Identification of Common Epitopes on a Conserved Region of NSs Proteins Among Tospoviruses of *Watermelon silver mottle virus* Serogroup

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ABSTRACT

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The NSs protein of *Watermelon silver mottle virus* (WSMoV) was expressed by a *Zucchini yellow mosaic virus* (ZYMV) vector in squash. The expressed NSs protein with a histidine tag and an additional Nla protease cleavage sequence was isolated by Ni²⁺-NTA resins as a free-form protein and further eluted after sodium dodecyl sulfate-polyacrylamide gel electrophoresis for production of rabbit antiserum and mouse monoclonal antibodies (MAbs). The rabbit antiserum strongly reacted with the NSs crude antigen of WSMoV and weakly reacted with that of a high-temperature-recovered gloxinia isolate (HT-1) of *Capsicum chlorosis virus* (CaCV), but not with that of *Calla lily chlorotic spot virus* (CCSV). In

contrast, the MAbs reacted strongly with all crude NSs antigens of WSMoV, CaCV, and CCSV. Various deletions of the NSs open reading frame were constructed and expressed by ZYMV vector. Results indicate that all three MAbs target the 89- to 125-amino-acid (aa) region of WSMoV NSs protein. Two indispensable residues of cysteine and lysine were essential for MAbs recognition. Sequence comparison of the deduced MAbs-recognized region with the reported tospoviral NSs proteins revealed the presence of a consensus sequence VRKPGVKNTGCKFTMHNQIFNP (denoted WNSscon), at the 98- to 120-aa position of NSs proteins, sharing 86 to 100% identities among those of WSMoV, CaCV, CCSV, and *Peanut bud necrosis virus*. A synthetic WNSscon peptide reacted with the MAbs and verified that the epitopes are present in the 98- to 120-aa region of WSMoV NSs protein. The WSMoV serogroup-specific NSs MAbs provide a means for reliable identification of tospoviruses in this large serogroup.

The family *Bunyaviridae* is a large and diverse group of viruses that infect animal and plant hosts. It is comprised of five genera: *Orthobunyavirus*, *Hantavirus*, *Nairovirus*, *Phlebovirus*, and *Tospovirus* (11). They all have a worldwide distribution and many members cause human illness ranging from mild asymptomatic infection to hemorrhagic fever and fatal encephalitis (44). *Watermelon silver mottle virus* (WSMoV) is a member of the only plant-infecting genus, *Tospovirus* (11), and is transmitted by *Thrips palmi* Karny in a persistent manner (5). The virus is one of the major viruses limiting the production of cucurbits in Eastern Asia (22,50). Virions of WSMoV are enveloped quasi-spherical particles containing three single-stranded nucleic acid segments denoted as L, M, and S RNAs (11,50). The complete WSMoV genomic sequence has been determined (7,9,51). The L RNA (8,917 nucleotides) of WSMoV is of negative polarity, encoding a putative RNA-dependent RNA polymerase (RdRp, 331.8 kDa) in the viral complementary strand (7). Both M and S RNAs contain two open reading frames (ORFs) in an ambisense organization. The M RNA (4,880 nucleotides) encodes a non-structural NSm protein (35 kDa) in the viral strand and a precursor (127.6 kDa) for the Gn and Gc glycoproteins in the viral complementary strand (9). The NSm protein plays a role for cell-to-cell and long-distance movement of nonenveloped ribonucleocapsid structures and forms tubule structures in infected tobacco

protoplasts (28,34); and the Gn and Gc glycoproteins compose spikes on the viral envelope (26,31). The S RNA (3,534 nucleotides) encodes a nucleocapsid protein (NP, 30.7 kDa), which encapsidates viral RNAs, in its viral complementary strand (48,51). A nonstructural NSs protein (49.7 kDa) encoded from the viral strand of WSMoV S RNA forms filamentous inclusion bodies in cytoplasm of infected plant cells (27).

Sequence homology of NPs is the most reliable and important descriptor for establishing a distinct species and separating tospoviruses into serogroups (14). Serological relationships and phylogenetic analysis of NPs indicate that the current 16 tospovirus species are classified into three major serogroups and four distinct serotypes (24,38). WSMoV, *Peanut bud necrosis virus* (PBNV) (42), *Watermelon bud necrosis virus* (WBNV) (23), *Capsicum chlorosis virus* (CaCV) (33), and *Calla lily chlorotic spot virus* (CCSV) (4) are clustered in the WSMoV serogroup (also known as serogroup IV). The WSMoV serogroup and the *Tomato spotted wilt virus* (TSWV) serogroup (also known as serogroup I), which contains five species, are two major serogroups in the genus *Tospovirus* (38). Members of the TSWV serogroup are most prevalent in Europe and America. Viruses in WSMoV serogroup cause severe devastation on *Cucurbitaceae* and *Solanaceae* crops and are economically important in the Eastern hemisphere, including Taiwan, Japan, India, Thailand, and Australia (24).

Plant viruses in many cases generate suppressors of RNA silencing to antagonize plant defense mechanisms for establishment of virus infection (40). The tospoviral NSs protein (3,45), similar to the NS3 protein of tenuiviruses (3), is a gene-silencing suppressor of negative sense RNA viruses responsible for the pathogenicity. Gene-silencing suppressors are found not only in plant viruses but also in animal viruses, for example, the B2

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protein of *Flock house virus* (35), the E3L protein of vaccinia virus, the NS1 protein of influenza virus (36), and the Tas of *Primate foamy virus* type 1 (32). The family *Bunyaviridae* consists of plant viruses as well as animal viruses. Their NSs proteins may play an important role for gene-silencing suppression in both plant and animal hosts.

Due to instability of tospoviruses, it is difficult to purify sufficient amounts of their individual proteins for antibody production directly from infected plant tissues. In planta expressions by viral vectors coupled with an efficient purification procedure have been applied for isolation of target proteins (10,13,16,29). Because of the high titer of the vector and fast growth of cucurbit hosts, various proteins have been successfully expressed by the *Zucchini yellow mosaic virus* (ZYMV) vector for different applications. These include β -glucuronidase (2) or jellyfish green fluorescent protein (GFP) (18) as a reporter; the herbicide-resistant bar protein (phosphinothricin acetyltransferase) for control of weeds (43); the multifunctional plant proteins MAP30 (Momordica anti-HIV protein, 30 kDa) and GAP31 (Gelonium anti-HIV protein, 31 kDa) for control against HIV-1 virus (1); the human interferon-alpha 2 (IFN) (2) and the dust mite allergen Der p 5 (18) for immunotherapy; and the plant viral proteins such as RdRp (12), coat protein (CP) (2), movement protein, and 2b protein (47) of *Cucumber mosaic virus*, and NPs of various tospoviruses (6).

A ZYMV vector driven by the *Cauliflower mosaic virus* 35S promoter was previously constructed in our laboratory. The expressed foreign proteins were fused with a histidine tag and an additional Nla protease cleavage sequence to facilitate purification and processing free proteins, respectively (18). In this study, the NSs ORF of WSMoV was cloned into the ZYMV vector and the expressed NSs protein was purified and used to immunize rabbits and mice for production of polyclonal antibodies (PAb) and monoclonal antibodies (MAbs), respectively. Various deletions of the NSs ORF of WSMoV were constructed, expressed, and tested against MAbs in western blotting for epitope scanning.

The sequence of MAbs-targeted region at the N terminus of NSs is conserved among all members in the WSMoV serogroup. Two critical amino acid residues, cysteine (C₁₀₈) and lysine (K₁₀₉), in the common region for MAbs recognition were identified. Also, the use of these antibodies for detection of other WSMoV serogroup members was demonstrated.

MATERIALS AND METHODS

Virus sources. WSMoV (50) and a newly reported CCSV isolated from calla lilies (4) were collected from Taiwan. A high-temperature-recovered gloxinia isolate (HT-1) of CaCV from the United States was previously reported (21). These three viruses are classified in the WSMoV serogroup (24,38). TSWV, a type member of TSWV serogroup isolated from tomato in New York (TSWV-NY), was provided by R. Provvidenti, New York State Experiment Station, Geneva. An isolate of *Groundnut ringspot virus* (GRSV) collected from infected tomato in Brazil was provided by D. Gonsalves, New York State Experiment Agricultural Station (39). *Impatiens necrotic spot virus* (INSV) isolated from impatiens in the United States (INSV-M) was provided by J. Moyer, North Carolina University, Raleigh (30). Peanut chlorotic fan-spot virus (PCFV) was isolated from peanut in Taiwan (8). All virus cultures were maintained in the systemic host *Nicotiana benthamiana* Domin. and the local lesion host *Chenopodium quinoa* Willd. by mechanical transmission. The TW-TN3 isolate of *Zucchini yellow mosaic virus* (ZYMV TW-TN3) (37) was maintained in the systemic host zucchini squash (*Cucurbita pepo* L.) and the local lesion host *C. quinoa*.

Expression of WSMoV NSs protein by ZYMV recombinant. The full-length NSs ORF was amplified from WSMoV S RNA using primers WNSs67KS and WNSs1383cK (sequences shown in Table 1) by reverse transcription-polymerase chain reaction (RT-PCR), and cloned into pCR2.1-TOPO by TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA) to generate pTOPO-WNSs. The DNA fragment corresponding to NSs ORF was

TABLE 1. Primers used to amplify individual DNA fragments corresponding to the full-length or deleted NSs open reading frames for construction of *Zucchini yellow mosaic virus* (ZYMV) chimeras

Primer name	Sequence ^a	Restriction site	Position at S RNA ^b	ZYMV chimera
Upstream				
P1 WNSs67KS	5'-GGGTACCGCATGCATGTCTACTGCAAAGAATGCTGCT-3'	<i>Sph</i> I	67–90	ZWSMoV-NSs, ZWNSsN, ZWNSsNM, ZWNSsNC, ZWNSsΔ89-125
P2 WNSs331KS	5'-GGCATGCTTCTGCGAGCATGAAATGAACTTAATT-3'	<i>Sph</i> I	331–357	ZWNSs89
P3 WNSs370S	5'-GGGCATGCGGTGTGAAGAACACAGGCTGCAAGTTC-3'	<i>Sph</i> I	370–396	ZWNSs102
P4 WNSs379S	5'-GGGCATGCAACACAGGCTGCAAGTTCACAATGC-3'	<i>Sph</i> I	379–403	ZWNSs105
P5 WNSs382S	5'-GGGCATGCACAGGCTGCAAGTTCACAATGCACA-3'	<i>Sph</i> I	382–406	ZWNSs106
P6 WNSs388S	5'-GGGCATGCTGCAAGTTCACAATGCACAATCAAATC-3'	<i>Sph</i> I	388–414	ZWNSs108
P7 WNSs394S	5'-GGGCATGCTTCACAATGCACAATCAAATCTTTA-3'	<i>Sph</i> I	394–418	ZWNSs110
P8 WNSs397S	5'-GGGCATGCACAATGCACAATCAAATCTTTAATC-3'	<i>Sph</i> I	397–421	ZWNSs111
P9 WNSs406S	5'-GGGCATGCAATCAAATCTTTAATCCAAATTTCCAAT-3'	<i>Sph</i> I	406–432	ZWNSs114
P10 WNSs442KS	5'-GGGTACCGCATGCATGACGCCCGGAACAATTTTCAGAA-3'	<i>Sph</i> I	442–465	ZWNSsM, ZWNSsMC
P11 WNSs844KS	5'-GGGTACCGCATGCGAAGGGGCTTCGCAAGGACTTTC-3'	<i>Sph</i> I	844–867	ZWNSsC
P12 WNSs442AvrII	5'-GGCCTAGGATGACGCCCGGAACAATTTTCAGAAG-3'	<i>Avr</i> II	442–466	ZWNSsΔ89-125
P13 WNSs844AvrII	5'-GCCTAGGGAAGGGGCTTTCGCAAGGACTTTC-3'	<i>Avr</i> II	844–867	ZWNSsNC
Downstream				
M1 WNSs537cK	5'-GGGTACCGCACTCATCCAAACACCATCCCGA-3'	<i>Kpn</i> I	514–537	ZWNSsN
M2 WNSs939cK	5'-GGGTACCTCATTACTGTTGTCAGCAACAGT-3'	<i>Kpn</i> I	916–939	ZWNSsM, ZWNSsNM
M3 WNSs1383cK	5'-GGGTACCTTCTGCTTTCACAACAAAGTGTG-3'	<i>Kpn</i> I	1360–1383	ZWSMoV-NSs, ZWNSsC, ZWNSsNC, ZWNSsMC, ZWNSs114, ZWNSs111, ZWNSs110, ZWNSs108, ZWNSs106, ZWNSs105, ZWNSs102, ZWNSs89, ZWNSsΔ89-125
M4 WNSs330cAvrII	5'-GGCCTAGGTGTTATGTCTAGTCCAAATTTTCAAAA-3'	<i>Avr</i> II	304–330	ZWNSsΔ89-125
M5 WNSs537cAvrII	5'-GCCTAGGGCACTCATCCAAACACCATCCCGA-3'	<i>Avr</i> II	515–537	ZWSMoV-NSs, ZWNSsNC

^a Restriction enzyme cleavage sequences added for cloning of DNA fragments into the ZYMV vector are underlined.

^b Numbers represent the nucleic acid positions of the viral-sense S RNA of *Watermelon silver mottle virus* (WSMoV).

released from pTOPO-WNSs using restriction enzymes *SphI* and *KpnI*, and then ligated with the *SphI/KpnI*-digested ZYMV vector p35SZYMVGFP_{His} (6,18). The plasmid of the ZYMV recombinant carrying NSs ORF was isolated by the mini-prep method (41), dissolved in TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0), and mechanically introduced with a glass spatula on *C. quinoa* leaves (10 µg in 10 µl per leaf) dusted with 600 mesh Carborundum. Local lesions that developed were individually transferred to cotyledons of single zucchini squash plants (6). Total RNAs extracted from symptomatic squash leaves using the ULTRASPECTM RNA isolation system (Biotex Laboratories, Houston, TX) and primers WNSs67KS and WNSs1383cK were used to check the presence of the insert in the recombinant by RT-PCR. PCR products were analyzed in 1.0% agarose gels by electrophoresis.

Purification of the expressed NSs protein. An affinity method of chromatography (12) was modified for purification of the ZYMV-expressed NSs protein from infected zucchini squash plants. Fifty grams of infected squash leaves was ground in 100 ml of buffer A (50 mM Tris-HCl [pH 8.0], 15 mM MgCl₂, 10 mM KCl, 20% [vol/vol] glycerol, 0.05% β-mercaptoethanol, and 0.1 mM phenylmethylsulphonyl fluoride [PMSF]) with a blender. Extracts were clarified by centrifugation at 3,000 × *g* for 10 min, and supernatants were filtered through Miracloth (Calbiochem, La Jolla, CA). The filtrates, treated with 1% Triton X-100 at 4°C for 30 min, were centrifuged at 30,000 × *g* for 30 min. The supernatants were filtered through 0.45-µm-pore-size filters (Millipore, Billerica, MA). Approximately 1 ml of Ni²⁺-NTA resins (Ni-NTA SUPERFLOW, Qiagen, Germany), pre-equilibrated in buffer B (50 mM Tris-HCl [pH 8.2], 15 mM MgCl₂, 20% [vol/vol] glycerol, 0.05% β-mercaptoethanol, and 0.1 mM PMSF) was added. The mixtures were gently shaken for 1 h at 4°C and loaded onto a column. After allowing the resins to settle, the unbound materials were discarded and the resins were washed with twofold bed volume of buffer B containing 5 mM imidazole. The proteins bound to the resins were eluted with 10 ml of buffer B containing 250 mM imidazole. The NSs protein was further purified by gel electrophoresis method (49). Each fraction of the purification steps was monitored by western blot using the MAb against the histidine tag (MAb-His, Amersham Pharmacia Biotech, Buckinghamshire, England).

The amount of purified NSs protein was estimated with standardized histidine-tagged GFP using the MAb-His (Amersham Pharmacia Biotech) in western blotting or by comparison with bovine serum albumin (BSA) in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and estimated by the software Spot Density of AlphaInnotech IS2000 (AlphaInnotech Corporation, San Leandro, CA) as previously described (6).

Production of rabbit antiserum. Antiserum was produced by injecting the purified ZYMV-expressed WSMoV NSs protein in a New Zealand white rabbit as described (49). The NSs protein (100 µg in 1 ml of phosphate-buffered saline [PBS]) was emulsified with an equal volume of Freund's complete adjuvant (Difco Laboratories, BD, Franklin Lakes, NJ) and injected subcutaneously into the rabbit. Subsequently, 100 µg of the immunogen in 1 ml of PBS emulsified with an equal volume of Freund's incomplete adjuvant (Difco Laboratories) was administered weekly for 3 weeks. The rabbit was bled weekly for 2 months, starting from 1 week after the fourth injection.

Production of mouse MAbs and ascitic fluids. Fifty micrograms of purified NSs protein in 250 µl of PBS emulsified with an equal volume of Freund's complete adjuvant (Difco Laboratories) was intraperitoneally injected into 6- to 8-week-old female BALB/cByJ mice (Academia Sinica, Taipei). The same amount of immunogen emulsified with Freund's incomplete adjuvant (Difco Laboratories) was used for two subsequent weekly intraperitoneal injections. Mice were sacrificed 3 days after a final injection with 50 µg of purified NSs protein in 250 µl of

PBS without adjuvant, and spleen cells were harvested for cell fusion with Fox-NY myeloma cells (American Type Culture Collection) following a method described previously (20). After fusion, cells were cultured in a 37°C incubator supplied with 6% CO₂. Cultured media were collected and screened for anti-NSs antibodies by indirect enzyme-linked immunosorbent assays (ELISA) using crude extracts prepared from leaf tissues of *N. benthamiana* plants infected with WSMoV. Subsequently, the antibody-secreting hybridoma cells were cloned by limiting dilution. Stable hybridoma cell lines were selected after three cycles of cloning. Pristane-primed BALB/cByJ mice were intraperitoneally injected with 1.0 × 10⁶ hybridoma cells each for production of ascitic fluids (19).

Western blot. Protein expression and purification, yield estimation of purified NSs protein, and virus detection were all monitored by western blotting as described (15). Crude extracts from leaves of tospovirus-infected *N. benthamiana* plants were analyzed at a 50-fold dilution in dissociation buffer (100 mM Tris-HCl [pH 7.2], 2% β-mercaptoethanol, 10% sucrose, 0.005% bromophenol blue, and 10 mM EDTA). Crude extracts of zucchini squash infected with wild-type ZYMV TW-TN3 or its recombinants were diluted to 20-fold in dissociation buffer. MAb-His (Amersham Pharmacia Biotech) was used at a 5.0 × 10⁻⁴ dilution to detect the ZYMV-expressed WSMoV NSs protein. The rabbit antiserum to ZYMV CP (37) was used at a 2.5 × 10⁻⁴ dilution to confirm infection by the recombinants. Ascitic fluids containing MAbs to WSMoV NP (134B1A8) or CCSV NP (335F9E7) (38) were used at a 1.0 × 10⁻⁵ dilution to verify the presence of tospoviruses. The alkaline phosphatase (AP)-conjugated goat anti-rabbit immunoglobulin G (IgG) (Jackson ImmunoResearch Laboratories, West Grove, PA) and the AP-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) were used at a 2.0 × 10⁻⁴ dilution as the secondary antibody for detection of rabbit and mouse antibodies, respectively. Reactions were visualized by the addition of chromogenic substrate (nitro-blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate paratoluidine salt in 100 mM NaCl, 5 mM MgCl₂, and 100 mM Tris-HCl [pH 9.5]).

Indirect ELISA. Indirect ELISA was employed according to a method described previously (49). Either 100-fold diluted crude extracts of tospovirus-infected *N. benthamiana* or 20-fold diluted crude extracts of ZYMV recombinant-infected zucchini squash were used as coating antigens. Cell culture media at a one-half dilution were used for screening antibody-secreting hybridoma cell lines. Ten-fold serial dilutions starting from a 1.0 × 10⁻³ dilution of the rabbit antiserum or ascitic fluids were used for titration. The AP-conjugated goat anti-mouse IgG and the AP-conjugated goat anti-rabbit IgG were used at a 2.0 × 10⁻⁴ dilution as the secondary antibody for detection of mouse and rabbit antibodies, respectively. The absorbance at 405 nm (A₄₀₅) was determined with a universal microplate reader (ELx800, Bio-Tek instrument, Winooski, VT) 10 to 40 min after the addition of AP substrate (Sigma 104; Sigma-Aldrich Fine Chemicals, Milwaukee, WI).

Epitope scanning of MAbs. DNA fragments corresponding to different portions of the NSs ORF were amplified by PCR using pTOPO-WNSs as a template. Sequences of individual primers used for amplification are listed in Table 1. The protocol of denaturing at 94°C for 30 s, reannealing at 58°C for 30 s, and synthesis at 72°C for 1 min for 30 cycles, with a final reaction at 72°C for 7 min, was used for PCR. Individual DNA fragments corresponding to deleted NSs ORFs were cloned into pCR2.1-TOPO by TOPO TA Cloning Kit (Invitrogen) for sequence confirmation. DNA fragments were released from pCR2.1-TOPO using restriction enzymes *SphI* and *KpnI*, and then ligated with the *SphI/KpnI*-digested ZYMV vector p35SZYMVGFP_{His} as previously described (6,18). The individual ZYMV recombinants derived from each plasmid were recovered from *C. quinoa* and

zucchini squash similar to the procedure described for the recombinant expressing the full-length NSs protein.

Comparison of the conserved region of tospoviral NSs proteins. Multiple sequence alignments of the conserved region of tospoviral NSs proteins were analyzed using ClustalW version 1.82 (46). Sequences of NSs proteins for analysis were obtained from databases under the following accession numbers: CCSV, AAW58114 (38); CaCV HT-1, AAC15500 (21); PBNV, AAB04144 (42); and WSMoV, AAB36955 (51).

Peptide synthesis and antiserum production. To confirm the common epitope identified by the produced MAbs, a 23-amino-acid (aa) peptide (VRKPGVKNTGCKFTMHNQIFNP) denoted as WNSscon coupled with multiple antigen peptides was synthesized by PTI Symphony (Protein Technologies, Inc., Tucson, AZ). The synthetic WNSscon peptide of 0.2 µg was analyzed by 15% SDS-PAGE, transferred to membranes, and reacted with the rabbit antiserum diluted to 1.0×10^{-3} and mouse MAbs diluted to 1.0×10^{-5} in western blotting. The synthetic WNSscon peptide also was injected into a New Zealand white rabbit to produce antiserum as described previously. The produced antiserum against WNSscon peptide was used at a 1.0×10^{-2} dilution to react with crude extracts from leaves of tospovirus-infected *N. benthamiana* plants in western blotting.

RESULTS

Establishment of the ZYMV recombinant expressing WSMoV NSs protein in squash plants. A cDNA construct of

Fig. 1. Symptom and protein expression of the *Zucchini yellow mosaic virus* (ZYMV) recombinant ZWSMoV-NSs carrying the full-length NSs open reading frame of *Watermelon silver mottle virus* (WSMoV) in zucchini squash plants. **A**, Symptoms developed on leaves of ZWSMoV-NSs- or wild-type ZYMV TW-TN3-infected plants 14 days postinoculation. **B**, Confirmation of the presence of the insert in the recombinant by reverse transcription-polymerase chain reaction (RT-PCR). Total RNAs isolated from symptomatic leaves were used as templates in RT-PCR using the specific primer pair WNSs67KS and WNSs1383cK. A DNA product of 1.3 kb was amplified from the ZWSMoV-NSs-infected plants. **C**, Monitoring of the NSs protein expression by western blotting. A free-form NSs protein of 52.2 kDa (arrow) in crude extracts of the ZWSMoV-NSs-infected squash plants was detected by monoclonal antibody against the histidine tag. **D**, Confirmation of the presence of the recombinant by the antiserum to the ZYMV coat protein (CP). A protein of 31.3 kDa corresponding to the ZYMV CP (arrow) was detected in the ZWSMoV-NSs- and ZYMV TW-TN3-infected squash plants.

ZYMV chimera carrying the full-length WSMoV NSs ORF was obtained and denoted p35SZWSMoV-NSs. The recombinant ZWSMoV-NSs derived from p35SZWSMoV-NSs induced typical local lesions on inoculated *C. quinoa* leaves and caused severe systemic symptoms of yellow mosaic and leaf distortion on zucchini squash plants 10 to 14 days postinoculation (Fig. 1A). The presence of ZWSMoV-NSs was confirmed by an RT-PCR product of 1.3 kb, corresponding to the complete NSs ORF, which was amplified with primers WNSs67KS and WNSs1383cK (Fig. 1B). A protein of 52.2 kDa, containing the additional residues of the histidine tag, proteolytic and cloning sites, slightly larger than the native WSMoV NSs protein (49.7 kDa) was detected in ZWSMoV-NSs-infected squash plants by western blotting using MAb-His (Fig. 1C). In addition, ZYMV CP (31.3 kDa) was detected in ZWSMoV-NSs-infected squash plants by western blotting with the rabbit antiserum to the CP of ZYMV (Fig. 1D).

Purification of the expressed NSs protein. Each step for purification of ZYMV-expressed NSs protein from infected squash tissues was analyzed by western blotting using MAb-His. A protein of 52.2 kDa in elution fractions was pooled and identified as the ZYMV-expressed NSs protein by MAb-His (Fig. 2). A trace amount of a larger protein of 104 kDa was also obtained. Proteins eluted from Ni^{2+} -NTA resins were further separated by gel electrophoresis to remove other plant proteins. An estimated 470 µg of purified NSs protein was obtained from 100 g of ZWSMoV-NSs-infected squash tissues by comparing with standardized histidine-tagged GFP in western blotting and quantified BSA in SDS-PAGE, and estimated by the Spot Density of Alpha-Innotech IS2000.

Production of antibodies. The purified NSs protein was used as an immunogen for production of rabbit PABs and mouse MAbs. The antiserum from immunized rabbits had a dilution endpoint of 1.0×10^{-5} as determined by ELISA. The antiserum was used at a 1.0×10^{-3} dilution in further investigations.

Additionally, three stable hybridoma cell lines (231E6D12, 238C2G10, and 239F1C11) were established by limiting dilution. Cells from individual lines were injected intraperitoneally into pristane-primed mice for production of ascitic fluids. The dilution endpoints of ascitic fluids from 231E6D12, 238C2G10, and 239F1C11 were all found to be 1.0×10^{-8} , and were used at a 1.0×10^{-5} dilution for further studies.

Fig. 2. Purification of NSs proteins expressed by the *Zucchini yellow mosaic virus* (ZYMV) recombinant ZWSMoV-NSs in infected zucchini squash plants by affinity chromatography. Monoclonal antibody against the histidine tag was used to detect the presence of ZYMV-expressed NSs proteins by western blotting in different fractions during purification by the Ni^{2+} -NTA affinity chromatography, including the pellet (3K-P) and the supernatant (3K-S) after $3,000 \times g$ low-speed centrifugation, the pellet (30K-P) after high-speed centrifugation at $30,000 \times g$, the flow-through (FT) of the supernatant of $30,000 \times g$ centrifugation, the flow-through of buffer containing 5 mM imidazole for washing (Wash), and the eluted fractions of buffer containing 250 mM imidazole (Elution). The purified histidine-tagged green fluorescent protein (GFP) expressed by the ZYMV vector was used as a positive control. The NSs protein in each fraction is indicated by an arrow.

Serological reactions of rabbit antiserum and mouse ascitic fluids. The rabbit antiserum reacted strongly with the NSs protein in the crude extracts of WSMoV-infected plants of *N. benthamiana* and *C. quinoa* and weakly with the crude extracts of CaCV-infected samples, but did not react with samples of TSWV, GRSV, INSV, CCSV, PCFV, and ZYMV TW-TN3 in western blotting (Fig. 3A). In indirect ELISA, the average readings of WSMoV (2.14) and CaCV (0.93) were 8.4- and 3.7-fold higher than that (0.25) of the mock control, respectively, but the average readings (0.24 to 0.29) of TSWV, GRSV, INSV, CCSV, and PCFV were not significantly different (defined as twofold higher) from that of the mock control (Fig. 3B).

Ascitic fluids produced from three hybridoma cell lines (231E6D12, 238C2G10, and 239F1C11) all reacted positively with crude extracts of *N. benthamiana* and *C. quinoa* plants individually inoculated with WSMoV, CaCV, or CCSV, but not with those from plants infected with TSWV, GRSV, INSV, PCFV, or ZYMV TW-TN3 in western blotting (Fig. 3C). In indirect ELISA, the MABs also reacted with crude antigens of WSMoV (an average reading of 1.84), CaCV (1.27), and CCSV (0.85), with readings 13.2-, 9.1-, and 6.1-fold higher than that (0.14) of the mock control, respectively, but not with those of TSWV (0.12), GRSV (0.13), INSV (0.12), and PCFV (0.13) (Fig. 3D). The identity and the presence of the tested tospoviruses were checked by MABs specific to the NP of WSMoV or CCSV in western blotting. WSMoV NP MAB 134B1A8 identified WSMoV and CaCV (Fig. 3E), whereas CCSV NP MAB 335F9E7 identified CCSV (Fig. 3F).

Determination of the MABs-recognized region. DNA fragments corresponding to different portions of the WSMoV NSs ORF were introduced into the ZYMV vector for expression of various truncated NSs proteins. The regions of NSs ORF expressed and their corresponding serological reactions with the

WSMoV NSs and His-MABs in western blotting are shown in Figure 4. The ZYMV recombinants expressing the N-terminal region (aa 1 to 157), the middle region (aa 126 to 291), the C-terminal region (aa 260 to 439), the N-terminal to middle region (aa 1 to 291), and the middle to C-terminal region (aa 126 to 439) of the WSMoV NSs protein were denoted ZWNSsN, ZWNSsM, ZWNSsC, ZWNSsNM, and ZWNSsMC, respectively. The N-terminal region (aa 1 to 157) and the C-terminal region (aa 260 to 439) were fused and expressed by the ZYMV vector denoted ZWNSsNC. Positive reactions with the three MABs were observed in samples of ZWNSsNM and ZWNSsNC, but not in those of ZWNSsN, ZWNSsM, ZWNSsC, and ZWNSsMC, indicating that the NSs MABs target the N-terminal region of the WSMoV NSs protein.

Based on the above results, recombinants expressing NSs proteins with various deletions in the N-terminal extensions were constructed. Samples of ZWNSs114, ZWNSs111, and ZWNSs110 expressing aa 114 to 439, aa 111 to 439, and aa 110 to 439 of the NSs protein, respectively, did not react with all three selected NSs MABs. However, these MABs reacted positively with samples of ZWNSs108, ZWNSs106, ZWNSs105, ZWNSs102, and ZWNSs89 expressing aa 108 to 439, aa 106 to 439, aa 105 to 439, aa 102 to 439, and aa 89 to 439 of the NSs protein, respectively. In addition, two DNA fragments corresponding to nucleotides 67 to 330 and nucleotides 442 to 1383 of WSMoV S RNA were ligated to generate the aa 89 to 125 deleted NSs protein that was expressed by the recombinant ZWNSsΔ89-125. This 89 to 125 deleted NSs protein reacted with MAB-His, but did not react with the NSs MABs. These results indicated that the epitopes recognized by the three MABs were located within the region of aa 89 to 125 of the NSs protein and that aa 108 to 109, C₁₀₈ and K₁₀₉, are two indispensable residues for the reactivity of the protein.

Fig. 3. Serological reactions of the rabbit antiserum and the mouse monoclonal antibody (MAB) 231E6D12 with NSs proteins of different tospoviruses in western blot and indirect enzyme-linked immunosorbent assay (ELISA). **A and B**, Reaction of the rabbit antiserum (As-WSMoV NSs) with crude extracts of tospovirus-infected *Nicotiana benthamiana* and *Zucchini yellow mosaic virus* (ZYMV)-infected squash plants in western blot and indirect ELISA, respectively. **C and D**, Reaction of *Watermelon silver mottle virus* (WSMoV) NSs MAB 231E6D12 with crude extracts of tospovirus-infected *N. benthamiana* and ZYMV-infected plants in western blot and indirect ELISA, respectively. **E**, Verification of WSMoV and Capsicum chlorosis virus (CaCV) by the MAB 134B1A8 to the nucleocapsid protein (NP) of WSMoV to prevent contamination. **F**, Verification of Calla lily chlorotic spot virus (CCSV) by the MAB 335F9E7 to the CCSV NP to prevent contamination. Crude extracts of mock-inoculated *N. benthamiana* plants were used as negative controls. *Peanut bud necrosis virus* (PBNV), *Tomato spotted wilt virus* (TSWV), *Impatiens necrotic spot virus* (INSV), *Groundnut ringspot virus* (GRSV), and *Peanut chlorotic fan-spot virus* (PCFV).

Comparison of the MAbs-targeted region with the NSs proteins among members of WSMoV serogroup. Amino acid sequences of the NSs proteins of CCSV, CaCV, PBNV, and WSMoV were compared. A consensus sequence, VRKPGVKNTGCKFTMHNQIFNPN (denoted WNSscon), present in the MAbs-targeted region at the position of aa 98 to 120 of WSMoV NSs protein, sharing high identities of 95, 91, and 86% with those of PBNV, CaCV, and CCSV, respectively, was noticed (Fig. 5).

Serological confirmation of the MAbs-recognized region. A 23 aa of WNSscon peptide (VRKPGVKNTGCKFTMHNQIFNPN) that reflects aa 98 to 120 of WSMoV NSs protein was synthesized to test the reactivities of the rabbit antiserum and the three mouse MAbs by western blotting. MAbs 231E6D12, 238C2G10, and 239F1C11 reacted with the synthetic peptide, but the rabbit antiserum did not (Fig. 6A).

The rabbit antiserum against the synthetic WNSscon reacted positively with crude NSs antigens of WSMoV, CaCV, and CCSV, but not with those of TSWV, GRSV, INSV, and PCFV (Fig. 6B). Our results indicate that tospoviral NSs proteins of WSMoV serogroup share a conserved sequence at the N-terminal

region, and that the antiserum raised from the consensus aa 98 to 120 synthetic peptide of NSs proteins recognized all tested members of the WSMoV serogroup.

DISCUSSION

The isolate of ZYMV TW-TN3 engineered as a plant viral vector in our laboratory has been successfully used to express the structural NPs encoded by S RNA of different tospoviruses including TSWV, INSV, WSMoV, PBNV, and WBNV (6). In this study, the nonstructural NSs protein encoded by the S RNA of WSMoV was expressed by the ZYMV vector in squash. Similar to our previous constructs (6,18), a full-length NSs ORF followed by a histidine tag and an additional N1a protease cleavage site were inserted between the P1 and helper component-proteinase (HC-Pro) cistrons of the ZYMV vector. The expressed NSs protein processed as a free soluble form and was efficiently purified by Ni²⁺-NTA affinity chromatography. Traces of a larger molecular weight (104 kDa) protein detected in the eluted fractions during purification (Fig. 2) were considered as the fu-

Fig. 4. Schematic representation of different constructs containing various deletions of NSs open reading frames (ORFs) expressed by the *Zucchini yellow mosaic virus* (ZYMV) vector for epitope mapping. Individual DNA fragments (black bars) corresponding to different portions of the NSs ORF (white bar) were amplified using primer pairs shown in Table 1, and then in-frame inserted into the ZYMV vector. The amino acid positions of NSs protein are numerically indicated. The various forms of NSs proteins in crude extracts of the recombinant-infected zucchini squash were monitored by monoclonal antibody (MAb) 231E6D12 to WSMoV NSs protein (MAb-NSs) and MAb to the histidine tag (MAb-His) in western blot. Positive and negative reactions are indicated by + and -, respectively. The recombinant names and the length of expressed NSs ORFs are indicated.

Fig. 5. Comparison of the monoclonal antibodies (MAbs)-targeted region of *Watermelon silver mottle virus* (WSMoV) NSs protein with those of tospoviruses in WSMoV serogroup. Sequences of the 89 to 125 amino acid region of NSs proteins of WSMoV, *Peanut bud necrosis virus* (PBNV), *Capsicum chlorosis virus* (CaCV), and *Calla lily chlorotic spot virus* (CCSV) are presented with divergent amino acids shown. A consensus sequence, VRKPGVKNTGCKFTMHNQIFNPN, indicated in bold was denoted WNSscon and synthesized for production of a rabbit antiserum. The two indispensable amino acids, C₁₀₈ and K₁₀₉, for MAB recognition in the NSs proteins are boxed.

sion protein of WSMoV NSs (52.2 kDa) and ZYMV HC-Pro (52.1 kDa). This is due to incomplete proteolysis as observed in our previous studies of GFP, Der p 5 allergen (18), and tospoviral NPs (6). The HC-Pro of potyviruses can be isolated by Ni²⁺-NTA resins (25), and the size of ZYMV HC-Pro is close to that of the expressed NSs protein. To remove HC-Pro, NSs-HC-Pro fusion protein, and other plant proteins, the expressed NSs protein was further purified by eluting it following SDS-PAGE. The protein expression by ZYMV vector coupled with the affinity column is an efficient method to obtain large amount of WSMoV NSs protein for the production of specific rabbit PAb and mouse MAb.

The rabbit antiserum and mouse MAb produced against ZYMV-expressed WSMoV NSs protein reacted differently with various tospoviral NSs antigens. In western blots and ELISA, all three MAb reacted with the NSs proteins of members in the WSMoV serogroup including WSMoV, CaCV, and CCSV (24, 38), whereas the rabbit antiserum reacted strongly with the NSs protein of WSMoV and weakly with that of CaCV, but not with that of CCSV (Fig. 3). This indicates that the three MAb secreted from the cloned hybridoma cell lines recognize common epitopes present on the NSs molecules of WSMoV, CaCV, and CCSV. The lack of serological reaction between the rabbit antiserum and CCSV NSs protein could be due to the presence of a major population of IgG targeting on epitopes that are different from those targeted by the three MAb. Results from present studies of serological analysis indicate that CaCV is closely, but CCSV is distantly, related to WSMoV, and yet they share common antigenic determinants revealed by the MAb.

Epitope scanning revealed that the MAb target epitopes at the N-terminal region from aa 89 to 125 of the WSMoV NSs protein. However, the N-terminal region (aa 1 to 157) of NSs protein expressed by ZWNSsN did not react with the MAb. When monitored by MAb-His, the truncated NSs proteins expressed by ZWNSsN, ZWNSsM, and ZWNSsC did not react with MAb-His (Fig. 4), but the presence of the inserts in the genome of these ZYMV recombinants was confirmed by RT-PCR (data not shown). This could be due to the instability of the truncated NSs

proteins produced in host cells. However, when the N-terminal aa 1 to 157 was coupled with the central aa 158 to 291 and the C-terminal aa 260 to 439, the proteins reacted with MAb-His, indicating that the central and C-terminal regions of the NSs molecule have a stabilizing effect on the expressed protein.

The two amino acid residues, C₁₀₈ and K₁₀₉, play an important role in the recognition of epitopes by the three MAb. The protein expressed by ZWNSs110 without these two C₁₀₈ and K₁₀₉ residues lost the ability to react with NSs MAb, whereas the protein expressed by ZWNSs108 containing C₁₀₈ and K₁₀₉ retained the serological reaction. The C₁₀₈ residue contains an SH group that can form a disulfide bond to stabilize the three-dimensional conformation of a protein or may be responsible for cross-linkage or protein-protein interactions. The K₁₀₉ residue carries a positive charge that normally participates strongly in protein interaction and nucleic acid binding. In addition, one C residue (at position 90), two K residues (positions 100 and 104), two histidine residues (positions 92 and 113, positively charged), and two structural proline residues (positions 101 and 119) are also conserved in the common epitopes of NSs proteins of WSMoV, PBNV, CaCV, and CCSV (Fig. 5), suggesting they may be involved in the stability and/or functional activity of this tospoviral gene-silencing suppressor. Peptides with different mutations in one or both of the critical residues, C₁₀₈ and K₁₀₉, or in other amino acids within the common epitopes will be synthesized to react with the MAb for investigating the importance of these residues in the binding of the MAb.

The recombinant ZWSMoV-NSs expressing the WSMoV NSs protein induced severe symptoms as those caused by the wild-type ZYMV TW-TN3, but developed in a shorter time (2 to 3 days earlier). In contrast, the individual recombinants with various deletions of NSs gene induced milder symptoms on inoculated zucchini squash plants (data not shown). NSs protein is the gene-silencing suppressor of tospoviruses (3,45), the full-length protein increases the virulence of the chimeric virus. However, the functions of HC-Pro are apparently counteracted by truncated NSs proteins that reduce virulence. The importance of the consensus sequences of tospoviral NSs proteins in the function of viral virulence remains to be further investigated.

The antibody specifically against the NSs protein of TSWV has been described earlier for detection of NSs inclusion bodies through microscopy (27). A 24-aa peptide, YFLSKTLEVLPK-NLQTMSYLDISIQ, developed from a conserved sequence at the C-terminal region of the NSs proteins of serogroup I to IV of tospoviruses was synthesized and rabbit antisera produced. The antisera broadly reacted in western blotting and ELISA with NSs proteins prepared from plant tissues infected with TSWV, GRSV, *Tomato chlorotic spot virus*, and INSV, but not with WSMoV (17). In this report, however, we describe a different conserved region that is present at the N terminus of NSs proteins and the MAb produced in this study specifically react with the members of WSMoV serogroup, WSMoV, CaCV, and CCSV. We did not show serological reactions of the MAb with the NSs proteins of another two members of WSMoV serogroup, PBNV and WBNV, because these two viruses are not present in Taiwan. Based on the high NSs identities of the WSMoV WNSscon with that of PBNV (95%), which are much higher than those with CaCV (91%) and CCSV (86%), we predict that the three MAb should also react with the NSs protein of PBNV. Although WBNV NSs protein sequences are not available for comparison, we also predict that the three MAb will also react with the NSs proteins of WBNV since it is closely related to PBNV (24).

For detection of tospoviruses from field and greenhouse samples, NP antisera are commonly used. Previously we reported the production of antiserum and MAb against the WSMoV NP and their application in field surveys (38). The WSMoV NP antiserum positively but weakly reacts with CCSV NP; in contrast, the WSMoV NP MAb do not react with CCSV NP (38). In

Fig. 6. Analysis of the epitopes in the NSs proteins of the *Watermelon silver mottle virus* (WSMoV) serogroup tospoviruses by western blotting. **A**, Confirmation of the epitopes recognized by the three monoclonal antibodies (MAb). The rabbit antiserum R-74 and the three mouse MAb 231E6D12, 239F1C11, and 238C2G10 were used to react with crude extracts of WSMoV-infected *Nicotiana benthamiana* plants and 0.2 µg of the synthetic WNSscon peptide. **B**, Serological reaction of the WNSscon antiserum reacted with NSs proteins of tospoviruses. The rabbit antiserum produced against the WNSscon peptide (As-WNSscon) reacted positively with crude NSs antigens of WSMoV, *Capisicum chlorosis virus* (CaCV), and *Calla lily chlorotic spot virus* (CCSV), but not with those of *Tomato spotted wilt virus* (TSWV), *Groundnut ringspot virus* (GRSV), and *Impatiens necrotic spot virus* (INSV). The positions of reacted NSs proteins and WNSscon peptide are indicated by an arrow.

titration assay of NSs antibodies or NP antibodies produced by our laboratory, the similar endpoints (1.0×10^{-5} for antisera and 1.0×10^{-8} for MAbs) and thus dilutions of 1.0×10^{-3} to 5×10^{-4} for NP or NSs antisera and 1.0×10^{-5} for NP or NSs MAbs were recommended for detection of field samples. The NSs protein forms inclusion bodies (27) and is an abundant viral protein in infected tissues; thus, our NSs MAbs provide a good tool for detection of all species of WSMoV serogroup. The NSs MAbs are being used for field surveys in Taiwan and India for tospoviruses belonging to WSMoV serogroup; the preliminary results indicate that our NSs MAbs are as sensitive as NP antibodies. However, the broader reactions with all members of WSMoV serogroup make it superior than the species-specific NP antibodies.

Results from current studies together with our previous report (6) demonstrate that the ZYMV vector system is an excellent tool that can be not only used as a heterologous protein expresser but also applied in epitope mapping. We have shown that the MAbs against the conserved region of NSs proteins can be prepared from a denatured antigen, and that they are useful tools for serological identification of tospoviruses belonging to the WSMoV serogroup. Furthermore, the NSs MAbs can be used to determine the proportion of transmitters in a thrips population. Also, if one has to screen transgenic plants expressing WSMoV NP, the NSs MAbs are useful in determining the titers of the challenge WSMoV after inoculation, since NP antibodies will detect the NP produced by the transgene and the virus. On the other hand, we have shown that it is feasible to synthesize a continuous peptide reflecting the conserved region of NSs proteins for the production of a broad-spectrum antiserum that will react with different viruses in the same serogroup.

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