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Identification of domains of the Tomato spotted wilt virus NSm protein involved in tubule formation, movement and symptomatology

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ABSTRACT

Deletion and alanine-substitution mutants of the Tomato spotted wilt virus NSm protein were generated to identify domains involved in tubule formation, movement and symptomatology using a heterologous Tobacco mosaic virus expression system. Two regions of NSm, G^{19}-S^{159} and G^{209}-V^{283}, were required for both tubule formation in protoplasts and cell-to-cell movement in plants, indicating a correlation between these activities. Three amino acid groups, D^{154}, $EYKK^{205–208}$ and $EEEEE^{284–288}$ were linked with long-distance movement in *Nicotiana benthamiana*. $EEEEE^{284–288}$ was essential for NSm-mediated long-distance movement, whereas D^{154} was essential for tubule formation and cell-to-cell movement; indicating separate genetic controls for cell-to-cell and long-distance movement. The region I^{57}-N^{100} was identified as the determinant of foliar necrosis in *Nicotiana benthamiana*, and mutagenesis of $HH^{93–94}$ greatly reduced necrosis. These findings are likely applicable to other tospovirus species, especially those within the 'New World' group as NSm sequences are highly conserved.

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Introduction

The family *Bunyaviridae* is composed of five genera: *Orthobunyavirus*, *Hantavirus*, *Nairovirus*, *Phlebovirus* and *Tospovirus* (Nichol et al., 2005). The genus *Tospovirus*, takes its name from the type species, Tomato spotted wilt virus (TSWV), and is the sole genus in the family *Bunyaviridae* with plant pathogenic species (Adkins, 2000). TSWV has a broad host range that includes 1090 plant species in 15 families of monocotyledonous plants, 69 families of dicotyledonous plants, and one family of pteridophytes, and is distributed across the world (Parrella et al., 2003). Symptoms caused by TSWV include chlorosis, necrosis, ringspots, stunting and ring/line patterns affecting leaves, stems and fruits (e.g. Adkins and Rosskopf, 2002; reviewed by Chiemsombat and Adkins, 2006; German et al., 1992; Mumford et al., 1996). As one of the most economically important plant viruses, TSWV causes worldwide losses surpassing 1 billion dollars annually (Adkins, 2000).

Like the other bunyaviruses, TSWV is an enveloped virus with three genomic RNAs denoted as L, M and S. The L RNA is negative sense and contains one large open reading frame (ORF) that encodes an RNA dependent RNA polymerase (RdRp). The S RNA is ambisense and encodes the nucleocapsid protein (N) and a non-structural protein (NSs). The M RNA is also ambisense and encodes the glycoproteins (Gn and Gc) and a second non-structural protein (NSm) (Adkins, 2000; Parrella et al., 2003).

A true reverse genetics system remains elusive for all members of the *Bunyaviridae*. However, advances in the molecular genetics of the animal-infecting members of the *Bunyaviridae* have been facilitated by the rescue of infectious Uukuniemi virus (genus *Phlebovirus*) from cloned cDNAs using an RNA polymerase I expression system (Flick and Pettersson, 2001) and a T7 polymerase-based Vaccinia virus system for Bunyamwera virus (genus *Orthobunyavirus*) (Bridgen and Elliott, 1996; Dunn et al., 1995) and Rift Valley fever virus (genus *Phlebovirus*) (Lopez et al., 1995).

In the plant-infecting members of the *Bunyaviridae*, a TSWV genome reassortment system was developed (Qiu et al., 1998). Using this approach and through analysis of nucleotide sequences, many features of TSWV biology have been assigned to the different genomic RNA segments and even to specific proteins (Hoffmann et al., 2001; Jahn et al., 2000; Margaria et al., 2007; Okuda et al., 2003; Sin et al., 2005), but it is still impossible to directly manipulate specific TSWV genes within the context of tospovirus genomes. TSWV infects the long-used experimental hosts, *Nicotiana benthamiana* (Christie and Crawford, 1978; Quacquarelli and Avgelis, 1975) and *Nicotiana*

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tabacum, causing distinctive symptoms of chlorosis, necrosis, ringspots and/or ring/line patterns (e.g. Goodin et al., 2008).

The unique NSm protein, which is not encoded by any of the animal-infecting members of the *Bunyaviridae*, is thought to be the result of the adaptation of tospoviruses to plants. NSm has characteristics typical of plant virus movement proteins (MPs), including expression during a short period early in systemic infection, association with nucleocapsid aggregates in the cytoplasm (Kormelink et al., 1994), intracellular localization close to plasmodesmata (Prins et al., 1997), localization in cellular fractions enriched for cell walls and cytoplasmic membranes (Kormelink et al., 1994; Prins et al., 1997; Storms et al., 1995), formation of tubule structures (Lewandowski and Adkins, 2005; Storms et al., 1995), plasmodesmata modification (Prins et al., 1997; Storms et al., 1998), RNA binding (Soellick et al., 2000) and interactions with a host trafficking protein (Paape et al., 2006). The first direct evidence that NSm functions as an MP was generated by complementation of a movement-deficient Tobacco mosaic virus (TMV) vector by heterologous expression of NSm (Lewandowski and Adkins, 2005), as a TSWV reverse genetics system is lacking.

In addition to demonstrating that NSm is able to support cell-to-cell movement in the absence of any other TSWV proteins, the TMV-based expression system also showed that NSm induced tubule formation in protoplasts, and supported long-distance movement and induced TSWV-like symptoms in *Nicotiana benthamiana* (Lewandowski and Adkins, 2005). The latter data are consistent with speculation that the aberrant, disease-like phenotype of NSm transgenic *Nicotiana tabacum* plants was due to a role of NSm as a symptom determinant (Prins et al., 1997; Rinne et al., 2005). The C-terminus of the NSm sequence was shown to be required for movement and tubule formation, but its absence did not prevent symptom expression (Lewandowski and Adkins, 2005). In the current study, using the strategies of deletion-mapping and alanine-substitution mutation, we identified essential NSm domains required for tubule formation, movement and symptom development. The possible relationship between these three NSm-mediated biological activities is discussed.

Results

Phylogenetic analysis of NSm proteins identifies commonalities for mutagenesis

Phylogenetic analyses of tospovirus NSm and N protein sequences has identified two major clusters, 'New World' and 'Old World' (e.g. Chiemsombat and Adkins, 2006; Silva et al., 2001). TSWV is a member of the 'New World' group (Silva et al., 2001). Comparison of the NSm sequence of the previously described Florida TSWV isolate 7-1 (GenBank accession no. AY956380; Lewandowski and Adkins, 2005) with the sequences of NSm proteins of other members of the 'New World' group indicated that the first 50 amino acids at the N-terminus are hypervariable, whereas many conserved regions including 'P/D-L' and 'D' motifs in the '30K superfamily' of viral MPs (Melcher, 2000; Mushegian and Koonin, 1993; Silva et al., 2001) are located in the central portion of the proteins (Fig. 1). Our computer analysis (data not shown) indicated that the central portion (N^{97}-C^{195}) of the TSWV NSm protein (Fig. 2A) and 11 other tospoviruses for which NSm sequence is available contain alternating hydrophobic and hydrophilic regions. These regions are similar to the Cowpea mosaic virus (CPMV) 48-kDa MP, which also forms tubules (Bertens et al., 2000). Moreover, a left-handed coiled-coil (Burkhard et al. 2001) was predicted to be well conserved near the C-terminus (Fig. 1).

Wild-type (wt) NSm from TSWV isolate 7-1 was expressed from transcripts of construct pTMVcpNSm (hereafter referred to as cpNSm), which contains the full-length *NSm* gene under the control of the TMV CP subgenomic promoter (Lewandowski and Adkins, 2005). Based on the above-described sequence analyses, alanine-substitution and deletion mutants of NSm were constructed in pTMVcpGFP, an MP- and CP-deficient TMV vector (Grdzelishvili et al., 2000). All mutants were sequenced to confirm the intended NSm mutagenesis was successful and to confirm that no additional mutations were introduced.

Eleven clusters of charged amino acid(s) which are conserved within identical or highly similar regions of 'New World' tospovirus NSm protein sequences were selected for alanine-substitution mutagenesis in TSWV isolate 7-1 (Fig. 1). The positions of the alanine substitutions in relation to the hydrophobicity of the linear protein sequence and NSm deletion mutants are shown on the *x*-axis in Fig. 2A. Notably, only the mutation in A154 is located within the central hydrophobic region of NSm, whereas all other alanine substitutions are located within hydrophilic regions. Mutations in A269–274 and A284–288 are located within the coiled-coil domain, although computer analysis of the mutant NSm proteins predicted that only the mutations introduced in A269–274 completely destroyed the predicted domain (data not shown).

Transcripts derived from TMV-based constructs encoding wt or mutant NSm proteins were used to transfect protoplasts derived from tobacco suspension cells. Northern blot analysis of viral RNAs extracted at 24 h post-inoculation (hpi) revealed that all deletion and alanine-substitution mutants replicated, although some accumulated increased or decreased levels of genomic and subgenomic RNAs as compared to parent construct cpNSm (Fig. 2B). Tobacco plants homozygous for the resistance gene *N* and the TMV MP [NN-MP(+)] were also inoculated with transcripts as a positive control for cell-to-cell movement. All mutants formed local lesions on leaves of NN-MP (+) plants 3–4 days post-inoculation (dpi; data not shown) indicating viability in plants.

Domains of NSm required for subcellular localization and tubule formation in protoplasts

Previous subcellular fractionation and labeling studies established that wt NSm localizes to cell walls, cytoplasmic membranes and plasmodesmata during both TSWV infection of *Nicotiana tabacum* plants and expression of wt NSm in the absence of other TSWV proteins in protoplasts and plants (Kormelink et al., 1994; Prins et al., 1997; Storms et al., 1995). To elucidate key domains involved in NSm localization, we examined whether mutagenesis of NSm affected localization by fractionating extracts of the transfected protoplasts into cytoplasmic (S30) and membrane (P30) fractions as previously described by Bertens et al. (2000). Western blotting showed that for all alanine-substitution mutants, the majority of NSm protein was present in P30 (Fig. 3, odd numbered lanes 5–26), with only a trace amount in S30 (Fig. 3, even numbered lanes 5–26). The relative proportion of mutant NSm in P30 vs. S30 fractions was similar to wt NSm (Fig. 3, lanes 1, 2, 27, and 28), suggesting that the alanine substitutions did not interfere with NSm localization. Moreover, the accumulated levels of wt NSm and the alanine-substitution mutants were similar, indicating that the amino acid substitutions did not affect stability in tobacco protoplasts. Surprisingly, most of the truncated protein expressed by the N-terminal deletion mutants (N50 and N97) and C-terminal deletion mutants (C248 and C195) was also found in P30 (Fig. 3, odd numbered lanes 31–38), suggesting that the central region might be responsible for NSm membrane association resulting in its presence in the P30 fraction. Western blot detection of NSm mutants with larger deletions (e.g. N147 and C146) was not successful, likely due to deletion of the epitopes recognized by the NSm antiserum (data not shown).

The effects of mutagenesis on the ability of NSm to form tubules in protoplasts as seen with wt NSm were examined by indirect immunofluorescence microscopy as described previously (Kikkert et al., 1997; Lewandowski and Adkins, 2005; van Lent et al., 1991). Our

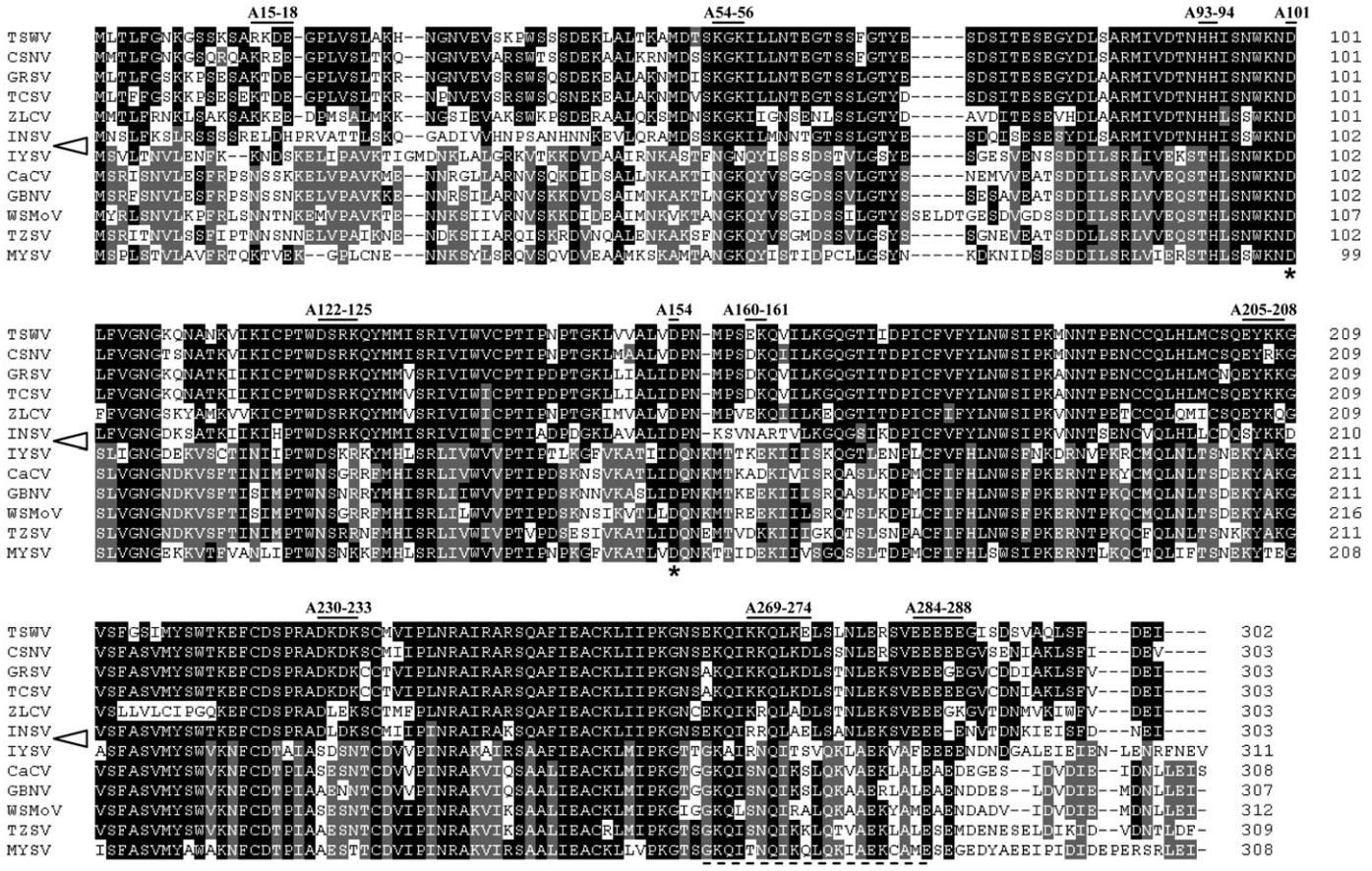

Fig. 1. Alignment of NSm proteins of 'New World' and 'Old World' tosposviruses reveals conserved amino acids targeted in TSWV NSm for mutagenesis. ClustalX alignment of NSm proteins of 'New World' tospoviruses (shown above triangle in sequence blocks): Tomato spotted wilt virus (TSWV, AY956380), Chrysanthemum stem necrosis virus (CSNV, AF213675), Groundnut ringspot virus (GRSV, AF213673), Tomato chlorotic spot virus (TCSV, AF213674), Zucchini lethal chlorosis virus (ZLCV, AF213676) and Impatiens necrotic spot virus (INSV, NC_003616), and 'Old World' tospoviruses (shown below triangle in sequence blocks): Iris yellow spot virus (IYSV, AF213677), Capsicum chlorosis virus (CaCV, NC_008303), Groundnut bud necrosis virus (GBNV, NC_003620), Watermelon silver mottle virus (WSMoV, NC_003841), Tomato zonate spot virus (TZSV, NC_010490) and Melon yellow spot virus (MYSV, NC_008307). Amino acid residues within a column and conserved in at least four of the six 'New World' tospoviruses or a majority of all species are shaded black; those conserved among at least four of the six 'Old World' tospoviruses are shaded gray. Aspartate residues in the conserved motifs of 'D/P-L' and 'D' found in '30K-superfamily' movement proteins are indicated below the alignment with asterisks. A potential coiled-coil domain is underlined with a broken line. Positions of TSWV NSm alanine-substitution mutants (A15–18, A54–56, A93–94, A101, A122–125, A154, A160–161, A205–208, A230–233, A269–274 and A284–288) are indicated above the alignment. Amino acid positions in NSm (gaps not counted) are indicated to the right of each sequence block.

prior study showed that deletion of the C-terminal 54 amino acids of NSm completely abolished tubule formation (Lewandowski and Adkins, 2005). Similarly, no tubule formation was observed in protoplasts transfected with the N-terminal deletion mutants, N50 and N97 (data not shown). In contrast, the alanine-substitution mutants showed a variety of tubule phenotypes (Fig. 4), in spite of there being no apparent differences in localization of the mutant NSm proteins after fractionation of infected protoplasts (Fig. 3).

Mutants A15–18 and A160–162 consistently formed tubules on the exterior surface of transfected protoplasts which resembled the wt NSm tubule phenotype (Fig. 4). Mutants A101, A205–208 and A284–288 consistently formed tubules that were shorter in length than those observed with wt NSm. Mutant A230–233 was severely impaired in tubule formation (Fig. 4), with a reduced number of short tubules observed on the surface of the protoplasts in only one of three experiments. No tubules were formed by mutants A54–56, A93–94, A122–125, A154 and A269–274 although several distinct patterns of subcellular localization were observed. NSm of mutant A154 appeared to be uniformly distributed within the cell (Fig. 4). Most of the NSm produced by mutants A93–94 and A269–274 was observed in and/or around the nucleus (Fig. 4). NSm produced by mutants A54–56 and A122–125 was more widely distributed in the cytoplasm (Fig. 4). Collectively, these data suggest that regions G^{19}-S^{159} and G^{209}-V^{283} of NSm are involved in tubule formation and indicate that amino acid groups **KGK**$^{54–56}$, **HH**$^{93–94}$, **DSRK**$^{122–125}$, and **KK**Q**LKE**$^{269–274}$ (bold font indicates amino acids replaced with alanine in the mutants) play an essential role in NSm subcellular distribution and tubule formation as their replacement with alanine altered NSm distribution within and/or eliminated tubule formation exterior to protoplasts.

Domains of NSm required for cell-to-cell movement

The C-terminus of NSm was previously shown to be essential for cell-to-cell movement (Lewandowski and Adkins, 2005) and this result was corroborated in the current study by the failure of mutants C195 and C248 to move cell-to-cell in *Nicotiana tabacum* cv. Xanthi nc (Xanthi nc) and *Nicotiana benthamiana* plants (Table 1). To determine the function of the N-terminus in cell-to-cell movement, Xanthi nc and *Nicotiana benthamiana* plants were inoculated with transcripts of mutants N50 or N97. Neither mutant induced local lesions on either host by 10 dpi (Table 1), indicating that sequences within the N-terminal 50 amino acids are required for cell-to-cell movement.

To more finely map domain(s) required for cell-to-cell movement, Xanthi nc plants were inoculated with transcripts of the alanine-substitution mutants. Five mutants (A15–18, A101, A160–161, A205–208 and A284–288) retained cell-to-cell movement function as evidenced by induction of local lesions in Xanthi nc (Table 1). Local

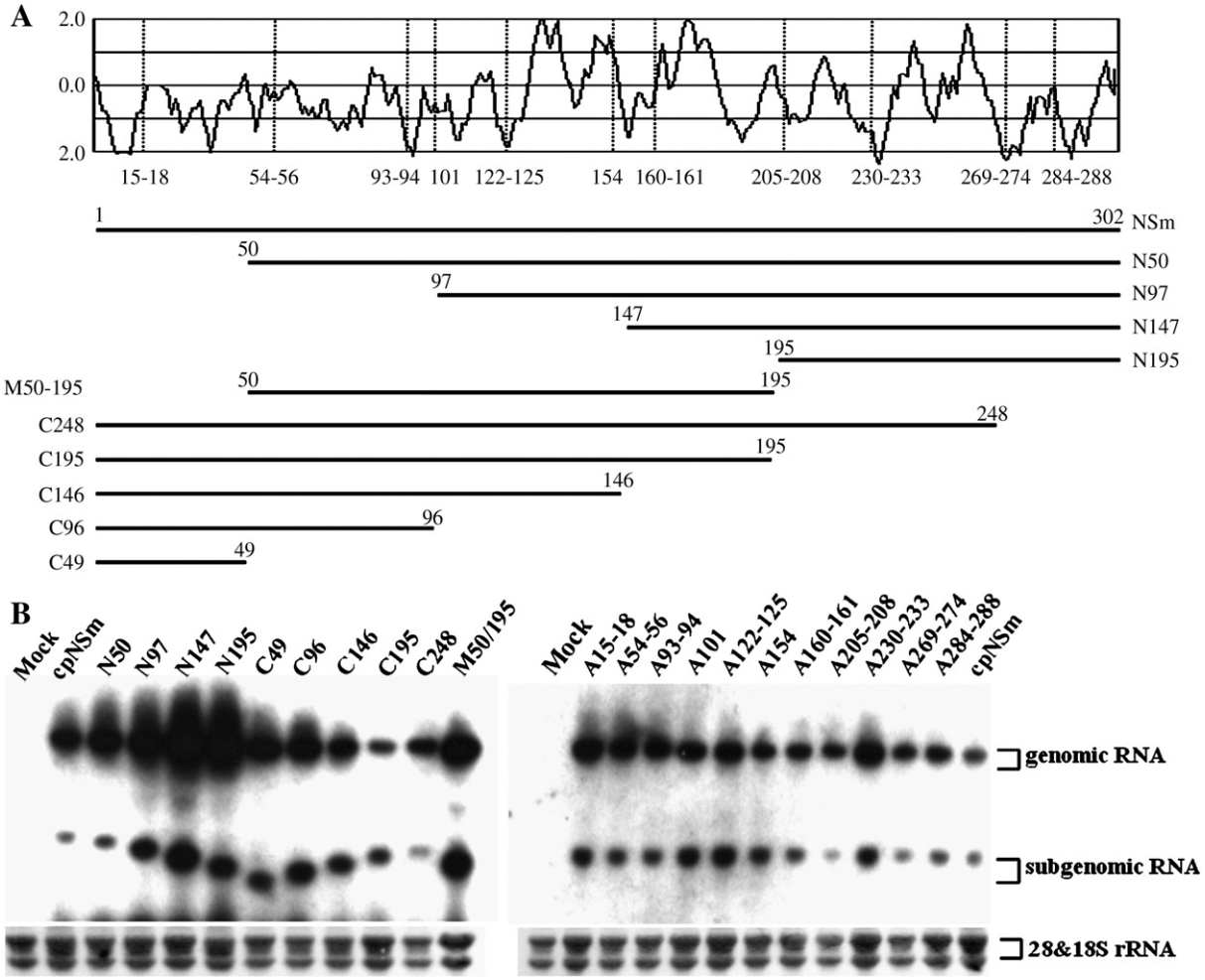

Fig. 2. Characterization and mutagenesis of TSWV NSm protein. (A) Hydropathy plot of the TSWV NSm protein with positions of alanine-substitution mutants shown on the *x*-axis. Horizontal lines below the plot represent the portion of NSm remaining in each deletion mutant (numbers denote positions of the N- or C-terminus of the corresponding mutants). (B) Northern blot hybridization analysis of total RNA from tobacco protoplasts transfected with transcripts of cpNSm (wt) and NSm mutants. Protoplasts were harvested at 24 hpi. Bands corresponding to genomic and subgenomic RNAs are indicated. The 18S and 28S rRNAs are shown to indicate the relative equivalency of samples loaded.

lesions induced by mutants A15–18, A160–161 and A205–208 appeared 5–6 dpi, similar to cpNSm, whereas the development of lesions by mutants A101 and A284–288 was delayed by approximately one additional day. The lesions induced by mutant A160–161 were larger (1.42 mm mean diameter at 10 dpi) than those induced by cpNSm (1.15 mm mean diameter at 10 dpi), and mutants A15–18, A205–208, A284–288 and A101 (Table 1). Mutant A101 induced the smallest lesions with a mean diameter of 0.77 mm, indicating that replacing aspartate with alanine at position 101 impaired, but did not abolish cell-to-cell movement. No lesions were induced by mutants A54–56, A93–94, A122–125, A154 and A269–274, all of which also failed to produce tubular structures in protoplasts (Fig. 4). Mutant

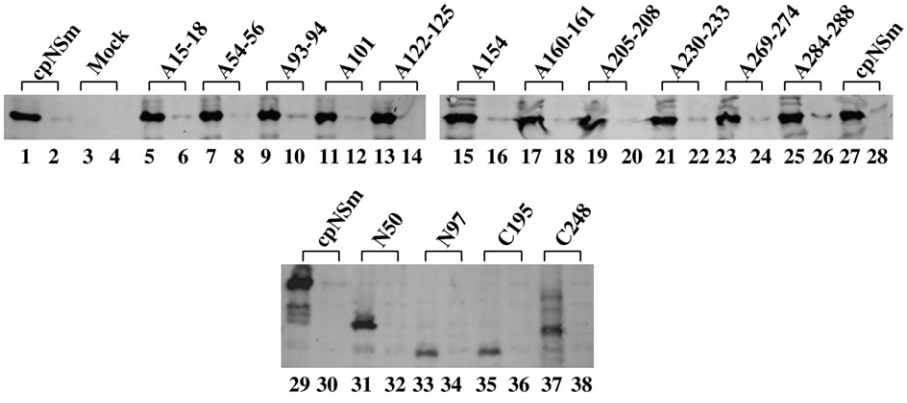

Fig. 3. Subcellular localization by Western blot analysis of NSm produced by the indicated mutants. Extracts from transfected tobacco protoplasts (24 hpi) were separated into S30 and P30 fractions by centrifugation. 5 μl of each sample were analyzed on a 12% SDS-polyacrylamide gel, transferred onto PVDF membrane, and then probed with TSWV NSm antiserum. Lanes marked with odd numbers contain P30 fractions and lanes with even numbers contain S30 fractions.

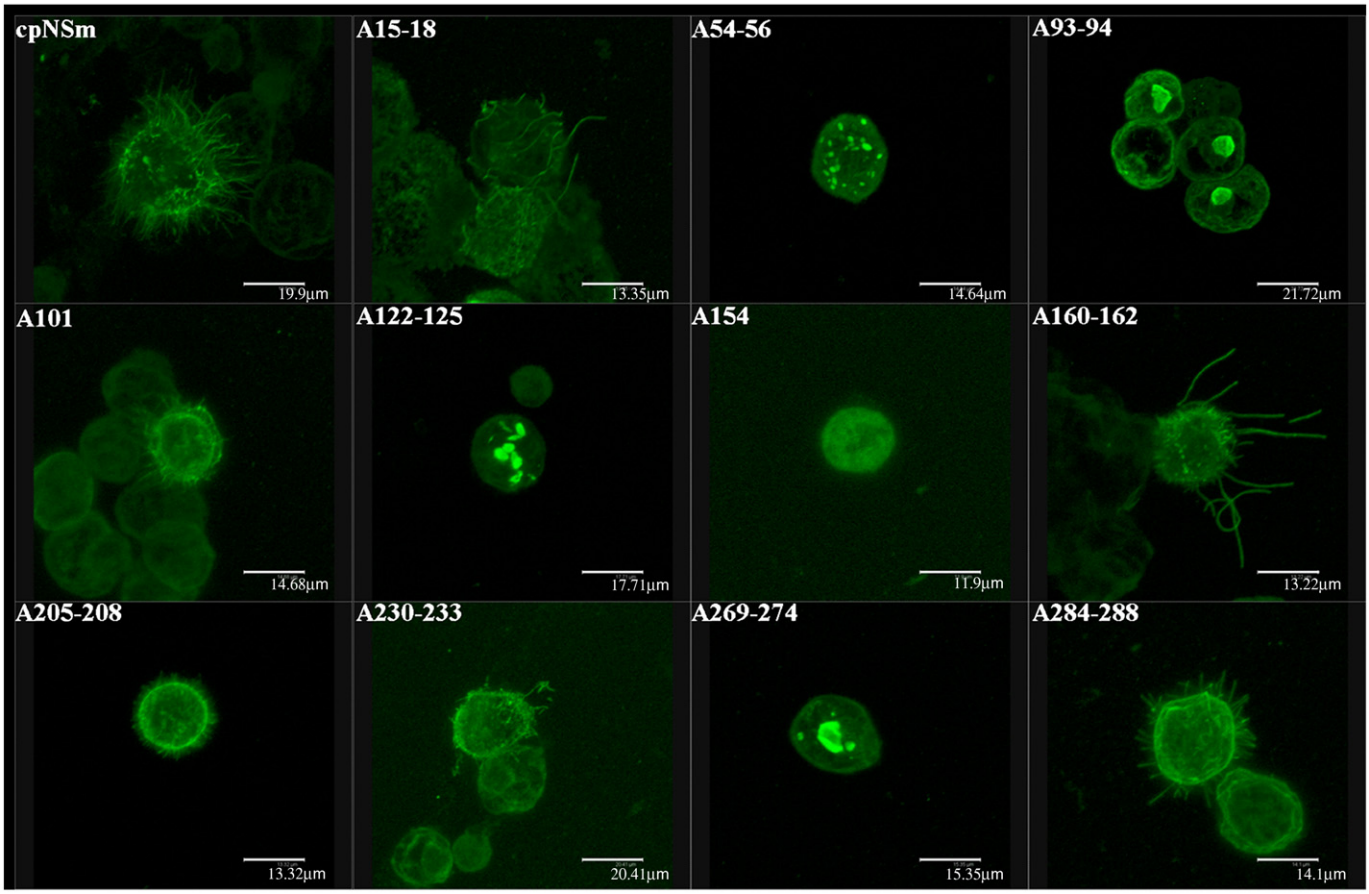

Fig. 4. Indirect immunofluorescence assay of tobacco suspension cell protoplasts transfected with transcripts of cpNSm and alanine-substitution mutants. Protoplasts were harvested at 24 hpi, fixed to glass slides, probed with TSWV NSm antiserum and FITC-conjugated secondary antibody and viewed with a confocal microscope. Weak fluorescence but no tubules were observed from the protoplasts transfected with wild-type TMV or mock-inoculated with water, as shown previously (Lewandowski and Adkins, 2005; Fig. 5C and D). A scale bar with measurement is presented with each micrograph.

A230–233 was distinct in that it occasionally formed tubules, but always failed to induce local lesions.

Consistent with the data from Xanthi nc plants, mutants A15–18, A101, A160–161, A205–208 and A284–288 also moved from cell to cell in *Nicotiana benthamiana* plants (Table 1, Fig. 5A). Symptoms appeared by 5–6 dpi on the leaves inoculated with transcripts of cpNSm or these five mutants. However, the types of symptoms in inoculated leaves at 10 dpi varied (Fig. 5A). Mutants A15–18, A160–161 and A205–208 induced necrotic spots similar to cpNSm, whereas mutant A101 induced necrotic lesions smaller than cpNSm, and mutant A284–288 produced dark spots. At 10 dpi mutants A15–18, A160–161, A205–208 and A284–288 accumulated levels of viral RNA similar to cpNSm in inoculated leaves (Fig. 5B). The accumulation of mutant A101 RNA was greatly reduced (Fig. 5B), consistent with the smaller lesions induced by this mutant in inoculated leaves of Xanthi nc (Table 1). Collectively, these data indicate a role for D^{101} in cell-to-cell movement. At 10 dpi, no viral RNA was detectable in the asymptomatic *Nicotiana benthamiana* leaves inoculated with mutants A54–56, A93–94, A122–125, A154, A230–233, or A269–274 (data not shown), all of which failed to induce local lesions in Xanthi nc.

Failure to move cell-to-cell in inoculated leaves of Xanthi nc and *Nicotiana benthamiana* plants suggests that mutagenesis disrupted functional domain(s) by replacement of one or more essential amino acids. The amino acids shown in bold are charged amino acids that when replaced with alanine abolished cell-to-cell movement: **K**G**K**$^{54–56}$, **HH**$^{93–94}$, **DSRK**$^{122–125}$, **D**^{154}, **DKDK**$^{230–233}$, and **KK**Q**LKE**$^{269–274}$. Because most of the mutant NSm proteins contained multiple amino acid substitutions, it is impossible to discriminate between local effects due to replacing one or multiple amino acid(s) from more global effects on protein folding that disrupted functional domains.

The behavior of both the deletion and alanine-substitution mutants indicated the requirement for the N- and C-termini of NSm for cell-to-cell movement. The alanine-substitution mutants further indicated the importance of the regions G^{19}-S^{159} and G^{209}-V^{283} for cell-to-cell movement, the same regions implicated as important for tubule formation.

Domains of NSm required for long-distance movement in Nicotiana benthamiana

Wild-type NSm protein supports long-distance movement of cpNSm in *Nicotiana benthamiana* (Lewandowski and Adkins, 2005). To determine whether the alanine substitutions affected long-distance movement, *Nicotiana benthamiana* plants inoculated with transcripts of the alanine-substitution mutants were observed for up to 20 dpi for development of symptoms in upper non-inoculated leaves. When no symptoms were observed, Northern blot hybridization was used to assay for the presence of virus (data not shown). Mutants A15–18 and A160–161 moved similarly to cpNSm into upper non-inoculated leaves of all plants, whereas mutants A101 and A205–208 were detected in upper non-inoculated leaves of only three and seven of 15 inoculated plants, respectively (Table 1). Although competent for cell-to-cell movement, long-distance movement of A284–288 was not detected by Northern blot hybridization in any *Nicotiana benthamiana* plant (Table 1) even up to 20 dpi. None of the mutants deficient in cell-to-cell movement (A54–56,

Table 1
Characteristics of TSWV NSm mutants in protoplasts and plants.

Mutant[a]	Tubules in protoplasts[b]	Local infection		Systemic Infection	
		Xanthi nc[c]	*Nicotiana benthamiana*[d]	*Nicotiana benthamiana*[e]	NB-MP(+)[e]
wt NSm					
cpNSm	+	1.15 ± 0.25	+	13/13	12/12
Deletion mutants					
N50	−	−	−	0/10	8/8
N97	−	−	−	0/10	0/8
C195	−	−	−	0/10	0/8
C248	−	−	−	0/10	0/8
Alanine-substitution mutants					
A15–18	+	1.06 ± 0.20	+	13/13	10/10
A54–56	−	−	−	0/10	10/10
A93–94	−	−	−	0/10	10/10
A101	+	0.77 ± 0.21	+	3/15	10/10
A122–125	−	−	−	0/10	10/10
A154	−	−	−	0/10	5/10
A160–161	+	1.42 ± 0.35	+	13/13	10/10
A205–208	+	0.99 ± 0.15	+	7/15	10/10
A230–233	+/−	−	−	0/10	10/10
A269–274	−	−	−	0/10	10/10
A284–288	+	0.97 ± 0.13	+	0/15	0/10

[a] RNA transcripts of corresponding mutants were used for transfection or inoculation as described in Materials and methods. Successful infection of plants was determined by symptom appearance and by Northern blot hybridization using RNA from the inoculated and systemic leaves. Results of at least three separate experiments were combined.
[b] Ability of NSm to produce tubules on protoplasts (24 hpi) derived from high temperature-adapted tobacco suspension cells. +, tubular structures regularly detected on the surface of protoplasts; +/−, tubules rarely detected; −, no tubules detected.
[c] Infectivity on *Nicotiana tabacum* Xanthi nc (Xanthi nc). Mean size of lesions on the inoculated leaves was measured 10 dpi (diameter in mm ± SD of at least 35 measurements). −, no lesions developed.
[d] Infection status of the inoculated leaves of *Nicotiana benthamiana* at 10 dpi. Ten plants were used for each mutant. Local infection was determined by symptom appearance and Northern blot hybridization of extracted RNA. −, no symptoms and no viral RNA detected; +, symptoms appeared and viral RNA accumulated.
[e] Systemic infection of *Nicotiana benthamiana* (20 dpi) or TMV MP-transgenic *Nicotiana benthamiana* [NB-MP(+)] (10 dpi) expressed as 'number of plants systemically infected/number of plants inoculated.'

A93–94, A122–125, A154, A230–233, and A269–274) were detected by Northern blot hybridization in upper non-inoculated leaves of *Nicotiana benthamiana* plants (Table 1).

It was unclear if mutations affecting cell-to-cell movement directly or indirectly affected long-distance movement. Transgenic expression of the TMV MP in *Nicotiana benthamiana* [NB-MP(+)] plants is able to support cell-to-cell movement of movement-defective TMV-NSm hybrids (Lewandowski and Adkins, 2005). Therefore, direct and indirect affects of NSm on long-distance movement could be analyzed using a combination of wt and NB-

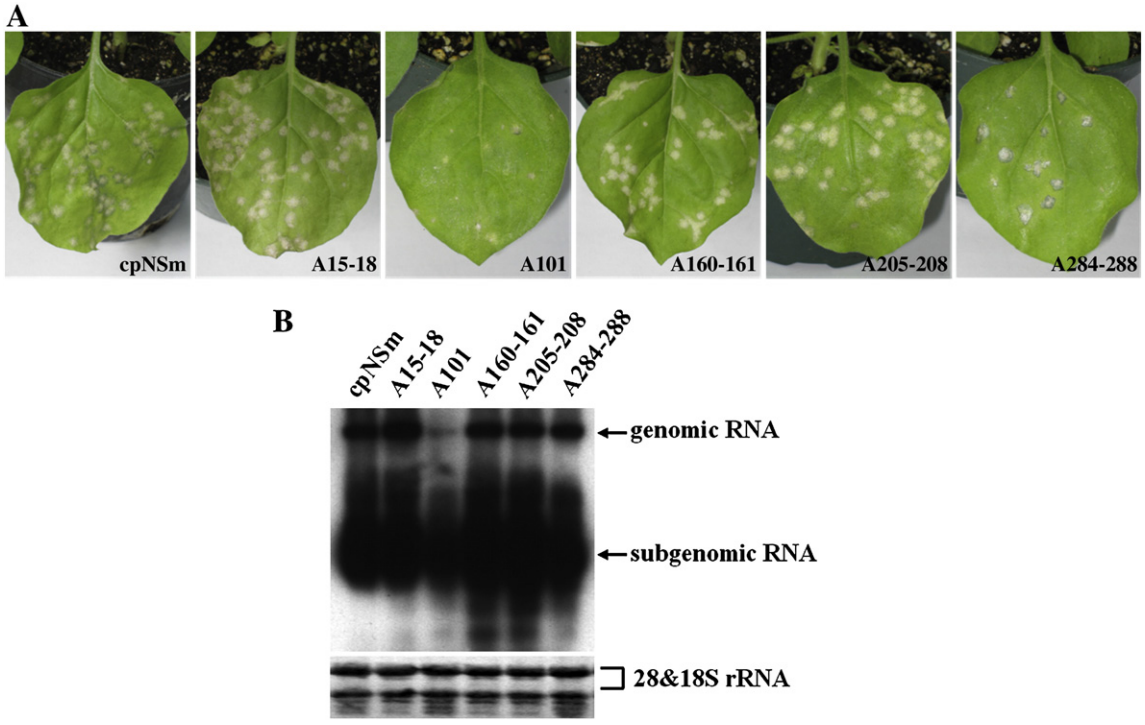

Fig. 5. Characteristic symptoms at 10 dpi on *Nicotiana benthamiana* inoculated with cpNSm and indicated mutants. (A) Local symptoms induced by the indicated alanine-substitution mutants. (B) Northern blot hybridization analysis of total RNA from leaves of *Nicotiana benthamiana* inoculated with the indicated mutants. RNA was extracted from leaves collected at 10 dpi. Bands corresponding to genomic and subgenomic RNAs are indicated. The 18S and 28S rRNAs are shown to indicate the relative equivalency of samples loaded.

MP(+) *Nicotiana benthamiana* plants. In addition to its role in cell-to-cell movement, the TMV MP also supports long-distance transport of TMV RNA in *Nicotiana benthamiana* in the absence of TMV CP (Knapp et al., 2001).

In our current study, NB-MP(+) plants inoculated with transcripts of TMVcpGFP (a MP- and CP-deficient vector analogous to TMVcpNSm but expressing the easily visible GFP marker) showed GFP fluorescence in the upper non-inoculated leaves only after 20 dpi (data not shown), demonstrating that the transgenic expression of TMV MP supports long-distance movement of TMVcpGFP and establishing the time-frame in which long-distance movement mediated by only the TMV MP will occur.

The wt NSm-expressing construct cpNSm (built from TMVcpGFP as described in Materials and methods) produced systemic symptoms after only 6–7 dpi in NB-MP(+) plants, whereas 13–15 dpi were required in *Nicotiana benthamiana* plants indicating an apparent *trans* effect when both the TMV MP and NSm were present. These results established a baseline for assessing long-distance movement of cpNSm in comparison with the derived mutants in the presence or absence of TMV MP.

NB-MP(+) plants were individually inoculated with one of the NSm mutants to examine the effects of mutations on long-distance movement that were independent of the ability to move cell to cell (Table 1). To lessen the possibility of confusing the role that the mutated NSm plays in facilitating long-distance movement with that of the transgenically-expressed TMV MP, symptoms were evaluated on all plants at 10 dpi and total RNA was extracted from upper leaves at 10 dpi for Northern blot analysis (data not shown).

Mutant N50 was detected in upper non-inoculated leaves 6–7 dpi, comparable to cpNSm, indicating that the N-terminal 49 amino acids are not required for long-distance movement. In contrast, mutants N97, C195 and C248 failed to move long-distance in all eight inoculated NB-MP(+) plants by 10 dpi.

Nine alanine-substitution mutants, six of which were previously found to be defective for cell-to-cell movement (mutants A54–56, A93–94, A122–125, A154, A230–233 and A269–274), accumulated in the upper non-inoculated leaves of all ten inoculated NB-MP(+) plants by 6–7 dpi (Table 1). In contrast, long-distance movement of cell-to-cell movement-defective mutant A154 was detected in the upper leaves of only five of ten NB-MP(+) plants by 10 dpi suggesting that D^{154} was less critical for long-distance movement although our previous results showed it to be essential for cell-to-cell movement. Mutant A284–288 was not detected in the upper non-inoculated leaves of any NB-MP(+) plant at 10 dpi or selected plants retained for 20 dpi. Thus, mutant A284–288 was the only cell-to-cell movement-competent mutant that did not move into the upper non-inoculated leaves of either NB-MP(+) or *Nicotiana benthamiana* plants, indicating that this mutation not only disrupted NSm-mediated long-distance movement but also eliminated the apparent *trans* effect provided by transgenic expression of the TMV MP.

Movement profiles of mutants A101 and A205–208 in wt and TMV MP-transgenic plants revealed some interesting differences. Less than 50% of *Nicotiana benthamiana* plants inoculated with each mutant showed systemic symptoms, indicating that the function of long-distance movement was impaired. However, each mutant moved efficiently in all ten inoculated NB-MP(+) plants. The reduced cell-to-cell movement of mutant A101 (as evidenced by smaller lesions in Xanthi nc plants; Table 1) might have contributed to the lower number of systemically infected *Nicotiana benthamiana* plants but this impairment was overcome by transgenic expression of TMV MP. In contrast, A205–208 moved from cell to cell more similarly to cpNSm in *Nicotiana benthamiana* plants, suggesting that the lower percentage of plants systemically infected was due to a defect at an interface between cell-to-cell and long-distance movement—a defect that could be complemented by transgenic expression of TMV MP.

Domains of NSm involved in symptom development

The domain(s) involved in NSm-mediated symptom development were identified by comparing symptoms induced by cpNSm and the NSm mutants on NB-MP(+) plants (Fig. 6). In asymptomatic leaves, infection was confirmed by Northern blot hybridization (data not shown). The two C-terminal deletion mutants, C248 and C195, induced TSWV-like necrotic ringspots in the inoculated leaves of NB-MP(+) plants (Fig. 6A). However, a larger C-terminal deletion, mutant C146, caused chlorotic spots with necrotic rings (Fig. 6A). When the C-terminal deletion was further extended, as in mutants C96 and C49, no symptoms were observed in inoculated leaves (Fig. 6A). The N-terminal deletion mutant N50 induced necrotic ringspots, whereas the more extensive N-terminal deletions in mutants N97 and N147 induced only chlorotic spots (Fig. 6A). The more extensive N-terminal deletion in mutant N195 did not induce symptoms in inoculated leaves (Fig. 6A).

Three NSm deletion mutants, N50, C248 and C195 induced milder TSWV-like necrotic ringspots than the necrotic spots caused by cpNSm. These mutants shared one common region defined by M^{50}-C^{195}. To test whether this central region alone was a determinant of necrosis, mutant M50–195 was constructed and transcripts were used to inoculate NB-MP(+) plants. However, the inoculated leaves were symptomless at 10 (Fig. 6A) and 15 dpi (data not shown). It is possible that the mutant M50–195 NSm protein was incorrectly folded or targeted, either of which could have prevented symptom induction.

Alanine-substitution mutants induced four distinguishable responses on the inoculated leaves (Fig. 6B). Mutants A15–18, A54–56, A122–125, A154, A160–161, A205–208 and A269–274 induced necrotic ringspots/spots that were comparable to the diffuse, tan colored necrotic spots induced by cpNSm, indicating that the substituted amino acids in these mutants had little or no effect on symptom development in the inoculated leaves. In contrast, mutants A101 and A230–233 induced larger, confluent areas of necrosis in inoculated leaves, whereas mutant A284–288 induced more restricted, darker necrotic spots that were easily distinguished from those induced by cpNSm (Fig. 6B), and mutant A93–94 induced only faint necrotic ringspots. These data indicate that amino acid(s) D^{101}, $DKDK^{230–233}$, and $EEEEE^{284–288}$ are involved in symptom phenotype and/or severity in inoculated leaves, whereas $HH^{93–94}$ is critical for symptom induction.

The systemic symptoms that developed at 10 dpi in NB-MP(+) plants inoculated with transcripts were also evaluated (Fig. 6C). Mutant A15–18, which produced local symptoms comparable to cpNSm, also consistently induced systemic symptoms of stunting, leaf necrosis and crinkling similar to cpNSm (Fig. 6C). Systemic symptoms similar to cpNSm were also induced by mutants A54–56, A122–125, A154, A160–161, A205–208 and A269–274 (data not shown). Mutants A101 (Fig. 6C) and A230–233 (data not shown) induced more severe symptoms in the upper leaves compared to cpNSm, consistent with the more severe symptoms observed in inoculated leaves. Mutant A93–94 induced stunting and systemic leaf crinkling, but not necrosis, further supporting the involvement of $HH^{93–94}$ in the induction of necrosis (Fig. 6C). No systemic symptoms were visible in NB-MP(+) plants inoculated with mutant A284–288 (Fig. 6C), which did not move beyond the inoculated leaves (Table 1).

Of the deletion mutants, only N50 systemically infected NB-MP (+) plants (Table 1), inducing systemic symptoms of crinkling and yellowing of the upper leaves at 10 dpi, but it did not induce necrosis (Fig. 6C). Within the central region defined by the deletion mutant M^{50}-C^{195} (described above), only mutation of amino acids $HH^{93–94}$

significantly ameliorated symptoms. Collectively, these results indicate that the N-terminal 49 amino acids and the C-terminal 107 amino acids of NSm are not directly related to symptom development. The region containing amino acid residues I^{57}-N^{100} was found to be the major determinant for development of foliar necrosis, with the amino acid residues $HH^{93–94}$ critical for this function.

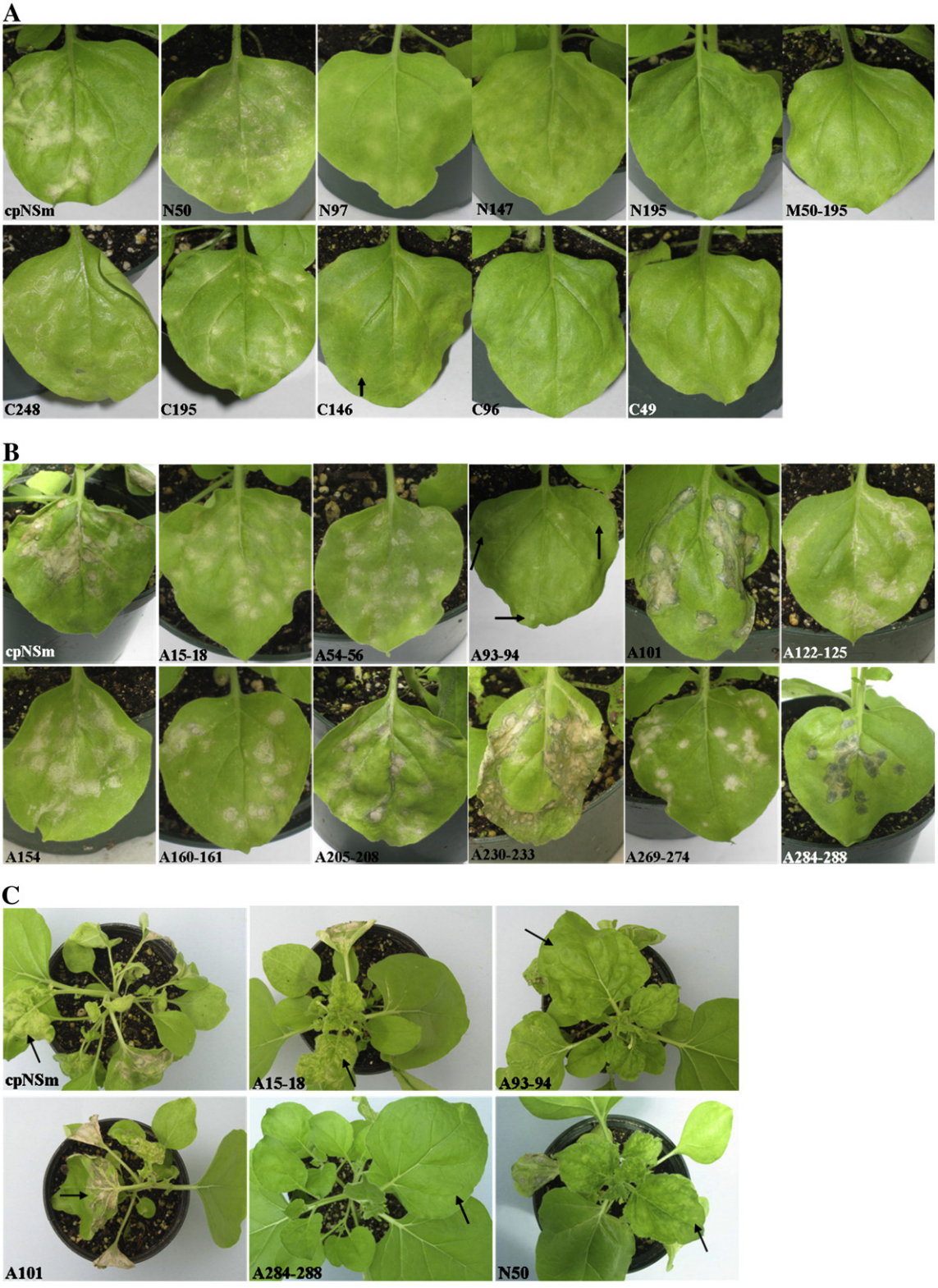

Fig. 6. Characteristic symptoms at 10 dpi on TMV MP-transgenic *Nicotiana benthamiana* inoculated with cpNSm and indicated mutants. (A) Local symptoms induced by the indicated NSm deletion mutants. Faint chlorotic spot surrounded by necrotic ring induced by mutant C146 is indicated with an arrow. (B) Local symptoms induced by the indicated alanine-substitution mutants. Faint necrotic ringspots induced by mutant A93–94 are indicated with arrows. (C) Systemic symptoms induced by the indicated NSm mutants. Symptoms of mutant A15–18 were comparable with those induced by mutants A54–56, A122–125, A154, A160–162, A205–208 and A269–274 (data not shown). Similarly, mutant A101 induced systemic symptoms comparable to those of mutant A230–233 (data not shown). No systemic symptoms were observed with A284–288 at 10 dpi. Upper leaves with (cpNSm, A15–18, A101 and N50) or without symptoms (A93–94 and A284–288) are indicated with arrows.

Discussion

When a tractable genetic system is not available, transient expression systems are considered a feasible approach to express and characterize heterologous ORFs or non-coding sequences from viruses. Using Potato virus X (PVX), the Tomato leaf curl New Delhi virus nuclear shuttle protein was characterized as a pathogenicity determinant and a target of host defense responses (Hussain et al., 2005), and the 5′ non-coding region of Grapevine chrome mosaic virus RNA-2 was shown to trigger a necrotic response in three *Nicotiana* spp. (Fernandez et al., 1999). Using a TMV expression system, it was demonstrated that the Groundnut rosette virus (GRV) ORF3-encoded protein is involved in long-distance RNA movement (Ryabov et al., 1999), viral RNA binding and formation of ribonucleoprotein complexes (Taliansky et al., 2003), and that the GRV ORF4-encoded protein induces tubule formation on the surface of protoplasts (Nurkiyanova et al., 2001). A TMV-based system was used to demonstrate that the TSWV NSm protein is an MP, forms tubules, and is a symptom determinant (Lewandowski and Adkins, 2005). Using this same expression system, we have now defined functional domains within NSm.

Cell wall and cytoplasmic membrane fractions of TSWV-infected tissues are known to be enriched with NSm (Kormelink et al., 1994; Storms et al., 1995). Indirect immunofluorescence assays in our current study showed that wt NSm was mainly associated with the plasma membrane and was organized into tubules protruding from the surface of tobacco protoplasts. In contrast, mutant NSm proteins expressed by constructs A54–56, A93–94, A122–125 and A269–274 did not form tubules and instead localized in the cytoplasm or nucleus. However, Western blot analysis showed that the majority of NSm expressed by these mutants was still present in the membrane and cell-wall containing P30 fraction. Deletion of the N-terminal 96 or the C-terminal 107 amino acids also did not affect NSm localization to the P30 fraction, suggesting that the central region bounded by N^{97} and C^{195} contains potential membrane-binding domain(s). Although the immunofluorescence and fractionation data are consistent with each other, we cannot unequivocally differentiate between NSm membrane association and NSm aggregation, both of which would likely result in the majority of NSm being found in the P30 fraction. However, our computer analysis of NSm with the Dense Alignment Surface method (Cserzo et al., 1997) predicted two transmembrane segments (R^{132}-V^{137} and P^{174}-Y^{180}) within the N^{97}-C^{195} region. MP transmembrane domain(s) have been well documented for TMV (Brill et al., 2000; Fujiki et al., 2006), Beet yellows virus (Peremyslov et al., 2004) and PVX (Mitra et al., 2003; Solovyev et al., 2000).

Though the mechanisms of viral symptom development are still largely unknown for most viruses (Culver and Padmanabhan, 2007), the necrotic spots we observed in inoculated leaves are likely induced by an interaction between NSm and a host component(s), and inoculation of the NB-MP(+) plants with the alanine-scanning mutants identified amino acids I^{57}-N^{100} of NSm as a major determinant for induction of foliar necrosis in *Nicotiana benthamiana*. Replacement of D^{101}, $DKDK^{230–233}$ or $EEEEE^{284–288}$ with alanine residues did not affect the induction of necrosis, but changed the lesion phenotype in inoculated leaves, whereas replacement of $HH^{93–94}$ with alanine residues greatly reduced development of necrosis, suggesting that these residues play a role in inducing this phenotype. It remains to be determined whether the altered localization of NSm expressed by mutant A93–94 observed in transfected protoplasts was related to milder symptoms *in planta*. However, the majority of NSm expressed by mutant A269–274 also accumulated around the nuclear membrane, yet this mutant induced necrotic symptoms similar to cpNSm, suggesting that the change in subcellular distribution of NSm alone is insufficient to attenuate symptoms. The more severe symptoms induced by mutants A101 and A230–233 might be caused by altered interactions between NSm and host component(s).

Tubule formation in protoplasts is induced by many viruses including Alfalfa mosaic virus (AMV) (Huang et al., 2001a; Kasteel et al., 1997a; Sánchez-Navarro and Bol, 2001), Brome mosaic virus (Kasteel et al., 1997a), GRV (Nurkiyanova et al., 2001), Cauliflower mosaic virus (CaMV) (Huang et al., 2001b; Kasteel et al., 1996; Perbal et al., 1993), CPMV (Bertens et al., 2000; Kasteel et al., 1996, 1997b; Lekkerkerker et al., 1996), Cucumber mosaic virus (CMV) (Canto and Palukaitis, 1999) and TSWV (Storms et al., 1995). Although it is unlikely that all of these viruses use identical processes for movement, the formation of tubular structures suggests that there are some common and/or functionally equivalent mechanism(s) for virus transport (Scholthof, 2005; Thomas and Maule, 1995). Despite the argument that the tubules are not necessary for CMV cell-to-cell movement (Canto and Palukaitis, 1999), a positive correlation between tubule formation and cell-to-cell movement has been demonstrated for CPMV (Bertens et al., 2000; Lekkerkerker et al., 1996) and AMV (Sánchez-Navarro and Bol, 2001). In the case of TSWV NSm, mutations within two domains (G^{19} to S^{159} and G^{209} to V^{283}) altered or abolished tubule formation and cell-to-cell movement, further establishing a connection between these two biological activities. All of the NSm mutants that failed to produce tubular structures in protoplasts failed to spread from cell to cell. Mutant A230–233, which occasionally produced short tubular structures, also failed to move from cell to cell, also suggesting that tubule formation is a prerequisite for cell-to-cell movement. A similar observation was made for CPMV 48-kDa protein mutant AM2 (Bertens et al., 2000) supporting the connection between tubule formation and cell-to-cell movement. One key difference is that virions have been observed within tubules produced by some viruses such as CPMV (van Lent et al., 1991), whereas the cpNSm RNA is presumably moving as a ribonucleoprotein complex with NSm.

Many of the mutations introduced in this study disrupted one or more functions of the NSm protein indicating multifunctional domains if not inter-related functions. Certain functions of NSm, such as long-distance movement and/or symptom expression by mutants defective in tubule formation or cell-to-cell movement were only discernable in TMV MP-transgenic *Nicotiana benthamiana* plants that overcame the failure of mutants to move cell-to-cell in inoculated leaves. The use of both wt and transgenic hosts in this study allowed the separation of mutations that specifically affected long-distance movement from functions related to tubule formation and cell-to-cell movement.

The N-terminal 49 amino acids were not required for long-distance movement, although deletion of this region abolished tubule formation and cell-to-cell movement. In contrast, the C-terminus and several additional amino acids or motifs were important for long-distance movement. Alanine-substitution mutations within NSm demonstrated that D^{154}, $EYKK^{205–208}$ and $EEEEE^{284–288}$, are involved with long-distance movement in *Nicotiana benthamiana*, but the respective mutants had distinct phenotypes. Amino acid D^{154} was essential for cell-to-cell movement, but the long-distance movement phenotype of mutant A154 was complex, with no systemic infection in *Nicotiana benthamiana* plants and systemic infection in only 50% of inoculated NB-MP(+) plants, indicating that restoration of cell-to-cell movement was not itself sufficient to restore a wt long-distance movement phenotype in either wt or transgenic plants. Mutagenesis of amino acids $EYKK^{205–208}$ resulted in a 50% reduction in the number of *Nicotiana benthamiana* plants systemically infected, but with no effect on systemic infection of NB-MP(+) plants. As with mutant A284–288, the effect on systemic infection by A205–208 was likely not due to an impairment in cell-to-cell movement, which was similar to wt NSm. One possible explanation for the "partial" expression of long-distance movement by mutants A154 and A205–208 is the presence of second site mutants or revertants in plants in which movement was detected. In contrast, amino acids $EEEEE^{284–288}$ within the C-terminus of NSm were required for long-distance movement in

both wild-type and NB-MP(+) plants but not because of an essential involvement in cell-to-cell movement.

Separation of cell-to-cell and long-distance movement functions has been reported previously with Tobacco etch virus (TEV) and Red clover necrotic mosaic virus (RCNMV). The TEV CP plays a role in both cell-to-cell and systemic movement (Dolja et al., 1994, 1995), but with distinct functional domains, as does the RCNMV MP (Wang et al., 1998). This should not be surprising because cell-to-cell and long-distance movement represent two connected, yet distinct pathways, both involving different types of plant cells and structures and both of which may require assistance from different host components (Carrington et al., 1996; Waigmann et al., 2004).

Interestingly, alanine substitution for amino acids EEEEE$^{284–288}$ also eliminated the apparent *trans* effect of transgenic expression of the TMV MP as measured by our failure to detect this mutant in the upper non-inoculated leaves of any NB-MP(+) plants at 10 dpi or selected plants retained for 20 dpi. This may indicate that the two proteins share a common interaction with or an interactive site on the viral RNA that is essential for long-distance movement. Further research to elucidate the mechanism for this inhibition of movement and the observed additive effect of co-expression of TSWV and TMV MPs could be useful to illuminate commonalities and differences in movement between tospoviruses and tobamoviruses.

Tospoviruses have been grouped into 'New World' and 'Old World' species (e.g. Chiemsombat and Adkins, 2006; Silva et al., 2001), and their NSm proteins share several conserved motifs such as 'P/D-L' and 'D' in the central and C-terminal regions (Silva et al., 2001). The role of the 'P/D-L' and 'D' motifs in cell-to-cell movement of CaMV (Thomas and Maule, 1995), CPMV (Bertens et al., 2000) and TSWV (results with mutants A101 and A154, this study) indicate that conserved domains in proteins from phylogenetically distinct viruses might share similar biological functions as previously postulated by Scholthof (2005). Beyond the conservation among the primary sequences, the NSm proteins of all currently recognized tospovirus species harbor a predicted transmembrane-spanning segment(s) within the central region. Except for Melon yellow spot virus the NSm proteins of all currently recognized tospovirus species were predicted to form a coiled-coil structure near the C-terminus. The central region between N^{97} and C^{195} appears to be involved in NSm membrane association, whereas the coiled-coil domain appears to be involved in tubule formation and cell-to-cell movement based on the biological activities of NSm mutant A269–274. In this current study, the substituted amino acid(s) are highly conserved within the 'New World' group but only some of these amino acids are also conserved with members of the 'Old World' group (e.g. A101 and A154 described above; see Fig. 1). Results of our mutational analysis demonstrate the importance of both NSm amino acids conserved only in 'New World' tospoviruses and those conserved in all tospoviruses. Although it is conceivable that the roles of the charged amino acids identified through mutational analysis of the TSWV NSm protein are more directly applicable to 'New World' tospovirus species, some are also likely important for 'Old World' tospovirus species. In addition, the structural similarity among NSm proteins of all tospoviruses suggests that the function(s) of predicted structures in TSWV NSm, such as transmembrane domain(s) and the coiled-coil may be conserved across the genus. Further exploration of this interesting protein is merited within both additional TSWV isolates and tospovirus species.

Table 2
Primers used for construction of NSm deletion and alanine-substitution mutants.

Construct name	Primer name	Primer sequence (5′-3′)
wt NSm		
pTMVcpNSm	5′EcoRV-NSm	AATT GATATC T ATGTTGACTCTTTTCGGTAAT
	3′XhoI-NSm	TTAC CTCGAG TTATATTTCATCAAAAGACAAC
Deletion mutants		
pTMVcpNSmN50	5′NSm148–169	GC GATATC T *ATG* GATACATCCAAAGGAAAG
pTMVcpNSmN97	5′NSm289–310	GC GATATC T *ATG* AACTGGAAAAATGATCTTTTTG
pTMVcpNSmN147	5′NSm439–460	GC GATATC T *ATG* AAACTTGTGGTTGCCTTGGTT
pTMVcpNSmN195	5′NSm583–602	GC GATATC T *ATG* TGCTGTCAGCTGCATTTG AT
pTMVcpNSmC248	3′NSm744–724	GA CTCGAG *TTA* AGATCTAGCTCTAATAGCTCT
pTMVcpNSmC195	3′NSm585–565	GA CTCGAG *TTA* GCAGTTTTCTGGAGTGTTATT
pTMVcpNSmC146	3′NSm438–419	GA CTCGAG *TTA* TCCTGTAGGGTTTGGTATAG
pTMVcpNSmC96	3′NSm288–268	GA CTCGAG *TTA* TGAGATATGATGGTTTGTATC
pTMVcpNSmC49	3′NSm147–128	GA CTCGAG *TTA* AGCTTTAGTCAAAGCAAGCT
pTMVcpNSmM50–195	5′NSm148–169/3′NSm585–565	
Alanine-substitution mutants		
pTMVcpNSmA15–18	5′RKDE15–18	CGATATCTATGTTGACTCTTTTCGGTAATAAGGGGTCTTCTAAGTCTGCC
		GCAGCGGC**C**CG**C**AGGTCCTTTAGTTTCACTT
pTMVcpNSmA54–56	5′KGK54–56	ACATCC**GC**AGGA**GC**GATACTGTTGAACACAGAG
	3′KGK54–56	GTTCAACAGTATC**GC**TCCT**GC** GGATGTATCCATAGCTTTAG
pTMVcpNSmA93–94	5′HH93–94	ACAAAC**GCTGC**TATCTCAAACTGGAAAAATG
	3′HH93–94	TTTCCAGTTTGAGATA**GCAGC**GTTTGTATCTACTATCATTC
pTMVcpNSmA101	5′D101	TCAAACTGGAAAAATG**C**TCTTTTTGTTGGCAAC
	3′D101	GTTGCCAACAAAAAGA**G**CATTTTTCCAGTTTGA
pTMVcpNSmA122–125	5′DSRK122–125	ACTTGGGCCAGCGCAGCACAATACATGATGATTTCCA
	3′DSRK122–125	CATGTATTGT**GC**T**GC**GCTG**G**CCCAAGTCGGACAGATCTTG
pTMVcpNSmA154	5′D154	GTGGTTGCCTTGGTTGCTCCCAACATGCCATCT
	3′D154	AGATGGCATGTTGGGA**G**CAACCAAGGCAACCAC
pTMVcpNSmA160–161	5′EK160–162	CCATCTG**C**A**GC**GCAAGTCATTCTGAAAGGTC
	3′EK160–162	TTTCAGAATGACTTGC**GCTG**CAGATGGCATGTTGGGATCA
pTMVcpNSmA205–208	5′EYKK205–208	AGCCAAG**C**ATAC**GC**G**GC**GGGGGTTTCTTTTGGTAGTA
	3′EYKK205–208	AGAAACCCCC**GCC**G**C**GTAT**G**CTTGGCTGCACATCAAATGC
pTMVcpNSmA230–233	5′DKDK230–233	GCTG**CTGC**AG**CTGC**AAGTTGCATGGTCATACCTC
	3′DKDK230–233	GACCATGCAACTT**GC**A**G**CT**GC**A**G** CAGCTCTGGGTGAATCACA
pTMVcpNSmA269–274	5′KKQLKK269–274	ATT**GC**A**GC**ACAGCTT**GC**AG**C**ATTGAGCTTAAATCTTGAGA
	3′KKQLKK269–274	GCTCAAT**GCTGC**AAGCTGT**GCTGC**AATCTGCTTTTCACTGTTTC
pTMVcpNSmA284–288	3′EEEEE284–288	ACTCGAGTTATATTTCATCAAAAGACAACTGAGCAACACTGTCAGAAAT
		CCCT**G**CC**G**CT**G**CT**G**CT**GC**AACTGATCTCTCAAGATTT

For deletion mutants, the start codon 'ATG' added to N-terminus of the N-terminal deletion mutants and the stop codon 'TAA' linked to C-terminus of the C-terminal deletion mutants are in italics. The altered bases in alanine-substitution mutants are shown in bold. The restriction recognition sequences of EcoRV and XhoI are underlined.

Materials and methods

Construction of NSm mutants

The NSm deletion mutants were constructed by stepwise deletion of nucleotides in increments encoding approximately 50 amino acid residues from the N- or C-terminus. Using one of the forward primers 5′NSm148–169, 5′NSm289–310, 5′NSm439–460 or 5′NSm583–602 with reverse primer 3′XhoI-NSm (Table 2), four N-terminal deletion constructs, pTMVcpNSmN50, pTMVcpNSmN97, pTMVcpNSmN147 and pTMVcpNSmN195, respectively, were created by polymerase chain reaction (PCR) with the *NSm7-1* gene as template. Five C-terminal deletion constructs, pTMVcpNSmC49, pTMVcpNSmC96, pTMVcpNSmC146, pTMVcpNSmC195 and pTMVcpNSmC248, were constructed with a similar PCR-based strategy using forward primer 5′ EcoRV-NSm with one of the reverse primers 3′NSm147–128, 3′ NSm288–268, 3′NSm438–419, 3′NSm585–565 or 3′NSm744–724, respectively (Table 2). pTMVcpNSmM50–195 is a construct that encodes the central region of NSm, and was generated with the primer pair 5′NSm148–169 and 3′ NSm585–565 (Table 2). The resulting PCR products were digested with EcoRV and XhoI and ligated into EcoRV–XhoI-digested pTMVcpGFP as described by Lewandowski and Adkins (2005).

Eleven alanine-substitution NSm mutants were generated with the primers listed in Table 2. The mutations in pTMVcpNSmA15–18 and pTMVcpNSmA284–288 were introduced by direct PCR amplification, whereas the mutations in pTMVcpNSmA54–56, pTMVcpNSmA93–94, pTMVcpNSmA101, pTMVcpNSmA122–125, pTMVcpNSmA154, pTMVcpNSmA160–161, pTMVcpNSmA205–208, pTMVcpNSmA230–233 and pTMVcpNSmA269–274 were introduced by overlapping PCR (Ho et al., 1989). The resulting PCR products were digested with EcoRV and XhoI and ligated into EcoRV–XhoI-digested pTMVcpGFP as above.

In vitro transcription and inoculation of protoplasts and plants

Transcripts produced from KpnI-linearized plasmids with T7 RNA polymerase (Lewandowski and Dawson, 1998) were used for transfection of protoplasts and inoculation of plants. Protoplasts were derived from a high temperature-adapted tobacco suspension cell line and transfected as previously described (Lewandowski and Dawson, 1998, 2000). At 24 hpi, an aliquot of protoplasts was used for indirect immunofluorescence analysis of tubule formation. The remaining protoplasts were collected by centrifugation and the pellets stored at −80 °C. Wild-type *Nicotiana benthamiana* and Xanthi nc, and TMV MP-transgenic NB-MP(+) and NN-MP(+) plants were grown and maintained in a greenhouse at 25 °C, and inoculated as previously described (Dawson et al., 1986).

Indirect immunofluorescence, Northern blotting and Western blotting

Indirect immunofluorescence analysis of NSm localization in protoplasts was as previously described (Kikkert et al., 1997; Lewandowski and Adkins, 2005; van Lent et al., 1991). Equivalent amounts of total RNA (quantified using a NanoDrop spectrophotometer; NanoDrop Technologies, Wilmington, DE) extracted from protoplasts or leaves were analyzed by Northern blotting using a TMV 3′UTR-specific probe (Lewandowski and Dawson, 1998). For Western blotting, the tobacco protoplasts were processed as described by Bertens et al. (2000), except that anti-NSm antiserum (kindly supplied by John Sherwood, University of Georgia) was used as the primary antibody. Briefly, the frozen protoplast pellet (~2×10^6 cells) was suspended in 200 µl extraction buffer (50 mM Tris-acetate, pH 7.4, 10 mM potassium acetate, 1 mM EDTA, 5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride) and fractionated by centrifugation at 30,000 ×*g* for 30 min at 4 °C. The resulting supernatant (S30) was collected and the pellet (P30) was suspended in 200 µl of the same buffer. 5 µl of S30 and P30 from protoplasts transfected with each mutant was analyzed by electrophoresis on 12% SDS-polyacrylamide gels, blotted to PVDF membrane and probed with NSm antiserum.

Computer analysis of TSWV NSm protein

Tospovirus NSm protein sequences were aligned with ClustalX 1.83 (Thompson et al., 1997) and viewed with GeneDoc (Nicholas and Nicholas, 1997). Hydrophobicity was analyzed with Kyte-Doolittle hydrophobic scale (http://www.vivo.colostate.edu/molkit/hydropathy/index.html) (Kyte and Doolittle, 1982). The coiled-coil domain was predicted by Coiled-coil predictions (http://www.russell.embl-heidelberg.de/cgi-bin/coils-svr.pl) (Lupas et al., 1991) and the transmembrane domain prediction was calculated by the Dense Alignment Surface method available on the DAS server (http://www.sbc.su.se/~miklos/DAS/tmdas.cgi) (Cserzo et al., 1997).

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