RNAi-Mediated Transgenic Tospovirus Resistance Broken by Intraspecies Silencing Suppressor Protein Complementation

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Extension of an inverted repeat transgene cassette, containing partial nucleoprotein (N) gene sequences from four different tomato-infecting *Tospovirus* spp. with a partial N gene sequence from the tomato strain of *Tomato yellow ring virus* (TYRV-t), renders transgenic *Nicotiana benthamiana* plants additionally resistant to this strain but not to the soybean strain of this *Tospovirus* sp. (TYRV-s), both strains exhibiting 14.4% nucleotide sequence divergence in their N genes. Surprisingly, coinoculation of the TYRV-t-resistant transgenic lines with both TYRV-t and TYRV-s resulted in rescue of the former. Mass-spectrometric analysis of the viral ribonucleocapsids accumulating in the transgenic plants showed the presence of the N proteins of both strains excluding hetero-encapsulation as rescue mechanism and indicating suppression of TYRV-t N gene transcript breakdown by RNA interference. Prior (*Potato virus X* [PVX]-vector-mediated) expression of the TYRV-s silencing suppressor (NS_s) gene also allowed TYRV-t to break the resistance. This phenomenon was also observed when the homologous (TYRV-t) NS_s gene was provided from a PVX replicon, demonstrating that TYRV can break RNA-mediated host resistance upon a priori expression of its NS_s protein. Remarkably, mixed inoculation of TYRV-t with other *Tospovirus* spp. or nonrelated viruses did not result in resistance breaking, indicating that the rescuing activity of NS_s—though based on suppressing RNA silencing—is species-dependent.

Sources of natural resistance to *Tospovirus* spp. suitable for commercial breeding are very limited and, therefore, extensive studies have been made to create engineered forms of host resistance against these pathogens (Goldbach et al. 2003). Among these are the use of plantibodies or aptamers (Prins et al. 2005; Rudolf et al. 2003) but, thus far, the most successful and widely studied strategy employs transgenic expression of partial or intact nucleoprotein (N) gene sequences, which has led to heritable immunity against homologous *Tospovirus* spp. (Gielen et al. 1991; Jan et al. 2000; Prins et al. 1996; Stoeva et al. 1998). It has been demonstrated that this form of resistance is based on RNA silencing or RNA interference (RNAi)

(Baulcombe 1999) (i.e., transgenically expressed viral RNA sequences provoke the antiviral RNA silencing mechanism in the plant). Analysis of transgenic plants containing sequences from different parts of the viral genome has shown that, in the case of *Tospovirus* spp., antiviral RNA silencing targets the viral transcripts rather than the (N protein-encapsidated) viral genome (Prins et al. 1996, 1997). To induce RNA silencing against *Tospovirus* spp., a minimal length of the N transgene sequence (236 to 387 bp) is required but, when fused to a non-target gene, a shorter sequence (59 to 110 bp) is sufficient (Jan et al. 2000; Pang et al. 1997).

A major drawback of RNA-mediated virus resistance is its high specificity. For instance, RNAi-mediated resistance against *Tomato spotted wilt virus* (TSWV) does not hold against *Groundnut ring spot virus* (GRSV) and *Tomato chlorotic spot virus* (TCSV) despite 78 to 80% genome sequence homology to TSWV (Prins et al. 1996). To circumvent this problem, broad *Tospovirus* spp. resistance has recently been created by designing a chimaeric transgene cassette containing multiple (partial) N gene sequences derived from different (tomato-infecting) *Tospovirus* spp. and triggering the silencing of all tospoviral targets simultaneously (Bucher et al. 2006). Moreover, because this transgene cassette, due to its inverted repeat topology, is expressed as a double-stranded (ds) hairpin RNA molecule, resistant lines were obtained at very high efficiency.

Recently, we have identified and characterized a new tomato-infecting *Tospovirus* sp. from Iran named *Tomato yellow ring virus* (TYRV) (Hassani-Mehraban et al. 2005). Further studies on this novel species have revealed the presence of two distinct strains; that is, a tomato strain (TYRV-t) and a soybean strain exhibiting an overall 20% sequence divergence in their S RNA segment (Hassani-Mehraban et al. 2007).

To further improve the existing (Bucher et al. 2006) transgene cassette, directed against four different tomato-infecting *Tospovirus* spp. (i.e., TSWV, TCSV, GRSV, and *Watermelon silver mottle virus* [WSMoV]), this cassette has now been extended with a partial N gene sequence of the fifth tomato-infecting *Tospovirus* sp., TYRV from tomato (TYRV-t), and tested for its performance in *Nicotiana benthamiana*. It is shown that transgenic resistance against TYRV-t does not hold against the soybean strain (TYRV-s) and, moreover is broken by TYRV-t when coinoculated with TYRV-s. Further investigations indicate a role of the viral silencing suppressor protein in this phenomenon.

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RESULTS

Performance of an extended tospoviral transgene cassette.

Previously chimaeric inverted repeat transgene constructs with *N* gene sequences from four different tomato-infecting *Tospovirus* spp. have been generated and shown to confer resistance to these viruses in *N. benthamiana* (Bucher et al. 2006). The resistance was shown to be based on RNA silencing of incoming viral RNA target sequences, induced by hairpin RNA molecules in planta transcribed from these transgene cassettes. To extend the resistance spectrum to a fifth tomato-infecting *Tospovirus* sp., TYRV (Hassani-Mehraban et al. 2005), the chimaeric *N* gene cassettes were provided with a partial *N* gene sequence of TYRV-t. Four constructs were made in which the five *N* gene sequences were cloned in different orientations (inward-directed [IN] versus outward-directed [OUT]) as inverted repeats separated by an intron sequence in sense (INTs) or antisense (INTas) polarity (Fig. 1A). For each of these four constructs, at least 15 independent transgenic *N. benthamiana* lines were generated and tested for resistance by mechanical

inoculation with TYRV-t. Transformed lines containing the *N* gene RNAi cassette with a sense intron sequence (IR-OUT-INTs and IR-IN-INTs) (Fig. 1A; Table 1) already showed high levels (up to 67 to 73%) of resistance in the F₁ generation, whereas no resistance levels were obtained when the intron sequence was present in antisense orientation. From the lines containing an *N* gene RNAi cassette with a sense-oriented intron sequence, the F₂ progeny showed 100% resistance to all five *Tospovirus* spp. (Table 1). Of these, line 31, containing an IR-IN-INTs construct, was used for further analysis.

The transgenic resistance is based on RNA silencing.

Analysis of small-interfering RNAs (siRNAs) in transgenic lines containing a multiple *N*-gene cassette demonstrated that the resistance was based on RNA silencing (Bucher et al. 2006). To confirm that this also applied for TYRV-t in transgenic plants containing the extended tospoviral *N*-gene cassette, transgenic TYRV-resistant plants were tested for the presence of TYRV-t-specific siRNAs. To this end, total RNA was purified from nonchallenged leaves of F₂ line 31.5 and leaves in-

Fig. 1. Schematic presentation of the inverted repeat *N*-gene cassette constructs used for transformation of *Nicotiana benthamiana*. **A**, Fragments approximately 150 nucleotides in length, specific for the *N* gene of *Tomato spotted wilt virus* (TSWV) (nucleotide position 462 to 623 relative to the ATG start codon at +1), *Groundnut ring spot virus* (GRSV) (nucleotide position 462 to 612), *Tomato chlorotic spot virus* (TCSV) (nucleotide position 312 to 462), *Wartmelon silver mottle virus* (WSMoV) (nucleotide position 4 to 158), and the tomato strain of *Tomato yellow ring virus* (TYRV-t) (nucleotide position 667 to 825) were polymerase chain reaction fused and cloned as an inverted repeat (IR) flanking an intron (IN) sequence in various orientations. **B**, Sequence alignment of the TYRV-t partial *N* gene, used for the tospoviral transgene cassette, to that of the soybean strain (TYRV-s).

oculated with TYRV-t or TYRV-s, and subsequently examined by Northern blot analysis for the presence of siRNAs. Indeed, TYRV *N*-gene-specific siRNAs were present in line 31.5 or plants challenged with TYRV-t + TYRV-s and TYRV-s, comigrating with those derived from TCSV and with a 21-nucleotide (nt) siRNA size marker (Fig. 2A and B), whereas no siRNA was detectable in nontransgenic *N. benthamiana*. Altogether, the results indicated that the obtained resistant phenotype was also for TYRV based on RNA silencing.

Rescuing of TYRV-t by mix inoculation with TYRV-s.

Previous studies have shown that transgenic resistant plants expressing (partial) TSWV *N* gene sequences are only resistant to strains and isolates of the homologous virus but not to other, closely related *Tospovirus* spp. (de Haan et al. 1992; Gielen et al. 1991; Prins et al. 1995). Recently, we have reported on the occurrence of two distinct strains of TYRV in Iran (i.e., TYRV-s in soybean along with TYRV-t in tomato), differing in several biological parameters and showing 13.8% sequence diversity within the TYRV-t partial *N* gene used for cloning and transformation (Fig. 1B). TYRV-t-resistant line 31.5 appeared susceptible to TYRV-s (Fig. 3) and showed an elevated level of TYRV-specific siRNAs after challenging with this strain compared with unchallenged line 31.5 (Fig. 2B). Moreover, when coinoculated with TYRV-s, the TYRV-t was able to systemically infect line 31.5, as demonstrated by reverse-transcription polymerase chain reaction (RT-PCR) detection (Fig. 4A), and leaves revealed further enhanced levels of TYRV-specific

siRNAs compared with line 31.5 only infected with TYRV-s. The rescue phenomenon was not observed when TYRV-t was coinoculated with two more distantly related *Tospovirus* spp., *Iris yellow spot virus* (IYSV) (24.3% *N* gene sequence divergence to TYRV-t) or *Impatiens necrotic spot virus* (INSV) (52.1%) (Fig. 4B and C), or with nonrelated viruses *Cucumber mosaic virus* (CMV) (Fig. 4D) and *Potato virus Y* (PVY) (data not shown).

Rescuing of TYRV-t by TYRV-s is not based on hetero-encapsidation.

The observation that TYRV-t could be rescued in transgenic resistant lines by TYRV-s implied a transcomplementation event involving a protein from TYRV-s. Because the *N* protein gene of TYRV-s was not silenced in line 31.5, it was tempting to assume that the TYRV-s *N* protein was able to hetero-encapsidate the TYRV-t genome, thus supporting its replication and systemic spread.

To test this possibility, nontransformed and resistant (line 31.5) *N. benthamiana* plants were inoculated singly or mixed

Table 1. Resistance levels in F₁ and F₂ progeny of transgenic *Nicotiana benthamiana* lines

Line (construct) ^a	Resistant/tested ^b	Resistant plants (%)	
		F ₁	F ₂
IR-OUT-INTs	11/15	73	100
IR-IN-INTs	10/15	67	100
IR-IN-INTas	0/15	0	0
IR-OUT-INTas	0/15	0	0

^a IR = inverted repeat, OUT = outward-directed orientation, INTs = sense polarity, IN = inward-directed orientation, INTas = antisense polarity.

^b Number of resistant F₁ lines per number of F₁ lines tested, indicating that all 10 plants for each line tested were resistant to the tomato strain of *Tomato yellow ring virus*.

Fig. 3. Pathogenicity on wild-type and transgenic resistant *Nicotiana benthamiana* plants (line 31.5) upon mechanical inoculation with the tomato strain of *Tomato yellow ring virus* (TYRV-t) (left plants) and the soybean strain (TYRV-s) (right plants). All plants show chlorosis and stunting, except line 31.5 challenged with TYRV-t, which remained healthy.

Fig. 2. Northern blot detection of **A**, viral specific small-interfering (si)RNAs in wild-type (WT) *Nicotiana benthamiana* and **B**, tomato strain of *Tomato yellow ring virus* (TYRV-t) transgenic resistant line 31.5 mock inoculated or challenged with TYRV-t or the soybean strain (TYRV-s). **A**, Northern blot detection of TYRV-t-specific siRNAs from mock-inoculated WT *N. benthamiana* or challenged singly or with a mixture of TYRV-t + TYRV-s (bottom part of the panel). Hybridization was performed with a digoxigenine-labeled DNA probe specific for TYRV-t; **B**, Northern blot detection of *Tomato chlorotic spot virus* (TCSV)- or TYRV-t-specific siRNAs from WT *N. benthamiana* or transgenic resistant line 31.5 either mock inoculated or challenged singly or with a mixture of TYRV-t + TYRV-s (bottom part of the panel). Hybridization was performed with a DNA-specific probe for TCSV (left part of the panel) and TYRV-t (right part of the panel), as indicated at the bottom of the panels. A 21-nucleotide siRNA size marker has been included at the left. A loading control was performed (upper part of the panels) to verify the input of similar RNA amounts.

with TYRV-s and TYRV-t and analyzed for the presence of N protein of either strain. Once leaves were systemically infected, these were harvested for the purification of viral ribonucleoproteins (RNP), after first having verified the presence of both strains by RT-PCR. Purified RNP preparations were resolved on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), N protein was excised from the gels, trypsin cleaved, and subsequently analyzed by mass spectrometry (MS). Analysis of TYRV-t and TYRV-s N protein from the nontransformed, singly infected control plants showed distinct peptide profiles, thus allowing the use of MS for distinguishing TYRV-t and -s N proteins, even in mixtures (Table 2).

Nucleoprotein purified from RNP preparations of a mixed infection of line 31.5 demonstrated the presence of TYRV-t-specific N protein peptides along with those of TYRV-s N peptides (Table 2, last column), ruling out a hetero-encapsidation as rescue mechanism.

TYRV-t is rescued by TYRV-s silencing suppressor protein.

The presence of TYRV-t N protein during a mixed infection of line 31.5 with TYRV-s indicated that silencing of the TYRV-t N gene transcript was hampered. This suggested that the presence of the TYRV-s-encoded silencing suppressor (NS_s) was crucial for rescuing TYRV-t. To test this possibility, the resistant

Fig. 4. Reverse-transcription polymerase chain reaction (RT-PCR) detection of the tomato strain of *Tomato yellow ring virus* (TYRV-t) from wild-type (WT) *Nicotiana benthamiana* and transgenic resistant lines upon a single or mixed inoculation with **A**, the soybean strain (TYRV-s); **B**, *Iris yellow spot virus* (IYSV); **C**, *Impatiens necrotic spot virus* (INSV); or **D**, *Cucumber mosaic virus* (CMV). Plants used are indicated at the top (WT or resistant line 31.5), and whether they were singly or mixed infected. On top of each lane, the virus detected by RT-PCR is indicated. During the single infections, this also refers to the virus used to challenge these plants. **A**, For RT-PCR detection of TYRV-t and TYRV-s, specific primer sets for each were used, rendering a 220-bp fragment specific for TYRV-t and a 496-bp fragment for TYRV-s. RT-PCR detection of the other viruses rendered fragments of approximately **B**, 800 bp for IYSV; **C**, 800 bp for INSV; and **D**, 1,100 bp for CMV. As size marker, λ DNA digested with *Pst*I was included at the left of each gel.

Table 2. Mass spectrometry (MS) data obtained from ribonucleoprotein (RNP)-derived N protein purified from the soybean and tomato strains of *Tomato yellow ring virus* (TYRV-s and TYRV-t, respectively) (singly) infected wild-type *Nicotiana benthamiana* and a mixed infection of transgenic line 31.5

RNP sample, unique peptide ^a	Position	m/z	Charge state [M+H] ⁺	Mascot score	TYRV-t + TYRV-s
TYRV-s					
K.MFMELIAVENVAEQQK.M	104 to 119	942.35	2+	96	x
K.LCELPMVSAAYGLKPSSK.F	124 to 140	639.6	3+	101	x
K.ILNDCTPGAAGAASLNK.F	222 to 238	851.79	2+	108	x
K.ALESTFGK.I	245 to 252	411.68	2+	45	x
TYRV-t					
K.MFMELIAVENASEQQK.M	104 to 119	948.34	2+	101	x
K.LCELPMVNAYGLKPSPK.F	124 to 140	627.25	3+	97	x
K.ILNDCTPGTAGAASLNK.F	222 to 238	836.85	2+	126	x
K.ALESFQK.I	245 to 252	426.68	2+	47	x

^a Tomato and soybean strain-specific peptides, their charge state, mass, and MASCOT score as identified by tandem MS/MS are shown, with the distinguishing amino acids indicated in bold.

line 31.5 was coinoculated with TYRV-t and a *Potato virus X* (PVX) vector expressing the RNAi suppressor-active, TYRV-s NS_S protein. Because the timing of TYRV-s NS_S expression from PVX was thought to be important, a time course experiment was performed in which line 31.5 plants were infiltrated with PVX-NS_S (from TYRV-s) at day 0 and infected with TYRV-t 2 days later. Using RT-PCR, the presence of TYRV-t was monitored in the primary and systemically infected leaves. Although previous use of the TYRV-t-specific primer set did not reveal any cross reaction (Fig. 4), during the analysis here, a large fragment of approximately 1,000 bp showed up in all samples. This fragment did not match the expected 220-bp TYRV-t-specific fragment and was absent from healthy leaf samples; therefore, the fragment was likely due to a cross-annealing event. In repeated inoculation experiments, TYRV-t was rescued when resistant plants were preinoculated with PVX-NS_S. Time-course analysis showed that, in this case, the rescuing of TYRV-t by PVX-NS_S was observed for a period of several days from 2 to 4 days postinoculation onward (Fig. 5A, upper panel). Western blot analysis of inoculated and systemic leaves revealed the absence of NS_S from the systemic leaves, and its transient presence in the inoculated leaf during the time span that TYRV-t was rescued (Fig. 5A, lower panel).

Given the high sequence similarity (94% identity) between the NS_S proteins of TYRV-t and TYRV-s, TYRV-t's own suppressor would also be expected to rescue TYRV-t on a resistant line. Indeed, this was observed (Fig. 5B). Control experiments in which TYRV-t and empty PVX vector were coinoculated on 31.5 were negative, as expected (data not shown).

DISCUSSION

Previously, it was shown that plants, transformed with a hairpin construct containing partial *N* gene sequences from four *Tospovirus* spp. (TSWV, GRSV, TCSV, and WSMoV) are resis-

tant to all of them, and that the underlying mechanism is based on post-transcriptional gene silencing (also referred to as RNAi) (Bucher et al. 2006). In the present work, this chimaeric *N*-gene cassette has been extended with a partial *N*-gene sequence of a fifth, tomato-infecting *Tospovirus* sp. from Iran, TYRV-t, and successfully shown to broaden the resistance spectrum to five different *Tospovirus* spp. after transformation to *N. benthamiana*. Four different inverted repeat constructs, spaced by an intron sequence, were made and—in the case of a sense intron insert—shown to generate a high frequency of resistance, up to 73%, already in the F₁ progeny. Resistance was shown to be based on RNA silencing of target sequences. When this *N*-gene cassette was provided with the intron insert in reverse orientation, the resistance frequency of the transgenic plants obtained was dramatically lower, implying that intron orientation did matter and contrasting earlier data from Heilersig and associates (2006). Interestingly, the resistance against TYRV-t did not hold against the soybean strain of this virus, showing 13.8% diversity within the cloned sequence. Mismatches between the *N* genes of both TYRV strains were found along the entire sequence, with nine mismatches in the first half of the fragment and 13 in the second half (Fig. 1B). Although TYRV-t and TYRV-s shared a stretch of 22 nt fully identical within the transformed 159-nt TYRV-t sequence, this appeared not sufficient to additionally render resistance against TYRV-s. From earlier studies on RNAi-mediated resistance against *Tospovirus* spp., it was known that RNA-mediated resistance is broken at approximately 20% sequence divergence (Prins et al. 1995, 1996); however, the current results indicate that isolates with even lower divergence can break this resistance. This not only, and again, stresses the high sequence specificity of RNA-mediated resistance (Baulcombe 1999; de Haan et al. 1992; Gielen et al. 1991; Goodwin et al. 1996; Prins et al. 1996) but also has consequences for the use of RNA-mediated resistance against *Tospovirus* spp. such as TYRV and IYSV, for which it is known that their *N* gene sequence may

Fig. 5. Time-course reverse-transcription polymerase chain reaction (RT-PCR) detection of the tomato strain of *Tomato yellow ring virus* (TYRV-t) during local infection of transgenic resistant line 31.5. **A**, RT-PCR detection of TYRV-t (using a specific primer set rendering a 220-bp fragment specific for TYRV-t) after transcomplementation with the soybean strain (TYRV-s) silencing suppressor (NS_S) protein expressed from *Potato virus X* (PVX), showing the appearance of a 220-bp specific TYRV-t fragment from 2 to 4 days postinfection (dpi) (upper panel), and coinciding with the expression of TYRV-s NS_S in the infiltrated leaves (Western blot, lower panel). **B**, RT-PCR detection of TYRV-t after *cis*-complementation with TYRV-t NS_S protein expressed from PVX, and coinciding with the expression of TYRV-t NS_S in the infiltrated leaves (Western blot, lower panel). As controls, total RNA purified from mock-infected (lane Healthy) and TYRV-t-infected *Nicotiana benthamiana* (lane TYRV-t) were included. In the first lane (M), λ DNA digested with *Pst*I has been included as size marker. Leaves were infiltrated with *Agrobacterium tumefaciens* containing pGR106-I- NS_S at day 0, and inoculated with TYRV-t 2 days later. Three days after infiltration (i.e., 1 dpi), local leaves were harvested each day for analysis up to 8 dpi.

vary between 10 and 15% at nucleotide level. More surprisingly, however, was the observation that, during a mixed (TYRV-t and TYRV-s) inoculation, strain TYRV-t also was able to systemically infect the transgenic, resistant plants. As a result, elevated levels of TYRV-(t+s)-specific siRNAs could be discerned in these plants. The presence of TYRV-t N protein in these plants, as confirmed by MS, indicated that TYRV-t N gene transcripts were not silenced any more and that TYRV-t genome replication and systemic spread in the resistant transgenics was not due to hetero-encapsidation by TYRV-s N protein. Rather, our further experimentation revealed that it is the action of the viral NS_s causing viral rescue on the resistant phenotype. At first sight, this explanation seems very plausible, because the resistance is based on RNA silencing which can be blocked by providing a suppressor protein of a nonsilenced virus.

Because TSWV NS_s has recently been shown to exhibit affinity for siRNAs and longer dsRNAs (*unpublished data*), it is likely that the rescuing of TYRV-t is due to a reduction in TYRV-t-sensing RISC complexes in the transgenic lines caused by the a priori presence of NS_s. Considering the high sequence homology of tospoviral NS_s proteins, the failure to rescue a TYRV-t infection in transgenic resistant lines by mixed infections with more distinct *Tospovirus* spp. is somewhat intriguing. However, it cannot be excluded that this might be due to differences in timing and amounts of NS_s expression.

Rescuing of TYRV-t was only transient and restricted to the inoculated leaf when NS_s was provided using a PVX vector, the absence of systemic rescue probably being due to the intrinsic instability of the PVX-vector construct (Lacorte 2006; Uhde et al. 2005; Ziegler et al. 2000). Earlier, a PVX-based vector expressing NS_s of TSWV was shown to render a mixture of PVX molecules with an intact, partially, or lost NS_s open reading frame in the progeny (Sonoda and Tsumuki 2004), a phenomenon also assumed to result from vector instability (Avesani et al. 2007). Indeed, Western immunoblot experiments to detect NS_s systemically were always negative, whereas the protein was easily detected in the inoculated leaf (Fig. 5A and B). Alternatively, a decrease of NS_s expression may have been caused by silencing of the PVX replicon through siRNAs generated after TYRV-t replication.

The failure of distinct *Tospovirus* spp. or nonrelated viruses to rescue TYRV upon mixed inoculation seems to indicate that the apparent blocking of antiviral RNAi is (intra)species spe-

cific. The molecular basis of this high specificity is not yet understood and requires further studies but, whatever the mechanism has been, our data unequivocally show that care should be taken when using RNAi-based transgenic resistance approaches when dealing with *Tospovirus* spp. exhibiting significant N gene sequence variation.

MATERIALS AND METHODS

Virus sources and maintenance.

Tospovirus spp. TSWV, GRSV, TCSV, WSMoV (de Ávila et al. 1992; Heinze et al. 1995), and TYRV (Hassani-Mehraban et al. 2007) were maintained on *N. benthamiana* by mechanical inoculation. For inoculation, infected leaf material was ground in 0.01 M phosphate buffer, pH 7.0, containing 0.01 M Na₂SO₃ and subsequently rubbed on Carborundum-dusted leaves of wild-type- or *Agrobacterium tumefaciens*-transformed *N. benthamiana*. Plants were kept under greenhouse conditions with a daily regime of 16 h of light and 8 h of darkness.

Cloning and construction of inverted-repeat constructs.

For the construction of a gene cassette containing partial N-gene sequences from five different *Tospovirus* spp., an earlier described N-gene cassette containing fragments of approximately 150 nt of the N genes from four different *Tospovirus* spp. (Bucher et al. 2006) was used and extended by PCR fusion with a 159-nt fragment of the TYRV-t N gene using TYRV-IR-F and N-Stop primers (Table 3). This fragment mapped to nucleotide positions 667 to 825 of the N gene and was specifically selected with no homology to the (partial) N-gene sequences within the gene cassette to avoid instability, due to intramolecular recombination in either bacteria or plants, of the construct. Clones in pGEM-T Easy vector (Promega Corp., Madison, WI, U.S.A.) were selected for both orientations using M13F and N-Stop primers (Table 3). To obtain a hairpin RNA construct of this gene cassette, two copies of the N-gene cassette were cloned as an inverted repeat by using the GATEWAY Cloning Technology according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, U.S.A.). To this end, the N-gene cassette was first flanked with recombination adapter sequences by PCR using PGateEasyF and PGateEasyR primers (Table 3) prior to introduction into donor vector pDONR207 by BP clonase. The cassette was subsequently transferred to destination vector

Table 3. List of primers used in this study

Primer ^a	Sequence (5' to 3') ^b
TYRV-IR-F	CCCGGATCCCTAAATGACTGTACTCCAGGG
N-Stop	CCCGGATCCGCACTCATTAATAATGCATC
FP01	CCCGCGGCCGCTCTAACGTTAAGCAGCTCACAG
M13F	TGTAAAACGACGGCCAGT
PGate Easy F	GGGGACAAGTTTGTACAAAAAAGCAGGCTACGACGGCCAGTGAATTGTA
PGate Easy R	GGGGACCATTGTGACAAGAAAGCTGGGTGACCATGATTACGCCAAGCTA
FP6	CATTGTTTGAGTCTCTTCTATGAGCTTAACTCTTATCATGCTATC
FP2	GAGATAACTTTCACAACTGCTTGACTTTCTGAAAAATCGCC
TYRV-t ND	GAATTCACCTTTCACTGGG
TYRV-s ND	CCCGGATCCTGTGGAAAAATGTGGCTG
T35S-2	CCTTATCTGGGAACCTACTCACACA
IY1	CCCGAGGATCCATCCATGGCTACCGTTAGGG
IY2	CCCGAGGATCCAAATTAATTATCTATCTTTCTTGG
J060	CATGGATCCTGCAGAGCAATTGTGTCA
J064	CTTTGCTTTTACGACAGTGCA
CMVCP-5	CCCCGGATCCTGGTCTCCTT
CMVCP-1	CCCCGGATCCACATCAAGTTTTRAGRTTCAATTC
NS _s F	CCCGCGGCCGATGTCTACCGTCAAAACA
NS _s R	CCCCGCGGCCGCTCACTGCACCTCTTCTAC

^a TYRV = *Tomato yellow ring virus*; F = forward; R = reverse; TYRV-t and TYRV-s = tomato and soybean strains, respectively; NS_s = silencing suppressor.

^b Nonviral sequences (restriction enzymes, *attB* 1-2 recombination sites, and fusion-polymerase chain reaction overlapping stretches) included in the primers are shown in italics.

pK7GWIWG2 by LR clonase to give rise to an inverted repeat construct in which the *N*-gene cassette repeats were separated by an *Arabidopsis* intron sequence (Karimi et al. 2002). Clones were selected containing an IN or OUT inverted repeat construct with an INTs or INTas sequence. Construct fidelity was verified by restriction enzyme digestion and sequence analysis prior to transformation into *A. tumefaciens* GV3101.

Transformation and regeneration of *N. benthamiana*.

Transformation and regeneration of in-vitro-grown *N. benthamiana* were performed by the leaf disk method according to Horsch and associates (1985). Transgenic tobacco shoots, selected for resistance to kanamycin (100 µg/ml), were rooted, potted in soil, and transferred to the greenhouse. In all, 40 lines of each construct were generated and, from these, 15 lines were challenged with TYRV-t by mechanical inoculation. Those lines showing a resistance phenotype were selected to produce seed for the F₂ generation.

Testing transgenic lines for *Tospovirus* spp. resistance.

Seed of the F₂ progeny were plated on selective MS medium (Murashige and Skoog, including vitamins, at 4.4 g/liter; 3% sucrose [wt/vol], pH 5.7 to 5.8; and agarose at 6 g/liter) (Bucher et al. 2006) containing kanamycin selection pressure (100 µg/ml). Young seedlings were transferred to pots and challenged by mechanical inoculation with TYRV-t, TYRV-s, TSWV, GRSV, TCSV, or WSMoV individually. In coinoculation studies, seedlings were challenged with TYRV-t in a combination with TYRV-s, IYSV, INSV, CMV, or PVY. Plants were monitored for symptom expression on a daily basis. After 3 to 4 weeks, plants were checked for the presence of *Tospovirus* spp. by double-antibody sandwich enzyme-linked immunosorbent assay.

Infections with TYRV-t or TYRV-s were verified by RT-PCR on total RNA extracted from systemically infected leaves 14 days postinoculation using Trizol agent (Invitrogen) according to the manufacturer's instructions. RT-PCR was performed on 2 µg of RNA using AMV reverse transcriptase (Promega Corp.) and primers TYRV-t ND and TYRV-s ND, specific for TYRV-t and TYRV-s, respectively, in combination with N-Stop (Table 3), rendering an *N*-gene fragment of 220 nt for TYRV-t and 496 nt for TYRV-s. In a similar fashion, specific primer sets were used to detect IYSV (IY1 and IY2), INSV (J060 and J064), and CMV (CMVCP-1 and CMVCP-5) (Table 3).

Detection of siRNA.

Total RNA was isolated from transgenic line 31.5 challenged with TYRV-t, followed by polyethylene glycol precipitation (Bucher et al. 2004). Approximately 20 µg of total RNA was resolved on a 12% polyacrylamide gel containing 7 M urea and 0.5 M Tris-borate EDTA buffer and subsequently transferred to Hybond N+ membrane (GE Healthcare UK Ltd., Buckinghamshire, U.K.). The RNA was screened with probes specific for detection of TCSV and TYRV-t. Probes were approximately 150 to 160 bp in length and digoxigenine labeled during PCR amplification using primers FP6 and FP2, and TYRV-IR-F and N-Stop, respectively (Table 3), in the presence of NTP-digoxigenine (Roche Diagnostics GmbH, Mannheim, Germany). Prehybridization and hybridization was carried out in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 48°C. After overnight hybridization, membranes were finally washed twice with 2× SSC and 0.2% SDS at 48°C. siRNA molecules were detected by chemiluminescence as described by Webster and Barker (1998).

PVX vector constructs.

The NS_S genes of TYRV-s and TYRV-t were RT-PCR amplified using primers NS_SF and NS_SR (Table 3) and subse-

quently cloned in the pGR106-I binary vector containing a PVX replicon. Clones containing the sense orientation were transformed to *A. tumefaciens* GV3110 containing the pSoup helper plasmid. *A. tumefaciens* was infiltrated on *N. benthamiana* plants for transient expression of TYRV-s and TYRV-t NS_S. Samples were taken from infiltrated leaves after 3 to 8 days postinfiltration (dpi) and analyzed for the expression of NS_S by Western immunoblotting using WSMoV NS_S monoclonal antibody.

A. tumefaciens clones expressing TYRV-s NS_S and TYRV-t NS_S protein were selected and used in an *Agrobacterium*-mediated transient transformation assay (Johansen and Carrington 2001) on TYRV-t-resistant, transgenic lines prior to inoculation with TYRV-t at 2 dpi. Plants were monitored for symptom expression on a daily basis and analyzed by RT-PCR for the rescuing and systemic spread of TYRV-t.

Tandem MS of RNP-derived nucleoprotein.

MS was performed on tospoviral N protein excised from Coomassie Brilliant Blue-stained SDS-PAGE gels loaded with purified RNP preparations (de Ávila et al. 1990). In-gel proteolytic digestion of simply-blue-stained protein was performed essentially as described (Lebbink et al. 2006) using trypsin (Roche Diagnostics, Penzberg, Germany). Samples were subjected to nanoflow liquid chromatography (LC) (Agilent 1100 series) and concentrated on a C18 precolumn (100 µm i.d., 2 cm column size). Peptides were separated on an analytical column (75 µm i.d., 20 cm column size) at a flow rate of 200 nl/min with a 60-min linear acetonitrile gradient from 0 to 80%. The LC system was directly coupled to a quantitative time-of-flight microtandem mass spectrometer (Micromass Waters, Manchester, U.K.). A survey scan was performed from 400 to 1,200 amu s⁻¹ and precursor ions were sequenced in MS/MS mode at a threshold of 150 counts. Data were processed and subjected to database searches using Proteinlynx Global Server (version 2.1; Micromass) or MASCOT software (Matrixscience, Boston) against SWISSPROT and the National Center for Biotechnology Information nonredundant database, with a 0.5-Da mass tolerance for both the precursor ion and fragment ion. The identified peptides were confirmed by manual interpretation of the spectra.

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