Resistance to Heterologous Isolates of Tomato Spotted Wilt Virus in Transgenic Tobacco Expressing Its Nucleocapsid Protein Gene

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ABSTRACT

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The nucleocapsid protein (NP) gene of the lettuce isolate of tomato spotted wilt virus (TSWV-BL), from the L serogroup, was synthesized and cloned into a plant expression cassette using polymerase chain reaction. Transgenic tobacco plant lines were obtained via *Agrobacterium tumefaciens*-mediated leaf disk transformation, and their progenies were tested for their ability to resist infections by TSWV isolates belonging to the L and I serogroups. Nearly all transgenic R_1 plants were resistant to the homologous TSWV-BL isolate. High levels of resistance to the heterologous isolates of the L serogroup were found in plants that accumulated little, if any, NP; plants that accumulated high levels of NP showed the best resistance to infection by an isolate of the I serogroup (Begonia isolate). However, all of these plant lines were susceptible to infection by a distinct Brazilian isolate that belongs to neither the L nor the I serogroup, although the plants that accumulated high levels of the NP did display a delay in symptom development. These results demonstrate that a fairly broad spectrum of resistance can be obtained by the expression of a TSWV-BL NP gene and that there are limits to this spectrum. In addition, the finding that this spectrum of resistance is dependent on the levels to which the NP accumulates suggests that different mechanisms may exist that mediate these different resistance modes.

Additional keywords: Nicotiana tabacum, virus resistance.

Tomato spotted wilt virus (TSWV) is unique among plant viruses because the nucleic acid-protein complex is covered by a lipoprotein envelope and because it is the only thrip-transmitted virus. This virus has recently been classified as the Tospovirus genus of the Bunyaviridae family (13). TSWV virions contain a 29K nucleocapsid protein (NP), two membrane-associated glycoproteins (58K and 78K) and a large 200K protein, presumably for the viral transcriptase (8,9,30,39). The virus genome consists of three negative-strand RNAs designated L RNA (8,900 nucleotides), M RNA (5,400 nucleotides), and S RNA (2,900 nucleotides) (8,10,11,29,41), each of which is encapsidated by the NP. The partial or full-length sequences of S RNAs from three TSWV isolates reveal the presence of two open-reading frames (ORF) with an ambisense gene arrangement (11,23,28). The larger ORF is located on the viral RNA strand and has the capacity to encode a 52K protein. The smaller ORF is located on the viral complementary RNA strand and is translated through a subgenomic RNA into the 29K NP.

Two TSWV serogroups, L and I, have been identified and characterized on the basis of serological analysis of the structural proteins and morphology of the cytopathic structures (22,40). They have serologically conserved G1 and G2 glycoproteins, but the NP of the I serogroup is serologically distinct from that of the L serogroup (22). Comparison of the NP between the L and I serogroups has shown 62 and 67% identities at nucleotide and amino acid levels, respectively (23).

TSWV has a wide host range, infecting more than 360 plant species of 50 families (13), and causes significant economic losses to vegetables and ornamental plants worldwide (3,14,22). The L serogroup has been found extensively in field crops such as vegetables and weeds (4), whereas the I serogroup has been largely confined to ornamental crops (22,23). A cucurbit isolate (20) has recently been identified as a distinct isolate, because it systemically infects watermelon and other cucurbits and its NP is serologically unrelated to that of either serogroup (S.-D. Yeh et al, *personal communication*). Although the spread of the TSWV disease can sometimes be reduced by breeding resistant plants or by using nongenetic approaches, complete control of the disease by these conventional methods has generally proven to be difficult (4).

Since 1986, numerous reports have shown that transgenic plants with the coat protein (CP) gene of a virus are often resistant to infection by that virus (2). This phenomenon is commonly referred to as CP-mediated protection. The degree of protection ranges from delay in symptom expression to absence of disease symptoms and virus accumulation. Two recent independent reports (15,27) showed that transgenic plants expressing the NP gene of TSWV are resistant to infection by the isolate from which the NP gene was isolated. However, since TSWV is widespread with many biologically diverse isolates, it is very important to test the effectiveness of the transgenic plants to resist infections by different TSWV isolates. Our results expand on those of the previous reports by demonstrating that our transgenic plants showed resistance to two heterologous isolates of the L serogroup and an isolate of the I serogroup. No resistance was observed to an isolate with the NP that was serologically distinct from the L and I serogroups. We also show that resistance to the two heterologous isolates of the L serogroup was mainly found in plants accumulating very low, if any, levels of NP, whereas transgenic plants that accumulated high levels of NP were resistant to the isolate of the I serogroup.

MATERIALS AND METHODS

Purification of TSWV and viral RNAs. The TSWV-BL isolate (42) was purified from *Datura stramonium* L. as described by Gonsalves and Trujillo (16). The purified virus was resuspended in a solution of 0.04% bentonite, 10 μg/ml of proteinase K, 0.1 M ammonium carbonate, 0.1% (w/v) sodium diethyldithiocarbanate, 1 mM EDTA, and 1% (w/v) sodium dodecyl sulfate (SDS), incubated at 65 C for 5 min, immediately extracted with H_2O-saturated phenol, and then extracted with chloroform-isoamyl alcohol (24:1). Viral RNAs were precipitated in 2.5 vol of ethanol and dissolved in distilled H_2O.

cDNA and polymerase chain reaction-based NP gene cloning. cDNA was synthesized from purified TSWV-BL RNAs as described by Gubler and Hoffman (17). Plasmid clones containing TSWV-BL S RNA cDNA sequences were identified by hybridizing against a ^{32}P-labeled oligomer (5′-AGCAGGCAAAACTCGCAGAA CTTGC) complementary to nucleotide positions 2008–2033 of the TSWV-CPNH1 S RNA (11). Several cDNA clones were selected, and clone pTSWVS-23 was determined to contain the largest cDNA insert, about 1.7 kb in length (Fig. 1).

The full-length NP gene was obtained by the use of polymerase chain reaction (PCR). First-strand cDNA synthesis was performed at 37 C for 30 min in a 20-μl reaction mixture using oligomer primer JLS90-46 (5′-AGCTAACCATGGTT AAGCTCACTAAGGAAAGC), which is complementary to the S RNA in the 5′ terminus of TSWV NP gene (nucleotide positions 2751–2773 of the TSWV-CPNH1). The reaction mixture contained 1.5 μg of viral RNAs, 1 μg of the oligomer primer, 0.2 mM each dNTP, 1× PCR buffer (the GeneAmp kit, Perkin-Elmer Cetus, Norwalk, CT), 20 units of RNasin ribonuclease inhibitor (Promega Corp., Madison, WI), 2.5 mM $MgCl_2$, and 25 units of AMV reverse transcriptase (Promega). The reaction was terminated by heating at 95 C for 5 min and cooled on ice. Then 10 μl of the cDNA/RNA hybrid was used to PCR-amplify the NP gene according to the manufacturer's instructions (Perkin-Elmer Cetus) using 1 μg each of oligomer primers JLS90-46 and JLS90-47 (5′-AGCAT TCCATGG TTAACACACTAAGCAAGCAC), the latter oligomer being identical to the S RNA in the 3′ noncoding region of the gene (nucleotide positions 1919–1938 of the TSWV-CPNH1). A typical PCR cycle was 1 min at 92 C (denaturing), 1 min at 50 C (annealing), and 2 min at 72 C (polymerizing). The sample was directly loaded and separated on a 1.2% agarose gel. The separated NP gene fragment was extracted from agarose gel, ethanol-precipitated, and dissolved in 20 μl of distilled H_2O.

Construction of plant expression and transformation vectors. The gel-isolated NP gene fragment was digested with the restriction enzyme *Nco*I and directly cloned into *Nco*I-digested plant expression vector pBI525 (obtained from William Crosby, Plant Biotechnology Institute, Saskatoon, Saskatchewan, Canada). The resulting plasmids were identified and designated as pBI525-NP$^+$ in the sense orientation relative to the cauliflower mosaic virus (CaMV) 35S promoter and as pBI525-NP$^-$ in the reverse orientation. The ability of this expression cassette to produce the NP was determined by transient expression of the NP gene in *Nicotiana tabacum* protoplasts, as described by Pang et al (32). The expression cassette containing the NP gene was then excised from pBI525-NP$^+$ by a partial digestion with *Hin*dIII/*Eco*RI (since the NP gene contains internal *Hin*dIII and *Eco*RI sites) and ligated into the plant transformation vector pBIN19 (Clontech Laboratories, Inc.) that had been cut with the same enzymes. The resulting vector pBIN19-NP$^+$ and the control plasmid pBIN19 were transferred to *A. tumefaciens* strain LBA4404, using the procedure described by Holsters et al (18).

Nucleotide sequence analyses. The nucleotide sequences of the inserts in clones pTSWVS-23 and pBI525-NP$^+$ were determined using the dideoxyribonucleotide method (35), T7 polymerase (Sequenase, U.S. Biochemicals, Cleveland, OH), and the double-stranded sequencing procedure described by Siemieniak et al (36). Nucleotide sequences were determined from both DNA strands, and this information was compared with the published sequences of TSWV isolates CPNH1 (11) using computer programs available from the Genetics Computer Group, Madison, WI (12).

***Agrobacterium*-mediated transformation.** Leaf disks of *N. tabacum* var. *Havana* '423' were inoculated with the *Agrobacterium* strain LBA4404 (ClonTech) containing the vector pBIN19-NP$^+$ or the control plasmid pBIN19, as described by Horsch et al (19). Transformed cells were selected and regenerated in MS medium containing 300 μg/ml of kanamycin, and roots were induced after transfer of plantlets to hormone-free medium. Rooted transformants were transferred to soil and grown under greenhouse conditions. Transgenic plants were self-pollinated, and seeds were selectively germinated on kanamycin medium.

Serological detection of proteins. Double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) (16,42) was used to detect the expression of NP gene in transgenic plants with polyclonal antibodies against the TSWV-BL NP. Each sample was prepared by grinding a leaf disk (about 0.05 g) from the top second leaf of the plant in 3 ml of an enzyme conjugate buffer (phosphate-buffered saline [5], 0.05% Tween 20, 2% polyvinylpyrrolidone 40, and 0.2% ovalbumin).

For Western blots, a leaf disk (about 0.05 g) was ground in 0.25 ml of 2× SDS/sample buffer (0.126 M Tris buffer, 20% glycerol, 2% SDS, 2% 2-mercaptoethanol, and 0.01 mg/ml of bromphenol blue). Proteins (10–20 μl of sample per lane) were separated and blotted as described by Ausubel et al (1). The membrane was processed following the manufacturer's instruction manual of immunoselect kit (Gibco BRL Life Technologies Inc.). Antibodies to the whole virion (42) were preabsorbed with cell extracts from healthy tobacco plants as described by Gonsalves and Trujillo (16) and were used in Western blot at a concentration of 2 μg/ml.

Serological reactions of TSWV isolates (TSWV-BL, Arkansas, 10W pakchoy, Begonia, or Brazil) were assayed in DAS-ELISA using antibodies raised against the TSWV-BL virion or the NP of TSWV-BL (42) or TSWV-I (22) (antiserum to TSWV-I NP was kindly provided by J. W. Moyer, North Carolina State University, Raleigh).

Inoculation of transgenic plants with TSWV isolates. Inocula were prepared by infecting *N. benthamiana* Domin. with different TSWV isolates and grinding infected leaves (0.5 g) of *N. benthamiana* plants (1–2 wk after inoculation) in 15 ml of a buffer (0.033 M KH_2PO_4, 0.067 M K_2HPO_4, and 0.01 M Na_2SO_3). The inoculum extracts were immediately rubbed on the corundum-dusted leaves of transgenic plants, and the inoculated leaves were subsequently rinsed with H_2O. Because TSWV is highly unstable after grinding, each batch of inoculum was used to first inoculate NP$^+$ plants containing the NP gene, and the last inoculated plants of each inoculum were always control NP$^-$ plants containing the vector sequence alone to assure that a particular virus inoculum was still infective at the end of inoculation. Data on local lesions and systemic infections were taken starting 7 days after inoculation.

RESULTS

Synthesis and cloning of the TSWV-BL S RNA cDNA and NP gene. The isolation of the TSWV-BL NP gene, which resides in the S RNA component of TSWV, was approached using two strategies. The cDNA cloning strategy yielded several clones containing cDNA inserts derived from TSWV-BL S RNA, as identified by hybridization against an oligomer probe complementary to the TSWV-CPNH1 S RNA (11). Clone pTSWVS-23 contained the longest insert, which mapped at about 1.7 kb in length (data not shown). The second strategy used the published sequence of TSWV-CPNH1 S RNA (11) and PCR (34) to amplify and engineer the NP gene for expression directly from total TSWV-BL RNA. Oligomer primers JLS90-46 and -47 were synthesized, with JLS90-46 being complementary to the S RNA in the 5′-coding region of the NP gene (positions 2751–2773 of the TSWV-CPNH1) and JLS90-47 being of the 3′-noncoding region of the NP gene (positions 1919–1938 of the TSWV-CPNH1) (Fig. 1). Both of the primers contain the recognition site for the restriction enzyme *Nco*I for subsequent cloning, and the primer JLS90-46 has a plant consensus translation initiation codon sequence (AAXXATGG) (26), which upon amplification was expected to fuse the translation initiation codon to the third codon (GTT) of the NP gene (Fig. 1). Fusion of the translation initiation codon to the third codon of the TSWV-BL NP gene was performed to preserve the *Nco*I recognition site while not incorporating any new amino acid codons (37). Thus, expression of the PCR-engineered TSWV NP gene would yield a TSWV-BL NP that was two amino acids (Ser-Lys) shorter at the N terminus than the native NP.

This specifically amplified DNA fragment, of about 850 bp,

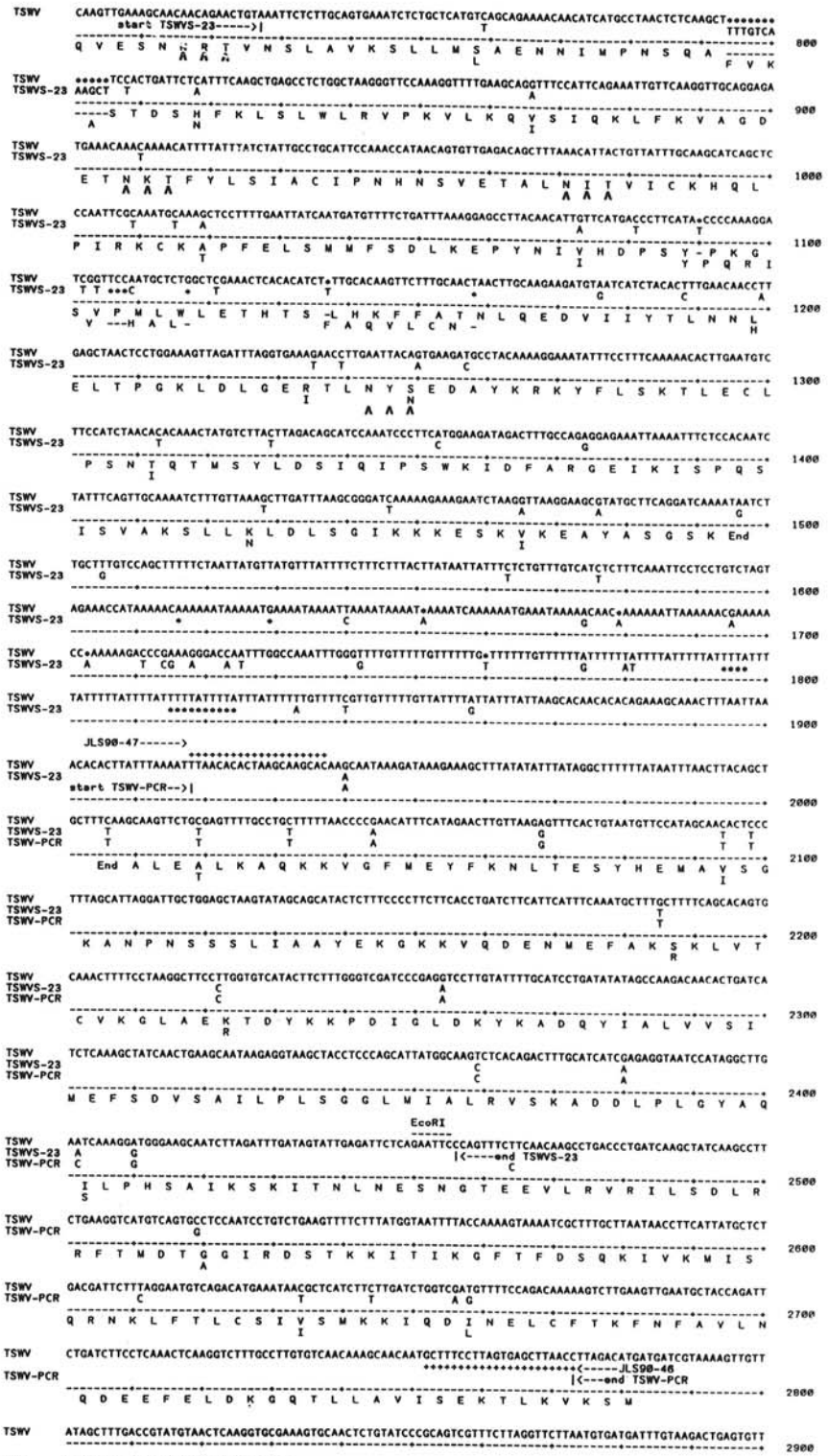

Fig. 1. Nucleotide and deduced amino acid sequences of cloned cDNA and polymerase chain reaction (PCR)-engineered insert of tomato spotted wilt virus (TSWV)-BL S RNA and their comparison with the nucleotide sequence of TSWV-CPHN1 S RNA. The nucleotide sequence of the TSWV-BL S RNA clones pTSWVS-23 (TSWV-23) and pBI525-NP^{+} (TSWV-PCR) were obtained using the double-stranded dideoxynucleotide sequencing procedure described by Siemieniak et al (36), and their sequences are compared with the relevant regions of the nucleotide sequence of the TSWV-CPNH1 S RNA reported by de Haan et al (11) (GenBank accession D00645). The DNA sequence of TSWV-CPNH1 S RNA is presented on the top line (TSWV), and only the nucleotide differences found in the sequences of TSWV-23 and TSWV-PCR are shown below. The complete deduced amino acid sequences of the ambisense open reading frames of TSWV-CPNH1 S RNA are presented below the nucleotide sequence counting line using the single letter code (7). Only amino acid sequence differences deduced from the TSWV-BL sequences are presented. The single letters representing amino acids have been placed at the first nucleotide position of each respective codon. The amino acid sequence of the 52K protein reads from the top, whereas that of the nucleocapsid protein (NP) reads from the bottom nucleotide strand (strand not shown). The starting and ending points for the cloned inserts TSWV-23 (positions 727–2456) and TSWV-PCR (positions 1918–2773) are shown, and together they represent a total of 2,023 bp of the TSWV-BL S RNA. Gaps have been used in both the nucleotide (represented by asterisks) and amino acid (represented by dashes) sequences. The location of the *Eco*RI site at the 3′ end of the TSWV-23 insert is shown above the nucleotide sequence line. The location that the two oligomer primers used for PCR-engineering the NP gene of TSWV-BL are shown above (5′ oligo JLS90-47) and below (3′ oligo JLS90-46) the sequence line; shared nucleotides are indicated by the plus symbol. Potential N-glycosylation sites are indicated by carets; the fifth potential N-glycosylation site (position 1243) is absent from the 52K protein of the BL isolate because of an amino acid replacement.

was digested with *Nco*I and cloned into the plant expression vector pBI525. The orientation of the TSWV-BL NP gene with respect to the CaMV 35S promoter was determined by restriction enzyme site mapping (*Eco*RI, *Hin*dIII, *Ava*I, and *Aiw*NI). Several clones were isolated that contain the insert in the proper orientation (pBI525-NP$^+$) and others that contain the insert in the opposite orientation (pBI525-NP$^-$). This restriction enzyme site-mapping data also showed that the inserts of clones pBI525-NP$^+$ contained restriction enzyme sites that were identical with those found in the TSWV-CPNH1 NP gene published by de Haan et al (11) (data not shown). The expression of TSWV-BL NP gene was thus controlled by a double CaMV 35S promoter fused to the 5′-untranslated leader sequence of alfalfa mosaic virus of the expression vector pBI525. Expression vectors that use the stacked double CaMV 35S promoter elements yield higher levels of mRNA transcription than similar vectors that use a single 35S promoter element (31).

Three pBI525-NP$^+$ clones were transiently expressed in tobacco protoplasts to confirm that the amplified DNA fragment encoded the NP. To achieve this, the clones were transferred into tobacco protoplasts by the PEG method (32), and after two days of incubation the expressed NP was detected by DAS-ELISA using antibodies against the whole TSWV-BL virion. High levels of NP were produced in tobacco protoplasts harboring the NP gene in plasmid pBI525-NP$^+$; no NP was detected in tobacco protoplasts transformed with the antisense NP sequence (pBI525-NP$^-$) (data not shown).

Nucleotide sequence analysis of TSWV-BL cDNA and PCR-engineered clones. The cDNA insert of the clone pTSWVS-23 and the PCR-engineered insert of clone pBI525-NP$^+$ were sequenced. These sequences are shown in Figure 1, where they are compared with the sequence of TSWV-CPNH1 S RNA (11). This comparison showed that cDNA insert of clone pTSWVS-23 included about 760 bp of the 52K protein viral component gene, the complete intergenic region (492 bp), and 450 bp of the NP gene (about half of the NP gene). This cloned insert had its 3′ end located exactly at an *Eco*RI recognition site (Fig. 1), which suggested incomplete *Eco*RI methylation during the cDNA cloning procedure. Although this clone did not contain the complete TSWV-BL NP gene, its sequence was of considerable importance since it had a 450-bp overlap with the sequence of the PCR-engineered NP gene (a total of 2,023 bp of the TSWV-BL S RNA is presented in Fig. 1). The sequence comparison between this TSWV-BL PCR-engineered and TSWV-CPNH1 (11) NP genes revealed a total of 21 nucleotide differences (2.7%), eight of which encode amino acid replacements (3.1%). Since this PCR-engineered NP gene was obtained using *Taq* polymerase, which is known to incorporate mutations (34), it is possible that some of these differences were introduced during PCR amplification. However, 15 of these nucleotide differences were located within the overlapping region between the TSWV-BL cDNA and PCR clones, and all but one of these nucleotide differences (position 2402 of Fig. 1) are shared by both TSWV-BL S RNA-derived clones. This comparison clearly showed that the PCR amplification did not contribute greatly, if at all, to the difference between the nucleotide sequences of these two cloned NP gene regions. The nucleotide difference at position 2402 resulted in the amino acid replacement of Ile with Ser, and even this difference could be due to the lack of homogeneity within the TSWV-BL isolate.

Analysis of transgenic tobacco lines. Transgenic plants were assayed using PCR and Northern and Western blots. These analyses showed that the NP gene sequences were integrated into the plant genome, and transgenic plants produced NP mRNA and polypeptide with the expected molecular weights (data not shown). The relative levels of NP expression were estimated in each R_0 transgenic line by DAS-ELISA using antibodies of the TSWV-BL NP. The ELISA readings were taken 150 min after the addition of substrate. Of the 23 NP$^+$ lines, 10 lines had OD_{405nm} readings of 0.2–0.5, six lines between 0.1–0.2, and the remaining seven lines less than 0.1. Healthy tobacco or transgenic NP$^-$ plants gave OD_{405nm} readings of 0.00–0.050. Seven NP$^+$ lines with different levels of NP accumulation and three NP$^-$ control lines were chosen to produce R_1 seeds for testing the ability of transgenic R_1 plants to resist infections by different TSWV isolates.

Protection of transgenic plants against TSWV isolates from different serogroups. Five TSWV isolates, which had previously been described by Wang and Gonsalves (42), were used to challenge R_1 plants: TSWV-BL, Arkansas, 10W pakchoy, Begonia, and Brazil. All of the isolates, with the exception of the Begonia isolate, produced systemic infections on tobacco. The Begonia isolate induced distinct local lesions on inoculated leaves but no systemic infection. The first three isolates were reactive to the antibodies against the whole virion and the NP of TSWV-BL (42) (Fig. 2) and belonged to the L serogroup. The Begonia isolate reacted strongly to the antibodies against the NP of TSWV-I (the I serogroup) but not to those raised against the TSWV-BL NP (Fig. 2). Therefore, the Begonia isolate belonged to the I serogroup. Interestingly, the Brazilian isolate gave weak but positive ELISA reactions to antibodies against TSWV-BL virion but not to the antibodies against the NP of either serogroup (Fig. 2). Moreover, this isolate caused systemic mottle and crinkle on the leaves of the infected tobacco and *N. benthamiana,* but it did not infect squash or cucumbers (data not shown), indicating that it is a distinct isolate from the cucurbit isolate (20).

Seedlings derived from seven R_0 lines were germinated on kanamycin medium and inoculated with the above TSWV isolates. Infectivity data were recorded daily starting 7 days after inoculation. Plants inoculated with TSWV-BL, Arkansas, 10W pakchoy, or Brazil isolates were rated susceptible if virus symptoms were observed on uninoculated leaves. Plants inoculated with the Begonia isolate were rated susceptible if local lesions were observed on inoculated leaves, since this isolate does not cause systemic infection in tobacco. The inoculation results are summarized in Table 1. All inoculated control NP$^-$ R_1 plants became infected by these five isolates. However, almost all NP$^+$ R_1 plants were resistant to the homologous isolate TSWV-BL (Fig. 3), whereas much lower percentages of NP$^+$ R_1 plants were resistant to heterologous isolates Arkansas (Fig. 3), 10W pakchoy, and Begonia. On the other hand, all NP$^+$ R_1 plants from the seven transgenic lines were susceptible to the Brazil isolate, even though a slight delay (1–2 days) in symptom expression was observed in some of the high NP-expressing NP$^+$ R_1 plants from line NP$^+$ 4.

Resistant R_1 plants remained symptomless throughout their life cycles. The inoculated leaves of 17 symptomless NP$^+$ plants were checked for the presence of the virus by back inoculation on leaves of *Chenopodium quinoa* plants. No virus was recovered from the inoculated leaves of symptomless NP$^+$ plants, suggesting that the virus could not replicate or spread in these NP$^+$ plants.

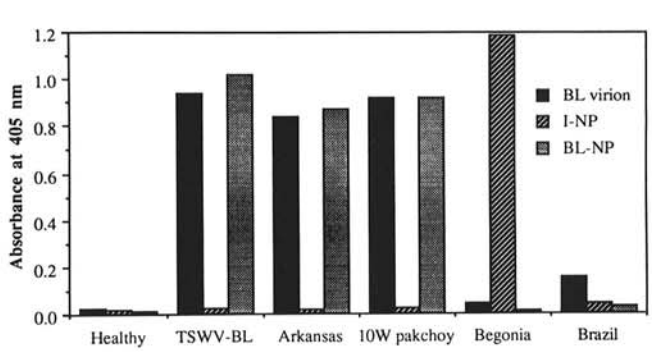

Fig. 2. Serological relationship of tomato spotted wilt virus (TSWV) isolates used in this study. *Nicotiana benthamiana* Domin. were infected with TSWV isolates (TSWV-BL, Arkansas, 10W pakchoy, Begonia, and Brazil). An infected leaf disk (0.05 g) was ground in 12 ml of the enzyme conjugate buffer and analyzed by double-antibody sandwich enzyme-linked immunosorbent assay using antibodies raised against TSWV-BL virion (BL virion) or the nucleocapsid protein of TSWV-BL (BL-NP) or TSWV-I (I-NP). Concentration of antibodies for coating: 1 μg/ml. Dilution of conjugates: 1:2,000 for BL virion, 1:250 for BL-NP, and 1:1,000 for I-NP. Results were taken 10 min (BL virion), 50 min (BL-NP), or 30 min (I-NP) after adding substrate.

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

R_0 line	ELISA[a]	Reactions[b] to TSWV isolates				
		BL	Arkansas	10W pakchoy	Begonia[c]	Brazil
NP^+ 2	0.015	0/20	4/25	3/24	29/40	36/36
NP^+ 4	0.386	6/30	21/23	18/21	9/48	42/42
NP^+ 9	0.327	0/20	NT[d]	20/20	NT	NT
NP^+ 14	0.040	0/20	NT	9/20	18/18	18/18
NP^+ 21	0.042	0/15	5/15	3/15	2/4	6/6
NP^+ 22	0.142	0/20	NT	15/20	31/36	36/36
NP^+ 23	0.317	0/20	NT	16/20	NT	NT
NP^-	< 0.050	42/42	24/24	62/62	66/66	54/54

[a] Enzyme-linked immunosorbent assay data of R_0 lines from which the R_1 plants were derived.
[b] Extracts of infected *Nicotiana benthamiana* were applied to the three leaves of plants at leaf stages 3–5. Each extract was always used to inoculate NP^+ plants followed by control NP^- plants. Data were taken about 12–15 days after inoculation and expressed as number of plants systemically infected over number of plants inoculated, except where indicated.
[c] Begonia isolate induced local lesions on the R_1 plants, and the resistance was expressed as number of plants producing local lesions over number of plants inoculated.
[d] Not tested.

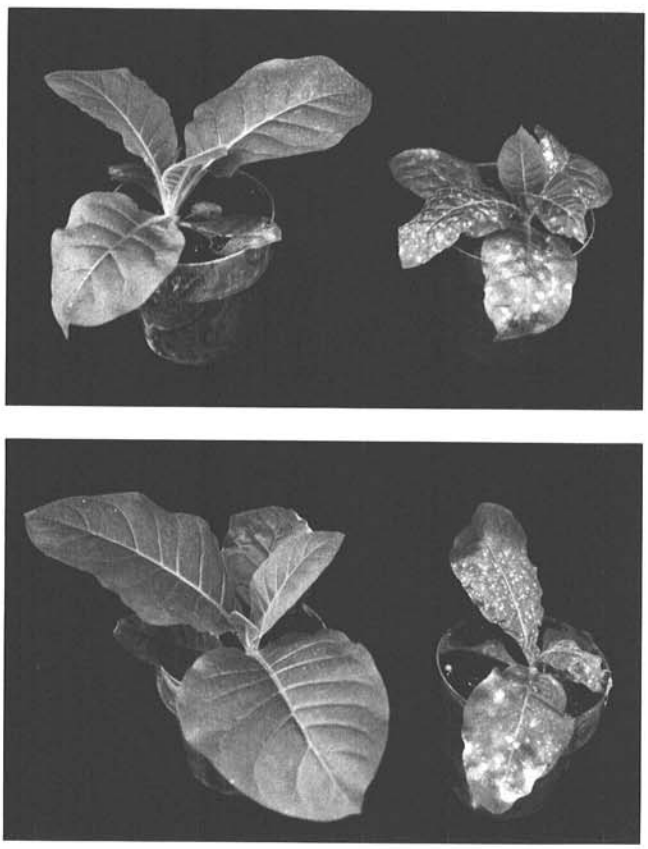

Fig. 3. Inoculation of R_1 transgenic tobacco plants with the TSWV-BL (top) and Arkansas (bottom) isolates of tomato spotted wilt virus. The plants were photographed 20 days after inoculation. Left, the R_1 plants of transgenic line NP^+ 2 containing the nucleocapsid protein gene; right, the control NP^- plants.

Relation between the level of NP accumulation in transgenic plants and the degree of resistance to heterologous TSWV isolates. Analysis of the data in Table 1 suggested that R_1 plants derived from R_0 lines with low levels of NP offered the best resistance to the heterologous isolates of the L serogroup (Arkansas and 10W pakchoy), whereas R_1 plants from a R_0 line with a high level of NP were resistant to the Begonia isolate, which belongs to the I serogroup. For example, in average 76% of inoculated R_1 plants from low NP-expressing lines NP^+ 2, 14, and 21 were resistant to infections by the Arkansas and 10W pakchoy isolates, whereas resistance to these isolates was observed in only 11% of similarly inoculated plants from high NP-expressing lines NP^+ 4, 9, and 23. On the other hand, the Begonia isolate infected 79% of R_1 plants from the low NP-expressing lines NP^+ 2, 14, and 21 but only 19% of plants from high NP-expressing line NP^+ 4.

Thus, it was of interest to accurately quantitate the relation of NP expression in individual plants with resistance to the heterologous isolates. In a number of our inoculation experiments reported in Table 1, we had taken leaf samples of transgenic plants before inoculating with the Arkansas and 10W pakchoy isolates. Samples were also taken from uninoculated leaves of plants inoculated with the Begonia isolate after we initially observed the apparent relation between NP expression levels and resistance. This latter method of sampling could be done without interference from infection by the Begonia isolate, because this isolate does not cause systemic infection in tobacco or react with antibodies to the TSWV-BL NP. All samples were assayed for relative NP levels by DAS-ELISA using antibodies raised to isolated NP of TSWV-BL. Figure 4 shows the relation between NP levels in transgenic R_1 plants (irrespective of the R_0 lines they came from) and their resistance to the Arkansas and 10W pakchoy isolates or to the Begonia isolate. Nearly all transgenic R_1 plants with very low or undetectable ELISA reactions (0–0.05 OD_{405nm}) were resistant to infections by the Arkansas and 10W pakchoy isolates (the L serogroup) but susceptible to the Begonia isolate (the I serogroup). In contrast, almost all R_1 plants that gave high ELISA reactions (0.4–1.0 OD_{405nm}) were resistant to the Begonia isolate but susceptible to the Arkansas and 10W pakchoy isolates.

DISCUSSION

Two previous reports (15,27) showed that transgenic tobacco plants expressing the TSWV NP gene are resistant to infection with the homologous isolate. In this study, we report that transgenic tobacco plants expressing the NP gene of the TSWV-BL isolate are highly resistant to infections by the homologous TSWV-BL isolate and heterologous isolates of the same serogroup (Arkansas and 10W pakchoy). More significantly, the resistance is effective to the Begonia isolate, which belongs to the I serogroup.

The evidence for resistance to the L-type isolates is clear because these isolates produced dramatic systemic infections. However, the results from inoculations with the Begonia isolate should be interpreted with some caution since this isolate caused only local lesion reactions. We have tested two other I-type isolates, but both induced only local infections or no symptom on tobacco (unpublished data). To circumvent this potential limitation, we are producing transgenic *N. benthamiana,* which is a systemic host to all TSWV isolates that we have tested.

Despite a slight delay of symptom expression, transgenic plants did not show resistance to the Brazil isolate of TSWV. Our serological results show that this isolate is distinct from the

L- and I-type isolates and biologically different from the cucurbit isolate. Moreover, we have sequenced 60% of the S RNA of the Brazil isolate and found that the sequence is significantly different from the published sequences of the L and I serogroups (unpublished data). The Brazil isolate may thus belong to still another serogroup of TSWV. In any event, our infectivity results show that it is unlikely that a single NP gene will provide resistance to all isolates in the Tospovirus genus.

We observed that transgenic plants that gave low or undetectable ELISA reactions (0–0.05 OD_{405nm}) were resistant to infection by the heterologous isolates (Arkansas and 10W pakchoy) of the L serogroup, whereas no protection against these isolates was found in plants accumulating high levels of the NP. Compared with the ELISA readings of control NP^- plants (0–0.05 OD_{405nm}), these transgenic plants may produce little, if any, TSWV-BL NP. Similar results have been observed by others (21,24,25,27,33,38), who reported that transgenic plants, in which the CP accumulation was not detected, were highly resistant to virus infection. The mechanism underlying this phenomenon is presently unknown. It is likely that this type of resistance might be attributed to interference of CP RNA molecules produced in transgenic plants with viral replication, presumably by hybridizing to negative-sense replicating RNA of the attacking virus, binding to essential host or viral factors (e.g., replicase) or interfering with virion assembly (25).

It should be noted, however, that the resistance to the homologous TSWV-BL isolate is apparently independent of the expression levels of the NP gene. Although we did not measure the relative NP levels of the individual R_1 plants inoculated with TSWV-BL, it is reasonable to assume that the NP produced in these inoculated R_1 plants (a total of 145 plants tested) ranged from undetectable to high, as was observed in Table 1 and Figure 4.

In contrast to the case for protection against the heterologous isolates of the L serogroup, protection against the Begonia isolate of the TSWV-I serogroup was found in the high NP-expressing R_1 plants. Comparison of published NP nucleotide sequence of the L serogroup with that of the I serogroup reveals 62 and 67% identity at the nucleotide and amino acid levels, respectively (23).

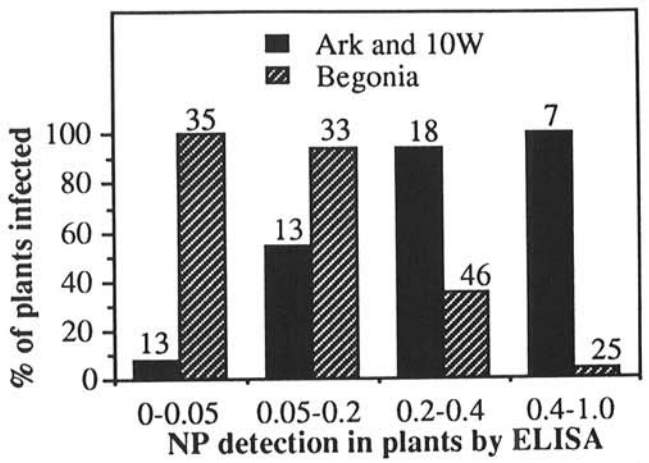

Fig. 4. Correlation of the level of nucleocapsid protein (NP) accumulation in transgenic plants with the degree of resistance to tomato spotted wilt virus (TSWV) isolates. Transgenic plants were assayed in double-antibody sandwich enzyme-linked immunosorbent assay for NP accumulation with antibodies raised against the NP of TSWV-BL. Plates were read 150 min after adding substrate, and the transgenic plants were grouped into four categories of OD_{405nm}: smaller than 0.050, between 0.050 and 0.200, between 0.200 and 0.400, and greater than 0.400. The OD_{405nm} readings of control NP^- plants were from zero to 0.05. The same plants were challenged with either the Arkansas (Ark) and 10W pakchoy (10W) isolates or the Begonia isolate and the susceptibility of each plant was recorded about 12 days after inoculation. The results were pooled from 51 R_1 NP^+ plants inoculated with the Arkansas and 10W pakchoy isolates and 139 R_1 NP^+ plants inoculated with the Begonia isolate. Numbers above bars represent total numbers of R_1 NP^+ plants tested.

The difference of NP genes of the two serogroups might be so great that the NP (the L serogroup) produced in transgenic plants acted as a dysfunctional protein on the attacking Begonia isolate of the I serogroup. Incorporation of this defective CP into virions may generate defective viruses that inhibit virus movement (6) or its further replication. This type of interaction is expected to require high levels of the NP for the protection. Alternatively, resistance to the Begonia isolate may also involve interference of NP transcripts produced in R_1 plants with viral replication. If this is true, more NP transcripts (because of the heterologous nature of two NP genes) may be required to inhibit replication of heterologous virus. Further research should be directed to elucidate the exact mechanism(s) governing the resistance of transgenic plants with different levels of the NP with respect to which TSWV isolates that a plant is challenged.

Although we have no obvious explanations for the results showing the relation of NP levels in individual R_1 plants to resistance to the heterologous isolates of the L and I serogroups, we believe that these are definite trends since the data were derived from a large number (190) of plants. In this respect, previous reports determined the relationship of CP or NP level of a given transgenic R_0 line to resistance by measuring the percent infectivity of its R_1 population. We believe that a measurement of CP or NP levels in individual plants may provide a more accurate way to relate NP or CP levels to resistance. By this form of data analysis, our results show that the resistance was more closely related to NP levels in each test plant than to the NP level of the R_0 line from which they were derived. For TSWV-BL NP gene in tobacco, at least, it appears that integration sites of the NP gene in plant chromosome may not be important for viral resistance.

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