A single chimeric transgene derived from two distinct viruses confers multi-virus resistance in transgenic plants through homology-dependent gene silencing

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We showed previously that 218 and 110 bp N gene segments of tomato spotted wilt virus (TSWV) that were fused to the non-target green fluorescent protein (GFP) gene were able to confer resistance to TSWV via post-transcriptional gene silencing (PTGS). N gene segments expressed alone did not confer resistance. Apparently, the GFP DNA induced PTGS that targeted N gene segments and the incoming homologous TSWV for degradation, resulting in a resistant phenotype. These observations suggested that multiple resistance could be obtained by replacing the GFP DNA with a viral DNA that induces PTGS. The full-length coat protein (CP) gene of turnip mosaic virus (TuMV) was linked to 218 or 110 bp N gene segments and transformed into *Nicotiana benthamiana*. A high proportion (4 of 18) of transgenic lines with the 218 bp N gene segment linked to the TuMV CP gene were resistant to both viruses, and resistance was transferred to R₂ plants. Nuclear run-on and Northern experiments confirmed that resistance was via PTGS. In contrast, only one of 14 transgenic lines with the TuMV CP linked to a 110 bp N gene segment yielded progeny with multiple resistance. Only a few R₁ plants were resistant and resistance was not observed in R₂ plants. These results clearly show the applicability of multiple virus resistance through the fusion of viral segments to DNAs that induce PTGS.

**Introduction**

Tomato spotted wilt virus (TSWV) and turnip mosaic virus (TuMV) are among the ten most important viruses worldwide that infect vegetables (Tomlinson, 1987). TSWV, the type species of the genus *Tospovirus*, has a wide host range and is transmitted by several species of thrip (German et al., 1992). The virus genome consists of three ssRNAs designated as small (S), medium (M) and large (L). The L RNA is of negative polarity and encodes a 331–5 kDa putative viral polymerase (de Haan et al., 1991), while the S and M RNAs contain two open reading frames, in an ambisense gene arrangement (de Haan et al., 1990; Maiss et al., 1991; Kormelink et al., 1992b), which are expressed via the synthesis of subgenomic mRNAs (Kormelink et al., 1992a). The M RNA encodes the precursor of the envelope glycoproteins G1 and G2 and the non-structural protein NS₄M (Kormelink et al., 1994). The S RNA encodes a non-structural protein, NS₅S, and the structural nucleocapsid (N) protein. TuMV is a member of the genus *Potyvirus* in the family *Potyviridae* (Shukla et al., 1994). The monopartite genome of potyviruses consists of a positive-sense ssRNA of about 10 kb with a 5′-linked virus-encoded protein (VPg) and a polyadenylate tract at the 3′ end (Dougherty & Carrington, 1988). The coat protein (CP) gene is localized at the 3′ end of the genome and the CP is generated by proteolytic cleavage of the virus-encoded polyprotein (Dougherty & Carrington, 1988).

Post-transcriptional gene silencing (PTGS) has been reported to be one of the major mechanisms of RNA-mediated resistance in transgenic plants (Dougherty & Parks, 1995; Baulcombe, 1996; Baulcombe & English, 1996; Dawson, 1996; Prins & Goldbach, 1996; Beachy, 1997; van den Boogaart et al., 1998). Recently, we showed that transgenes consisting of ~400 bp segments of the N gene of TSWV...
conferred resistance to TSWV in transgenic plants through PTGS (Pang et al., 1997), but N gene segments of 92–235 bp did not. However, transgenic plants expressing transgenes consisting of 218 or 110 bp N gene segments linked to the 720 bp green fluorescent protein (GFP) gene were resistant to TSWV (Pang et al., 1997). Further work showed that transgenic plants with transgenes consisting of N gene segments of 59 or 24 bp similarly linked to GFP were not resistant to TSWV, even though the transgene showed PTGS (Jan et al., 2000). Collectively, these data suggested that segments of the N gene of 110 bp or more could confer resistance when linked to a transcribed DNA (designated ‘silencer’ DNA) that induces PTGS. This observation opened the possibility of developing multiple virus resistance in transgenic plants by using viral DNA as a ‘silencer’ and linking it to segments of other viral DNA.

In order to test this strategy for multiple virus resistance, we linked the 867 bp TuMV CP gene (Jan et al., 1999) to a 218 bp N gene segment of TSWV and transferred the construct into Nicotiana benthamiana. The CP gene of TuMV was used as a ‘silencer’ DNA because TuMV is considered to be the most important virus of cultivated cruciferous cash crops (Green & Deng, 1985) and it is known that the CP gene of TuMV induces PTGS and confers resistance to TuMV (Jan et al., 1999). Here, we report that transgenic plants expressing a transgene consisting of TuMV CP fused with a 218 bp N gene segment of TSWV are resistant to TSWV and TuMV via the PTGS mechanism.

**Methods**

**Vector construction and plant transformation.** The N gene of the lettuce isolate of TSWV (TSWV-BL; Pang et al., 1992) and the CP gene of the garden balsam isolate of TuMV (TuMV-ESC8; Jan et al., 1999) were used as templates for constructing hybrid transgenes with N gene segments of various lengths fused to the CP gene (Fig. 1). The terminology of the gene segments was described previously and indicates their size and location (Pang et al., 1997). The 3/4 N gene fragment corresponds to nt 400–600 of the 866 nt N gene (Pang et al., 1997), while the 5/8 N gene fragment corresponds to nt 400–500. The forward primers for the N gene segments were designed to contain an out-of-frame ATG and/or stop codon to ensure the production of untranslatable N gene transcripts (Pang et al., 1997). The 3/4 N gene segment (Pang et al., 1997) was amplified by PCR with oligomer primers 92-55 (5' AGATTCCTCTAGACCATGGTGACTTGATGAGCAAAG-TCTGTGAGGCTTGC), which is identical to the 5' RNA (Pang et al., 1992) of TSWVBL at nt 2373–2354, and 93-86 (5' TCTTGAGGATCCATGGATCCTGATATATAGCCAAGA), which is 5' untranslated leader (UT) of AlMV and the 3' untranslated sequence of the nopaline synthase gene (NOS-T).

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**TuMVCP+3/4N**

![TuMVCP+3/4N](pBI525)

**TuMVCP+5/8N**

![TuMVCP+5/8N](pBI525)

**Fig. 1.** Maps of the transgenes used in this study. Non-translatable N gene segments were fused transcriptionally with the TuMV CP gene. Each transgene was driven by a double-enhanced (Enh) CaMV 35S promoter (35S-P), the 5' untranslated leader (UT) of AlMV and the 3' untranslated sequence of the nopaline synthase gene (NOS-T).
Results

Generation of transgenic plants expressing N gene segments fused with TuMV CP

The untranslatable 3/4 N and 5/8 N gene segments of 218 and 110 bp were cloned as transcriptional fusions in the sense orientation into the XbaI/BamHI sites downstream of the TuMV CP gene in the plant expression vector pBI525-TuMVCP + (Fig. 1; Jan et al., 1999). Several clones with the proper insert were isolated, identified by restriction enzyme site mapping and sequenced to confirm their identity. The constructs containing the 3/4 N or 5/8 N gene segment fused to the TuMV CP were designated pBI525-TuMVCP +3/4N and pBI525-TuMVCP +5/8N, respectively (Fig. 1). Expression of those fusions is thus controlled by the double-enhanced cauliflower mosaic virus (CaMV) 35S promoter, the 5′ untranslated region from alfalfa mosaic virus (AlMV) and the 3′ untranslated region of the nopaline synthase (NOS) gene (Fig. 1). The expression cassettes were then moved to the plant transformation vector pBIN19 and used to obtain transgenic N. benthamiana plants via Agrobacterium-mediated transformation.

Table 1. Inoculation of TuMV and TSWV to R1 plants expressing the 3/4 N or 5/8 N gene segment linked to TuMV CP

TuMV or TSWV were applied to three upper leaves of the plants at the 5–7 leaf stage. Observations were made every other day for at least 45 days. The reactions were grouped into three phenotypes: susceptible (S), typical systemic symptoms were observed at 4–8 days after inoculation; delayed (D), systemic symptoms were delayed by 3–10 days compared with control susceptible plants; and resistant (R), the plants remained symptomless over the 45 day observation period. A total of 18 lines with CP +3/4N and 14 with CP +5/8N transgenes were screened for resistance to TSWV and TuMV.

<table>
<thead>
<tr>
<th>Line</th>
<th>Reaction to TSWV</th>
<th>Reaction to TuMV</th>
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<tbody>
<tr>
<td></td>
<td>n</td>
<td>S</td>
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<tr>
<td>CP + 3/4N</td>
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</tr>
<tr>
<td>55-4</td>
<td>11</td>
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<td>14</td>
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<td>55-21</td>
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</tr>
<tr>
<td>Nine other lines</td>
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<td>137</td>
</tr>
<tr>
<td>CP + 5/8N</td>
<td></td>
<td></td>
</tr>
<tr>
<td>125-12</td>
<td>20</td>
<td>14</td>
</tr>
<tr>
<td>Thirteen other lines</td>
<td>182</td>
<td>182</td>
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</table>

Fig. 2. Northern blot and nuclear run-on transcription analyses of R1 plants carrying the CP +3/4N fusion gene. (a) Northern blot analysis of R1 plants. RNA (10 µg per lane) was isolated from transgenic plants before inoculation and subsequently probed with the N gene of TSWV-BL (top) or the CP gene of TuMV (bottom). Plants were inoculated and scored as resistant (R) or susceptible (S). Lanes: 1–17, R1 plants of lines 55-4 (lanes 1–4), 55-6 (5–8), 55-9 (9–12) and 55-15 (13–17); 18, non-transgenic control. (b) Nuclear run-on transcription analysis of R1 plants. Labelled nuclear RNAs were hybridized to restriction enzyme-digested fragments of the nptII (lane 1), TSWV N (2), TuMV CP (3) and actin (4) genes separated on an agarose gel and blotted onto a membrane. The nuclei used in the assays were isolated from two resistant plants of line 55-9, two susceptible plants of line 55-15 and a non-transgenic control (NT).

A total of 23 R0 transgenic plants with TuMVCP +3/4N and 21 with TuMVCP +5/8N were initially confirmed to be transgenic by PCR analysis and nptII ELISA. Northern analysis of some R0 plants showed that the level of TuMV CP transcript...
Table 2. Inoculations of TSWV and TuMV to R2 progeny of TuMV- or TSWV-resistant R1 plants of lines 55-9, 55-21 and 125-12

See Table 1 for explanation of codes S, D and R. TuMVR or TSWVR refer to TuMV- or TSWV-resistant R1 plants that served as the seed source for the plants that were inoculated. ND: Not done.

<table>
<thead>
<tr>
<th>Progeny source</th>
<th>Expt 1*</th>
<th>Expt 2*</th>
<th>Expt 3</th>
</tr>
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<tr>
<td></td>
<td>TSWV (Inoc. 1)</td>
<td>TuMV (Inoc. 1)</td>
<td>TSWV (Inoc. 2)</td>
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<tr>
<td></td>
<td>S</td>
<td>D</td>
<td>R</td>
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<tr>
<td>R1 plants of line 55-9 with TuMV CP + 3/4N gene</td>
<td>4</td>
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<td>2</td>
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<tr>
<td>TuMVR-1</td>
<td>10</td>
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<td>3</td>
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<tr>
<td>TuMVR-2</td>
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<td>1</td>
</tr>
<tr>
<td>TuMVR-3</td>
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<tr>
<td>TuMVR-4</td>
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<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td>36</td>
<td>16</td>
<td>19</td>
</tr>
<tr>
<td>From 10 R1 plants of line 55-21 with TuMV CP + 3/4N gene</td>
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<td>752</td>
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<tr>
<td>TuMVR</td>
<td>80</td>
<td>44</td>
<td>0</td>
</tr>
<tr>
<td>From four R1 plants of line 125-12 with TuMV CP + 5/8N gene</td>
<td>53</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

* Plants were inoculated with TSWV or TuMV (Inoc. 1) and then TSWV-resistant plants were inoculated with TuMV and TuMV-resistant plants were inoculated with TSWV (Inoc. 2).
† Results are given as no. resistant/no. inoculated.

was tightly correlated with that of the N gene transcript, indicating that they are transcribed as a single transcription unit as designed (data not shown). All of the R1 plants were self-pollinated to produce R2 seeds for inoculation tests.

A TSWV N gene segment fused to the TuMV CP gene confers resistance to both viruses

Prior to testing for resistance, R1 seedlings from the transgenic lines were screened by nptII ELISA to identify transgenic and non-transgenic plants in the populations. Unlike negative-control plants, which were all susceptible to the virus, a proportion of R1 plants expressing the TuMVCP + 3/4N and TuMVCP + 5/8N transgenes were resistant to TuMV and TSWV. Table 1 summarizes the reactions of the inoculated R1 plants to TSWV-BL and TuMV-ESC8. The reactions could be grouped into three different categories: (i) resistant to both TuMV and TSWV, (ii) resistant to TuMV but susceptible to TSWV and (iii) susceptible to both TuMV and TSWV. No resistance to TSWV and TuMV was observed in a total of 262 plants examined from nine of the 18 TuMVCP + 3/4N transgene lines (Table 1). Some plants from four of the other nine lines (55-4, 55-6, 55-9, 55-17) displayed resistance to TSWV and TuMV while, interestingly, the five remaining lines (55-8, 55-11, 55-16, 55-20, 55-21) had plants that were resistant to TuMV but susceptible to TSWV (Table 1). In contrast, plants resistant to TSWV and TuMV were found in only one of the 14 tested lines with the TuMVCP + 5/8N transgene (Table 1).

Northern and nuclear run-on experiments showed that TuMV- and TSWV-resistant plants displayed PTGS of the TuMV CP and TSWV N genes (Fig. 2a). Selected TuMV- or TSWV-resistant plants of lines 55-4, 55-6 and 55-9 (Table 1) had relatively low RNA accumulation of CP and N gene transcripts, while selected susceptible plants from the same lines and plants from the susceptible line 55-15 showed high levels of CP and N gene transcripts (Fig. 2a). Nuclear run-on experiments showed that the transgene was being actively transcribed in the virus-resistant but low-message-accumulating plants of line 55-9 (Fig. 2b; line 55-9) (Fig. 2a) compared with plants of the susceptible line 55-15 (Fig. 2a, b). These results suggested that the reduced steady-state levels of the transcripts and virus resistance were due to PTGS.

Multiple resistance in R2 plants

Progeny (R2 plants) from several TSWV- or TuMV-resistant R1 plants from lines 55-9, 55-21 and 125-12 were evaluated for resistance to both viruses. Line 55-9 contained the CP + 3/4N gene and had R1 plants resistant to both TuMV and TSWV. Line 55-21 had the same gene as line 55-9, but the R1 plants were resistant only to TuMV. Line 125-12 had the CP + 5/8N gene and had R1 plants resistant to both viruses (Table 1). Plants were evaluated in two ways. In one set of
experiments, plants were inoculated with either TSWV or TuMV and observed for 34 days and then the resistant plants were inoculated with the virus that had not been inoculated previously. In the other experiments, plants were inoculated with extracts containing a mixture of both viruses. Results from the three experiments were similar and are summarized in Table 2. A proportion of the progeny from five of the six R₁ plants of line 55-9 were resistant to both viruses. Plants that were resistant to the first virus were also resistant to the second virus. These results showed clearly that multiple virus resistance was carried into the R₂ plants. A proportion of the progeny (R₂ plants) from 10 R₁ plants of line 55-21 were resistant to TuMV, but none of them were resistant to TSWV. This was consistent with the data generated from the R₁ plants (Table 1). In contrast, R₂ plants of line 125-12 with the CP + 5/8N gene had almost no resistance (Table 2). Only two of the 104 plants that were tested showed resistance, one to TSWV and the other to TuMV. Furthermore, while a number of plants from lines 55-9 and 55-21 showed delayed symptom development following virus inoculation, symptom delay was not observed in R₂ plants from line 125-12.

Discussion

We showed recently that transgenic plants with a transgene of GFP fused to small segments of the N gene of TSWV are resistant to TSWV through the mechanism of PTGS (Pang et al., 1997; Jan et al., 2000). Thus, multiple resistance was likely to be obtained if a viral gene was substituted for the GFP gene. We show now that transgenic plants containing a transgene consisting of the TuMV CP gene fused to a short TSWV N gene segment are resistant to both viruses via the PTGS mechanism. This is the first report of multiple virus resistance obtained using this approach.

Transgenic plants with multiple resistance have also been generated previously by combining the entire CP genes of more than one virus, with each gene being driven by a promoter and a terminator. Transgenic potato expressing the CP genes of potato virus X and potato virus Y were resistant to both viruses (Lawson et al., 1990; Kanievski et al., 1990). Similarly, transgenic tobacco with resistance to three tospoviruses (TSWV, tomato chlorotic spot virus and groundnut ringspot virus) (Prins et al., 1995), transgenic squash with resistance to two or three viruses [cucumber mosaic virus (CMV), watermelon mosaic virus 2 (WMV2) and zucchini yellow mosaic virus (ZYMV)] (Fuchs & Gonsalves, 1995; Tricoli et al., 1995; Fuchs et al., 1998), transgenic cantaloupe with resistance to CMV, ZYMV and WMV2 (Fuchs et al., 1997) and transgenic tomato with resistance to CMV isolates of subgroups I and II (Kanievski et al., 1999) have been developed. Our approach differs from these in that we need only one transgene with a promoter and a terminator and the transgene may contain only a segment of one of the viral genes.

PTGS is homology dependent and is the apparent mechanism for RNA-mediated virus resistance. It is, thus, reasonable to think that transgenic plants with resistance to three or more viruses can be obtained simply by transforming plants with a transgene consisting of fused gene segments of different viruses linked to a ‘silencer’ DNA (e.g. GFP, TuMV CP). Recent data from our laboratory provide support for this approach (unpublished results). N. benthamiana plants with a chimeric transgene consisting of three small N gene segments of three different tospoviruses linked to GFP DNA were resistant to all three tospoviruses. This novel approach has several advantages for controlling viruses. Firstly, multiple resistance could be generated to desired viruses. Secondly, the small non-translatable segments minimize the risks of recombination, transcapsidation, synergism or complementation, which have been raised as disadvantages of some strategies that use full-length viral genes. Thirdly, a chimeric transgene requires only limited genetic elements for expression in transgenic plants, thus reducing the demand for genetic elements to engineer multiple traits into a single biotech product. Finally, the CP gene has been shown to be very effective in conferring resistance and many viral CP genes have been identified and are readily available. In addition, it is likely that this strategy could be used in functional genomics to down-regulate multiple genes coordinately in plants, to identify genes responsible for biochemical pathways or to develop new biotech products with multiple traits.

Similar co-suppression of two genes in a chimeric transgene has been described in a few reports. Introduction in tomato of a chimeric gene construct containing 244 bp of the 5’ end of the polygalacturonase gene linked to the 5’ end of a 1320 bp pectinesterase gene was able to trigger co-suppression of both genes (Seymour et al., 1993). Gene constructs consisting of the β-glucuronidase gene (uidA) linked to the full-length chalcone synthase (chsA) cDNA or the 5’ half or the 3’ half triggered PTGS of both genes in transgenic petunia (Van Blokland et al., 1994; Stam et al., 1997). These results, along with our own, show that silencer sequences are effective when they are located either at the 5’-end (Fig. 1; Van Blokland et al., 1994; Stam et al., 1997; Jan et al., 2000) or the 3’-end (Seymour et al., 1993) of chimeric transgenes. Moreover, our results are consistent with observations from other groups, who have shown that there are multiple target sites for PTGS along the transgene. Marano & Baulcombe (1998) showed that transgenic tobacco plants containing the TMV-U1 5.4 KDa replicase gene were resistant to potato virus X vectors containing the first half, the middle half or the second half of the replicase gene. Jacobs et al. (1999) reported that sequences throughout the basic β-1,3-glucanase mRNA coding region are targets for PTGS in transgenic tobacco.

We observed that the 110 bp N gene segment was much less efficient than the 218 bp segment in conferring resistance to TSWV (Table 2), confirming our previous observations (Pang et al., 1997; Jan et al., 2000). This also suggests that the
minimum N gene length for conferring resistance is between 59 and 110 bp, since the former length does not confer resistance (Jan et al., 2000). However, the minimum gene segment length for conferring resistance to other viruses or for inactivating other homologous mRNAs probably differs from our observations with the N gene. Recently, Créte & Vaucheret (1999) showed that transformation of chimeric nii1–uidA, uidA–nii1 and nii1–uidA–nii1 transgenes carrying 186 bp of the 5′ end and/or 241 bp of the 3′ end of the tobacco nitrite reductase nii1 cDNA fused with the uidA gene did not trigger co-suppression of endogenous nii1 genes in transgenic tobacco. A possible reason for these results might be that the minimum length of nii1 gene required to trans-inactivate the nii1 genes is larger than 241 bp. Alternatively, the 5′- and 3′-terminal regions of the nii1 gene may be relatively inefficient targets for PTGS, as was recently reported by Jacobs et al. (1999) for transgenic tobacco with the gnt1 β-1,3-glucanase gene.

Interestingly, we also obtained plants that were resistant to TuMV (e.g. line 55-21 in Tables 1 and 2) and susceptible to TSWV, but not vice versa. These results suggest that there are differences between regions of a transgene in the effectiveness at conferring resistance within the same plant. Logically, one would assume that the chances of obtaining resistance to a particular virus via PTGS get higher as the length of the homologous DNA segment, and thus the target area for degradation, increases. Further analyses of these plants should shed light on the reasons for the occurrence of plants with resistance only to TuMV. Such studies will provide insights into the most effective gene segment lengths to use in developing multiple virus resistance by the above approach.

In summary, we have shown that transgenic plants with resistance to both a potyvirus and a tospovirus can be obtained by fusing a segment of the tospovirus N gene to the potyvirus CP gene. This provides a simple approach to develop plants with resistance to two viruses. More importantly, this work provides strong evidence that transgenic plants with resistance to three or more viruses can be obtained by transforming plants with a transgene consisting of viral segments linked to a DNA silencer, which could also be a viral gene. The practical value of multiple virus resistance is obvious, since crops are frequently infected with more than one virus.

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References


Multiple resistance via a single transgene


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