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Research Note

Engineered RNA-Mediated Resistance to Tomato Spotted Wilt Virus Is Sequence Specific

Marcel Prins,[1] Renato de Oliveira Resende,[1] Corine Anker,[1] Alie van Schepen,[2] Peter de Haan,[2] and Rob Goldbach[1]

[1]Department of Virology, Wageningen Agricultural University, Binnenhaven 11, 6709 PD Wageningen; and [2]S&G Seeds, Division of Technology, Westeinde 62, P.O. Box 26, 1600 AA Enkhuizen, The Netherlands
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Transgenic plants were produced that expressed a wide range of randomly chosen sequences of the tripartite tomato spotted wilt virus (TSWV) RNA genome or its complement. Testing the progenies of these plants revealed that only transgenic expression of N or NS_M gene sequences resulted in resistance to TSWV.

Additional keywords: movement protein, *Nicotiana tabacum,* nucleoprotein, tospovirus, virus resistance.

Since the first description of pathogen-derived resistance (PDR) against a plant virus (Powell Abel et al. 1986) many reports of coat protein-mediated resistance to plus-trand RNA viruses have been published (for references see, e.g., Wilson 1993). Transgenic expression of the nucleoprotein of the negative-strand RNA virus TSWV also resulted in resistance (Gielen et al. 1991; MacKenzie and Ellis 1992; Pang et al. 1992). In addition, PDR could by obtained by transgenic expression of other (modified) viral genes, e.g., genes encoding replicases, movement proteins, and other nonstructural proteins of various viral sources (for references see, e.g., Beachy 1993). Many of these reports indicate the involvement of the transgenically produced protein. However, transgenic plants also exhibit resistance when using untranslatable sequences derived from the coat protein gene of potyviruses (Lindbo and Dougherty 1992; Van der Vlugt et al. 1992), the nucleoprotein gene of TSWV (De Haan et al. 1992), and the RdRp gene of PVX (Mueller et al. 1995), indicating an RNA-mediated type of resistance. This RNA-mediated resistance operates via a mechanism similar to cosuppression or posttranscriptional gene silencing (Lindbo et al. 1993; Dougherty et al. 1994; Mueller et al. 1995; English et al. 1996).

Here we investigate further whether the induction of RNA-mediated resistance against TSWV is gene specific or if any randomly selected sequence of the TSWV genome would confer such resistance upon transgenic expression. For this purpose 17 different viral cDNA constructs were made. Together with the previously analyzed N gene constructs (Gielen et al. 1991; De Haan et al. 1992), this set spans over 70% of the TSWV genome, covering virtually the entire M and S segments and a large part of the L segment (Fig. 1). Between 7 and 25 original transformants (*Nicotiana tabacum* var. SR1) were obtained for each construct (Table 1), for a total of 325

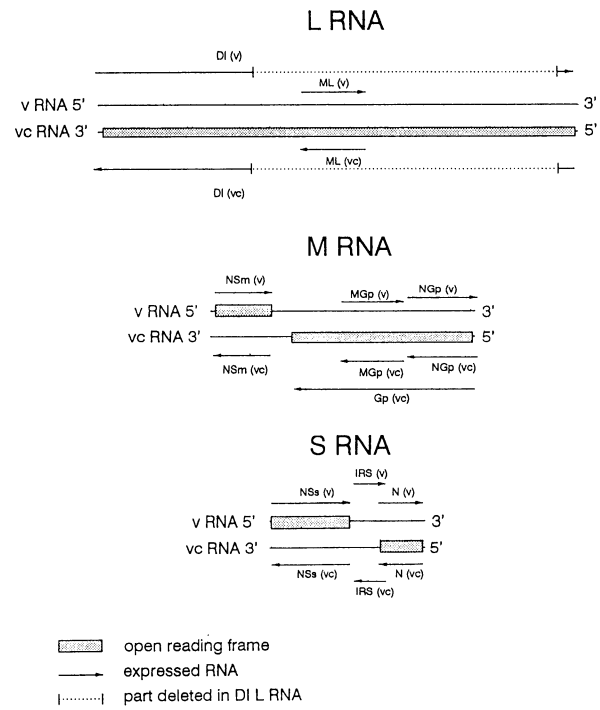

Fig. 1. Viral constructs of tomato spotted wilt virus (TSWV) relative to its genome. DI encompasses L RNA sequences found in a naturally occurring defective interfering RNA (Resende et al. 1991), ML spans the middle of the L ORF including the majority of the conserved polymerase motifs. Clone NSm contains the entire NS_M ORF. NGp comprises the N-terminal third of the precursor to the glycoproteins, MGp the central part of this precursor, while Gp transcribes the complete glycoprotein ORF. N and NSs clones include the respective genes with their viral 5′ untranslated leader sequences. IRS contains the cDNA of the highly internally base-paired intergenic region of the S RNA. For all genomic fragments, except for Gp, both viral (v) and viral complementary sequences (vc) were used. The sequences, supplied with a CaMV 35S promoter and a nos terminator, were cloned in the binary vector pBIN19 (Bevan 1984) and subsequently used for *Agrobacterium tumefaciens*-mediated transformation of *Nicotiana tabacum* var. SR1 leaf disks.

Corresponding author: R. Goldbach:
E-mail: rob.goldbach@medew.viro.wau.nl

Present address of R. de Oliveira Resende: Departmento de biologia celular-IB, Universidade de Brasilia, Brasilia DF, Brazil.

transformed plants in addition to the 48 lines previously tested. The S1 progenies of these plants were assayed for resistance to TSWV. Subsequently, the S2 progenies of surviving plants were also inoculated with the virus. All plants were inoculated twice within a 2-wk interval, using a high virus titer (TSWV strain BR-01).

Of all 373 plant lines tested, the only lines exhibiting high levels of virus resistance were those which expressed N or NS_M sequences. The resistance or lack thereof was irrespective of whether the sequences were expressed in a sense or antisense direction or whether or not they could be translated. The S2 progenies of the resistant plants expressing antisense N sequences and those expressing sequences of the NS_M gene, were completely immune to high doses of virus. These resistant S2 plants remained virus free, as verified by ELISA. The TSWV resistant plants were, however, fully susceptible to the closely related tospoviruses TCSV and GRSV, indicating the narrow spectrum of RNA-mediated resistance (data not shown). All lines expressing other parts of the TSWV genome were completely susceptible to the virus, indicating that for TSWV, the RNA-mediated pathogen-derived resistance is sequence specific and restricted to plants expressing N or NS_M gene sequences (Table 1).

Expression of transgenic RNA in S1 lines was checked by Northern blot analyses and was comparable for all constructs. All lines showed low levels of RNA accumulation when compared to viral RNA levels reached during virus infection. No correlation was found between the level of RNA accumulation and the degree of resistance (data not shown). However run-on assays, as exemplified in Figure 2 for N gene transformed lines, show that despite the lack of correlation between the steady-state RNA levels and the observed resistance, there was a clear correlation with the nuclear transcription levels and the resistance, as demonstrated for potyviral CP sequences by Lindbo et al. (1993) and Goodwin et al. (1996). Obviously, plants that display high nuclear transgene expression, yet have low steady-state RNA levels, must have an active mechanism for specific breakdown of these (transgenically expressed) viral RNA sequences, consequently resulting in resistance to the virus.

The observation that RNA-mediated resistance against TSWV is not effective with most parts of the viral genome is quite unexpected and seems to contradict some of the results obtained with positive-strand RNA viruses. For potato virus X (PVX) for instance, it has been found that even nonviral (GUS) sequences can induce RNA-mediated virus resistance provided only that this sequence has been introduced in the viral genome (English et al. 1996). A possible explanation why only the N and NS_M sequences of TSWV are capable of introducing transgenic resistance may be that its genomic RNA segments, like those of all negative-stranded RNA viruses, remain packaged with nucleocapsid protein throughout the infection cycle. The cosuppression-like RNA-mediated resistance might therefore not operate on (encapsidated) genomic level but rather on (nonencapsidated) viral mRNA level. Along this line the relative importance of the viral protein encoded by the different tospoviral mRNAs may be crucial for the effectiveness of several of the constructs used. Indeed both the N protein (regulator of transcription-to-replication switch) and NS_M protein (putative movement protein, Kormelink et al. 1994; Storms et al. 1995) are essential for systemic host plant infection, while inhibition of some other functions (e.g., G1/G2 and NS_S) may only interfere with replication in (Wijkamp et al. 1993) or spread by (Resende et al. 1991) the insect vector. Sequences derived from the putative viral RNA dependent RNA polymerase gene (L) did not confer resistance, although this protein is obviously necessary for replication. The limited number of polymerase molecules already present in the virus particle (Van Poelwijk et al. 1993)

Table 1. Resistance levels in S1 and S2 progenies of transgenic tobacco plants expressing parts of the TSWV genomic RNAs

Line	(# Resistant S1 lines/# S1 lines tested)	Resistant plants (%) S1	S2
N (vc/ORF)[a]	4/25	25–90	100
N (vc/AUG-)[b]	4/23	30–80	100
N (v)	4/24	10–50	100
IRS (v)	0/17	0	...
IRS (vc)	0/17	0	...
NS_s (v/ORF)	0/10	0	...
NS_s (vc)	0/22	0	...
NG (v)	0/22	0	...
NG (vc)	0/18	0	...
MG (v)	0/15	0	...
MG (vc)	0/20	0	...
G1/G2 (vc/ORF)	0/21	0	...
NS_M (v/ORF)	4/33	10–30	100
NS_M (v/AUG-)	1/26	50	100
NS_M (vc)	2/24	15–50	100
ML (v)	0/11	0	...
ML (vc)	0/25	0	...
DI (v)	0/13	0	...
DI (vc)	0/7	0	...

[a] Gielen et al. 1990.
[b] De Haan et al. 1991.

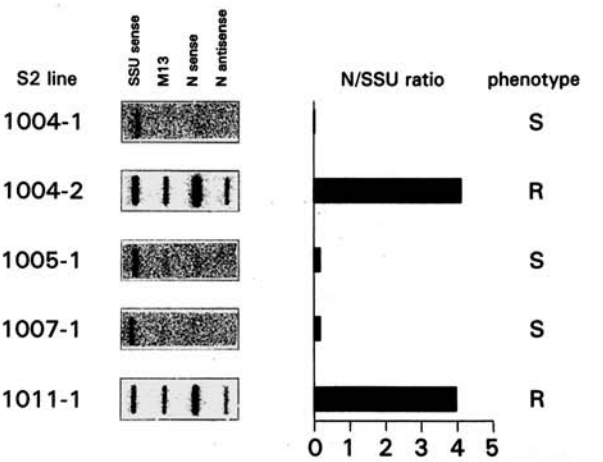

Fig. 2. Transgene transcription levels in isolated nuclei of tomato spotted wilt virus (TSWV) N gene-transformed plants. Steady-state RNA levels in all lines except segregant line 1004-1, lacking the transgene, are similar. SSU indicates the nuclear expression of the small subunit of rubisco, used as an internal standard. Amount of sense and antisense transcripts of TSWV N gene are indicated. N/SSU ratio indicates the relative expression level of the transgene. No transcription of antisense N sequences (induced, e.g., by a plant promoter) was observed, indicating that direct interaction of transgenically produced antisense RNAs with the viral gene sequence does not play an important role in the resistance mechanism. Hybridization of the probe to M13 DNA is nonspecific background. R and S are resistant and susceptible phenotypes of the transgenic plants.

might be able to carry out the initial rounds of replication and be sufficient for overcoming the inhibition of virus replication in transgenic plants. Alternatively, a small number of catalytic L protein molecules, sufficient for viral replication, may be produced despite the suppression of the mRNA.

A second explanation for the presented data might be that, for TSWV, the majority of the genome does not suit yet-unknown criteria needed for induction of or accessibility to posttranscriptional transgene silencing, like, e.g., short stretches of primary or secondary structure in the expressed RNA. Perhaps only the N or NS_M gene sequences or parts thereof have these capabilities, resulting in resistance of host plants to TSWV. This view is supported by the observation that, despite a large number of lines tested, no (cosuppression-like) RNA-mediated resistance to potyviruses was observed in plants expressing potyviral CI and NIb sequences (Maiti et al. 1993; Audy et al. 1994). Furthermore, in numerous transgenic plant lines expressing chalcone isomerase sequences no plants were observed that displayed cosuppression (J. Kooter, personal communication). Obviously, further experiments need to be carried out to support or exclude the alternative interpretations for sequence-specific RNA-mediated resistance against TSWV.

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LITERATURE CITED

Audy, P., Palukaitis, P., Slack, S. A., and Zaitlin, M. 1994. Replicase-mediated resistance to potato virus Y in transgenic tobacco plants. Mol. Plant-Microbe Interact. 7:15-22.

Beachy, R. N., ed. 1993. Transgenic resistance to plant viruses. Sem. Virol. 4:327-416.

Bevan, M. 1984. Binary *Agrobacterium* vectors for plant transformation. Nucleic Acids Res. 12:8711-8722.

De Haan, P., Gielen, J. J. L., Prins, M., Wijkamp, I. G., Van Schepen, A., Peters, D., Van Grinsven, M. Q. J. M., and Goldbach, R. 1992. Characterization of RNA-mediated resistance to tomato spotted wilt virus in transgenic tobacco plants. Bio/Technology 10:1133-1137.

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

English, J., Mueller, E., and Baulcombe, D. 1996. Suppression of virus accumulation in transgenic plants exhibiting silencing of nuclear genes. Plant Cell 8:179-188.

Gielen, J. J. L., De Haan, P., Kool, A. J., Peters, D., Van Grinsven, M. Q. J. M., and Goldbach, R. W. 1991. Engineered resistance to tomato spotted wilt virus, a negative-strand RNA virus. Bio/Technology 9:1363-1367.

Goodwin, J., Chapman, K., Swaney, S., Parks, T. D., Wernsman, E. A. and Dougherty, W. G. 1996. Genetic and biochemical dissection of transgenic RNA-mediated virus resistance. Plant Cell 8:95-105.

Kormelink, R., Storms, M., Van Lent, J., Peters, D., and Goldbach, R. 1994. Expression and subcellular localization of the NS_M protein of tomato spotted wilt virus (TSWV), a putative viral movement protein. Virology 200:56-65.

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

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

MacKenzie, D. J., and Ellis, P. J. 1992. Resistance to tomato spotted wilt virus infection in transgenic tobacco expressing the viral nucleocapsid gene. Mol. Plant-Microbe Interact. 5:34-40.

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

Mueller, E., Gilbert, J., Davenport, G., Brigneti, G., and Baulcombe, D. C. 1995. Homology-dependent resistance: Transgenic virus resistance in plants related to homology-dependent gene silencing. Plant J. 7:1001-1013.

Pang, S-Z, Nagpala, P., Wang, M., Slightom, J. L., and Gonsalves, D. 1992. Resistance to heterologous isolates of tomato spotted wilt virus in transgenic tobacco expressing its nucleocapsid protein gene. Mol. Plant Pathol. 82:1223-1229.

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

Resende, R. de O., De Haan P., Avila, A. C. de, Kitajima, E. W., Kormelink, R., Goldbach, R., and Peters, D. 1991. Generation of envelope and defective interfering RNA mutants of tomato spotted wilt virus by mechanical passage. J. Gen. Virol. 72:2375-2383.

Storms, M. M. H., Kormelink, R., Peters, D., Van Lent, J. W. M. and Goldbach, R. W. 1995. The nonstructural NSm protein of tomato spotted wilt virus induces tubular structures in plant and insect cells. Virology:485-493.

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

Van Poelwijk, F., Boye, K., Oosterling, R., Peters, D., and Goldbach, R. 1993. Detection of the L protein of tomato spotted wilt virus. Virology 197:468-470.

Wijkamp, I., Van Lent, J., Kormelink, R., Goldbach, R., and Peters, D. 1993. Multiplication of tomato spotted wilt virus in its insect vector, *Frankliniella occidentalis*. J. Gen. Virol. 74:341-349.

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