; Wilson 1993).

The mechanism of CP-MR is not understood at this time. A model describing a mechanism by which transgenic CP may inhibit infection of the tobacco following inoculation with TMV has been put forth (reviewed in Register and Nelson 1992; Reimann-Philipp and Beachy 1993). The evidence suggests that TMV CP expression in plants prior to infection appears to block or limit the initial uncoating of the infecting virus. This protection mechanism may not account for all the protection afforded by TMV in natural cross-protection. Yamaya et al. (1988) demonstrated that expression of the entire TMV genome in transgenic plants provided a much stronger protection against viral infection after challenge with TMV RNA than had been reported by Nelson et al. (1987) for the expression of the TMV CP alone. Recently, Lindbo et al. (1993b) have provided evidence for a different mechanism involved in protection against potyvirus infection. In their model, specific cytoplasmic mRNAs, of transgene or viral origin, are targeted for degradation. The signal for the induction of this degradation pathway may be the overexpression

Corresponding author: R. S. Nelson;
E-mail: RNELSON@aardvark.ucs.uoknor.edu

of foreign potyvirus CP transgene transcript (Lindbo et al. 1992a; Dougherty et al. 1994; Smith et al. 1994). This mechanism is used to explain the lack of continued virus accumulation in plants which initially showed symptoms but continued to grow and eventually recovered. The mechanism by which the cell senses the foreign RNA message levels and induces this type of resistance is not known.

The CP gene from PStV has been cloned and sequenced (Cassidy et al. 1993). To successfully engineer resistance to PStV in peanuts an efficient transformation procedure must first be developed. Some progress has been made toward this end (Lacorte et al. 1991; Franklin et al. 1993; Ozias-Akins et al. 1993; Brar et al. 1994), but as yet attempts to express the PStV CP gene in peanut plants have been unsuccessful. Alternatively, we have studied the potential for effective pathogen-derived resistance against PStV in a model system. In this paper we report the production of two transgenic *N. benthamiana* lines expressing PStV CP gene constructs capable of being translated which develop no visible symptoms and accumulate no detectable PStV virus in systemic tissues. We define these lines as being extremely resistant or "immune" to PStV challenge. We also report that all the plant lines capable of producing all or a portion of the PStV CP, which were initially susceptible to infection by PStV (26 lines total), recovered from the infection. This observation has been reported by a few other researchers employing potyvirus



Fig. 1. Plasmid subclones of the peanut stripe virus (PStV) coat protein (CP) open reading frame and 3′ nontranslated region (NTR) used for *Nicotiana benthamiana* transformation. Each PStV CP coding region and 3′ NTR was subcloned into the *NcoI/XbaI* sites of the shuttle vector, pRTL2 (Restrepo et al. 1990). The flanking *Hind*III sites were used to subclone each construct into the binary vector, pGA482 (An 1987) for *Agrobacterium*-mediated plant transformation. The expected peptide lengths in amino acids (aa) are included in the open bars. The deletion introduced to make the CP-ATG minus construct resulting in an out-of-frame translation product is shown. 35S = cauliflower mosaic virus 35S transcript; FL = full length.

pathogen-derived resistance using the CP gene or its antisense (Regner et al. 1992; Lindbo et al. 1992a; Hammond and Kamo 1993; Maiti et al. 1993; Ravelonandro et al. 1993). Significantly, it was also determined that plant lines expressing the transgene missing the engineered CP translation initiation codon did not recover once they were infected. We believe that the induced resistance shares significant similarity to the cosuppression or gene-silencing phenomenon (Smith et al. 1994; see reviews by Register and Nelson 1992; Lindbo et al. 1993a) and discuss the possibility that a translatable message aids in achieving this phenotype.

## RESULTS

### PStV CP expression constructs.

To define the elements of the PStV CP gene that are required for protection, a number of expression constructs were made (Fig. 1). A construct containing the complete PStV CP coding region and 3′ nontranslated region (full-length CP = CP-FL) was cloned between an enhanced 35S promoter with a tobacco etch virus (TEV) leader sequence and the 35S transcription terminator. The CP-Δ16 construct contained a deletion of the first 16 amino acids at the N terminus and removed the DAG amino acid sequence required for aphid transmission (Atreya et al. 1991). The CP-Δ106 construct contained the carboxy terminal two-thirds of the potyvirus CP having the highest degree of identity with other potyviral CPs. We have previously demonstrated that each of the CP gene constructs synthesize the expected PStV CP polypeptide in *E. coli* (Cassidy et al. 1993). To distinguish between the protein and RNA contributions to the protection, a plasmid identical to the full-length CP construct (CP-FL) but with the engineered ATG codon deleted was produced (CP-ATG minus, Fig. 1). This modification resulted in the potential synthesis of an out-of-frame 37 amino acid peptide initiating 38 nucleotides downstream from the original start of translation. Throughout this manuscript we will refer to PStV CP gene constructs capable of synthesizing authentic PStV CP peptides as ATG plus, whereas the full-length CP gene construct from which the engineered ATG initiation codon had been removed will be referred to as ATG minus (although additional ATG codons do occur further downstream).

### Analysis of transgene and challenge of ATG plus transgenic plants with PStV.

*N. benthamiana* was transformed with these constructs to test their ability to confer resistance to PStV infection. Genomic DNAs from primary transformants were analyzed by PCR for the presence of the expected size copy of the CP gene (data not shown). Plants testing positive by PCR were allowed to self-pollinate and the $R_1$ seed was collected. Segregation ratios were determined by analyzing extracts from each plant for the expression of the NPTII protein and PStV CP by ELISA and Western blot analysis, respectively (data not shown). The plants expressing the CP-FL and CP-Δ16 gene varied in the levels of CP accumulation (i.e., from undetectable levels to as much as 2% of total soluble protein). No CP was detected from the plants expressing the CP-Δ106 construct. This result is similar to that reported by Silva-Rosales et al. (1994) for the expression of a TEV CP gene construct with a large deletion. As discussed by these authors,
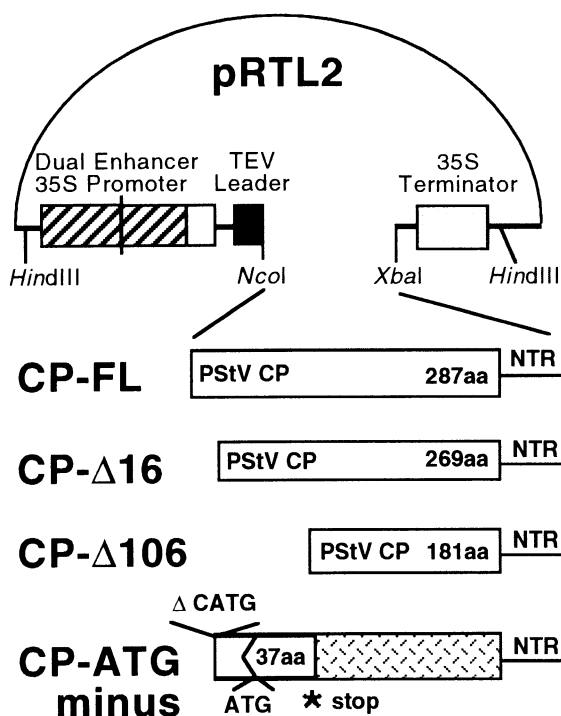
it is possible that the deletion results in a lack of CP stability and therefore undetectable amounts of CP accumulation in transgenic plants. To test for resistance to PStV, $R_1$ plants were mechanically inoculated and visible symptoms were scored on a daily basis. The appearance of symptoms on nontransgenic *N. benthamiana* (control) and transgenic plants was plotted as a function of time (Fig. 2). The plants transformed with each of the PStV CP genes (FL, Δ16, Δ106, and ATG minus) showed a distinct delay of 2 to 5 days in the appearance of symptoms. The delay is in relation to symptom

development in nontransgenic as well as transgenic plants which have segregated negative for the PStV CP gene in $R_2$ populations. It is possible that the delay is a result of a nonspecific effect of the vector sequence as was reported by Ravelonandro et al. (1993). However, in most cases where other investigators have compared results from plants segregating negative for the transgene or plants transformed with a binary vector alone with nontransgenic plants, a delay in symptom development or virus accumulation was not observed. Within lines which were delayed in symptom appear
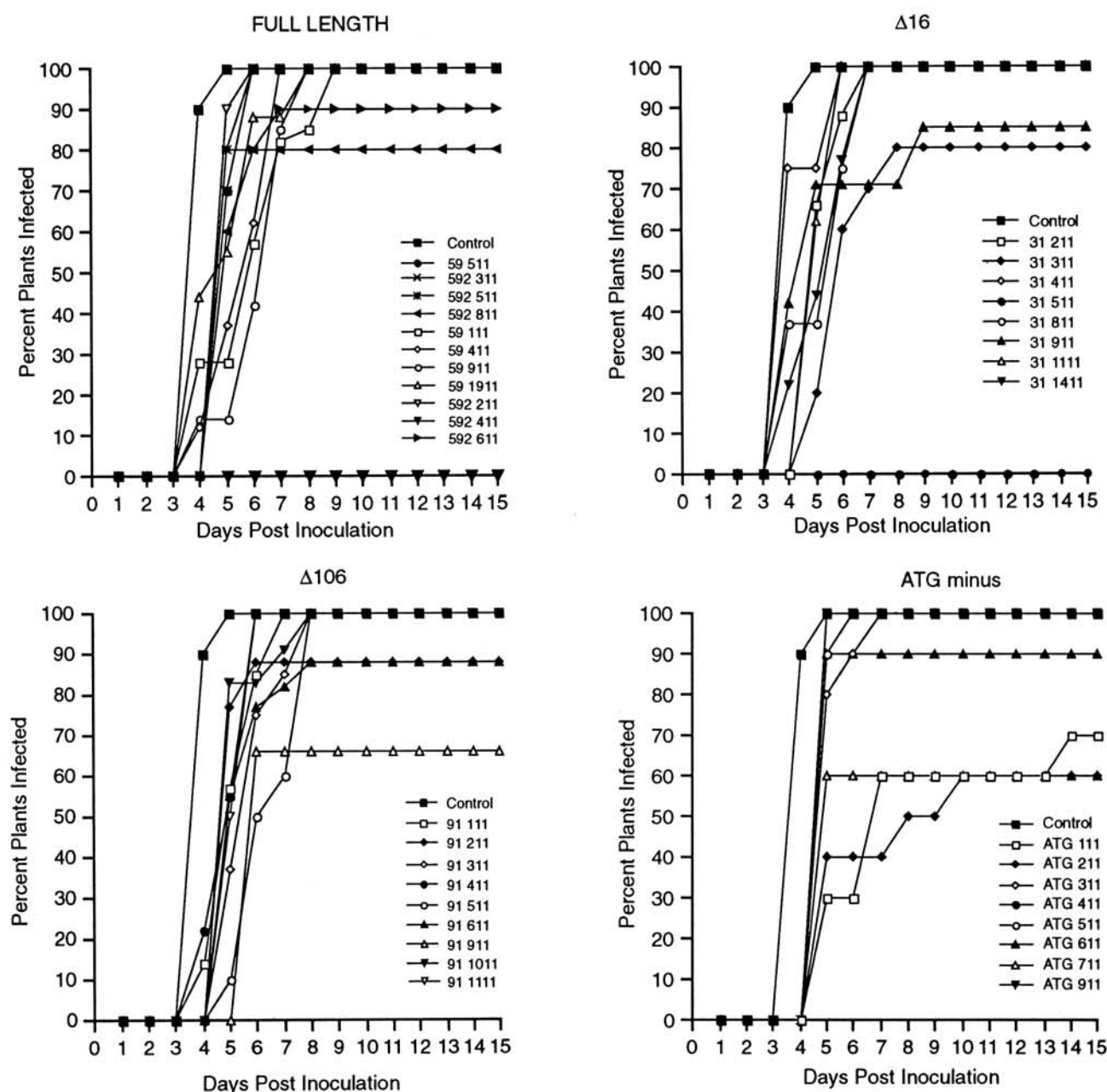


**Fig. 2.** Appearance of symptoms following virus inoculation of plant lines expressing four different peanut stripe virus (PStV) coat protein (CP) gene constructs. Visible symptoms were scored daily following virus inoculation. Each plant line represents an independent transformation event. The individual plant line designation for each CP construct is shown in the graph insert. Control = nontransgenic plants and transgenic plants segregating negative for the CP construct. Full Length = the complete PStV CP coding region and 3′ nontranslated region; Δ16 = the full-length construct minus the first 16 amino acids at the N terminus; Δ106 = the full-length construct minus the first 106 amino acids at the N terminus; ATG minus = the full-length construct minus an engineered ATG codon at the N terminus.

ance, occasionally there were a few plants that did not develop symptoms.

Two lines, CP-FL 592-411 and CP-Δ16 31-511, were found by visual inspection to be "immune" to PStV infection. Plant lines 592-411 and 31-511 were positive for the integrated CP gene construct by PCR and positive for NPTII protein by ELISA but produced no detectable PStV CP polypeptides. Plants that showed no signs of virus were tested by Western blot analysis for the presence of PStV to judge whether visible symptoms accurately reflected the presence of virus. Virus was found only in tissue that had distinct visible symptoms (data not shown).

Homozygous $R_2$ lines were selected from lines segregating 3 plus:1 minus for PStV CP expression. The homozygous progeny from $R_1$ lines which displayed the recovery phenotype also displayed the recovery phenotype (data not shown). Homozygous $R_2$ progeny from the "immune" plant line, CPΔ16 31-511, were all "immune" to PStV (data not shown). This indicates that plants hemizygous for the PStV gene in the $R_1$ generation displayed a visual phenotype similar to that displayed by $R_2$ generation plants homozygous for the PStV gene.

### Recovery of infected ATG plus transgenic plants.

Plants from lines expressing the translated CP messages (CP-FL, CP-Δ16, or CP-Δ106) that became infected with PStV produced new growth which gradually showed fewer visible symptoms from the viral infection (i.e., a recovery phenotype). The development of viral symptoms was increasingly limited to the distal portion of each successive new leaf until new leaves developed that were symptomless (Fig. 3). This transition occurred between 9 and 15 days postinoculation. Leaf tissues of equivalent area and weight were analyzed for virus accumulation from recovering plant lines and nontransgenic control plants. Samples from recovering leaves contained both symptomatic and asymptomatic areas (Fig. 3, leaf 2). The presence of virus was detected only in regions where symptoms were visually apparent and thus viral accumulation per leaf was less in those leaves showing fewer symptoms (Fig. 3). Plants that did not contain the transgenes due to segregation and nontransgenic plants contained similar and significant amounts of virus in each of the systemic leaves tested (Fig. 3, control).

### Down regulation of PStV transgene products following virus challenge.

The level of transgene CP accumulation prior to viral inoculation from 10 plants within a representative plant line (CP-FL 592-911 [9-2-26]) is shown in Figure 4A. Half of the plants were inoculated with PStV. After the virus-inoculated plants displayed the recovery phenotype, leaves from inoculated and uninoculated plants were analyzed again to determine the level of transgene CP accumulation. The level of CP accumulation in the "recovered" leaves was greatly reduced compared with the levels found in analogous leaves from uninoculated plants and in some instances was undetectable (Fig. 4B). This decrease is not due to changes in CP accumulation during the development of the plant as the level of CP accumulation in the uninoculated plants remained unchanged from the lower leaf to the upper leaf (compare lanes 6 to 10 in A versus B, Fig. 4). The levels of NPTII protein, as measured by ELISA before and after recovery, remained unchanged (data not shown). Each of the lines that demonstrated the recovery phenotype were tested in this way with identical results.

In addition to analysis of transgenic CP accumulation, the steady-state levels of transgene CP mRNA in transgenic plants expressing the ATG plus CP constructs (i.e., full-length, Δ16, or Δ106) were measured in leaves prior to inoculation and in upper leaves from plants following recovery (Fig. 5). The steady-state levels of CP mRNA in the upper leaves were found to be reduced as much as 10.8-fold based on equal levels of ribosomal RNA (Fig. 5; line no. CP-FL 1911, plant 6).
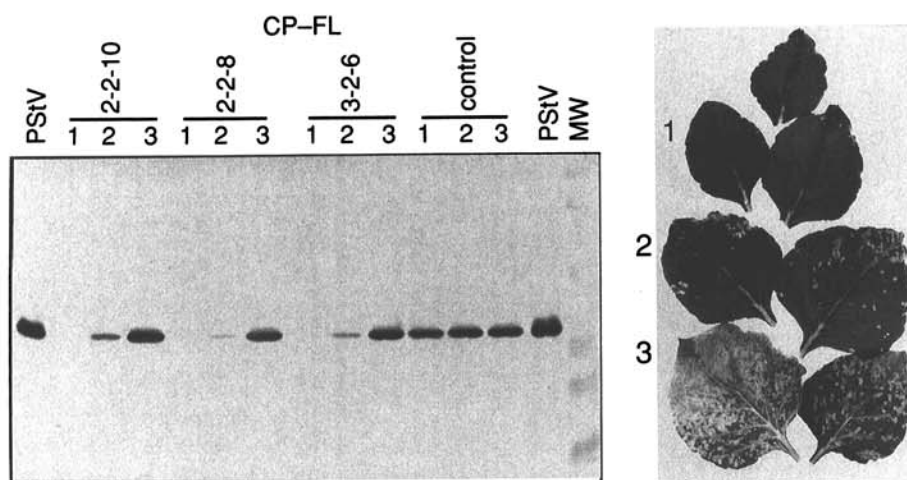


**Fig. 3.** The relationship between viral coat protein (CP) accumulation and visible symptoms in plants displaying the recovery phenotype. Leaf samples from representative plants expressing the full-length peanut stripe virus (PStV) CP gene construct (progeny from plant line CP-FL 592-211 [2-2-10, 2-2-8] and CP-FL 592-311 [3-2-6]) or from a nontransgenic plant (control) were harvested (bottom leaf to top leaf: 3, 2, 1) and analyzed for viral CP accumulation by Western blot analysis. Leaves from a representative plant displaying the recovery phenotype are shown with leaves representative of those sampled for viral CP determinations labeled. Samples from "recovered" leaves contained both symptomatic and asymptomatic areas. Purified PStV was used as a marker for the location of PStV CP (PStV). The location of prestained molecular weight marker (MW) is indicated (molecular weights for bands from bottom to top: 18.5, 27.5, 32.5, 49.5, 80, and 106 kDa).

## Challenge of recovered leaves.

An experiment was conducted to determine whether the recovery phenotype was due to impaired movement of the virus from initially infected tissues or resistance of the recovered tissue to PStV. Transgenic seedlings (plant line CP-FL511 [5-2-3]) were inoculated with either PStV or inoculation buffer alone (mock, Fig. 6). Following the recovery of the PStV-inoculated plants, upper leaves of both mock- and PStV-inoculated plants were inoculated with PStV or TEV. After 5 days, symptoms were visible in the uppermost leaves of all the TEV-inoculated plants. Transgenic plants previously inoculated with PStV did not develop symptoms of virus infection in the upper systemic leaves after inoculation of the "recovered" leaves with PStV. Western blot analysis of the CP

accumulation from these challenged plants confirmed that PStV could not establish an infection in "recovered" tissues, whereas TEV could (Fig. 6).

## Analysis of transgene and challenge of ATG minus transgenic plants with PStV.

As expected, no CP was detected from the transgenic plants expressing the ATG minus CP gene. A wide range of mRNA levels were detected in transgenic plants expressing the ATG minus CP transgene (Fig. 7). After inoculation with PStV, some plants expressing the ATG minus CP gene constructs showed no systemic symptoms (e.g., plants 1 and 5 of plant line 711, RNA levels shown in Fig. 7) but most showed a delay in the appearance of viral symptoms similar to that observed for plants expressing the ATG plus CP gene constructs (e.g., plant 9 of line 711, plants 2 and 6 of line 211 and plants 3 and 7 of line 111, shown in Fig. 7). There was no apparent correlation between the steady-state level of RNA in the sampled leaves versus the timing of appearance or severity of symptoms produced in the plants (compare observations noted above with steady-state RNA levels shown in Fig. 7; data not shown). All control plants and plant 6 of line 711 (Fig. 7) in this experiment showed visible symptoms with no delay compared with the other plants. The visible symptoms on plants expressing the ATG minus CP gene construct were altered compared with nontransgenic plants or plants from ATG plus lines (Fig. 8). Visible symptoms in nontransgenic plants appeared in the second systemic leaf above the inoculated leaves beginning at the base of the leaf and then appeared throughout the rest of the developing upper leaves as they expanded (Fig. 8A). In the ATG minus plants where symptoms appeared, the position of the first visible symptoms was similar to that observed for nontransgenic plants but the time of appearance was delayed and the coverage within each leaf was impaired (Fig. 8C). Unlike the nontransgenic or ATG plus (Fig. 8B) plants, symptoms could not be seen throughout an entire leaf until the fifth or sixth systemic leaf above the inoculated leaves (Fig. 8). In no instance did infected ATG minus plants exhibit a recovery phenotype through 60 days postinoculation.
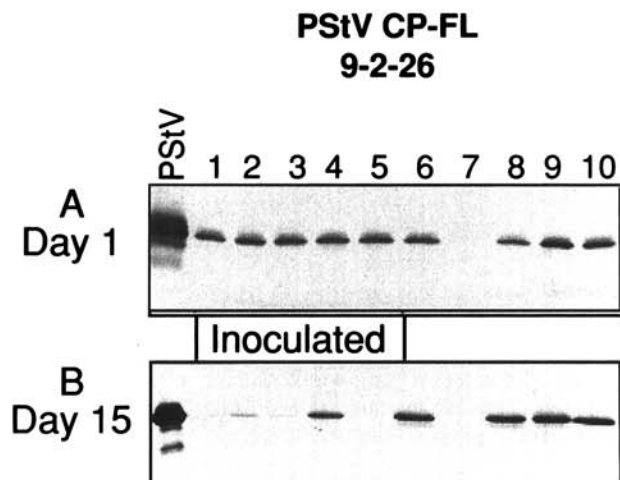
**PStV CP-FL**
**9-2-26**



**Fig. 4.** Accumulation of transgene peanut stripe virus (PStV) coat protein (CP) in plants displaying the recovery phenotype. **A** shows, by Western blot analysis, the level of transgene CP accumulation in 10 plants from the progeny of segregating line CP-FL 59-911 [9-2-26] prior to inoculation with PStV. Plants 1 to 5 were then inoculated with PStV. Fifteen days postinoculation (**B**, day 15) equal size samples from upper leaves were tested for the accumulation of the transgene PStV CP from both inoculated (1 to 5) and uninoculated (6 to 10) plants. Plant number 7 was a segregant negative for NPTII protein and PStV CP.
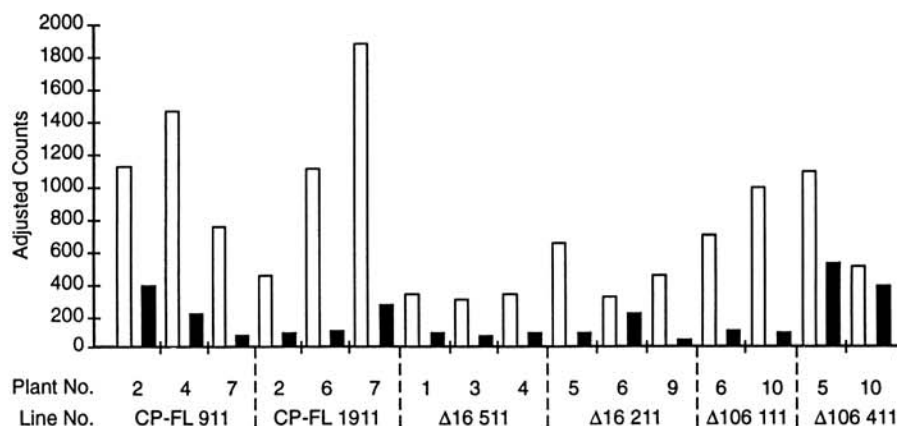


**Fig. 5.** Quantitation of peanut stripe virus (PStV) coat protein (CP) mRNA accumulation in transgenic plants expressing ATG plus CP gene constructs before virus infection and following recovery. The open and solid bars represent the CP mRNA levels before infection and following recovery, respectively. The representative plant lines and individual plant numbers have been marked. The adjusted counts represent the hybridization signal of the CP mRNA normalized to the signal detected by a ribosomal probe to the same samples.

## Challenge of plant lines immune to PStV with other viruses.

The transgenic plant lines "immune" to PStV infection, CP-FL 592-411, and CP-Δ16 31-511, were tested for resistance to three other potyviruses; peanut mottle virus, tobacco etch virus, and potato virus Y. In each case, mechanical inoculation of the heterologous potyvirus resulted in visible symptoms as severe as that observed in nontransgenic *N. benthamiana* (data not shown). These lines were also tested for resistance to the tobamovirus, tobacco mosaic virus. The transgenic lines were not resistant and soon died from the infection.

## DISCUSSION

Results from this study indicate that transgenic *N. benthamiana* plants expressing ATG plus PStV CP constructs of various lengths or a PStV CP full-length ATG minus construct all were protected to varying degrees after challenge with PStV. As has been reported in many cases involving CP-MR, the level of protection did not correlate with the level of CP expression (see review by Lindbo et al. 1993a). In our experiments, the plant lines that were immune to PStV infection, FL-CP 592-411 and CP-Δ16 31-511, did not express detectable levels of protein, whereas plant lines producing CP at a level of 2% of total soluble protein were not immune. The majority of plant lines expressing ATG plus constructs with or without detectable levels of CP responded to the initial viral infection through what appears to be an inducible system resulting in the recovery of the infected plants from PStV infection, and a reduction in transgene-derived CP and CP mRNA accumulation. Such a recovery phenotype for plants expressing sense or antisense constructs of potyvirus CP sequences has been observed previously (Regner et al. 1992; Hammond and Kamo 1993; Lindbo et al. 1992a, Ravelonandro et al. 1993; Dougherty et al. 1994). The lack of CP accumulation in all leaves of the "immune" lines may be due to the presence of the protection mechanism identical to that

activated in systemic leaves of recovered plants as suggested by Lindbo et al. (1993b). Although our plants expressing translatable constructs were "immune" or recovered, plants expressing the ATG minus construct (eight lines) never displayed the recovery phenotype. Although these plants did not recover, the visible phenotype in transgenic plants expressing the ATG minus construct indicates a reduction in viral infectivity. This could be attributed to an effect on viral replication, movement, or both. We have not completed experiments to determine the mechanism involved in this symptom phenotype.

Although the mechanism by which the level of transgene-derived CP mRNA is controlled is unknown, several examples of mRNA regulation by specific degradation within eukaryotic cells have been described (see reviews by Sullivan and Green 1993; Matzke and Matzke 1993; Peltz et al. 1994) and in some instances related to the potyvirus protection phenomenon (Lindbo et al. 1993b, Dougherty et al. 1994, Smith et al. 1994, also see reviews by Register and Nelson 1992; Lindbo et al. 1993a). In the Matzke and Matzke (1993) review, *trans*-inactivation is described as involving the inhibition of expression of one gene by another gene that has regions homologous to the first. One type of *trans*-inactivation, referred to as cosuppression, involves the down regulation of an endogenous gene and a transgene by the presence of the transgene. Although cosuppression has generally been described for mRNA from endogenous genes or stably integrated transgenes, the phenomenon has been observed for transcripts produced in a transient expression system. (Hobbs et al. 1993). The relationship of this observation to that of the potyvirus protection phenomenon, where the virus would be acting as a transient expression vector, is intriguing. Lindbo et al. (1993b) observed a reduction in the steady-state levels of TEV CP transgene mRNA in recovered tissue of tobacco, and we obtained the same result in our studies with PStV (Fig. 5). Using nuclear run-off analyses, they determined that transcription rates were not impaired for the transgene, indicating that the control of mRNA levels is posttranscriptional and
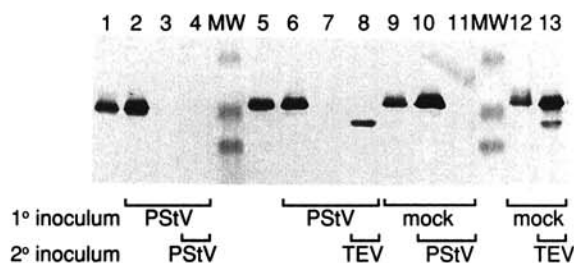


**Fig. 6.** Accumulation of peanut stripe virus (PStV) and tobacco etch virus coat protein (CP) in leaves above those showing "recovery" and challenged with PStV. Lanes 1 and 5 contain samples from lower leaves prior to primary (1°) virus inoculation and lanes 9 and 12 contain samples from upper leaves of mock-inoculated plants prior to secondary (2°) virus inoculation. Lanes 2, 6, and 10 contain samples from leaves showing symptoms of PStV infection following PStV inoculation. Lanes 3, 7, and 11 contain samples from leaves displaying the recovery phenotype. Lanes 4 and 10 and 8 and 13 are from upper systemic leaves following the secondary inoculation with PStV and TEV, respectively. The PStV CP migrates as a 33-kDa protein and the TEV CP migrates as a 30 kDa protein. The molecular weight markers (MW) are Sigma prestained low molecular weight markers (top to bottom—49.5, 32.5, and 27.5 kDa).
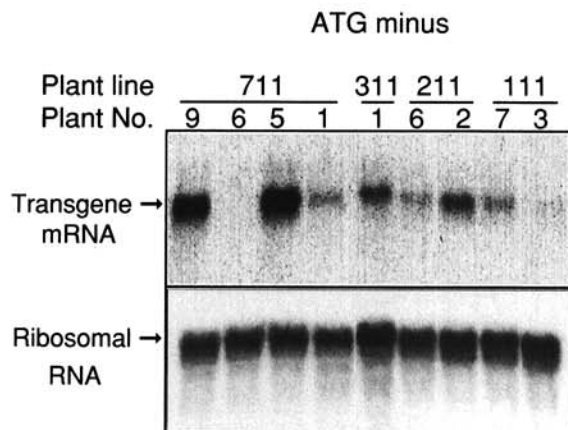


**Fig. 7.** Analysis of the accumulation of transgene mRNA from representative plants expressing the ATG minus gene construct in $R_1$ segregating plant lines. Approximately 5 µg of total RNA from each transgenic plant was probed with a peanut stripe virus (PStV) coat protein (CP) riboprobe (top panel, transgene mRNA). The same blot was stripped and reprobed with a random-primed rRNA probe (lower panel, ribosomal RNA). Plant number 6 of line 711 was segregating negative for NPTII expression.

probably takes place in the cytoplasm. It will be important to determine whether the plant lines that are immune to PStV infection represent lines where the induced recovery mechanism has already been activated, resulting in the immediate degradation of the viral inoculum and immunity. The difference between recovery and immunity may be as simple as the active gene copy number or level of transcript as suggested by Barker et al. (1993) for coat protein gene-mediated resistance against potato leafroll virus or by de Carvalho et al. (1992) for cosuppression.

It has been suggested that translation is not necessary for the induction of the protection (Lindbo et al. 1993a); however, examples exist where translation has an important role in both the induction and specificity of the protection. An example of such a linked system is the specific degradation of β-tubulin mRNA in response to tubulin subunit concentrations (Theodorakis and Cleveland 1992). In this system, recognition of β-tubulin mRNA occurs through a cotranslational event requiring the synthesis of only the amino terminal tetrapeptide, resulting in the specific regulation of β-tubulin mRNA levels. Any change in the first four amino acids abolished the regulation of the mRNA levels (Yen et al. 1988). For plants displaying pathogen-derived resistance, the necessity for amino terminal sequence conservation was demonstrated for alfalfa mosaic virus where a single amino acid substitution at position 2 within the transprotein was sufficient to eliminate the protection afforded by the CP construct in trangenic tobacco plants (Tumer et al. 1991). Recently, Taschner et al. (1994) have extended this work showing that expression of the mutant CP gene construct in plants was sufficient to give protection against a virus containing the mutated CP gene. They interpreted these results as indicating that the CP and not the CP mRNA was responsible for protection. In our study, 26 tobacco lines that expressed ATG-plus constructs and were not immune to PStV showed a recovery phenotype, while all eight lines that expressed ATG-minus constructs did not recover. Thus, it appears that the CP mRNA alone is insufficient to induce the elements required for the plant to recover from the initial viral infection. Most previous observations on potyvirus CP sense constructs indicate that those constructs that have four or more amino acids synthesized displayed a recovery phenotype while those that were ATG minus were not characterized for this phenotype (Lindbo and Dougherty 1992a, 1992b; Regner et al. 1992; Ravelonandro et al. 1993; van der Vlugt et al. 1992; Farinelli and Malnoe 1993; Lindbo et al. 1993a). Very recently, however, Smith et al. (1994) published work where the CP of potato virus Y potyvirus (PVY) was not expressed, at least from the N-terminal position, due to a frameshift and stop only seven nucleotides downstream from the engineered AUG. Only a dipeptide, with no similarity to the viral sequence in this region, may be produced by this construct before translational termination. Some plant lines expressing this construct are highly resistant to PVY challenge, having no accumulation of virus in the inoculated leaves. One may conclude from this work that specificity for the protection is in the RNA sequence; however, the possibility that translation initiation per se helps to induce the protection system cannot be discounted. Our results suggest that it is important for an engineered AUG to be present to obtain the recovery phenotype.
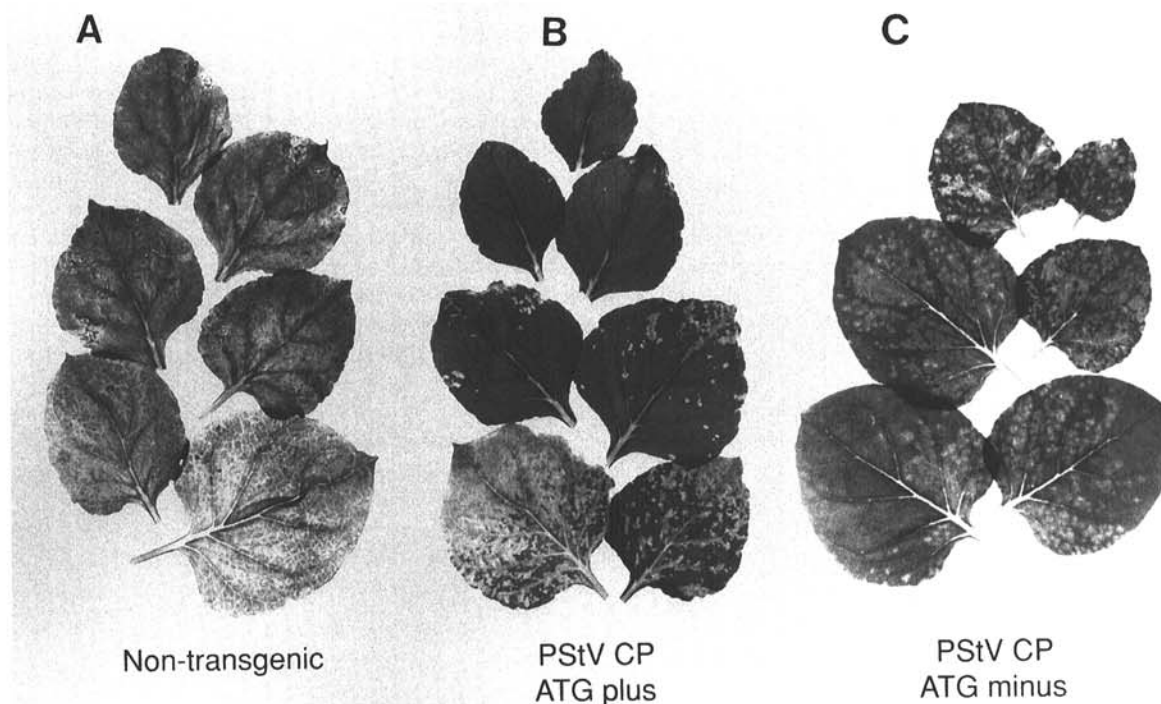


**Fig. 8.** Symptoms of peanut stripe virus (PStV) infection on representative transgenic and nontransgenic *Nicotiana benthamiana*. Plants were photographed at the same time after inoculation. Beginning two leaves above the inoculated leaf, leaves were removed from the main stem and positioned in ascending order as they were on the plant. The leaves from a nontransgenic plant (nontransgenic, **A**) show a full PStV infection. The leaves from the ATG plus plant (PStV CP ATG plus, **B**) show the development of the recovery phenotype. The leaves from the ATG minus plant (PStV CP ATG minus, **C**) show the delayed and limited initial infection followed by a limited, full infection and lack of recovery.

Whether this is simply due to a stabilization of transcript to allow the mRNA surveillance system to be induced or is necessary for specifying sequences for degradation requires further study.

## MATERIALS AND METHODS

### Vector construction and plant transformation.

Cloning of the PStV CP gene has been described previously (Cassidy et al. 1993). The full-length PStV CP construct (FL, previously referred to as 5'–3'), Δ16 and Δ106 constructs were subcloned into the vector pRTL2 (Restrepo et al. 1990; Fig. 1) and subsequently into the binary vector, pGA482 (An 1987) utilizing the *Hind* III sites at each end. The plasmid, CP-ATG (ATG minus), was made by digesting the plasmid, CP-FL, with *Nco*I, blunting the overhanging ends with mung bean nuclease and religating (Fig. 1). The four-nucleotide deletion, including the ATG, was confirmed by sequencing the resulting plasmid.

Leaf disks of sterile *N. benthamiana* were transformed using *Agrobacterium tumefaciens* strain LBA4404 harboring the pGA482 binary vector with each of the PStV CP constructs (Horsch et al. 1988). DNA extracted from transgenic plants was analyzed for the presence of the transgene by PCR using a primer to the 35S promoter and the 3' end of the PStV CP gene. Primary transformants with intact transgenes were allowed to self pollinate and the $R_1$ seed collected.

### Viral inoculations and observations.

Plants approximately 8 weeks old were mechanically inoculated with sap from a previously infected *N. benthamiana* plant. Seedlings that were 4 weeks old were used for experiments that required a secondary inoculation. The younger plants responded to the primary virus infection in a similar fashion as older plants. A disk from a leaf of a systemically infected plant was cut with a #7 cork borer (~15 mg fresh weight) and ground in 1.4 ml of inoculation buffer (50 mM sodium phosphate, pH 7.5, 1 mM ethylenediametetraacetic acid [EDTA]). The first leaf with a midrib larger than 5 cm and the next leaf below were each dusted with Carborundum and 50 µl of sap was spread over the surface using 10 strokes with a gloved hand. TEV (a gift from J. Carrington, Texas A&M University) and PVY (a gift from J. Sherwood, Oklahoma State University) were propagated in *N. benthamiana* and sap inocula were similarly made. The dilutions of sap which consistently gave 100% infection on nontransgenic *N. benthamiana* were used as inoculum in the protection experiments with the transgenic plants. Purified TMV was inoculated at a concentration of 2 µg/ml. Virus symptoms were visually scored by noting the time of the onset of symptoms.

### Coat protein analysis.

The level of neomycin phosphotransferase II (NPTII) and PStV CP in each of the transgenic plants tested for protection was determined. The NPTII levels were determined using an enzyme-linked immunosorbent assay (ELISA) kit (5 Prime-3 Prime, Inc., Boulder, Col.) with a modification of the suggested procedure. Instead of grinding each leaf sample in Tris/phenylmethylsulfonylfluoride (PMSF) buffer the disks were cut to fit into a microtiter well where they were incubated overnight at 4°C in 100 µl of Tris/PMSF buffer. The

following day the disks were shaken from the wells and the plate processed as suggested by the manufacturer of the ELISA kit. The color reaction was measured using a Dynatech MR5000/7000 Microplate reader (Dynatech, Chantilly, Va.). Each of the transgenic plants was also tested for CP expression by Western analysis (Towbin et al. 1979). PStV was detected with rabbit polyclonal serum made against the PStV virion or CP expressed in *E. coli* from either J. Sherwood (Oklahoma State University, (Culver et al. 1989) or D. V. R. Reddy (ICRISAT, India), respectively. Initial screening of the transgenic plants for the presence of the transgene CP was done by extracting total soluble proteins from a leaf disk cut with a #7 cork borer in 150 µl of Laemmli buffer (Laemmli 1970) and subsequently for virus CP by extracting the same size leaf disk in phosphate-buffered saline (Sambrook et al. 1989). TEV CP was detected using the polyclonal antisera from D.V.R. Reddy which cross-reacts with TEV (unpublished results).

### RNA analysis.

Total nucleic acids were isolated from leaf tissue by grinding in liquid nitrogen and extracting with a buffered phenol/chloroform solution (0.1 M glycine, 50 mM NaCl, 10 mM EDTA, 2% SDS, 1% sodium lauryl sarcosine, pH 9.0 [extraction buffer] mixed 50:50 with phenol:chloroform [24:1]). The nucleic acids were then precipitated with an equal volume of isopropanol and 1/10 volume of 3 M sodium acetate pH 5.0. The DNA was spooled out using a glass rod and the RNA was collected by centrifugation. Both the DNA and RNA were resuspended in extraction buffer and reextracted once with phenol:chloroform, once with chloroform and reprecipitated. The final pellets were washed with 70% ethanol and vacuum dried briefly. The pellets were then resuspended in 20 to 50 µl of an RNAse free solution containing 10 mM Tris and 1 mM EDTA. The DNA was used in PCR analysis for determining the intactness of the PStV CP transgene. The RNA was used in both slot blot and Northern blot analyses using GeneScreen Plus (Du Pont, Boston, Massachusetts) performed according to the procedure recommended by the manufacturer. Labeled in vitro transcript complementary to nucleotides 8944 to 10,059 of the viral genome was first hybridized to the blot to detect the PStV CP mRNA and then to a random primed rRNA probe (Eckenrode et al. 1985) to normalize the hybridization signal for differences in RNA loading. The hybridization signals from the RNA blots were quantitated using a Molecular Dynamics PhosphoImager SF (Sunnyvale, Calif.).

## LITERATURE CITED

Adalla, C. B., and Natural, M. P. 1988. Peanut stripe virus disease in the Philippines. ICRISAT, Patancheru, India. p. 9.

An, G. 1987. Binary Ti vectors for plant transformation and promoter analysis. Methods Enzymol. 153:292-305.

Atreya, P. L., Atreya, C. D., and Pirone, T. P. 1991. Amino acid substitu-

tions in the coat protein result in loss of insect transmissibility of a plant virus. Proc. Natl. Acad. Sci. USA 88:7887-7891.

Barker, H., Reavy, B., Webster, K. D., Jolly, C. A., Kumar, A., and Mayo, M. A. 1993. Relationship between transcript production and virus resistance in transgenic tobacco expressing the potato leafroll virus coat protein gene. Plant Cell Rep. 13:54-58.

Brar, G. S., Cohen, B. A., Vick, C. L., and Johnson, G. W. 1994. Recovery of transgenic peanut (Arachis hypogaea L.) plants from elite cultivars utilizing ACCELL® technology. Plant J. 5:745-753.

Cassidy, B., Sherwood, J. L., and Nelson, R. S. 1993. Cloning of the capsid protein gene from a blotch isolate of peanut stripe virus. Arch. Virol. 128:287-297.

Culver, J. N., Sherwood, J. L., and Sanborn, M. R. 1989. Use of monoclonal antibodies in detection and serological classification of peanut stripe virus. Peanut Sci. 16:63-66.

de Carvalho, F., Gheysen, G., Kushnir, S., Van Montagu, M., Inze, D., and Castresana, C. 1992. Suppression of β-1,3-glucanase transgene expression in homozygous plants. EMBO J. 11:2595-2602.

Demski, J. W., Reddy, D. V. R., Sowell, J. G., and Bays, D. 1984. Peanut stripe virus—a new seed-borne potyvirus from China infecting groundnut (Arachis hypogaea). Ann. Appl. Biol. 105:495-501.

Dougherty, W. G., Lindbo, J. A., Smith, H. A., Parks, T. D., Swaney, S., and Proebsting, W. M. 1994. RNA-mediated virus resistance in transgenic plants: Exploitation of a cellular pathway possibly involved in RNA degradation. Mol. Plant-Microbe Interact. 7:544-552.

Eckenrode, V. K., Arnold, J., and Meagher, R. B. 1985. Comparison of the nucleotide sequence of soybean 18S rRNA with the sequences of other small-subunit rRNAs. J. Mol. Evol. 21:259-269.

Farinelli, L., and Malnoe, P. 1993. Coat protein gene-mediated resistance to potato virus Y in tobacco: Examination of the resistance mechanisms—Is the transgenic coat protein required for protection? Mol. Plant-Microbe Interact. 6:284-292.

Franklin, C. I., Trieu, T. N., Cassidy, B. G., Dixon, R., and Nelson, R. 1993. Genetic transformation of green bean callus via Agrobacterium mediated DNA transfer. Plant Cell Rep. 12:74-79.

Hammond, J., and Kamo, K. K. 1993. Transgenic coat protein and antisense RNA resistance to bean yellow mosaic potyvirus. Acta Hortic. 336:171-178.

Hobbs, S., Warkentin, T., and DeLong, C. 1993. Transgene copy number can be positively or negatively associated with transgene expression. Plant Mol. Biol. 21:17-26.

Horsch, R. B., Fry, J. E., Hoffmann, N. L., Neidermeyer, J., Rogers, S. G., and Fraley, R. T. 1988. Plant Molecular Biology Manual. Kluwer Academic Publishers, Dordrecht, Belgium. A5:1-5.

Hull, R., and Davies, J. W. 1992. Approaches to nonconventional control of plant virus disease. Crit. Rev. Plant Sci. 11:17-33.

Lacorte, C., Mansur, E., Timmerman, B., and Cordeiro, A. 1991. Gene transfer into peanut (Arachis hypgaea L.) by Agrobacterium tumefaciens. Plant Cell Rep. 10:354-357.

Laemmli, E. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

Lindbo, J. A., and Dougherty, W. G. 1992a. Pathogen-derived resistance to a potyvirus: Immune and resistant phenotypes in transgenic tobacco expressing altered forms of a potyvirus coat protein nucleotide sequence. Mol. Plant-Microbe Interact. 5:141-153.

Lindbo, J. A., and Dougherty, W. G. 1992b. Untranslatable transcripts of the tobacco etch virus coat protein gene sequence can interfere with tobacco etch replication in transgenic plants and protoplasts. Virology 189:725-733.

Lindbo, J. A., Silva-Rosales, L., and Dougherty, W. G. 1993a. Pathogen derived resistance to potyviruses: Working, but why? Sem. Virol. 4:369-379.

Lindbo, J. A., Silva-Rosales, L., Proebsting, W. M. and Dougherty, W. G. 1993b. Induction of a highly specific antiviral state in transgenic plants: Implications for regulation of gene expression and virus resistance. Plant Cell 5:1749-1759.

Maiti, I. B., Murphy, J. F., Shaw, J. G., and Hunt, A. G. 1993. Plants that express a potyvirus proteinase gene are resistant to virus infection. Proc. Natl. Acad. Sci. USA 90:6110-6114.

Matzke, M., and Matzke, A. J. M. 1993. Genome imprinting in plants: Parental effects and trans-inactivation phenomena. Annu. Rev. Plant Physiol. Plant Mol. Biol. 44:53-76.

Mishra, A., Gohel, V. R., Valand, G. B., Patel, J. G., and Shukla, D. D. 1993. Peanut stripe virus disease of groundnut—a review. Int. J. Pes-

tic. Mngmt. 39:210-215.

Nelson, R.S., Powell-Abel, P., and Beachy, R.N. 1987. Lesions and virus accumulation in inoculated transgenic tobacco plants expressing the coat protein gene of tobacco mosaic virus. Virology 158:126-132.

Ozias-Akins, P., Schnall, J. A., Anderson, W. F., Singsit, C., Clemente, T. E., Adang, M. J., and Weissinger, A. K. 1993. Regeneration of transgenic peanut plants from stably transformed embryogenic callus. Plant Sci. 93:185-194.

Peltz, S. W., He, F., Welch, E., and Jacobson, A. 1994. Nonsense-mediated mRNA decay in yeast. Progr. Nucleic Acid Res. Mol. Biol. 47:271-298.

Powell Abel, P., Nelson, R. S., De, B., Hoffmann, N., Rogers, S. G., Fraley, R. T., and Beachy, R. N. 1986. Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. Science 232:738-743.

Ravelonandro, M., Monsion, M., Delbos, R., and Dunez, J. 1993. Variable resistance to plum pox virus and potato virus Y infection in transgenic Nicotiana plants expressing plum pox virus coat protein. Plant Sci. 91:157-169.

Register, J. C., and Nelson, R. S. 1992. Early events in plant virus infection: Relationships with genetically engineered protection and host gene resistance. Sem. Virol. 3:441-451.

Regner, F., da Camara Machado, A., da Camara Machado, M. L., Steinkellner, H., Mattanovich, D., Hanzer, W., and Katinger, H. 1992. Coat protein mediated resistance to plum pox virus in Nicotiana clevelandii and N. benthamiana. Plant Cell Rep. 11:30-33.

Reimann-Philipp, U., and Beachy, R. N. 1993. The mechanism(s) of coat protein-mediated resistance against tobacco mosaic virus. Sem. Virol. 4:349-356.

Restrepo, M. A., Freed, D. D., and Carrington, J. C. 1990. Nuclear transport of plant potyviral proteins. Plant Cell 2:987-998.

Riechmann, J. L., Laín, S., and Garcia, J. A. 1992. Highlights and prospects of potyvirus molecular biology. J. Gen. Virol. 73:1-16.

Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Silva-Rosales, L., Lindbo, J. A., and Dougherty, W. G. 1994. Analysis of transgenic tobacco plants expressing a truncated form of a potyvirus coat protein nucleotide sequence. Plant Mol. Biol. 24:929-939.

Sullivan, M. L., and Green, P. J. 1993. Post-transcriptional regulation of nuclear-encoded genes in higher plants: The roles of mRNA stability and translation. Plant Mol. Biol. 23:1091-1104.

Smith, H. A., Swaney, S. L., Parks, T. D., Wernsman, E. A., and Dougherty, W. G. 1994. Transgenic plant virus resistance mediated by untranslatable sense RNAs: Expression, regulation, and fate of nonessential RNAs. Plant Cell 6:1441-1453.

Taschner, P. E. M., Van Marle, G., Brederode, F. T., Tumer, N. E., and Bol, J. F. 1994. Plant transformed with a mutant alfalfa mosaic virus coat protein gene are resistant to the mutant but not to wild-type virus. Virology 203:269-276.

Theodorakis, N. G., and Cleveland, D. W. 1992. Physical evidence for cotranslational regulation of β-tubulin mRNA degradation. Mol. Cell. Biol. 12:791-799.

Towbin, H., Staehelin, T., and Gordon, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. USA 76:4350-4354.

Tumer, N. E., Kaniewski, W., Haley, L., Gehrke, L., Lodge, J. K., and Sanders, P. 1991. The second amino acid of alfalfa mosaic virus coat protein is critical for coat protein-mediated protection. Proc. Natl. Acad. Sci. USA 88:2331-2335.

van der Vlugt, R. A. A., Ruiter, R. K. and Goldbach, R. 1992. Evidence for sense RNA-mediated protection to PVY^N in tobacco plants transformed with the viral coat protein cistron. Plant Mol. Biol. 20:631-639.

Wilson, T. M. A. 1993. Stategies to protect crop plants against viruses: Pathogen-derived resistance blossoms. Proc. Natl. Acad. Sci. USA. 90:3134-3141.

Yamaya, J., Yoshioka, M., Meshi, T., Okada, Y., and Ohno, T. 1988. Cross protection in transgenic tobacco plants expressing a mild strain of tobacco mosaic virus. Mol. Gen. Genet. 215:173-175.

Yen, T. J., Machlin, P. S., and Cleveland, D. W. 1988. Autoregulated instability of β-tubulin mRNAs by recognition of the nascent amino terminus of β-tubulin. Nature 334:580-585.

Zeyong, X. 1988. Research on peanut stripe virus disease in the People's Republic of China. ICRISAT, Patancheru. p. 6.