A minimum length of N gene sequence in transgenic plants is required for RNA-mediated tospovirus resistance

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We showed previously that transgenic plants with the green fluorescent protein (GFP) gene fused to segments of the nucleocapsid (N) gene of tomato spotted wilt virus (TSWV) displayed post-transcriptional gene silencing of the GFP and N gene segments and resistance to TSWV. These results suggested that a chimeric transgene composed of viral gene segments might confer multiple virus resistance in transgenic plants. To test this hypothesis and to determine the minimum length of the N gene that could trans-inactivate the challenging TSWV, transgenic plants were developed that contained GFP fused with N gene segments of 24–453 bp. Progeny from these plants were challenged with: (i) a chimeric tobacco mosaic virus containing the GFP gene, (ii) a chimeric tobacco mosaic virus with GFP plus the N gene of TSWV and (iii) TSWV. A number of transgenic plants expressing the transgene with GFP fused to N gene segments from 110 to 453 bp in size were resistant to these viruses. Resistant plants exhibited post-transcriptional gene silencing. In contrast, all transgenic lines with transgenes consisting of GFP fused to N gene segments of 24 or 59 bp were susceptible to TSWV, even though the transgene was post-transcriptionally silenced. Thus, virus resistance and post-transcriptional gene silencing were uncoupled when the N gene segment was 59 bp or less. These results provide evidence that multiple virus resistance is possible through the simple strategy of linking viral gene segments to a silencer DNA such as GFP.

Introduction

The concept of pathogen-derived resistance (PDR) states that pathogen genes that are expressed in transgenic plants may confer resistance to infection by the homologous or related pathogens (Sanford & Johnston, 1985). Numerous studies have shown that PDR is an effective means of producing virus-resistant plants and can be used for a number of different plant virus groups with various viral genes (Grumet, 1995; Lomonossoff, 1995). PDR is mediated either by the transgene protein (protein-mediated) or by the transgene RNA (RNA-mediated) (Baulcombe, 1996a; Lomonossoff, 1995; van den Boogaart et al., 1998; Wilson, 1993).

Post-transcriptional gene silencing (PTGS) is considered to be one of the dominant mechanisms of RNA-mediated protection in transgenic plants (Dawson, 1996; Goodwin et al., 1996; Lindbo et al., 1993; Mueller et al., 1995; Pang et al., 1996, 1997; Prins et al., 1996; Sijen et al., 1996; Smith et al., 1994; Tanzer et al., 1997). RNA-mediated virus resistance is also known as homology-dependent resistance (Mueller et al., 1995). Several models have been postulated to account for the mechanism of PTGS and the resulting virus resistance (Baulcombe, 1996b): (i) the direct production of antisense RNA model (Grierson et al., 1991); (ii) the expression threshold model (Dougherty & Parks, 1995; Smith et al., 1994); (iii) the aberrant RNA (ectopic pairing) model (English et al., 1996; Baulcombe & English, 1996) and (iv) the double-stranded RNA-induced model (Metzlaff et al., 1997; Montgomery & Fire, 1998; Prins & Goldbach, 1996; Waterhouse et al., 1998).

Tomato spotted wilt virus (TSWV) is the type species of the genus Tospovirus. The virus genome consists of three single-stranded RNAs that are designated as L (~ 8900 nucleotides), M (~ 5000 nucleotides) and S (~ 2900 nucleo-
The S RNA encodes a 52 kDa nonstructural protein (NS) in the viral RNA strand and the 29 kDa nucleocapsid (N) protein in the viral complementary RNA strand. The M RNA encodes the precursor to the envelope glycoproteins G2 (58 kDa) and G1 (78 kDa) in the viral complementary RNA strand and a 34 kDa nonstructural protein (NSM), which possibly functions as a virus cell-to-cell movement protein, in the viral RNA strand (Kormelink et al., 1994). The L RNA is of negative polarity and encodes a 330 kDa putative viral polymerase (de Haan et al., 1991).

We previously reported that transgenic plants expressing a large segment (longer than 387 bp) of the N gene of TSWV conferred virus resistance through PTGS while N transgene segments smaller than 235 bp did not (Pang et al., 1997). However, resistance to TSWV was obtained when small N gene segments were fused to the non-target green fluorescent protein (GFP) gene. These data suggested that GFP was triggering PTGS of the chimeric gene while the TSWV N gene segment of the chimeric gene conferred resistance to the attacking TSWV. This system should thus allow us to determine the minimum unit of viral transgene that can confer resistance via the PTGS mechanism and to test whether a chimeric gene made of short virus segments could provide a simple way to develop transgenic plants that are resistant to multiple viruses. Here we demonstrate that minimum lengths of 59–110 bp were required, fused with a silenter DNA, for RNA-mediated tospovirus resistance. In addition, the same lines expressing the hybrid transgene are protected against both TSWV and tobacco mosaic virus (TMV)–GFP, demonstrating the feasibility of this simple strategy for engineering multiple virus resistance in transgenic plants.

Methods

■ Virus constructs. An infectious cDNA clone (designated pTMV-GFP), known as p4GD-PL (Casper & Holt, 1996), of the TMV common strain with a GFP gene sequence was used for construction of infectious TMV cDNAs carrying foreign inserts. The clone was a kind gift from F.-J. Jan (formerly of the Scripps Institute, La Jolla, CA, USA). The construct pTMV-GFP contains the virus–GFP sequence inserted in pBluescript KS(+) with a bacteriophage T7 RNA polymerase promoter to allow for in vitro production of viral RNAs. A construct, pTMV-GFP-NP, containing the N gene of TSWV (Fig. 1a) was constructed in our laboratory (Jan, 1998) and also used to produce transcripts for some inoculation tests.

■ In vitro transcription and virus infection. Plasmids pTMV-GFP and pTMV-GFP-NP were linearized with KpnI at the 3′ end of TMV cDNA and made blunt-ended by removing the 3′ overhang with Klenow polymerase (Promega). In vitro transcription was performed essentially as described by Holt & Beachy (1991). Capped in vitro transcripts were diluted 1:1 with 20 mM sodium phosphate buffer, pH 7.0, and the mixture was applied to carborundum-dusted leaves of Nicotiana benthamiana. Plants were rinsed with water immediately after inoculation and placed in a greenhouse for observation of symptoms. Inoculations of TSWV-BL were done as described previously (Pang et al., 1992). Systemic symptoms were recorded daily for at least 30 days.

■ Cloning and transformation. Maps of the transgenes used in this study are shown in Fig. 1(b). The 9/16N gene segment was amplified by PCR by using oligomer primers 96–5 (5′ TCTTGGAGATCCATGGGATAAAGGTAAGCTACTCT) and 96–4 (5′ CACTTGTGAATCCATGATGCAAGCTGCTGAG), which is complementary to the S RNA of TSWV-BL at positions 2323–2341, and 92–54 (5′ TACATTCTCTAGAACCAGGATGATGCAAGCTGCTGAG), which is complementary to the S RNA at positions 2359–2378. The 17/32N gene segment was generated by annealing oligomer primers 96–5 (5′ CTAGACCAAATTGGGATTGCAATGTGGAGGCTTG) and 96–4 (5′ GATCCAGGCTCCAGCACTTGCATTGATCTCAG), which is complementary to the S RNA at positions 2335–2378. The 17/32N gene segment was located at positions 2335–2378 of S RNA with NcoI site and Xhol overhang sequences in the 5′ end and a BamHI overhang sequence in the 3′ end. The 9/16N and 17/32N segments were cloned in the sense orientation into the Xhol/BamHI sites of a plant expression vector pBI525 (Pang et al., 1992). For construction of N gene segment fusions with GFP, the translatable GFP ORF was amplified with primers (Pang et al., 1997) from the plasmid pGFP (Clontech) and cloned alone or as a transcriptional fusion into the NcoI site upstream of the N gene segments 9/16N and 17/32N in pBI525. The resulting plant expression vectors were digested with HindIII and EcoRI and the HindIII–EcoRI

Fig. 1. Structures of recombinant viruses and transgenes. (a) Schematic diagrams of TMV–GFP and TMV–GFP–NP. The N gene sequence of TSWV was transcriptionally fused with GFP at the NcoI site of TMV–GFP (Casper & Holt, 1996). (b) Maps of the transgenes used in this study. Non-translatable N gene segments were expressed alone or as transcriptional fusions with the GFP gene. Each transgene was flanked by a double-enhanced CaMV 35S promoter, the 5′ untranslated leader of alfalfa mosaic virus and the 3′ untranslated sequence of the nopaline synthase gene. N gene fragments are represented by 2/2N, 3/4N, 5/8N, 9/16N and 17/32N for the second half, the third quarter, the 5/8th, the 9/16th and the 17/32nd segment of the N gene of TSWV-BL (Pang et al., 1997).
segments containing the corresponding gene cassettes were isolated and introduced into the same sites of pBIN19 (Clontech). The resulting binary vectors were transferred into Agrobacterium tumefaciens LBA4404 and cultures of A. tumefaciens containing the vectors were used to inoculate leaf discs of N. benthamiana plants, essentially as described by Horsch et al. (1985).

Transgenic plants containing the 2/2N, 3/4N and 5/8N gene segments fused with GFP were described by Pang et al. (1997).

Nuclear run-on transcription assays, ELISA and Northern blot analyses of transgenic plants. Isolation of nuclei and nuclear run-on transcription assays were described previously by Pang et al. (1996). Double-antibody sandwich ELISA was used to detect the neomycin phosphotransferase (npt II) enzyme in transgenic plants by using an nptII ELISA kit (5 Prime to 3 Prime Inc.). For estimation of RNA transcript levels in transgenic plants by Northern blot, total plant RNAs were isolated as described by Napoli et al. (1990) and Northern blotting was performed as described by Pang et al. (1993). Ten μg total RNA per lane was separated on formaldehyde-containing agarose gels (Sambrook et al., 1989) and the agarose gels were stained with ethidium bromide to monitor the uniformity of total plant RNA in each lane. Since the N gene segments in transgenic plants containing GFP + 9/16N and GFP + 17/32N were too short (59 and 24 bp, respectively) to be detected when using probes made by random priming (Feinberg & Vogelstein, 1983), an oligonucleotide probe was used to detect the N gene in transgenic plants containing GFP linked to 17/32N or 9/16N. The oligonucleotide probe was 32P-labelled as described by Sambrook et al. (1989). Hybridizations were performed essentially as described in the manufacturer’s protocol for GeneScreen Plus membrane (Dupont) except that the hybridization was at 42 °C and washing was at 48 °C. Images of some autoradiograms were photographed with a COHU CCD camera model 4915–2000 (COHU Inc.). Signals were quantified by using the US National Institutes of Health–Image program version 1.59.

Results

Transgenic plants with post-transcriptionally silenced GFP transgene resist infection by TMV–GFP and TMV–GFP–NP

It has been shown that transgenic plants with a silenced transgene can resist infection by a chimeric virus containing an RNA sequence homologous to the silenced transgene (Sijen et al., 1996; English et al., 1996; Marano & Baulcombe, 1998). To determine whether the transgenic plants with a silenced GFP transgene could inactivate chimeric TMV containing a GFP sequence or a GFP–N gene segment, 19 GFP-transgenic lines were produced and R1 plants of 12 lines were inoculated with in vitro transcripts of TMV–GFP or TMV–GFP–NP (Fig. 1a) and observed for infection. Transgenic R1 seeds were germinated and assayed by nptII ELISA to identify the non-transgenic segregants from the populations. A number of R1 plants were inoculated with TSWV or infectious transcripts of TMV–GFP (Table 1). Unlike control plants, various proportions of progeny from four of 12 tested lines were resistant to TMV–GFP and TMV–GFP–NP. Resistance to TMV–GFP was correlated with resistance to TMV–GFP–NP. For example, seven of eight and seven of nine plants from line 5L were resistant to TMV–GFP and TMV–GFP–NP, respectively. RNA gel blot analysis of inoculated leaves showed that TMV–GFP genomic RNA accumulated to high levels in the susceptible line 3 and non-transformed N. benthamiana plants, while little or no TMV–GFP genomic RNA was detected on the resistant line 5L (data not shown).

Northern blot and nuclear run-on assays were performed to determine whether the protection of GFP-transgenic plants against TMV–GFP and TMV–GFP–NP was due to the PTGS mechanism. Results of hybridization analysis showed a correlation between the resistance phenotype and low levels of GFP RNA transcript accumulation (Fig. 2a). In addition, the resistant plants with low steady-state RNA accumulation had high RNA transcription rates in nuclei (Fig. 2b). These results suggest collectively that PTGS not only suppressed GFP expression but also trans-inactivated the replication of the chimeric virus containing the homologous GFP sequence.

Fusion of N gene segments with GFP confers resistance to TMV–GFP and TSWV

We reported previously that non-translatable constructs containing segments of the TSWV-BL N gene that were fused to the GFP gene (designated as GFP + 2/2N, GFP + 3/4N and GFP + 5/8N) conferred resistance to TSWV in transgenic N. benthamiana via PTGS (Pang et al., 1997). Experiments were done to determine whether these resistant lines could resist infection by TMV–GFP as well as TSWV. Transgenic R1 and R2 seeds were germinated and assayed by nptII ELISA to identify the non-transgenic segregants from the populations. A number of R1 plants were inoculated with TSWV or infectious transcripts of TMV–GFP (Table 1). Unlike control plants, which were susceptible to TSWV and TMV–GFP, a portion of the R1 transgenic plants from lines with GFP + 2/2N, GFP + 3/4N and GFP + 5/8N gene fusions were resistant to TSWV and TMV–GFP (Table 1). Thus, for line 29 of GFP + 2/2N, 17 of 18 plants tested were resistant to TSWV and six of seven plants tested were resistant to TMV–GFP. Similarly, all plants of GFP + 3/4N line 24 tested were resistant to TSWV (20/20) and TMV–GFP (7/7) (Table 1).

In another set of experiments, self-pollinated R2 plants from selected TSWV-resistant R1 plants (Pang et al., 1997) were inoculated with TMV–GFP, TMV–GFP–NP and TSWV. The R2 plants with GFP–N fusions (GFP + 2/2N, GFP + 3/4N and GFP + 5/8N) were resistant to TSWV, TMV–GFP and TMV–GFP–NP (Table 2). With the N gene or GFP gene sequences as probes, Northern blots of some R2 plants showed that resistance to TSWV and TMV–GFP was accomplished by low to medium levels of accumulation of the fusion gene...
Fig. 2. Northern blot and nuclear run-on transcription analyses of the GFP transgene. (a) Northern blot analysis of R1 plants with GFP gene. Total RNAs were isolated from transgenic plants (10 µg per lane) and analysed by Northern blots that were probed with the GFP gene. Lanes: 1–2, R1 plants of non-silenced line 3; 3–4, R1 plants of silenced line 5L; 5–6, R1 plants of non-silenced line 8; 7, non-transgenic plant. Signal densities were quantified by using the NIH-Image program. Transgenic plants that gave density readings between 129 and 187 were rated as high expressors (H) while transgenic plants with density readings between 0 and 27 were rated as low expressors (L). (b) Nuclear run-on transcription analysis of R1 plants. Labelled nuclear RNAs were hybridized to restriction enzyme-digested GFP, nptII and actin (from N. benthamiana) DNA segments, which had been separated on an agarose gel and blotted onto membrane. The nuclei used in the assays were isolated from an R1 plant of line 5L and an R1 plant of line 3. The ratios are the relative GFP and nptII transcription rates normalized to the transcription rate of actin from N. benthamiana. An actin DNA segment from N. tabacum was loaded in the lane between nptII and GFP and the ratio of its transcription rate is not given, since this DNA did not react with the labelled nuclear RNAs from N. benthamiana.

Table 1. Reactions of R1 transgenic plants with GFP + 2/2N, GFP + 3/4N and GFP + 5/8N transgenes to inoculation with TSWV and TMV–GFP

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<tr>
<td>GFP + 5/8N</td>
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<td>6</td>
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<td>12</td>
<td>1</td>
<td>11</td>
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TSWV-BL-infected leaf extracts (1/30) (Pang et al., 1997) or infectious transcripts of TMV–GFP produced by *in vitro* transcription (Holt & Beachy, 1991) were applied to three upper leaves of N. benthamiana at the 5–7-leaf stage. Plants susceptible (S) to TSWV showed typical systemic symptoms 5–10 days p.i. Plants susceptible to TMV–GFP showed green fluorescence on inoculated leaves 2–5 days p.i. and on upper leaves 4–6 days p.i. and then developed systemic virus symptoms (mosaic) 6–14 days p.i. Highly tolerant (HT) plants showed a delay in systemic symptoms for more than 10 days p.i. Resistant (R) plants remained symptomless at 30 days p.i. n, Total plants inoculated.
Table 2. Reactions of R<sub>2</sub> progeny of transgenic plants with GFP + 2/2N, GFP + 3/4N and GFP + 5/8N transgenes to inoculation with TSWV, TMV–GFP and TMV–GFP–NP

Self-pollinated R<sub>2</sub> plants from TSWV-resistant R<sub>1</sub> plants (Pang et al., 1997) were used in this experiment. Plants susceptible to TMV–GFP–NP showed systemic mosaic symptoms 14–30 days p.i. See Table 1 for further definitions and inoculation procedures. NT, Not tested.

<table>
<thead>
<tr>
<th>Transgene</th>
<th>Line</th>
<th>TSWV</th>
<th>TMV–GFP</th>
<th>TMV–GFP–NP</th>
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<tr>
<td></td>
<td></td>
<td>n</td>
<td>R</td>
<td>S</td>
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<tr>
<td>GFP + 2/2N</td>
<td>8 (373)</td>
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<td>12</td>
<td>9</td>
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<tr>
<td>GFP + 3/4N</td>
<td>5 (193)</td>
<td>26</td>
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<tr>
<td>GFP + 5/8N</td>
<td>6 (250)</td>
<td>35</td>
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![Image](https://microbiologyresearch.org/fig3.png)

Fig. 3. Northern analysis of R<sub>2</sub> plants containing the GFP–N gene fusions. Leaves were harvested for RNA isolation when the plants were at the 5–7-leaf stage. Ten µg per lane total RNA was used for the Northern blot, which was probed with the GFP gene (top) or the N gene of TSWV-BL (bottom). Lanes: 1–4, susceptible (S) plants of line GFP + 2/2N 8 (373); 5–8, resistant (R) plants of GFP + 2/2N 8 (373); 9–12, resistant plants of line GFP + 3/4N 5 (193); 13–16, resistant plants of line GFP + 5/8N 1 (3); 17, a non-transformed plant. Signal intensities were quantified as Fig. 2. The transgenic plants giving density readings below 34, between 67 and 93 and above 106 were rated as low (L), medium (M) and high (H) expressers, respectively.

transcripts (Fig. 3). These results demonstrate collectively that the hybrid transgene was capable of inactivating both the N gene-containing tospoviruses and the GFP-containing TMV through the PTGS mechanism.

**A minimum length of N gene segment is required to inactivate the incoming tospovirus**

Two lines of evidence suggested that the minimum length of the N gene that confers resistance when fused with GFP was close to 110 nucleotides. Firstly, only one of 13 R<sub>n</sub> lines with the GFP + 5/8N transgene was resistant to TSWV compared with six of eight lines with the GFP + 3/4N transgene (Pang et al., 1997). Secondly, only one of 18 progeny from the resistant R<sub>n</sub> plant containing GFP + 5/8N was resistant to TSWV infection while five of six progeny were resistant to TMV–GFP (Table 1).

To determine more precisely the minimum length of the TSWV N gene that is required to confer resistance in the GFP + N plants that show PTGS, transgenic N. benthamiana plants were engineered to express 110–24 bp of the N gene alone or fused with the GFP gene (Fig. 1b). In one set of experiments, R<sub>n</sub> plants expressing these fusions were inoculated with infectious transcripts of TMV–GFP or TMV–GFP–NP and observed for symptoms. Control transgenic plants with only the 9/16N or 17/32N gene segments were similarly inoculated. Plants that showed resistance to TMV–GFP or TMV–GFP–NP or a recovery phenotype (symptoms developed initially but new leaves were symptomless at 14–30 days p.i.) were then inoculated with TSWV (Table 3). Five of 12 R<sub>n</sub> plants tested that expressed the 110 bp segment of the N gene linked to GFP (GFP + 5/8N) showed resistance or recovery following inoculation with TMV–GFP or TMV–GFP–NP. Two of these five lines were also resistant to TSWV (Table 3). In contrast, six of 12 GFP + 9/16N (59 bp) and five of 12 GFP + 17/32N (24 bp) lines were resistant to TMV–GFP or TMV–GFP–NP, but none of these 11 resistant lines showed resistance to TSWV. All six plants with 9/16N or 17/32N gene segments alone were susceptible to TMV–GFP or TMV–GFP–NP.

In a second set of experiments, progeny of GFP + 9/16N and GFP + 17/32N from another twelve independent R<sub>n</sub> lines were similarly inoculated with TMV–GFP and TSWV. As shown in Table 4, a proportion (61 of 170) of the R<sub>n</sub> plants with GFP + 9/16N or GFP + 17/32N were resistant to TMV–GFP but all 469 plants that were inoculated with TSWV-BL became infected.

Some of the R<sub>n</sub> plants expressing the fusions were analysed by Northern blot with N gene or GFP gene sequences as probes. The results showed that the resistance observed correlated with low accumulation of the fusion gene transcripts (data not shown). Collectively, these results show that
Table 3. Reactions of R0 transgenic plants with GFP + 5/8N, GFP + 9/16N and GFP + 17/32N transgenes to inoculation with TMV–GFP, TMV–GFP–NP and TSWV-BL

See Table 1 for inoculation procedures and definition of reactions. Recovery (Rec) plants initially developed symptoms similar to susceptible plants but new leaves were symptomless 14–30 days p.i. See Fig. 1 for definitions of N gene segments of TSWV-BL. NT; Not tested; R, resistant; S, susceptible.

<table>
<thead>
<tr>
<th>Transgene</th>
<th>TMV–GFP or TM–GFP–NP</th>
<th>TSWV</th>
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<tr>
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<tr>
<td>GFP + 5/8N</td>
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* Plants that showed resistance or recovery phenotype were then inoculated with TSWV-BL-infected leaf extracts diluted 1:30.

Table 4. Reactions of R1 transgenic lines with small N gene segments alone or linked to GFP

See Table 1 for inoculation procedures and Fig. 1 for definitions of N gene segments of TSWV-BL. Lines were described as resistant (R) if 40% or more of the plants remained symptomless 35 days p.i. Other lines were considered susceptible (S).

<table>
<thead>
<tr>
<th>Transgene</th>
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<td>Control</td>
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GFP + 9/16N and GFP + 17/32N can induce gene silencing and confer resistance to TMV–GFP and TMV–GFP–NP but not resistance to TSWV, indicating that an N gene DNA sequence of 59 or 24 bp is insufficient to trans-inactivate the incoming tospovirus.

Discussion

We showed previously that plants with transgene constructs of GFP fused to short N gene segments (200–110 bp) of TSWV were resistant to TSWV, while plants with transgenes consisting of only these short N gene segments were susceptible (Pang et al., 1997). The underlying resistance mechanism was PTGS. These results suggested that plants resistant to multiple viruses could be obtained by transforming them with a single chimeric transgene consisting of gene segments from different viruses. In this study, we provide evidence that this concept does work by showing that a GFP + N gene segment transgene conferred resistance to both TMV–GFP and TSWV. The resistance was inherited through the Rg progeny. We also further define the minimum length of the transgene required to confer resistance against TSWV and presumably other tospoviruses by showing that gene segments of 59 bp or less fail to confer resistance, even though the transgene is post-transcriptionally silenced. This is the first report that RNA-mediated virus resistance and PTGS can be uncoupled if the virus transgene sequence is below a minimum size.

The simplest explanation for our results is that the 720 bp GFP DNA acts as a ‘silencer’ DNA because it is large enough to induce PTGS, in contrast to N gene segments of 200 bp or less, which are not sufficiently large to induce PTGS. The silencer DNA (GFP in this case) may simply stabilize the transgene fusions or the GFP gene may provide RNA sequence...
elements required for activating PTGS. Resistance to TSWV is obtained in transgenic plants when these N gene segments (200–110 bp) are linked to GFP because they are part of a transgene that is post-transcriptionally silenced. Our previous work also showed that N gene segments of more than 400 bp could serve as ‘silencer’ DNAs. We speculate that the putative antisense molecules that are produced from the region of the mRNAs of N gene segments that are 59 bp or less are either too small or not sufficiently abundant to degrade the incoming virus effectively and induce a resistant phenotype.

Our findings demonstrate the feasibility of, and define some of the important parameters for, developing multiple virus-resistant transgenic plants by using a chimeric transgene with a ‘silencer’ DNA linked to small segments that originate from target plant viruses. This novel strategy could have significant practical value because most crops are susceptible to more than one virus and a single crop is often exposed to infections by multiple viruses in a growing season. It is also likely that this strategy could be used to down-regulate multiple genes in plants and obtain transgenic plants with virus resistance and other interesting traits, for example, delayed ripening (Gray et al., 1992).

A potato virus X (PVX) vector has been used to show that silencing of a non-viral transgene can suppress a virus with inserted sequences homologous to the silencing transgene (English et al., 1996). Tobacco plants displaying PTGS of β-glucuronidase (GUS) or npt were resistant to PVX.GUS and PVX.NPT, respectively, and tomato plants with PTGS of polygalacturonase (PG) were resistant to PVX.PG. Sijen et al. (1996) also observed that N. benthamiana plants with PTGS of the movement protein (MP) of cowpea mosaic cowumor (CPMV) displayed resistance to PVX.MP.

N. benthamiana infected with TMV–GFP–NP did not show green fluorescence and the appearance of systemic symptoms was delayed by 6–8 days compared with plants infected with TMV–GFP. Casper & Holt (1996) reported that the approximately 200 bp 3’ UTR in the GFP cDNA inhibited GFP expression drastically from TMV. Thus, the N gene sequence at the 3’ end of the GFP gene might have severely inhibited transcription and/or translation of the virus subgenomic mRNA.

Studies with CPMV and PVX suggest that there are preferential sites on the transgene mRNA molecules that act as signals for sequence-specific degradation (Sijen et al., 1996; English et al., 1996). The 640 bp 3’ region of the transcribed MP transcript of CPMV and the 700 bp 3’ region of the GUS transgene might have been responsible for elimination of the incoming chimeric PVX in those studies (PVX.MP and PVX.GUS, respectively). However, our data show that a single transgene confers resistance to both a tospovirus and TMV–GFP, suggesting either that there are multiple signals on the mRNA molecule for degradation or that, once the degradation process is triggered by some specific secondary structure or feature of the RNA, the process is capable of degrading any RNA molecule that shares sequence similarity with the transgene. The latter hypothesis does require a minimum length of homologous sequence to be effective, which is consistent with the data presented here. A similar result was reported by Seymour et al. (1993), who showed that the PG and pectinesterase (PE) genes were coordinately down-regulated in transgenic tomato plants transformed with a chimeric gene construct containing the 244 bp 5’ end of PG fused to the 5’ end of an 1320 bp PE. Moreover, our results are consistent with the recent observation of Marano & Baulcombe (1998), who showed that transgenic tobacco plants containing the TMV-U1 54 kDa replicase gene were resistant to PVX vectors containing the first half, middle half or second half of the replicase gene.

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