Resistance in plants transformed with the P1 or P3 gene of tobacco vein mottling potyvirus

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Tobacco plants were transformed with genes encoding the tobacco vein mottling potyvirus (TVMV) P1 or P3 protein. When compared with vector-transformed or P1 transgenic lines, seedlings of P3 transgenic lines (except a low expressor line) developed poorly, suggesting a detrimental effect of P3 on the plant. All P1 and P3 transgenic lines were protected against the homologous TVMV strain and showed variable proportions of two resistance phenotypes: asymptomatic plants or symptomatic plants that recovered from infection. The resistance was effective against a high inoculum dose but had a narrow spectrum. The heterologous strain TVMV-S was able to overcome resistance in most P1 lines but did not break the resistance of most P3 lines. No line showed resistance to another potyvirus (potato virus Y) or to potato virus X. These features and the low levels of transgene expression in resistant plants suggest that protection in P1 and P3 lines is RNA-mediated. In contrast with most reports on virus-activated gene silencing, some P3 lines with the predominant ‘recovery’ phenotype showed silencing of the transgene that was activated at a certain developmental stage of the plant independently of virus infection.

Introduction

Numerous examples of transformation of plants with sequences or genes of viral origin that result in protection against viral diseases have been reported (Wilson, 1993; Lomonossoff, 1995; Beachy, 1997). This pathogen-derived resistance (PDR) operates by a number of different mechanisms that have begun to be unravelled in the past few years (Baulcombe, 1996). Resistance can be protein-mediated, as reported first by Powell-Abel et al. (1990) where expression of a native coat protein (CP) and not its coding sequence is responsible for resistance by interfering with virus disassembly and movement in the vascular system. Other viral proteins, such as movement proteins, only confer resistance when modified versions of the protein are expressed (Cooper et al., 1995), acting as dominant negative mutants (Herskowitz, 1987) that impair a particular step of the infection cycle. Therefore, viral protein-mediated resistance confers protection by different mechanisms that depend on the protein inserted, but a common characteristic is that resistance usually correlates with high levels of expression of the transgene.

Another mechanism of PDR to plant viruses is mediated by RNA and is typically associated with transgenic lines with low or no detectable transgene expression. The resistance manifested is strong but has a narrow spectrum due to its sequence-dependent nature and presents similarities with the post-transcriptional gene silencing (PTGS) phenomenon observed in plants transformed with non-viral genes (Baulcombe, 1996; Prins & Goldbach, 1996; van Kammen, 1997). PTGS results in the suppression of RNA accumulation in a sequence-specific manner. When a plant is transformed with a viral sequence, PTGS will suppress accumulation of the transgene mRNA and of any viral RNA with sequence similarity with the transgene, leading to protection (English et al., 1996). PTGS probably occurs by cytoplasmic degradation of the targeted RNAs (Lindbo et al., 1993; Sijen et al., 1996; Tanzer et al., 1997) and a similar phenomenon has been described recently in non-transgenic, virus-infected plants (Ratcliff et al., 1997).

We are interested in testing viral genes for transgenic resistance to potyviruses and in studying environmental factors that may affect resistance. Potyviruses, the largest and most destructive group of plant viruses (Ward & Shukla, 1991), have a single-strand plus-sense RNA genome of about 10 kb with a single open reading frame (ORF). The translation product of this ORF is processed by three virus-encoded
proteinases to yield a CP and at least seven non-structural proteins (Riechmann et al., 1992; Dougherty & Semler, 1993). CP-mediated resistance has been described for many potyvirus–host combinations (Fitchen & Beachy, 1993; Hackland et al., 1994). Also, the expression of untranslatable versions of the CP gene of a potyvirus led to the first demonstration of RNA-mediated resistance in plant viruses (Lindbo et al., 1993) and has been extensively studied (Dougherty et al., 1994; Cassidy & Nelson, 1995; Goodwin et al., 1996). The ability of some potyvirus non-structural genes to induce resistance is beginning to be investigated. RNA-mediated resistance has been described for the 6K2-Nla portion of the genome (Swaney et al., 1995) and for the NiB coding region (Guo & García, 1997). On the other hand, none of several transgenic lines expressing the CI gene was found to be resistant (Maiti et al., 1993), but these lines were specifically selected for being high expressors and no low-expressor lines were examined.

We have assessed in this study whether transgenic expression of the P1 or P3 coding region of the genome of a potyvirus, tobacco vein mottling virus (TVMV), could interfere with virus infection. TVMV P3 protein is detected in the membrane fractions of infected tissue and computer analysis of its deduced amino acid sequence suggests that P3 may be an integral transmembrane protein (Rodríguez-Cerezo & Shaw, 1991). The function of P3 in the infection cycle is not known, although small insertions in the TVMV p3 coding region abolish virus replication (Klein et al., 1994). The potyvirus P1 protein is a protease that originates from the N terminus of the polyprotein from which it is released by cleaving at its own C terminus (Mavankal & Rhoads, 1991; Verchot et al., 1991, 1992). The P1 protein has an accessory role in potyvirus genome amplification also (Verchot & Carrington, 1995). Here we show that by expressing sequences of the P1 or P3 coding region of TVMV we obtain transgenic tobacco plants that have features consistent with RNA-mediated resistance and, for one transgene (P3), consistent with developmentally regulated transgene silencing. Expression of the P3 gene in transgenic tobacco plants had a strong detrimental effect on seedling development.

Methods

Virus sources. TVMV and TVMV-S were obtained from John G. Shaw and Thomas P. Pirone, respectively (University of Kentucky, Lexington, KY, USA). PVY was obtained from F. Ponz (CTI-INIA, Madrid) and PVX from D. C. Baulcombe (The Sainsbury Laboratory, Norwich, UK). All viruses were maintained in Nicotiana tabacum L. cv. KY14 plants to be used as inocula for resistance experiments.

Transgene constructions and plant transformation. cDNAs representing the P1 and P3 coding regions of TVMV RNA were generated using synthetic oligonucleotides as primers for PCR in which the template was the full-length infectious clone pXBS7 (Domier et al., 1989). The P1 coding region was amplified with oligonucleotides A and B and the P3 region with oligonucleotides C and D described in Rodríguez-Cerezo & Shaw (1991). The PCR-amplified fragments were digested with Ncol and BamHI and ligated into similarly digested pBS-AIMV5', a derivative of pBS containing the 5'- untranslated region of alfalfa mosaic virus (AIMV) RNA 4 (Maiti et al., 1993). Fragments containing the P1 or P3 coding region downstream from the AIMV leader sequence were excised by digestion with XhoI and SfiI and cloned into pKYLX713S5 (Maiti et al., 1993). The resulting plasmids, pKYLXP1 and pKYLXP3 (Fig. 1), were used for direct DNA transformation of Agrobacterium tumefaciens GV3850. A. tumefaciens cultures were used to transform By21 tobacco leaf disks. Control tobacco line IDK was transformed with vector pKYLX713S5 alone. Transformants (T0) were selected in kanamycin (100 µg/ml) and a total of eight or five plants transformed with pKYLXP1 or pKYLXP3, respectively, were regenerated. All plants were self-fertilized and the T1 seed was used for inoculation experiments.

Inoculation and evaluation of transgenic plants. Seedlings...
derived from T1 seeds were selected on kanamycin-containing agar plates and transferred to soil 4 weeks post-germination. Plants (at least 20 plants per line) were mechanically inoculated on the second or third leaf from the bottom (approximately 6 weeks post-germination) after dusting the leaf with carborundum. Inoculum consisted of fresh sap from tobacco plants infected with TVMV, TVMV-S, PVY or PVX, prepared by grinding a piece of young infected leaf in 50 mM sodium phosphate, pH 7.0, in a ratio of 1:3 (w/v). Inoculated plants were kept in a greenhouse and examined daily for symptom development. The relative amount of virus in inoculated plants was estimated by direct double-antibody sandwich ELISA (Clark & Adams, 1977). Samples were taken 2 and 4 weeks post-infection (p.i.) and corresponded usually to leaves 4 and 6 from the bottom of the plant. These leaves, and leaf 2 sampled prior to inoculation, were also analysed for the presence of viral or transgene-derived RNAs as described below.

### Extraction and analysis of RNA from plants

Total nucleic acids were extracted from leaf tissue samples as described (Klein et al., 1994). After LiCl precipitation, the enriched RNA fraction was collected by centrifugation and dissolved in sterile water. Northern blot analysis of RNAs electrophoresed in 1% agarose-formaldehyde gels was as described by Sambrook et al. (1989). Nucleic acids (5 µg) were transferred to Hybond membranes (Amersham) by the capillary blot procedure. Transgene-derived P3 RNA was detected with a 32P-labelled complementary RNA (cRNA) probe prepared by in vitro transcription of a subclone of pXB7 (Domier et al., 1989) containing a 758 bp HindIII fragment (residues 2093–2851 of TVMV RNA). For detecting P1 transgene-derived RNAs in Northern blots, a fragment of pXS7 was amplified by PCR with oligonucleotides A and B (Rodríguez-Cerezo & Shaw, 1991) and cloned in pBS. This subclone was used to prepare P1-specific 32P-labelled cRNA probes. RNAs obtained from plants were also analysed by RT–PCR for detection of P1 transgene-derived RNA. RNAs were treated with ribonuclease-free DNase (Promega) and first-strand cDNA was produced from 1 µg total RNA in a 20 µl reaction with 2 units of avian myeloblastosis virus reverse transcriptase (Seikagaku America) and minus-sense oligonucleotide B, following standard procedures (Sambrook et al., 1989). To distinguish, if necessary, between P1 transgene- and TVMV infection-derived RNAs, the cDNA generated was used in PCR reactions using oligonucleotide B and the plus-sense oligonucleotide ALMV18 (5′ CTCTTCAATACTTCCACCA 3′) corresponding to part of the ALMV leader sequence present in the transgene. As a control for genomic DNA contamination, PCR reactions were performed on total RNA omitting the reverse transcriptase step.

### Extraction and analysis of proteins from plants

Total protein extracts from leaf samples (0.2 g) from transgenic or control plants were prepared as described (Rodríguez-Cerezo & Shaw, 1991) at different times post-germination. For Western blot analysis, proteins were separated in 12% polyacrylamide gels containing SDS and transferred to nitrocellulose membranes. Detection of the P1 and P3 proteins of TVMV was accomplished using the antisera described by Rodríguez-Cerezo & Shaw (1991), diluted 250-fold.

### Results

#### Transformation of tobacco with the P1 or P3 gene of TVMV

Tobacco (Nicotiana tabacum L. cv. By21) was transformed with translatable versions of the P1 or P3 gene of TVMV. The corresponding regions of the genome (Fig. 1) were amplified by PCR using as template the full-length TVMV infectious clone pXB7 (Domier et al., 1989) and cloned downstream from the sequence of the 5′-terminal untranslated region of ALMV RNA 4 in a derivative of pBS (Maiti et al., 1993). The modified genes were then inserted into the transformation vector pKLYX7135S (Maiti et al., 1993) which contains a modified 35S promoter with a duplicated enhancer region.

The P1 transgene (Fig. 1) was designed to express a protein identical to amino acid residues 1–245 of the TVMV polyprotein, except for an Ala residue at position 2 (a Ser in TVMV). For this, we considered the first in-frame initiation codon of TVMV RNA to be at nucleotide position 206 (Domier et al., 1986). Recent re-examination of the sequence of the infectious clone pXB7 (Nicolas et al., 1996) revealed an additional G residue after position 196, which places two additional AUG codons in-frame at positions 153 and 166. The initiation codon of the P1 protein of TVMV in vivo is not known, but preliminary evidence indicates that translation initiation at any of the three AUG codons mentioned results in viable virus infection (K. Levay & J. G. Shaw, personal communication). The P1 proteinase of TVMV begins at the N terminus of the polyprotein and releases itself by cleaving at a Phe–Ser site. Experiments in vitro indicate that the Phe–Ser residues at amino acid positions 256–257 may be the cleavage site of TVMV P1 (Mavankal & Rhoads, 1991). Thus, the P1 transgene may lack 11 amino acids of the C terminus when compared with the P1 protein present in infected plants (Fig. 1). The P3 transgene was designed to express a protein identical to residues 714–1112 of the TVMV polyprotein except for the additional Met residue at the N terminus.

Eight P1- and five P3-transgenic plants were regenerated in the presence of kanamycin and self-fertilized to obtain T1 progeny seeds. Segregation analysis of T1 seed for kanamycin resistance showed that a single active locus was present in each progeny seeds. Segregation analysis of T1 seed for kanamycin resistance showed that a single active locus was present in each of the P1 or P3 lines (not shown). T1 seeds of P1 lines gave rise to normal seedlings indistinguishable from control plants. In contrast, seedlings derived from T1 seeds of P3 lines were chlorotic and developed very slowly until 4–5 weeks post-germination (not shown). Subsequently some, but not all, P3 seedlings developed sufficiently to be transferred to soil for virus inoculation experiments. The exception was line P3-3, with seedlings presenting no chlorosis and normal growth rate (not shown).

#### Responses of transgenic lines to TVMV

Kanamycin-resistant seedlings from the T1 generation were used in all inoculation experiments. Control lines included non-transformed By21 tobacco and vector-transformed By21 tobacco (line IDK). Plants at the 3–4-leaf stage were inoculated with extracts of TVMV-infected plants. The inoculum dose was at least 100 times higher than the dose needed to infect all inoculated control tobacco plants (not shown). All plants from control lines became infected and showed systemic vein...
clearing 1 week p.i. followed by systemic vein mottling in subsequently developed leaves (phenotype S, for susceptible). Two phenotypes in addition to the S phenotype were observed in the transgenic lines. The most frequently observed phenotype was plants that showed initial vein clearing symptoms at the same time as susceptible control plants, but never developed typical vein mottling in the upper leaves. Instead, these plants displayed interveinal chlorotic spots that became less numerous as new leaves developed, and eventually disappeared (phenotype RC, for recovery). RC plants had similar virus titres as control plants at 2 weeks p.i. (as estimated by ELISA, not shown) but were usually symptom-free at 3–4 weeks p.i., with no virus antigen detectable by ELISA. In leaves showing chlorotic spots, detection of TVMV antigen was restricted to the chlorotic areas (not shown). The third phenotype was plants that remained symptomless during the experiment and had ELISA values (not shown) indistinguishable from uninoculated plants when monitored at 2 or 4 weeks p.i. (phenotype R, for resistance). The proportion of plants reacting with each phenotype to TVMV varied depending on the transgenic line (Fig. 2, TVMV). Lines P1-51 and P3-3 were the most resistant, with very high frequencies of R plants. A second group of lines had a predominantly RC response (lines P1-6, P1-9 and P1-52, and P3 lines other than P3-3) and a third group of lines did not have a clearly predominant phenotype (P1-2).

In the highly resistant line P1-51, plants scored as RC (about 10%, Fig. 2, TVMV) showed recovery from infection about 1–2 weeks earlier than the rest of the lines. We tested if such a quick recovery could also be occurring in P1-51 plants scored as R, by analysing virus titre 1 week p.i. in the inoculated leaves. Most P1-51 plants scored as R did not accumulate detectable amounts of TVMV virus in the inoculated leaf (as estimated by ELISA). However, about 20% of the plants tested (4 out of 17) had virus titres in the inoculated leaf similar to those of control plants (not shown). These plants had no detectable virus in upper, non-inoculated leaves (tested at 2 and 4 weeks p.i.) and never displayed symptoms.

**Specificity of the resistance**

The specificity of the resistance conferred by the P1 or P3 transgene was tested by challenging the plants with the S strain of TVMV (Fig. 2, TVMV-S). TVMV-S is a strain that overcomes resistance to TVMV conferred by the recessive va gene present in some tobacco cultivars (Gibb et al., 1989). TVMV-S genomic RNA has been fully sequenced and the overall sequence identity with TVMV is about 89% (Nicolas et al., 1996). As shown in Fig. 2, inoculation with TVMV-S broke the resistance of the P1 transgenic lines, with the exception of the most resistant line (P1-51) in which the predominant phenotype was RC. In the region covered by our P1 transgene construct, nucleotide sequence identity between TVMV and TVMV-S was 82%. The P1 lines did not show any resistance when challenged with another potyvirus, potato virus Y (PVY), or with the potexvirus potato virus X (PVX) (not shown). In contrast with the results obtained for the P1 lines,
the P3 lines showed similar phenotypes when challenged with either TVMV strain, with the exception of line P3-19 in which the RC phenotype was broken by TVMV-S (Fig. 2). It should be noted that the P3 gene nucleotide sequence is more conserved than the P1 region between the TVMV strains, with about 90% sequence identity. Again, no resistance was found to PVY or to PVX in the P3 lines.

Transgene expression in inoculated and uninoculated plants

The steady-state levels of accumulation of RNA transcripts of the P1 transgene were examined just before inoculation and 2–4 weeks p.i. For all P1 lines, P1 transcripts were not detectable by Northern blot hybridization of total RNA extracts from leaves at any of these time points (not shown). Fig. 3(A) summarizes these results with examples of P1 plants showing each of the three phenotypes described. As expected, the probe used to detect the P1 transgene-derived RNA hybridized with TVMV genomic RNA and the levels of viral RNA were consistent with the phenotypes observed. S and RC plants contained similar levels of viral RNA at 2 weeks p.i. (leaf 4 from bottom) but no RNA was detected by 4 weeks p.i. in RC plants (leaf 6 from bottom). Viral RNA was undetectable in R plants at the time-points studied. To verify the expression of the transgene in P1 lines, total RNA samples taken from plants at the stage suitable for inoculation were analysed by RT–PCR with P1 transgene-specific primers (Fig. 3B). P1 transgene expression could be detected by RT–PCR in all P1 lines and the PCR products did not originate from amplification of contaminant plant DNA (compare RT+ with RT− lanes in Fig. 3B). Although P1 transgene expression could be detected by RT–PCR even 4 weeks p.i. (not shown) we did not attempt to develop a PCR quantitative assay to study changes in transgene expression levels among lines or during the development of infection. As expected from the low levels of P1 transcript present in transgenic plants, no P1 protein-related polypeptides were detected in extracts of any of the P1 lines by Western blot analysis (not shown).

Transgene expression was analysed by Northern blot for representative plants of each of the P3 lines (Fig. 4). Steady-state levels of P3 transgene expression were measured with a probe that detected both transgene- and viral-derived RNA. For each line we analysed plants inoculated with TVMV (Fig. 4, blots TVMV) or uninoculated plants (Fig. 4, blots C) at three time-points, the earliest one being when plants had the developmental stage typical for inoculation. At this stage (lanes p in Fig. 4), the expression of the P3 transgene was detectable but levels varied between lines. The most resistant line (P3-3) had almost undetectable levels of transgene expression while line P3-28 had the highest level of expression. Transgene expression was then analysed 2 and 4 weeks p.i. (lanes 2 and 4 in Fig. 4). Neither transgene mRNA nor TVMV genomic RNA were detected in resistant line P3-3. The rest of the lines, when inoculated with TVMV, showed predominantly the RC phenotype and this correlated with a decrease in viral and transgene RNA accumulation that was evident by 4 weeks p.i. (blots TVMV in Fig. 4). However, and in contrast to many reports of transgene silencing induced by virus infection in plants with RC phenotypes, the decrease of transgene expression observed in the P3 lines occurred also in plants that had not been inoculated (blots C in Fig. 4), indicating that a silencing response is manifested at certain stage of development of the plant regardless of virus infection. A weak transgene signal remained in uninoculated plants at the end of the experiment when blots were overexposed (see lanes 4* in
Fig. 4. Analysis of P3 transgene expression (Northern blot) on total RNA samples from transgenic tobacco plants inoculated with TVMV (TVMV blots) or uninoculated (C blots). Samples were taken prior to inoculation (lanes p) or 2 and 4 weeks p.i. (lanes 2 and 4). The probe was designed to hybridize with both TVMV RNA and P3 transgene-derived RNA. Line pKYLX is a control vector-transformed tobacco line. Autoradiograms were exposed for 1 week except for lane 4* of P3-28 plants, which was exposed for 3 weeks.

Since line P3-28 was the highest expressor of P3 transgene we attempted to detect the protein product by Western blot analysis with an antiserum specific for TVMV P3 (Rodriguez-Cerezo & Shaw, 1991). Most of the P3 protein produced in TVMV-infected tissue is thought to be cleaved by a viral proteinase (NiA) to yield a 37 kDa protein and a putative 5–6 kDa polypeptide not detectable with our antiserum (Rodriguez-Cerezo & Shaw, 1991). The P3 (42 kDa) and 37 kDa proteins were detected as previously described in TVMV-infected tissue from control plants (Fig. 5). A protein with a mobility similar to P3 (42 kDa) was detected in extracts from uninfected P3-28 plants (Fig. 5) at the developmental stage suitable for inoculation (about 45-50 days post-germination). Only the full-length P3 protein (42 kDa) was observed in transgenic, non-inoculated plants in which viral proteinases were absent (Fig. 5). Transgenic P3 protein was not detectable by our analytical procedures in other P3 transgenic lines or in later stages of development of P3-28 plants (not shown).

Discussion

Our results show high levels of protection in 13 transgenic tobacco lines transformed with translatable versions the P1 or P3 coding region of a potyvirus, TVMV. Two resistance phenotypes were observed: a highly resistant, asymptomatic phenotype (R) and the development of abnormal disease symptoms (appearing without any delay as compared with controls) followed by development of symptomless leaves (a ‘recovery’ phenotype, RC). The resistance observed was effective against highly infectious inocula but had a narrow spectrum. A strain of TVMV (TVMV-S) was able to overcome the TVMV P1 transgene resistance in most lines but did not break the resistance of most P3 lines. The lower sequence identity between strains in the P1 transgene region (82%) compared with the P3 region (90%) should be noted. No resistance was found against another potyvirus, PVY. These features, and the relation between resistance and low levels of transgene expression, suggest that the resistance is mediated by RNA in P1 and P3 transgenic lines.

Transgenic expression of functional tobacco etch virus P1 protein (as shown by complementation studies) has been reported and seems not to interfere with virus infection (Verchot & Carrington, 1995). There is a report of resistance based on expression of the P1 gene of PVY in potatoes (Pehu et al., 1995). In these plants, the expression levels of the P1 transgene were not characterized, but the plants showed a high level of protection that was sequence identity-dependent, suggesting an RNA-mediated mechanism. The same mechanism has been proposed to explain the resistance and recovery exhibited by lines containing the P1 plus part of the HC-Pro cistrons of plum pox virus (Tavert-Roudet et al., 1998). The N and C termini of the TVMV P1 protein have not been determined in vivo, but since the possibility exists that our
transgene could produce a slightly smaller version of the protein produced in vivo (Fig. 1), we cannot rule out that undetectable amounts of a P1-related polypeptide could be interfering with virus infection in our P1 lines, although the strength and specificity of the resistance observed argues against this possibility.

This is the first report of transgenic expression or resistance based on the potyviral P3 gene. As noted in our results, plants from P3 transgenic lines were chlorotic and initially developed slowly, with the exception of the lowest expressor line, P3-3. This suggests a detrimental effect of the P3 protein on the plant. P3 is an integral membrane protein in infected tissue and its expression stops the growth of bacterial cultures shortly after inducible expression (our unpublished results). The detrimental effect of P3 may lead to indirect selection of lines with no expression (like P3-3) or with a high transgene expression level that is silenced when plants reach a certain stage, independently of virus infection (like line P3-28). Establishing lines with durable expression of the P3 protein to assess whether it can confer protein-mediated resistance may be difficult. Lines like P3-28, inoculated at a young stage or at the protoplast level, could also be used to study whether the putative role of P3 on replication (Klein et al., 1994) is complementable in trans. Our results also support the notion that the abundant 37 kDa protein detected with anti-P3 serum in TMV-infected tissues (Rodriguez-Cerezo & Shaw, 1991) is produced by cleavage of P3 by a viral protease, since only the full-length P3 protein is detected in transgenic plants.

The phenotypes observed in our P1 and P3 lines coincide with the two resistance phenotypes that have been associated with PTGS in other systems (Dougherty et al., 1994; Goodwin et al., 1996). These are the ‘immunity’ or ‘resistant’ phenotype (R; no symptoms and no accumulation of virus) and the ‘recovery’ phenotype (RC; systemic symptoms that gradually disappear in newly developed leaves some time after inoculation). Recently Tanzer et al. (1997) have shown that R lines inoculated at earlier developmental stages are indeed susceptible to virus infection followed by quick recovery from symptoms, suggesting that both R and RC phenotypes are reversible by meiosis and are manifestations of the same process. Slight differences in the developmental stage could explain our results with line P1-51, which showed mostly asymptomatic, virus-free plants but also had some virus-containing plants with different degrees of quick recovery. The recovery in some P1-51 plants was fast enough to generate symptomless phenotypes scored as R. This ‘quick recovery’ in P1-51 was clearly delayed when plants were inoculated with the heterologous strain TVMV-S indicating that, in addition to the developmental stage of the plant, sequence identity with the incoming virus can modulate the speed of the recovery process.

Tanzer et al. (1997) have proposed that the primary difference between R and RC lines would reside in the need for virus infection to activate silencing. In this model, R lines are associated with silencing of the transgene prior to virus infection. In contrast, RC lines show initially high levels of gene expression and gene silencing is induced by inoculation with a virus that has sequence identity with the transgene (Lindbo et al., 1993; Cassidy & Nelson, 1995; Goodwin et al., 1996; Guo & Garcia, 1997). It has been proposed that triggering of the silencing mechanisms is only induced after the combination of transgene and viral RNA levels reaches a critical threshold (Lindbo et al., 1993; Goodwin et al., 1996). This is not the case for some P3 lines described here (Fig. 4), where the silencing is triggered simultaneously both in inoculated and non-inoculated plants, and seems to occur at a certain developmental stage. A similar example of development-associated silencing has been reported recently for a viral transgene, the tospoviral N gene (Pang et al., 1996) and there are also examples of this phenomenon for non-viral transgenes (Elmayan & Vaucheret, 1996; Kunz et al., 1996). TMV infection of our P3 lines may have an overlapping role resulting in a final degree of gene silencing that is more complete in inoculated than uninoculated plants (Fig. 4, lanes 4* of P3-28). On the other hand, all our P1 lines had very low steady-state levels of transgene mRNA but most of them were not fully resistant, but rather showed an homogeneous RC response. Since P1 transgene expression was only detectable by RT–PCR, this prevented us from studying whether these lines were further silenced by infection with the homologous virus.

It appears that most coding regions of the potyvirus genome have the ability to induce RNA-mediated resistance when expressed in transgenic plants. Few studies have screened systematically a complete viral genome for regions able to induce RNA-mediated resistance, an important question for mechanistic and practical reasons. For the tospovirus tomato spotted wilt virus, only the expression of sequences of the N or NSm genes resulted in resistance (Prins et al., 1997). Evidence exists for a minimal length of the transgene needed (Sijen et al., 1996) but clearly further effort is necessary to identify other features of the RNA sequence that may influence its ability to trigger gene silencing. In the task of designing transgenes for potyvirus resistance, and in view of our results, it seems important to select for regions of near 90% identity between strains to obtain a wider resistance. Since the expression of some viral genes (such as P3) can be detrimental for the plant, it would be important to use untranslatable or frameshift versions of the gene.

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References


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