The tubule-forming NSm protein from *Tomato spotted wilt virus* complements cell-to-cell and long-distance movement of *Tobacco mosaic virus* hybrids

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Abstract

A Florida isolate of *Tomato spotted wilt virus* (TSWV) was able to complement cell-to-cell movement of a movement-defective *Tobacco mosaic virus* (TMV) vector expressing the jellyfish green fluorescent protein (GFP). To test for complementation of movement in the absence of other TSWV proteins, the open reading frame for the NSm protein was expressed from TMV constructs encoding only the TMV replicase proteins. NSm was expressed from either the coat protein or movement protein subgenomic promoter, creating virus hybrids that moved cell to cell in inoculated leaves of tobacco, providing the first functional demonstration that NSm is the TSWV movement protein. Furthermore, these CP-deficient hybrids moved into upper leaves of *Nicotiana benthamiana*, demonstrating that NSm can support long-distance movement of viral RNAs. Tubules, characteristic of the NSm protein, were also formed in tobacco protoplasts infected with the TMV–TSWV hybrids. The C-terminus of the NSm protein was shown to be required for movement. TMV–TSWV hybrids expressing NSm and GFP moved within inoculated leaves. Our combination of single-cell and intact plant experiments to examine multiple functions of a heterologous viral protein provides a generalized strategy with wider application to other viruses also lacking a reverse genetic system.

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Introduction

*Tomato spotted wilt virus* (TSWV), genus *Tospovirus*, family Bunyaviridae is the causal agent for large economic losses in a wide variety of plant hosts. TSWV has an ambisense RNA genome divided between three RNAs designated S, M, and L (Fig. 1; Adkins, 2000). The M RNA encodes the NSm protein in the virion-sense and the complement encodes the glycoprotein precursor. The enveloped virions contain the viral RNAs, which are associated with the nucleocapsid (N) protein, and several other viral proteins including the RNA-dependent RNA polymerase (RdRp). The complexity of virions and the unusual genome organization and replication strategy have so far prevented the development of a cloned genetic system for the genus *Tospovirus*. Lack of a genetic system has made it impossible to conduct standard mutagenesis for functional studies of some viral gene products. In contrast, with the development of infectious clones of *Tobacco mosaic virus* (TMV) nearly 20 years ago (Dawson et al., 1986), powerful reverse genetics approaches have been used to dissect functions of viral proteins and sequence elements (reviewed by Scholthof, 2004).

Plant viruses encode one or more proteins that are required for movement within plants (Hull, 2002). Plant virus movement proteins (MP) have been shown to nonspecifically bind to nucleic acids, modify plasmodesmata, and in certain genera to induce the formation of tubules on the surface of protoplasts (reviewed by Waigmann et al., 2004). The first viral protein shown to have a movement
function is the TMV 30-kDa protein (Deom et al., 1987; Meshi et al., 1987), which remains the prototypical plant virus MP (Citovsky, 1999). The TMV MP localizes to (Tomenius et al., 1987), and modifies the size exclusion limit of plasmodesmata (Wolf et al., 1989), binds RNA non-specifically (Citovsky et al., 1990), associates with microtubules (Heinlein et al., 1995), requires phosphorylation for activity (Citovsky et al., 1993; Watanabe et al., 1992), and binds to pectin methyl esterase (Chen et al., 2000; Dorokhov et al., 1999).

There are many examples of a heterologous viral MP complementing movement of another, sometimes unrelated virus, which can be delivered through stable or transient expression or by co-infection (Waigmann et al., 2004). Although many of the complementation experiments involved tobamoviruses (Malysenko et al., 1989; Atabekov et al., 1999), complementation of movement between different viral genera has been documented, even between viruses with differing numbers of MPs (Solovyev et al., 1996; Taliantsky et al., 1982). Perhaps not surprisingly, regions of sequence similarity have been identified between tubule- and non-tubule-forming MPs (Melcher, 2000). Trans-complementation of movement is not a universal phenomenon and the ability of one virus to complement is not a prediction of the reciprocal complementation (Atabekov et al., 1999; Maule, 1991). Furthermore, trans-complementation of movement may require other viral proteins or replication of the helper virus (Lauber et al., 1998; Morozov et al., 1997; Tamai et al., 2003).

Although the TSWV NSm protein performs many of the functions attributed to plant virus MPs including association with, and modification of plasmodesmata (Kormelink et al., 1994; Prins et al., 1997; Storms et al., 1998), tubule formation (Storms et al., 1995), RNA binding activity and interaction with N protein and host factors (Soellick et al., 2000), NSm has not been functionally demonstrated to be a
movement protein. We show that TSWV is capable of complementing movement of an MP-deficient TMV vector, thus providing a heterologous genetic platform with which to test MP functionality. The NSm open reading frame (ORF) from a Florida isolate of TSWV was cloned in place of the TMV 30-kDa MP to test for its ability to complement TMV movement. When full-length NSm was expressed from either the TMV MP or coat protein (CP) subgenomic (sg) promoter, the virus hybrids moved cell to cell in plants. Expression of NSm also complemented long-distance movement of these CP-deficient hybrids. TMV hybrids expressing NSm were capable of forming tubules on the surface of infected tobacco protoplasts. Furthermore, we present a general strategy combining viral vectors, protoplasts, wild-type and MP-transgenic plants to dissect functions of MPs of viruses lacking a reverse genetics system.

**Results**

**Characterization of TSWV isolate FL 7-1**

The Florida TSWV isolate FL 7-1 produced local lesions on *Nicotiana glutinosa* 5 days post-inoculation (p.i.). FL 7-1 produced typical TSWV symptoms on *N. rustica* (chlorotic/necrotic rings on inoculated leaves; systemic leaf deformation and necrosis), *Datura stramonium* (chlorotic rings on inoculated leaves; systemic chlorosis and leaf deformation), *Nicotiana benthamiana* (chlorotic spots on inoculated leaves; systemic chlorosis and leaf deformation), *Petunia × hybrida* (necrotic local lesions on inoculated leaves), and *Solanum bahamense* (chlorotic rings on inoculated leaves; systemic leaf deformation). Nucleotide and deduced amino acid sequences of a 579 base pair region of an RT-PCR product were 95–98% and 96–98% identical, respectively, to TSWV N gene sequences in GenBank.

**TSWV complements a movement-defective TMV vector**

TSWV isolate FL 7-1 was tested for its ability to complement cell-to-cell movement of a movement-defective TMV vector 30B-GFPC3/TE1 (Fig. 1A; Rabindran et al., 2005), which expresses a modified green fluorescent protein (GFP) from *Aequorea victoria*, but is unable to move beyond initially infected cells. 30B-GFPC3/TE1 did not move cell to cell in inoculated leaves of *N. benthamiana* demonstrating that the inoculum did not contain revertants that regained a functional TMV MP (Fig. 1B). However, cell-to-cell movement of 30B-GFPC3/TE1 was complemented in transgenic *N. benthamiana* plants expressing TMV MP [NB-MP(+)] (Fig. 1C). To test for complementation of movement, *N. benthamiana* plants inoculated with FL 7-1 were subsequently inoculated with 30B-GFPC3/TE1 1 to 4 days later. FL 7-1 was able to complement cell-to-cell movement of the MP-deficient TMV vector, as evidenced by the appearance of foci expressing GFP in inoculated leaves (Fig. 1D). This result suggested that one or more TSWV proteins functionally substituted for the TMV 30-kDa MP. Inoculation with TSWV 2 or 3 days prior to inoculation with 30B-GFPC3/TE1 gave the optimal complementation based on the number of GFP foci formed.

To confirm that fluorescence in co-inoculated leaves of *N. benthamiana* was due to GFP expression from the TSWV-complemented movement of 30B-GFPC3/TE1, leaf disks were excised from fluorescing and non-fluorescing areas of co-inoculated leaves. Northern blot hybridization of total RNA extracted from leaf disks revealed a strong 30B-GFPC3/TE1 hybridization signal from areas of the leaf expressing GFP, yet only a weak signal from non-fluorescing areas (Fig. 1F). This result indicated that 30B-GFPC3/TE1 moved in inoculated leaves. To confirm that movement of the vector was due to TSWV complementation and not reversion of the movement defect, fluorescing tissue from inoculated leaves, stems, petioles, and upper non-inoculated leaves was homogenized in a high pH buffer suitable for TMV transfer, but not conducive for TSWV transmission. *N. benthamiana* and NB-MP(+) plants were inoculated with sap either immediately after homogenization or following a 1-h incubation at room temperature. One week after inoculation, no GFP expression was observed on the wild-type *N. benthamiana* assay plants. However, similar numbers of GFP spots were present on NB-MP(+) plants from sap inoculated immediately or after a 1-h incubation. These results demonstrate that 30B-GFPC3/TE1 had moved beyond inoculated leaves and had not reverted.

**NSm supports TMV cell-to-cell movement in tobacco**

The NSm ORF from the FL 7-1 TSWV isolate was cloned and sequenced (GenBank accession no. AY956380) and was found to be 93–98% and 94–100% identical at the nucleotide and amino acid levels, respectively, to TSWV NSm sequences available in GenBank. The NSm ORF was cloned behind the TMV CP and MP sg promoters in cpNSm and mpNSm, respectively (Fig. 2A). cpNSm encodes the native NSm protein, whereas mpNSm encodes a fusion protein consisting of the first nine amino acids of the TMV MP plus two additional amino acids fused to the N-terminus of NSm.

To test for infectivity of the TMV–TSWV hybrids, pTMVmpNSm and pTMVcpNSm were linearized with *Kpn*I, and in vitro RNA transcripts were produced with T7 RNA polymerase and used to transfect tobacco suspension cell protoplasts. Northern blot hybridization of total RNA extracted at 22 h p.i. revealed that mpNSm and cpNSm both replicated and produced a novel sg RNA of ~1.4 kb, as expected (Fig. 2B, lanes 2 and 3).

To test whether the NSm protein alone was sufficient to complement TMV movement in *N. tabacum*, transcripts of mpNSm and cpNSm were used to independently inoculate expanded leaves of susceptible (Xanthi) and resistant
As a positive control for cell-to-cell movement, tobacco plants homozygous for the resistance gene \( N \) and the TMV MP [NN-MP(\(+\)) were also inoculated. Both hybrids spread cell to cell and produced local lesions on the control NN-MP(\(+\)) tobacco plants indicating that the hybrids were viable in plants (Fig. 3A). cpNSm and mpNSm also moved cell to cell in Xanthi nc tobacco (Fig. 3A) indicating that NSm alone can complement TMV movement. cpNSm also produced local lesions on Xanthi tobacco, suggesting that NSm is a symptom determinant in tobacco.

In an attempt to disrupt the movement function of NSm, sequences encoding the C-terminal 54 (cpNSm248) and 107 (cpNSm195) amino acids of the NSm ORF were deleted from cpNSm (Fig. 2A). Both hybrids replicated in tobacco protoplasts and produced smaller sg RNAs than cpNSm (Fig. 2B, compare lanes 3–5). To determine whether the truncated NSm protein was able to support cell-to-cell movement, tobacco plants were inoculated with infectious RNA transcripts. cpNSm195 and cpNSm248 moved cell to cell and formed local lesions in inoculated leaves of the control NN-MP(\(+\)) plants. cpNSm195 did not form lesions on Xanthi nc tobacco suggesting that there was no cell-to-cell movement. However, cpNSm248 formed tiny local lesions in Xanthi nc tobacco, suggesting that removal of 54 amino acids greatly debilitated function(s) required for movement.

**NSm supports long-distance movement of TMV hybrids in the absence of CP**

TMV CP deletion mutants move inefficiently in tobacco (Dawson et al., 1988), but can move long distances in \( N. \) benthamiana (Knapp et al., 2001). To test whether NSm could support movement of TMV hybrids lacking the TMV CP, \( N. \) benthamiana plants were inoculated with infectious transcripts of cpNSm or mpNSm. Both hybrids moved beyond the inoculated leaves and induced symptoms on upper non-inoculated leaves, indicating that NSm can also support long-distance movement of TMV mutants that do not form virions (Fig. 4E and data not shown). cpNSm produced more severe symptoms than mpNSm in upper leaves. TMV CP is not required for movement into upper non-inoculated leaves of \( N. \) benthamiana (Fig. 4F), suggesting that the TMV 30-kDa MP and NSm are functionally equivalent to move TMV RNAs in this host.

**NSm induces symptoms in \( N. \) benthamiana**

cpNSm produced necrotic lesions on inoculated leaves of \( N. \) benthamiana beginning 5–6 days p.i. (Fig. 4A). C-terminal deletions of the NSm protein resulted in hybrids that did not produce symptoms in inoculated leaves of \( N. \) benthamiana (Fig. 4D) indicating that these hybrids did not move in \( N. \) benthamiana or that the infection was symptomless. However, in NB-MP(\(+\)) plants, cpNSm248 formed chlorotic lesions (data not shown), whereas cpNSm195 formed concentric necrotic rings (Fig. 4B) indicating that N-terminal sequences are involved in symptom expression in \( N. \) benthamiana.

To determine whether the lack of symptoms in \( N. \) benthamiana was due to lack of movement, total RNA was extracted from the leaves of \( N. \) benthamiana and NB-MP(\(+\)) plants inoculated with cpNSm195, cpNSm248, cpNSm, or the TMV CP deletion mutant S3-28 (Dawson et al., 1988) and analyzed by Northern blot hybridization. Inoculated leaves of control \( N. \) benthamiana plants ino-
lated with cpNSm or S3-28 accumulated high levels of viral RNA (Fig. 4C, lanes 1 and 6, respectively). In contrast, cpNSm195 and cpNSm248 accumulated high levels of viral RNA only in the control NB-MP(+) plants (Fig. 4C, lanes 2 and 4, respectively), and did not accumulate detectable levels of viral RNA in inoculated leaves of *N. benthamiana* (Fig. 4C, lanes 3 and 5, respectively), indicating that C-terminal deletion of at least 54 amino acids rendered NSm non-functional for movement in *N. benthamiana*.

**TMV hybrids expressing NSm produce tubules**

Tubules are formed in TSWV-infected protoplasts or protoplasts transiently expressing NSm (Storms et al., 1995, Kikkert et al., 1997). To determine whether the TMV hybrids were a useful system to examine tubule formation, protoplasts were transfected with cpNSm, mpNSm, or wild-type TMV, or mock inoculated. Protoplasts were harvested at 22–24 h p.i. and processed for indirect immunofluorescence to detect NSm expression. Weak background fluorescence and no tubules were observed for mock-inoculated (Fig. 5C) and TMV-infected protoplasts (Fig. 5D). However, cpNSm- and mpNSm-infected protoplasts had numerous tubules emanating from the cell surface (Figs. 5A and B) and had bright fluorescence on the cell surface and surrounding the nucleus. These results indicate that NSm induced the formation of tubules in protoplasts transfected with cpNSm and mpNSm.

**TMV–TSWV hybrid vectors express GFP**

As another means to visualize cell-to-cell movement, a cassette consisting of the TMV CP sg promoter and GFP was inserted into cpNSm and mpNSm, creating cpNSm-
GFP and mpNSm-GFP, respectively (Fig. 2A). N. benthamiana and Xanthi tobacco inoculated with cpNSm-GFP or mpNSm-GFP were observed at various times post-inoculation under UV illumination. Both cpNSm-GFP and mpNSm-GFP moved cell-to-cell and expressed GFP (data not shown).

Discussion

Demonstrating viral protein function in the absence of a reverse genetics system is complicated because of the inability to do even simple mutagenesis experiments. It has proven difficult to create an infectious clone of viruses with negative-strand or ambisense genomes, such as those within the genus Toospovirus. Despite several lines of evidence showing that the TSWV NSm protein has characteristics of plant virus movement proteins, including sequence similarity, expression during the early stages of infection, RNA-binding activity, and the ability to induce the formation of tubules (Kormelink et al., 1994; Melcher, 2000; Soellick et al., 2000; Storms et al., 1995, 1998), a movement function has never been proven. Taking an approach that combined in planta complementation experiments, cloning and mutagenesis of the putative MP, and the construction of virus hybrids, we overcame the lack of a
reverse genetics system for TSWV and demonstrated that
NSm is indeed the TSWV movement protein.

Certain plant viruses are capable of complementing cell-
to-cell movement of other unrelated viruses (Taliansky et
al., 1982; reviewed by Maule, 1991 and Waigmann et al.,
2004). We showed that a typical Florida isolate of TSWV
could complement cell-to-cell and long-distance movement
of a movement-defective TMV vector expressing GFP.
Success with this approach depends upon having a common
host, in this case \textit{N. benthamiana}. While not essential, the
movement-defective 30B-GFPC3/TE1 vector and a suitable
MP-transgenic host for its propagation made it easier to
optimize inoculation conditions. We found that allowing
TSWV to replicate and move for 2–3 days gave optimal
complementation of 30B-GFPC3/TE1 movement in \textit{N.
benthamiana}. Complementation results were verified by
Northern blot analysis from GFP-expressing and non-
fluorescent leaf tissue (Fig. 1F). Back-inoculation to \textit{N.
benthamiana} and NB-MP(+) plants demonstrated that virus
was present in non-inoculated tissues and that the vector
remained movement defective.

Replacement of the TMV MP with the TSWV NSm
protein resulted in hybrid viruses that moved in plants. The
formation of local lesions in Xanthi nc tobacco by cpNSm
and mpNSm indicated that NSm functionally substituted for
the TMV 30-kDa protein. C-terminal truncation of NSm
greatly reduced the ability to move cell to cell, indicating
that this region is essential for movement. We observed tiny
local lesions in cpNSm248-inoculated Xanthi nc tobacco
plants, suggesting that removal of the C-terminal 54 amino
acids of NSm greatly affected, but did not completely
destroy the movement function. However, removal of 107
amino acids totally abolished viral movement.

TMV does not require CP for cell-to-cell movement
(Dawson et al., 1988) or long-distance movement in certain
hosts, such as \textit{N. benthamiana} (Knapp et al., 2001). cpNSm
and mpNSm moved beyond inoculated leaves of \textit{N.
benthamiana} indicating that NSm was sufficient to comple-
tment long-distance movement of these hybrids. A similar
pattern of movement in \textit{N. benthamiana} was observed for
the CP deletion mutant S3-28, which retains the TMV MP
(Knapp et al., 2001; Fig. 4F). The TSWV N and NSm
proteins co-localize with tubules and interact (Soellick et al.,
2000; Storms et al., 1995), which suggests that nucleopro-
tein complexes of viral RNA and N protein normally move
through tubules. Our hybrids do not express TSWV N

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5.png}
\caption{Tubule formation in protoplasts infected by TMV hybrids expressing NSm. Tobacco suspension cell protoplasts transfected with infectious transcripts of
cpNSm (A), mpNSm (B), wild-type TMV (D), or mock-inoculated with water (C) were harvested at 22 h p.i. NSm was detected by indirect
immunofluorescence with anti-NSm antiserum and FITC-conjugated secondary antibody and viewed on a confocal microscope. Scale bar = 8.0 \mu m.}
\end{figure}
protein or TMV CP indicating that virion formation is not required for NSm to traffic TMV RNAs. This raises the question of whether TSWV RNAs naturally move in the absence of N.

TSWV produces dramatic symptoms on many hosts, yet the symptom determinant has not been mapped. Progress in this area has been slowed by the lack of an infectious clone and has been restricted to sequence analysis of different isolates and the selection of reassortants (Qiu et al., 1998). Interestingly, cpNSm caused necrosis or necrotic local lesions on Xanthi tobacco and N. benthamiana, responses not typical of TMV infection in these hosts. Although deletion of the C-terminal 54 amino acids almost abolished movement, it was possible to examine the effects of this deletion on symptom expression through the inoculation of N. benthamiana plants expressing the TMV MP. On NB-MP(+)-plants, TMV MP supplied in trans complemented the cell-to-cell movement defect of the hybrids expressing truncated NSm protein. cpNSm248 formed chlorotic lesions that eventually became necrotic, whereas cpNSm195 formed concentric necrotic rings. In contrast, cpNSm195 and cpNSm248 failed to accumulate to detectable levels in inoculated leaves of N. benthamiana (Fig. 4C), consistent with the failure to induce symptoms. These results indicate that C-terminal NSm sequences are required for cell-to-cell movement and suggest that N-terminal sequences have a role in TSWV symptom expression in N. benthamiana.

In contrast, mpNSm did not induce necrosis on Xanthi tobacco, and produced milder symptoms on both inoculated and systemically infected leaves of N. benthamiana. cpNSm expresses the authentic NSm protein, whereas mpNSm expresses the NSm protein fused to the N-terminus of the TMV MP. TMV MP and CP expression is differentially regulated, both temporally and quantitatively; CP is expressed later and accumulates to much greater levels than MP (Lehto et al., 1990). Any of these factors may have contributed to the milder symptoms observed for mpNSm.

The relative simplicity of our system, which is based on virus hybrids containing three viral proteins, coupled with the availability of TMV MP transgenic plants that complemented movement of all hybrids tested is a robust system to examine the role of NSm in symptomatology. The NSm alone is capable of inducing tubule formation in protoplasts (Storms et al., 1995). NSm was expressed from both the TMV CP and MP sg promoters in tobacco protoplasts. Protoplasts infected with TMV–TSWV hybrids cpNSm or mpNSm reacted with anti-NSm antiserum and produced the tubules characteristic of NSm and TSWV infection. No tubules were observed with cpNSm248- or cpNSm195-infected protoplasts although fluorescence was observed (data not shown). The TMV hybrid approach offers the ability to rapidly mutate the MP ORF and quickly ascertain the effects in vivo in protoplasts and plants.

The GFP ORF was inserted into the original hybrids as a means to visualize vector movement. GFP was expressed in plants inoculated with cpNSm-GFP and mpNSm-GFP, but was restricted to inoculated leaves. The approach of inserting gene cassettes consisting of a viral sg promoter and reporter gene enables the rapid and systematic addition of genes into these constructs and subsequent testing. Further development of these constructs as vectors would include a similar approach to the re-introduction of the CP gene to facilitate long-distance movement.

We have developed an approach to dissect multiple functions of a protein from a virus lacking a reverse genetics system. We showed through a complementation assay with a movement-defective viral vector that TSWV could complement TMV movement. Next, we exploited the power of the TMV reverse genetics system to create virus hybrids expressing the putative TSWV MP. Through in planta experiments, we functionally demonstrated that NSm is indeed the TSWV MP. We have provided the first evidence of the ability of NSm to confer virus movement in the absence of other TSWV proteins. Using the same virus hybrids and a protoplast system, we also demonstrated the utility of this system to examine the role of NSm function in tubule formation. While this function has been known (Storms et al., 1995), we now have a system and the ability to mutagenize the NSm ORF and examine several interrelated functions associated with virus movement.

Mutagenic approaches have identified functional domains within other tubule-forming MPs including Cowpea mosaic virus (Bertens et al., 2003) and Cauliflower mosaic virus (Thomas and Maule, 1995), although infectious clones existed for these viruses. Stable and transient expression approaches have also been used to demonstrate the ability of putative MPs to support cell-to-cell movement of mutants or unrelated viruses. Huang et al. (2005) recently identified the MP from a negative-stranded RNA virus using particle bombardment of leaf tissue with a construct expressing the putative MP of Rice yellow stunt rhabdovirus. However, trans-complementation approaches such as these have the limitation of being restricted to examining cell-to-cell movement and will likely require additional cloning to examine other functions such as cellular localization or...
tubule formation in protoplasts. In contrast, virus hybrids such as those from this study enable using a single construct to examine multiple functions.

Transgenic plants expressing the TMV MP were valuable as controls for infectivity, but perhaps more importantly as tools that may help to dissect the symptom determinants of NSm or other viral proteins. Following mutagenesis of the NSm ORF, movement and symptom expression can be monitored on pairs of transgenic and non-transgenic local lesion and systemic hosts, respectively.

Our results conclusively demonstrated that NSm is the movement protein of TSWV and that NSm can functionally substitute for the TMV 30-kDa MP. Furthermore, this approach has a wider application to other viruses lacking a reverse genetics system. The minimum requirements are two viruses with an overlapping host range, an infectious clone of one virus or vector that has been rendered movement-defective, and a vector to construct the hybrids containing the putative MP. Complementation experiments can even be established without knowing the identity of the experimental virus as we have recently found several unknown viruses that were able to complement movement of 30B-GFPC3/TE1 much better than TSWV. Although not essential, MP transgenic plants provide both an easy source to propagate the movement-defective viral-vector and serve as a positive control for infectivity of virus hybrids. The latter is particularly important in the absence of a protoplast system. It may even be possible to use available transgenic plants expressing a heterologous MP, if those plants complement movement of the vector. Protoplast studies have the potential to separate functional domains, such as those required for tubule formation from those associated with movement and symptomatology.

**Materials and methods**

**Plant material**

Wild-type *N. benthamiana* and transgenic *N. benthamiana* homozygous for the TMV MP, designated NB-MP(+) (Giesman-Cookmeyer et al., 1995), were grown and maintained in a growth room at 25°C under a 14-h light/10-h dark regimen. F1 progeny from a cross between *N. tabacum* cv. Xanthi line 26c transgenic for the TMV MP (Gera et al., 1995) and *N. tabacum* cv. Xanthi nc (carrying the *N* gene for resistance to TMV) were selfed. Seed from F2 progeny determined to be homozygous for both the TMV MP and the resistance gene *N* against tobamoviruses gave rise to a line designated NN-MP(+) tobacco. *N. tabacum* cv. Xanthi, *N. tabacum* cv. Xanthi nc, NN-MP(+) tobacco, *N. rustica*, *N. glutinosa*, *Solanum americanum*, *S. bahamense*, and *D. stramonium* plants were grown in a greenhouse. TSWV host range studies were conducted in a greenhouse and plants inoculated with TMV hybrids were maintained in a growth room.

**Isolation and characterization of TSWV isolate FL 7-1**

Eight isolates of TSWV were collected in May 2001 from tomato and pepper research plots in Bradenton, FL at the University of Florida’s Gulf Coast Research and Education Center. Isolate 7, collected from a tomato plant with leaf purpling and curling symptoms, was selected for further study. An *N. glutinosa* plant was inoculated with tomato tissue homogenized in 0.5% (w/v) sodium sulfate. Isolate FL 7-1 was derived from the first local lesion collected from *N. glutinosa*. This single local lesion was ground and used to inoculate single American black nightshade (*S. americanum*) and jimsonweed (*D. stramonium*) plants. Following development of systemic chlorosis and necrosis in *S. americanum* and chlorotic rings in *D. stramonium*, the virus was propagated in additional plants of the same species.

Several indicator host plants were inoculated with sap from either infected *S. americanum* or *D. stramonium*. Presence of TSWV was confirmed by enzyme-linked immuno-nosorbent assay using a commercially available kit (Agdia, Elkhart, IN) and RT-PCR amplification of a fragment of the nucleocapsid (N) gene with primers TSWV722 (TSWV S RNA nts 2098–2118) and TSWV723 (complement of TSWV S RNA nts 2698–2717) (Adkins and Rosskopf, 2002). The sequence of the cloned RT-PCR product was compared to TSWV N gene sequences in GenBank.

**Complementation of TMV-GFP movement with TSWV**

To test for TSWV complementation of TMV movement, pairs of *N. benthamiana* plants were inoculated with sap from TSWV FL 7-1-infected *D. stramonium* leaves. One, 2, 3, or 4 days p.i., the same leaves were inoculated with 30B-GFPC3/TE1 (Rabindran et al., 2005), a movement-defective derivative of TMV vector 30B-GFPC3 (Shivprasad et al., 1999). Additional *N. benthamiana* plants were inoculated with 30B-GFPC3/TE1 or TSWV FL 7-1. As a positive control for 30B-GFPC3/TE1 movement, NB-MP(+) plants were inoculated. Plants were evaluated for symptoms daily for 1 week and thereafter weekly until their collapse. GFP expression was visualized by illumination with a hand-held long-wave UV lamp as previously described (Shivprasad et al., 1999).

Single GFP spots (1 cm) from leaves pre-inoculated with TSWV and subsequently inoculated with 30B-GFPC3/TE1 were ground in glycine phosphate buffer containing celite (Knapp et al., 2001). One pair of *N. benthamiana* and NB-(MP+) plants was inoculated immediately and a second set of plants was inoculated after incubating the sap for 1 h at room temperature. Seven days p.i., plants were evaluated for the total number of GFP fluorescing spots. Total RNA from GFP-expressing and non-fluorescing regions of inoculated leaves was analyzed by Northern blotting using GFP- (Shivprasad et al., 1999) and TMV-3′UTR probes (Lewandowski and Dawson, 1998).
Cloning of NSm ORF and construction of TMV–TSWV hybrids

Cloning and construction of viral hybrids used established procedures (Ausubel et al., 1987). Total RNA was isolated from infected \textit{D. stramonium} leaves (Navas-Castillo et al., 1997). A 1-kb product containing the entire NSm open reading frame (ORF) and flanking sequences was amplified by RT-PCR using primers corresponding to nts 59–81 and complementary to nts 1040–1055 of the TSWV M RNA. The PCR product was cloned into pGEM-T (Promega) and sequenced on an ABI3700 automated sequencer at the USHRL Genomics Laboratory. The complete NSm ORF was amplified with PCR using forward primer 5'-GTTGAATATATTGAGACTCTTTCGGTAA-3' that added an \textit{EcoRV} site (underlined) and extra T residue (bold) upstream of the ATG, and reverse primer 3’-XhoII-NSm2 (5’T-TAAATCGAGTATTATTTTCAAAAGACAAAC-3’) that added an \textit{XhoII} site (underlined) immediately downstream of the stop codon, to create pT-NSm.

pTMV-GFP5750 contains the GFP ORF inserted between TMV nts 5750 and 5892 and is one of a series of derivatives of pTTT-GFP301 (Grdzelishvili et al., 2000) constructed by overlapping-PCR (Grdzelishvili and Lewandowski, unpublished). pTMVcpGFP is an MP-deficient derivative of pTMV-GFP5750 containing the CP sg promoter, GFP ORF and 3' proximal sequences from pTMVGFPS5750 inserted as a \textit{SalI}/\textit{BsiWI} fragment into \textit{XhoII}/\textit{BsiWI} fragment of NSm expression. Drops containing ca. 5 \times 10^4 protoplasts were spotted onto poly-L-lysine-coated slides (Polysciences, Inc., Warrington, PA). Samples were processed as described (van Lent et al., 1991; Kikkert et al., 1997) except that the anti-NSm antiserum (kindly supplied by John Sherwood) and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit antiserum (Sigma-Aldrich Corp., St. Louis, MO) were diluted 1:1000 and 1:40, respectively, in 150 mM NaCl, 2.7 mM KCl, 10 mM Na_2PO_4, 2 mM KH_2PO_4, pH 7.4 containing 1% bovine serum albumin and slides were incubated at 37 °C. After washing, samples were covered with a drop of Fluoromount G (Electron Microscopy Sciences, Hatfield, PA) and a cover slip and viewed with a TCS SL Confocal Microscope (Leica, Exton, PA).

In vitro transcripts

In vitro transcripts were produced from \textit{KpnI}-linearized plasmids with T7 RNA polymerase (Lewandowski and Dawson, 1998). Infectious transcripts were used to inoculate plants (Dawson et al., 1986) or transfect protoplasts derived from a high-temperature adapted tobacco suspension cell line (Lewandowski and Dawson, 1998, 2000). Total RNA was extracted 22 h p.i. and analyzed by Northern blotting using a TMV 3'-UTR-specific probe (Lewandowski and Dawson, 1998).

To determine whether the NSm deletion mutants moved in plants, total RNA from leaves of \textit{N. benthamiana} and NB-MP(+) plants inoculated with cpNSm, cpNSm195, cpNSm248, or S3-28 (Dawson et al., 1988) was extracted at 13 days p.i. and analyzed by Northern blot hybridization using a TMV 3'-UTR-specific probe (Lewandowski and Dawson, 1998).

Immunofluorescence

Protoplasts were harvested at 22–24 h p.i. for analysis of NSm expression. Drops containing ca. 5 \times 10^4 protoplasts were spotted onto poly-L-lysine-coated slides (Polysciences, Inc., Warrington, PA). Samples were processed as described (van Lent et al., 1991; Kikkert et al., 1997) except that the anti-NSm antiserum (kindly supplied by John Sherwood) and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit antiserum (Sigma-Aldrich Corp., St. Louis, MO) were diluted 1:1000 and 1:40, respectively, in 150 mM NaCl, 2.7 mM KCl, 10 mM Na_2PO_4, 2 mM KH_2PO_4, pH 7.4 containing 1% bovine serum albumin and slides were incubated at 37 °C. After washing, samples were covered with a drop of Fluoromount G (Electron Microscopy Sciences, Hatfield, PA) and a cover slip and viewed with a TCS SL Confocal Microscope (Leica, Exton, PA).

Cloning of NSm ORF and construction of TMV–TSWV hybrids

To develop a system to visualize movement of the TMV–TSWV hybrids, cpNSm and mpNSm were modified to express GFP. To create pTMVΔCla152, pTMVcpΔ–518/–245 (Grdzelishvili et al., 2000), was digested with \textit{SalI}, end-filled with T4 DNA polymerase, and digested with \textit{KpnI} and the resulting 0.94-kb fragment was ligated into pTMVΔCla digested with \textit{ClaI}, end-filled with T4 DNA polymerase, and digested with \textit{KpnI}. pTMV409 contains the \textit{ClaI}/\textit{BsiWI} fragment (nts 5662–6542) from p30B-GFP (Shivprasad et al., 1999) ligated into similarly digested pTMVΔCla152. pTMV415 contains the \textit{XhoII}/\textit{KpnI} fragment (nts 5892–6395) from pTMVΔ5713–5891 (Grdzelishvili et al., 2000) ligated into similarly digested pTMV409. To construct pTMVcpNSm-GFP and pTMVmpNSm-GFP, the \textit{SalI}/\textit{BsiWI} fragment from pTMV415 was ligated into \textit{XhoII}/\textit{BsiWI}-digested pTMVcpNSm and pTMVmpNSm, respectively. Wild-type TMV transcripts were prepared from pTMV004 (Lewandowski and Dawson, 1998).

Inoculation of plants and protoplasts with in vitro transcripts

In vitro transcripts were produced from \textit{KpnI}-linearized plasmids with T7 RNA polymerase (Lewandowski and Dawson, 1998). Infectious transcripts were used to inoculate plants (Dawson et al., 1986) or transfect protoplasts derived from a high-temperature adapted tobacco suspension cell line (Lewandowski and Dawson, 1998, 2000). Total RNA was extracted 22 h p.i. and analyzed by Northern blotting using a TMV 3'-UTR-specific probe (Lewandowski and Dawson, 1998).
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