The Biological Properties of a Distinct Tospovirus and Sequence Analysis of Its S RNA

Sheng-Zhi Pang, Jerry L. Slightom, and Dennis Gonsalves

First and third authors: Department of Plant Pathology, Cornell University, NYSAES, Geneva, NY 14456; second author: Molecular Biology Research, Unit 7242, The Upjohn Company, Kalamazoo, MI 49007.

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ABSTRACT

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A tospovirus isolate from Brazil, designated TSWV-B, was first identified as a unique isolate based on our observation that transgenic plants expressing the N gene of the lettuce strain of tomato spotted wilt virus (TSWV-BL) were susceptible to TSWV-B but showed resistance to both TSWV (L type) and impatiens necrotic spot virus (INSV). TSWV-B was serologically distinct from TSWV and INSV. TSWV-B generally incited symptoms resembling those caused by other TSWV isolates, except TSWV-B systemically infected *Petunia hybrida*, which is a local-lesion host of TSWV. Unlike the cucurbit isolate TSWV-W, TSWV-B did not infect *Cucumis sativus* and only occasionally induced systemic infections on *C. metuliferus*. The complete nucleotide sequence of the S RNA of TSWV-B was determined with cDNA clones to be 3,049 nucleotides long. The genome organization of this S RNA was similar to those of TSWV and INSV. The alignment of the S RNA nucleotide and deduced amino acid sequences with the homologous sequences of TSWV (isolates CNPH1, L3, and BL) and INSV revealed that TSWV-B was related more closely to all the TSWV isolates than to INSV. There was a higher degree of identity among the TSWV isolates than with TSWV-B. Thus, TSWV-B appears to be a distinct tospovirus; however, a precise classification requires additional biological and molecular information on this isolate as well as comparison to other tospovirus isolates.

Tospoviruses have a wide host range and cause significant economic losses to vegetables and ornamental plants worldwide (2,4,15–17,25). Based on serological affinities and nucleotide sequence identity, tomato spotted wilt virus (TSWV, formerly L type) and impatiens necrotic spot virus (INSV, formerly TSWV-I) have recently been classified as two separate viruses within the *Tospovirus* genus (17). Several other TSWV-like viruses (1,5,8,18,22,23,34,39) have been reported as serologically distinct from TSWV and INSV and may represent additional virus species of the *Tospovirus* genus (17,27).

The tospovirus genome consists of three single-stranded RNAs designated S RNA (2,900 nucleotides), M RNA (~5,000 nucleotides), and L RNA (8,900 nucleotides) (10,11,31,37). From the S RNA, a 52-kDa nonstructural (NS_s) protein is translated from the large ORF (open-reading frame) on the viral RNA strand, and the 29-kDa nucleocapsid (N) protein is translated from the small ORF on the viral complementary RNA strand (12,26,29). This ambisense-coding arrangement is also a characteristic of the INSV M RNA, which encodes 58- and 78-kDa membrane-associated glycoproteins in one ORF and a 35-kDa NS_s protein in the other ORF (27). The TSWV L RNA encodes a large 200-kDa protein, presumably for the viral transcriptase (9).

Transgenic *Nicotiana tabacum* L. plants expressing the N gene of TSWV are resistant to homologous isolates (19,28). Subsequent study in our laboratory (33) demonstrated that transgenic tobacco plants expressing the N gene of TSWV-BL displayed a broad spectrum of resistance not only to heterologous isolates of TSWV but also to a begonia isolate of INSV. However, these transgenic plants showed no resistance to a Brazilian isolate (TSWV-B), first described by Wang and Gonsalves (38), although the plants that accumulated high levels of the N protein did display a delay in symptom expression (33). ELISA (enzyme-linked immunosorbent assay) with polyclonal antibodies against the N protein of TSWV and INSV showed that the TSWV-B N protein is not serologically related to either TSWV or INSV (33). TSWV-B biologically differs from a cucurbit isolate (TSWV-W; [22,39]) because it usually does not induce systemic infection on cucurbits (33). Here we report the biological properties of TSWV-B, the nucleotide and deduced amino acid sequences of its S RNA, and sequence comparisons among tospovirus S RNAs.

MATERIALS AND METHODS

Host-range tests. TSWV-B was isolated originally from *Lycopersicon esculentum* Mill. and was first described by Wang and Gonsalves (38). TSWV-B was subsequently transferred to and maintained in *Nicotiana benthamiana* Domin. To characterize its biological properties, plants from several species representing four families were used for a host-reaction test. Inoculum was

prepared by grinding leaf tissue (0.5 g) from *N. benthamiana* infected with TSWV-B in 15 ml of cold inoculation buffer (0.033 M KH_2PO_4, 0.067 M K_2HPO_4, and 0.01 M Na_2SO_3). The inoculum extracts immediately were rubbed onto corundum-dusted leaves of test plants, and the inoculated leaves were subsequently rinsed with water. Inoculated plants were grown in the greenhouse and observed daily for at least 3 wk. In cases of ambiguous results, extracts from the test plants were checked on systemic hosts, *N. benthamiana* and *N. tabacum*, for the presence of the virus. In addition, because we did not have antibodies to detect this virus by ELISA, PCR (polymerase chain reaction) was used to analyze the inoculated plants for the virus infection. Total plant RNA was isolated according to the method of Napoli et al (32). The isolated total RNA was subjected to first-strand cDNA synthesis and subsequent PCR-based amplification as described by Pang et al (33). The synthetic oligonucleotides used for both first-strand cDNA synthesis and PCR amplification were 5′-TACTTATCTAGAACCATGGTCATGTCTAAGGTCAAGCTCAC and 5′-TCTTGAGGATCCATGGCTATTATGCAACACCAGCAATTTTGGC, corresponding to the 5′ (nucleotide positions 2,879–2,899 of the TSWV-B S RNA) and the 3′ (nucleotide positions 2,122–2,145 of the TSWV-B S RNA) ends of the TSWV-B N gene, respectively.

Viral RNA purification. TSWV-B nucleocapsids were purified from *N. benthamiana* plants infected with TSWV-B as described by Law and Moyer (25). The purified nucleocapsids were resuspended in a solution of 0.04% bentonite, proteinase K at 10 μg/ml, 0.1 M ammonium carbonate, 0.1% (w/v) sodium diethyldithiocarbanate, 1 mM EDTA, and 1% (w/v) sodium dodecyl sulfate (SDS), were incubated at 65 C for 5 min, and were extracted immediately with H_2O-saturated phenol, followed by another extraction with chloroform/isoamyl alcohol (24:1). The single-stranded (ss) viral RNAs were precipitated in 2.5 volumes of ethanol in the presence of 0.3 M sodium acetate and were dissolved in distilled H_2O. Double-stranded (ds) viral RNAs also were isolated from the infected leaf tissue by a combination of methods (24,30), which has been successfully used for isolating dsRNA from tissues infected with grapevine leafroll virus (21). These two methods were chosen for viral RNA purification because we were unable to purify the virus particle from the infected tissue by the method of Gonsalves and Trujillo (20).

cDNA synthesis and cloning. To construct cDNA libraries specific to the TSWV-B S RNA, both viral ssRNAs and dsRNAs were separated on 0.7% agarose gels, and the S ssRNA (comigrated with the S RNA of TSWV-BL) and the S dsRNA (determined by its molecular weight) were removed from gels. The gel slices were incubated at −20 C, crushed, loaded into a 5-ml syringe with glass wool at the bottom, and centrifuged for 5 min at 3,000 rpm. The RNA solutions collected were precipitated with 2.5 volumes of ethanol in the presence of 0.3 M sodium acetate. The isolated S ssRNA and dsRNA were denatured by methylmercury treatment and subjected to a cDNA-synthesis procedure, as described in the *Protocols and Applications Guide* provided by Promega (Madison, WI). The cDNA synthesis was primed by random primers, and the cDNA fragments were cloned via an *Eco*RI adapter into the *Eco*RI-digested λ ZAPII according to the manufacturer's instructions (Stratagene, San Diego, CA). Two libraries were constructed, one from the S ssRNA and the other from the S dsRNA. Both cDNA libraries were screened with the cDNA probes prepared by reverse transcription of gel-purified S RNA. Positive clones were further characterized by restriction enzyme site mapping and by Southern DNA analysis. Hybridizations were performed essentially according to the manufacturer's protocol (GeneScreen Plus, E. I. Du Pont De Nemours & Co., Inc., Boston, MA).

The 3′ terminus of the TSWV-B S RNA was isolated from the dsRNA library by screening with an end-labeled synthetic oligomer (5′-TTCTGGTCTTCTTCAAACTCA) corresponding to the 2,816–2,836 nucleotide positions of the TSWV-B S RNA. The 5′ extreme end of the S RNA was obtained with the 5′ RACE system from GIBCO BRL (Gaithersburg, MD). Both ssRNA of TSWV-B and total RNAs isolated from tobacco plants infected with TSWV-B were used to synthesize first-strand cDNA with an oligonucleotide (5′-CTGTAGCCATGAGCAAAG) complementary to the 746–763 nucleotide positions of the TSWV-B S RNA. The 3′ end of the first-strand cDNA was tailed with dCTP with terminal deoxynucleotidyl transferase. Tailed cDNA was amplified by PCR with an anchor primer that anneals to the homopolymeric tail and an oligonucleotide (5′-TTATATCTTCTTCTTGGA) that anneals to the 512–529 nucleotide positions of the TSWV-B S RNA. The PCR-amplified fragment was gel-purified and directly cloned into the T-vector pT7Blue, provided by Novagen (Madison, WI), for sequence analysis.

Nucleotide sequencing and comparative analyses. DNA sequencing was performed by the dideoxyribonucleotide method (35), T_7 polymerase (Sequenase, U. S. Biochemical Corp., Cleveland, OH), and the double-stranded sequencing procedure described by Siemieniak et al (36). The nucleotide sequences were determined from both DNA strands, first by the universal and reverse primers and then by the internal primers specifically designed for sequencing the TSWV-B S RNA. Sequence data were analyzed with computer programs available from the Genetics Computer Group (GCG, Madison, WI, [13]).

RESULTS AND DISCUSSION

Properties of TSWV-B. Host reactions to TSWV-B are summarized in Table 1. TSWV-B caused systemic infections on all the test plants in Amaranthaceae and Solanaceae. Symptoms usually included local necrotic lesions, systemic mottling, leaf distortions, and plant stunting. TSWV-B induced only local lesions on *Chenopodium quinoa* Willd. Test plants in Cucurbitaceae responded differently to inoculations of TSWV-B. On *Cucumis sativus* L., neither local lesions nor systemic symptoms developed; however, five of 32 *Cucumis metuliferus* E. Meyer ex Naudin plants showed symptoms of systemic mottling and leaf distortions. Uninoculated leaves of all test plants also were analyzed for the presence of the virus by back inoculation on *N. benthamiana* and *N. tabacum* and by PCR amplification with the synthetic primers specific to the TSWV-B N gene. Results from these tests were consistent with visual symptom observations (data not shown).

Comparisons of the symptomatology of TSWV-B and two other tospoviruses (Table 1; [39]) showed that TSWV-B differed from TSWV-NY, in that TSWV-B systemically infected *Petunia hybrida* Hort. Vilm.-Andr., which is a local-lesion host of TSWV-NY. INSV incited only local lesions on *L. esculentum*, *N. tabacum*, and *P. hybrida* (Table 1; [7,25]), and TSWV-B infected all of them systemically. Unlike TSWV-W, which primarily infects cucurbits (22,23,39), TSWV-B did not systemically infect *C. sativus* and only occasionally induced systemic infections on *C. metuliferus*. Viral RNAs and structural proteins also were analyzed on agarose and polyacrylamide gels, respectively, and they were similar in size to the corresponding counterparts of TSWV and INSV (data not shown).

Nucleotide sequence analysis of TSWV-B cDNA clones. Both viral ssRNAs and dsRNAs were isolated from the infected leaf tissue of *N. benthamiana* and separated on agarose gels. The TSWV-B S RNA was gel-purified and copied into cDNA with the random primers. Two cDNA libraries were constructed: one from the S ssRNA purifed from TSWV-B nucleocapsids and the other from the S dsRNA. Positive clones were identified from both cDNA libraries by the cDNA probes prepared from the TSWV-B S RNA. Three overlapping clones containing the largest inserts (L1, L22, and L30) were selected from the cDNA library constructed from the S ssRNA for nucleotide sequence analysis.

Nucleotide sequence analysis of the inserts in clones L1, L22, and L30 revealed 1,994-, 2,368-, and 1,576-bp inserts, respectively. The comparison with the TSWV-CNPH1 S RNA (12) indicated that the assembled sequence of 2,842 bp represented 93% of the S RNA genome, excluding both 5′ and 3′ ends of the S RNA (Fig. 1). Together these three clones contained the NS_s ORF, the complete intergenic region (629 nucleotides), and 738 nucleotides of the N gene (only 39 nucleotides at the N-terminus of

TABLE 1. Host reactions of three distinct tospoviruses[a]

Host plants	Reaction to TSWV-B		Reaction to TSWV-NY		Reaction to INSV	
	Local	Systemic	Local	Systemic	Local	Systemic
Amaranthaceae						
Gomphrena globosa	+	NS,M,LD	+	NS,M	+	VN
Chenopodiaceae						
Chenopodium quinoa	+	−	+	−	+	−
Solanaceae						
Datura stramonium	+	NS,LD	+	NS,M	+	(CS)
Lycopersicon esculentum	−	M,LD,S	−	CS,M	+	−
Nicotiana benthamiana	−	M,LD,S	−	M,W	+	VN,LD
N. tabacum	−	LD,S			+	−
N. glutinosa	+	LD,S	+	NS,M	+	VC,LD,M
N. rustica	+	LD,S,CS	+	NS,M	+	(M)
Petunia hybrida	−	W	+	−	+	−
Cucurbitaceae						
Cucumis metuliferus	−	[M,LD,S]	+	−		
C. sativus	−	−	+	−	+	−

[a] Reactions to tomato spotted wilt virus (TSWV-NY) and impatiens necrotic spot virus (INSV) were reported by Yeh et al (39) and de Avila et al (7), respectively. Abbreviations for symptoms: +, local lesions; −, no symptoms; CS, chlorotic spots; LD, leaf distortions, M, mosaic; NS, necrotic spots; S, stunting; VC, vein clearing; VN, vein necrosis; W, wilting; ( ), erratic systemic infection; [ ], sporatic systemic infection (only five of 32 *C. metuliferus* plants were systemically infected).

the N protein was not represented in the cDNA clones). The 3′ region of the S RNA was isolated from the original cDNA library constructed from the S dsRNA with an end-labeled synthetic oligomer corresponding to the 3′ end of cDNA clone L22 (Fig. 1). Five clones that hybridized with the synthetic oligomer were sequenced with the primer. Among the five clones, only two, designated S6 and S7, contained the N-terminal nucleotide sequence of the N gene. In addition, clone S7 also contained the extreme consensus 3′ end of the TSWV-B S RNA (Figs. 1 and 2). The 5′ end of the S RNA was obtained with the 5′ RACE system and T-vector pT7Blue Cloning system (Novagen, Madison, WI), as described previously. Eight independent clones were sequenced with an oligomer primer (5′-GTTCTGAGA-TTTGCTAGT) close to the 5′ region of the S RNA (nucleotide positions 40–57 of the TSWV-B S RNA). Six contained the 5′ extreme end of the S RNA, and the 5′-terminal nucleotide sequence from these six clones was identical. Thus, the complete nucleotide sequence of the TSWV-B S RNA was 3,049-nucleotides long, as shown in Figure 2.

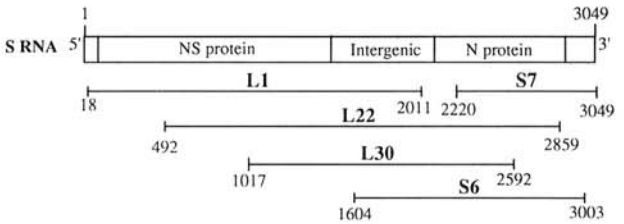

Fig. 1. Location of the sequenced cDNA clones in the tomato spotted wilt virus (TSWV)-B S RNA. The five overlapping cDNA clones are shown to scale below an S RNA map of TSWV-B. The five clones (L1, L22, L30, S6, and S7) were synthesized with random primers from the S RNA isolated from *Nicotiana benthamiana* plants infected with TSWV-B.

Genomic organization of TSWV-B S RNA. Sequence analysis revealed that the complete nucleotide sequence of the TSWV-B S RNA was 3,049-nucleotides long, 134 nucleotides longer than the S RNA of TSWV-CNPH1. This difference was mainly attributed to the elongated intergenic region of the TSWV-B S RNA. The 5′ and 3′ extreme ends (Fig. 2, 15 nucleotides in italic lowercase letters) of the TSWV-B S RNA were conserved (almost identical to those of TSWV-CNPH1 and INSV S RNAs) and complementary, except a mismatch at nucleotide position 11. A similar mismatch was previously observed in the S RNAs of TSWV-CNPH1 (11,12) and in animal viruses (3,14) in the family Bunyaviridae but at nucleotide position nine. The significance of this discrepancy is unknown. Nevertheless, conservations of the terminal sequences among tospoviruses and of the mismatch within the family Bunyaviridae indicate their possible roles in replication and encapsidation (3,14).

The sequence analysis of TSWV-B S RNA revealed two ORFs with an ambisense arrangement (Fig. 2), similar to other tospoviruses. The large ORF (1,404 nucleotides) was localized on the viral RNA strand originating at nucleotide 88 and terminating at nucleotide 1,491 (Fig. 2). This ORF encoded a protein of 467 amino acids with a predicted molecular mass of 52.6 kDa, presumably for the NS_s protein. The deduced amino acid sequence of this ORF contained four potential glycosylation sites, three of which (the last three from the N-terminus) were located at the same positions as those of TSWV-CNPH1 and -L3. The small ORF (777 nucleotides), located on the viral complementary strand, was defined by an initiation codon at nucleotide 2,898 and by a termination codon at nucleotide 2,122 (Fig. 2). This ORF encoded an N protein of 258 amino acids with a predicted molecular mass of 28.7 kDa. Unlike the N proteins of TSWV and INSV, which do not contain an N-glycosylation site, the deduced amino acid sequence of TSWV-B N protein contained one potential N-glycosylation site at amino acid residue 68 (Fig. 2).

The two ORFs were separated by an intergenic region of 629 nucleotides that was, because of several insertions, 126 and 44 nucleotides longer than its counterparts in TSWV-CNPH1 and -L3, respectively. The complex and stable hairpin structures (data not shown) that potentially could be generated by internally base-pairing U-rich stretches with A-rich stretches of the intergenic region seem to be similar to those produced in TSWV-CNPH1 and -L3, in both structure and stability (as indicated by minimal free-energy values; data not shown). It has been suggested that this internally base-paired structure acts as a transcription termination signal (3).

Comparative analyses of the S RNA sequences of tospoviruses. Pairwise comparisons of the entire TSWV-B S RNA with the published S RNA regions of tospoviruses (Table 2) showed that it shares its greatest nucleotide sequence identities with TSWV isolates CHNHI, L3, and BL (75.8–76.4%, overall; 77.5–78.0%, N gene; 72.4–76.4%, intergenic region; and 79.0–80.0%, NS_s gene), which are, nevertheless, much lower than those found among the TSWV isolates (94.8–96.4%, overall; 96.8–97.3%, N gene; 89.2–95.9%, intergenic region; and 95.6%, NS_s gene). A similar pairwise comparison between the homologous S RNA sequences of TSWV-B and INSV revealed a much lower degree of shared identity (63%).

Consistent with our results from ELISA (33) and Western analysis (data not shown), the amino acid sequences of the N and NS_s genes from TSWV isolates CNPH1, L3, and BL were more closely related to each other (96.9–98.5%, N gene, and 89.4%, NS_s gene) than to TSWV-B (79.1–79.9%, N gene, and 78.3–82.0%,

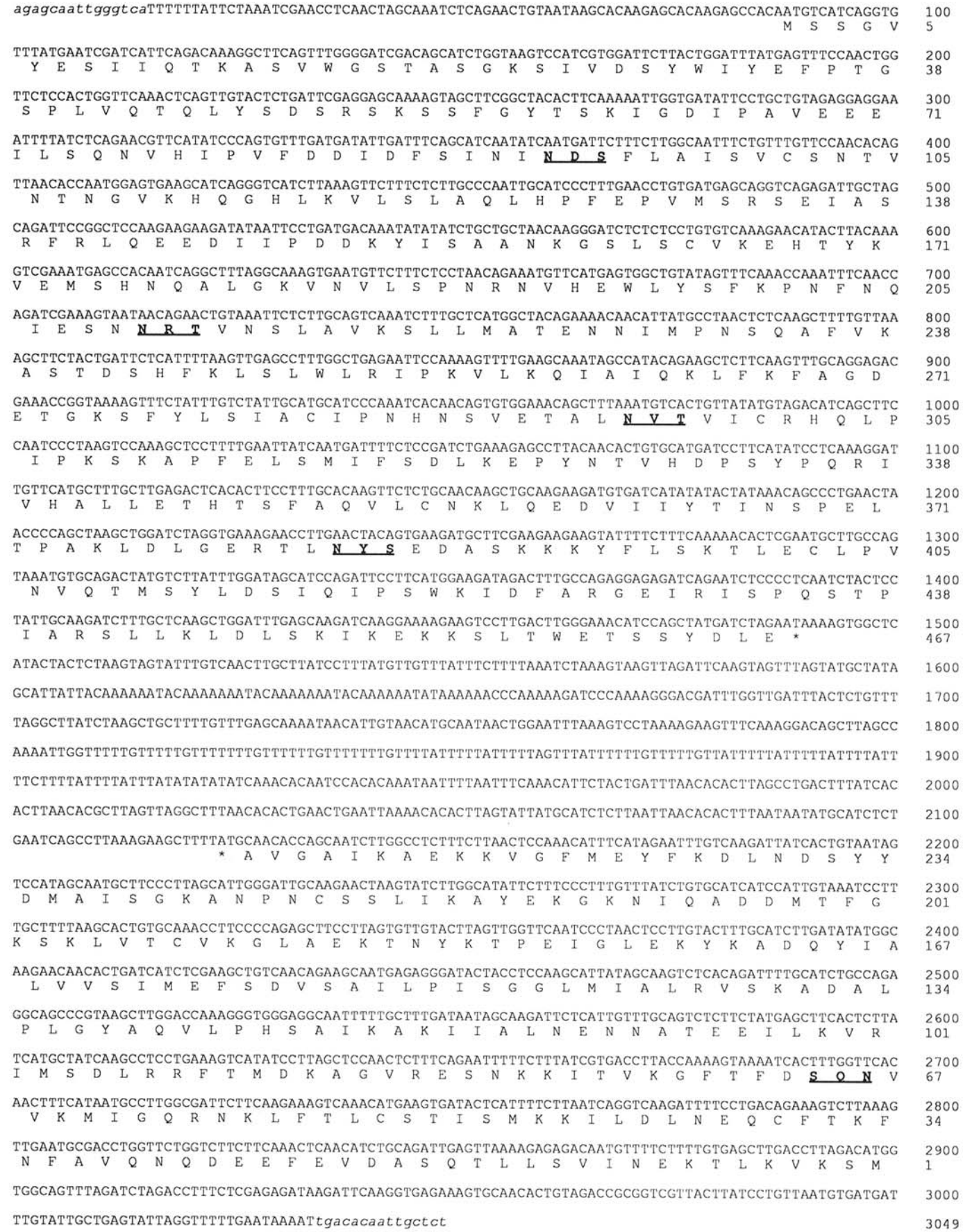

Fig. 2. The complete nucleotide sequence of the cDNA copy of tomato spotted wilt virus (TSWV)-B S RNA and the deduced amino acid sequences of the nonstructural and nucleocapsid proteins. The cDNA sequence of the TSWV-B S RNA was determined from both DNA strands by the primer-directed dideoxy chain termination reactions with double-stranded cDNA templates. The first nucleotide residue is numbered at the 5′ end of the S RNA, and the amino acid residues are numbered from the first methionine of proteins. Potential N-linked glycosylation sites are bold and underlined, and the 15 nucleotides that represent the conserved 5′ and 3′ terminal sequences of the S RNA are shown in italic lowercase letters.

TABLE 2. Pairwise comparisons of aligned nucleotide (nt) and deduced amino acid (aa) sequences of the S RNA of tospoviruses

Comparison[a]	Overall nt[b] (%)	52-kDa protein gene (NS_s gene) nt (%)	52-kDa protein gene (NS_s gene) aa (%)	Intergenic nt (%)	29-kDa protein gene (N gene) nt (%)	29-kDa protein gene (N gene) aa (%)
B/CNPH1	76.4	80.0	78.3	72.4	77.5	79.1
B/L3	75.8	79.0	82.0	76.4	78.0	79.9
B/BL	76.3	...	...	72.8	77.6	79.5
B/INSV	63.0	...	...	...	63.1	55.3
CNPH1/L3	94.8	95.6	89.4	89.2	96.8	98.5
CNPH1/BL	96.4	...	...	95.9	97.2	96.9
L3/BL	95.1	...	...	92.6	97.3	98.5
INSV/CNPH1	62.7	...	...	...	60.8	55.1
INSV/L3	60.9	...	...	...	60.9	55.1
INSV/BL	61.7	...	...	...	60.9	53.9

[a] The partial or complete S RNA sequences of isolates of tomato spotted wilt virus (TSWV)-CNPH1 (2,916 kb), -L3 (2,837 kb), -BL (2,037 kb), and impatiens necrotic spot virus (INSV; 1,144 kb) were published by de Haan et al (12), Maiss et al (29), Pang et al (33), and Law et al (26), respectively, and were used for pairwise comparisons with the S RNA sequence of TSWV-B (3,049 kb) or with each other.

[b] Percent identity was calculated by comparing nucleotide or predicted amino acid sequences with the BESTFIT program of the GCG sequence-analysis software package.

NS_s gene). In contrast, a much lower degree of amino acid identity was observed between the N proteins of TSWV-B and INSV (55.3%), with a value similar to those found between the N proteins of INSV and TSWV isolates (53.9–55.1%). Furthermore, the N gene of INSV was longer, encoding 262 amino acids, than those of the TSWV isolates and TSWV-B, which encoded 258 amino acids. The amino acid sequence alignment suggested that the four additional residues in the INSV N protein were the result of two insertions: a three amino acid insertion (residues 83 to 85) and an amino acid insertion at residue 110 (data not shown).

Our data show that the N gene of TSWV-B is much more distantly related to the N gene of INSV (~63%) than to that of TSWV (~78%) isolates (Table 2). Should TSWV-B be considered as a new tospovirus? After this manuscript had been submitted for publication, we learned from the dissertation of de Avila (6) that the N genes of two tospovirus isolates from South Africa (SA-05) and Brazil (BR-03) have been sequenced, and our analyses show 94.4 and 81.5% identities with that of TSWV-B, respectively. Thus, data accumulated on isolates of tospoviruses should allow the classification of TSWV-B in the near future.

In summary, the entire S RNA of TSWV-B was sequenced from cDNA clones. The genomic size and organization of its S RNA is similar to those of other tospoviruses. The comparative analyses (Table 2) suggest that TSWV-B is more closely related to the TSWV isolates than to INSV but is much less similar to the TSWV isolates than the TSWV isolates are to each other. To our surprise, transgenic plants expressing the N gene of TSWV-BL (L type) are resistant to INSV but not to TSWV-B (33). Although our ELISA results show that TSWV-B does not react with antibodies against the N protein of either TSWV-BL or INSV (33), weak cross-reactions to antibodies against the N protein of both TSWV-BL and INSV were clearly observed in Western analysis (data not shown). Thus, it appears that TSWV-B is a distinct tospovirus; however, a precise classification requires additional comparisons of biological and molecular information of this isolate with other tospovirus isolates.

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