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Functional Analysis of the Tomato Spotted Wilt Virus (TSWV) NSm Protein by Using Immunoblotting and Immunogold Labelling Assay

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The genome of tomato spotted wilt virus (TSWV) is composed of three RNA segments, S, M, and L RNA and the 5.0 kb M RNA encodes two glycoproteins G1, G2 and NSm protein of unknown function. In an effort to investigate the function of the NSm protein, antibody was raised against NSm fusion protein overexpressed in *Escherichia coli*. This antibody was used to detect the NSm protein by using western blot analysis and electron microscopic observation after immunogold labelling. For the cloning of the NSm gene, total RNA extracted from a TSWV infected plant was used for cDNA synthesis and polymerase chain reaction (PCR) instead of going through time-consuming virus purification. A protein band specifically reacting to the NSm antibody was detected from TSWV inoculated plants. The NSm protein was detected in the cell wall fraction and in pellet from low speed centrifugation when the infected plant tissue was fractionated into 4 fractions. In the immuno-electron microscopic observation, gold particles were found around the plasmodesmata of infected plant tissue. These results suggest that the NSm protein of TSWV plays some role in cell-to-cell movement of this virus.

Tomato spotted wilt virus (TSWV) is the type species of the genus tospovirus of the Bunyaviridae family (13). More than 400 species in 50 plant families including tomato, pepper and lettuce are known as the hosts of TSWV (15, 3, 4, 28). The virus is an enveloped spherical particle of 80-110 nm in diameter, and has projections consisting of two glycoproteins G1 (78 kDa) and G2 (58 kDa) (19, 23). This virus is transmitted only by thrips and looses some part of the genome after repeated mechanical transmission (20, 2, 22).

There are three RNA fragments covered by nucleocapsid proteins (N protein, 29 kDa) inside of the particle. Nucleotide sequences of these RNAs have been determined recently (12, 14). The S RNA (2.9 kb) is ambisense and encodes a nonstructural protein (NSs, 52.4 kDa) in positive sense and the N protein in viral complementary sense (10). The M RNA (5.0 kb) is also ambisense and encodes another nonstructural protein (NSm, 33.6 kDa) in positive sense and G1G2 protein precursor in viral complementary sense (16). The L RNA (8.9 kb) is negative sense and encodes L protein (> 300 kDa) (19), whose polymerase activity has been recently characterized (1).

Key words: TSWV, NSm, PCR, immunobotting, immunogold labelling, movement protein

Among the 6 proteins encoded by the viral genome, the exact functions of the two nonstructural proteins, NSs and NSm, have not been understood. Because they are not components of the virus particle, they may play other important roles during virus replication, transmission, and movement.

There are six genera in the Bunyaviridae family, *Bunyavirus, Phleovirus, Hantavirus, Nairovirus, Uukuvirus*, and *Tospovirus*. Among these genera, *Bunyavirus* and *Phlebovirus* genera have the NSm gene in positive sense while the *Tospovirus* have the NSm protein gene in negative sense. The exact function of the NSm protein in these virus genera is not known.

Various molecular biological strategies including sequence comparison and transgenic plants have been used to determine the function of viral proteins (27, 5). In an effort to determine the function of the NSm protein, antibody was raised against overexpressed NSm protein, and this antibody was used to locate the NSm protein in infected plants.

MATERIALS AND METHODS

Virus and Plants

TSWV L-strain originated from Hawaii was maintained in *Emilia fosbergii* Nicolson by thrips transmission, and

infected plants were used as inoculum for mechanical transmission on *Datura stramonium* L.

RNA Extraction

One gram of TSWV infected Datura leaves was ground in 4 ml of 1 M Tris·HCl, pH 8.0 and centrifuged for 2 min at 14000 rpm with a microcentrifuge. Two hundred microliter of extraction buffer (1 M Tris·HCl, pH 8.0, 200 mM $LiCl_2$, 2% SDS, 20 mM EDTA) was added to the same volume of the supernatant and vortexed. To the mixture, 400 µl of phenol/chloroform mixture (24:1) was added, and then incubated at 65°C for 5 min After centrifugation for 5 min, 350 µl of supernatant was taken and same volume of phenol/chloroform mixture was added. This mixture was kept on ice for 3 min. After centrifugation, phenol/chloroform extraction of the supernatant was repeated 3 more times, and 300, 250, and 200 µl of supernatant was taken from each extraction, respectively to avoid any contamination. The RNA was precipitated with ethanol and the final pellet was resuspended with 50 µl of RNase-free distilled water.

cDNA Synthesis and Polymerase Chain Reaction

The primers for cDNA synthesis and PCR amplification were designed based on the published M RNA sequence of the Brazilian isolate (16). The primer NSm5 (5'-gctgacgagtcat**ATG**TTGACTCTTTTCGG-3') is viral sense and contains the translation initiation codon (bold), a *Nde* I restriction site (underlined), and extra nucleotides for an efficient restriction digestion (lower cases). The primer NSm6 (5'-cggaggtcggatccCTATATTTCATCAAAG-3') is viral complementary sense and contains a stop codon of the NSm protein (bold), a *Bam*HI restriction site for cloning (underlined), and extra nucleotides for an efficient restriction digestion (lower case).

For the first strand synthesis, two microliter of viral RNA was mixed with 50 pmole of NSm6 primer in a total volume of 10 µl. After denaturation for 10 min at 70°C, the mixture was chilled on ice, added to 30 µl of reverse transcription mixture. Reaction was conducted for one hour at 42°C, followed by heat inactivation for 10 min at 70°C. Aliquots of 2.5 µl out of 40 µl first strand cDNA synthesis mixture was used for PCR amplification without further purification.

For the second strand synthesis, the primers NSm5 and NSm6 were used for PCR amplification of the cDNA, and the PCR reaction conditions were as follows: 5 min pre-heating at 95°C, a denaturation step at 90°C for 30 sec, an annealing step at 55°C for 45 sec and a extension step at 75°C for 1 min. Forty cycles of PCR reactions were followed by a 3 min extension reaction at 72°C.

Overexpression of NSm Fusion Protein and Antibody Production

The PCR product was purified, digested with *Nde*I and *Bam*HI, and cloned into expression vector pET11a (Novagen), generating the plasmid pETNSM. This plasmid was used to transform *E. coli* strain BL21(DE3) and positive clones were used for overexpression. The insert of the pETNSM was subcloned into pBluescript KS+ (Stratagene, La Jolla, CA) and the nucleotide sequence of the insert was determined by dideoxynucleotide chain termination methods (21). Since there was host protein with similar molecular weight as the pETNSM fusion protein, the *NdeI/BamHI* fragment was subcloned into *BamHI* digested pET11c after insertion of a *BamHI* linker in the *Nde*I site, resulting the clone pETNSMB. The 0.8 kb *BamHI* fragment of plasmid pETNSMB was subcloned into another expression vector pGEX-2T (Pharmacia, Piscataway, NJ). The resulting clone pGEXM was used for overexpression and antibody production. Expression of NSm fusion protein was induced with 1 mM IPTG and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, gels were stained with copper chloride and the appropriate band was excised (18). The protein was electrophoretically eluted, dialyzed overnight in PBS buffer at 4°C, and concentrated using a Centricon 30 spin column (Amicon, Beverly, MA). After concentration of purified protein was measured, antibody was prepared by injecting the protein into a rabbit. The produced polyclonal antibody was tested for its specificity before use.

Detection of NSm Protein from Infected Plants

Datura stramonium L., *Emilia fosbergii* Nicolson, *Nicotiana benthamiana* Domin, and *N. rustica* L. were mechanically inoculated with TSWV and leaves showing typical symptoms were harvested 5-7 days after inoculation. Leaves from healthy plants were used as a negative control. The leaves were ground in liquid nitrogen and 500 µl extraction buffer (30 mM KH_2PO_4, 400 mM NaCl, 10 mM β-mercaptoethanol, 1% sarcosyl) was added to 500 mg powder in an 1.5 ml microcentrifuge tube. The mixture was shaken with a bead beater for 2 min and kept on ice for 30 min. After another 2 min shaking with the bead beater, the mixture was centrifuged for 10 min at 4°C with a microcentrifuge. Twenty microliter of the supernatant was boiled with Laemmli loading buffer (17), and separated using 10% SDS-PAGE. The separated proteins were transferred onto a nitrocellulose membrane and immunoblot analysis was conducted by ECL system (Pharmacia) by using the polyclonal antibody against NSm and goat-anti-rabbit antibody conjugated with horseradish peroxidase (Pharmacia).

Fractionation of Plant Tissue

Infected plant cell components were fractionated into 4 fractions as described by Deom *et al.* (11). Infected *D. stramonium* was harvested 5-7 days after inoculation and healthy plant was used as a negative control. Ten grams of leaves were ground in liquid nitrogen and resuspended in 20 ml extraction buffer (100 mM Tris·HCl, pH 8.0, 10 mM EDTA, 5 mM DTT). The slurry was filtered

through 200 mesh nylon filter to get cell wall components (CW) by washing the remaining tissue materials with extraction buffer with 2% Triton X-100 twice. The filtrate was centrifuged for 10 min at 1000 g and the pellet was saved (P1). The supernatant was centrifuged again for 30 min at 30,000 g to obtain a P30 pellet and S30 supernatant. Fractions were boiled in Laemmli loading buffer, and separated on 10% SDS-PAGE and western blot was performed with NSm antibody.

Immunocytological Methods

For immunoelectron microscopy, young leaves of *Emilia fosbergii* Nicolson showing typical symptoms were harvested 7 days after inoculation. The leaves were cut into 1 mm slices, and processed and embedded in LR white (EMS, Fort Washington, PA) as described by Westcot *et al.* (25). Thin sections of embedded tissue were made with a RMC MT-7 ultramicrotome (Research and Manufacturing Co., Inc., Tuscon, AZ) and mounted on 200 mesh nickel grids coated with 0.25% carbon stabilized formvar film. After 15 min blocking with 5% fetal bovine serum in 0.05 M phosphate buffered saline with 0.2 M glycine, pH 7.4 (FBS-PBS-GLY), the grids were floated on a 6 μl drop of antiserum diluted 1:100 in FBS-PBS-GLY for 45 min at room temperature. The grids were washed 4 times with 10% FBS-PBS-GLY for 5 min and incubated for 30 min at room temperature on a 6 μl drop of 10 nm protein A-gold conjugate diluted 1:15 in 5% FBS-PBS-GLY. The grid was washed as above, fixed with 2% glutaldehyde in water for 15 min and washed 4 times with water. The grid was stained with 2% uranyl acetate and lead citrate, and viewed in a Zeiss 10A transmission electron microscope (Carl Zeiss, Germany).

RESULTS AND DISCUSSION

PCR Amplification of the NSm Gene

Most of the enveloped plant viruses such as tospovirus and rhabdovirus are fragile and need large amount of plant material to get a sufficient amount of the virus because yield is very low. In order to avoid time consuming virus purification processes, total RNA of infected plant tissue was extracted without virus purification and used for cDNA synthesis and PCR amplification. A major DNA band of expected size was detected on the agarose gel (Fig. 1). Later, the band was cloned into pBluescript KS+ and sequenced for confirmation. This method was specific, very quick and needed only a small amount of plant material. This method could also be used for rapid detection of other viruses using PCR from small samples.

Overexpression of NSm Fusion Protein and Antibody Production

The amplified NSm gene was cloned into a *E. coli* expression vector pET11a resulting a plasmid pETNSM. On a Coomassie blue stained gel, a protein band of 33 kDa was detected from *E. coli* having the plasmid pETNSM. However, there were other *E. coli* proteins of similar size and the fusion protein could not be separated and purified for antibody production. In order to construct another fusion protein which would allow easy separation, the plasmid pETNSM was digested with *NdeI*, blunted with T4 DNA ligase (Promega, Madison, WI) and a *BamHI* linker was introduced. The 0.8 kb *BamHI* fragment was cloned into *BamHI* digested pET 11c and pGEX-2T, which resulted in the plasmid pETNSMB and pGEXM, respectively. The fusion protein from pETNSMB contains extra amino acids from the cloning vector and is little bigger than the fusion protein from pETNSM (Fig. 2, lane pETNSMB). Also, there is a part of glutathione-S-transferase in the pGEX-2T vector in front of the cloning site and the fusion protein from pGEXM is about 63 kDa instead of 33 kDa. Proteins of expected size were detected from *E. coli* containing those plasmids (Fig. 2, lane pGEXM and NSm). The fusion protein made from the plasmid pGEXM was purified and used for antibody production. The raised antibody specifically reacted to the fusion protein made from the plasmid pETNSM, pETNSMB, and pGEXM, and did not show any non-specific reaction to the N protein and part of the L protein made from *E. coli* (Fig. 2).

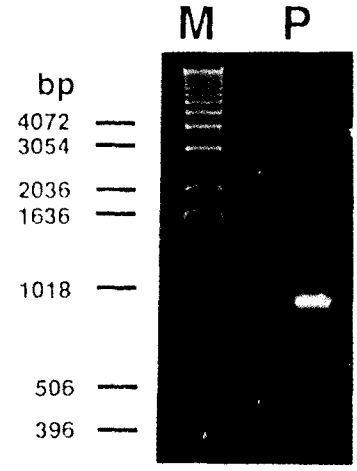

Fig. 1. PCR amplification of TSWV NSm gene.
Total RNA was extracted from infected *D. stramonium* and cDNA was synthesized with NSm gene-specific primer. The cDNA was PCR amplified with two NSm gene specific primer and 5 μl out of 100 μl reaction was electrophoresised on 1% agarose gel. A single band of about 800 bp was obtained. M, DNA size marker with size on left; P, amplified PCR product.

Detection of the NSm Protein in Infected Plants

Two nonstructural proteins, NSs and NSm are encoded by S and M RNA of TSWV, respectively (14). The nonstructural NSs protein has been detected from

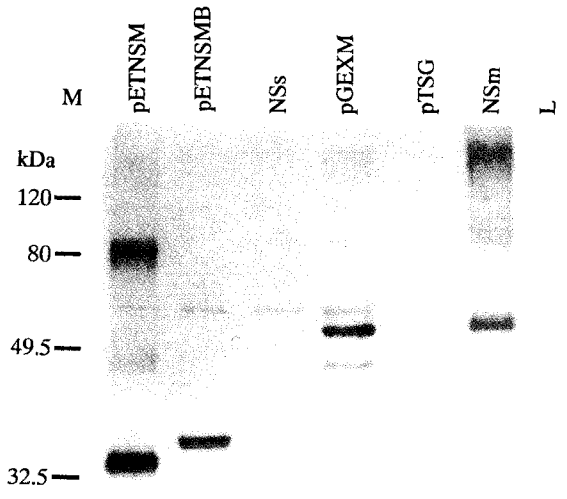

Fig. 2. Specificity of NSm antibody.
Antibody raised against purified NSm fusion protein was tested for specificity. pETNSM, NSm gene in pET11a vector; pETNSMB, NSm gene pET11c which has extra amino acid derived from the cloning vector; NSs, purified NSs protein made from *E. coli*; pGEXM, NSm gene in pGEX-2T vector which has extra 30 kDa polypeptides from the vector; pTSG, G2 gene in pET11a; NSm, gel purified NSm protein from the pGEXM plasmid; L, portion of L gene expressed in *E. coli*.

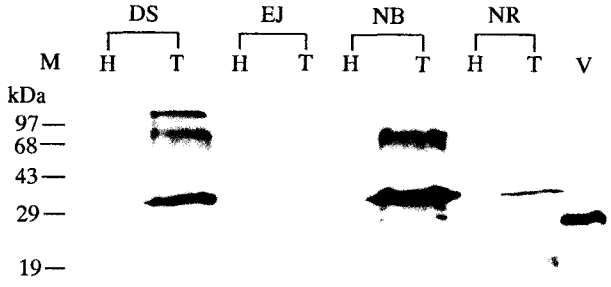

Fig. 3. Western blot detection of NSm protein from infected plant.
Total protein was extracted from TSWV infected plants (T) and healthy control (H) and tested for the presence of the NSm protein. DS, *D. stramonium*; EJ, *E. fosbergii*; NB, *N. benthamiana*; NR, *N. rustica*; v, purified virus.

the salivary gland of the thrips where virus replication occurs. This indicates that the NSs protein plays some role during virus replication (11, 26). However, the function of the NSm protein has not been understood. In order to confirm the presence of the NSm protein in the infected plant and its role in viral replication processes, immunoblotting was conducted with total protein extracted from healthy and TSWV infected plants. As shown in Fig. 3, proteins of about 33 kDa were detected from three plant species inoculated with TSWV. No protein was detected from inoculated *E. fosbergii* in which may the virus titer be

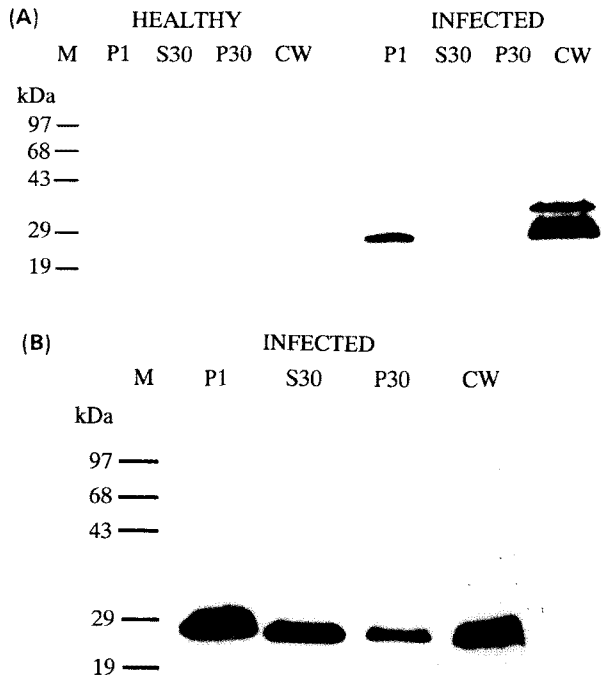

Fig. 4. Immunoblot analysis of fractionated plant tissue.
Tissues from healthy and TSWV infected plants were fractionated into four fractions, separated by SDS-PAGE. Proteins were transferred onto nitrocellulose membrane and western blot analysis was performed with anti-NSm antibody (A) and anti-N antibody (B). P1, pellet from low speed centrifuge; S30, supernatant from 30,000 g centrifugation; P30, pellet from 30,000 g centrifugation; CE, cell wall fraction.

low. No protein was detected from healthy plants, which indicates that the protein detected in the infected plants is a virus-specific NSm protein. Protein bands of over 68 kDa were detected from infected *D. stramonium* and *N. benthamiana*, and another protein of over 97 kDa was detected from *D. stramonium*. However, these protein bands were not detected from healthy plants. Those extra bands may be multimeric forms of the NSm protein.

Location of the NSm Protein in Plant Tissue Fractions

Infected plant tissue was fractionated into 4 fractions and immunoblotting was performed with NSm antibody. The NSm protein was detected from cell wall fraction and pellet from the first centrifugation which contains large organelles and some part of cell wall components. These two fractions contain most of the plant cell wall components, and the presence of NSm protein in these fractions indicates that most of the protein is associated with the cell wall components. By contrast, the N protein was detected from all four fractions (Fig. 4B). No protein band was detected with NSm antibody from any fraction of healthy plant (Fig. 4A). The association of NSm protein with plants cell wall components suggests that

Fig. 5. Electronmicrograph of thin section of TSWV infected plants immunogold labelled with NSm antibody.
Gold particles are concentrated on or near the plasmodesmata (arrow). Scale bar indicate 100 nm.

this protein has some functions in virus movement.

Localization of NSm Protein by Immuno-gold Labelling

An electron microscope was been used to locate specific proteins in cells or tissue by labelling the proteins with antibody-conjugated gold particles of 5-15 nm in diameter. The presence of NSm protein in the cell wall components was confirmed by immunoblotting, and an immunocytopathological observation was performed to find the exact location of this protein in cell wall components. As shown in Fig. 5, plasmodesmata connecting the cells were specifically and heavily labelled by the gold particles. This result consists with the results shown in Fig 4. Although the exact mechanisms of the cell-to-cell movement of plant viruses are not known, there have been some plant viral movement proteins reported (6, 7). These viral movement proteins are usually associated with the plasmodesmata, the cell to cell connecting channel of plant tissue, and are known to change the morphology of the plasmodesmata so that the viral RNA can move through this structure (8). The association of the NSm protein with the plasmodesmata indicates that this protein is a viral movement protein. This could be confirmed by additional experiments such as construction of transgenic plants expressing the NSm protein and a complementation test of movement defective mutants.

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