

# Molecular Basis of Gene-Specific RNA-Mediated Resistance to Tomato Spotted Wilt Virus in Transgenic Tobacco Plants

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*Transgenic resistance to tomato spot wilt virus (TSWV) has been accomplished by expressing the viral nucleocapsid (N) protein gene in transgenic tobacco. Similar levels of protection, i.e., complete immunity to the virus in homozygous S2 plants, have been observed when an untranslatable N protein gene was expressed, indicating that the N protein gene-based resistance is mediated by the expressed transgene viral RNA. To test whether this RNA-mediated resistance is limited to plants that express viral N protein RNA sequences, 325 transgenic plant lines were produced that expressed a wide range of parts of the TSWV RNA-genome or its complement, spanning over 70% of the TSWV genome, including the entire S and M RNA sequences. The progenies of these plants were screened for resistance to TSWV. Remarkably, only plants that expressed N or NSm gene sequences displayed immunity to the virus. The functions of these proteins may be blocked early in the infection process, suggesting an important role for these proteins in early stages of systemic virus infection. The mechanism by which the expression of transgenic RNAs can cause immunity in transformed plants is discussed.*

The genome of TSWV consists of three RNA species, of which the L RNA (8.9 kb) is of complete negative polarity; whereas, both the M RNA (4.8 kb) and S RNA (2.9 kb) have an ambisense coding strategy. The five viral open-reading frames (ORF) are translated from viral mRNAs (Kormelink et al. 1992b) and specify a total of six mature viral proteins: two nonstructural proteins (NSm and NSs of 52.4 kDa and 33.4 kDa, respectively); the two envelope glycoproteins (G1 and G2 of 78 kDa and 58 kDa, respectively); the putative viral polymerase (L of 331.5 kDa); and the N protein of 28.8 kDa (de Haan et al. 1990, 1991; Kormelink et al. 1992a).

In some cases the appearance of defective interfering RNAs (DI RNAs) derived from the L RNA have been observed in tospovirus-infected plants (Resende et al. 1991, 1992). Engineered resistance to TSWV has been accomplished previously by expressing the viral N protein gene in transgenic tobacco (Gielen et al. 1991) and was confirmed by others (MacKenzie and Ellis 1992; Pang et al. 1992). Recently, engineered TSWV resistance has been introduced in tomato plants (Kim et al. 1994)

and tomato hybrids (Ultzen et al. 1995). This N-gene mediated resistance appeared to be virus-specific and is broken by other tospoviruses. By simultaneous introduction of several tospoviral N protein gene sequences into one genetic locus in the plant genome, broad-spectrum immunity to several tospoviruses was obtained (Prins et al. 1995).

Besides the use of the nucleoprotein for tospoviruses and the widespread use of coat-protein sequences for positive strand RNA viruses (Wilson 1993), other, nonstructural gene sequences have been used to confer engineered virus resistance, including replicase, protease, and movement protein genes (reviewed in Beachy 1993).

Many of these reports indicate the involvement of the transgenically produced protein. However, a number of reports have shown that transgenic plants also exhibit resistance when using untranslatable forms of coat protein sequences (Lindbo et al. 1992; van der Vlugt et al. 1992), as was shown for sequences derived from the N protein gene of TSWV (de Haan et al. 1992). Resistance in plants expressing untranslatable forms of coat-protein and nucleoprotein genes indicate an RNA-mediated type of resistance. Most of the suggested mechanisms for this RNA-mediated resistance propose inhibition of viral replication by an anti-sense effect, or competition for factors involved in the replication process. Lindbo et al. (1993) proposed a process similar to cosuppression (Matzke and Matzke 1995) as a possible mechanism for this RNA-mediated resistance based on their observation of suppression of transgenic RNA levels after infection with the corresponding virus in resistant transgenic plants.

We report here that high levels of resistance to TSWV can be obtained in transgenic tobacco plants, not only by expressing N-gene sequences, but also by expressing sequences derived from its putative viral movement protein gene, NSm.

## Materials and Methods

### Viruses and plants

The different tospovirus strains, i.e., TSWV strain BR-01, tomato chlorotic spot virus (TCSV) strain BR-03, and groundnut ringspot virus (GRSV) strain SA-05, have been described by de Ávila et al. (1990, 1992, 1993) and were maintained on systemic hosts *Nicotiana rustica* var. America or *N. tabacum* var. SR1. Recipient plants used in the transformation experiments were *N. tabacum* var. SR1 plants. All manipulations with transgenic plant material were carried out under conditions (PKII) imposed by the Dutch (Voorlopige Commissie Genetische Modificatie).

### Construction of expression vectors

TSWV cDNA fragments situated in the genome at positions indicated in Figure 1

were cloned as *Pst*I fragments, either directly or after addition of *Pst*I linkers in the single *Pst*I site in expression vector pZU-A (Gielen et al. 1991) immediately downstream of a CaMV 35S promoter and flanked at their 3' ends by a nopaline synthase terminator. All expression cassettes containing TSWV cDNA inserts were finally cloned in pBIN19 transformation vector (Bevan 1984).

### **Transformation of tobacco**

The pBIN19-derived vectors were introduced in *Agrobacterium tumefaciens* strain LB4404 (Ditta et al. 1980) by triparental mating using pRK2013 (Horsch et al. 1985) as a helper plasmid. *N. tabacum* var. SR1 plants were transformed and regenerated as described by Horsch et al. (1985).

### **Analysis of protection of transgenic plants against TSWV**

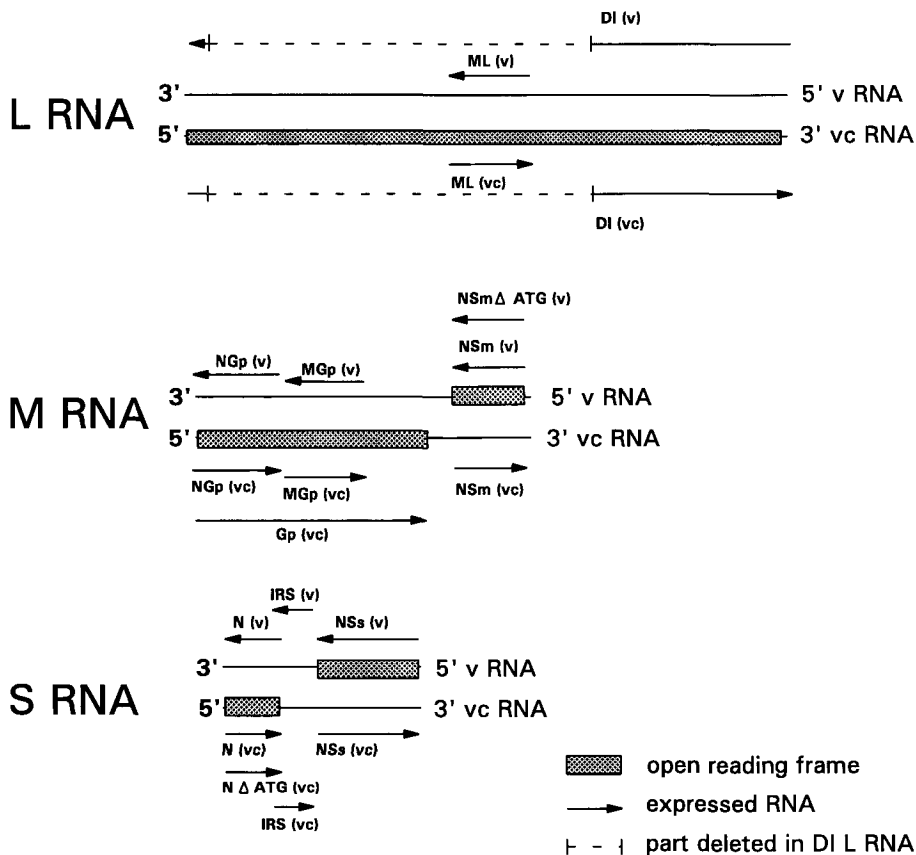
S1 progeny seeds were collected from 325 transformed plants. Resulting S1 lines were assayed for resistance to TSWV and resistant plants were maintained for seed production. Twenty plants from the S2 progeny lines were subsequently inoculated with TSWV. Finally, plants from TSWV-resistant S2 lines (expressing NSm or N sequences) were also challenged with TCSV and GRSV. Inoculations were done according to Gielen et al. (1991). The appearance of systemic symptoms was monitored on a daily basis until day 35 after inoculation. Plants were scored susceptible when leaves younger than the inoculated leaf showed characteristic tospovirus-induced symptoms, i.e., severe stunting and chlorosis, usually followed by death of the plant within a week. Leaf samples from visually healthy plants were collected to check for the presence of the NSs gene product by enzyme-linked immunosorbant assay (ELISA), using a polyclonal antiserum directed against TSWV NSs protein (Kormelink et al. 1991). This antiserum recognizes the NSs proteins of established TSWV, TCSV, and GRSV and is indicative of viral replication (Wijkamp et al. 1993).

## **Results**

### **Transformation of tobacco with TSWV sequences**

A total of 17 clones containing randomly chosen genomic RNA sequences of TSWV were constructed. Together with the previously analyzed N protein gene constructs (Gielen et al. 1991; de Haan et al. 1992) these clones span over 70% of the TSWV genome, covering virtually the entire M and S segments and a large part of the L segment. The positions of the tospoviral RNA sequences expressed from these constructs in the RNA genome of TSWV are indicated in Figure 1.

All genomic fragments, supplied with a CaMV 35S promoter and a nos terminator sequence, were cloned in binary vector pBIN19 (Bevan 1984) and subsequently used for *A. tumefaciens*-mediated transformation of *N. tabacum* var. SR1 leaf discs.



**Figure 1.** Position on the TSWV genome of the viral cDNA constructs used for transformation of tobacco plants. DI encompasses L RNA sequences found in a naturally occurring defective interfering RNA, ML spans the middle of the L ORF including the majority of the polymerase motifs, NGp comprises the N terminal third of the precursor to the glycoproteins, MGp the central part of this precursor, and Gp transcribes the complete glycoprotein ORF. Clone NSm encodes the entire NSm ORF, N and NSs clones also include their respective viral 5' untranslated leader sequences attached to their gene sequences. IRS contains the cDNA of the highly base-paired intergenic region of the S RNA.

### Resistance levels in transgenic tobacco plants

Between 7 and 35 original transformants were obtained for each construct (Table 1) to give a total of 325 transformed plants in addition to the 48 N-gene expressor lines tested in previously described experiments (Gielen et al. 1991; de Haan et al. 1992). The S1 progenies of these plants were assayed for resistance to TSWV. Subsequently, the S2 progenies of surviving S1 plants were also inoculated with the virus. To obtain highly resistant progeny, i.e., RNA-mediated resistance phenotype, all plants were inoculated twice within a 2-week interval, using a high titer of

**Table 1.** Resistance levels inoculation in S1 and S2 progenies of transgenic tobacco plants expressing parts of the TSWV genomic RNAs.<sup>a</sup>

Line	Resistant lines/ Total lines	Percentage of resistant plants within resistant lines	
		S1	S2
N <sup>b</sup> (vc/ORF)	4/25	25–90	100
N <sup>c</sup> (vc/ATG-)	4/23	30–80	100
N (v)	4/24	10–50	100
IRS (v)	0/17	0	–
IRS (vc)	0/17	0	–
NS <sub>s</sub> (v/ORF)	0/10	0	–
NS <sub>s</sub> (vc)	0/22	0	–
NG <sub>p</sub> (v)	0/22	0	–
NG <sub>p</sub> (vc)	0/18	0	–
MG <sub>p</sub> (v)	0/15	0	–
MG <sub>p</sub> (vc)	0/20	0	–
G <sub>p</sub> (vc/ORF)	0/21	0	–
NSm (v/ORF)	4/33	10–30	100
NSm (v/ATG-)	1/26	50	100
NSm (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	–

<sup>a</sup>V viral RNA; VC viral complementary RNA.

<sup>b</sup>Gielen et al. 1990.

<sup>c</sup>de Haan et al. 1991.

TSWV strain BR-01. In addition to the previously described N-gene expressor lines (Gielen et al. 1991; de Haan et al. 1992), only plants expressing the antisense N or NSm (both sense and antisense) remained free of virus, whereas all lines expressing other parts of the TSWV genome were susceptible to the virus, indicating that for TSWV, the RNA-mediated pathogen-derived resistance is sequence specific and restricted to plants expressing N or NSm gene sequences (Table 1).

### Expression of transgenic RNA and protein

The transcriptional expression of transgenes was checked by Northern blot, using a <sup>32</sup>P-dATP labelled double-stranded cDNA probe. In most lines, transgenically produced RNA was detected, but in much lower levels than those observed in virus-infected plants. Possible expression of the NSs and G1 and G2 when translatable RNAs were expressed in transgenic lines was assayed by antigen-coated plate and

triple-antibody sandwich ELISA, respectively, using NSs polyclonal antiserum (Kormelink et al. 1991) and monoclonal antibodies against glycoproteins (Huguenot et al. 1990). Detectable amounts of protein could not be shown in any of the S1 lines that expressed translatable NSs or glycoprotein RNAs, despite the presence of transgenic RNAs. In leaf extracts from NSm-transformed plants no NSm protein could be detected by Western blots or by ELISA techniques. NSm protein, however, may have been present in undetectably low levels.

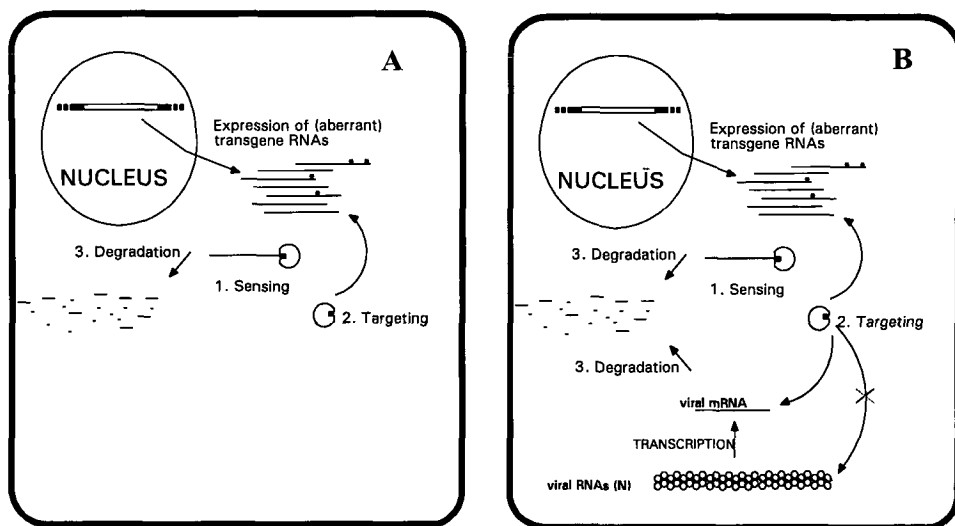
### **Resistance to other tospoviruses**

The antisense N and NSm expressing S2 progeny plants, which were completely immune to infection with TSWV, were also challenged with two other closely related tospoviruses: TCSV strain BR-03 and GRSV strain SA-05 (de Ávila et al. 1993). All inoculated plants appeared to be completely susceptible to both viruses, similar to previous observations with the TSWV-resistant sense N expressing plants (de Haan et al. 1992)

### **Discussion**

We show that, besides expression of N-gene sequences of tospoviruses (Gielen et al. 1991; de Haan et al. 1992; Prins et al. 1995), expression of sequences derived from the NSm gene, the putative tospoviral movement protein gene, confers resistance in transgenic tobacco plants. Observed resistance in plants that expressed the NSm gene sequence reached similarly high levels to those observed in plants that expressed N protein gene sequences. The manifestations of the RNA-mediated resistance phenomenon induced by NSm sequences is similar to that of the previously described N gene-mediated resistance. Resistance induced by NSm and N sequences both result in immunity to TSWV in homozygous S2 progeny lines. Moreover, they both share the same spectrum of resistance, and only hold their resistance against TSWV from which the transgene sequence was derived. Similar characteristics have also been found for RNA-mediated resistance to other viruses, e.g. potyviruses TEV (Lindbo et al. 1992) and PVY (van der Vlugt et al. 1992).

A number of theories have been proposed for the mechanism of such an RNA-mediated resistance. Lindbo et al. (1993) proposed a mechanism that implies the induction of an antiviral state in the cytoplasm of transgenic plants, similar to the sense- or cosuppression phenomenon observed in transgene expression studies of endogenous genes in plants (for a review see Matzke and Matzke 1995). This cosuppression causes dramatically decreased endogenous RNA steady-state levels upon expression of homologous transgene sequences as a result of a post-transcriptional RNA-degrading mechanism. RNA-mediated resistance in plant lines expressing the TEV coat protein RNA sequences that were immune to the virus was suggested to be a result of increased RNA turnover of (transgene) viral sequences, because nuclear de novo synthesis in run-on assays remained high in these plants;



**Figure 2.** Proposed mechanism of transgene silencing (A) (modified from Lindbo et al. 1993) and RNA-mediated resistance to tospoviruses (B). (A) In silenced plants, transgenes are expressed to an “unacceptable” level and may contain aberrations, which is sensed (1) by a cytoplasmic factor that is able to specifically target the transgene mRNAs (2) and subsequently degrade it (3). (B) After entry of the virus, the (unencapsidated) viral mRNAs, which have the same sequence as the transgene (i.e., N or NSm), are also targeted by the cytoplasmic factor and degraded, rendering the virus unable to carry out essential functions in replication and movement, respectively.

whereas, cytoplasmic steady-state RNA levels were low (Dougherty et al. 1994). Our preliminary results with run-on studies also suggest that TSWV resistance operates through a cosuppression-like mechanism similar to that described for potyviruses (Lindbo et al. 1993; Dougherty et al. 1994).

Only sequences derived from the N and NSm gene regions of the TSWV genome appear to be able to confer resistance to transgenic plants. All other RNA sequences used in this extensive experiment, covering most of the TSWV genome and all genes, were unable to confer resistance to transgenic plants. The explanation for the sequence specificity of the resistance should reside in the mode of its action. Possibly, the mechanism that causes RNA-mediated resistance (Figure 2A) cannot interfere with the viral genomic RNA because it remains encapsidated in the N protein throughout the infection cycle, but only with the (nonencapsidated) viral mRNAs (Figure 2B). The relevance of the viral proteins, encoded by these RNAs, may be crucial for the effectiveness of several of the constructs used.

Because only N and NSm sequences can confer resistance, this would imply that both N and NSm proteins play essential roles in tospoviral infection of plants;

whereas, others proteins do not. Indeed, the G1/G2 and NSs proteins may not be essential for mechanical inoculation and subsequent replication and spread of the virus in the plant. Resende et al. (1992) already indicate that this is true for the glycoproteins, because envelop-deficient mutants of TSWV, lacking the capacity to produce glycoproteins, are perfectly capable of infecting plants systemically. The role of the NSs protein has not been studied in great detail, but comparison of the NSs gene sequences reveals it is the least conserved of all tospovirus genes (only 8% between TSWV and groundnut bud necrosis virus; Heinze et al. 1995), which indicates that it is not involved in a basic process like genome replication or cell-to-cell movement in plants.

Sequences derived from the putative viral RNA-dependent RNA polymerase gene (L) seem to be unable to confer resistance, although this protein is obviously indispensable for replication. Perhaps the complete ORF, or at least most of the RNA sequence, must be expressed in plants to induce resistance and not only the partial gene sequences used in this experiment. Another possibility may be that the limited number of polymerase molecules already present in the virus particle (van Poelwijk et al. 1993) is sufficient to support the initial rounds of replication, which may be essential to overcome the inhibition of virus replication in transgenic plants.

This paper demonstrates that two different regions of the tospoviral genome, i.e. the N and NSm protein genes, can be successfully exploited to engineer high levels of (RNA-mediated) host-plant resistance. RNA-mediated resistance can tolerate quite a number of mutations in the target virus. A combination of these two effective regions into one chimaeric transformation vector may even further increase this tolerance, thereby creating a very durable form of resistance.

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