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Tobacco plants transformed with an untranslatable form of the coat protein gene of the *Potato virus Y* are resistant to viral infection

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Abstract

The coat protein (CP) gene of the *Potato virus Y* common strain (PVY^O), rendered untranslatable by introduction of a stop codon immediately after the initiation codon, was cloned into an *Agrobacterium tumefaciens* transformation vector. The regenerated transgenic tobacco plants were analyzed for the presence of the CP gene. Although transgene transcripts were observed in all transgenic lines tested, three different phenotypes of resistance were observed, including high resistance, partial resistance, and susceptibility. An inverse correlation between the transgene transcript steady-state levels and resistance of transgenic plants to PVY^O was observed. The results obtained suggest that the resistance observed in the transgenic plants is essentially due to the presence of multiple PVY transgenes that accumulate low levels of the transgene transcript. A post-transcriptional RNA degradation process appears to support the transgene transcript reduction within the RNA-mediated resistance mechanism.

Introduction

Potato virus Y (PVY), a well-characterized aphid-transmitted potyvirus, is the type species of the genus Potyvirus in the family Potyviridae. It contains a positive single-stranded RNA molecule of 10 kilobases (kb), coding for a single, large polyprotein that is cleaved by at least three virus-specific encoded proteinases (Dougherty and Carrington, 1988; Riechmann et al., 1992). The complete nucleotide (nt) sequences have been determined for several potyviruses, including PVY-N (necrotic strain) (Robaglia et al., 1989). It was initially demonstrated that expression of the coat protein (CP) gene of *Tobacco mosaic virus* (TMV) in transgenic plants could provide effective protection against this virus (Powell-Abel et al., 1986). Since then, similar results have been obtained with several virus groups (for reviews, see Beachy et al., 1990; Gadani et al., 1990; Nejidat et al., 1990; Wilson, 1993). In addition to the CP-mediated mechanism, there are several other cases of virus defense in plants, where the resistance has been demonstrated to occur at the RNA level (Lindbo and Dougherty, 1992a,b). A translation product does not appear to be necessary for the resistant state (de Haan et al., 1992; Lindbo and Dougherty, 1992a,b; Van der Vlugt et al., 1992; Pang et al., 1993). Introduction of an untranslatable mRNA effectively generated transgenic plants resistant to several potyviruses for example to *Tobacco etch virus* (TEV) (Dougherty et al., 1994), to PVY (Van der Vlugt et al., 1992), and to *Peanut stripe virus* (PStV) (Cassidy and Nelson, 1995). Resistance observed in the transgenic plants seems to be based on the presence of CP RNA sequences rather than on the accumulation of viral CP. A phenomenon known as gene silencing, co-suppression, sense suppression, or RNA interference, has occurred in many transgenic plants.

The introduction of virus-derived sense or antisense RNA in transgenic plants conferring RNA-mediated virus resistance, induces a post-transcriptional gene silencing (PTGS) (Baulcombe, 1996; Stam et al., 1997). Homology-dependent gene silencing appears to be an innate immune response to virus infection in plants (Waterhouse et al., 1998; Baulcombe, 1999).

To explain the effectiveness of transgene mRNA silencing of viral genomic RNA, it has been suggested that a plant-encoded RNA-dependent RNA polymerase makes a complementary strand from the transgene mRNA and that the small cRNAs potentiate the degradation of the target RNA (Ruiz et al., 1998; Baulcombe, 1999). Moreover, Han et al. (1999) suggested that resistance against PVY is the consequence of PTGS directed by homologous transgenes and cRNAs are synthesized to mediate the degradation. A ribonuclease activity was detected which appeared to be specific for this transcript in the PVY-resistant transgenic plants.

The model proposed by Waterhouse et al. (1998) provides a mechanism by which dsRNA can direct sequence specific degradation of RNAs in the cytoplasm. In support of the proposed relationship between PTGS and natural virus resistance, it has been shown that a species of small antisense RNA (25 nucleotides) in plants is likely synthesized from an RNA template and may represent the specificity determinant of PTGS (Hamilton and Baulcombe, 1999). PTGS is often associated with multicopy T-DNA loci and the repetitive nature of a locus appeared essential for eliciting such mechanism (Stam et al., 1997).

Here, we provide analysis of a series of transgenic tobacco plants that contain an untranslatable form of the CP gene of PVY. A high level of resistance towards mechanical inoculation with PVY^O was observed in one transgenic line. However, in three other transgenic lines, symptoms were developed with a delay in their appearance and with a reduction in virus accumulation. The phenomenon of recovery was not observed in our experiments. The mRNA accumulation and steady-state RNA levels of the transgene-derived transcript present in different members of the genetic series supported a post-transcriptional RNA degradation process as the underlying mechanism for transgene transcript reduction and virus resistance.

Materials and methods

Virus and gene construct

Potato virus Y common strain (PVY^O) was propagated in tobacco (*Nicotiana tabacum* cv. *Xanthi*) by mechanical inoculation under greenhouse conditions. Virus purification was as described by Dougherty and Hiebert (1980) with some modifications. The extracted RNA was stored at $-80\,^{\circ}C$ until further use.

A cDNA copy of the PVY^O CP cistron with the 3′-non translated region (3′-NTR) was synthesized from PVY^O genomic purified RNA by reverse transcription coupled with the polymerase chain reaction (PCR). To introduce an ATG initiation codon, followed by a frameshift mutation at the beginning of the CP coding sequence, we have synthesized an oligonucleotide containing the first 20 nucleotides of the PVY-CP sequence, engineered with an ATG start codon in an optimal context of translation initiation (Kozak, 1981), and followed by three stop codons (see Figure 1) (kh1: 5′-CGCCAGG**ATG**GCA^{T}AATGA CACAATTGATGC-3′). The untranslated form of the PVY CP sequence was amplified by PCR, using the kh1 and an oligodT primers. The amplified DNA was purified by preparative agarose gel electrophoresis and cloned into the T-vector of pBluescript prepared previously as recommended by Marchuk et al. (1991).

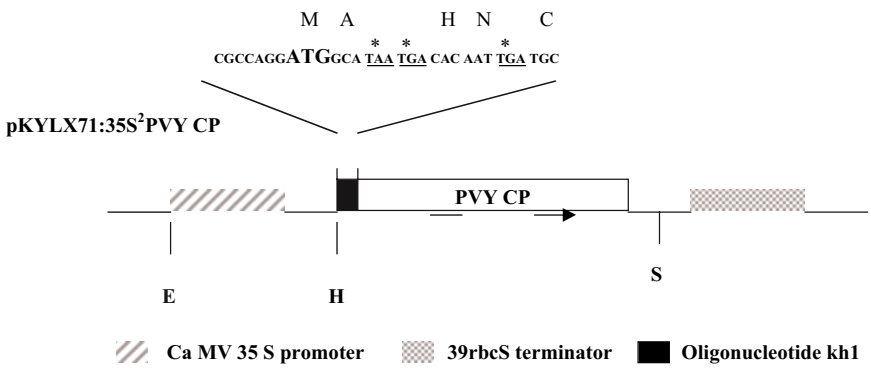

Figure 1. Schematic representation of the plant transformation vector pKYLX71 : $35S^2$ PVY CP, containing the untranslatable PVY^O CP gene inserted between the enhanced CaMV 35S promoter and the 3′rbcS terminator region. E = *EcoR*I, H = *Hind*III, S = *Sst*I.

The resulting plasmid pBS-PVY CP was digested with *Hind*III-*Sst* I, and the appropriate fragments were cloned into the plant gene expression vector pKYLX71 : 35S^2, containing two enhanced 35S promoter in tandem (Maiti et al., 1993). The resulting pKYLX71 : 35S^2-PVY CP construct contained the PVY CP ORF in the sense orientation but with three stop codons downstream from the initiation codon. The sequence of the modified start region was confirmed by sequence analysis (data not shown). These mutations should prevent production of the capsid protein. The recombinant transformation vector pKYLX71 : 35S^2-PVY CP was mobilized into *Agrobacterium tumefaciens* strain LBA4404 by electroporation according to Mersereau et al. (1990).

Plant transformation and analysis

For the transformation experiments, tobacco plants (*Nicotiana tabacum* cv. *Xanthi*) were grown in MS medium in a growth chamber at 25 °C with a day length of 16 h under 3000 lux light intensity.

Nicotiana tabacum cv. *Xanthi* leaf disks tissue were transformed and regenerated into whole plants essentially as described by Horsch et al. (1985). Plantlets which were able to root on (100 mg/l) kanamycin, were screened for the presence of the capsid gene by PCR, Southern, and Northern blots.

To detect the PVY CP gene, PCR was performed with two convergent primers complementary to the 5′ and 3′ sequences of the CP gene. The denaturation, annealing, and primer extension steps of the PCR program were 94 °C for 1 min, 50 °C for 1 min and 72 °C for 2 min, respectively for 30 cycles, followed by a final extension step at 72 °C for 10 min. PCR products were separated on 1% agarose gel.

Plant genomic DNA was extracted from young leaves according to the CTAB method (Aitchitt et al., 1993). Ten micrograms of DNA was digested with *Hind*III restriction enzyme that cut the DNA at a single site within the transferred DNA, separated on 1% agarose gel and blotted to a nylon Hybond N+ membrane (Amersham). Total RNA was isolated from young leaves of transgenic plants by LiCl precipitation (Verwoerd et al., 1989).

Fifteen micrograms of total RNA was loaded in 1.2% formaldehyde agarose gel, transferred to a nylon Hybond N+ membrane (Amersham). Southern and Northern blots were hybridized with a random-primed α-^{32}P-dCTP labeled probe spanning the full length of the CP gene. Southern and Northern blotting experiments were carried out essentially as described by Sambrook et al. (1989). Autoradiograms were exposed for one week at −70 °C with two intensifying screens.

Virus resistance assays

Six transgenic tobacco progeny lines (TCP-1, -2, -5, -10, -13, and -14), randomly selected and showing normal phenotype were screened for virus resistance after mechanical inoculation with PVY^O. For each transgenic line, 10 plants were mechanically inoculated with a 1 : 10 dilution of PVY^O infected plant sap and 10 plants for the control (NT). Plants were observed daily and scored for the development of systemical symptoms. All inoculated plants were checked for the presence of virus every week by a double antibody sandwich (DAS)–ELISA, using a polyclonal rabbit antiserum against purified virus, until five weeks post-inoculation.

Results

Transgenic tobacco plants containing PVY CP sequence

Tobacco plants regenerated on kanamycin media were micro-propagated and transferred to the greenhouse. All the transferred plants showed normal phenotypes and set seed after self-pollination. In order to investigate the integration of the transgene in all kanamycin-resistant tobacco plants, PCR analysis was carried out. PCR was performed with two convergent primers complementary to PVY CP gene spanning a 1130 bp sequence of the transgene. Figure 2 shows that the six selected transformant lines derived from different leaf disks amplified the expected fragment.

Challenging assays

To analyze the development of infection by PVY^O in greenhouse, 10 transgenic plants for each line and 10 control plants were screened for virus resistance after mechanical inoculation with PVY^O and observed daily for the appearance of virus-induced symptoms. The presence of the virus was confirmed by DAS–ELISA in all plants, before inoculation and after one week from the inoculation and at a frequency of seven days. All control plants were completely

infected three weeks after inoculation and showed severe mosaic. Three different PVY-specific responses were noted: (1) susceptibility (lines TCP-1 and TCP-2), (2) partial resistance (lines TCP-5, -10, and -13), and (3) high resistance (line TCP-14) (Table 1). The tested progeny of lines TCP-1 and TCP-2 are similar to the control untransformed plants with no delay in symptom appearance and no significant decrease in virus concentration (as estimated by DAS–ELISA, average OD405nm is 1.2–1.3 three weeks post-inoculation. The standard deviation (std) is 0.118). Progeny from lines TCP-5, TCP-10, and TCP-13 showed between 80% and 90% partial protected plants. Symptom appearance was delayed by 2–3 weeks and the virus concentration was much reduced (average OD405nm is 0.3–0.4, std is 0.039). The virus was uniformly spread through the plants, as quantified by ELISA and no recovered phenotype was observed similarly to those reported by Ratcliff et al. (1999). Interestingly neither virus nor symptoms could be detected in progeny of line TCP-14 (average OD405nm is 0.05–0.06, std is 0.004) (Figure 3).

Less than 17% of the transgenic lines encoding untranslatable CP transgene mRNA, were immune to PVY^O, but 42% of the lines showed partial resistance (Table 1). Gathering these data, the sense CP mRNA is effective in eliciting PVY resistance.

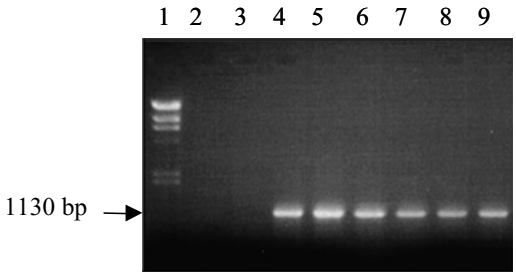

Figure 2. PCR analysis of the transgenic tobacco lines: lane 1, λ-DNA markers digested with *Hind*III; lane 2, H_2O; lane 3, control tobacco plants; lanes 4–9, transgenic lines TCP-1, -2, -5, -10, -13, and -14, respectively. The arrow at the left indicates the position to which a 1130-bp migrated.

Correlation between molecular analysis and resistant phenotypes

Genetic analysis of the six transformant lines was conducted by Southern and Northern blot. Genomic DNA was digested with the restriction enzyme *Hind*III that cut the DNA at a single site within the transferred DNA, and analyzed by Southern hybridization using the PVY CP gene as a probe (Figure 4). In lines TCP-1 and TCP-2, only one band was observed. This suggests that both plant lines contain one single copy. Lines TCP-5, -10, -13, and -14 harbor multiple copies. As shown in Figure 4, it was difficult to determine the exact copy of these plants, however lines TCP-5 and

Table 1. Molecular and phenotypic characterization of the transgenic tobacco plants

Plant line	Kanamycin resistance (%)	Transgene copy numbers	Transgene RNA level[d]	PVY phenotype (%)		
				Susceptible[a]	Partial resistance[b]	Highly resistance[c]
NT	0	0	ND	100	—	—
TCP-1	100	1	High	100	—	—
TCP-2	100	1	High	100	—	—
TCP-5	100	2[e]	High	20	80	—
TCP-10	100	3	High	10	90	—
TCP-13	100	6	Low	20	80	—
TCP-14	100	4	Low	—	—	100

[a]In the susceptible phenotype, typical PVY^O-induced symptoms were observed. Average OD405nm after three weeks post inoculation with PVY^O is 1.2–1.3.
[b]In the partial resistance phenotype, symptoms were delayed in their appearance and plants displayed fewer symptoms. Average OD405nm after three weeks post inoculation with PVY^O is 0.3–0.4.
[c]In the highly resistance phenotype, no symptoms were observed. Average OD405nm after three weeks post-inoculation with PVY^O is 0.05–0.06.
[d]The steady-state level of RNA transcripts derived from PVY CP open reading frame is presented. Representative levels can be viewed in Figure 5. ND, not detected. We have arbitrary classified RNA steady-state levels as high (lines TCP-1, -2, -5, and -10), and low (lines TCP-13 and TCP-14) and report these levels in Table 1.
[e]A major band likely containing multiple copies of the transgene in tandem.

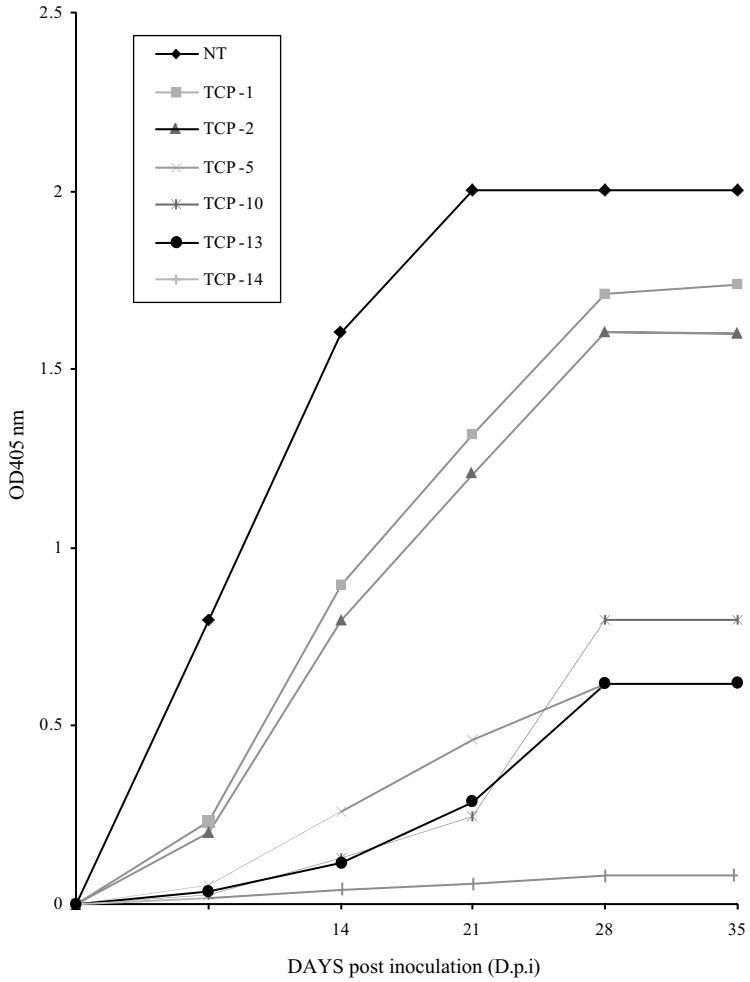

Figure 3. Evaluation of PVY^O accumulation in tobacco lines transformed with untranslatable CP gene at various days post-inoculation. Two leaves per plant were inoculated mechanically with one-tenths dilution of PVY^O-infected plant sap. Ten plants of each line were used for inoculation test. Leaves from each plant belonging to the TC-X line were collected separately and tested with DAS–ELISA. The data represent the average of ELISA (OD405nm) values determined for 10 plants of transgenic tobacco lines: TCP-1, -2, -5, -10, -13, and -14; with untransformed plants as a control (NT). The kinetics of virus accumulation was determined by DAS–ELISA and expressed as the OD405nm values. →: Average OD is 0.1 for healthy plants.

TCP-10 contain two or three copies when compared to plants TCP-13 and TCP-14 that showed more copies. There was an apparent correlation between the high transgene copy number and the resistance phenotype shown by the plant lines (Lindbo and Dougherty, 1992a,b). Therefore, the number of transgene copies in the plants showing partial resistance or immune phenotype was estimated to be more than two.

To determine if the expected transgene transcripts were accumulated in the transgenic lines, Northern hybridization analysis was performed. Total RNA was extracted and analyzed in Northern blot hybridization studies. The expected 1300 nt transgene transcript was observed in all transgenic lines tested (Figure 5). As expected no band was detected in the control plants. The observed differences in level of the accumulated transcript could be due to the copy number of transgenes. We sought to determine if the elevated levels of PVY CP transcript accumulation correlates with transgene copy number. Lines TCP-5 and TCP-10 that likely contain a multiple transgene copy number showed a

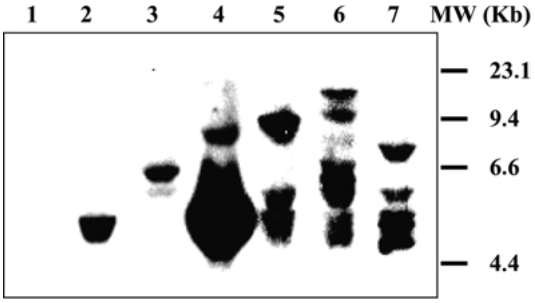

Figure 4. Southern blot analysis of total DNA from transgenic tobacco leaf tissue expressing the untranslatable PVY CP transgene. Genomic DNA (20 μg) was digested with the restriction endonuclease *Hind*III and hybridized with ^{32}P-labeled DNA of the PVY CP sequence. Lane 1, untransformed tobacco genomic DNA; lanes 2–7, transgenic lines TCP-1, -2, -5, -10, -13, and -14, respectively; lane MW, λ-DNA markers digested with *Hind*III.

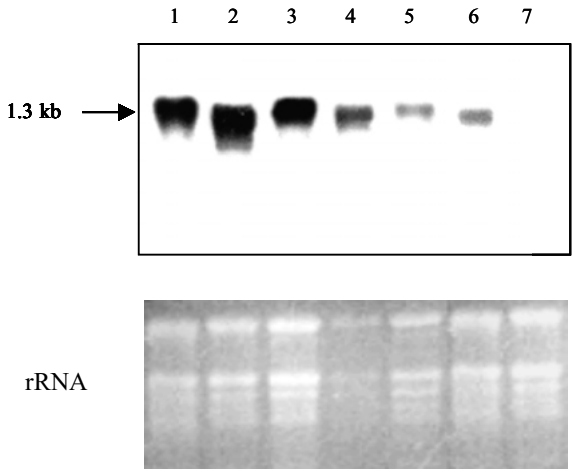

Figure 5. Northern blot analysis of transcripts derived from transgenic tobacco plants expressing the untranslatable PVY CP transgene. Total RNA was separated by electrophoresis in 1.2% agarose gel with formaldehyde and hybridized with ^{32}P-labeled DNA of the PVY CP sequence. The arrow at the left indicates the position to which a 1300-nt transcript migrated. Lanes 1–6, RNA from transgenic lines TCP-1, -2, -5, -10, -13, and -14, respectively, lane 7, RNA from control tobacco plants. Ethidium bromide staining of an electrophoresed gel shows the ribosomal RNA (rRNA) loading of the samples.

transcription level that was approximately similar to those found in susceptible TCP-1 and TCP-2 lines. Lines TCP-10 and TCP-13 which are partially resistant, accumulated respectively a high and a low level of PVY CP transcript. In contrast, the line TCP-14 immune to PVY infection shows a low level of mRNA transcript. These results suggest that F1 progeny of TCP-5, -10, and -13 are segregating plants when referred to line TCP-14 that is homogeneous.

An inverse correlation was observed between the level of the transcript mRNA and the high copy number of viral transgenes excepted for lines TCP-5, -10, and -13 that appeared as segregating lines (Figure 5).

Discussion

There have been a number of detailed reports regarding the generation of PVY-resistant tobacco plants expressing untranslatable PVY CP RNA (Lindbo and Dougherty, 1992a,b; van der Vlugt et al., 1992; Dougherty et al., 1994). In this paper, we described the generation of transgenic plant lines of tobacco expressing untranslatable PVY CP RNA. Engineered protection against PVY^{O} infection was obtained and responses of the different transgenic lines to infection with PVY^{O} were characterized. Three phenotypes were observed, including high resistance in line TCP-14, partial resistance manifested as an attenuation of symptoms with a delay of three weeks in their appearance in three other lines (TCP-5, -10, and -13) and susceptibility manifested in lines TCP-1 and TCP-2.

Our result using the sense gene construct derived from the CP gene of PVY, confirm the findings of other researchers about the inverse correlation between the degree of protection and the level of transcription of the introduced virus genome-derived sequence (Mueller et al., 1995; Lindbo et al., 1993). The results also demonstrate a correlation between high transgene copy number along with virus immunity and that the immunity is not mediated by the transgene protein. Our data are consistent with PTGS as reported by Waterhouse et al. (1998), in which the silenced gene is still actively transcribed but the messenger RNAs are degraded rapidly. Han et al. (1999) also suggested that resistance against PVY is the consequence of PTGS directed by homologous transgenes and cRNAs are synthesized to mediate the degradation. Furthermore, it has been found that lines TCP-5 and TCP-10 which accumulate the transcript at a higher level are partially resistant to PVY. These findings could be explained by the mode of action suggested by Van der Vlugt et al. (1992) and Smith et al. (1994): the transgene-derived RNA may hybridize to viral negative-sense RNA replication intermediates, thereby blocking further virus replication. Numerous models have been proposed to explain the RNA-mediated virus resistance, but there is no direct evidence to rule out any of the model.

Extreme virus resistance conferred by virus genome-derived sequences has been shown to be mediated by a mechanism of PTGS (Baulcombe, 1996; Sijen et al., 1996). Similarly to the elegant experiments of English et al. (1996), who directly connected RNA-mediated resistance and PTGS, the PVY immunity observed in line TCP-14 could be mediated by the highly specific degradation of both the transgene CP mRNA and the target RNA, which contains either the same or complementary nucleotide sequences. It has been shown that the co-suppression occurred in transgenic tobacco is depending of the multicopy transgenes that are required in an inverted repeat arrangement so that transcriptional read-through will produce mRNAs with self-complementarity (Waterhouse et al., 1998).

The RNA-mediated resistance against RNA plant viruses has been observed in transgenic plants at varying frequencies. Accordingly, Sijen et al. (1996), proposed that PTGS is correlated with RNA-mediated resistance. Gene silencing affects both the target and the expressed sequence that triggeres the silencing and appears to be an inducer of a surveillance system that can detect and eliminate or inactivate unwanted RNA species within the plant (Waterhouse et al., 1998).

Previously, an immune phenotype against PVY^O, induced by the expression of an heterologous potyvirus CP from *Lettuce mosaic virus* (LMV), was observed either in tobacco plants (Dinant et al., 1993) or in commercial potato cultivars (Hassairi et al., 1998). This could be explained by the inhibition of PVY replication or long distance movement. Lindbo and Dougherty (1992a) suggested that CPs from heterologous potyviruses may protect, as they are dysfunctional in the heterologous system which results in limited systemic movement of the virus.

In the present study, transgenic tobacco lines challenged for PVY^O protection showed significant levels of protection to PVY^O in four lines, while two other lines are sensitive. It appears that multiple transgenes and accumulated steady-state levels of the transcript to low levels is important to obtain the highly resistant phenotype.

Acknowledgements

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