Nucleotide Sequence of the N Gene of Watermelon Silver Mottle Virus, a Proposed New Member of the Genus *Tospovirus*

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ABSTRACT

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A virus isolated from watermelon was previously identified as tomato spotted wilt-like virus in the genus *Tospovirus* of the family *Bunyaviridae*. The virus, designated Tospo-W, contains a nucleocapsid (N) protein serologically unrelated to tomato spotted wilt virus (TSWV) or impatiens necrotic spot virus (INSV). In this study, double-stranded RNAs of Tospo-W were extracted from virus-infected tobacco and used for cDNA cloning. A cDNA clone, pWN12, that was selected from a cDNA library to the separated S dsRNA, contained 1,200 nucleotides including the entire N ORF that encodes a protein of 275 amino acids with a predicted M_r of 30,657. Comparison of the nucleic acid sequence of the Tospo-W N gene with those of the other five tospoviruses revealed that their identity percentages were 54.4–55.9%. The degrees of similarity at the amino acid level were only 39.6–46.1%. In vitro transcription and translation of the clone pWN12 generated a protein of 31 kDa, similar to the authentic N protein and reacted specifically with the antibody to the Tospo-W N protein. A nick-translated DNA probe from the insert of pWN12 reacted with RNAs extracted from plants infected with Tospo-W or with a tospovirus isolate from melon, but not with RNAs from plants infected with three TSWV isolates or three INSV isolates. The low-degree homology of the N gene with other tospoviruses coupled with the lack of cross hybridization using a Tospo-W–specific cDNA probe suggests that Tospo-W, renamed as watermelon silver mottle virus, is a distinct member of the genus *Tospovirus*.

Additional keywords: nucleotide sequence, watermelon tospovirus.

Viruses in the *Tospovirus* genus of the arthropod-borne *Bunyavirus* family are unique in that they infect plants (14). Tomato spotted wilt virus (TSWV) is the type member of the genus *Tospovirus* (15). It is characterized by quasi-spherical enveloped particles containing three linear ssRNA species, denoted small (S) RNA, medium (M) RNA and large (L) RNA (34,47). The three genomic RNAs form pseudocircular structures associated with a nucleocapsid (N) protein of 29 kDa and a few copies of a large protein (L protein), the putative viral polymerase. Two membrane glycoproteins of 78 kDa (G1) and 58 kDa (G2) were considered to form spikes on the viral envelope (35,44). The complete genomic nucleotide sequence of a Brazilian isolate of TSWV has been determined. The L RNA (8,897 nucleotides) is of negative polarity and encodes a putative RNA polymerase of 331.5 kDa (10,26). The other two genomic RNAs use ambisense coding strategies. The M RNA (4,821 nucleotides) contains two ORFs, one in the viral sense that encodes a nonstructural (NSm) protein of 33.6 kDa and the other in the viral complementary sense that encodes the protein of 127.4 kDa as a precursor for the G1 and G2 glycoproteins (25). TSWV S RNA (2,916 nts) also has two ORFs; one in the viral sense encodes a nonstructural (NSs) protein of 52.4 kDa and the other in the viral complementary sense encodes the N protein of 29 kDa (12,27). Although the intracellular location of the NSs protein has been determined, its function remains unknown (27).

Serological classification of tospovirus isolates distinguished them into serotypes or serogroups. Most of the TSWV isolates reported in the literature belong to the "L-serotype" (or common-serotype) (1,9,17,20,38,39,48). A different serotype, designated I-serotype, was reported by Law and Moyer (28). The N proteins of the I-serotype isolates were not serologically related to those of the L-serotype, but the two envelope proteins (G1 and G2) were found conserved between the two serotypes (28). The I-serotype has been renamed as impatiens necrotic spot virus (INSV) and is considered a new species in the genus *Tospovirus* distinct from TSWV (29). The M and S RNAs of INSV have also been shown to have an ambisense structure similar to that of TSWV (29,30). Using polyclonal antibodies against the N proteins to compare 20 isolates from different geographical areas and crops, a third serogroup distantly related to TSWV and distinct from INSV was documented (9). Further comparison of the nucleotide sequences of the N genes of this serogroup with TSWV and INSV indicated they are distinct tospoviruses. Therefore, two new tospovirus species, tomato chlorotic spot virus (TCSV) and groundnut ringspot virus (GRSV) were proposed (8). Recently, the sequence of S RNA of a biologically and serologically distinct tospovirus originating in Brazil, denoted as TSWV-B, has been determined; TSWV-B appears to be a distinct tospovirus (37).

A tomato spotted wilt-like virus, designated as Tospo-W, which causes significant losses in watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai) in Taiwan, was recently identified as a tospovirus based upon particle morphology, host reactions, thrips transmission, and serological relationships (49). Serological tests by enzyme-linked immunosorbent assay (ELISA) and Western blotting using polyclonal and monoclonal antibodies demonstrated that the watermelon virus is serologically related to the tospovirus causing silver mottle on watermelon in Okinawa, Japan (23), and a peanut isolate causing bud necrosis in India (2,38), but distinct from TSWV and INSV (2,49).

The serological classification for different serotypes in the genus *Tospovirus* has mainly been based upon the characteristics of the N protein. In this investigation, we determined the nucleotide sequence of the N gene of Tospo-W and compared it with those of the other tospovirus isolates to clarify the taxonomic position of the virus. Furthermore, we developed a dot blot hybridization assay for detection and diagnosis of Tospo-W using a cloned cDNA probe.

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MATERIALS AND METHODS

Virus sources. The tomato spotted wilt-like virus Tospo-W was previously isolated from watermelon (*C. lanatus*) in Taiwan (49). A typical isolate of TSWV, denoted TSWV-NY, was isolated from tomato in New York State (kindly provided by R. Provvidenti, Cornell University) and used for comparison throughout the study. A melon isolate, designated as Tospo-M, was isolated from a melon field in Fengsun, Taiwan. Other isolates of typical TSWV and INSV kindly provided by J. Moyer (TSWV-M and INSV-M from the United States), G. Adam (TSWV-A from Brazil and INSV-A from Germany), and J. Cho (INSV-C from New Guinea) were also used for hybridization comparison.

All isolates were maintained in the systemic hosts *Nicotiana rustica* L., *N. benthamiana* Domin., and *Datura stramonium* L. The plants at the stage of 2–4 fully expanded leaves were mechanically inoculated with inoculum prepared by grinding leaf tissue in 0.1 M potassium phosphate buffer (pH 7.0) containing 0.01 M sodium sulfite.

Isolation of total viral dsRNAs. ds RNA was isolated essentially as described by Valverde et al (46). Leaves of *N. rustica* (10 g), 12–14 days after inoculation with Tospo-W, were ground in liquid nitrogen with a mortar and pestle. The plant tissue powder was mixed with extraction buffer [45 ml of sodium-Tris-EDTA (STE, 0.1 M NaCl, 0.05 M Tris, 0.001 M EDTA), 15 ml of 10% sodium dodecyl sulfate (SDS), 0.1 ml of NH_4OH, 0.8 ml of 4% EDTA-treated bentonite, and 1 ml of 2-mercaptoethanol] and stirred at room temperature (RT) for 10 min. The mixture was further extracted by addition of 25 ml of STE-saturated phenol/chloroform, and stirred at RT for 45 min. The aqueous phase was removed after centrifugation at 8,000 *g* for 15 min, and 95% ethanol was added to a final concentration of 16.5%. The dsRNA was absorbed by addition of 2 g CF 11 cellulose powder (Whatman) and the mixture was shaken at RT for 45 min. Then the cellulose solution was poured into a column and washed by 16.5% ethanol in STE. The dsRNA was eluted from the column with STE and precipitated by ethanol. The pellet was washed with 80% ethanol, dried under vacuum, and dissolved in sterilized distilled water.

Separation of S dsRNA. Samples containing total viral dsRNAs were loaded on low-melting agarose gels (0.8%). Electrophoresis was conducted at 80 V and gels were stained by ethidium bromide (0.5 μg/ml). The bands corresponding to S dsRNA of TSWV were excised under UV light and RNA was removed using the Gelase recover protocol (Protech) and dissolved in 0.1% diethyl pyrocarbonate (Sigma)–treated distilled water.

Construction of a cDNA library. S dsRNA was first denatured according to the methylmercuric hydroxide (MeHg) method of Bailey and Davidson (4). The denatured dsRNA was immediately diluted with 100 μl of polyadenylation reaction mixture (50 mM Tris-HCl, pH 8.2, 10 mM $MgCl_2$, 250 mM NaCl, 2.5 mM MnCl2, 0.25 mM ATP, 1 mM DTT, 250 μg of bovine serum albumin, 100 units of RNasin (Amersham), 2.5 units of *Escherichia coli* poly (A) polymerase (Amersham) and 13 μl of [^{32}P]-ATP(3,000 Ci/mmole, Amersham)). After incubation for 15 min at 37 C, polyadenylated RNA was extracted with phenol/chloroform and precipitated with 3 volumes of ethanol and 1/10 volume of 3 M sodium acetate, pH 5.5 (7,43).

The polyadenylated S dsRNA was denatured again by the MeHg method and used to construct a cDNA library according to the protocols for ZAP-cDNA synthesis and cloning (18). After digestion with *Xho* I, the ds DNA was inserted into the *Xho* I/*Eco* RI site of lambda ZAP-II vector (Stratagene), packaged by capsid proteins (Stratagene, Gold Packaging Extract), and plated on bacterial lawns of *E. coli* strain PLK-F′, followed by amplification in strain XL1-Blue.

Plaque hybridization and in vivo excision. The cDNA clones were screened by hybridization according to the standard method of Maniatis et al (33). The cDNA probe used for plaque screening was synthesized from the MeHg-denatured viral S dsRNA with random primers (33). Lambda plaques selected by hybridization were in vivo excised by the helper phage VCSM 13 according to the method described by Short et al (42). The plasmids were extracted by miniboiling according to the method of Holmes and Quigley (19) and digested with *Eco* RI/*Xho* I to release the inserted DNA. The digests were analyzed by agarose-gel electrophoresis. Three clones, designed as pWN5, pWN11, and pWN12, which contained inserts of 0.5 kb, 1.1 kb, and 1.2 kb, respectively, were selected for further studies.

Subcloning and sequencing. The selected clones pWN5, pWN11, and pWN12 were found to have overlapping sequences with a similar A-rich sequence at one end. The cDNA inserts were digested with *Eco* RI/*Xho* I and subcloned into pBluescript II KS(-) vector to generate three clones, pWNK5, pWNK11 and pWNK12, with an opposite orientation corresponding to pWN5, pWN11, and pWN12, respectively. Two clones (PND8-1 and PND8-2) that contained restriction fragments of pWN12 were constructed according to standard protocols (33).

DNA sequencing was performed by the dideoxynucleotide chain-termination method (41), using ssDNA templates generated by VCSM13 (Stratagene) and ds DNA templates prepared from the selected clones (19). Sequence data were assembled and analyzed by the PC/GENE software (IntelliGenetics, Inc.). The aligned sequences were compared with those of the published tospoviruses, including TSWV (12), INSV (29), GRSV and TCSV (8), and TSWV-B (37).

In vitro expression of the N gene. Two clones, pWN11 and pWN12, containing complementary viral strand DNA corresponding to the entire N gene, were selected for in vitro transcription and translation analysis. RNA transcripts were synthesized from the transcription vector pBluescript SK(-) by runoff transcription using T3 RNA polymerase according to the manufacturer's directions (Stratagene). The molecular size and quantity of capped RNA transcripts were estimated by electrophoresis in 1% formaldehyde agarose gel. The transcripts were directly used as templates for in vitro translation.

In vitro translation was performed using the rabbit reticulocyte translation system (Stratagene). The translation mixtures containing [^{35}S] methionine (> 1,000 Ci/mmole, Amersham) were incubated at 30 C for 60 min. Protein products were analyzed by electrophoresis in 12% polyacrylamide gels followed by autoradiography.

Immunoprecipitation. Translation products were diluted with 4 volumes of immunoprecipitation buffer (0.1 M NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.5, 1% Nonidet P-40) containing 10 mM methionine. The solution was then mixed with 1/2 volume of the undiluted antiserum against the Tospo-W N protein (kindly provided by R. J. Chiu, Chung Hsing University) or normal serum. After incubation at RT for 2 h, 2 volumes of *Staphylococcus aureus* strain II bacteria suspension (10%, Sigma), which were prewashed with immunoprecipitation buffer, were added to the mixture and incubated at RT for 1 h (24). Antibody-precipitated proteins were collected by centrifugation at 6,000 *g* for 5 min. The pellets were thoroughly resuspended in immunoprecipitation buffer and the suspension was subjected to centrifugation again. This washing process was repeated three times. The final pellets were dissolved in an original volume of SDS-sample buffer followed by heating at 100 C for 3 min. The total translation proteins and the immunoprecipitated products were analyzed by 10% SDS-polyacrylamide gel electrophoresis and autoradiography.

Total RNA extraction and hybridization. Nucleic acids for RNA dot blot assay were extracted from virus-infected tobacco (*N. rustica*) and watermelon (*C. lanatus*) 6–10 days after inoculation, using the phenol/SDS extraction method (3).

The total RNA extracted from plants and dsRNAs denatured as described were resolved by electrophoresis in 1% formaldehyde agarose gel. The separated RNAs were transferred to nylon membranes with a PosiBlot pressure blotter (Stratagene). Alternatively, the RNAs were spotted directly onto nylon membranes by fivefold serial dilutions (40). The hybridization condition followed the protocol described previously (33). A DNA fragment of pWN12 digested with *Eco* RI/*Xho* I was radiolabeled with [^{32}P]-dATP by nick translation according to Maniatis et al (33). The DNA (1 μg) was incubated in 48 μl of nick translation buffer

(0.05 M Tris-HCl, pH 7.2, 0.01 M $MgSO_4$, 0.1 mM dithiothreitol, 50 μg/ml of BSA) containing 2 μM unlabeled dNTPs(-dATP), 3.5 μl of [^{32}P]-dATP (3000 Ci/mmole), 1 μl of DNase I (2 ng/ml), and 1 unit of DNA polymerase I according to the instructions of Boehringer Mannheim (Germany). The reaction was performed at 16 C for 1 h, stopped by the addition of 2 μl of 0.5 M EDTA, and followed by ethanol precipitation.

RESULTS

Cloning and sequence determination of the Tospo-W N gene. Three cDNA clones were selected by hybridization with the [^{32}P]-labeled cDNA probes derived from S dsRNA of Tospo-W by random priming. They were designated as pWN5, pWN11, and pWN12, containing inserts of 0.5 kb, 1.1 kb, and 1.2 kb, respectively. Nucleotide sequences were determined from the three clones and subclones of the restriction fragments of pWN12 (Fig. 1). Each nucleotide was sequenced from both orientations with an average of 3.5 determinations. The assembled nucleotide sequence of 1,200 residues of the longest clone pWN12 is shown in Fig. 2. Nucleotide sequences of pWN5 and pWN11 were included in pWN12. Computer comparison of pWN5, pWN11, and pWN12 revealed that the cDNA synthesis was all primed from an A-U-rich region of the viral complementary strand of Tospo-W S dsRNA and covered the complete ORF of the N gene. The conserved sequence at the 3′ termini of the S RNAs of tospoviruses (12,29) was absent from the clone pWN12, suggesting that the 3′ end of the noncoding region was not cloned in its entirety.

X N H H E cDNA
pWN12
pWN11
pWN5
pND8-1
pND8-2
N protein RNA(Vc)
0 200 400 600 800 1000 1200

Fig. 1. Complementary DNA clones constructed from dsRNA of Tospo-W in pBluescript II SK(-) vector. The three clones (pWN12, pWN11, and pWN5) were selected by hybridization using a [^{32}P]-labeled random primed cDNA probe derived from S dsRNA of Tospo-W. Clones pND8-1 and pND8-2 were subclones of the restriction enzyme fragments of pWN12. The nucleotide sequence of the N gene of Tospo-W was elucidated from the ssDNA template of these clones. The viral complementary (Vc) RNA strand is presented and the box corresponds to the nucleocapsid open reading frame. Restriction enzymes are abbreviated X, *Xho* I; N, *Nhe* I; H, *Hind* III; E, *Eco* RI.

Predicted protein of the Tospo-W N gene. Computer prediction for the possible reading frames of the viral (V) and viral complementary (Vc) RNA strands of the clone pWN12 revealed that the Tospo-W N ORF starts at an initiation codon from nucleotide positions 98–100 from the 5′ terminus of the Vc RNA (nt 1,101–1,103 of V RNA) and a termination codon at nucleotide positions 923–925 of Vc S RNA (nt 276–278 of V RNA) (Fig. 2). No other ORFs of significant length larger than 5 kDa could be found. The amino acid sequence derived from this ORF is also shown in Fig. 2. The predicted gene product contains 275 amino acids and has a calculated M_r of 30,657. The length of this ORF indicated that it encodes a protein with molecular size similar to the putative 32 kDa N protein described previously (2,49).

Comparison of the Tospo-W N gene with those of the other tospovirus isolates. The complete ORF of the N gene of Tospo-W is 825 nucleotides long (Fig. 2), 39 or 51 nucleotides longer than those of the reported tospovirus isolates (8,12,29,32,37). Comparison of the nucleotide sequence of the Tospo-W N gene with those of the other five tospovirus isolates revealed that they share 54.4–55.9% identity. The amino acid sequence of Tospo-W N protein showed low degrees of identity, only 26.4–33.3% (Table 1), with those of the other tospoviruses. The percentages of amino acid similarity were slightly higher, between 39.6–46.1% (Table 1). The alignment of the amino acid sequence of the Tospo-W N protein with those of the other tospoviruses is shown in Fig. 3. The Tospo-W N protein is 13 or 17 amino acids longer than those reported for TSWV, GRSV, TCSV, TSWV-B, and INSV (8,12,29,32,37). Although the putative N protein of Tospo-W shared low degrees of similarity with those of the other tospovirus isolates, some consensus residues are still present. The C-terminal region (23 amino acids) of the N proteins of Tospo-W is highly variable and shares no similarity with any of the tospoviruses compared (Fig. 3).

In vitro expression of the N gene. Vc sense RNA transcripts of 1.1 kb and 1.2 kb were synthesized from the two clones pWN11 and pWN12, respectively, (Fig. 4A) and translated in rabbit reticulocyte lysate. The translation products were analyzed by

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   1  GGGTTTTTTGGAATTTTTTATATATATATATATTGTTTATTTATTTGTTTATTTATTTTATTGTTTTTATTTATTTTATTTATTTATTTTATATCTATAT

 101  AAGCACACACATAGGTATAACTCTATAATAGCTACTCGTATAATTAAAATAAGCATATAGTTATATAAGCACACACAAAAAACTTAAACAAACATAAATA

 201  GATTTATTAAATTATATAGTTAAGCATAAACACAAACACACATGTGCACATATATGCACGGTTGACACTTCAAAGTTACACTTCCAAAGAAGTGCTGGGC
                                                                                     *  V  E  L  S  T  S  P
 301  TTTGAACTTTTCTTTGAATGATCACCATAATCATCCACAGAAAGGTTAGCACTGAAGACCCTGTCCATGTACTTAACTTGATCCTCATATTTCTTGAGCG
      K  S  S  K  K  S  H  D  G  Y  D  D  V  S  L  N  A  S  F  V  R  D  M  Y  K  V  Q  D  E  Y  K  K  L  S
 401  AAATAGAGCTAGCTGTTCCAGGGTTACTTTCACTGAGTATTTTGACAGCCTGTTTGAACATAACATCCAAATCATCCTTAAATTCAACCTGTGAAGCAGA
        I  S  S  A  T  G  T  N  S  E  S  L  I  K  V  A  Q  K  F  M  V  D  L  D  D  K  F  E  V  Q  S  A  S
 501  AAGAACCTTAGCCACCTTACAAACTTGCTCATAGGTGGAGAAGTTCTTTATACCCAACTTTTCTTTCTTTATATTTTGGTAATAAGCTAAAGGGAAAATA
       L  V  K  A  V  K  C  V  Q  E  Y  T  S  F  N  K  I  G  L  K  E  K  K  I  N  Q  Y  Y  A  L  P  F  I
 601  ATCGGTGCCAGTCCCCTTATACTAGATAAAAGAGGTAGAGGTCCTCCAATACATAACATCATCCTCAAGGCACAAGAATCATAAGATGCGGGCACATTCA
      I  T  A  L  G  R  I  S  S  L  L  P  L  P  G  G  I  C  L  M  M  R  L  A  C  S  D  Y  S  A  P  V  N  L
 701  ACCCATAAGCAGCAACTAATGGTAGTTCCATGATTTTCCCATACATTTCCTGTCTAGCAGCTTCATTCTTGCTTTTCTCTACCATACTAACTATTTTTGT
        G  Y  A  A  V  L  P  L  E  M  I  K  G  Y  M  E  Q  R  A  A  E  N  K  S  K  E  V  M  S  V  I  K  T
 801  CCTGATAAAAGCTTCTGTCCTCTTGAATGTCCAGTCCTCAGGTCCGACGTCAACACTTGTGGCAACAATATTTTTACCGCAAAAATTATACTTACCGCTC
       R  I  F  A  E  T  R  K  F  T  W  D  E  P  G  V  D  V  S  T  A  V  I  N  K  G  C  F  N  Y  K  G  S
 901  TTGCAGGCTGCAAAAATCTGTTTCCTGCATTTTAGGATATTCAAGCAGTTTGTAAAGGTTATCTCTATATTTTTGTTGTTGTAAAAAAAAGCTTTGAAGC
      K  C  A  A  F  I  Q  K  R  C  K  L  I  N  L  C  N  T  F  T  I  E  I  N  K  N  N  Y  F  F  A  K  F  S
1001  TAAATCCGGGAGTGGAGTCCTCTGTTTCAATTTCAACATCTGCTGTACCACCAGCCAAAAGTTCCTTGATTTTCTTTTCTGTGAGCTGCTTAACGTTAGA
        F  G  P  T  S  D  E  T  E  I  E  V  D  A  T  G  G  A  L  L  E  K  I  K  K  E  T  L  Q  K  V  N  S
1101  CATGGTGTTTACAAAGGAAACGTCTGATATGTTGTTTGAACTGATATTATTAGCGCCTCGATTGCTCTCTCTCTCTCTCCTCGTGCCGATTCGACGAG
       M
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Fig. 2. Nucleotide sequence and deduced amino acid sequence of the cDNA clone pWN12. The amino acid sequence was predicted from the complementary sense of the nucleotide sequence shown.

polyacrylamide-gel electrophoresis. One discrete translation product was obtained, with an M_r of 31 kDa (Fig. 4B, lanes 2, 3). This translation product reacted with the antibody to the Tospo-W N protein (Fig. 4B, lanes 5, 6) but not with normal serum (Fig. 4B, lane 7). This result clearly indicated that the ORF of the viral complementary strand of the Tospo-W cDNA clones encodes the nucleocapsid protein. One additional product of 38 kDa was also observed from the transcript of pWN12 (Fig. 4B, lane 3) and reacted with the antibody to the Tospo-W N protein (Fig. 4B, lane 6). The leader sequence of pWN12 is only 61 nucleotides longer than that of pWN11 (Fig. 1). This span could not generate a protein 7 kDa larger than the 31 kDa protein. We speculate that the 38 kDa protein may be derived from the binding of the 31 kDa N protein to the leader sequence of the RNA transcript derived from pWN12.

Dot blot assay. The results of the dot blot using the nick-translated probe derived from pWN12 are shown in Fig. 5A&B. The probe detected Tospo-W RNAs in as little as 8 ng of total RNA extracted from infected tobacco, and also reacted strongly with the total RNA extracted from tobacco infected with Tospo-M.

TABLE 1. Comparison of the N gene of Tospo-W with those of the other five reported topsoviruses

Viruses[a]	TSWV	GRSV	TCSV	TSWV-B	INSV
Tospo-W	55.3[b]	55.1	55.9	55.5	54.4
	32.9[c]	33.3	26.4	31.4	30.2
	46.1[d]	44.5	39.6	44.2	40.1
TSWV	...	76.7	76.8	77.5	60.5
	...	78.3	76.7	79.1	54.7
	...	87.2	86.8	88.0	67.1
GRSV		...	81.0	94.5	61.5
		...	81.0	95.0	54.3
		...	88.8	95.8	67.1
TCSV			...	82.2	62.8
			...	84.1	53.1
			...	91.9	68.6
TSWV-B				...	62.9
				...	55.0
				...	67.8

[a]TSWV = tomato spotted wilt virus (12), GRSV = groundnut ringspot virus (8), TCSV = tomato chlorotic spot virus (8), TSWV-B (37), and INSV = impatiens necrotic spot virus (29).
[b]Percentage of nucleotide identity of the ORF. Nucleotide identity analyzed by the NALIGN program of the PC/GENE software (IntelliGenetics, Inc.).
[c]Percentage of amino acid identity. Amino acid identity and similarity analyzed by the PALIGN program of the PC/GENE software (IntelliGenetics, Inc.).
[d]Percentage of amino acid similarity.

The results indicated that Tospo-W and Tospo-M isolates possess a high degree of homology. Moreover, the Tospo-W back-inoculated watermelon plants also reacted positively when as little as 1.6 ng of total RNA was used. The total RNAs extracted from TSWV-NY- or INSV-infected tobacco failed to hybridize with the Tospo-W-specific probe (Fig. 5A). In the other dot blot test, the nucleic acid probe did not react with the three typical isolates of TSWV and the three typical isolates of INSV tested (Fig. 5B).

DISCUSSION

The enveloped tospovirus virions are difficult to purify in adequate amounts. Different purification methods (6,11,28,45) were tried to purify Tospo-W virions for the extraction of the genomic ssRNAs to construct a cDNA library, but we failed to obtain a sufficient amount of virus particles. Alternatively, we used dsRNA from Tospo-W-infected plants as templates for synthesis of cDNA. This method has been successfully applied for viruses in the genus *Reovirus* that possess genomic double-stranded RNAs (7,21) and for other plant (+) ssRNA viruses that contain dsRNAs as replicative forms (13,22,46). In this study, cDNA clones corresponding to the nucleocapsid protein of Tospo-W were successfully obtained by the dsRNA cloning technique. Our method of extracting dsRNAs from plants infected with enveloped viruses provided a fast and simplified procedure for cDNA cloning of tospovirus.

An A-U-rich intergenic region located in S RNA separating the two ORFs of N and NSs has been reported for TSWV and INSV isolates (11,12,29). A similar A-U-rich sequence was also found in the region downstream from the Tospo-W N ORF. This region has the potential to form a secondary structure and may be involved in transcription termination (12). Analysis of three selected cDNA clones synthesized from S dsRNA of Tospo-W revealed that the clones were all primed from the same area of the internal A-rich region of the viral complementary RNA strand and subsequently elongated toward its 5′ end. No clones primed from the 3′ terminus of the viral sense RNA were obtained. It is possible that the 3′ end of MeHg-denatured S dsRNA was not efficiently polyadenylated by poly A polymerase, or the A-rich region located on Vc S RNA has a stronger affinity for the oligo d(T) primer.

The sequence analysis of the pWN12 revealed only one large ORF located on the viral complementary RNA strand. The N ORF of Tospo-W contains 825 nucleotides. Computer comparison of the predicted N ORF with those of the other TSWV, GRSV, TCSV, TSWV-B, and INSV isolates (8,12,29,32,37) showed nucleic acid identity of 54.4–55.9% and amino acid similarity of less than 46.1%. These degrees of identity and similarity at the nucleic acid and amino acid levels are much lower than the values between TSWV and INSV (Table 1). This, coupled with the

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TSWV      MSKVK-LTKESIVALLTQGK-DLEFEEDQNLVAFNFKTFCLENIDQIKKMSVISCLTFLKNRQSIMKVIKQSDFTFGKITIKK---TSDRIGGTDM   91
GRSV      MSKVK-LTKENIVSLLTQSE-DVEFEEDQNQVAFNFKTFCQENLDLIKKMSITSCLTFLKNRQSIMKVVKQSDFTFGKVTIKK---NSERVEAKDM   91
TCSV      MSKVK-LTRENIISLLTQAG-EIEFEEDQIKATFNFEDFCGENLDSIKKMSITSCLTFLKNRQSIMKVVNLCDFTFGKITIKK---NSGRVGANDM   91
TSWV-B    MSKVK-LTKENIVSLLTQSA-DVEFEEDQNQVAFNFKTFCQENLDLIKKMSITSCLTFLKNRQGIMKVVNQSDFTFGKVTIKK---NSERVGAKDM   91
INSV      MNKAK-ITKENIVKLLTQSD-SLEFEETQNEGSFNFTDFFTNNREKIQNMTTASCLSFLKNRQSIMRVIKSADFTFGSVTIKKTRNNSERVGVNDM   94
Tospo-W   MSNVKQLTEKKIKELLAGGTADVEIETEDSTPGFSFKAFFYNNKNI--EITFTNCLNILKCRKQIFAACKSGKYNFCG---KNIVATSVDVGPEDW   91
          *...* .* ..*  **. .   ..*.*...   .*.*..*  .* .    ...  .**..** *. *. . .   ...*        *.   .*  .. .*

TSWV      TFRRLDSLIRVRLVE--ETGNSEN-LNTIKSKIASHPLIQAYGLPLDDA-KSVRLAIML--GGSLPLIASVDSFEMISVVLAIYQDAKYKDLGIDP  181
GRSV      TFRRLDSMIRVKLIE--ETANNEN-LAIIKAKIASHPLVQAYGLPLDDA-KSVRLAIML--GGSIPLIASVDSLEMISVVLAIYQDSQVQELGIEP  181
TCSV      TFRRLDSMIRVKLIE--ETGKAEN-LAIIKSKIASHPLVQAYGLPLTDA-KSVRLAIML--GGSIPLIASVDSFEMISIILAIYQDAKYKDLGIEP  181
TSWV-B    TFRRLDSMIRVKLIE--ETANNEN-LAIIKAKIASHPLVQAYGLPLADA-KSVRLAIML--GGSIPLIASVDSFEMISVVLAIYQDAKYKELGIEP  181
INSV      TFRRLDAMVRVHLVG--MIKDNGSALTEAINSLPSHPLIASYGLATTDL-KSCVLGVLL--GGSLPLIASVLNFEIAALVPAIYQDAKHVELGIDM  185
Tospo-W   TFKRTEAFIRTKIVSMVEKSKNEAARQEMYGKIMELPLVAAYGLNVPASYDSCALRMMLCIGGPLPLLSSIRGLAPIIFPLAYYQNIKKEKLGI--  185
          **.*  ....*.....        ....        ... . **...***     .  .*  *  ..*  **..**..*. ...        * **.  .   .***

TSWV      KKYDTKEALGKVCTVLKSKAFEMNEDQVKKGKEYAAILSSSNPNAKGSVAMEHYSETLNKFYEMFGV-----------KKQAKLAEL--A  258
GRSV      TKYNTKEALGKVCTVLKSKGFTMDDAQDNKGKEYAKILSSSNPNAKGSIAMDYYSDNLEKFYEMFGV-----------KKEAKIAGV--A  258
TCSV      SKYNTKEALGKVCTVLKSKGFTMDEEQVQKGKEYATILSSCNPNAKGSIAMEHYSEHLDKFYAMFGV-----------RKEAKISGV--A  258
TSWV-B    TKYNTKEALGKVCTVLKSKGFTMDDAQINKGKEYAKILSSCNPNAKGSIAMDYYSDNLDKFYEMFGV-----------KKEAKIAGV--A  258
INSV      SKFSTKEAVGKVCTVLKSKGYSMNSVEIGKAKQYADILKACSPKAKGLAAMDHYKEGLTSIYSMFNA-----------TIDFGKNDS--I  262
Tospo-W   KNFSTYEQVCKVAKVLSASQVEFKDDLDVMFKQAVKILSESNPGTASSISLKKYEDQVKYMDRVFSANLSVDDYGDHSKKSSKPSTSLEV  275
          ....* *..  **  .**...    ....        . *. . **....*  .  .  ... *.. ..  .    .*..                ..
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Fig. 3. Alignment of the deduced amino acid sequence of the nucleocapsid protein of Tospo-W with those of five other reported tospovirus isolates. Sequence data were obtained from de Haan et al (12) for TSWV, Law et al (29) for INSV, de Avila et al (8) for TCSV and GRSV, and Pang et al (37) for TSWV-B. The alignment was performed by the CLUSTAL program of the PC/GENE software. Perfectly conserved residues are indicated by an asterisk and highly conserved residues by a dot.

serological unrelatedness to TSWV and INSV based on the N proteins (2,49), indicates that the Tospo-W isolate is a distinct virus in the *Tospovirus* genus.

The predicted N gene product of Tospo-W has an M_r value of 30,657 and contains 275 amino acids. This gene product is larger than the known N gene products of TSWV (258 amino acids) (12,32) and INSV (262 amino acids) (29). The molecular size of the Tospo-W N gene product was also determined by cell-free translation of the in vitro transcripts derived from clones pWN11 and pWN12. Although the leaders of the two clones were different lengths, a major protein with a similar M_r of 31,000 was generated from both RNAs. This protein was specifically precipitated by the antibody to the Tospo-W N protein, and its size agreed with that previously described for the Tospo-W N protein (49). The results prove that the entire ORF of the N gene has been cloned, even though the intact 3′ end of the noncoding region was not intact. This conclusion is further supported by the fact that the predicted N-terminal sequence of the N protein of Tospo-W aligned well with those of other tospoviruses compared (Fig. 3). The 5′ and 3′ termini of S RNA of TSWV contain inverted complementary repeats that are probably involved in RNA replication and in the formation of circular nucleocapsid cores in virions (11). Whether or not this structure is present in the Tospo-W isolate remains to be determined.

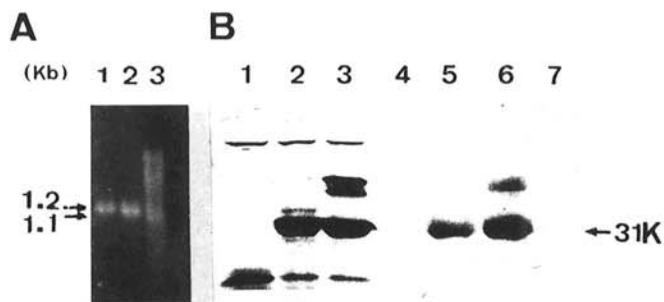

Fig. 4. Identification of the product of the Tospo-W N ORF by in vitro transcription and translation. **A,** RNA transcripts were transcribed from *Xho* I-linearized pWN12 (lane 1) and pWN11 (lane 2), and analyzed in a 1% agarose gel. CMV genomic RNAs were used as markers (lane 3). **B,** translation products without RNA transcript (lane 1, 4), and with the transcript from pWN11 (lane 2 & 5) or pWN12 (lane 3, 6 & 7). Translation products were precipitated with Tospo-W NP antibody (lane 4-6) or normal serum (lane 7).

GRSV, TCSV, and TSWV-B are three viruses recently claimed as possible distinct members of the genus *Tospovirus* (8,37). In this investigation, we found that the N protein of TSWV-B has 94.5% amino acid identity and 95.0% amino acid similarity with that of GRSV. The high degrees of sequence homology indicate that they should be considered two strains of the same virus. When viruses are classified by sequence homology, the amino acid similarity should be considered important since structurally similar amino acids provide similar protein conformation, which results in similar functions and closely-related serological properties. If this is taken into consideration, TCSV and TSWV-B should also be considered strains of the same tospovirus since their N proteins share a high degree of amino acid similarity (91.9%, Table 1). Thus, TSWV-B becomes an intermediate strain to link GRSV and TCSV together. Moreover, the N proteins of these three virus isolates share a high degree of amino acid similarity with TSWV (86.8–88.0%) and have the same number (258) of amino acids as that of TSWV. This, coupled with the serological relatedness of the viruses (8,9,37), suggests that GRSV, TCSV, and TSWV-B should all be considered strains of TSWV rather than distinct tospoviruses. Since more tospovirus isolates are available for comparison, major criteria to define a distinct member of tospovirus are essential.

The N protein of INSV (262 amino acids) is larger than that of TSWV, GRSV, TCSV, and TSWV-B (258 amino acids). This fact, coupled with low amino acid similarity (Table 1) and serological unrelatedness (28), indicated that INSV is a distinct tospovirus.

The N protein of Tospo-W virus has been shown to be serologically unrelated to TSWV and INSV and has a larger M_r of 32,000 (2,49). The virus has been classified as a third major serotype of tospoviruses that includes groundnut bud necrosis virus (GBNV) from India (2,38), a watermelon tospovirus from Japan that incites a watermelon silver mottle disease and was originally thought to be TSWV (23), and a tomato tospovirus isolated from Taiwan (2). Like most TSWV isolates, GBNV causes local infection in cucurbits (38,39), while Tospo-W induces severe systemic symptoms in most cucurbit species (49). Moreover, Tospo-W does not infect peanut at all (S.-D. Yeh, *unpublished*). Also, serological differences between Tospo-W and GBNV in immunoblot were observed (2). From the differences in host reactions and serology, it appears that Tospo-W is different from GBNV. However, the actual relationship between the two viruses will be clarified after the nucleotide sequence of the N gene of GBNV is elucidated.

Based on the evidence provided by the studies on host reactions, serological relationships, patterns of double-stranded genomic

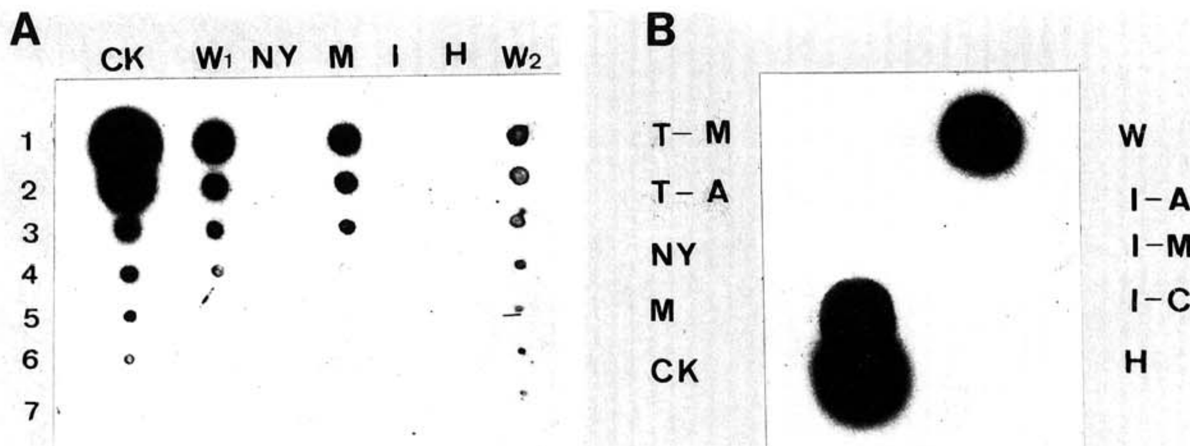

Fig. 5. Dot blot hybridization with the [^{32}P]-labeled nick-translated probe derived from the insert of pWN12. **A,** serial dilutions of total RNA (1 μg, 200 ng, 80 ng, 40ng, 8 ng, 1.6 ng, and 320 pg) were spotted in rows 1–7. Total RNAs were extracted from *N. rustica* infected with Tospo-W (watermelon isolate), TSWV-NY, or Tospo-M (melon isolate) and healthy *N. rustica*, in order from the left to right, indicated as W_1, NY, M, and H, respectively. The total RNA was also extracted from INSV-infected *N. benthamiana* (an isolate provided by J. Moyer) and from Tospo-W back-inoculated watermelon (lane W_2). DNA of pWN12 was used as control (lane CK). **B,** total RNAs from healthy plants (H), or from *N. benthamiana* infected with TSWV-M (T-M), TSWV-A (T-A), TSWV- NY (NY), Tospo-M (M), Tospo-W (W), INSV-A (I-A), INSV-M (I-M), and INSV-C (I-C), respectively. DNA of pWN12 was used as control (CK).

RNAs, molecular size of the N protein, nucleic acid identity and protein similarities of the N gene, ambisense gene expression strategy, and hybridization relationships, we concluded that Tospo-W, renamed as watermelon silver mottle virus (WSMV), should be regarded as a distinct virus member in the genus *Tospovirus*. On the other hand, the melon isolate Tospo-M, which is serologically identical to Tospo-W (Yeh et al, 1992), also showed a close relationship with Tospo-W at the nucleic acid level, as reflected in the hybridization test, indicating that the two isolates belong to the same new species.

The watermelon tospovirus WSMV now appears widely spread throughout Taiwan and is considered one of the major limiting factors for the production of melons and watermelons. Our results showed that the viral nucleic acids extracted from Tospo-W-inoculated plants could be easily detected by dot blot assay with a [^{32}P]-labeled cDNA probe. This cDNA probe, labeled with either a radioactive or nonradioactive element, provides a sensitive tool for diagnosis of the diseases caused by WSMV.

Transgenic tobacco plants that express the N gene of TSWV lacked systemic symptoms and showed little or no systemic accumulation of virus after mechanical challenge with severe TSWV (16,31,36). The mechanism of resistance mediated by the nucleocapsid gene has been shown by de Haan et al to be RNA-mediated (5). The clone that contains the entire ORF of the N gene of Tospo-W can be used to generate transgenic melons or watermelons for control of the disease caused by WSMV.

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