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Nontarget DNA sequences reduce the transgene length necessary for RNA-mediated tospovirus resistance in transgenic plants

(pathogen-derived resistance/cosuppression)

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ABSTRACT **RNA-mediated virus resistance has recently been shown to be the result of post-transcriptional transgene silencing in transgenic plants. This study was undertaken to characterize the effect of transgene length and nontarget DNA sequences on RNA-mediated tospovirus resistance in transgenic plants. Transgenic *Nicotiana benthamiana* plants were generated to express different regions of the nucleocapsid (N) protein of tomato spotted wilt (TSWV) tospovirus. Transgenic plants expressing half-gene segments (387–453 bp) of the *N* gene displayed resistance through post-transcriptional gene silencing. Although smaller *N* gene segments (92–235 bp) were ineffective in conferring resistance when expressed alone in transgenic plants, these segments conferred resistance when fused to the nontarget green fluorescent protein gene DNA. These results demonstrate that (*i*) a critical length of *N* transgene (236–387 bp) is required for a high level of transgene expression and consequent gene silencing, and (*ii*) the post-transcriptional gene silencing mechanism can trans-inactivate the incoming tospovirus genome with homologous transgene segments that are as short as 110 bp. Therefore, the activation of post-transcriptional transgene silencing requires a significantly larger transgene than is required for the trans-inactivation of the incoming viral genome. These results raise the possibility of developing a simple new strategy for engineering multiple virus resistance in transgenic plants.**

Gene silencing was discovered when introduction of a transgene resulted in suppression of the homologous host gene (1, 2). Subsequent studies showed that transgenes can also be silenced when multiple copies of the transgene were introduced into plants or when a single copy of the transgene became homozygous (3–10). Gene silencing can occur at post-transcriptional or transcriptional levels (3, 11–14). Post-transcriptional gene silencing results from an increase in RNA turnover (3, 15). Transcriptional gene silencing usually involves extensive methylation of the affected genes (4, 7, 9, 12, 16–18). However, transgene methylation is not limited to transcriptional gene silencing and has recently been reported to be also associated with post-transcriptional gene silencing (14, 19–22).

Post-transcriptional silencing has recently been found to be the cause of RNA-mediated virus resistance in transgenic plants (20–28). This type of virus resistance is also referred to as homology-dependent resistance (25, 29). RNA-mediated resistance is effective against RNA viruses that replicate and accumulate in the cytoplasm of the infected cells. It is therefore likely that the mechanism of gene silencing and RNA-mediated virus resistance takes place in the cytoplasm. Several models have been postulated to explain observed gene silencing and the resulting virus resistance, which include the expression threshold model (8, 20, 24, 30, 31) and the ectopic pairing model (22, 31, 32).

Previously, we generated engineered resistance to tomato spotted wilt virus (TSWV) in lettuce (27), tobacco (33, 34), *Nicotiana benthamiana* (35), and tomato (unpublished work). The genome of tospoviruses consists of three single-stranded RNAs: S RNA (2,900 nucleotides), M RNA (≈5,000 nucleotides), and L RNA (8,900 nucleotides). Both S and M RNAs contain two open reading frames (ORFs) of an ambisense gene arrangement (36–41), which are expressed by means of the synthesis of subgenomic mRNAs (42). The S RNA encodes a 52-kDa nonstructural protein (NSs) in the viral RNA strand and the 29-kDa nucleocapsid (N) protein in the viral complementary RNA strand, while the M RNA encodes a 34-kDa nonstructural protein (NSm) in the viral RNA strand and the precursor to the 58-kDa (G2) and 78-kDa (G1) membrane-associated glycoproteins in the viral complementary RNA strand. The L RNA is of negative polarity and encodes a large 200-kDa protein, presumably for the viral transcriptase (43).

We are studying post-transcriptional gene silencing in relation to the consequent tospovirus resistance in transgenic plants. Previously, we reported that this type of resistance is affected by transgene dosage and the stage of plant development in transgenic lettuce expressing full-length *N* gene sequences (27). Here we demonstrate that the RNA-mediated tospovirus resistance is dependent on the length of the *N* transgene. Unlike large *N* gene segments (387–453 bp), small *N* transgene segments (92–235 bp) were not silenced in transgenic plants unless they were transcriptionally fused to the green fluorescent protein gene (*GFP*). The implication of this observation is discussed in relation to the possible mechanism of post-transcriptional gene silencing as well as a possible strategy for engineering multiple virus resistance in transgenic plants.

MATERIALS AND METHODS

Cloning and Transformation. The *N* gene (33) of the lettuce isolate of tomato spotted wilt virus (TSWV-BL) was used as the template for construction of the *N* gene segments of various lengths using the primers listed in Table 1. The forward primers for the *N* gene segments were designed to contain an out-of-frame ATG and/or stop codons to ensure the production of untranslatable *N* gene transcripts. The PCR-amplified *N* gene segments (Table 2) were cloned in the sense orientation into the *Nco*I site of the plant expression vector pBI525 (33).

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Abbreviations: TSWV, tomato spotted wilt virus; N, nucleocapsid protein; GFP, green fluorescent protein; INSV, impatiens necrotic spot virus; GRSV, groundnut ringspot virus.

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Table 1. Primers used in cloning *N* gene segments of TSWV and the entire GFP gene

Name	Nucleotide positions*	Sequence
		For the *N* gene fragments
91-84	2766–2744	5′-AGCTAATCTAGAACC**AT**GG**ATG**ACTCAC**TAA**GGAAAGCATTGTTGC
93-89	2669–2650	5′-TACAGTTCTAGAACC**ATG**GTCTGGAAAACCT**TGA**CCAG
93-85	2576–2556	5′-TACAGTTCTAGAACC**ATG**GT**AA**AGCGATTTTACTTTTGGTA
92-55	2373–2354	5′-AGATTCTCTAGACC**ATG**G**TGA**CTTG**ATG**AGC**AAA**GTCTG**TGA**GGCTTGC
93-91	2266–2248	5′-TACAGTTCTAGAACC**ATG**GAAAATACAAGGATCTCGGG
93-87	2153–2133	5′-TACAGTTCTAGAACC**ATG**GT**AG**AAGGGGAAAGAGTATGCTG
90-47	1918–1937	5′-AGCATTGGATCCATGGTTAACACACTAAGCAAGCAC
93-86	2158–2177	5′-TCTTGAGGATCCATGGCTGATCTTCATTCATTTCAA
93-90	2269–2288	5′-TCTTGAGGATCCATGGATCCTGATATATAGCCAAGA
92-53	2383–2402	5′-TACAGTGGATCCATGGTTAAGGTAATCCATAGGCTTGAC
93-84	2577–2597	5′-TCTTGAGGATCCATGGCTTAATAACCTTCATTATGC
93-88	2671–2690	5′-TCTTGAGGATCCATGGAAAAGTCTTGAAGTTGAATG
94-265	2556–2530	5′-AGCTAATCTAGAACC**ATG**G**ATG**AAAAATTACCA**TAA**AGAAAACTTCAGAC
94-266	2182–2206	5′-AGCATTGGATCCATGGTTAGTTACCTAGTTTTCTTTTCAGCACAGTGCAAACT
		For the *GFP* gene
5′ GFP	NA	5′-TGAACATCTAGAACCATGGGTAAAGGAGAAGAACTTTTCACTGG
3′ GFP	NA	5′-TGAACAGGATCCATGGTCTACGAATGCTATTATTTGTATAGTTC

NA, not applicable.

*Nucleotide position number of each primer for the *N* gene segments was as published by Pang *et al.* (33). The *N* gene and GFP gene sequences are underlined and the rest of the sequences correspond to the primers used for cloning. Out-of-frame ATG and stop codons for the untranslatable versions in the 5′ primers are shown in boldface.

For construction of various *N* gene segment fusions with *GFP*, the translatable *GFP* ORF was amplified with *GFP* primers (Table 1) from the plasmid pGFP (CLONTECH) and cloned as transcriptional fusions into the 5′ *Nco*I site of the *N* gene segments 2/2 *N*, 3/4 *N*, and 5/8 *N* in pBI525. The resulting *GFP*/*N* fusions contained translatable *GFP* ORF followed by untranslatable *N* gene segments of different lengths as the 3′ untranslated regions of the *GFP* gene.

The resulting plant expression vectors were digested with *Hin*dIII and *Eco*RI (partial digestion where necessary), and the *Hin*dIII–*Eco*RI segments containing the corresponding gene cassettes were isolated and introduced into the same sites of pBIN19. The resulting binary vectors were transferred into *Agrobacterium tumefaciens* LBA4404, and the *A. tumefaciens* containing the vectors were used to inoculate leaf discs of *Nicotiana benthamiana* plants, essentially as described by Horsch *et al.* (44).

ELISA and Northern Blot Analyses of Transgenic Plants. Double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) was used to detect the *npt*II enzyme in transgenic plants using an *npt*II ELISA kit (5 Prime → 3 Prime). Northern blotting was performed as described previously (34).

Inoculation of Transgenic Plants. Inoculations were done as described previously (33). Systemic symptoms were recorded every other day for at least 2 months.

Isolation of Nuclei and Nuclear Run-off Transcription Assays. Isolation of nuclei and nuclear run-off transcription assays were previously described (27).

Table 2. Inoculations of TSWV-BL to R_1 *N. benthamiana* plants expressing *N* gene segments

Gene	Size, bp	No. tested		No. of resistant lines	Reactions of test plants*		
		Lines	Plants		HS	HT	HR
1/2 *N*	387	5	99	2	75	4	20
m/2 *N*	411	6	108	6	36	17	55
2/2 *N*	453	7	118	5	63	26	29
1/4 *N*	194	12	134	0	134		
2/4 *N*	193	5	64	0	64		
3/4 *N*	218	12	145	0	145		
4/4 *N*	235	7	89	0	89		
1/8 *N*	102	14	170	0	170		
2/8 *N*	92	8	63	0	63		
5/8 *N*	110	7	85	0	85		
6/8 *N*	108	13	162	0	162		
Control			239		239		

*TSWV-BL-infected leaf extracts (1/30) were applied to three upper leaves of *N. benthamiana* at the 5- to 7-leaf stage. Reactions were grouped into three phenotypes: (*i*) highly susceptible (HS), typical systemic symptoms were observed 5–10 days after inoculation; (*ii*) highly tolerant (HT), systemic symptoms were delayed more than 10 days after inoculation; and (*iii*) highly resistant (HR), plants remained symptomless. All R_1 plants were tested by *npt*II ELISA to identify transgenic plants. Nontransgenic plants are in the control row; the other rows consist of transgenic plants. See Fig. 1 for definitions of *N* gene segments of TSWV-BL.

RESULTS

Small *N* Gene Segments (92–235 bp) Do Not Induce RNA-Mediated Tospovirus Resistance. The untranslatable *N* gene segments 92–453 bp in length (Table 2) were PCR amplified using appropriate sets of the 5′ and 3′ primers (Table 1). The 5′ primers were designed to contain an out-of-frame ATG followed by stop codons to prevent the translation of truncated N protein. The *N* gene segments, representing various regions of the entire *N* gene (Fig. 1; Table 2), were cloned in the sense orientation into the plant expression vector pBI525. This vector utilized the enhanced cauliflower mosaic virus (CaMV) 35S promoter, the 5′ untranslated region from alfalfa mosaic

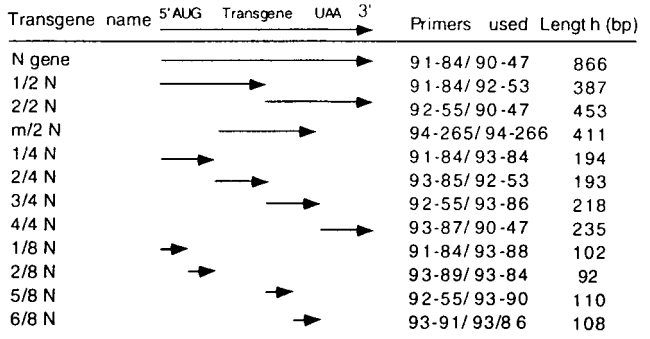

FIG. 1. Map of the *N* transgenes of TSWV-BL that were used in this study.

virus (AlMV), and the 3′ untranslated region of the nopaline synthase gene. The resulting expression cassettes were used to obtain transgenic *N. benthamiana* plants by *Agrobacterium*-mediated transformation for this study.

Transgenic R_1 seedlings were initially screened with the *npt*II ELISA to identify nontransgenic segregants from the R_1 transgenic populations. To exclude the possibility of escapes and avoid human bias during inoculation, nontransgenic segregants from each line were inoculated as internal controls along with transgenic R_1 plants. Additionally, nontransformed *N. benthamiana* plants were also included in each inoculation experiment. Reactions of the inoculated R_1 plants to TSWV-BL are summarized in Table 2. Unlike negative control plants, which were completely susceptible to the virus, expression of large *N* gene segments (387–453 bp, one-half *N* gene) conferred high levels of resistance to TSWV-BL in 20–51% of R_1 plants and tolerance to tospovirus infection in 4–22% of R_1 plants. The R_1 plants were also shown to be resistant to a closely related TSWV-10W isolate but not to the distantly related impatiens necrotic spot virus (INSV) (39) or groundnut ringspot virus (GRSV) (41) isolates (data not shown). Northern analysis on selected lines showed correlation of the resistance phenotype with low levels of the *N* gene segment transcript accumulation (Figs. 2 and 3*A*). To confirm that the reduced steady-state mRNA levels of the *N* gene segments were due to post-transcriptional down-regulation of the transgene, nuclear run-off transcription analysis was performed. Using the endogenous actin as a control, the *N* gene segments were found to be transcribed in the silenced progenies at higher rates than in the nonsilenced high-expressing progenies (Fig. 3*B*). These results collectively suggested that the resistance that we observed was the result of post-transcriptional transgene silencing, which affected the steady-state mRNA accumulation but not transcription rate.

R_1 plants expressing the small *N* gene segments (92–235 bp, one-fourth to one-eighth *N* gene) were similarly inoculated with TSWV-BL and other tospovirus isolates, and all of them were susceptible to tospovirus infection (Table 2). Northern blot analysis of selected R_1 plants showed that the transcript level of the *N* gene segments correlated with the length of transgenes. As shown in Fig. 4, the transcripts of large transgenes generally accumulated at much higher levels than those of small transgenes in the nonsilenced R_1 plants. This result suggested that the small *N* gene segments were not effectively transcribed in the nuclei, were transcribed at similar rates but

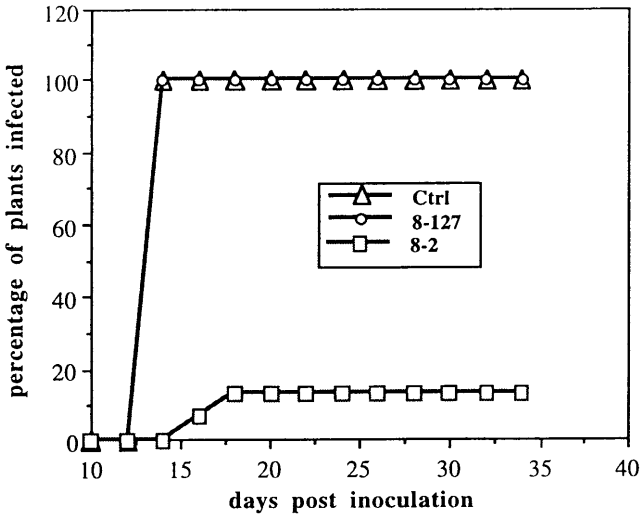

FIG. 2. Protection of transgenic plants against infection by TSWV-BL. R_1 plants of selected lines (8-127, a 1/2 *N* gene high expressor and 8-2, a 1/2 *N* gene low expressor, as determined by Northern blotting) were assayed for *npt*II using an *npt*II ELISA kit. The ELISA negative nontransgenic segregates (0.00–0.02 OD_{405}) were used as controls. The same plants were then challenged with TSWV-BL. Plants were examined every other day for the appearance of systemic symptoms, and any plant displaying symptoms on noninoculated leaves was recorded as systemic infection.

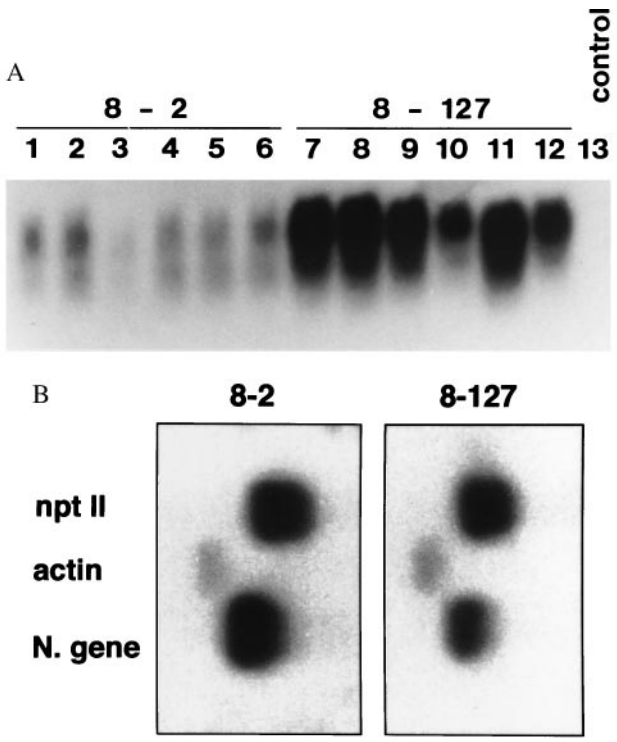

FIG. 3. Northern blot and nuclear run-off transcription analyses of 1/2 *N* gene low expressor (8-2) and high expressor (8-127) lines. (*A*) Northern blot analysis of 8-2 and 8-127 R_1 plants. Total RNAs were isolated from transgenic plants (15 μg per lane) and analyzed by Northern blotting using as the probe the *N* gene of TSWV-BL. Lanes 1–6, R_1 plants of line 8-2; lanes 7–12, R_1 plants of line 8-127; lane 13, a nontransgenic control. Size of 1/2 *N* gene transcript is ≈670 bp. (*B*) Nuclear run-off transcription analysis on R_1 plants. Labeled nuclear RNAs were hybridized to restriction enzyme-digested *N* gene, *npt*II, and actin fragments separated on agarose gels and blotted onto membranes. The nuclei used in the assays were isolated from an 8-2 R_1 plant and an 8-127 R_1 plant.

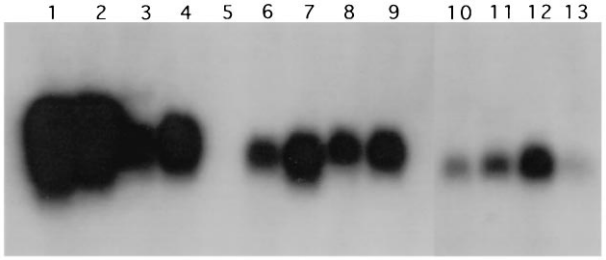

FIG. 4. Comparison of transcript accumulations in plants expressing *N* gene segments of different lengths that had been tested for resistance and post-transcriptional gene silencing. Ten micrograms per lane of total RNAs isolated from transgenic plants was used in Northern blots which were probed with the *N* gene of TSWV-BL. Lanes 1 and 2, two nonsilenced, virus-susceptible R_1 plants with the full-length *N* gene; lane 3, a silenced, virus-resistant R_1 plant with 2/2 *N* gene; lane 4, a nonsilenced, virus-susceptible R_1 plant with 2/2 *N* gene; lane 5, a nontransformed control; lanes 6–9, four nonsilenced, virus-susceptible R_1 plants with 3/4 *N* gene; lanes 10–13, four nonsilenced, virus-susceptible R_1 plants with 5/8 *N* gene. Sizes of transcripts for full-length *N* gene, 2/2 *N* gene, 3/4 *N* gene, and 5/8 *N* gene are ≈1150 bp, ≈730 bp, ≈500 bp, and ≈390 bp, respectively.

Table 3. Inoculations of TSWV-BL to R_0 *N. benthamiana* plants transformed with *GFP* gene fused to *N* gene segments

Genes	*GFP* + *N*, bp	No. of lines	Reactions of test plants HS	HT	HR
GFP	720 + 0	8	8		
GFP + 5/8 *N*	720 + 110	13	11	1	1
GFP + 3/4 *N*	720 + 218	8	2		6
GFP + 2/2 *N*	720 + 453	14	5	1	8

See Table 2 for abbreviations and inoculation procedures. See Fig. 1 for definitions of *N* gene segments of TSWV-BL.

were not properly processed or transported, or were not stable in the cytoplasm. As a result, there was no induction of transgene silencing in those small-transgene plants. In fact, a number of representative lines expressing the small *N* gene segments were analyzed by nuclear run-off transcription analysis, and the results showed no evidence that post-transcriptional silencing took place in any of those tested lines (data not shown).

Fusions of the Small *N* Gene Segments with *GFP* Confer RNA-Mediated Tospovirus Resistance. It was possible that the inability of the small *N* gene segments to confer RNA-mediated resistance was because the transgenes were too small to be expressed and accumulate at high levels (e.g., inefficient transcription, process, improper transport, or less stability) or because the small *N* gene segments fell below the minimal length of homology for trans-inactivation of the incoming virus genome. Various *N* gene segments (110, 218, and 453 bp) were fused to the 3′ end of the *GFP* gene, as described in *Materials and Methods*. Expression of such fusions in plant cells produces transcripts consisting of functional *GFP* ORF immediately followed by the respective *N* segments as the 3′ untranslated region. R_0 plants expressing these fusions were inoculated with the homologous isolate TSWV-BL, and inoculation results are summarized in Table 3. As a control, transgenic R_0 plants expressing *GFP* alone displayed typically systemic symptom at 5–10 days post inoculation. On the other hand, all *GFP*/*N* fusions conferred various levels of resistance to TSWV-BL (Table 3), including the small *N* segments (110 bp and 218 bp) which provided no protection against TSWV-BL when expressed alone in plants (Table 2). R_1 progenies from selected R_0 lines were similarly inoculated, and they showed similar levels of protection against TSWV-BL (Table 4). The R_1 plants were also shown to be resistant to a closely related TSWV-10W isolate but not to the distantly related INSV or GRSV isolates (data not shown). These results suggest that the *GFP* gene triggered gene silencing which degraded *GFP* and the small targeted *N* gene segments, and the homologous sequences of the incoming virus, resulting in the resistant state of the plant. However, the smallest *GFP*-5/8 *N* fusion (110 bp of homology) was less effective against TSWV-BL, as reflected by the lower number of R_0 plants protected and the quality of protection (Tables 3 and 4), indicating that the 110 bp of nucleotide sequence may approach the shortest homology required for trans-inactivation and consequent virus resistance. In addition, some of R_1 plants expressing the fusions were analyzed by Northern blotting using both *N* gene and *GFP* gene as probes (Fig. 5), and the results showed that the observed resistance again correlated with low accumulation levels of the fusion gene transcripts, suggesting that the same resistance mechanism operates in plants expressing the *N* gene segments alone or the *GFP*/*N* fusions.

Table 4. TSWV-BL inoculations of R_1 *N. benthamiana* plants transformed with *GFP* gene fused to *N* gene segments

Gene	Line no.	No. of plants tested	Reactions of test plants HS	HT	HR
GFP + 5/8 *N*	1	18	10	7	1
	3	14	14		
GFP + 3/4 *N*	5	17		8	9
	6	18		6	12
	7	18	15		3
	22	20			20
	23	20	16	1	3
	24	16			16
GFP + 2/2 *N*	8	15	9	1	5
	9	18	18		
	10	16	16		
	26	22	19	3	
	27	19	19		
	28	21	18	1	2
	29	21		1	20
Control		64	64		

See Table 2 for abbreviations and inoculation procedures. See Fig. 1 for definitions of *N* gene segments of TSWV-BL.

DISCUSSION

We have generated a number of plant lines that express different regions of TSWV *N* gene alone or fused with a nonviral sequence, *GFP*. Our studies have shown that transgenes smaller than one-quarter (235 bp) of the *N* gene were ineffective when expressed alone but were effective when fused to the *GFP* gene for post-transcriptional inactivation of the homologous, incoming, tospovirus. This result suggests that the inability of the small *N* transgenes alone to induce homology-dependent virus resistance was due not to their insufficient lengths of homology to the silenced transgene (in

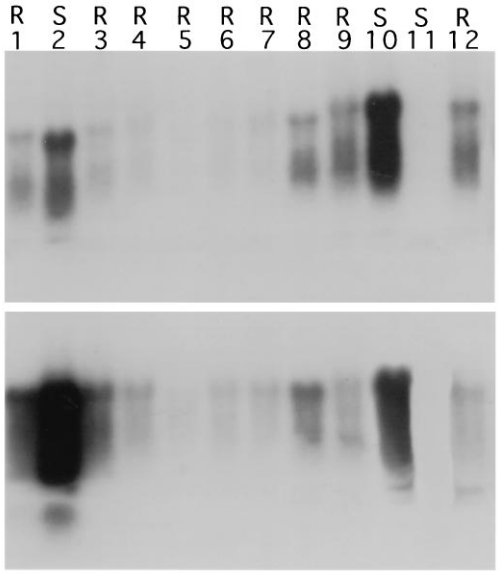

FIG. 5. Northern analysis of R_1 plants containing the *GFP*/*N* gene fusions. Leaves were harvested for RNA isolation when the plants were at the 5- to 6-leaf stage. Ten micrograms per lane of total RNAs was used for Northern blots, which were probed with the *N* gene of TSWV-BL (*Upper*) or the *GFP* gene (*Lower*). After removal of leaf samples for RNA analysis, the plants were inoculated with TSWV-BL and rated as either resistant (R) or susceptible (S), depending on the presence or absence of systemic symptoms at 14 days after inoculation. Lanes 1 and 2, *GFP* + 5/8 *N* line 1 R_1 plants; lanes 3–6, *GFP* + 3/4 *N* line 5 R_1 plants; lane 7, *GFP* + 3/4 *N* line 6 R_1 plants; lane 8, *GFP* + 3/4 *N* line 23 R_1 plants; lanes 9, 10, and 12, *GFP* + 2/2 *N* line 8 R_1 plants; and lane 11, a nontransformed plant. Transcripts for *GFP* linked to 5/8 *N*, to 3/4 *N*, or to 2/2 *N* are ≈1110 bp, ≈1220bp, and ≈1450 bp, respectively.

this case the virus genome) but because they are incapable of inducing gene silencing. Thus, this study differentiates the ability to induce transgene silencing from the ability to provide homology-dependent trans-inactivation.

Inability of the small *N* gene segments to induce transgene silencing can be due to their inefficient transcription in nuclei, inefficient processing/transport, and/or less stability of their transcripts in the cytoplasm. This conclusion is consistent with our Northern blot analysis, showing that the transcript of the small transgenes accumulated at much lower levels in the cytoplasm than the level of the large transgenes (Fig. 4). The reduced transcript accumulation of the small transgenes may result from steric interference of transcription initiation machinery with the transcription termination machinery. Alternatively, the small *N* gene transcripts may lose critical RNA sequence elements required for effective mRNA processing, transport, and/or stability, resulting in much less mature mRNA accumulation in the cytoplasm (45). The addition of the *GFP* gene to the small transgenes may simply enhance their ability to transcribe the transgene fusions, or the *GFP* gene may provide RNA sequence elements required for accumulation of the mature mRNA.

On the other hand, trans-inactivation of the silenced transgene requires much shorter sequence of homology. In this case, when expressed as a fusion with *GFP*, transgenes as short as 110 bp can trans-inactivate the incoming virus genome. This short homologous sequence presumably interacts with the incoming virus to form RNA duplex, which serves as a target for cellular degradation. The observation that nearly all of the transgenic plants with the smallest fusion transgene, *GFP*–5/8 *N*, were susceptible to TSWV (Tables 3 and 4) indicates that 110 bp (1/8 *N*) sequence of homology may be approaching the minimal length of homology required for trans-inactivation of the silenced genes (in this case the virus genome). This result is consistent with the recent observation of Sijen *et al.* (21). They showed that a small homologous sequence of only 60 nucleotides was sufficient to tag a recombinant potato virus X molecule for the gene-silencing-mediated elimination process. As we reported here, they also showed that the frequency and quality of resistance appeared to depend on both the length of the homologous sequence and the concentration of the inoculum.

Our study also shows that any half of the *N* gene (first, middle, or second) can confer post-transcriptional gene silencing-derived viral resistance (Table 2). This result suggests that the specific RNA secondary structure of the *N* gene sequence might not be necessary for inducing transgene silencing and viral resistance. Small segments (110–235 bp) were ineffective when expressed alone but were effective when fused to the *GFP* gene for post-transcriptional gene silencing and viral resistance. Taken together, the results indicated that the post-transcriptional gene silencing-derived virus resistance is dependent on the transgene length, but not on the specific sequences within the *N* gene of TSWV. These results also provide us the hint that any small part of the viral genome larger than a certain length might confer resistance when fused with a silencer DNA (e.g., *GFP*). Thus, we suggest that any viral sequence longer than 110 bp could confer RNA-mediated resistance when fused to stably expressed normal-length nonviral transgene. If it is true, it would significantly facilitate the engineering of viral resistance because isolation of a specific viral gene such as coat protein gene or replicase gene can be very tedious, especially if the viral genome organization is not well characterized. It should be pointed out that the coat protein gene will continue to be one of the best choices for RNA-mediated resistance because it is highly expressed and its transcript is presumably very stable in the infected cells.

Post-transcription gene silencing-derived resistance is an effective way for developing virus resistant transgenic plants. Furthermore, there are several advantages for inducing the gene silencing state with a chimeric transgene consisting of a silencer DNA (e.g., *GFP*) fused with one or more small nontranslatable segments of a viral genome(s). First, the silencer DNA can enhance the induction of gene silencing. Second, linking several segments of the viral genes could provide multiple virus resistance. Third, nontranslatable construction produces no protein, thus reducing the possible complementation of naturally occurring mutants and transencapsidation of other viruses. And fourth, the small segment also reduces the possibility of recombination with other viral genomes.

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