Resistance of Transgenic *Nicotiana benthamiana* Plants to Tomato Spotted Wilt and Impatiens Necrotic Spot Tospoviruses: Evidence of Involvement of the N Protein and N Gene RNA in Resistance

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ABSTRACT

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Transgenic *Nicotiana benthamiana* plants containing the nucleocapsid (N) protein gene of the lettuce isolate of tomato spotted wilt virus (TSWV-BL) have been produced. Analyses of these transgenic plants showed that plants producing small amounts of the N protein were highly resistant to both the homologous isolate and closely related isolates, whereas plants producing large amounts of the N protein possessed moderate levels of protection against both the homologous isolate and two isolates of distantly related impatiens necrotic spot virus (INSV). However, the latter protection was highly dependent on inoculum strength. Despite a delay in symptom appearance in the high expressors, none of the transgenic plants were protected against the more closely related, newly named groundnut ringspot virus originally from Brazil. These results substantiated and extended previous observations in transgenic tobacco. Comparative analyses of transgenic plants expressing either the translatable or the untranslatable N gene showed that protection against the homologous isolate and closely related isolates in plants expressing low levels of the N gene was due to the presence of N gene RNA, whereas protection against the homologous isolate and distantly related INSV isolates in plants expressing high levels of the N gene was due to the accumulation of N protein. Thus, different mechanisms appeared to be involved in protection against infection by tospoviruses that share different levels of N gene sequence identities. Interestingly, both N gene RNA- and N protein-mediated protections are effective against the homologous isolate. These mechanisms cannot operate simultaneously in the same transgenic plant, but they must operate in different plants, because a low level of N gene transcription is required for RNA-mediated protection, whereas a high level of N protein is required for protein-mediated protection. We also compared the nucleotide sequence of the N gene among the test tospovirus isolates, and the information obtained from this comparative analysis was used to develop hypotheses that may account for the two protection patterns.

Additional keywords: coat protein-mediated protection, pathogen-derived resistance.

Viruses in the Tospovirus genus infect a wide range of plant species worldwide, particularly tobacco, peanut, vegetables, and ornamental plants (3,12,13). The viral genome 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 of an ambisense gene arrangement (10,21,23,24,28,30) that is expressed via the synthesis of subgenomic mRNAs (22). The S RNA encodes a 52-kDa nonstructural protein in the viral RNA strand and the 29-kDa nucleocapsid (N) protein in the viral complementary RNA strand, whereas the M RNA encodes a 34-kDa nonstructural protein in the viral RNA strand and the precursor to the 58- and 78-kDa membrane-associated glycoproteins in the viral complementary RNA strand. The tomato spotted wilt virus (TSWV) L RNA encodes a large 330-kDa protein, presumably for the viral transcriptase (9).

Two virus species, TSWV (serogroup I) and impatiens necrotic spot virus (INSV, serogroup II), are recognized within the Tospovirus genus (13). Several other tospoviruses distinct from TSWV and INSV have been reported (1,4,6,7,14,18,19,30,33,38,39) and may represent additional virus species of the Tospovirus genus (13). For example, de Avila et al (6) considered the serogroup II isolates BR-03 and SA-05 as distinct species within the Tospovirus genus, and the names tomato chlorotic spot virus and groundnut ringspot virus (GRSV) have been proposed, respectively. The N gene of a recently characterized Brazilian isolate (30) is more closely related (94% at the nucleotide sequence level) to the serogroup II isolate SA-05; thus, we refer to this isolate as GRSV-BR. GRSV-BR was previously called the Brazil isolate (29) or TSWV-B (30,31).

Engineered resistance to tospoviruses was first reported by Gielen et al (15) and MacKenzie and Ellis (27), who showed that transgenic tobacco plants expressing the N gene of TSWV were resistant to infection by the homologous isolate. A subsequent study (8) suggested that the protection previously observed in transgenic plants expressing the translatable N gene was due to the presence of N gene transcripts and not the N protein. Our studies, however, have shown that transgenic tobacco plants expressing the N gene of TSWV-BL (the lettuce isolate of TSWV) displayed resistance to both TSWV and INSV (29), and the protection appears to be mediated by the N protein against the distantly related INSV and by the N gene RNA against the homologous and closely related TSWV isolates (31). However, protection against INSV was measured in a local-lesion host (29).

In this study, we demonstrated a similar INSV-resistance phenotype in a systemically infected host. In addition, we also reported that the level of N protein accumulation in transgenic tobacco plants is closely correlated with resistance to different tospoviruses (29). The correlation of N protein or coat protein level to resistance has not been observed for tospoviruses or other plant viruses by other researchers (20,25–27,32,37). Therefore, it is important

to determine if the correlation between N protein accumulation and resistance can be found in other host plants. Here, we produced transgenic *Nicotiana benthamiana* Domin. plants, a systemic host of all reported tospoviruses, and tested them for their reactions to five tospoviruses. Results from comparative sequence analyses of the five test tospovirus N genes are used to aid the interpretation of the observed protection patterns.

MATERIALS AND METHODS

***Agrobacterium tumefaciens*-mediated leaf-disk transformation.** Plasmid BIN19-N^+ was constructed and transferred to *A. tumefaciens* strain LBA4404 (from ClonTech, Palo Alto, CA) as described by Pang et al (29). *A. tumefaciens*-mediated transformation of *N. benthamiana* leaf disks and subsequent tissue culture were as described by Pang et al (29).

Enzyme-linked immunosorbent assay (ELISA) and Northern analyses of transgenic plants. Double antibody sandwich-ELISA (DAS-ELISA; [16,29,38]) was used to detect the production of N protein in transgenic plants with antibodies against the TSWV-BL N protein (38). Northern blot was performed as described previously (31).

Tospovirus isolates and inoculation of transgenic plants. The following five tospoviruses used in the inoculation study were described by Wang and Gonsalves (38) and by Pang et al (29,30): the lettuce isolate of TSWV (TSWV-BL); the 10W pakchoy of TSWV (TSWV-10W); the begonia (INSV-Beg) and Long Island (INSV-LI) isolates of INSV; and the Brazilian isolate of serogroup II (GRSV-BR). Inoculation was done as described previously (29). Systemic symptoms were recorded daily for at least 2 mo. To avoid the possibility of escapes, control N^- plants were included in each experiment, and each inoculum extract was used to inoculate N^+ plants first, followed by inoculation of control N^- plants.

Preparation, transfection, and analysis of *N. benthamiana* protoplasts. Preparation and transfection of protoplasts were described previously (31). After various incubation intervals (0, 19, 30, and 42 h), the cultured protoplasts were assayed by DAS-ELISA, using antibodies against the INSV N protein (antiserum to INSV N protein was provided by J. W. Moyer, North Carolina State University, Raleigh) to detect the replication of the attacking virus or against TSWV-BL N protein to monitor the expression level of the transgene in transgenic protoplasts.

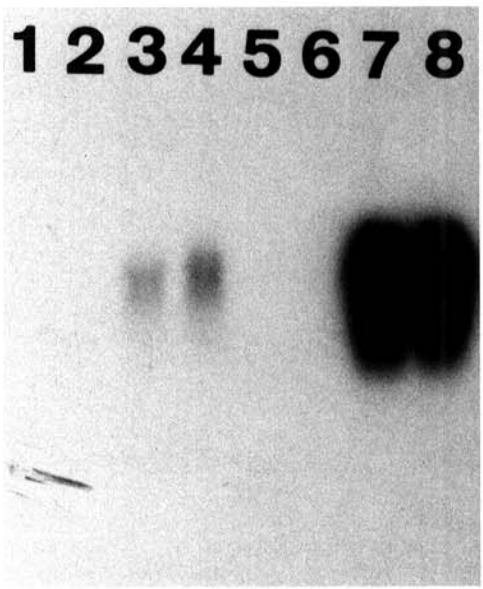

Fig. 1. Northern analysis of the R_0 *Nicotiana benthamiana* lines used for inoculation tests. Total RNAs isolated from transgenic plants (15 μg per lane) were analyzed by Northern blot (31). Lanes 1 and 2, control N^--2 and -6 plants, respectively; lanes 3 and 4, N^+-21 plant (considered as a low expressor); lanes 5 and 6, N^+-28 plant (considered as a non-expressor); lanes 7 and 8, N^+-34 and -37 plants (considered as high expressors), respectively.

Cloning and sequencing N genes of tospoviruses. The dsRNAs of TSWV-10W, INSV-Beg, and INSV-LI were isolated from infected *N. benthamiana* plants as described previously (30). The full-length N genes were obtained by reverse transcription and polymerase chain reaction (RT-PCR), as described by Pang et al (29). The N gene of TSWV-10W was synthesized with oligomer primers BL-A (5′-AGCTAACCATGGTTAAGCTCACTA-AGGAAAGC) and BL-B (5′-AGCATTCCATGGTTAACA-CACTAAGCAAGCAC), corresponding to the 5′ coding and 3′ noncoding regions of the TSWV-BL N gene (29), respectively. The N genes of INSV-Beg and -LI were amplified with oligomer primers INSV-A (5′-TACTTATCTAGAACCATGGACA-AAGCAAAGATTACCAAGG) and INSV-B (5′-TACAGTG-GATCCATGGTTATTTCAAATAATTTATAAAAGCAC), hybridizing to the 5′ coding and 3′ noncoding regions of the N gene of an INSV isolate (23), respectively. The amplified N gene fragments were purified on a 1.2% agarose gel (30), digested with the restriction enzyme *Nco*I, and directly cloned into the *Nco*I site of the modified pBluescript vector (Stratagene, La Jolla, CA). DNA sequencing was performed, using the dideoxyribonucleotide method (35), T7 polymerase (Sequenase, United States Biochemicals, Cleveland), and the double-stranded sequencing procedure described by Siemieniak et al (36). The nucleotide sequences were determined from both DNA strands and were compared with the published sequences of TSWV-BL (29), INSV (23), and GRSV-BR (30) by computer programs available from the Genetics Computer Group (GCG, Madison, WI) (11).

RESULTS

Analysis of transgenic plants. A total of 24 N^+ (transformed with pBIN19-N^+) and 18 N^- (transformed with vector pBIN19) transgenic *N. benthamiana* plants was transferred to soil and grown in the greenhouse. All N^+ lines were confirmed by PCR at the four- to five-leaf stages to contain the N gene sequence (*data not shown*). The relative level of N protein accumulation was estimated in each independent R_0 transgenic clonal line by DAS-ELISA with antibodies of the TSWV-BL N protein. Of the 24 N^+ lines, two had OD_{405} readings of 0.50–1.00, 17 between 0.02 and 0.10, and the remaining five less than 0.02. Healthy *N. benthamiana* or transgenic N^- plants gave OD_{405} readings of 0.00–0.02. All the R_0 plants were self-pollinated, and the seeds from the following transgenic lines were germinated on kanamycin (300 μg/ml) selection medium for inoculation tests: 1) N^--2 and -6, control transgenic lines containing vector pBIN19 alone; 2) N^+-28, a transgenic line that produced an undetectable amount of the N protein (OD_{405} = 0.005); 3) N^+-21, a transgenic line producing a low level of the N protein (OD_{405} = 0.085); and 4) N^+-34 and -37, two transgenic lines accumulating high levels of the N protein (OD_{405} between 0.50 and 1.00). These six lines were analyzed by Northern hybridization; the intensity of N gene transcripts correlated well with the levels of ELISA reactions (Fig. 1).

Challenge inoculation of transgenic plants with tospoviruses. Transgenic seedlings from the six R_0 lines were selected by germinating seeds on kanamycin selection medium; the seedlings were inoculated with the five tospoviruses (Table 1). The inoculated R_1 plants were rated susceptible if virus symptoms were observed on uninoculated leaves. To exclude the possibility of escapes, transgenic control N^- plants were used in each inoculation of transgenic N^+ plants. In addition, each inoculum extract was used to first inoculate N^+ plants, followed by inoculation of control N^- plants.

All R_1 plants from control lines N^--2 and -6 showed systemic symptoms 5–8 days after inoculation with all the viruses tested, without a single control plant escaping the infection or showing any delay in symptom appearance. None of the R_1 plants from line N^+-28 produced detectable levels of the N protein, and all were susceptible to the viruses, except for one plant inoculated with INSV-Beg (Table 1). ELISA assays of leaf disks from the

TABLE 1. Reactions of R_1 plants expressing the nucleocapsid (N) protein gene of tomato spotted wilt virus (TSWV) to inoculation with tospoviruses

R_0 line	ELISA[a]	No. of plants infected/no. of plants inoculated[b]				
		TSWV-BL	TSWV-10W	INSV-Beg	INSV-LI	GRSV-BR
N^--2/-6	<0.02	32/32	32/32	32/32	20/20	32/32
N^+-28	0.005	16/16	16/16	15/16		16/16
N^+-21	0.085	9/40	17/40	39/40	18/20	40/40
N^+-34	0.715	25/28[c]	28/28	23/28[c]		28/28
N^+-37	0.510	26/28[c]	22/22	21/28[c]	16/20[c]	22/22

[a] Enzyme-linked immunosorbent assay data of R_0 lines from which the R_1 plants were derived.
[b] Thirtyfold-diluted leaf extracts of infected *Nicotiana benthamiana* plants were applied to the three leaves of plants at the three- to five-leaf stages. Each extract was first used to inoculate N^+ plants followed by inoculation of control N^- plants. Data were taken daily for at least 2 mo after inoculation and were expressed as the number of plants systemically infected over the number of plants inoculated. INSV = impatiens necrotic spot virus; GRSV = groundnut ringspot virus.
[c] The underlined fractions indicate that nearly all susceptible R_1 plants displayed a significant delay of symptom appearance (Fig. 2A and C).

N^+-28 R_1 plants sampled before inoculation clearly showed that the plant possessing the INSV-Beg–resistant phenotype accumulated a high level of the N protein (OD_{405} = 0.78 compared to OD_{405} < 0.02 for all other N^+-28 R_1 plants).

The low N gene expressing line N^+-21 showed the best resistance against the homologous (78%), closely related TSWV-10W (57%) isolates and very little resistance to the two INSV isolates (3 and 10%); only three N^+-21 plants showed the resistant phenotype when inoculated with the INSV isolates. Leaf samples from these INSV-resistant N^+-21 R_1 plants gave much higher ELISA reactions (OD_{405} ranging from 0.5 to 1.00) and, thus, gave higher amounts of the N protein than did the susceptible N^+-21 plants (OD_{405} ranging from 0.02 to 0.20).

On the other hand, the high N gene expressing lines N^+-34 and -37 showed the highest resistance to INSV isolates (18–25%), followed by the homologous TSWV-BL isolate (7 and 11%), whereas none of the plants showed resistance to TSWV-10W. However, the N^+-34 and -37 R_1 plants that became infected with INSV or TSWV-BL did show various lengths of delays in symptom expression. None of the R_1 plants from these four transgenic N^+ lines were resistant to GRSV-BR (Table 1), but some of the R_1 plants from the N^+-34 and -37 lines showed a slight delay in symptom appearance (Fig. 2).

Relationship between the level of N protein accumulation and resistance to tospoviruses in transgenic plants. Our previous report (29) showed that the level of N protein production in N^+ R_1 plants was associated with resistance to different tospoviruses. To determine if this relationship holds true in transgenic *N. benthamiana* plants, the inoculated N^+ R_1 plants in Table 1 were reorganized into four groups based on the intensity of the ELISA reactions of tissues taken before inoculation, irrespective of the original R_0 plants. The N^+ R_1 plants that expressed low levels of the N protein (0.02–0.20 OD_{405}) showed high resistance (100 and 80%) to TSWV-BL and -10W but were all susceptible to INSV-Beg and -LI, showing no detectable delay in symptom expression relative to control N^- plants (Fig. 2A–C). In contrast, nearly all the N^+ R_1 plants with high levels of the N protein (0.20–1.00 OD_{405}) showed various levels of protection against TSWV-BL, INSV-Beg, and INSV-LI, ranging from a short delay in symptom expression to complete resistance (Fig. 2A and C), with most of these plants showing various lengths of delay in symptom development relative to control N^- plants. No protection was observed in the high expressors against TSWV-10W (Fig. 2B). In addition, none of the N^+ R_1 plants were resistant to GRSV-BR regardless of the level of N gene expression; however, a briefly delayed symptom appearance was observed in the N^+ R_1 plants producing high levels of the N protein (Fig. 2D). All control N^- R_1 plants and transgenic N^+ R_1 plants with undetectable ELISA reactions (0.00–0.02 OD_{405}, ELISA readings were equal to or smaller than those of negative control plants) were susceptible to all the tospoviruses tested (Fig. 2).

Effect of inoculum strength on pathogen-derived resistance. R_1 plants from two lines, N^+-21 and -37, were analyzed by DAS-ELISA and were subsequently inoculated with various dilutions of TSWV-BL or INSV-Beg. As shown in Figure 3, none of the low expressors (based on actual ELISA values) became infected when challenged with different strengths of TSWV-BL inoculum, ranging from 30- to 1,920-fold dilutions. In contrast, the level of resistance in the high expressors (based on actual ELISA values) to either TSWV-BL or INSV-Beg was inversely related to inoculum strength. None of the N^- R_1 control plants escaped from infection, even when the most diluted (1,920-fold) inoculum extracts were used.

Inhibition of replication of a distantly related INSV in *N. benthamiana* protoplasts expressing the TSWV-BL N gene. Whole INSV-LI virions were used to infect protoplasts isolated from three transgenic lines to investigate how the products of the transgene affect replication of the incoming virus. Viral replication was determined by measuring the level of the N protein of the infecting INSV in transgenic protoplasts with antibodies specific to the INSV N protein. DAS-ELISA analyses showed that all

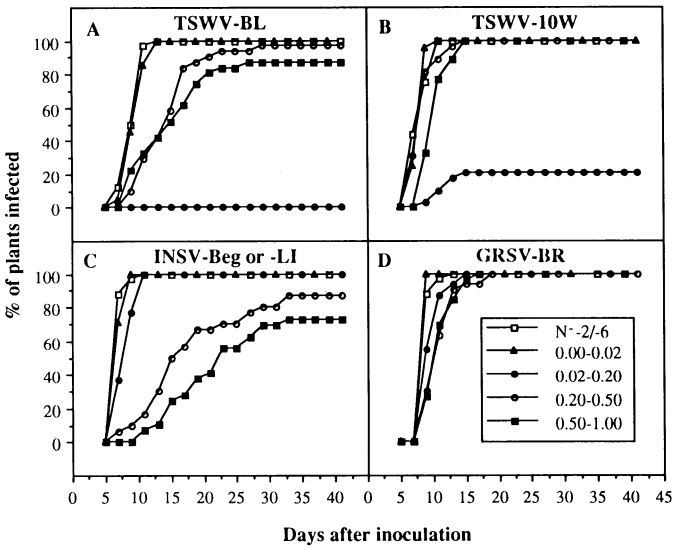

Fig. 2. Protection of transgenic *Nicotiana benthamiana* plants against infections by tospoviruses. Transgenic plants were assayed by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) for N protein accumulation, using antibodies raised against the N protein of the lettuce isolate of tomato spotted wilt virus (TSWV-BL). Plates were read 150 min after adding substrate, and the transgenic plants were grouped into four categories: OD_{405} < 0.02, 0.02–0.20, 0.20–0.50, and 0.50–1.00. Healthy control plants gave ELISA reactions of 0.00–0.02 OD_{405}. The same plants were challenged with isolates **A**, TSWV-BL; **B**, TSWV-10W (10W pakchoy); **C**, impatiens necrotic spot virus from begonia (INSV-Beg) or Long Island (INSV-LI); and **D**, groundnut ringspot virus from Brazil (GRSV-BR). Plants were examined daily for the appearance of systemic symptoms, and any plant displaying symptoms on uninoculated leaves was recorded as symptomatic. Reactions of transgenic plants to INSV-Beg or -LI were nearly identical and are presented together. A total of 617 R_1 plants were inoculated, and each curve was derived from results of 20–32 R_1 plants (Table 1).

progenies from a given line were relatively uniform, and nearly all R_1 progeny gave an expression level of transgenic N gene similar to their parental transgenic line (*data not shown*). These results allowed us to predict the expression level of R_1 populations based on those of their parental lines in this study. As shown in Figure 4, protoplasts derived from R_1 plants of the low-expressor line N^+-21 supported the replication of INSV-LI, whereas protoplasts from R_1 plants of the high-expressor line

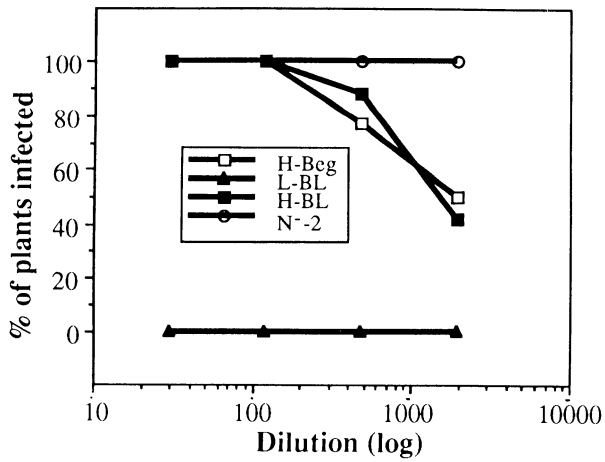

Fig. 3. Effect of inoculum strength on pathogen-derived resistance. R_1 plants in this test were individually assayed by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) and grouped into either high or low expressors before inoculation with different dilutions of the inoculum extract from *Nicotiana benthamiana* plants infected with either the lettuce isolate of tomato spotted wilt virus (TSWV-BL) or impatiens necrotic spot virus from begonia (INSV-Beg). Infectivity data were recorded daily for a period of 6 wk. Each curve was generated from 80 R_1 plants. Abbreviations: H-Beg, the high expressors (based on actual ELISA values of R_1 plants) inoculated with INSV-Beg; L-BL, the low expressors inoculated with TSWV-BL; H-BL, the high expressors inoculated with TSWV-BL; N^--2, the control R_1 plants inoculated with either INSV-Beg or TSWV-BL.

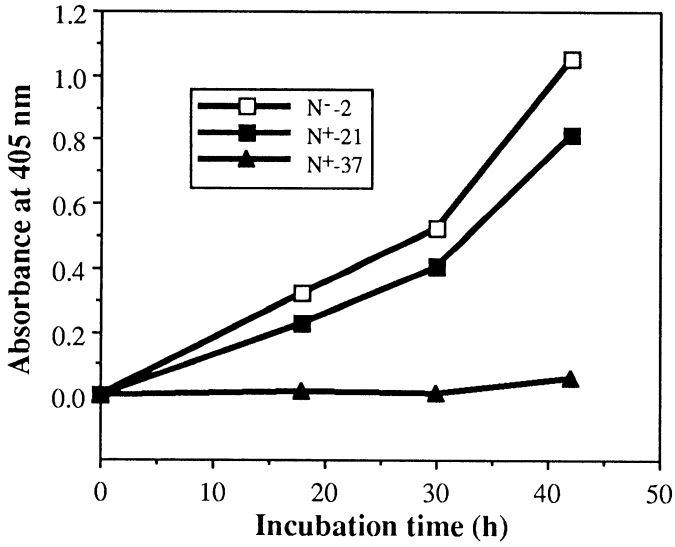

Fig. 4. Inhibition of viral replication in *Nicotiana benthamiana* protoplasts expressing the lettuce isolate of tomato spotted wilt virus (TSWV-BL) N gene. Protoplasts were isolated from R_1 plants derived from a control line N^--2, the low-expressor line N^+-21, and the high-expressor line N^+-37 (Table 1). About 6×10^6 isolated protoplasts of each line were transformed with the purified impatiens necrotic spot virus from Long Island (INSV-LI) virions (0.68 OD_{260}). The transformed protoplasts were cultured at 26 C for 0, 19, 30, and 42 h before lysis in the enzyme conjugate buffer for enzyme-linked immunosorbent assay, using antibodies against the INSV N protein. This experiment was repeated three times and data were consistent across experiments.

N^+-37 did not support replication until 42-h postinoculation, at which time low levels of viral replication were observed. The same protoplasts, at various time intervals (0, 19, 30, and 42 h), also were assayed by DAS-ELISA, using antibodies specific to the TSWV-BL N protein to monitor the expression level of the transgene. As expected, protoplasts from N^+-21 R_1 plants produced relatively low levels (0.338–0.395 OD_{405}), whereas protoplasts from N^+-37 R_1 plants accumulated high levels (0.822–0.865 OD_{405}). The expression level was consistent at all time intervals.

Sequence comparisons between the N genes of the test tospoviruses. To better understand the mechanisms by which transgenic plants resist infections by different tospoviruses, the N genes of TSWV-10W, INSV-Beg, and INSV-LI were sequenced, and their nucleotide sequences were compared with the corresponding sequences of TSWV-BL (29) and GRSV-BR (30). Nucleotide and deduced amino acid sequence comparisons are shown in Figure 5, and the comparative results are summarized in Table 2. Consistent with the serological classification (29,38), the N gene of TSWV-BL showed nucleotide and amino acid sequence identities closest to TSWV-10W (99.0 and 98.1%, respectively), nucleotide and amino acid sequence identities moderately close to GRSV-BR (77.6 and 79.5%, respectively), and nucleotide and amino acid sequence identities least close to INSV-Beg (60.4 and 53.9%, respectively) and INSV-LI (62.1 and 54.3%, respectively). As expected, the N gene of the INSV isolate reported by Law et al (23) displayed a high degree of nucleotide and deduced amino acid sequence identities to INSV-Beg (98.7 and 96.6%, respectively) and INSV-LI (99.1 and 98.9%, respectively). The N gene of GRSV-BR shared 63.2 and 64.2% nucleotide and 56.3 and 55.6% amino acid identities with INSV-Beg and -LI, respectively. Thus, TSWV-BL was more closely related to GRSV-BR than to INSV-Beg or -LI. When similar amino acids were taken into account, the amino acid similarity between the N protein of TSWV-BL and GRSV-BR was even greater (90.3%). In contrast, the N protein of INSV-Beg or -LI was only about 68.8% similar to that of TSWV-BL.

DISCUSSION

This study has shown that transgenic *N. benthamiana* plants that accumulate low amounts of the TSWV-BL N protein are highly resistant to the homologous and closely related (TSWV-10W) isolates, whereas plants that accumulate high amounts of this protein possess moderate levels of protection against both

TABLE 2. The comparison of resistance to different tospoviruses in transgenic plants expressing various forms of the tomato spotted wilt virus (TSWV-BL) N gene

	Homology to TSWV-BL N gene[a]			N^b		mN^b	
Tospovirus	nt	aa (identity)	aa (similarity)	Low	High	Low	High
TSWV-BL	100	100	100	I	R	I	S
TSWV-10W	99.0	98.1	98.4	I	S[c]	I	S
INSV-Beg[d]	60.4	53.9	68.8	S	R	S	S
INSV-LI	62.1	54.3	68.8	S	R		
GRSV-BR	77.6	79.5	90.3	S	S	S	S

[a]The N genes of TSWV-BL and groundnut ringspot virus (GRSV-BR) were reported by Pang et al (29,30). Percent homology was obtained by the BESTFIT program of the GCG package. nt = nucleotide; aa = amino acid.

[b]Transgenic *Nicotiana benthamiana* plants expressing low and high levels of the intact N gene (N) of TSWV-BL were challenged with tospoviruses, and inoculation results were obtained. Inoculation results of transgenic tobacco plants expressing low and high levels of the untranslatable N gene (mN) were reported by Pang et al (31). I = immune (resistance not dependent on inoculum strength); R = resistant (resistance highly dependent on inoculum strength); S = susceptible (plants as susceptible as nontransformed plants).

[c]The result was obtained from plants inoculated with 30-fold diluted inoculum. They may resist infection by TSWV-10W if more diluted inoculum is tested.

[d]Impatiens necrotic spot virus.

the homologous and distantly related (INSV-Beg and -LI) viruses. The level of protection observed in the low expressors was very high and independent of inoculum strength, whereas the level of protection found in the high expressors against both the homologous and distantly related viruses was highly dependent on the inoculum concentration. These results validate and extend our previous observations (29). More importantly, the finding that transgenic *N. benthamiana* plants (a systemic host of INSV) are protected against INSV-Beg and -LI is consistent with our previous result, based on local-lesion reactions of INSV-Beg in transgenic tobacco (29).

In this study, most of the high N gene expressors showed various levels of protection against the homologous isolate, ranging from a delay in symptom expression to complete resistance, and the level of protection was highly dependent on inoculum strength. This type of protection also was observed in transgenic tobacco

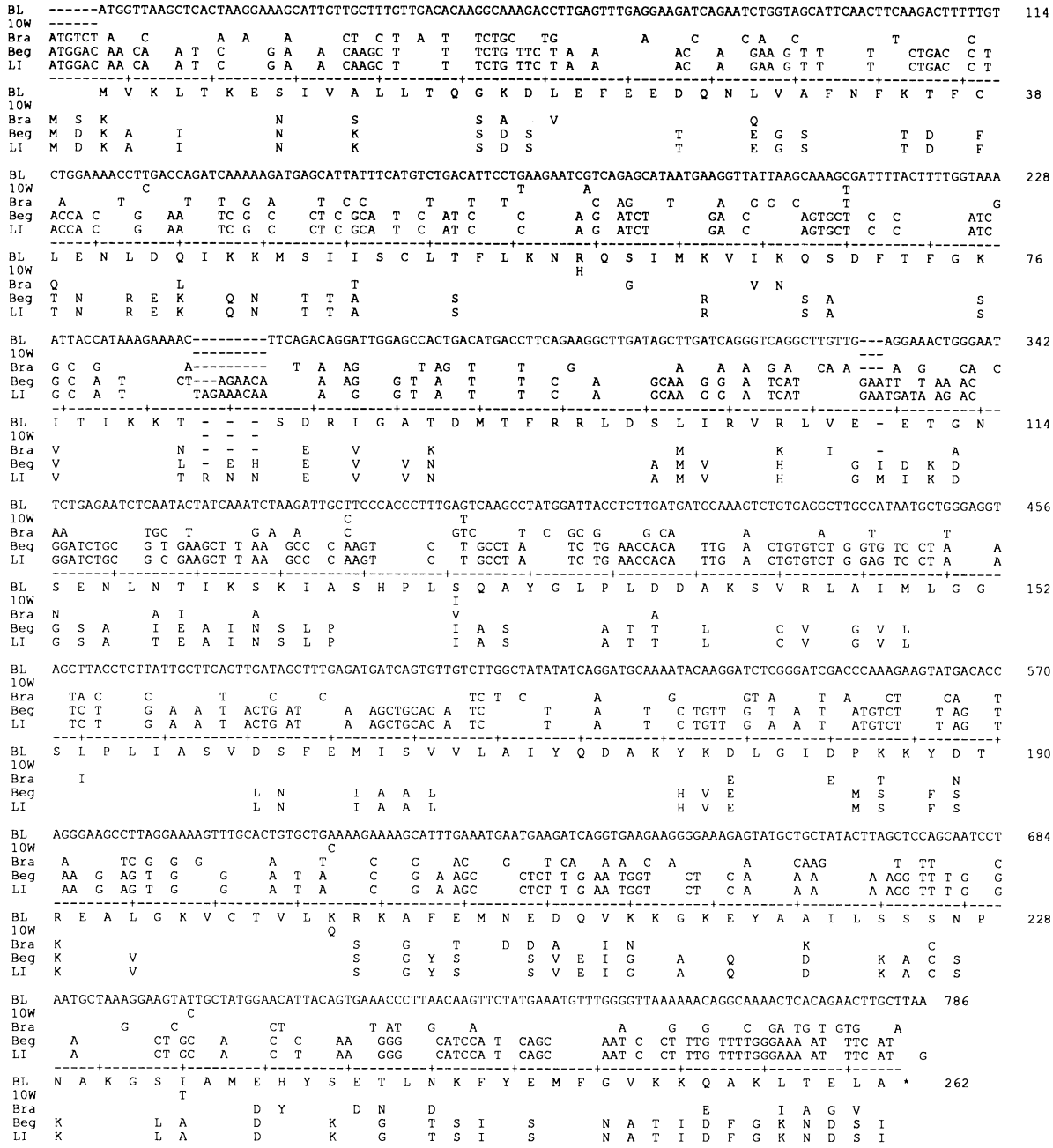

Fig. 5. The nucleotide and the deduced amino acid sequence comparisons of the nucleocapsid protein genes from the lettuce isolate of tomato spotted wilt virus (TSWV-BL), TSWV-10W (10W pakchoy), groundnut ringspot virus from Brazil (GRSV-BR), impatiens necrotic spot virus from begonia (INSV-Beg) and INSV-LI from Long Island. The N genes of TSWV-10W, INSV-Beg, and INSV-LI were cloned directly from viral RNAs by reverse transcription and polymerase chain reaction (RT-PCR). The nucleotide sequences were obtained by the double-stranded dideoxynucleotide sequencing procedure described by Siemieniak et al (36) and are compared with the nucleotide sequences of the TSWV-BL and GRSV-BR N genes reported by Pang et al (29,30). The nucleotide sequence of the TSWV-BL N gene is presented on the top line (BL), and only the nucleotide differences found in the N genes of the TSWV-10W (10W), GRSV-BR (BR), INSV-Beg (Beg), and INSV-LI (LI) are shown below. The deduced amino acid sequence of the TSWV-BL N gene is presented below the nucleotide sequence-counting line with a single letter code (5). Only amino acid sequence differences deduced from the other four tospoviruses are presented. The single letters representing amino acids have been placed at the first nucleotide position of each respective codon. Gaps are represented by (-) hyphens in both the nucleotide and amino acid sequences. The nucleotide sequences encoding two N-terminal amino acid residues of the N genes from TSWV-BL and -10W were excluded by PCR cloning to preserve the *Nco*I recognition site, while not incorporating any new amino acid codon for the subsequent cloning and expression. GenBank accession numbers for new sequences are L20887 (TSWV-10W), L20885 (INSV-Beg), and L20886 (INSV-LI).

plants expressing high levels of the translatable N gene of TSWV-BL (29) but not in those expressing high levels (Fig. 1) of an untranslatable N gene (31; Table 2), which clearly suggests a role for N protein-mediated protection. The mechanism for this N protein-mediated protection is unknown. One possibility is that the N protein produced in transgenic plants, as proposed for protection against tobacco mosaic virus, prevents the uncoating of the invading virus and, thus, disrupts the virus infection cycle (2,17,34). If this is the case, the protection is expected to also be effective against closely related TSWV isolates. However, this type of protection was not observed against the closely related TSWV-10W isolate (Fig. 2B). We noticed that TSWV-10W is more virulent than TSWV-BL. Therefore, it is possible that protection would have been observed if high N gene expressors had been inoculated with TSWV-10W at inoculum strengths lower than those used in our experiments.

Very high levels of protection against the homologous and closely related isolates were observed in transgenic plants expressing low levels of the translatable N gene of TSWV-BL (29; Fig. 2) and in transgenic plants with low levels of the untranslatable form of the same gene (31; Table 2). These results suggest the involvement of the N gene transcript rather than the N protein in the protection. This N gene RNA-mediated protection may result from interference of the N transcript produced in transgenic plants with viral replication, presumably by hybridizing to the genomic S RNA molecule of the attacking virus even before initiation of the first round of replication in the initially infected cells (31). If this is the case, the protection is expected to be most effective against the homologous and closely related (99.0% nucleotide sequence identity in the case of TSWV-10W) isolates and not against distantly related isolates (GRSV-BR, INSV-Beg, and INSV-LI).

We observed that transgenic N^+ plants giving undetectable ELISA reactions (ELISA readings were equal to or smaller than those of negative control plants) were completely susceptible to all tested tospoviruses, including TSWV-BL and -10W. Northern blots of the leaves sampled from some of those plants before inoculation showed that these ELISA-negative plants also produced undetectable amounts of N gene RNA, even when the autoradiogram was overexposed (*data not shown*). This observation clearly shows that a minimal amount of N gene RNA is required for N gene RNA-mediated protection against the homologous and closely related isolates. Thus, our previous observation that transgenic tobacco plants with undetectable ELISA reactions were resistant to the homologous and closely related isolates (29) was probably due to the limited sensitivity of our ELISA detection and not to the complete absence of N gene expression. The requirement for the presence of a minimal level of N gene RNA for resistance to the homologous and closely related isolates was further supported by our other study (31) that showed that transgenic plants containing the promoterless N gene (and, thus, no N gene RNA) were completely susceptible to all tospoviruses tested.

Surprisingly, the N gene RNA-mediated protection does not operate effectively in transgenic plants expressing high levels of the translatable or untranslatable N gene ([31]; Table 2). The breakdown in protection for transgenic plants with high levels of N gene RNA production remains a mystery because it does not correlate with the hypothetical protection mechanism suggested for transgenic plants with low levels of N gene RNA. It is possible that factors other than or in addition to the N gene RNA are involved. One factor could be the plant's own defense system(s), in which case low levels of N gene RNA could elicit a defense response to combat the expected incoming virus. On the other hand, high levels of the N gene RNA may interfere with the host defense mechanism by creating a dilution or masking effect that allows the incoming virus to escape detection. For example, high levels of the N gene RNA produced in transgenic plants may interfere with the ability of the host defense molecules to find the attacking virus. If this is the case, then the RNA-RNA duplex mechanism must be relatively weak because it does not protect the high expressors, which suggests that the host defense mechanism may be more effective.

Protection against the distantly related viruses INSV-Beg and -LI was found in plants that accumulated high levels of the TSWV-BL N protein and was achieved through inhibition of viral replication. We did not observe this resistant phenotype in transgenic plants producing untranslatable TSWV-BL N transcript when inoculated with INSV-Beg or -LI, regardless of the amount of N transcript produced ([31]; Table 2), which clearly suggests the involvement of the N protein in this protection mechanism. As proposed by Pang et al (31), the TSWV-BL N protein produced in these transgenic plants may act as a dysfunctional N protein when incorporated into the distantly related attacking virus particle, such as INSV-Beg and -LI. The resulting heteroencapsidated virus may lose some important biological properties required for replication. If this is true, then N protein-mediated protection can only be expected against virus isolates that cannot functionally exchange their own N proteins with those produced in transgenic plants and in transgenic plants that produce sufficient levels of "dysfunctional" N protein to compete with the N protein of the attacking virus. The lack of N protein-mediated protection against infection by the more closely related GRSV-BR virus (Table 1) suggests that the TSWV-BL N protein can form at least partially functional heteroencapsidated GRSV-BR viral particles; they share 90% amino acid similarity. However, functional heteroencapsidation of TSWV-BL and GRSV-BR RNAs remains to be demonstrated.

In summary, the results of this study substantiate our previous observations (29) and show that different mechanisms are involved in protecting tobacco and *N. benthamiana* plants against tospoviruses of different serogroups. It will be interesting to determine if these observations hold true for other plants, such as tomato, lettuce, and chrysanthemum. Last, our results with tobacco and *N. benthamiana* show that it will be difficult to produce a single transgenic plant that is resistant to all tospoviruses because the different apparent mechanisms require either a low level of N gene RNA or a high level of N protein. Thus, it is unlikely that a single N gene will provide resistance to all tospoviruses. Engineering of crops with more than one N gene (or N gene fragment) may be desirable to provide a wide spectrum of protection against tospoviruses present in a particular field environment.

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