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The Nonstructural NSm Protein of Tomato Spotted Wilt Virus Induces

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The expression and subcellular location of the 33.6-kDa nonstructural protein NSm of tomato spotted wilt virus (TSWV) was analyzed in *Nicotiana rustica* plants and protoplasts as a function of time. Immunofluorescent studies in protoplasts isolated from TSWV-infected *N. rustica* leaves showed that this protein could first be detected close to the periphery of the cell, near the plasmamembrane, and later in tubular structures emerging from the cell surface. *In situ,* these tubules appeared specifically in the plasmodesmata, suggesting their involvement in cell-to-cell movement of the virus during systemic infection. In protoplasts transfected with an expression vector containing the NSm gene, similar tubules were formed, indicating that NSm has the ability to form these structures in the absence of other virus-specific components. To test whether plant-specific components were involved in tubule formation, the NSm gene was also expressed in a heterologous expression system, i.e., insect cells. *Spodoptera frugiperda* and *Trichoplusia ni* cells were infected with a recombinant baculovirus expressing the NSm-gene (AcNPV/NSm). The efficient formation of NSm-containing tubules emerging from the surface of both cell types indicate that no plant-specific cell structures or proteins are involved in their development.
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INTRODUCTION

Viruses of the family Bunyaviridae are characterized by an enveloped particle morphology and a tripartite RNA genome. Most members infect animals (genera *Bunyavirus, Hantavirus, Nairovirus,* and *Phlebovirus*), but some are capable of infecting plants. These latter viruses are propagatively transmitted by thrips species (Wijkamp *et al.,* 1993; Ullman *et al.,* 1993) and are classified into a separate genus, *Tospovirus,* named after the type species *to*mato *spo*tted wilt virus (TSWV; Francki *et al.,* 1991). The tripartite RNA genome of TSWV contains five open reading frames, from which six functional proteins are derived (De Haan *et al.,* 1990, 1991; Kormelink *et al.,* 1991, 1992). Comparison of the genome of tospoviruses, exemplified by TSWV, with those of the animal-infecting members of the Bunyaviridae reveals the presence of an extra cistron in the tospoviral genome. This cistron is located on the M RNA segment and encodes a nonstructural protein of 33.6 kDa, denoted NSm (Kormelink *et al.,* 1991). Although some animal-infecting bunyaviruses also specify a protein referred to as NSm (e.g., bunyamwera virus, genus *Bunyavirus*), this polypeptide does not represent a separate gene product but forms part of the glycoprotein precursor and is assumed to have a function in virion assembly at the Golgi complex (Elliott, 1990; Nakitare and Elliott, 1993). Hence, the extra NSm gene of TSWV may well represent a genetic adaptation of this bunyavirus to plants as hosts. Kormelink *et al.* (1994) showed the early and transient appearance of the NSm protein in TSWV-infected plant tissue and its exclusive association to nucleocapsid aggregates and apparently modified plasmodesmata containing an electron-dense extension. Based on these results, it was suggested that the NSm protein represents the tospoviral movement protein involved in cell-to-cell movement of the virus. In this paper we report on the further analysis of the structural and functional properties of the NSm protein when expressed not only in plant, but also in insect cells. It is shown that tubular structures are specifically induced upon expression of NSm in both the plant and animal (insect) cell system.

MATERIALS AND METHODS

Virus isolate and antisera

In all experiments the Brazilian isolate BR-01 of TSWV was used (De Avila *et al.,* 1992). Polyclonal antisera against the nucleocapsid (N) and the nonstructural NSm protein, encoded by the viral M RNA, was prepared as previously described (Kormelink *et al.,* 1994; De Avila *et al.,* 1992). All antisera were used in a dilution of 1:1000.

Protoplast isolation and immunofluorescence microscopy

Nicotiana rustica plants were mechanically inoculated with TSWV-BR01. Protoplasts were isolated from system-

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ically infected leaves at daily intervals between 3 and 11 days postinoculation (Hibi *et al.,* 1975; Van Beek *et al.,* 1985). The protoplasts were processed for immunofluorescence microscopy immediately after isolation and studied with a Leitz Laborlux S epifluorescent microscope and a Bio-Rad MRC 500 confocal scanning laser microscope (CSLM) containing an argon ion laser and a BHS 488-nm excitation filter essentially as described by Van Lent *et al.* (1991).

Construction of pMon999/NSm and transfection of protoplasts

In order to clone the NSm gene without the 5′ untranslated sequence, the gene was PCR-amplified using oligonucleotides Zup 051 (dGGGAATTCTTTTCGGTAACAAGAGGCC) and Zup014 (dCCCTGCAGGATCCGAAATTTAAGCTTAAATAAGTG). After PCR amplification, the DNA fragment was digested with *Eco*RI, ligated to a *Bam*HI–*Eco*RI adaptor (containing the nucleotide sequence GGATCCGGCAACGAAGGTACCATGGGAATTC) and digested with *Bam*HI to generate the NSm gene as a *Bam*HI fragment. Subsequently, after purification from an agarose gel, the fragment was cloned into the *Bam*HI site of pMon999 between an enhanced 35S promoter and the nopaline synthase terminator (Sambrook *et al.,* 1989). The integrity of the *Bam*HI fragment containing the NSm gene was verified by sequence analysis. For transfection studies aliquots of one million *N. rustica* protoplasts were inoculated with 10 μg of pMon999/NSm subsequently adding 100 μl 0.6 *M* mannitol containing 10 m*M* $CaCl_2$, 0.5 ml 40% polyethyleneglycol MW 6000 containing 0.5 *M* mannitol and 0.1 *M* $Ca(NO_3)_2$, 4.5 ml 0.5 *M* mannitol containing 15 m*M* $CaCl_2$ and 0.1% 2[*N*-morpholino]ethanesulfonic acid, pH 5.6. The transfected protoplasts were incubated in nutrition medium for 16 and 24 hr postinoculation (p.i.), respectively, and prepared for immunofluorescence and negative staining electron microscopy as described by Van Lent *et al.* (1991).

Insect cell infection, immunofluorescence, and electron microscopy

The wild-type baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV) and the recombinant baculovirus AcNPV/NSm (Kormelink *et al.,* 1994) expressing the NSm protein were used to infect *Spodoptera frugiperda* (Sf 21) and *Trichoplusia ni* (T. ni 368) cultured insect cells.

For immunofluorescent localization of the NSm protein, Sf 21 and T. ni 368 insect cells were grown on sterile glass cover slips, infected with buffer, wild-type, and recombinant virus, and incubated in Hinks medium (Hink *et al.,* 1970) at 27°. At 16, 24, 36, and 48 hr p.i., the attached cells were fixed by immersion in acetone at −70° for 15–20 min. The cells were rinsed in phosphate-buffered saline (PBS, pH 7.2) for 15 min, blocked with PBS containing 1% (w/v) BSA for 1 hr at room temperature, and subsequently immunolabeled as described by Van Lent *et al.* (1991). The cells were analyzed as described above for protoplasts.

For *in situ* immunoelectron microscopical analysis, Sf 21 and T. ni 368 cells were infected with buffer, wild-type, or recombinant virus. At 48 hr p.i., cells were carefully pelleted and fixed, low-temperature dehydrated, and embedded in LR gold resin at −25°. Immunogold localization using preimmune serum (control), anti-NSm serum, and protein A-gold probes with a diameter of 5 nm followed by silver enhancement was carried out on ultrathin sections of embedded insect cells. Both embedding and immunogold localization were done as described by Van Lent *et al.* (1990). Alternatively, insect cells were fixed and embedded in gelatine (Van Lent *et al.,* 1990) and infiltrated with 2.3 *M* sucrose in phosphate/citrate buffer at 4° for 16 hr. Small blocks of gelatine embedded insect cells were cryo-fixed by immersion in liquid nitrogen and sectioned in a Reichert FCS ultracryotome. Cryosections were immunolabeled as described for plastic sections and stained with a mixture containing 0.5% uranyl acetate and 0.7% methylcellulose in double-distilled water. For negative staining electron microscopy insect cells were sampled at various hours after infection and stained with 2% PTA, pH 5.5, as described in detail by Van Lent *et al.* (1991) for the visualization of cowpea mosaic virus movement tubules.

RESULTS

Immunofluorescent detection of nucleocapsid (N) and NSm proteins in protoplasts isolated from infected leaves

To follow the expression and intracellular location of the NSm protein during TSWV infection of leaf tissue, samples of systemically infected *Nicotiana rustica* leaves were taken at daily intervals between 3 and 11 days p.i. The leaf samples were processed to protoplasts and the N and NSm proteins were detected by immunofluorescent staining. Cells were scored for the presence of N and NSm proteins. Table 1 shows the percentage of infected mesophyll cells (protoplasts) in systemically infected equivalent sampled leaves as a function of time p.i. At Day 4 p.i. the first infected cells (0.3%) were observed on the basis of N protein detection. The number of infected cells increased rapidly to 87% at Days 8 to 9 p.i. At 5–6 days p.i., the first NSm-positive cells were detected revealing a clear fluorescent signal in the cytoplasm at the periphery of the cell (Table 1, Fig. 1A). At Day 7, the number of NSm-positive cells had increased to 31%. About 4% of these cells exhibited the peripheral cytoplasmic location of the NSm protein, while in 27% of the cells (representing 87% of the total number of cells containing the NSm protein) the NSm protein appeared

TABLE 1

The Percentage of Infected Mesophyll Protoplasts[a] Seropositive for the N and NSm Protein of TSWV

		anti-NSm serum	
Days p.i.	anti-N serum	% Periphery appearance[b]	% Tubular structures[b]
4	0.3	0	0
5	3	0.3	0
6	24	7	0
7	64	4	27
8	84	0	4
9	87	0	0

[a] The protoplasts were isolated from systemically infected *N. rustica* leaves at daily intervals between 4 and 9 days postinoculation.

[b] The NSm expressing cells have been subdivided into a category containing NSm in the periphery of the cytoplasm (see Fig. 1A) and a category containing NSm aggregating into tubules extending from the cell surface (see Fig. 1B). The percentages are averages of three experiments.

in tubule-like protrusions extending from the cell surface (Table 1, Fig. 1B). At Day 8, the number of NSm-positive cells had decreased again to 4%, all cells only showing fluorescent signal in tubule-like protrusions. From Day 9 p.i. on, no NSm-positive protoplasts could be detected anymore, while in contrast the number of N-positive cells remained detectable at a constant level throughout the course of the infection. The expression of both the N and NSm protein as described in Table 1 coincided with the symptom development on the systemically infected leaves. From 5 days p.i. on, clear systemic symptoms occurred at the base of the leaf slowly migrating in 3 days time to the top of the leaf. Once the leaf was completely saturated with symptoms (9 days p.i.) and mature virus particles were present in almost every cell of the leaf (confirmed by electron microscopy; data not shown), detectable levels of the NSm protein were no longer present.

Tubular structures are also present in plasmodesmata of TSWV-infected leaf tissue

Having established that NSm is able to form tubular structures as a temporary phenomenon during the infection, the next question is the specific location of these structures in the infected leaf. In a previous study we were able to show that in infected plant cells NSm associates to free, nonenveloped nucleocapsids and to plasmodesmata (Kormelink *et al.,* 1994), though clear tubular structures were not discerned. Taking into account the very temporary appearance of NSm in infected *N. rustica* leaves, tissue was prepared for further immunocytological analysis at Days 6, 7, and 8 after inoculation and carefully examined for the presence of tubules. At 6 days p.i., immunogold labeling probed on ultrathin sections revealed that NSm was associated to morphologically unmodified plasmodesmata (not shown). However, at Days 7 and 8 p.i. tubular structures could be found. These

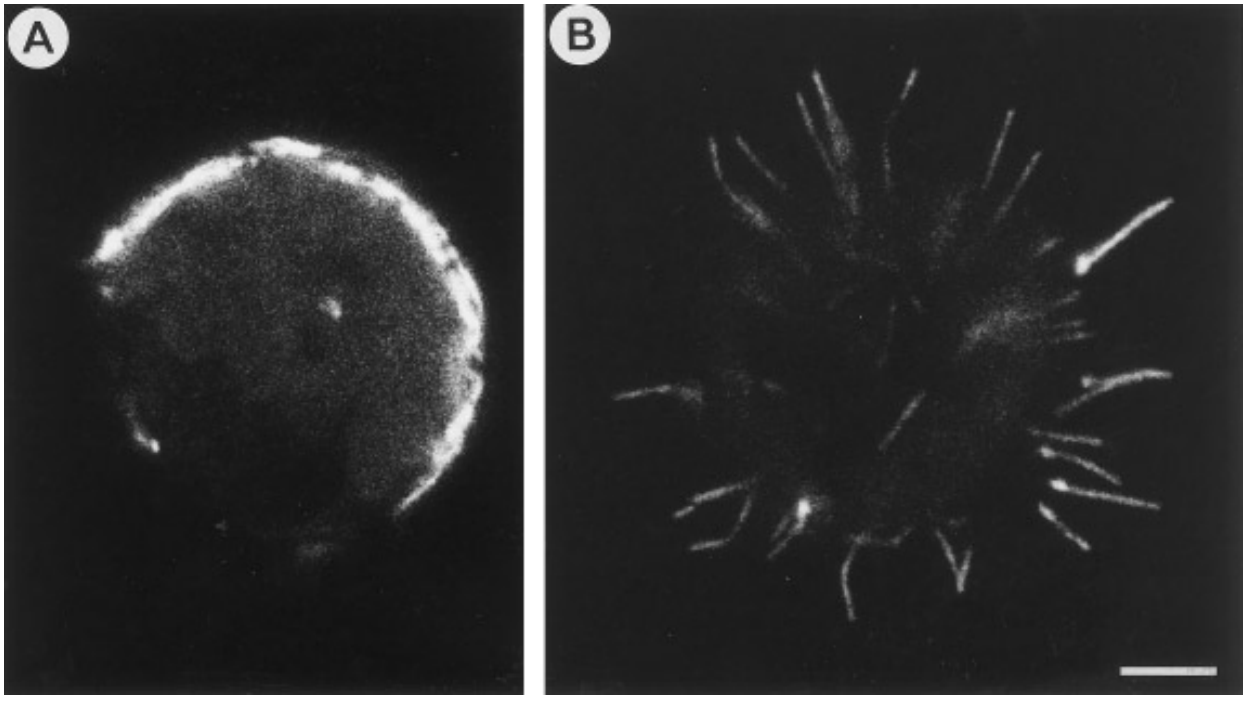

FIG. 1. Immunofluorescent staining of the NSm protein in protoplasts isolated from systemically infected leaves of *Nicotiana rustica.* (A) Localization of NSm in the cytoplasm of *N. rustica* protoplasts at 6 days p.i. (B) NSm-containing tubular structures emerging from the protoplast surface at 7 days p.i. Scale bar represents 10 μm.

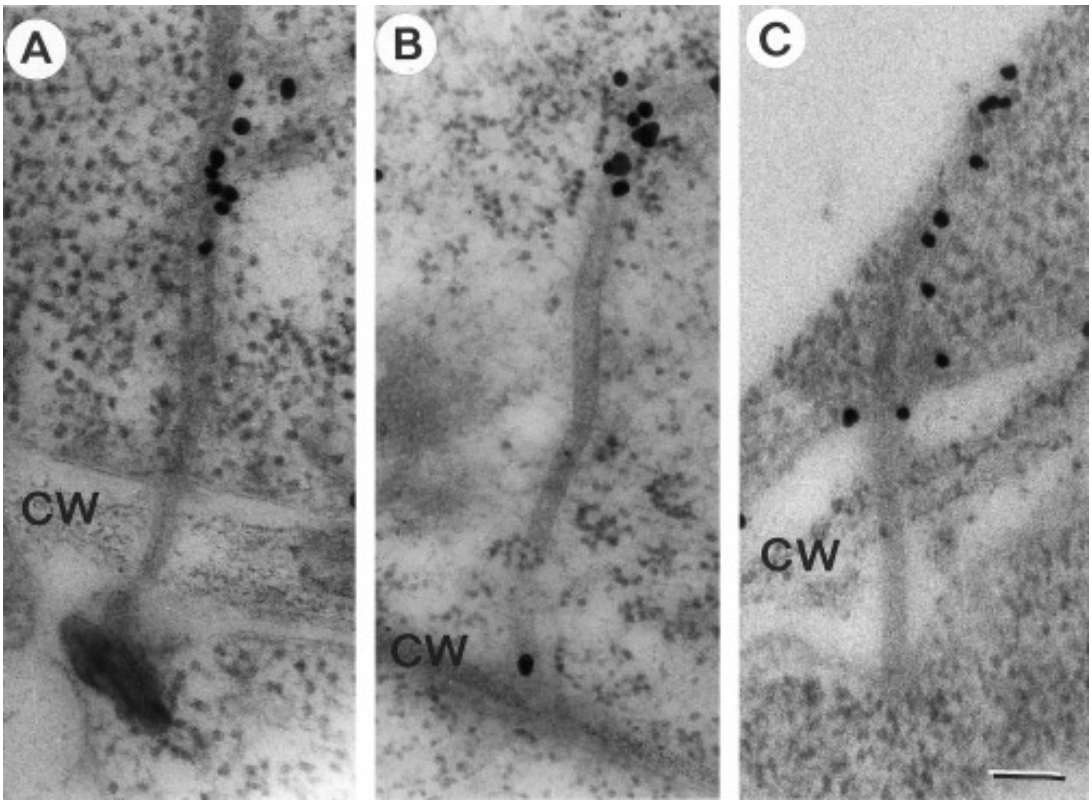

FIG. 2. *In situ* localization of NSm-induced tubular structures in systemically infected *N. rustica* mesophyll tissue at 7 days p.i., immunogold labeled with anti-NSm (A, B, C). Scale bar represents 100 nm. CW, cell wall.

structures, which specifically labeled with anti-NSm serum, had an estimated width of 40–45 nm and were exclusively found in association with plasmodesmata extending on only one side of the cell wall into the cytoplasm (Fig. 2).

Expression of the NSm gene in protoplasts transfected with pMon999/NSm

To verify whether the formation of tubule-like protrusions as described above was specifically induced by the NSm protein, the NSm gene was expressed in *N. rustica* protoplasts using an expression vector containing the NSm gene under the control of the CaMV 35S promoter (pMon999/NSm; Fig. 3A). Sixteen hours post-transfection, the NSm protein was exclusively found in the cytoplasm at the periphery of the cell (data not shown), while from 24 hr p.i. onwards tubule-like extensions with a length up to 40 μm could be detected at the cell surface (Fig. 3B). Control transfections with the pMon999 vector alone did not show NSm-specific immunofluorescence (data not shown). Transmission electron microscopy of negative-stained preparations confirmed that the NSm protein specifically aggregates into these structures (Fig. 3C) and that their morphology consists of a filamentous substructure (Fig. 3D). The diameter of the tubules, apparently enclosed by the plasmamembrane, was estimated to be 40–50 nm. These results indicate that the NSm gene has the capability to form tubular structures in the absence of other virus-specific components and that NSm is most likely the only viral gene involved in the tubule formation.

Expression of NSm in insect cells

To investigate whether involvement of possible host components is critical for the formation of tubular structures, *S. frugiperda* 21 and *T. ni* 368 insect cells were infected with baculovirus recombinant AcNPV/NSm containing the TSWV NSm gene behind the polyhedrin promoter. Immunofluorescent staining of NSm showed an accumulation of the protein in the cytoplasm and more specifically at the periphery of the cell at 16 and 24 hr p.i. (Fig. 4A). At 36 and 48 hr p.i. the protein apparently had aggregated into numerous tubule-like extensions emerging from the insect cell surface, but was also present in aggregates located in the cytoplasm (Fig. 4B) and around the nucleus. Immunogold electron microscopy localized the NSm protein more specifically to the plasmamembrane (Fig. 5A), to membranes of cytoplasmic vesicles (Fig. 5B) and to fibrillar aggregates present in the cytoplasm (Fig. 5C). Tubule-like structures, easily observed on the surface of immunofluorescent stained cells, were also found in thin cryo sections of infected

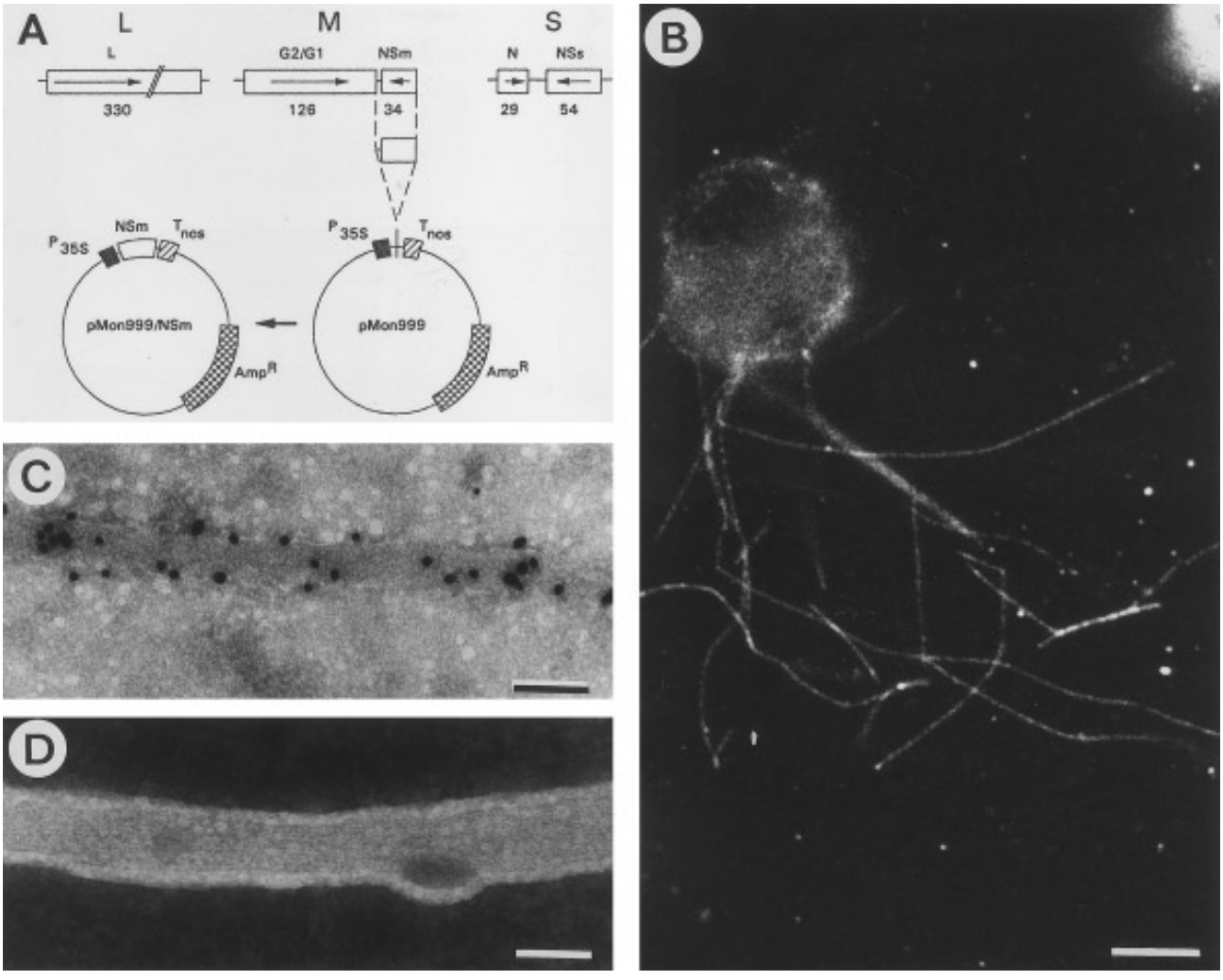

FIG. 3. Expression of the NSm gene in *N. rustica* protoplasts transfected with vector pMon999/NSm. (A) The TSWV NSm gene was cloned into plasmid pMon999 between an enhanced 35S promoter and the nopaline synthase (*nos*) terminator. L, M, and S represent the genomic RNA segments of TSWV in which the various genes are indicated. (B) Immunofluorescent CSLM image of tubular structures emerging from the surface of a protoplast 24 hr after transfection with pMon999/NSm and visualized by anti-NSm serum. (C, D) Electron micrographs of negatively stained pMon999/NSm-induced tubules. (C) NSm was detected by treatment with anti-NSm serum and protein A-gold labeling. Scale bars represent 10 μm (B), 100 nm (C), and 50 nm (D), respectively.

cells. These structures appeared as bundles of filaments with various diameters, protruding from the cell surface and surrounded by an extension of the plasmamembrane. The NSm protein was specifically associated with these tubules, confirming the viral origin of these structures (Fig. 5D). Negative staining electron microscopy of infected Sf 21 and T. ni 368 cells also showed numerous tubule-like structures (Fig. 6A), containing the NSm protein (Fig. 6B). Although the plasmamembrane could clearly be observed around the tubule in negative-stained samples of transfected protoplasts, the membrane was often absent from tubules observed in negative-stained samples of insect cells (Fig. 5). It is plausible that the plasmamembrane detached from the tubules during preparation of the samples for electron microscopy.

DISCUSSION

Previously, we showed that in infected plant tissue the NSm protein is transiently present and associated with viral nucleocapsids and plasmodesmata (Kormelink *et al.,* 1994). Based on these cytological findings, it was suggested that the NSm protein could be involved in the intercellular translocation of TSWV. Our present immunofluorescent data of protoplasts isolated from systemically infected leaf tissue confirm the transient nature of the NSm protein. Unlike the N protein, which accumulates to high levels in an increasing number of cells with progressing infection of the leaf, the NSm protein could only be detected in a short period between 6 and 8 days p.i. in systemically infected leaves from parallel inoculated plants. The transient appearance of the protein during

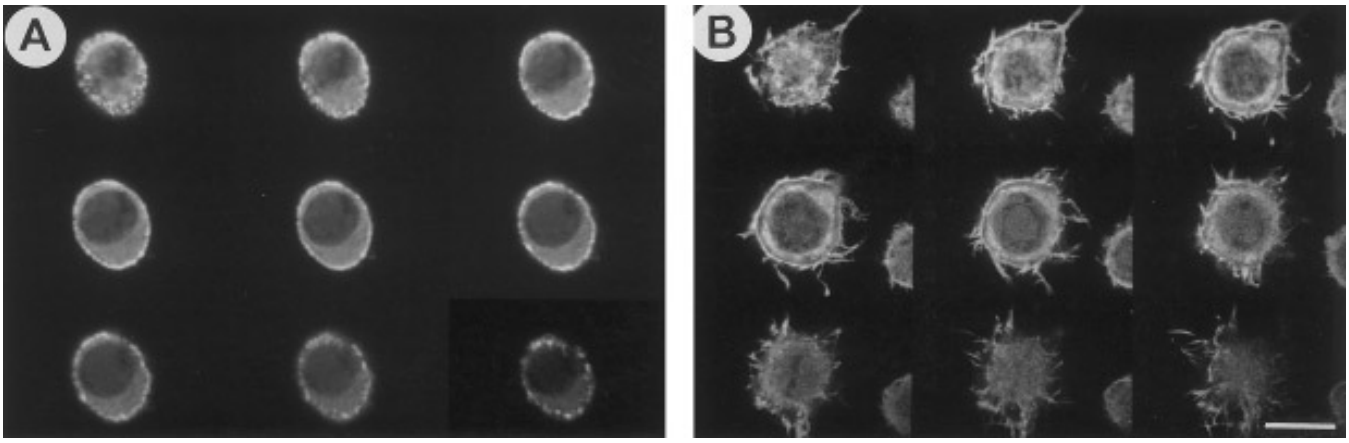

FIG. 4. Immunofluorescent CSLM-images showing the NSm protein in *T. ni* insect cells. (A) At 16 hr p.i. NSm is specifically present at the cell periphery, while (B) at 36 hr p.i. tubule-like extensions occur. Scans are made from top (upper left corner) to bottom (bottom right corner) with an optical section thickness of 1.1 μm in sequential focal planes. Scale bar represents 10 μm.

establishment of the infection points to an early and transient function of the protein. The expression of the NSm gene appears to be linked to the development of symptoms on the systemically infected leaves. From 5 days p.i. on, clear systemic symptoms occurred at the base of the leaves slowly migrating in 3 days to the top. Once the leaves were visually saturated with symptoms (9 days p.i.) and mature virus particles were present in almost every cell, NSm was no longer detectable. These kinetics of expression fit our previous suggestion (Kormelink *et al.,* 1994) that NSm has a function in intercellular translocation of TSWV.

In this paper we have furthermore shown that the NSm protein of TSWV has the property to assemble into tubule-like structures when expressed in single plant cells. These tubules are enveloped by the cell membrane, protruding from the cell surface outwards, suggesting a functional polarity of the structure. Tubules are not only found in protoplasts isolated from TSWV-infected plant tissue, but also in protoplasts transfected with the NSm gene alone. This is evidence of the exclusive involvement of the protein in the tubule-forming process, independent from virus replication or other viral proteins. Having established the tubule-forming capability of NSm, the question arises how this protein can function during cell-to-cell movement of TSWV as previously suggested by Kormelink *et al.* (1994). From extended immunocytological studies at the level of electron microscopy it has become evident that the tubular structures, morphologically similar to the NSm-induced tubules in transfected protoplasts, specifically penetrate plasmodesmata. This *in vivo* manifestation of the NSm protein (targetting to plasmodesmata and tubule assembly in plant tissue and protoplasts) shows great similarity to that of the 48-kDa protein of cowpea mosaic virus (CPMV) and the P1 protein of cauliflower mosaic virus (CaMV), of which the function in viral cell-to-cell movement has now been well established (Wellink and van Kammen, 1989; Van Lent *et al.,* 1990, 1991; Wellink *et al.,* 1993; Kasteel *et al.,* 1993; Perbal *et al.,* 1993). Nevertheless, differences exist between the TSWV- and CPMV-induced tubule with respect to their substructure. The tubules formed upon expression of NSm in plant protoplasts as well as in insect cells show a clear filamentous substructure, while this has never been observed in 48-kDa-induced structures. In this aspect the NSm-induced tubule resembles the CaMV P1-induced tubule which also exhibits a filamentous substructure (A. J. Maule and D. Kasteel, personal communication). In the case of CPMV it has been shown that mature virus particles (diameter 28 nm) are translocated through the tubules. It is obvious, however, that this would not hold for TSWV since the diameter of its enveloped virion (80–110 nm) not only greatly exceeds the functional diameter of plasmodesmata (effective size exclusion limit is 5 nm; Lucas *et al.,* 1993), but also that of the NSm-induced tubules (40–45 nm). Thus, if TSWV is translocated through tubules, it will probably move as a free, nonenveloped nucleocapsid structure. Three arguments support this interpretation. First, the NSm protein has been found in association with free nucleocapsids in the cytoplasm, but never with mature, enveloped particles (Kormelink *et al.,* 1994). This association occurs prior to the maturation of virus particles and supports the hypothesis that NSm may have a function in targetting of nucleocapsids to the plasmodesmata. Second, envelope-deficient mutants of TSWV, though nontransmittable by thrips, are able to infect plants systemically at wild-type speed (Resende *et al.,* 1991, 1993). And third, mature enveloped particles, despite their conspicuous structure, have never been observed inside the tubular structure.

Hence, the exact mechanism of plant virus translocation remains still unclear. Two major mechanisms for plant virus movement have thus far been proposed (Goldbach *et al.,* 1990; Maule, 1991; Deom *et al.,* 1992). One

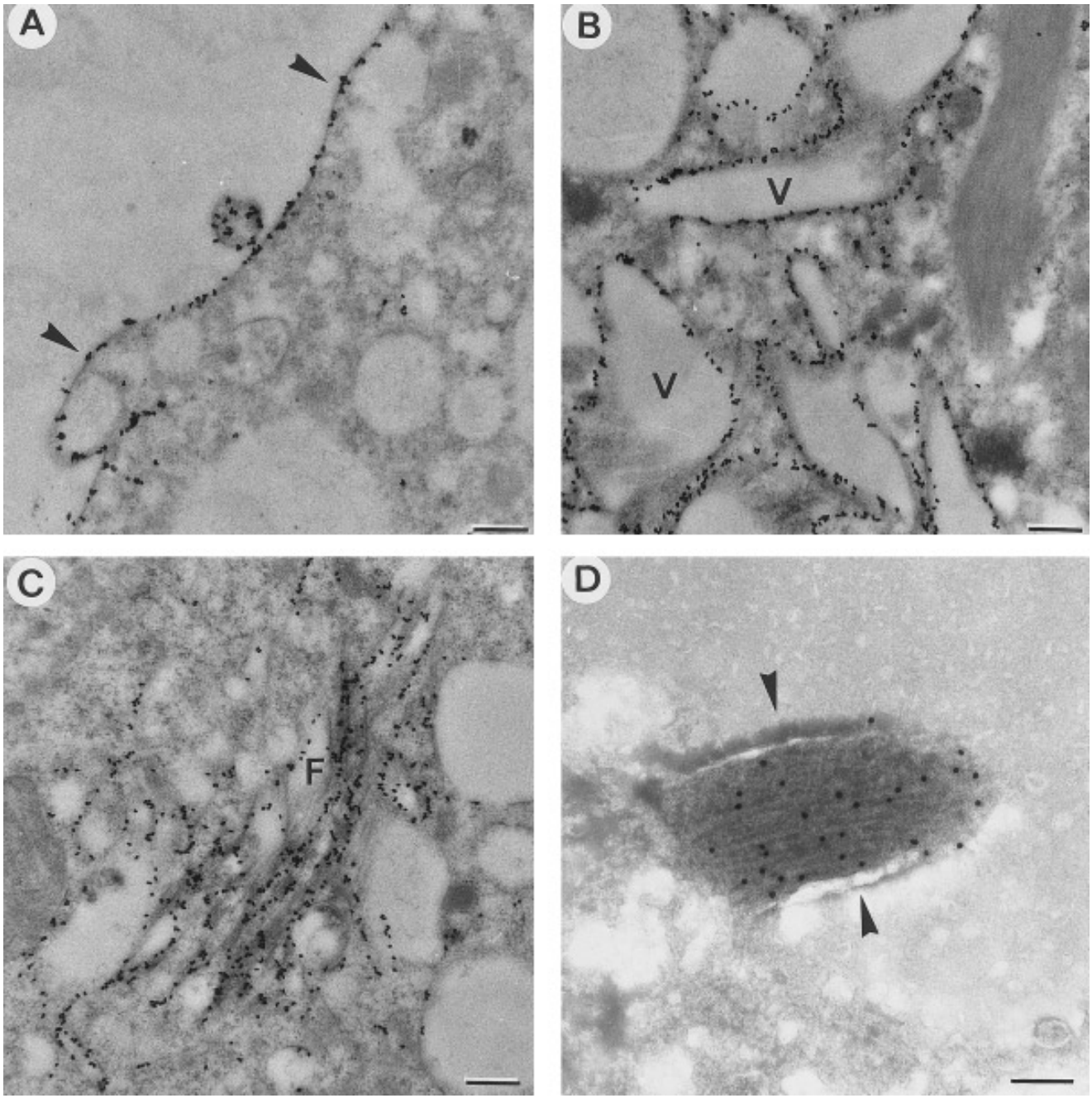

FIG. 5. Immunogold localization of NSm in *T. ni* (A, B, C) and *S. frugiperda* (D) insect cells at 48 hr p.i. (A) NSm specifically localized to the plasmamembrane, (B) membranes of cytoplasmic vesicles but also to (C) fibrillar aggregates in the cytoplasm. (D) Cryo-section of a tubule-like structure. Immunogold labeling shows the presence of NSm in this structure emerging from the cell surface, made up of filaments. Scale bars represent 200 nm (A, B, C) and 50 nm (D). F, fibrillar aggregates; V, cytoplasmic vesicles; arrowheads indicate plasmamembrane.

mechanism, exemplified by tobamovirus tobacco mosaic virus, employs the transport of a nonvirion form of the virus complexed with a movement protein through plasmodesmata which do not undergo extensive structural modifications (Deom *et al.,* 1992; Lucas *et al.,* 1993). The second mechanism involves the movement of complete virions along tubular structures as exemplified by the comoviruses, caulimoviruses, and nepoviruses (Van Lent *et al.,* 1990, 1991; Perbal *et al.,* 1993; Wieczorek and Sanfaçon, 1993). If TSWV is indeed translocated through the NSm-induced tubular structures it will probably move as free nucleocapsids, thus representing an intermediate type of intercellular transport, i.e., a tubule-guided transport of a nonmature form of the virus.

As *in situ* the NSm-induced tubular structures are found in a typical plant intercellular organelle, the plasmodesmata (Kormelink *et al.,* 1994, this paper), the involvement of one or more plant-specific host compo-

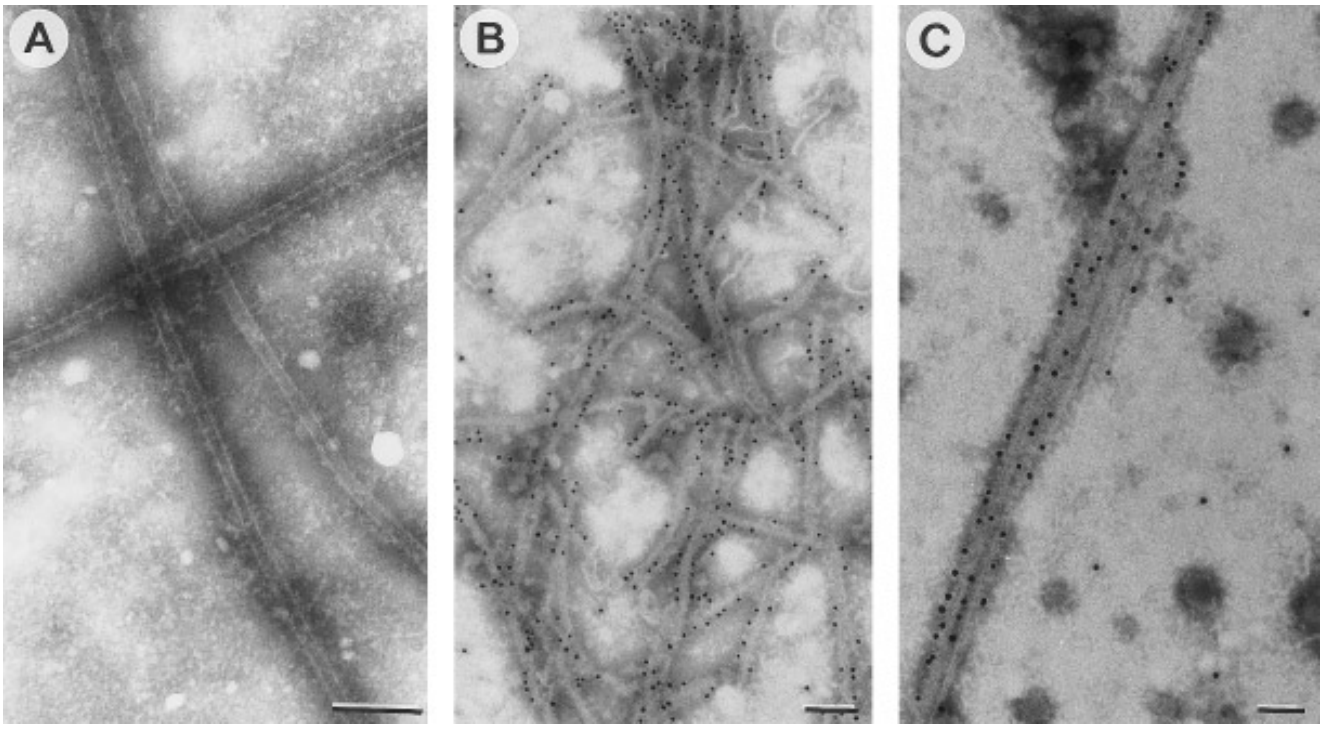

FIG. 6. Electron micrographs of negative stained *T. ni* cells infected with the recombinant AcNPV/NSm at 48 hr p.i. (A) Tubule-like structures (B, C) specifically labeled with anti-NSm serum conjugated with protein A-gold (10 nm). Scale bars represent 200 nm (A, B) and 100 nm (C), respectively.

nents (e.g., plasmodesmal proteins) in a tubule-mediated cell-to-cell movement mechanism cannot be ruled out. To this end, the expression and subcellular behavior of NSm has been analyzed in a heterologous animal (insect) cell system. In *S. frugiperda* and *T. ni* insect cells infected with a NSm-expressing baculovirus vector, similar phenomena were recorded as were found for expression of the NSm protein in plant cells. The protein was targetted to the plasmamembrane and assembled into tubules protruding from the cell surface. The great similarity in behavior of the NSm protein in plant and animal (insect) cells suggests that host components are not essential for tubule targetting and assembly, unless such a component(s) is (are) of a very conserved nature.

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