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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