# The role of $NS_M$ during tomato spotted wilt virus infection

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Proefschrift
Ter verkrijging van de graad van doctor
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# Stellingen

1. Het door het tomatenbronsvlekkenvirus (TSWV) gecodeerde $NS_M$-eiwit is een viraal transporteiwit.
   Dit proefschrift.

2. Het $NS_M$–eiwit van het tomatenbronsvlekkenvirus heeft geen functie in tripsen.
   Dit proefschrift.

3. Het verdient aanbeveling om resultaten verkregen met microinjecties waarbij druk wordt gebruikt met meer voorzichtigheid te interpreteren.
   Dit proefschrift.

4. Plasmodesmata zijn geen eenheidsstructuren maar organellen met variabele karakteristieken.

5. De conclusie dat KNOTTED1 zichzelf transporteert is slecht verdedigbaar.
   Lucas *et al.* (1995) Science **270**, 1980-1983.

6. De aanname van Derrick *et al.* (1992) dat het tabaksratelvirus (TRV) in bladharen van tabak een verhoging van de doorlaatbaarheid van plasmodesmata induceert was prematuur en onjuist, aangezien niet onderzocht is wat de standaard "size exclusion limit" voor bladharen is (Waigmann and Zambryski, 1995).
   Derrick *et al.* (1992) Plant Cell **4**, 1405-1412; Waigmann and Zambryski (1995) Plant Cell **7**, 2069-2079.

7. De gelijkstelling van primair met lineair[1] en secundair[2] met vertakt in de huidige plasmodesmata-terminologie is onjuist.
   [1]Ding *et al.* (1992) Plant Cell **4**, 915-928; Ding *et al.* (1993) Plant Journal **4**, 179-189; [2]Ehlers and Kollmann (1996) Planta **199**, 126-138.

8. De vorming van buisvormige transportstructuren is wellicht een algemene, intrinsieke eigenschap van virale transporteiwitten gezien het toenemende aantal plantenvirussen waarbij buisvorming wordt waargenomen.
   Van Lent *et al.* (1991) J. Gen. Virol. **72**, 2615-2623; Perbal *et al.* (1993) Virology **195**, 281-285; Wieczorek and Sanfacon (1993) Virology **194**, 734-742; Storms *et al.* (1995) Virology **214**, 485-493; Ritzenthaler *et al.* (1995) MPMI **8** (3), 379-387; Kasteel *et al.* (1997) J. Gen. Virol. **78**, 2089-2093.

9. Het systeem van peer-review ter bewaking van de kwaliteit van wetenschappelijke artikelen kan misbruik van voorkennis in de hand werken.

10. Het verdient geen aanbeveling de kwaliteit van wetenschappelijk onderzoek te kwantificeren.

11. De toenemende invloed van het bedrijfsleven op de wetenschap ondermijnt het fundamenteel wetenschappelijk onderzoek.

12. Werken op projectbasis kan welzijnsverlagend zijn voor werknemers.

13. De ontwikkeling van wetenschappelijke subculturen met ieder hun eigen vaktaal kan het interdisciplinaire onderzoek in sterke mate bemoeilijken.

14. Celbiologisch onderzoek ontbeert een microscoop met de resolutie van een transmissie electronenmicroscoop voor bestudering van ongefixeerd, levend materiaal.

15. Gezien het structuurvaste karakter van prionen dringt de vraag zich op of deze eiwitten geen toepassing verdienen bij de ontwikkeling van biologische chips/ICs.

16. De grote populariteit van programma's als Star Trek en Voyager kan ertoe leiden dat de grens tussen science fiction en werkelijkheid in de beleving ervan steeds kleiner wordt.

17. De ervaringswaarheden "zien is geloven" en "geloven is zien" illustreren tesamen de voorwaardelijkheid van de menselijke waarneming.

18. De toenemende invloed van Aziatische bedrijven en produkten op de Europese en Amerikaanse markt kan gezien worden als een economische vorm van neo-kolonialisme.

19. Omdat koersontwikkelingen op de beurs wereldwijd sterk onderhevig zijn aan sentimenten en voorspellingen dringt de vraag zich op of beursafgeleide economische ontwikkelingen en voorspellingen nog wel betrouwbaar zijn.

20. Het laatste decennium is er sprake van dualisme binnen Europa welke zich manifesteert in het streven naar (financieel-economische) eenwording in West-Europa en het omgekeerde proces in een aantal Oost-Europese landen.

21. Het voornemen van vooraanstaande Amerikaanse wetenschappers om binnenkort mensen te gaan klonen in commerciële klinieken (De Volkskrant dd 08-01-1998) onderstreept dat een verschuiving van ethische normen een tijdsafhankelijk fenomeen is.

22. Voetbal is oorlog, maar zonder voetbal zou er waarschijnlijk meer oorlog zijn.

23. Het gebruik van sigaren als genotmiddel heeft een extra dimensie gekregen sinds de affaire Clinton-Lewinski.

Stellingen behorende bij het proefschrift:

**The role of $NS_M$ during tomato spotted wilt virus infection**

Wageningen, 2 december 1998 Marc M.H. Storms

# Voorwoord

Zittend achter de PC in mijn nieuwe woon- en werkomgeving is hier het moment gekomen dat ik nog één keer achterom kijk naar mijn leven in Wageningen, waarvan in dit proefschrift de laatste vijf wetenschappelijke jaren zijn opgetekend. Vanzelfsprekend zou deze dissertatie niet tot stand zijn gekomen zonder de onmisbare steun van velen op de werkvloer, maar ook daarbuiten. Op deze plaats een dankwoord aan allen die mij de afgelopen jaren behulpzaam zijn geweest.

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Tenslotte wil ik ook een aantal mensen van "buiten de vakgroep" bedanken voor hun getoonde belangstelling en noodzakelijke afleiding. Allereerst de mensen van het Wageningse Volko(o)ren en van tafeltennisvereniging De Stuiterd. In het bijzonder wil ik Antonie, Marcel, Guus, Mieke, Istvan en David van het Queen-team bedanken voor de

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Eindhoven, juli 1998

# Contents

# 1

# General Introduction

The spread of virus from an initially infected cell to surrounding cells and vascular tissue is essential for a successful systemic infection in plants. Most viruses bridge long distances between plant tissues and organs by phloem-dependent transport through the vascular system. However, long distance movement does not provide the means for infection of plant cells that are not directly connected with vascular elements. Therefore, plant viruses must be able to spread from cell-to-cell by passing the plant specific cell wall through the plasmodesmata (for recent reviews see Lucas, 1995; Carrington *et al.*, 1996). As the physical space provided by plasmodesmata for movement of macromolecules is limited (defined as the size exclusion limit (SEL)) and plant virus particles or genomes have a size that exceeds the SEL (Figure 1.1.), movement of virus requires a structural modification of plasmodesmata. The mechanisms by which such modifications are achieved are far from being fully understood. Research in the last decade has invariably shown the essential involvement of virus-encoded "movement proteins" in cell-cell translocation, most likely in co-operation with other viral proteins and host factors. In addition, insight has been obtained regarding the plasmodesmal structure and functioning during virus transport. In this thesis, the role of the $NS_M$ protein in cell-to-cell movement of tomato spotted spotted wilt virus (TSWV) has been studied. Besides gaining insight into the fundamental aspects that underlie this crucial step of the virus infection cycle, unravelling of the cell-to-cell movement mechanism may contribute to the development of resistance strategies for control of virus disease in economically important crops.

### 1.1. Tomato spotted wilt virus (TSWV)

Tomato spotted wilt virus (TSWV) represents the type species of the genus Tospovirus within the large family Bunyaviridae (Francki *et al.*, 1991; Murphy *et al.*, 1995), which further consists of animal infecting viruses. Tospoviruses, like all other Bunyaviruses, have spherical particles (diameter 80-110 nm) with a lipid envelope containing two types of glycoproteins (Figure 1.2.). This envelope encompasses the tripartite, single stranded RNA genome, which is tightly associated with nucleoprotein (N) and 10-20 copies of the viral polymerase (Van Poelwijk *et al.*, 1993). The RNA segments contain five open reading frames (ORFs) which are translated into six functional proteins (De Haan *et al.*, 1990, 1991; Kormelink *et al.*, 1991, 1992).

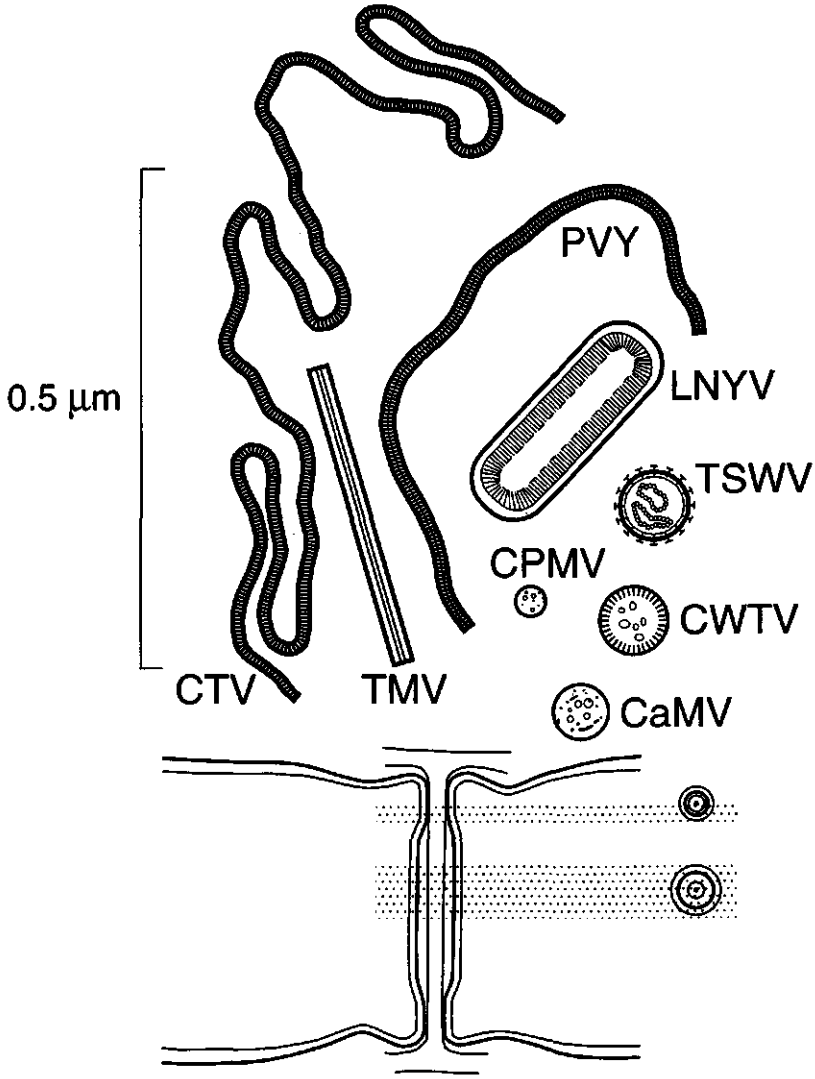


**Figure 1.1.** Representation of the relative sizes of various plant viruses in relation with the size of a plasmodesma. (CTV) citrus tristeza virus (2 µm x 10 nm); (TMV) tobacco mosaic virus (300 nm x 18 nm); (PVY) potato virus Y (750 nm x 10 nm); (LNYV) lettuce necrotic yellows virus (220 nm x 80 nm); (TSWV) tomato spotted wilt virus (80-110 nm); (CWTV) clover wound tumor virus (70 nm); (CaMV) cauliflower mosaic virus (50 nm); (CPMV) cowpea mosaic virus (28 nm). (Modified after Gibbs, 1976).

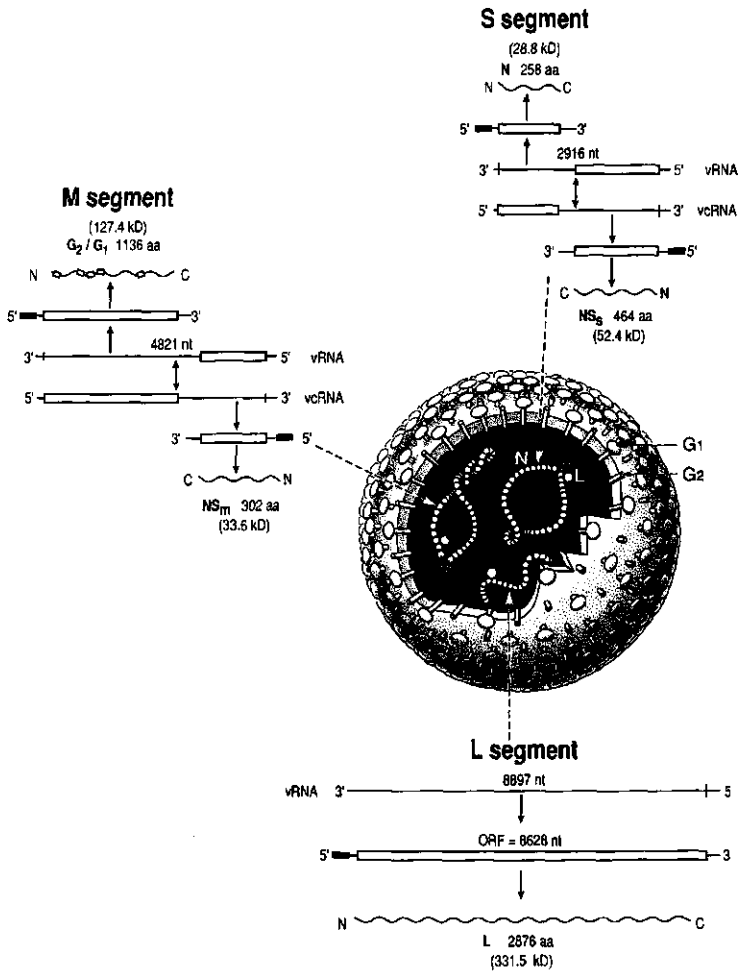


**Figure 1.2.** Morphology and genomic organisation of tomato spotted wilt virus. Heterologous sequences at the 5' ends of the viral messengers are indicated by black boxes. The L-RNA is of complete negative polarity and encodes for the viral RNA dependent RNA polymerase of 331.5 kDa (L-protein) (De Haan *et al.*, 1991). Both the S- and M-RNA have an ambisense gene arrangement (De Haan *et al.*, 1990; Maiss *et al.*, 1991; Kormelink *et al.*, 1992), containing one ORF in the viral (v) strand and one in the viral complementary (vc) strand. The S-RNA encodes for the nucleoprotein of 28.8 kDa in the vc-RNA, and a non-structural protein $NS_S$ of 52.4 kDa in the v-RNA of which at present the function is unknown. The M-RNA encodes for a glycoprotein precursor of 127.4 kDa in the vcRNA and a non-structural protein $NS_M$ of 33.6 kDa in the vRNA. The glycoprotein precursor is cleaved into G1 (78 kDa) and G2 (58 kDa) proteins which are glycosylated and present as spike projections at the envelope. The $NS_M$ gene finally is proposed to encode a movement protein (this thesis).

TSWV ranks among the ten most harmful plant viruses in the world causing an estimated annual crop loss of over 1 billion US dollars. Responsible for the importance of TSWV as plant pathogen is its broad host range. Currently, TSWV is known to infect more than 650 different plant species, monocotyledons as well as dicotyledons, belonging to more than 70 botanical families (Goldbach and Peters, 1994). Among these plants are economically important crops like tomato, potato, pepper, celery, lettuce, pineapple and ornamentals like chrysanthemum, dahlia, gerbera, impatiens and iris. In addition, the importance of TSWV as plant pathogen is also determined by the efficient, propagative transmission of the virus by a number of thrips species of which the Western flower thrips, *Frankliniella occidentalis*, represents the major viral vector (Gardner *et al.*, 1935; Dal Bó *et al.*, 1995).

After introduction into a plant cell by the thrips vector, the virus is relieved of its membrane and infectious nucleocapsids are released into the cytoplasm. Next, the viral RNA is transcribed and replicated. In analogy to other negative stranded viruses (Baudin *et al.*, 1994), it is thought that the concentration of the N protein in the cytoplasm regulates a functional switch of the viral polymerase. At the start of the infection, when the N protein level is low, the viral polymerase initiates the production of viral messengers, which are translated into the structural and non-structural viral proteins. Later in infection, when the N protein concentration has increased, the polymerase mediates the replication and production of viral genomic RNAs. The newly produced viral RNAs associate with N protein and also to few copies of the viral polymerase (Van Poelwijk *et al.*, 1993), resulting in the formation of nucleocapsids. As part of the maturation, these viral nucleocapsids bud into the Golgi complex, which contains the viral glycoproteins G1 and G2. Based on cytological studies (Kitajima *et al.*, 1992; Kikkert *et al.*, 1997), it is hypothesised that the glycoproteins are polarised at only one side (the trans membrane) of the Golgi stack. Budding at the trans membrane may result in doubly enveloped particles that contain the G1 and G2 spike projections at the inner membrane only. These doubly enveloped particles may fuse with the endoplasmatic reticulum (ER). This explains the large amounts of mature TSWV particles in the cisternae of the ER, a common observation for the later stages of infection. An essential step within the viral infection cycle is the spread of the virus to neighbouring cells and tissues. At the start of this PhD project, it was not known whether TSWV spreads as non enveloped nucleocapsids or as a mature virus particle, nor by which mechanism this occurs.

## 1.2. The various strategies for cell-cell movement

To spread from cell-to-cell, plant viruses have to cross the rigid cell wall through plasmodesmata. Much of the current knowledge on plasmodesmal functioning has in fact

been obtained from analyses of the plant virus movement mechanisms (e.g. Lucas *et al.*, 1993; Carrington *et al.*, 1996). Plasmodesmata exhibit different structural and functional characteristics. Two classes of plasmodesmata can be roughly distinguished. Primary plasmodesmata are formed during cytokinesis and are lined with the plasmalemma that is contiguous with both adjacent cells. Inside the plasmodesma, the desmotubule or appressed ER (AER) connects the endomembrane systems of the neighbouring cells. Some plasmodesmata contain a central cavity which is an enlarged area between the AER and the plasmamembrane (e.g. Ding *et al.*, 1992). The existence of a central cavity is characteristic for secondary plasmodesmata, which are formed by *de novo* synthesis through pre-existing cell walls or by branching of primary plasmodesmata.

The space between the AER and the plasmamembrane allows for cytoplasmic continuity between cells, but is limited in its ability to translocate macromolecules and plant viruses to adjacent cells. In general, the functional diameter of plasmodesmata, the size exclusion limit (SEL), limits the movement of molecules larger than 1 kDa (Goodwin, 1983; Terry and Robards, 1987; Wolf *et al.*, 1989). This corresponds to a Stokes' radius of less than 2 nm. All plant viruses, and even their non-encapsidated genomes, have a size that exceeds this SEL (Figure 1.1.). To enable their transport over the cell wall, the plasmodesma must therefore be modified. To achieve such modifications, plant viruses employ different strategies invariably involving viral (movement) proteins and presumably also host factors. The various strategies are discussed in the next sections of this paragraph.

One strategy is employed by the tobamoviruses, e.g. tobacco mosaic virus (TMV), and comprises the movement of a complex of viral RNA and viral encoded movement protein. The coat protein (CP) is not required for this process (Figure 1.3.A). As part of the intracellular trafficking from the cytoplasm to the plasmodesmata, the TMV RNA-MP complex associates to microfilaments and microtubules (Heinlein *et al.*, 1995; McLean *et al.*, 1995; Langford, 1995; St Johnson, 1995; Carrington *et al.*, 1996). The intercellular transport of the RNA-MP complex, which occurs at the plasmodesmata, can be subdivided into three steps, i.e. the binding of the RNA-MP complex to the plasmodesma, the subsequent transit of the complex through the plasmodesma and the release of the viral RNA-MP complex into the cytoplasm of the adjacent cell. Most likely this is an energy dependent process as the TMV MP binds to GTP (Li and Palukaitis, 1996), but it may also require cycles of phosphorylation and dephosphorylation of the MP (Citovsky *et al.*, 1993). To allow translocation of viral RNA, the SEL of plasmodesmata is increased to a level that is on average 10 fold higher than for plasmodesmata in control plants as determined by microinjection of fluorescent probes. This significant increase in SEL has been first described for mesophyll plasmodesmata in transgenic plants that constitutively express the TMV MP (Wolf *et al.*, 1989; Deom *et al.*, 1990). Moreover it has

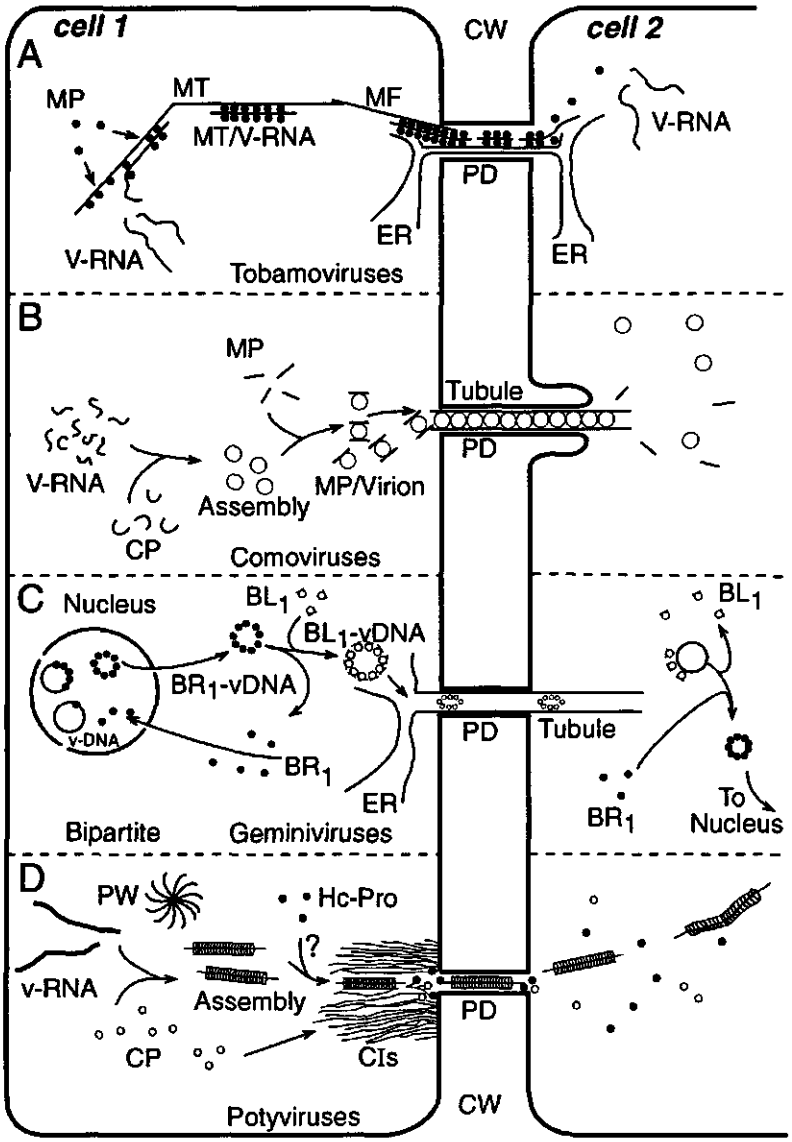


**Figure 1.3.** Model for the intra- and intercellular movement of the tobamoviruses (A), the comoviruses (B), the bipartite geminiviruses (C) and the potyviruses (D)(modified after Carrington *et al.*, 1996). MT: microtubule; MF: microfilament; MP: movement protein; PD: plasmodesma; CW: cell wall; CP: capsid protein; ER: endoplasmatic reticulum; v-RNA: viral RNA; v-DNA: viral DNA; CIs: cylindrical inclusions; PW: pinwheel.

been shown that the MP itself is able to traffic between adjacent mesophyll cells (Waigmann *et al.*, 1994). Recently, in *Nicotiana tabacum* plants infected with a TMV mutant expressing a fusion between the MP and the green fluorescent protein (GFP), a SEL increase was exhibited for only epidermal plasmodesmata within the characteristic fluorescent halo of the MP-GFP expression (Oparka *et al.*, 1997). This indicates that plasmodesmal gating is under temporal control and forms a substantial part of the cell-to-cell movement of TMV.

A second strategy of cell-cell movement is reported for both ssRNA (i.e. como- and nepoviruses; Van Lent *et al.*, 1990; 1991, Wieczorek and Sanfaçon, 1993, Ritzenthaler *et al.*, 1995) and dsDNA (i.e. caulimoviruses; Perbal *et al.*, 1993) plant viruses and involves a "tubule-guided" transport of mature virions (Figure 1.3.B). For cowpea mosaic comovirus (CPMV) it is proposed that upon infection of a plant host, the 48 kDa viral MP associates to newly produced virions in the cytoplasm. The virion-MP complex is then intracellularly targeted, through possible interactions with cytoskeletal components, to the cell periphery and specifically localises to plasmodesmata. Here, the MP induces a structural modification of the plasmodesma, by exchanging the AER by a tubular structure. This tubule, which is built up by the MP only (Wellink *et al.*, 1993; Kasteel *et al.*, 1997) is unidirectionally extending into the adjacent plant cell thereby revealing a donor-target principle (Van Lent *et al.*, 1990, 1991). As tubular structures devoid of mature virions have never been observed in CPMV-infected tissue (Van Lent *et al.*, 1990, 1991), it is likely that the virion is permanently co-aligned with the tubule and is transported into the neighbouring cell by a unidirectional basipetal growth of the tubule. The virus particle may then be released into the cytoplasm by proteolytic breakdown of the tubule.

A third mechanism for cell-cell movement is employed by the bipartite geminiviruses and comprises the transport of a viral DNA-MP complex possibly by a tubule-guided mechanism, for which in this case the viral CP is not essential (Figure 1.3.C). In contrast to the ssRNA viruses, which represent the majority of the plant viruses, bipartite ssDNA geminiviruses replicate in the nucleus. Intra- and intercellular movement of newly synthesised viral genomes therefore involves the passage of both the nuclear membrane boundary and the cell wall. For this reason geminiviruses encode two MPs, BR1 and $BL_1$, which co-operatively function in cell-cell movement (Von Arnim and Stanley, 1992; Pascal *et al.*, 1993). The $BR_1$ protein contains nuclear localisation signals which direct the protein to the nucleus in infected and transfected cells and it has the ability to bind to ssDNA (Pascal *et al.*, 1994; Sanderfoot *et al.*, 1996). Moreover, the $BR_1$ protein of bean dwarf mosaic virus (BDMV) mediates the movement of single and double stranded DNA out of the nucleus to the cytoplasm (Noueiry *et al.*, 1994). Based on these findings, it is proposed that the $BR_1$ protein is targeted to the nucleus where it binds to viral ssDNA and transports the complex through the nuclear pore to the cytoplasm. Next, the second

MP, $BL_1$, targets the infectious entity to the cell periphery and associates with the cell wall. This protein translocates extensively from cell-to-cell, increases the SEL of mesophyll plasmodesmata and facilitates the movement of double stranded DNA to adjacent cells (Noueiry *et al.*, 1994). Apparently, the $BL_1$ protein mediates the intracellular trafficking of the viral DNA (present in the replicative form of the virus (dsDNA)) to the plasmodesmata and further transport of the DNA-$BL_1$ complex through the plasmodesmata by a yet unknown, energy dependent mechanism. Recently, the $BL_1$ protein of squash leaf curl virus (SqLCV) was found specifically associated to tubular structures in systemically infected pumpkin leaves (Ward *et al.*, 1997). These tubules are supposedly derived from the endoplasmatic reticulum and penetrate exclusively the walls of procambial cells. For Euphorbia mosaic geminivirus, tubules have been detected in the cytoplasm and plasmodesmata of infected *Datura stramonium* leaf tissue (Kim and Lee, 1992). It is suggested that these tubules are involved in cell-to-cell movement of the viral genome, although the role of the tubule herein has yet to be specified.

A fourth strategy of cell-to-cell movement is employed by the potyviruses and comprises intercellular transport without the involvement of tubular structures, but there is a requirement of the CI protein, the CP and the helper component proteinase (HC-Pro)(Figure 1.3.D). Viruses of this family, e.g. tobacco etch virus (TEV) or potato virus Y (PVY), induce substantial physical and morphological changes to the plasmodesmata in infected tissue. Plasmodesmata of potyvirus infected cells contain so called cylindrical inclusions (CIs) which appear on both sides of the plasmodesmata of adjacent cells (Langenberg, 1986; Lesemann, 1988). These bidirectionally-orientated projections consist of the viral CI protein, which contains helicase and ATP-ase activity (Lain *et al.*, 1990, 1991). Based on this, the CI protein is thought to have an energy providing function in guidance of virions or RNA-protein complexes through plasmodesmata. Recently, the CP of tobacco vein mottling potyvirus (TVMV) was detected near or inside the cylindrical inclusions (CIs) in the early stages of TVMV-infected *Nicotiana tabacum* mesophyll tissue (Rodriguez-Cerezo *et al.*, 1997). Moreover, also viral RNA could be detected in association with the CIs and the CP. Later in infection, the CIs become detached from the cell wall and can be subsequently found in the cytoplasm bound to ER-cisternae forming the characteristic pinwheel structures (Lesemann *et al.*, 1988).

Mutational analyses on the CP of TEV revealed that the core domain is essential for both encapsidation of viral RNA and cell-to-cell movement, while the N and C-terminal domains are required for virus entrance and exit of vascular cells (long distance movement)(Dolja *et al.*, 1994, 1995). Microinjection studies performed with the CP of bean common mosaic necrosis virus (BCMNV) and lettuce mosaic virus (LMV) showed that both CPs trafficked from cell-to-cell, thereby increasing the plasmodesmal SEL of *N.*

*tabacum* mesophyll tissue up to 10 kDa dextran levels. Moreover, the CPs also facilitated the transport of viral RNA (Rojas *et al.*, 1997).

The helper component proteinase (HC-Pro) of BCMNV, which is known to function in cleavage and processing of the large viral polyprotein, is also required for cell-cell movement. Previous indications that HC-Pro is involved in movement were based on the presence of a nucleic acid binding domain in PVY HC-Pro (Maia and Bernardi, 1996) and the ability to mediate cell-cell movement of a movement defective geminivirus. Microinjection studies showed that HC-Pro moves to surrounding cells, thereby increasing the SEL of mesophyll plasmodesmata up to 40 kDa dextran levels. Furthermore, HC-Pro mediated the trafficking of BCMNV and LMV CP RNA in *N. benthamiana* and lettuce mesophyll cells (Rojas *et al.*, 1997).

Although evidence has been obtained about the involvement of the CIs, CP and HC-Pro in cell-cell movement, it is not clear in what way they interact in movement. Based on the presence of filamentous, helical particles at the CIs (Rodriguez-Cerezo *et al.*, 1997) and the ability of the BCMNV HC-Pro to enlarge the SEL of mesophyll plasmodesmata up to 40 kDa dextran levels (equivalent Stokes' radius ~ 8 nm) (Rojas *et al.*, 1997), it is possible that potyviruses (approx. width ~ 10 nm) move as virions.

Besides the potyviruses, also the potex-, carla-, hordei-, and furoviruses require the CP for cell-cell movement without the involvement of tubular structures. However, the last four groups of viruses differ from the potyviruses in that they contain three partially overlapping internal open reading frames, known as the triple gene block (TGB), which are all involved in movement (Petty and Jackson, 1990; Beck *et al.*, 1991). The TGB encodes three non-structural proteins of respectively 24-26 kDa, 12-14 kDa and 7-11 kDa, of which the specific functions have not yet been identified completely. Although no significant homology has been found between the TGB proteins and e.g. the TMV MP, there are similarities in function and location. For example, nucleic acid- and NTP-binding domains present in the TMV MP (Citovsky *et al.*, 1990; Saito *et al.*, 1988) are also shared for the 24-26 kDa protein of the potexviruses. Moreover, viruses containing TMV-like MPs can be complemented by the MPs of TGB viruses and vice versa (Malyshenko *et al.*, 1989). Microinjection studies performed in *N. clevellandii* trichome cells demonstrated that movement of PVX is accompanied by a SEL increase and that this plasmodesmal modification requires a functional 25 kDa protein (Angell *et al.*, 1996), although this protein is not found in association with plasmodesmata.

In addition to the requirement of the TGB proteins in cell-cell movement, the CP is also essential in this process (Chapman *et al.*, 1992; Forster *et al.*, 1992; Baulcombe *et al.*, 1995; Oparka *et al.*, 1996). The CPs of the potexviruses foxtail mosaic virus (FMV), cactus virus X (CVX) and PVX are specifically localised in the plasmodesmata of infected cells (Rouleau *et al.*, 1995; Oparka *et al.*, 1996). However, the CP is not targeted to the

plasmodesmata in the absence of other virus components nor does it alter the plasmodesmal SEL (Oparka *et al.*, 1996).

At this point it is not clear whether the carla-, furo-, hordei- and potexviruses move as a mature virion or as a RNA-MP complex. On one side, several observations point to the transport of virions. Fibrillar material with a mean diameter of 13 nm is present in the central cavity of plasmodesmata in PVX infected cells (Oparka *et al.*, 1996). As the diameter of this fibrillar material is equivalent to the width of PVX particles (13 nm; Koenig and Lesemann, 1989), it is suggested that intact PVX virions enter and pass through the plasmodesma. On the other hand, for the potexviruses FMV and CVX only the CP but no intact virions have been detected in the plasmodesmata (Rouleau *et al.*, 1995). Moreover, movement of PVX is associated with an increase in SEL (Angell *et al.*, 1996). As this alteration of SEL is not sufficient to allow the passage of 20 kDa F-dextran, which corresponds to a Stokes radius of 3.1 nm, it is possible that PVX moves as RNA-MP complex and not as virion (diameter 13 nm).

### 1.3. Outline of this thesis

At the onset of this research, there was no information about the cell-to-cell movement process of TSWV. It was only speculated that the $NS_M$ gene, the extra cistron in the tospoviral genome, could be the viral MP gene allowing tospoviruses to pass through plasmodesmata. To investigate the function of $NS_M$, the expression kinetics and subcellular location of this protein was studied in systemically infected *Nicotiana rustica* leaves (Chapters 2 and 3) and infected *N. rustica* protoplasts (Chapter 4). Moreover, the $NS_M$ gene was also studied in *N. rustica* tobacco protoplasts in the absence of other viral components by achieving its expression using a plant expression vector (Chapter 3).

An important characteristic observed for several MPs is the ability to change the functional diameter, or size exclusion limit (SEL), of plasmodesmata (e.g. Wolf *et al.*, 1989; Ding *et al.*, 1992, 1995; Fujiwara *et al.*, 1993; Waigmann *et al.*, 1994; Rojas *et al.*, 1997). To investigate whether this is also true for TSWV $NS_M$, the $NS_M$ gene was transgenically expressed in *Nicotiana tabacum* SR1 plants. The plants were then examined for $NS_M$ protein expression, subcellular localisation of the protein and effect on macromolecular diffusion properties of the plasmodesmata (Chapter 5). The latter was assessed by microinjection of fluorescently labelled probes exhibiting various diameters employing two different microinjection techniques, one by which the influx of probes is achieved by a pressure pulse and a second using a diffusion mediated delivery system (iontophoresis). The outcome of these experiments is discussed in Chapter 6.

To investigate the requirement for plant specific components in tubule formation, $NS_M$ was expressed in heterologous insect cells and mammalian (baby hamster kidney, BHK) cells (Chapter 7). To explore the possibility that $NS_M$ also has a function in the insect part of the viral life cycle, the expression and location of $NS_M$ was analysed in all developmental stages of the TSWV-infected thrips *Frankliniella occidentalis* (Chapter 8). In Chapter 9, the results are summarised and concluding remarks are drawn.

# 2

# Expression and subcellular location of the $NS_M$ protein of tomato spotted wilt virus, a putative viral movement protein.

## 2.1. SUMMARY

The 33.6 kDa non-structural $NS_M$ protein gene, located on the ambisense M RNA segment of tomato spotted wilt virus (TSWV), was cloned and expressed using the *Escherichia coli* pET-11t expression system. The protein thus produced was purified and used for the production of a polyclonal antiserum. Western immunoblot analyses of TSWV-infected *Nicotiana rustica* plants, using this antiserum, showed $NS_M$ synthesis during only a short period early in systemic infection. Although $NS_M$ was found associated with cytoplasmic nucleocapsids, it was absent from purified virus particles. Analyses of subcellular fractions from young, systemically infected leaves showed the presence of $NS_M$ in fractions enriched for cell walls and cytoplasmic membranes, respectively. The data obtained provide evidence that $NS_M$ represents the viral movement protein of TSWV, involved in cell-to-cell movement of non-enveloped ribonucleocapsid structures.

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## 2.2. INTRODUCTION

Tomato spotted wilt virus (TSWV) is the type species of the genus Tospovirus, in which a number of plant-infecting Bunyaviruses have been classified (De Haan *et al.*, 1989; Elliott, 1990; Milne and Francki, 1984; Francki *et al.*, 1991). Whereas animal-infecting members of the Bunyaviridae are mainly transmitted by ticks, mosquitoes and sandflies (Elliott, 1990), TSWV is exclusively transmitted by thrips in a persistent manner (Sakimura, 1962).
The complete nucleotide sequence of the genome of TSWV has become available (De Haan *et al.*, 1990, 1991). The TSWV L RNA is 8897 nucleotides (nt) and completely of negative polarity, encoding the putative viral RNA polymerase (De Haan *et al.*, 1991). The M RNA is 4821 nt and has an ambisense gene arrangement. It encodes a putative non-structural protein ($NS_M$) of 33.6 kDa in viral (v) sense and the precursor to the glycoproteins (G1 and G2) of 127.4 kDa in viral complementary (vc) sense. The S RNA is 2916 nt and has, similar to the M RNA, an ambisense gene arrangement. This genome segment encodes a non-structural protein ($NS_S$) of 52.4 kDa in v sense and the nucleocapsid (N) protein of 28.8 kDa in vc sense (De Haan *et al.*, 1990).
Comparison of the TSWV genome with those of animal-infecting bunyaviruses (Elliot, 1990; Francki *et al.*, 1991) reveals the presence of one extra gene in the former, i.e. the $NS_M$ gene located on the M RNA segment. This extra gene may reflect an adaptation of bunyaviruses to botanical hosts. In order to investigate the function of the $NS_M$ protein in the infection cycle of TSWV, this protein was expressed in the *E. coli* pET-11t system to enable the production of a specific polyclonal antiserum. Using this antiserum both the synthesis and the intracellular location of $NS_M$ during TSWV multiplication was analysed.

## 2.3. MATERIALS AND METHODS

### 2.3.1. Virus, plants and cDNA clones

TSWV isolate BR-01 was maintained in *Nicotiana rustica* "America" plants by thrips transmission and mechanical inoculation. Virus was purified according to the method of Tas *et al.* (1977). Nucleocapsids were isolated from infected leaf tissue as described by de Avila *et al.* (1990), omitting the sucrose gradient step. Complementary DNA clones representing the M RNA of TSWV BR-01 have been described previously (Kormelink *et al.*, 1992). Wild type (wt) and recombinant *Autographa californica* nuclear polyhedrosis viruses (AcNPV) were grown in monolayers of *Spodoptera frugiperda* 21 cells (Vaughn *et al.*, 1977) in TNMFH medium (Hink, 1970) containing 10% fetal calf serum.

### 2.3.2. Construction of AcNPV recombinant virus

The baculovirus *Autographa californica* Multiple Nuclear Polyhedrosis Virus (AcNPV) was used for eukaryotic expression of $NS_M$ in *Spodoptera frugiperda* insect cells. Complementary DNA clone pTSWV28 containing the complete open reading frame (ORF) of $NS_M$ was digested with *Bam*HI and cloned in the *Bam*HI site of pAc33DZ1 (Kormelink *et al.*, 1992; Zuidema *et al.*, 1990). The resulting transfer vector, pAc33DZ1/$NS_M$+L (+L denotes the presence of the TSWV-specific 5'-untranslated sequence), contained the complete ORF of $NS_M$ including the TSWV viral 5'-untranslated sequence.

For convenient cloning of the $NS_M$ gene without the viral 5'-untranslated sequence, the gene was amplified by polymerase chain reaction (PCR) using oligonucleotides Zup51 (dGGGAATTCTTTTCGGTAACAAGAGGCC), containing 27 nucleotides of which 21 are identical to nucleotides 109 to 129 of the viral (v) strand of M RNA, and Zup14 (dCCCTGCAGGATCCGAAATTTAAGCTTAAATAAGTG), having 22 nucleotides complementary to nucleotides 1022 to 1043 of the vRNA strand (Figure 2.1.A). After PCR-amplification, the DNA was digested with *Eco*RI, a *Bam*HI-*Eco*RI adaptor (containing the nucleotide sequence GGATCCGGCAACGAAGGTACCATGGGAATTC), with an internal start codon, ligated in order to restore the $NS_M$ ORF, and subsequently digested with *Bam*HI to generate the $NS_M$ gene as a *Bam*HI fragment. This fragment was purified from an agarose gel, cloned in the *Bam*HI site of plasmid pAc33DZ1 resulting in transfer vector pAc33DZ1/$NS_M$ (Figure 2.1.B). Recombinant baculoviruses were produced by co-transfection of *S. frugiperda* (Sf) cells with a mixture of *BSu*36I digested AcNPV PAK6 DNA and pAc33DZ1/$NS_M$+L or pAc33DZ1/$NS_M$ DNA according to Kitts and Possee (1993).

Recombinant baculoviruses were plaque purified (Brown and Faulkner, 1977) and subsequently grown in high titer stocks. Analysis of proteins from infected *S. frugiperda* on SDS-PAGE were as described previously (Kormelink, *et al.*, 1991).

### 2.3.3. Construction of pET-11t/$NS_M$

For cloning of the $NS_M$ gene in pET-11t the PCR-amplified DNA, obtained as described above, was digested with restriction enzymes *Eco*RI and *Bam*HI, leaving an $NS_M$ gene lacking the ATG start codon. The fragment was subsequently cloned in frame with the ATG start codon of pET-11t, resulting in plasmid pET-11t/$NS_M$. The nucleotide sequences at the insertion sites were verified by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977). Due to the use of oligonucleotide Zup51, containing an altered TSWV sequence for introduction of an *Eco*RI cloning site, two amino acids at the N-terminus of the $NS_M$

protein were changed, i.e. the second amino acid (leucine to glycine) and the third (threonine to isoleucine).

### 2.3.4. Expression in *Escherichia coli*

For analysis on $NS_M$ expression, BL21 cells were transformed with the pET-11t/$NS_M$ construct and grown overnight in LB medium and ampicillin selection pressure (100 mg/ml). A fresh flask was inoculated with 1/100 volume of the overnight culture and the cells were grown until an $OD_{600}$=0.5 was reached. IPTG was added to a final concentration of 0.4 mM and growth was prolonged for an additional 2-3 hr. The cells were collected, resuspended in lysis buffer (50 mM Tris-HCl pH 8.0; 5% SDS; 15 mM 2-mercaptoethanol) and boiled for 15 min.

For SDS-PAGE analyses of the proteins, the cells were subsequently boiled in 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 2% (w/v) SDS, 10% (v/v) glycerol, 0.001% bromophenol blue, 5% (v/v) beta-mercaptoethanol (protein loading buffer). Samples were analysed on a 12.5% polyacrylamide gel containing SDS (Laemmli, 1970).

### 2.3.5. Purification of $NS_M$ protein

For large-scale preparation of the TSWV $NS_M$ protein, proteins from 4 ml IPTG-induced *E. coli* cells transformed with pET-11t/$NS_M$ were resolved on 0.75-mm-thick preparative 12.5% polyacrylamide gels containing SDS (Protean II system, Bio-Rad). The $NS_M$ protein was purified as described previously for $NS_S$ (Kormelink *et al.*, 1991).

### 2.3.6. Preparation of antibodies to the $NS_M$ protein

Portions of 50 to 100 mg purified $NS_M$ protein were emulsified in Freund's incomplete adjuvant (Difco Laboratories) and injected into the hind legs of a rabbit at days 1, 13, 26 and 46. From day 41 on, the rabbit was bled several times and gamma-globulin fractions isolated according to Clark and Adams (1977) and tested with protein blots.

### 2.3.7. Western immunoblot analyses

Samples from TSWV-infected *N. rustica* were prepared by homogenizing 0.1 g of systemically infected leaves in 0.1 ml PBS containing 0.05% Tween-20. After combining the extract with 4x protein loading buffer, 10 ml of healthy- and TSWV-infected *N. rustica* extracts were applied on a 12.5% SDS-polyacrylamide gel. After SDS-PAGE, proteins were

transferred to Immobilon membrane (Millipore) and screened with polyclonal antisera as described previously (Kormelink *et al.*, 1991).

### 2.3.8. Preparation of subcellular extracts from TSWV-infected leaves

Subcellular extracts of systemically infected leaves were prepared essentially as described by Deom *et al.* (1990). In brief, TSWV-infected leaves were harvested 6 to 7 days after inoculation. The central nerves were removed and 10 g of leaves ground to a fine powder under liquid nitrogen. The material was subsequently resuspended in 20 ml grinding buffer (GB: 100 mM Tris-HCl pH 8.0; 10 mM EDTA; 5 mM dithiothreitol) and filtered through two layers of cheesecloth. The extract was centrifuged for 10 min at 1000 g to obtain a crude cell wall pellet (Pe-1). To obtain a Pe-1 fraction mainly consisting of cell wall material, the pellet was washed for two times in GB plus 2% Triton X-100. The supernatant obtained after the first centrifugation was subsequently centrifuged for 30 min at 30,000 g to obtain a Pe-30 pellet and S-30 supernatant. Half of the S-30 sample was layered on a 30% sucrose-cushion (in GB) and centrifuged for 60 min at 40,000 rpm in a Ti45 rotor, to obtain a pellet (S-30P, mainly containing the cytoplasmic nucleocapsids) and supernatant (S-30S) fraction. The proteins of the S-30 and S-30S fractions were concentrated by precipitation with 50% ammonium sulfate, resuspended in a smaller volume and dialysed prior to preparation for SDS-PAGE. All samples were concentrated 20-fold with respect to the original extract. Five ml of each fraction was applied on a 12.5% SDS-polyacrylamide gel.

## 2.4. RESULTS

### 2.4.1. Construction of recombinant baculovirus AcNPV/$NS_M$

To express the $NS_M$ gene in the baculovirus/insect cell system a cDNA fragment from clone pTSWV28 (Kormelink *et al.*, 1992) containing the complete ORF and most of the 5'-untranslated sequence of the TSWV $NS_M$ gene (nucleotides 10 to 1065 of the vRNA strand of TSWV M RNA) was cloned into plasmid pAc33DZ1 (Zuidema *et al.*, 1990), and transferred to baculovirus AcNPV by co-transfection of *S. frugiperda* cells with a mixture of *BSu*36 I digested AcNPV PAK6 DNA (Kitts and Possee, 1993) and pAc33DZ1/$NS_M$+L (+L stands for the presence of the TSWV-specific 5'-untranslated sequence). In addition, a $NS_M$ construct lacking the viral 5'-untranslated sequence was PCR-amplified using oligonucleotides Zup51 and Zup14 (Figure 2.1.A; Materials and Methods), cloned into pAc33DZ1 (Figure 2.1.B) to form transfer vector pAc33DZ1/$NS_M$, and transferred to AcNPV. Recombinants of AcNPV containing the $NS_M$ gene with (AcNPV/$NS_M$+L) or

Clones containing the $NS_M$ gene were selected and analysed by restriction enzyme analyses. The nucleotide sequence in the resulting pET-11t/$NS_M$ construct (Figure 2.1.C) was verified in order to confirm the intactness of the open reading frame.
The production of $NS_M$ protein in pET-11t/$NS_M$ transformed BL21 cells was induced with IPTG, and analysed by SDS-PAGE. A protein band corresponding to the expected size of the $NS_M$ protein was clearly visible (Figure 2.3.A, lane pET-11t/$NS_M$). The $NS_M$ protein produced in this way was purified (Figure 2.3.A, lane $NS_M$) and subsequently used for the production of antibodies.

### 2.4.3. Antiserum to $NS_M$ protein

Purified $NS_M$ protein was injected into rabbits four times at intervals of 1-2 weeks. After the third injection, blood was collected, the immunoglobulin fraction isolated, and the antiserum tested for the presence of antibodies against the $NS_M$ protein. A sample of pET-11t/$NS_M$ transformed BL21 cells, induced with IPTG, was subjected to electrophoresis, transferred to Immobilon membrane, and analysed by immunoblot analysis using 1 μg/ml of $NS_M$ antiserum. The results demonstrate the presence of antibodies against denatured $NS_M$ (Figure 2.3.B, lane pET-11t/$NS_M$), although also immunoglobulins against proteins co-purified from BL21 cells were present (Figure 2.3.B, lane pET-11t). Moreover, the antiserum detected specifically very low amounts of a protein with the expected size of $NS_M$ in insect cells infected with the AcNPV/$NS_M$+L recombinant baculovirus (Figure 2.2.B, lane SfxAcNPV/$NS_M$+L), which was clearly absent from healthy or wt AcNPV-infected insect cells. A higher level of expression of this protein was found when the TSWV $NS_M$ 5'-untranslated sequence was absent from the baculovirus construct (Figure 2.2.B, lane SfxAcNPV/$NS_M$). These results showed that the protein detected originated from the TSWV $NS_M$ coding sequences in AcNPV/$NS_M$, and demonstrated the specificity of the anti-$NS_M$ serum. Also, the absence of any cross-reaction with proteins from healthy or wt baculovirus-infected insect cells indicated that the contamination of immunoglobulins against proteins co-purified from BL21 cells did not interfere with immunological analyses. Hence it was anticipated that these contaminations would not interfere with further immunological analyses in plant systems. Some smaller protein bands, probably stable degradation products, were often seen in AcNPV/$NS_M$-infected insect cells that were reacting with anti-$NS_M$ serum (Figure 2.2.B, lane SfxAcNPV/$NS_M$; Figure 2.6.A, lane SfxAcNPV/$NS_M$).

Analysis of the nucleotide sequence of the $NS_M$ gene demonstrated the presence of two potential glycosylation sites in the $NS_M$ protein (Kormelink *et al.*, 1992). Western immunoblot analysis of $NS_M$ protein produced from the AcNPV/$NS_M$ recombinant in the presence of tunicamycin (25 μg/ml) revealed no detectable shift in migration of this

protein indicating that, at least in insect cells, this viral protein is not glycosylated (Figure 2.2.B, lane SfxAcNPV/$NS_M$ "+").

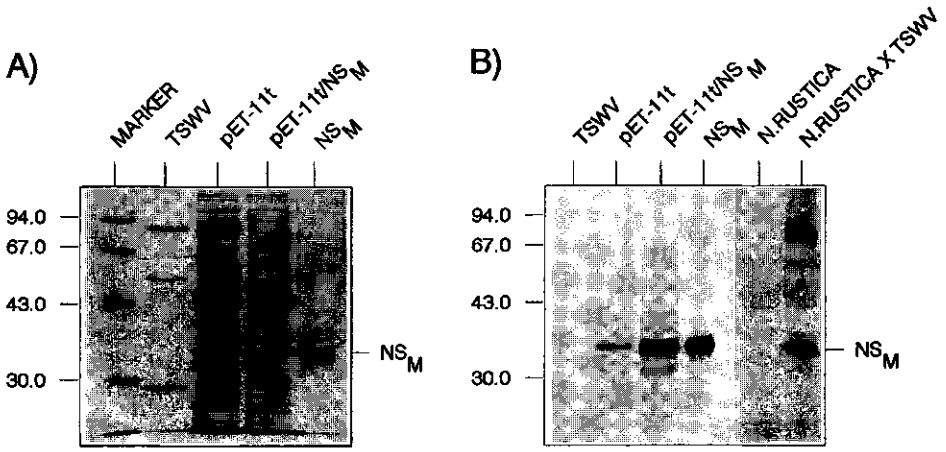


**Figure 2.3.** Production of TSWV $NS_M$ protein in *E. coli* **(A)** and specificity of the antibodies raised against TSWV $NS_M$ protein **(B)**. The production of $NS_M$ in *E. coli* was induced with IPTG, and analysed on Coomassie brilliant blue stained SDS-polyacrylamide gel **(A)**. Purified $NS_M$ protein used for the production of antibodies is shown in the fifth lane. As a control pET-11t transformed BL21 cells was included. Low-molecular-weight size markers (Pharmacia) are indicated at the left. The $NS_M$ protein expressed in *E. coli* is indicated. Similar protein samples were analyzed on a Western blot to test the specificity of antibodies raised against the $NS_M$ protein **(B)**. A sample of 250 ng purified TSWV BR-01 virus and 10 ml portions of extracts from healthy- and TSWV-infected *N. rustica* plants were included. The Western blot was analyzed using 1 µg/ml $NS_M$ antiserum.

### 2.4.4. Detection of $NS_M$ protein in TSWV-infected plant material

In order to establish the actual production of $NS_M$ during the TSWV infection cycle, plant extracts from healthy and systemically infected *N. rustica* leaves were analysed on Western blots using the antiserum raised against *E. coli* expressed $NS_M$. In extracts from infected plants a protein could be detected with the expected size of $NS_M$ (Figure 2.3.B, lane *N. rustica* x TSWV) which was absent from healthy plant extracts (Figure 2.3.B, lane *N. rustica*), confirming the viral origin of the protein detected in TSWV-infected plants. Additionally, a protein of about 67 kDa specifically reacted with antiserum against $NS_M$ in TSWV-infected *N. rustica*, most likely representing a dimer of $NS_M$. No reaction was obtained with purified TSWV particles (Figure 2.3.B, lane TSWV), demonstrating that this viral protein indeed represents a nonstructural protein.

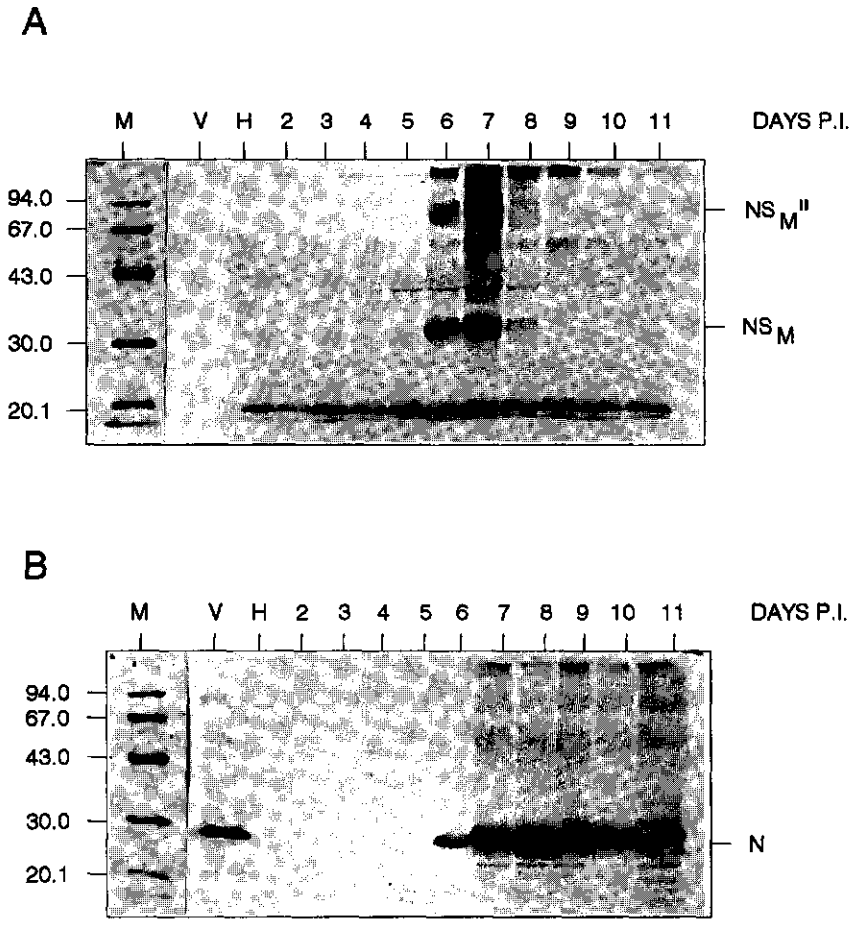


**Figure 2.4.** Time course of synthesis of $NS_M$ **(A)** and N **(B)** protein in TSWV-infected *N. rustica*. The plant extracts were prepared from systemically infected leaves at different times after inoculation, indicated at the top of the figure, as described in Materials and Methods. Ten ml of plant extract was applied on a 12.5% SDS-polyacrylamide gel. As controls, 250 ng purified TSWV virus (V), and an extract of healthy *N. rustica* (H) were included. After Western blotting on Immobilon membranes, the filters were screened with 1 µg/ml anti-$NS_M$ **(A)** or anti-TSWV serum **(B)**. Low-molecular-weight size markers (Pharmacia) are indicated at the left. The positions of N, $NS_M$ and a potential dimer of $NS_M$ (denoted as $NS_M$") are indicated.

To follow the synthesis of $NS_M$ during infection of *N. rustica*, plant extracts, prepared from systemically infected leaves at different times after inoculation, were analysed on Western immunoblots. The results revealed that the production of $NS_M$, and the putative $NS_M$ dimer ($NS_M$"), was maximal at days 6 and 7 p.i., coinciding with the appearance of systemic symptoms, and drastically decreased at 8 days p.i. (Figure 2.4.A). The kinetics of $NS_M$ accumulation is therefore clearly distinct from that of N protein and $NS_S$, the amounts of

which accumulated throughout later stages of infection (Figure 2.4.B,C). These data indicate a transient character of $NS_M$ and the involvement in an early process during TSWV infection. During the course of infection, another protein of about 40 kDa weakly cross-reacted with the anti-$NS_M$ serum. The identity of this band is not clear, however, its presence in extracts of healthy *N. rustica* (Figure 2.3.B, lane *N. rustica*) suggests that this protein is of host origin.

### 2.4.5. Subcellular localization of $NS_M$

To investigate the intracellular location of the $NS_M$ protein, different fractions from infected leaf material were isolated and analysed for the presence of the $NS_M$ protein. To this end, cytoplasmic nucleocapsid (TSWV-nu) fractions were prepared from TSWV-infected *N. rustica* at day 6 and day 9 p.i. Additionally, by another method, subcellular fractions from TSWV-infected leaves were prepared according to Deom *et al.* (1990), and the resulting fractions (Pe-1, Pe-30 and S-30) applied on a SDS-polyacrylamide gel and transferred to Immobilon membranes. Western immunoblot analysis revealed that $NS_M$ co-purified with nucleocapsids, and that it disappeared from those structures in later stages during the infection cycle (Figure 2.5.A, lane TSWV-NU 6 days p.i. and 9 days p.i.). A control Western immunoblot was analysed with anti-N serum to confirm the presence of equal amounts of nucleocapsids in both samples (data not shown). The $NS_M$ protein present in nucleocapsid extracts, though, migrated slower than $NS_M$ produced in the baculovirus system (Figure 2.5.A, lane SfxAcNPV/$NS_M$). This migration difference was not genuine but rather due to a difference in protein content, phosphorylation of the protein or ionic strength of the samples.

Using a different fractionation protocol, it could be demonstrated that the $NS_M$ protein was mainly present in the Pe-1 and Pe-30 fractions (Figure 2.5.B). A similar Western blot treated with antiserum against the TSWV N protein revealed highest amounts of N protein in the S-30 fraction (data not shown), indicating that the $NS_M$ protein detected in the Pe-1 and Pe-30 samples was specifically associated with these subcellular fractions, which contained enriched amounts of cell wall and cytoplasmic membrane material (Deom *et al.*, 1990). The presence of high amounts of nucleocapsids in the S-30 fraction suggests that the low amounts of $NS_M$ protein detected in this fraction was probably due to its association with nucleocapsids. In order to test this, the S-30 fraction was further fractionated, via a sucrose-cushion, into a pellet (S-30P) that was enriched for nucleocapsids, and a supernatant (S-30S) fraction. Subsequent Western immunoblot analysis showed the presence of $NS_M$ in the S-30P fraction and the absence in the S-30S fraction (Figure 2.5.B, lane S-30P and S-30S). The subcellular fraction data, therefore, are in agreement with the electron microscopical data presented in Chapter 3 that showed a close association of $NS_M$ with plasmodesmata and nucleocapsid aggregates.

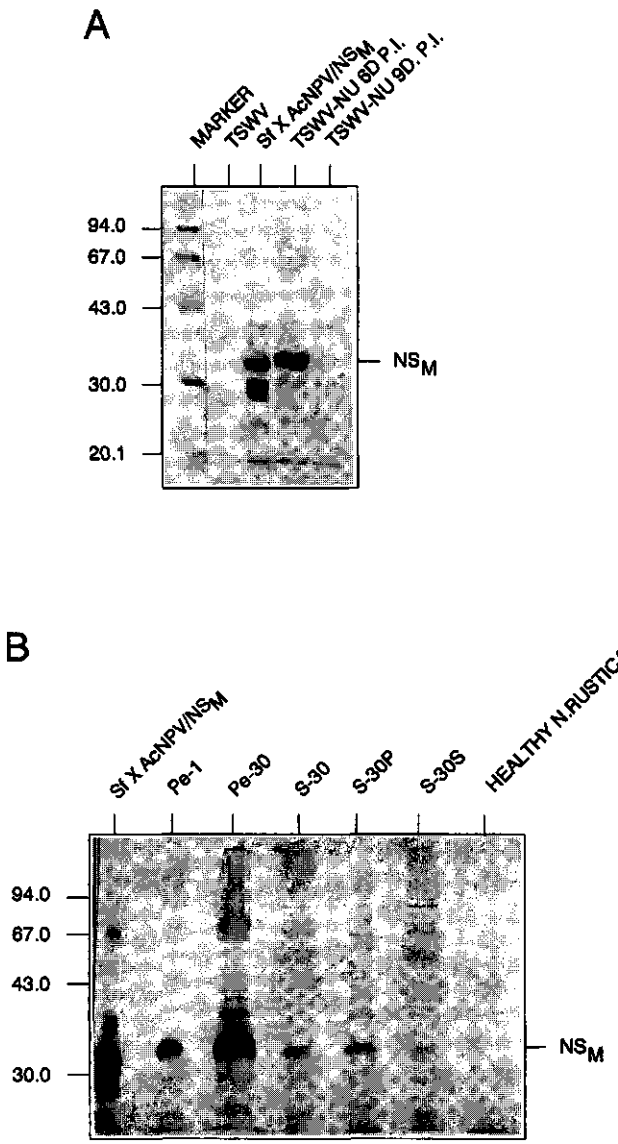


**Figure 2.5.** Presence of $NS_M$ in nucleocapsid fractions **(A)** and subcellular fractions of TSWV-infected *N. rustica* **(B)**. Purification of nucleocapsids and preparation of different fractions of TSWV-infected *N. rustica* leaf material were as described in Materials and Methods. Ten ml of nucleocapsid preparations (TSWV-NU) and 5 ml of the subcellular fractions (Pe-1, Pe-30, S-30, S-30P and S-30S) were applied on a 12.5% SDS-polyacrylamide gel. The proteins were transferred to Immobilon membrane and the filter subsequently screened with 1 µg/ml antiserum against $NS_M$. As controls were included, 250 ng purified TSWV virus, a protein extract of AcNPV/$NS_M$-infected *S. frugiperda* (Sf) cells and an extract of healthy *N. rustica*. The position of $NS_M$ is indicated.

## 2.5. DISCUSSION

For a growing number of plant-infecting positive strand RNA viruses a virally-encoded movement protein, involved in cell-to-cell movement of the pathogen, has been identified either based on biochemical evidence or on protein sequence analysis (for reviews see Deom *et al.*, 1992; Hull, 1991; Koonin *et al.*, 1991; Maule, 1991). Also various mechanisms for the process of cell-to-cell movement of these viruses, or their genomes, have been proposed (Goldbach, *et al.* 1990; Citovsky *et al.*, 1990; Deom *et al.*, 1992), which always involve the modification of plasmodesmata. For negative strand RNA viruses of plants, e.g. rhabdo- and tospoviruses, no information is available about their mode of cell-to-cell movement or about the possible involvement of viral gene products therein.

Comparison of the genome of TSWV with those of animal-infecting members of the Bunyaviridae already suggested that $NS_M$ represents a possible candidate for a viral movement protein, as it is specified by an extra open reading frame, not present in the genome of animal-infecting bunyaviruses. In order to gain insight into the function of this protein, an antiserum was produced against $NS_M$ expressed in *E. coli* and its actual synthesis demonstrated in infected *N. rustica*. Time course analyses showed the presence of $NS_M$ only during a short period early in the infection cycle (Figure 2.4.A), always coinciding with the first appearance of systemic symptoms, in contrast to other TSWV specific proteins that further accumulated from that moment on (Figure 2.4.B,C). This timing may be a first indication that the $NS_M$ protein could actually represent the putative viral movement protein, in agreement with the suggestion by Atabekov and Dorokhov (1984) that virus movement is an early function which is eventually switched off. Similar transient expression has been demonstrated for several other (putative) movement proteins, e.g. the TMV 30 K protein (Watanabe *et al.*, 1984; Lehto *et al.*, 1990) and the AlMV P3 protein (Berna *et al.*, 1986).

Subcellular localization studies on TSWV-infected *N. rustica* 6 to 7 days p.i. demonstrated that the $NS_M$ protein was found located in the fractions relatively enriched for cytoplasmic membranes (Figure 2.5.B, lane Pe-30) and cell wall residues (Figure 2.5.B, lane Pe-1), respectively. The presence of $NS_M$ in these fractions suggests that the protein might somehow be associated with specific structures present in these fractions e.g. the intracellular translocation channels or plasmodesmata present in cell wall residues (see also Chapter 3). The $NS_M$ protein was also found in low amounts in the cytoplasmic S-30 fraction. Its presence could be explained by its association to nucleocapsids in this fraction. Hence, the time course analysis and the fractionation studies provide indications for the involvement of the $NS_M$ protein, as the putative viral movement protein, in the cell-to-cell movement of TSWV.

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# The non-structural protein of tomato spotted wilt virus induces tubular structures in plant cells.

## 3.1. SUMMARY

To study the function of the 33.6 kDa non-structural protein $NS_M$ of tomato spotted wilt virus (TSWV), the expression and subcellular location of this protein was analysed in *Nicotiana rustica* plants and protoplasts as a function of time. Immunofluorescent detection of $NS_M$ in protoplasts isolated from TSWV infected *N. rustica* leaves revealed that this protein first accumulates near the periphery of the cell and near the plasmamembrane, and later in tubular structures emerging from the cell surface. In protoplasts transfected with an expression vector, containing the $NS_M$ coding sequence, similar tubules were formed, indicating the potential of this protein to form these structures in the absence of other viral specific components. Electron microscopical analyses of systemically infected leaves demonstrated that the $NS_M$ protein was associated to viral nucleocapsids in the cytoplasm, plasmodesmata and tubular structures that assemble at the plasmodesmata. It is concluded that the $NS_M$ protein may be involved as movement protein in a tubule-guided cell-to-cell virus translocation mechanism.

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## 3.2. INTRODUCTION

In the previous chapter it was shown that the $NS_M$ protein of TSWV is only transiently present early in the infection of plant tissue. During its transient appearance, $NS_M$ co-purifies with enriched cell fractions and cytoplasmic nucleocapsids (Kormelink *et al.*, 1994). Based on these studies it was suggested that $NS_M$ represents the viral MP of TSWV involved in viral passage through the cell wall. A characterisic observation of most, if not all, plant viral MPs is their specific association to plasmodesmata (Chapter 1; e.g. Carrington *et al.*, 1996). To investigate if this is also true for $NS_M$, its intracellular behaviour was studied in systemically infected *N. rustica* leaves. Here we show by microscopical analyses that during infection $NS_M$ is associated with non-enveloped nucleocapsids in the cytoplasm and with plasmodesmata, where it assembles into plasmodesma-penetrating tubular structures. The observation that $NS_M$ specifically forms tubules, not only in single plant cells (protoplasts) but also in infected leaf tissue, provides evidence for a tubule-guided cell-to-cell movement mechanism reminiscent to that described for como- and nepoviruses (Van Lent *et al.*, 1991; Wellink *et al.*, 1993; Wieczorek and Sanfaçon, 1993).

## 3.3. MATERIALS AND METHODS

### 3.3.1. Virus isolate, plant material and antisera.

In all experiments the Brazilian isolate BR-01 of tomato spotted wilt virus (TSWV) was used (De Avila *et al.*, 1992). In all studies *Nicotiana rustica* plants or protoplasts were involved. Polyclonal antisera against the $NS_M$ and nucleocapsid (N) protein, used in the immunolocalisation studies, were prepared as previously described (Kormelink *et al.*, 1991, 1994; De Avila *et al.*, 1992).

### 3.3.2. Protoplast isolation and immunofluorescence microscopy.

*N. rustica* plants were mechanically inoculated with TSWV-BR01. Protoplasts were isolated from systemically infected leaves at daily intervals between 3 and 11 days post inoculation (Hibi *et al.*, 1975; Van Beek *et al.*, 1985). The protoplasts were processed for immunofluorescence microscopy and studied with a Bio-Rad MRC 500 confocal scanning laser microscope (CSLM) containing an argon ion laser and a BHS 488 nm excitation filter (Van Lent *et al.*, 1991).

### 3.3.3. Construction of pMon999/$NS_M$ and transfection of protoplasts.

In order to clone the $NS_M$ gene without the 5' untranslated sequence, the gene was PCR-amplified using oligonucleotides Zup 051 (dGGGAATTCTTTTCGGTAACAAGAGGCC) and Zup 014 (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 $NS_M$ 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 (Maniatis *et al.*, 1982). The integrity of the *Bam*HI fragment containing the $NS_M$ gene was verified by sequence analysis. For transfection studies aliquots of one million *N. rustica* protoplasts were inoculated with 10 mg of pMon999/$NS_M$ under addition of 100 ml 0.6 M mannitol (containing 10 mM $CaCl_2$), 0.5 ml 40% poly-ethyleneglycol ((PEG; MW: 6000) containing 0.5 M mannitol and 0.1 M $Ca(NO_3)_2$), 4.5 ml 0.5 M mannitol (containing 15 mM $CaCl_2$) and 0.1% 2[N-morpholino]ethanesulfonic acid ((MES) pH 5.6). The transfected protoplasts were incubated in nutrition medium and prepared for immunofluorescence and negative staining electron microscopy as described by Van Lent *et al.*, 1991.

### 3.3.4. Electron microscopy.

Leaf samples of systemically infected and healthy leaves of *N. rustica* were taken daily between 6 and 9 days p.i. The samples were fixed in 2% (w/v) paraformaldehyde/ 3% (w/v) glutaraldehyde in phosphate citrate buffer, dehydrated and embedded in London Resin Gold at -25°C. After UV-polymerisation, thin sections were made and processed for immunogold labeling using antiserum against the $NS_M$ and N protein (Van Lent *et al.*, 1990, 1991). The specimens were examined by use of a Philips CM12 electron microscope.

## 3.4. RESULTS

### 3.4.1. Immunofluorescent detection of the $NS_M$ protein in *N. rustica* protoplasts isolated from infected leaves.

To follow the expression and intracellular behaviour of the $NS_M$ protein during the TSWV infection cycle, samples of systemically infected *N. rustica* leaf tissue were taken at daily intervals between 3 and 11 days post inoculation (p.i) and processed for the isolation of single

The percentage of infected mesophyll protoplasts seropositive for the N and $NS_M$ protein of TSWV.

| | anti-N serum | anti $NS_M$-serum | |
|---|---|---|---|
| Days p.i. | | % periphery appearance | % tubular structures |
| 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 |

The percentages are averages of three experiments.

**Figure 3.1.1.** Quantification of $NS_M$ and N protein expressing protoplasts isolated from TSWV-infected *N. rustica* leaves at daily intervals between 4 and 9 days p.i. as determined by immunofluorescence microscopy. The $NS_M$ expressing cells have been subdivided into a category containing $NS_M$ in the cytoplasm at the periphery of the cell (as visualised in Figure 3.2.A) and a category containing $NS_M$ aggregating into tubules extending from the cell surface (as is shown in Figure 3.2.B).

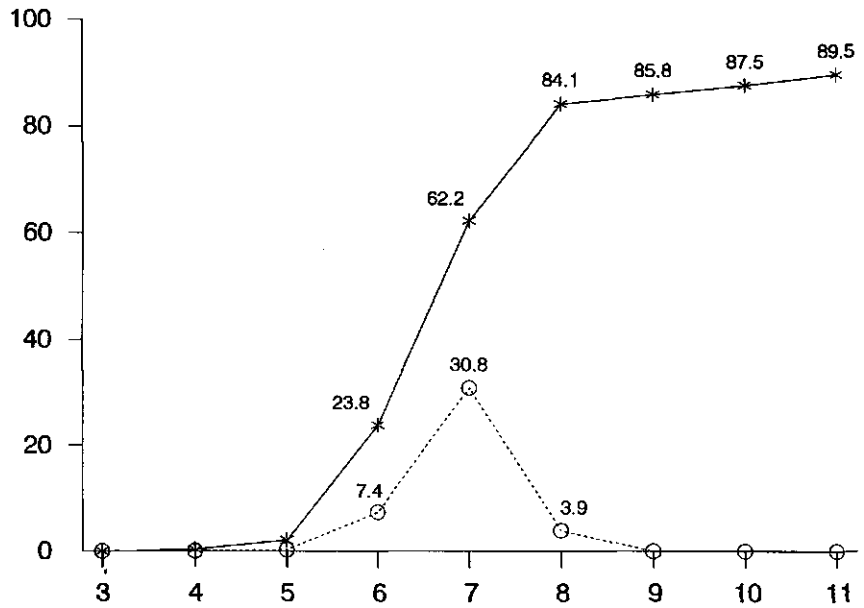


**Figure 3.1.2.** Graphic representation of the proportional amounts of protoplasts seropositive for N (*) and $NS_M$ (O) proteins. X-axis: days post infection; Y-axis: percentage of protoplasts.

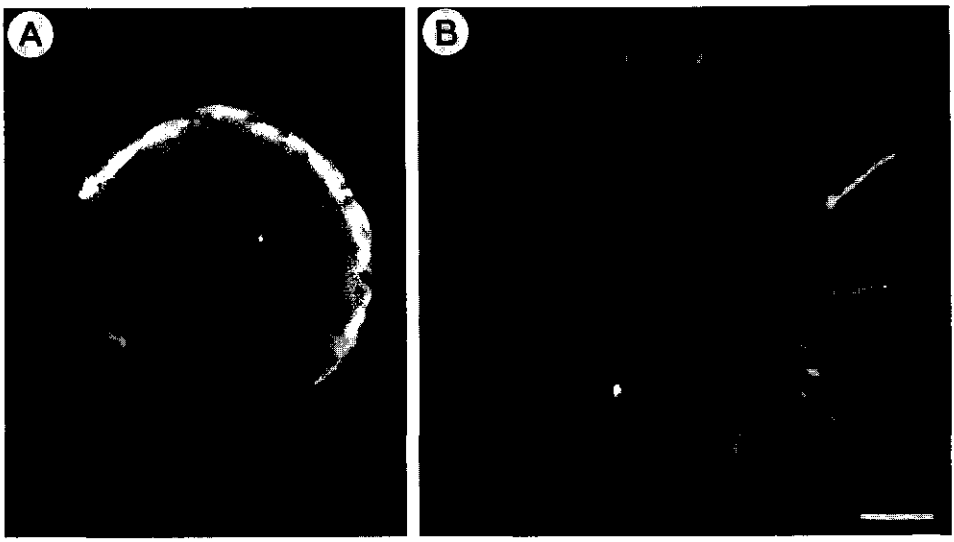


**Figure 3.2.** Immunofluorescent staining of the $NS_M$ protein in protoplasts isolated from systemically infected leaves of *Nicotiana rustica*.
**(A)** Localisation of $NS_M$ in the cytoplasm of *N. rustica* protoplasts at 6 days p.i. **(B)** $NS_M$-containing tubular structures emerging from the protoplast surface at 8 days p.i. Scale bar represents 10 µm.

cells (Hibi *et al.*, 1975; Van Beek *et al.*, 1985). Immunofluorescence microscopy probed on these cells revealed detectable amounts of $NS_M$ only between 6 and 8 days after inoculation confirming the transient character of this protein as was previously reported (Kormelink *et al.*, 1994; Chapter 2). Figure 3.1. shows the percentage of isolated, infected protoplasts as a function of time after inoculation. Infected cells were scored on the basis of N and $NS_M$ protein detection using polyclonal antisera against both viral proteins (Kormelink *et al.*, 1991, 1994; De Avila *et al.*, 1992). At 5-6 days p.i., the first $NS_M$-positive cells were observed and these exclusively revealed a clear fluorescent signal in the cytoplasm at the periphery of the cell (Figures 3.1.1. + 3.2.A). At day 7, the number of $NS_M$-positive cells had increased to 31% and, strikingly, in 27% of the cells (i.e. 87% of the $NS_M$ expressing cells) the $NS_M$ protein appeared to be present in tube-like protrusions extending from the cell surface, while in only 4% of the cells (i.e. 13% of the $NS_M$ expressing cells) $NS_M$ was detected in the periphery of the cytoplasm (Figures 3.1.1. + 3.2.B). At day 8, $NS_M$-positive cells only showed tube-like protrusions at the cell surface while the total amount of $NS_M$-positive cells had drastically decreased to 4% to reach 0% at the days hereafter. The percentage of cells producing the N protein increased sigmoid between day 5 and day 8 to reach a final maximum of about 80 to 90%. The kinetics of N and $NS_M$ protein expression, as shown in Figure 3.1.2., also illustrates that the amount of $NS_M$ drastically drops between days 7 and 8

p.i., while the N protein synthesis further continues. The expression of both the N and $NS_M$ protein as described in Figure 3.1.2. 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 its top. 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 $NS_M$ protein were no longer present.

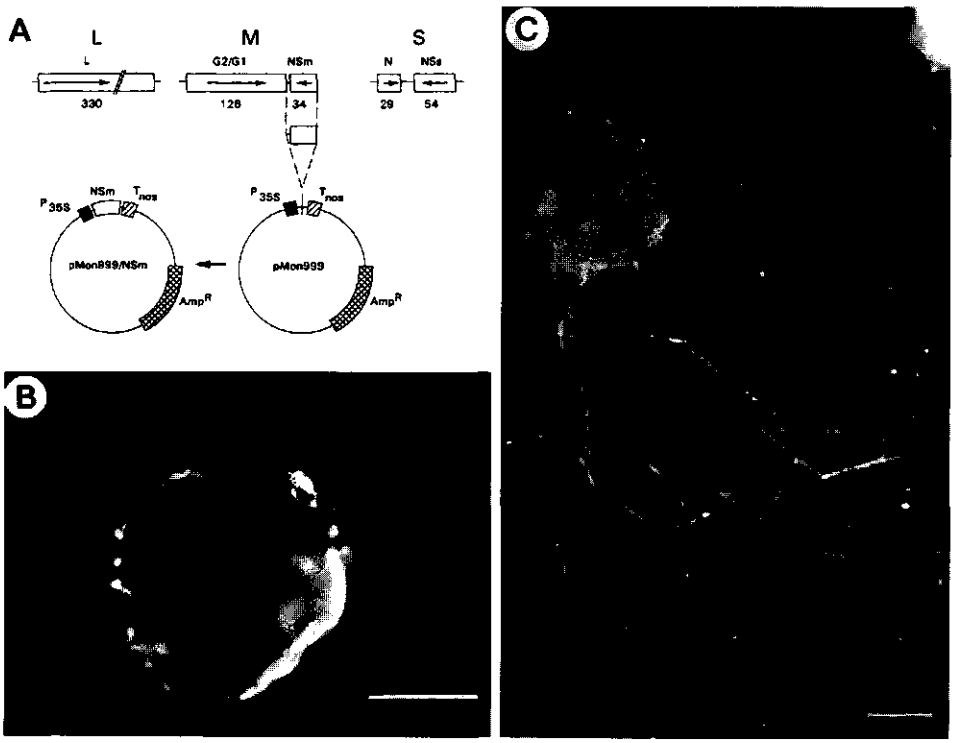


**Figure 3.3.** Expression of the $NS_M$ gene in *N. rustica* protoplasts transfected with vector pMon999/$NS_M$.

**(A)** The TSWV $NS_M$ 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 an early peripheral expression of $NS_M$ 16 hours after transfection. **(C)** Immunofluorescent CSLM image of tubular structures emerging from the surface of a protoplast 24 hours after transfection with pMon999/$NS_M$ and visualised by anti-$NS_M$ serum. Scale bars represent 10 µm.

### 3.4.2. Expression of the $NS_M$ gene in transfected *N. rustica* protoplasts.

To verify whether the formation of tubules was specifically induced by $NS_M$, this protein was expressed in protoplasts without the pathological background of virus infection. To this end, protoplasts of *N. rustica*, isolated from primary leaves of 2 week-old non-infected plants, were transfected with vector pMon999/$NS_M$, containing the $NS_M$ gene under the control of the CaMV 35S promoter (Figure 3.3.A). Sixteen hours post transfection, the expressed $NS_M$ protein was exclusively found along the periphery of the cell (Figure 3.3.B), while at 24 hours p.i. tube-like extensions with a length up to 40 µm (i.e. approximately twice the diameter of the cell) could be detected extending from the cell surface (Figure 3.3.C). In control transfections, using an empty pMon999 vector, neither immunofluorescence nor tubule formation was observed (data not shown). Transmission electron microscopy of negative stained preparations confirmed the specific aggregation of the $NS_M$ protein into tubular structures (Figure 3.4.A). The diameter of the tubule was estimated to be 40-50 nm, while the thickness of the tube wall was approximately 5 nm (Figure 3.4.B). In addition, the surface of the tubule appeared to contain a fibrous striping parallel along the length of the tubule. The transfection studies demonstrate that the $NS_M$ protein is capable of assembling into a tubular structure without further virus specific requirements. However, the involvement of plant specific components as a prerequisite for the formation of these tube-like extensions can not be ruled out and is further discussed in Chapter 7.

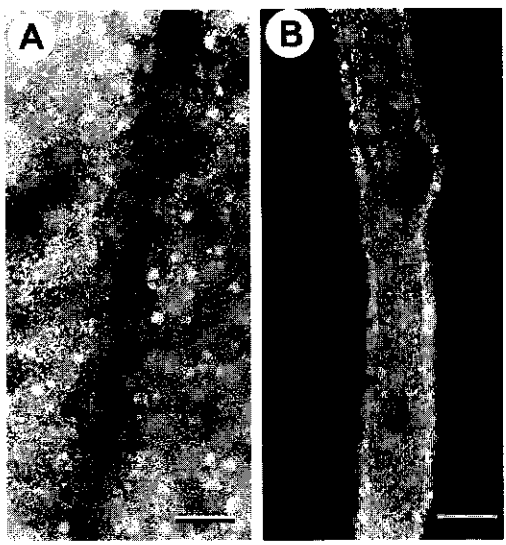


**Figure 3.4.** Electron micrographs of negatively stained pMon999/$NS_M$ induced tubules. **(A)** Treatment with and **(B)** without specific immunogold labeling using anti-$NS_M$ serum. Scale bars represent 100 nm **(A)** and 50 nm **(B)** respectively.

### 3.4.3. Intracellular localisation of the $NS_M$ protein in TSWV-infected leaf tissue.

To determine the intracellular position of the $NS_M$ protein and to get a clue about its function during the TSWV infection cycle, ultrathin sections of systemically TSWV-infected *N. rustica* leaf tissue at 6, 7, or 8 days p.i. were prepared for immunolocalisation at the level of electron microscopy. At 6 days p.i. $NS_M$ was found associated with electron dense aggregates (Figure 3.5.A), previously identified as non-enveloped viral nucleocapsid aggregates (Kitajima *et al.*, 1992). Labeling with antiserum against the N protein confirmed the composition of this electron dense material (Figure 3.5.B.). In contrast, $NS_M$ could not be detected in association with mature enveloped virions. Immunocytological analyses of TSWV-infected cells showed that in cells with relatively high amounts of $NS_M$ no enveloped virus particles could be detected, while in cells that contained enveloped particles, hardly any or no $NS_M$ protein was observed. Additionally at 6 and 7 days p.i., the $NS_M$ protein was found associated with morphologically unmodified plasmodesmata (Figure 3.5.C,D). However, at days 7 and 8 p.i. the $NS_M$ label appeared frequently arranged along electron dense extensions or tubular structures (Figure 3.6.A-D). These tubules with an estimated width of 40-50 nm appeared morphologically similar to the tubular structures observed on infected or $NS_M$-transfected protoplasts. Furthermore, they were exclusively found associated to plasmodesmata extending at only one site of the cell wall into the cytoplasm. However, no obvious nucleocapsid structures or mature (enveloped) virus particles could be discerned inside the tubule.

## 3.5. DISCUSSION

Previously, it was shown that the $NS_M$ protein of TSWV has an early and transient character during the infection of plant tissue (Kormelink *et al.*, 1994; Chapter 2). Unlike the N protein, which accumulates to high levels in an increasing number of cells with progressing infection of the leaf, the $NS_M$ protein could only be detected during a short period between 6 and 8 days p.i. in systemically infected leaves from synchronously inoculated plants. The transient appearance of the protein during establishment of the infection points to an early and transient function of the protein. The expression of the $NS_M$ 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 leaf 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, $NS_M$ was no longer detectable. This kinetic of expression fits our hypothesis that $NS_M$ has a function in spread of the infection and possibly intercellular translocation of TSWV.

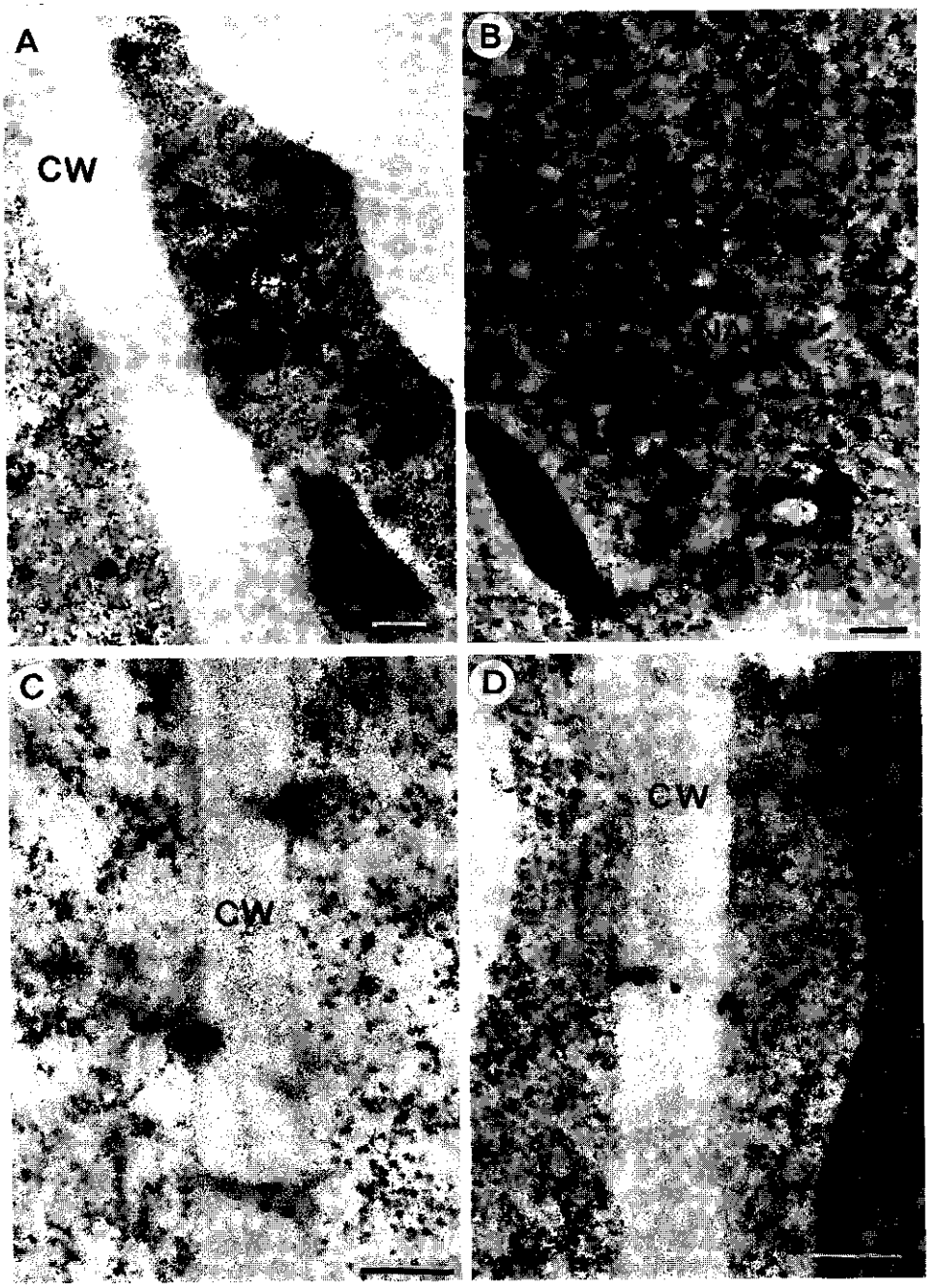


**Figure 3.5.** Electron micrographs of TSWV-infected *N. rustica* leaf tissue immunogold labeled with antiserum against the $NS_M$ protein **(A,C,D)** and N protein **(B)**. $NS_M$ specifically associates to viral nucleocapsids in the cytoplasm **(A)** and with plasmodesmata **(C,D)**. Scale bars represent 150 nm **(A)**, 300 nm **(B)** and 100 nm **(C,D)** respectively. CW: cell wall; NA: nucleocapsids.

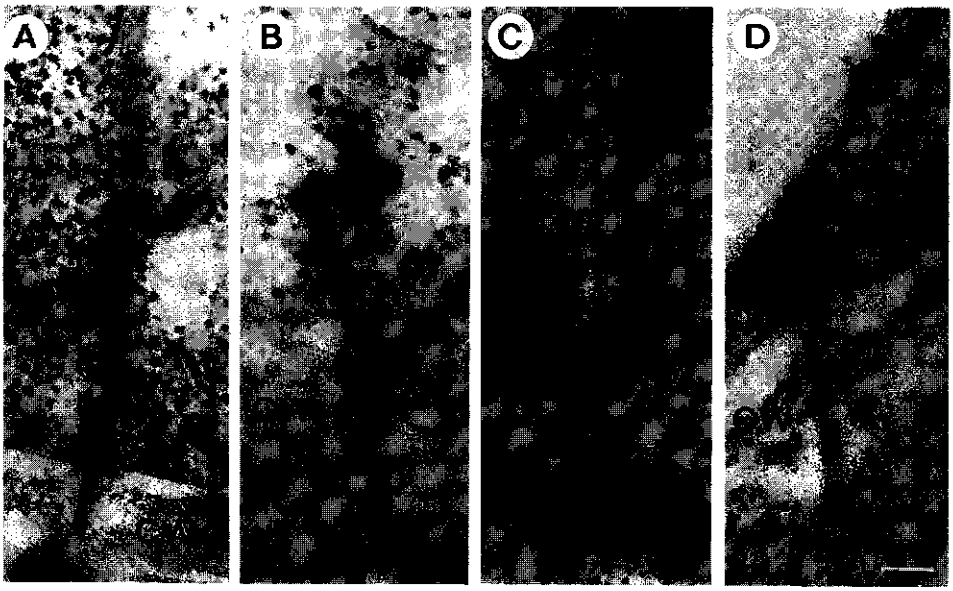


**Figure 3.6.** *In situ* localisation of $NS_M$-induced tubular structures in systemically infected *Nicotiana rustica* mesophyll tissue, immunogold labeled with anti-NSm serum **(A-D)**. Scale bar represents 100 nm. CW: cell wall

Within its transient appearance, the $NS_M$ protein is localised *in situ* to cytoplasmic nucleocapsids and plasmodesmata. Furthermore, tubular structures appear to extend from plasmodesmata into the cytoplasm at only one side of the cell wall. Moreover, it has been shown that the $NS_M$ protein has also the property to assemble into tubular 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 tubule. Tubules are not only found in protoplasts isolated from TSWV-infected plant tissue, but also in protoplasts transfected with the $NS_M$ gene alone. This is evidence for the exclusive involvement of the protein in the tubule forming process, independent from virus replication or other viral proteins.

Having established that the $NS_M$ protein associates to viral nucleocapsids, plasmodesmata and assembles into tubular structures, the question arises whether this protein has indeed a function during cell-to-cell movement of TSWV and how $NS_M$ functions during this process. Several lines of evidence support the idea that $NS_M$ represents the viral MP involved in tubule-guided cell-to-cell movement of TSWV. These are, firstly, the observation that the $NS_M$ gene represents an additional cistron only present in the genome of plant-infecting bunyaviruses, reflecting the adaptation of the standard bunyaviral genome to botanical hosts (Figure 3.7.; Kormelink *et al.*, 1992; Law *et al.*, 1992). Secondly, distinct

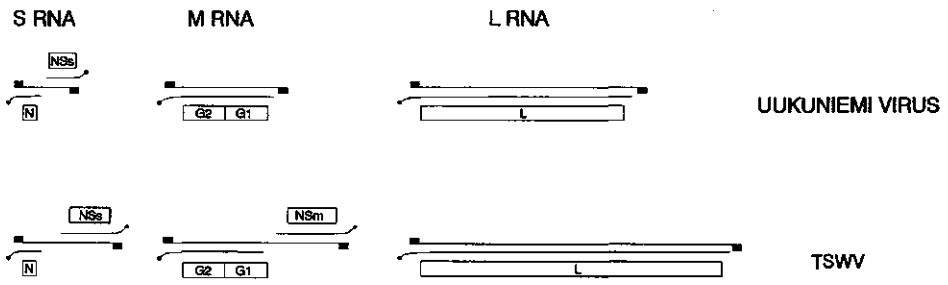


**Figure 3.7.** Comparison of an animal- and plant-infecting bunyavirus, exemplified by Uukuniemi virus (genus *Phlebovirus*) and tomato spotted wilt virus (TSWV; genus *Tospovirus*). For both virus species, the L RNA is of complete negative polarity and encodes the putative viral polymerase (L). The S-RNA segment of both viruses has an ambisense gene arrangement, encoding a nucleocapsid protein (N) in the viral-complementary strand and a nonstructural protein ($NS_S$) in the viral strand. Whereas the M RNA of all animal-infecting bunyaviruses is negative stranded, the M RNA of tospoviruses has an ambisense character and contains an additional cistron ($NS_M$) in the viral sense (Kormelink *et al.*, 1992; Law *et al.*, 1992). Furthermore, the M RNA segment contains the genetic information for the glycoproteins (G1 and G2).
n : complementary ends of the genomic RNA segments; • : capped cellular leader sequence of the viral mRNAs; Open bars indicate the translation products.

homology has been reported between $NS_M$ and MPs of several positive strand RNA viruses (Mushegian and Koonin, 1993). A final argument for the $NS_M$ directed tubule-guided translocation of tospoviruses is the close resemblance of the tospoviral tubules with those involved in como- and caulimovirus cell-to-cell movement (Van Lent *et al.*, 1990, 1991; Wellink *et al.*, 1993; Kasteel *et al.*, 1993; Perbal *et al.*, 1993). In case of CPMV it has been shown that the mature, spherical virus particles (diameter: 28 nm) are translocated through the tubules (Van Lent *et al.*, 1990, 1991; Wellink *et al.*, 1993; Kasteel *et al.*, 1993). 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 (5 nm; Lucas *et al.*, 1993), but also that of the $NS_M$-induced tubules (40-45 nm). Thus, if TSWV is translocated through the tubule, it will probably move as a non-enveloped nucleocapsid structure (Chapter 4). Three arguments are supportive for this interpretation. First, the $NS_M$ protein associates to nucleocapsids but never to mature, enveloped particles. This association occurs prior to the onset of virus particle maturation and supports the hypothesis that $NS_M$ may have a function in targeting of nucleocapsids to the plasmodesma. Secondly, envelope-deficient mutants of TSWV, though non-transmittable by thrips, are able to infect plants systemically at wild type speed (Resende *et al.*, 1991, 1993). And thirdly, mature enveloped particles, despite their conspicuous structure, have never been observed inside the tubules.

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# 4

# Evidence for translocation of non-enveloped viral nucleocapsids through transport structures of tomato spotted wilt virus.

## 4.1. SUMMARY

During systemic infection of plants by tomato spotted wilt virus (TSWV) the viral movement protein $NS_M$ assembles into tubular structures which penetrate through plasmodesmata in order to establish a cell-cell pathway for the virus. Here it is shown, based on immunofluorescence studies and immuno-electron microscopy, that the viral nucleoprotein is present inside these tubules, but not the envelope glycoproteins. These data support the idea that non-enveloped, viral nucleocapsids are transported during cell-to-cell movement of TSWV.

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## 4.2. INTRODUCTION

During systemic infection of plants the $NS_M$ protein of TSWV associates with viral nucleocapsids and with plasmodesmata, in which it assembles into tubular structures (Chapters 2, 3; Kormelink *et al.*, 1994; Storms *et al.*, 1995). This suggests that TSWV employs a so called tubule-guided movement mechanism. Intercellular virus movement through tubular structures is a well established phenomenon which has been described for a number of isometric plant viruses, e.g. the como- (Van Lent *et al.*, 1990; 1991), nepo- (Wieczorek and Sanfaçon, 1993; Ritzenthaler *et al.*, 1995), and caulimoviruses (Perbal *et al.*, 1993). These viruses are non-enveloped spherical particles, with a diameter of 25-40 nm and are translocated through the tubules as mature virions. Tomato spotted wilt virus, however, is an enveloped virus with a diameter of 80-110 nm, thereby exceeding the tubule diameter twice (40-50 nm; chapter 3; Storms *et al.*, 1995). As enveloped particles are not essential for systemic infection of plants (Resende *et al.*, 1991, 1993), it is plausible that TSWV is translocated through the tubule in the form of viral RNA in tight association with the nucleoprotein (nucleocapsids) and not as mature enveloped virions (De Haan *et al.*, 1990, 1991; Kormelink *et al.*, 1994; Storms *et al.*, 1995). To investigate the contents of the TSWV movement tubule, as to find clues about the transport structure of TSWV, we have exploited the *Nicotiana rustica* protoplast system (Kikkert *et al.*, 1997) which allows analyses of large numbers of tubules that extend from the synchronously infected cells. It is shown by immunofluorescence and immuno-electron microscopy that the nucleoprotein is present inside the tubules, but not the viral glycoproteins. This supports the hypothesis that TSWV is transported through the tubule as viral nucleocapsid and not as mature particle.

## 4.3. MATERIALS AND METHODS

### 4.3.1. Virus, plant material and antisera

The Brazilian isolate BR-01 of TSWV was used in all experiments. The virus was maintained in *N. rustica* and *D. stramonium* plants by thrips transmission and mechanical inoculation. For detection of the $NS_M$, N and G1, G2 proteins, polyclonal antisera were used at a concentration of 1μg/ml (Kormelink *et al.*, 1994; De Avila *et al.*, 1992; Kikkert *et al.*, 1997).

### 4.3.2. Isolation and purification of TSWV particles

Isolation and purification of TSWV occurred as essentially described by Gonsalves & Trujillo (1986) and Kikkert *et al.* (1997). Systemically infected *N. rustica* or *D. stramonium* leaves were harvested and stored at 4°C prior to the isolation of TSWV particles. Herefore, harvested leaves were grinded in 3 ml of extraction buffer (0.01M $Na_2SO_3$; a mixture of 0.1M $NaH_2PO_4$ and 0.1M $Na_2HPO_4$ at pH 7.0) for each gram of leaf material using a Philips blender. The homogenate was filtered though cheese clothe and centrifuged at 10.000 g in a Beckman JLA 10.500 rotor during 15 minutes. The supernatant was discarded while the pellet was gently homogenised with a stirrer in 1 ml resuspensionbuffer (freshly prepared 0.01M $Na_2SO_3$) for each gram leaf material. The suspension was collected, centrifuged at 8000 g in a Beckman JA 25.50 rotor during 15 minutes and the supernatant collected and subsequently concentrated at 100.000 g in a Beckman 45 Ti rotor during 30 minutes. The pellet was homogenised in 5 ml resuspensionbuffer per 100 grams of the initial leaf material and was layered on top of a 10-40% sucrose gradient. Subsequent centrifuging at 100.000 g for 45 minutes in a Beckman SW28 rotor revealed a clear virus band at the opalescent zone of the sucrose gradient. The virus band was collected with a 90° curled injection needle, diluted 1:1 in resuspension buffer and centrifuged for 1 hour at 100.000 g using a SW41 Beckman rotor. The virus pellet was resuspended in 200 µl double distilled water and the virus concentration estimated by a BioRad protein assay kit. The freshly purified virus was either directly used for infection of protoplasts or stored at -80°C after rapid plunge freezing in liquid nitrogen.

### 4.3.3. Isolation and infection of protoplasts

Primary leaves of 1-week-old healthy, non-infected *N. rustica* plants grown in a greenhouse under normal light conditions were used for the isolation of protoplasts. Isolation occurred by removal of the abaxial epidermis and incubation of the stripped leaf on a cell wall digesting solution as described by Hibi *et al.* (1975) and Van Lent *et al.* (1991). Aliquots of $1.10^6$ cells were inoculated with 10 µg of ice cold, preferably freshly isolated TSWV. Simultaneously 0.5 ml of cold 40% (w/v) polyethylene glycol in double distilled water was added and the mixture was vigorously shaken for 15 seconds. Subsequently 4.5 ml of 0.6 M mannitol containing 10mM $CaCl_2$ (pH 5.6) was added and the suspension was incubated for 20 minutes at room temperature. After washing the cells three times in washing buffer (0.6 M mannitol; 10mM $CaCl_2$), protoplasts were incubated in nutrition medium (Kikkert *et al.*, 1997). At 22 and 40 hours p.i. samples were taken and analysed by immunofluorescence and immunogold electron microscopy.

### 4.3.4. Immunofluorescent detection of viral proteins

At 22 and 40 hours p.i. 50 µl samples of protoplasts were taken from the nutrition medium, spotted on 0.05% poly-L-lysine coated glass slides (Huang *et al.*, 1983) and fixed in 70% ethanol for 15-20 minutes. After washing the glass slides in PBS, the protoplasts were blocked in 5% (w/v) BSA in PBS for 30 minutes and subsequently treated with 1µg/ml polyclonal antisera against the $NS_M$, N and G1, G2 proteins for 1 hour at 30°C. Antigen-antibody complexes were detected by 10 µg/ml horse anti-rabbit FITC-conjugated secondary antibodies after incubation for 1 hour at 30°C in the dark. Samples were analysed with a Leitz Laborlux S fluorescence microscope.

### 4.3.5. Immunogold analyses of infected protoplasts

Non-infected and TSWV-infected *N. rustica* protoplasts at 22 and 40 hours p.i. were primarily fixed in 0.75% (w/v) glutaraldehyde in nutrition medium for 30 min at room temperature, centrifuged at 250 g in a IEC Centra CLD centrifuge for 5 minutes and resuspended in 3% (w/v) glutaraldehyde/2% (w/v) paraformaldehyde in phosphate citrate buffer containing 10 mM $CaCl_2$. After fixing for 30 minutes at room temperature the protoplasts were rinsed in phosphate-citrate washing buffer (PC- buffer) containing 10 mM $CaCl_2$ and concentrated in 5% (w/v) gelatine in PC- buffer by centrifuging at 250 g. The gelatine was irreversibly solidified in 3% (w/v) glutaraldehyde/2% (w/v) paraformaldehyde in PC-buffer, sliced in small pieces and processed for further fixation, dehydration and embedding in London Resin Gold and White. Ultrathin sections were made on a Reichert ultracut S ultramicrotome and probed with primary and protein-A gold conjugated secondary antibodies. The specimen were examined with a Philips CM12 transmission electron microscope. Alternatively, infected protoplasts at 22 and 40 hours p.i. were processed for immunocytochemistry by negative staining electron microscopy as described by Van Lent *et al.* (1991).

## 4.4. RESULTS

### 4.4.1. Immunofluorescent detection of the N protein in $NS_M$ tubular structures of TSWV

At 22 and 40 hours post inoculation, TSWV-infected *N. rustica* protoplasts were sampled and TSWV proteins were detected by immunofluorescence light microscopy using antisera against the nucleoprotein, the two glycoproteins G1 and G2 or against the $NS_M$ protein.

Approximately 95% of the inoculated protoplasts were infected with TSWV, as was determined by immunofluorescent staining of the N-protein. At 22 hours p.i. 90% of these infected protoplasts showed numerous tubular structures upon fluorescent staining with anti-$NS_M$ (Figure 4.1.A). At 40 hours p.i. tubules were observed in only 60% of the infected protoplasts. When anti-N serum was used, at both time points thin thread-like structures were discerned protruding from the surface of the protoplasts in a similar fashion as the $NS_M$ tubules (Figure 4.1.B), indicating the presence of viral nucleoprotein in the transport tubules. The $NS_M$ tubules did not label with antisera against the G1 or G2 glycoproteins, although the glycoproteins were abundant in the cytoplasm (data not shown; Kikkert *et al.*, 1997).

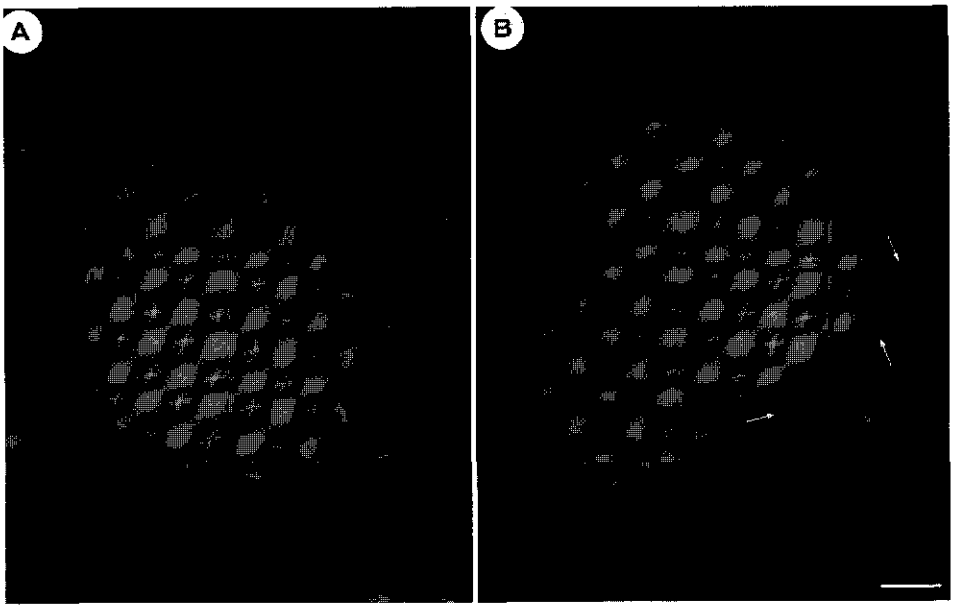


**Figure 4.1.** Immunofluorescent image of TSWV-infected *N. rustica* protoplasts at 22 hours p.i. probed with anti-$NS_M$ serum **(A)** and anti-N serum **(B)**. Arrows indicate thin tubule threads visualised with anti-N serum. Scale bar represent 5 µm.

### 4.4.2. Immunogold localisation of the N-protein in tubular structures of TSWV

The structure and chemical content of the tubules was further investigated by electron microscopy. Infected protoplasts at 22 hours and 40 hours p.i. were negatively stained or fixed, dehydrated and embedded in LR Gold, sectioned and immunogold labelled as described by Van Lent *et al.* (1990). In negatively stained whole mounts of infected protoplasts numerous tubular structures were again observed, loosely enveloped by the

plasmamembrane (Figure 4.2.). Occasionally, two or more tubules were enclosed by one membrane (Figure 4.2.A). This phenomenon was also previously reported for other plant viruses that form tubular structures on protoplasts (Van Lent *et al.*, 1991; Kasteel *et al.*, 1997). In negative stained samples, tubules labelled extensively with gold when treated with anti-$NS_M$ serum (Figure 4.2.B,C). The $NS_M$ label was only present at places where the plasmamembrane was disrupted or absent (Figure 4.2.B,C). Close observation of these tubule fragments showed a filamentous substructure, similar to that described previously for tubules formed in protoplasts and insect cells upon expression of the sole $NS_M$ gene (Storms *et al.*, 1995).

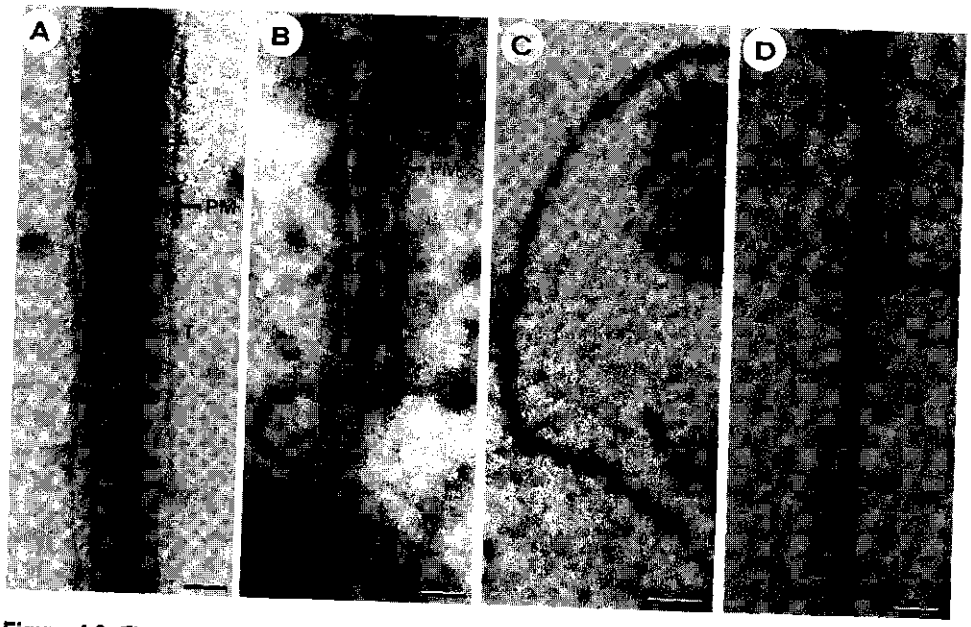


**Figure 4.2.** Electron micrographs of negatively stained tubular transport structures of TSWV at 22 hours p.i. Tubular structures loosely enveloped by the plasmamembrane **(A)** and treated with anti-$NS_M$ serum **(B,C)** and anti-N serum **(D)** conjugated with protein A-gold (10 nm). PM: plasmamembrane; T: tubular structure. Scale bars represent 100 nm **(A,B)** and 200 nm **(C,D)** respectively.

In thin sections the tubules appeared to emerge from the protoplast surface, as was already found by immunofluorescent staining. No plasmamembrane could be observed around the tubules or the cell, possibly due to extraction of lipids during the procedure of dehydration and resin infiltration. Immunogold labelling with anti-$NS_M$ serum showed intense labelling of those tubule fragments that were exposed to the section surface (Figure 4.3.A,B). Neither in thin sections nor in negative stained preparations a conspicuous viral structure

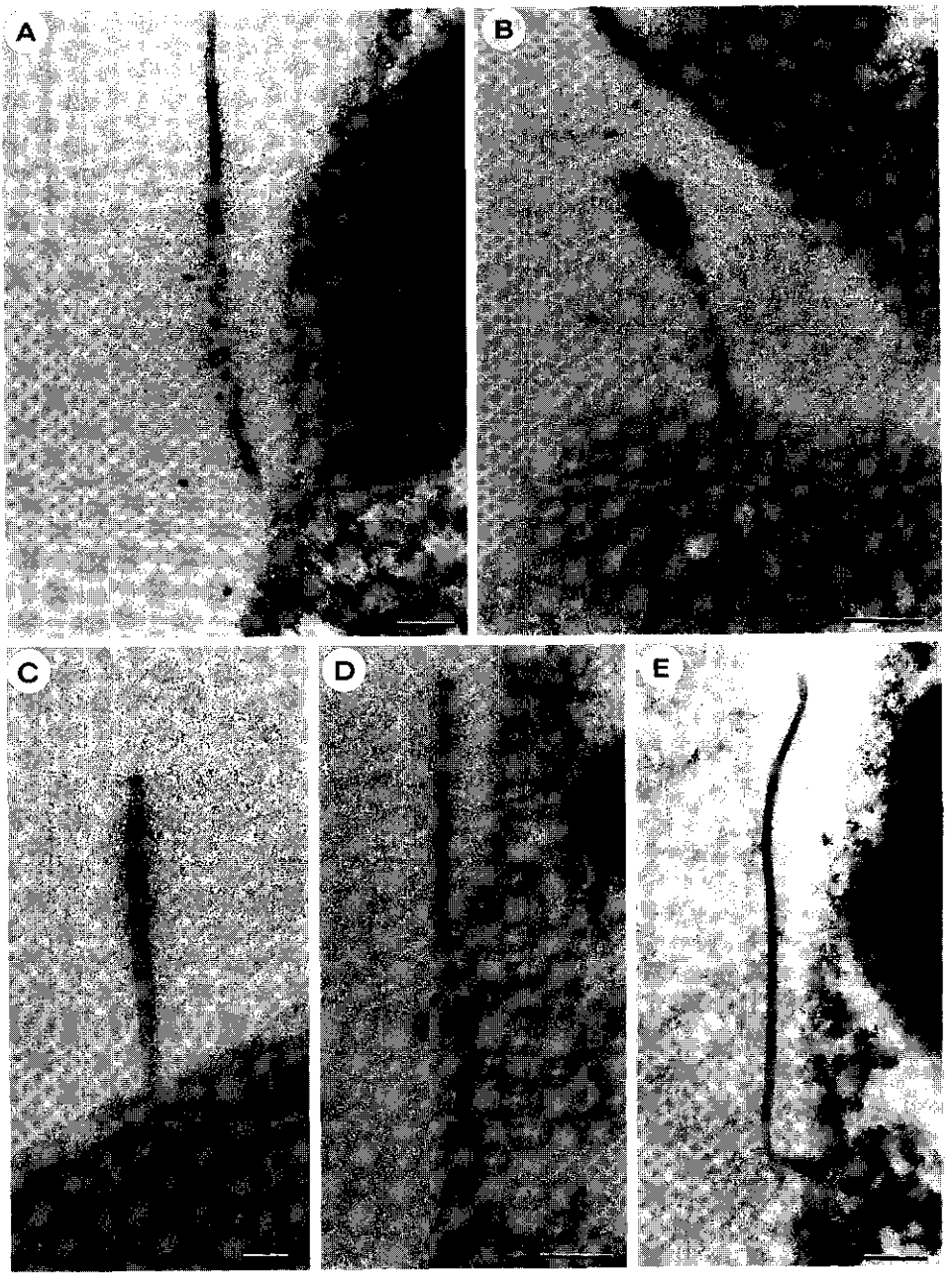


**Figure 4.3.** Electron micrographs of immunogold labelled TSWV-infected *N. rustica* protoplasts at 22 hours p.i. probed with anti-$NS_M$ serum **(A,B)** and anti-N serum **(C,D,E)**. Scale bars represent 100 nm **(C)** and 200 nm **(A,B,D,E)** respectively.

could be observed within the tubular structures. However, when anti-N serum was used, specific gold labelling was obtained in negatively stained specimen though only at those places where the tubule was deteriorated or broken (Figure 4.2.D). Furthermore, specific gold label was found in ultrathin sections at places where the tubule was sliced (Figure 4.3.D,E,F). Incubation of ultrathin sections and negative stained preparations with antiserum against the G1 and G2 glycoproteins did not result in any significant labelling of tubular structures while at these stages of infection the cytoplasm contained maturating virus particles at the Golgi complex (22 hours p.i) and numerous mature, enveloped particles in the endoplasmatic reticulum (40 hours p.i) which readily labelled with these antibodies (data not shown). This confirms that only the nucleoprotein is present inside the tubules, which indicates that nucleocapsids are translocated rather than enveloped particles. In view of the genome polarity of TSWV, the translocated complex should also contain the viral polymerase (L protein). Attempts to localise this protein in both the tubules and the cytoplasm have failed, probably due to the limited number of protein copies (Van Poelwijk *et al.*, 1993).

## 4.5. DISCUSSION

The data presented here reveal that only the viral $NS_M$ and N proteins, but not the viral glycoproteins, are present in the transport tubules of TSWV. Considering that mature enveloped virions have never been observed inside the tubules, in spite of their conspicuous morphology, it is therefore likely that it is the non-enveloped nucleocapsid, which is translocated to establish systemic TSWV infection in the plant. This view is supported by several additional observations. Firstly, the presence of the virion membrane is not a prerequisite for virus movement as envelope-deficient mutants of TSWV have the ability to infect plants at the speed of a wild-type infection (Resende *et al.*, 1991, 1993). Secondly, in the infected cell the $NS_M$ protein is only found in association with viral nucleocapsids but never with mature virus particles (Kormelink *et al.*, 1994). This association occurs prior to the maturation of virus particles and suggests that $NS_M$ has also a function in targeting of the nucleocapsids to the plasmodesmata.

The data presented in this paper, together with previous studies on the $NS_M$ protein support a transport mechanism for TSWV as visualised in Figure 4.4. In the infected plant cell, $NS_M$ becomes associated with newly formed nucleocapsids in the cytoplasm, which are targeted to the plasmamembrane and the plasmodesmata (Kormelink *et al.*, 1994). At the plasmodesmata, $NS_M$ assembles into a tubular structure (Storms *et al.*, 1995) occluding the nucleocapsids (this paper). Subsequently, the tubule extends into the neighbouring cell where the nucleocapsids are released by an unknown mechanism (e.g.

proteolytic breakdown of the tubule). As the nucleocapsids are transported in a non-enveloped form, binding of $NS_M$ to nucleocapsids may prevent maturation into enveloped virus particles through budding into the Golgi complex (Kikkert *et al.*, 1997).

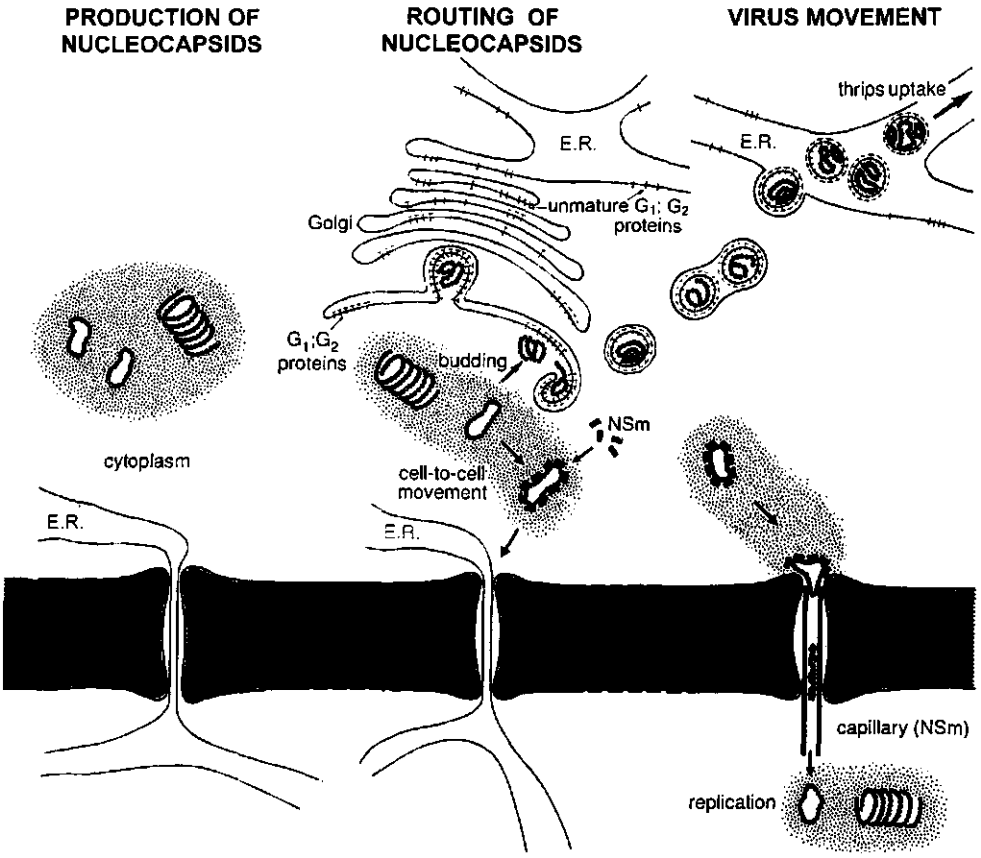


**Figure 4.4.** Model for the intercellular translocation pathway of TSWV. After entry at the initial infection side, the virus starts to replicate and produces viral nucleocapsids. These progeny nucleocapsids become associated with the $NS_M$ protein and are targeted to the plasmodesmata. The $NS_M$ protein assembles into a tubular structure that penetrates the plasmodesmata. To retain infectivity, a few L protein copies (not shown in the figure) should be associated to the translocated nucleocapsids. Nucleocapsids which are not associated with the $NS_M$ protein bud through the Golgi complex and may be translocated into the ER.

**Acknowledgements**

The authors wish to acknowledge Piet Kostense and Ab Haasdijk for the artwork and thank Joop Groenewegen for technical assistance. This research was financially supported by the Netherlands Foundation for Scientific Research (NWO) with grants from the Netherlands Foundation for Chemical Research (SON), the Netherlands Technology Foundation (STW) and the Joint Dutch-Israeli Agricultural Research Program (DIARP).

# 5

# Transgenic tobacco plants expressing the putative movement protein of tomato spotted wilt virus exhibit aberrations in growth and appearance.

## 5.1. SUMMARY

Transgenic tobacco plants have been obtained expressing the $NS_M$ protein of tomato spotted wilt virus (TSWV) from a constitutive promoter. Detectable amounts of the $NS_M$ protein could be observed in plants from nine different lines. The protein was only detectable in fractions enriched for cell wall material. Detailed immunogold labeling studies revealed specific association of $NS_M$ protein with plasmodesmata. Plants accumulating the $NS_M$ protein to detectable levels developed aberrations in growth, resulting in a significant reduction of size and accelerated senescence. In addition, these plants are restricted in their capacity to produce flowers. The results presented provide additional evidence that the $NS_M$ protein, by modifying plasmodesmata, represents the cell-to-cell movement function of tospoviruses. Furthermore the phenotype of the $NS_M$ transgenic plants suggests involvement of the $NS_M$ gene product in TSWV symptom expression.

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## 5.2. INTRODUCTION

For positive strand RNA plant viruses, information about the functions of viral proteins can be obtained by studying the effect of mutations in these proteins using infectious cDNA clones (reverse genetics). For negative strand RNA viruses, like TSWV, such studies have been hampered by the impossibility to recover infectivity from cloned DNA copies. Alternatively, transgenic host plants are a potential source of information on the contribution of viral proteins or protein domains in the virus infection cycle. We have therefore transformed tobacco plants with the $NS_M$ gene of TSWV. The observations made on such plants support the hypothesis that the $NS_M$ protein is involved in the spread of tospoviruses through plant hosts.

## 5.3. MATERIALS AND METHODS

All manipulations involving DNA or RNA were done according to standard procedures (Sambrook *et al.*, 1989). Preparation of subcellular extracts of transgenic plants and Western immunoblot analyses were according to Kormelink *et al.* (1994).

### 5.3.1. Viruses and plants

Tospovirus strains, BR-01 (TSWV), BR-03 (TCSV), and SA-05 (GRSV), have been described by Àvila *et al.* (1990, 1992 and 1993) and were maintained on systemic hosts *Nicotiana rustica* var. America or *N. tabacum* var. SR1.
Recipient plants used in the transformation experiments were *N. tabacum* var. SR1 plants. All manipulations with transgenic plant material were carried out under conditions (PKII) imposed by the Dutch authorities (VROM/COGEM).

### 5.3.2. Construction of $NS_M$ gene sequence expression vectors

$NS_M$ gene sequences of TSWV (Kormelink *et al.*, 1992), were modified using PCR in such a way that an EcoRI site was generated immediately downstream of the original start codon, using primer Zup051 (dGGGAATTCTTTTCGGTAACAAGAGGCC) located at position 108 to 129 of the viral M RNA and Zup014 (dCCCTGCAGGATCCGAAATT-TAAGCTTAAATAAGTG) located at position 1043 to 1023 of the viral complementary M RNA. The resulting PCR fragment was digested with EcoRI and a EcoRI/BamHI linker including an internal KpnI site and an in frame start codon was ligated. The slightly modified $NS_M$ gene (starting with amino acid sequence Met.Leu.Ile... in stead of Met.Thr.Val...) was

5' GATCCGGCAACGAAGGTACCATGGG 3'
3' GCCGTTGCTTCCATGGTACCCTTAA 5'
BamHI KpnI NcoI EcoRI

cloned in pMON999 and successfully checked for tubule inducing ability in transfected protoplasts (Storms *et al.*, 1995), although an effect of these single aminoacid changes on other functions of the protein can not be completely excluded. Simultaneously, the fragment was ligated in pUC18 as a BamHI restriction fragment. The resulting plasmid was linearised using KpnI, and PstI linkers were ligated after creating blunt ends using T4 DNA polymerase. In addition, the exonuclease activity of T4 DNA polymerase was used to create an untranslatable $NS_M$ sequence. The resulting clones were checked by sequence analysis and beside a translatable clone, a clone was selected in which the original start codon was mutagenised to CTG. The PstI restriction fragments, were ligated in plant transformation vector pZU-A (Gielen *et al.*, 1991) between the CaMV 35S promoter and the nopaline synthase (nos) terminator. In addition, the translation enhancing TMV untranslated leader (Gallie *et al.*, 1987) was ligated in front of the translatable $NS_M$-A construct. Finally, the $NS_M$ constructs were inserted in binary vector pBIN19 (Bevan, 1984), yielding pTSWV $NS_M$-A (translatable) and pTSWV $NS_M$-B (untranslatable). Details of this cloning schedule are presented in Figure 5.1.

### 5.3.3. Transformation of tobacco

The pBIN19-derived vectors pTSWV $NS_M$-A and $NS_M$-B were introduced into *Agrobacterium tumefaciens* strain LB4404 (Ditta *et al.*, 1980) by triparental mating, using pRK2013 (Horsch *et al.*, 1985) as a helper plasmid. *N. tabacum* var. SR1 plants were transformed and regenerated as described by Horsch and co-workers (1985).

### 5.3.4. Immunocytochemistry

Both transgenic *N. tabacum* plants expressing the TSWV $NS_M$ protein as well as non-transgenic tobacco plants were used for immunocytological analyses at the level of electron microscopy. Leaf samples were fixed in 2% (w/v) paraformaldehyde/ 0.01% glutaraldehyde (w/v) in phosphate citrate buffer containing 10 mM $CaCl_2$, dehydrated, embedded in London Resin Gold at -25$^0$C and UV-polymerized. Ultrathin sections were made and processed for immunogold labeling using antiserum against the $NS_M$ protein (Van Lent *et al.*, 1990; Kormelink *et al.*, 1994). Specimens were examined using a Philips CM12 transmission electron microscope.

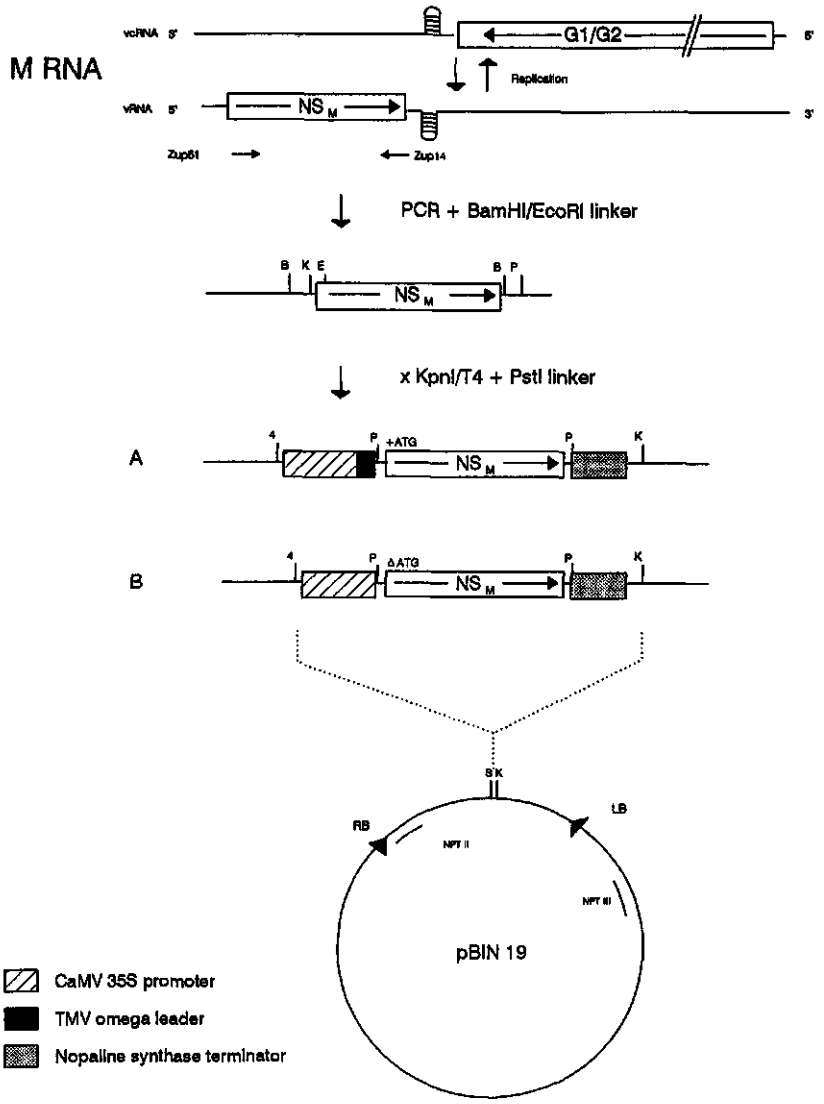


**Figure 5.1.** Construction of plant transformation vectors pTSWV $NS_M$-A and pTSWV $NS_M$-B 1: $NS_M$ sequences are PCR amplified from a genomic cDNA clone of TSWV, using primers that add a BamHI restriction site to the 3' end of the gene (Zup014) and a EcoRI site immediately downstream of the original start codon (Zup051). 2: An oligonucleotide linker sequence containing an in frame start codon is ligated to the 5' end of the PCR fragment after BamHI digestion, thereby restoring the $NS_M$ ORF. Two nucleotides upstream of the ATG a unique KpnI site is present. By treating KpnI linearised DNA differentially with T4 DNA polymerase, blunt ends are created either leaving the start codon intact (A) or destroying it (B). Finally, the $NS_M$ constructs were cloned in the binary vector pBIN19. B=BamHI; E=EcoRI; K=KpnI; P=PstI; 4=blunt after treatment with T4 DNA polymerase. RB and LB are right and left border sequences, respectively. The DATG indicates removal of the ATG start codon.

## 5.4. RESULTS

### 5.4.1. Transformation of tobacco plants with $NS_M$ gene sequences

Two different DNA constructs containing cDNA sequences derived from the TSWV $NS_M$ gene under the control of a CaMV 35S promoter were made, pTSWV $NS_M$-A, and pTSWV $NS_M$-B (Figure 5.1.). Construct pTSWV $NS_M$-A contained the intact $NS_M$ gene in a translatable form, whereas in the pTSWV $NS_M$-B construct the original ATG-start codon of the $NS_M$ gene was replaced by CTG, creating a non-translatable form of this gene. The first alternative ATG codon is located out of frame and translation would result in a peptide of only 4 amino acid residues. In addition, the 5' leader sequence of TMV RNA (Gallie *et al.*, 1987) was cloned in front of the pTSWV $NS_M$-A gene construct, to enhance translation of the $NS_M$ ORF. The two $NS_M$ gene sequence expressor cassettes were cloned into the binary vector pBIN19, and transferred to the genome of *Nicotiana tabacum* var. SR1 plants, via *A. tumefaciens*-mediated leaf disk transformation.
In total, 61 transformed plants were obtained that expressed $NS_M$-derived sequences, as verified by Northern blotting (data not shown). Of these plants, 35 contained $NS_M$-A sequences and 26 the $NS_M$-B insert. All original transformants were maintained for seed production.

### 5.4.2. Phenotype of transgenic plants

All plants transformed with the $NS_M$-B construct, original transformants as well as S1 and S2 progenies, had phenotypes that were indistinguishable from untransformed *N. tabacum* var. SR1 plants. Twenty-nine out of the 35 original transformants of the $NS_M$-A type developed normally. The other six pTSWV $NS_M$-A transformed plants (A14, A16, A26, A29, A31 and A35) developed extensive chlorosis of leaf tissue during their development (Figure 5.2.A,B). Two of these plants (A26 and A31) were unable to set seed, therefore no progeny of these plants could be tested. As will be discussed in paragraph 5.4.4., no $NS_M$ protein was produced in the $NS_M$-A plants that developed normally, in contrast to the plants with a deviating phenotype.

Progeny plant lines derived from self-pollination of the original pTSWV $NS_M$-A transformants revealed a more complicated picture. Nine S1 lines (A2, A11, A13, A14, A20, A23, A25, A29, and A35) exhibited morphologically altered plants, in a ratio close to 1:3 or 3:1 relative to the morphologically unmodified plants within these lines. These ratios indicate the involvement of a single translationally active gene insertion in the aberrant phenotype. This was confirmed by kanamycin resistance studies (results not shown). Among other symptoms such as necrosis and wilting, tobacco plants infected with TSWV show

chlorotic symptoms and a marked delay in growth which, based on the results obtained with the $NS_M$ transgenic plants, may be related to $NS_M$ expression.

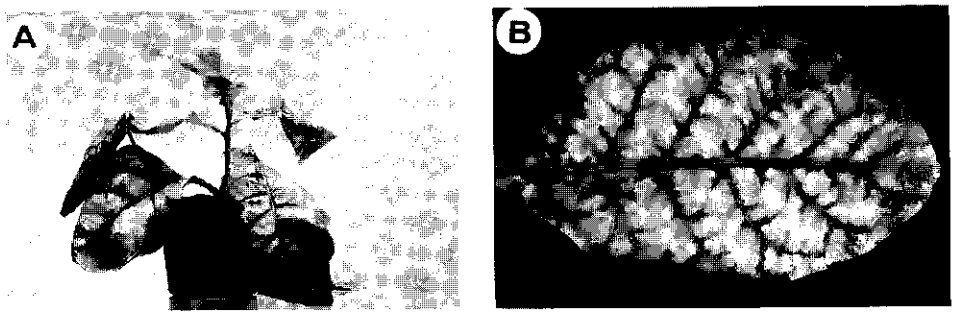


**Figure 5.2. (A)** Extensive chlorosis of developing transformant A14. Similar observations were made on transformants A16, A26, A29, A31 and A35. **(B)** Close-up of a leaf of transformant A14.

### 5.4.3. Development and further characterisation of S1 progeny of lines A23 and A25

Growth of plants from S1 lines A23 (segregation ratio 1:3) and A25 (segregation ratio 3:1) was monitored during the development of these plants. Sizes of plants were measured twice a week for a period of eight weeks after sowing, by adding up the length of the two largest leaves plus the length of the entire stem. A dramatic difference in growth rate within segregating progenies of lines A23 and A25 was observed (Figure 5.3.A,B). Representative selections of plants of lines A23 and A25 are shown approximately 7 weeks after sowing (Figure 5.3.C,D). The presence or absence of TSWV $NS_M$ protein in plants of lines A23 and A25 was verified by Western blot analysis of purified cell-wall material. In plants of line A25 the $NS_M$ protein was only detected in small plants and not in the segregates with wild type phenotype (results not shown). The presence of the $NS_M$ gene in the genome of plants of line A23 alone appeared not to be sufficient for inducing modifications in morphology. Only one out of four plants developed growth aberrations, indicating that in this line only plants homozygous for the $NS_M$ gene could reach sufficient $NS_M$ expression levels to result in retardation in growth. Indeed, kanamycin resistance, indicating the presence of the transgene, occurred in two-third of the plants that developed normally in this experiment. Somaclonal variation as a possible explanation for the observed phenomenon can be ruled out, since the occurrence of aberrations strictly coincides with the presence of the $NS_M$ gene product. This view is confirmed by the observation that plants transformed with untranslatable $NS_M$-B sequences never exhibited aberrations in morphology.

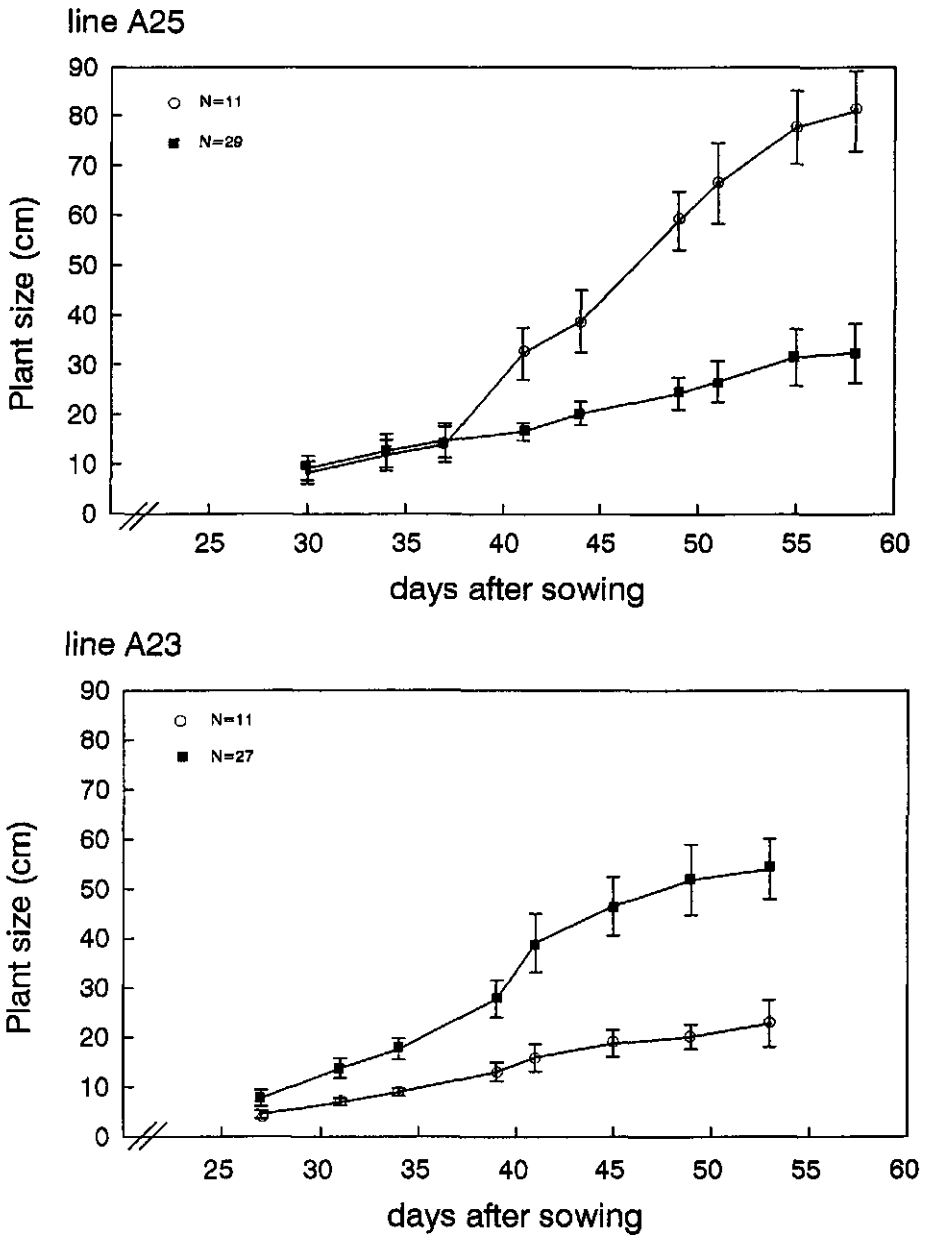


**Figure 5.3.** Growth curves of S1 plants of two $NS_M$ expressing *N. tabacum* var. SR1 lines displaying a segregation in size in two categories, i.e. those developing as wild type and those showing an aberrant phenotype. **(A)** Sizes of 38 individual plants of line A23 were measured twice weekly and stopped when the first plants started flowering. Plants of this line display a 3:1 segregation ratio with respect to size. **(B)** Of line A25, forty individual plants were monitored for development. This line displayed a 1:3 segregation ratio.


**Figure 5.3. (C, D)** Representative selections of plant from lines A23 and A25, respectively, approximately seven weeks after sowing.

### 5.4.4. Expression of $NS_M$ specific RNA and protein in transgenic plants

The presence of $NS_M$ gene-specific transcripts in S1 lines was checked by Northern blot analysis of pooled leaf material, using a $^{32}$P-dATP labeled double-stranded $NS_M$ cDNA probe. Transgenically produced $NS_M$ RNA could be detected in all lines tested (results not shown), albeit at low levels when compared to levels reached in TSWV infected plants.

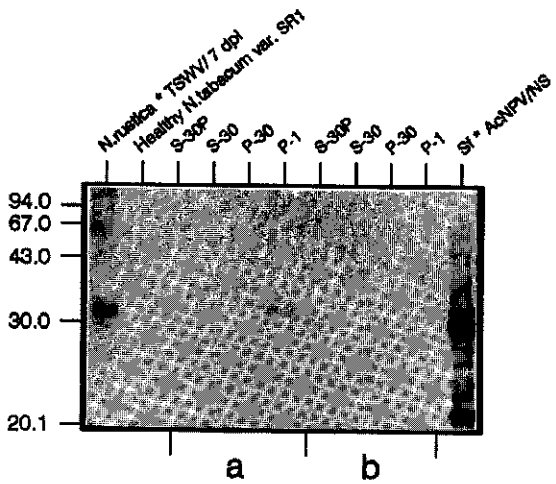


**Figure 5.4.** Western blot analysis of subcellular fractions of a morphologically aberrant plant, using $NS_M$ specific antiserum. **(A)** Morphologically aberrated plant of S1 line A25.
**(B)** unmodified plant from the same line. Various subcellular fractions of crushed leaves as described by Kormelink *et al.* (1994), P-1: crude cell wall material collected at 1,000 *g*; P-30 pellet of previous supernatant after 30,000 *g* centrifugation; S-30 supernatant of this fraction; S-30P pellet of previous fraction after 125,000 *g* centrifugation through a 30% sucrose-cushion (reveals nucleocapsids in virus infected plants).

In leaf extracts from $NS_M$-A transformed plants, $NS_M$ protein could neither be observed by Western blot analysis, or when ELISA techniques were used. Only after fractionation of these extracts into cellular components, $NS_M$ protein could be observed in cell wall-enriched fractions, but not in other fractions, indicating that this protein accumulated to low levels in cell wall material (Figure 5.4.). This was further confirmed by immunocytological data.

### 5.4.5. The $NS_M$ protein of TSWV is specifically targeted to plasmodesmata in transgenic *N. tabacum* tissue.

To gain insight as to how the transgenically expressed $NS_M$ protein could have led to the observed phenotypic aberrations, cytological analyses of leaf tissue of $NS_M$ protein-expressor plants were performed at the electron microscopical level using immunocytochemistry as well as structural analysis of the tissue morphology. Ultrathin sections of transgenic leaf tissue probed with antiserum against the $NS_M$ protein clearly illustrated that the $NS_M$ protein was only detectable in the cell wall, confirming the Western immunoblot analyses. More specifically, the protein was exclusively found in clear association with plasmodesmata (Figure 5.5.).

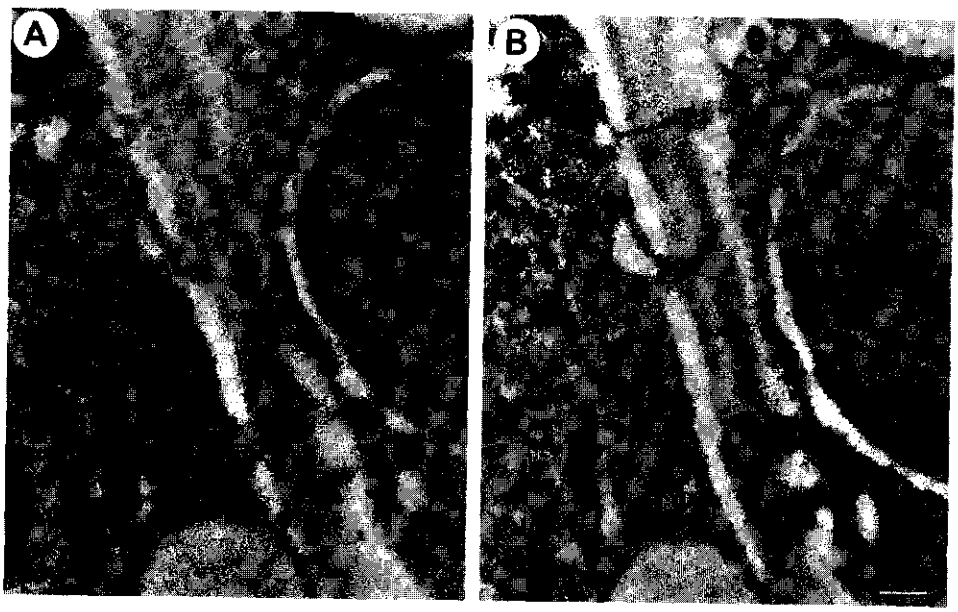


**Figure 5.5.** Immunogold decoration studies on approximately seven weeks old plant leaf tissue, using anti-$NS_M$ serum. Specific association of the transgenically expressed $NS_M$ protein with plasmodesmata in subsequent serial sections **(A,B).** Scale bar represents 300 nm.

Inspection of numerous ultrathin sections revealed that over 80% of the plasmodesmata in mesophyll, phloem parenchyma, epidermis, bundle sheath cells and trichomes of the leaf tissue of each of the tobacco lines were immunolabelled. Since some regions of the plasmodesmata in the section were not exposed to the antibodies (plasmodesmata diameter is smaller than section thickness), it is likely that all plasmodesmata contained the $NS_M$ protein. The $NS_M$ label was found both in branched and unbranched plasmodesmata, but the association did not result in morphological modifications of these structures. For example, no filamentous aggregates of viral protein were found in plasmodesmal cavities as is the case for the movement protein of TMV (Ding *et al.*, 1992; Moore *et al.*, 1992). The absence of such filamentous material or other modifications in $NS_M$ transgenic plants may, however, be due to the low level of $NS_M$ in these plants. During the actual viral infection process, where the early and transient expression of $NS_M$ results in tubular structures penetrating through plasmodesmata, the accumulation of $NS_M$ is significantly higher compared to the $NS_M$ transgenic plants (Storms *et al.*, 1995; Prins *et al.*, 1997). Since the constitutive though low expression of $NS_M$ has a dramatic effect on plant development that leads to growth distortion and malformations of the plant, higher expression levels may have been lethal to regenerants.

## 5.5. DISCUSSION

Morphological aberrations have been observed in transgenic tobacco plants expressing $NS_M$, the putative MP, of tomato spotted wilt virus. Modifications in growth and appearance were only observed in plant lines expressing translatable $NS_M$ RNA ($NS_M$-A lines). The majority of the $NS_M$-A transformed plants did not accumulate detectable amounts of $NS_M$ protein. Plants that did express $NS_M$ protein to a detectable level always developed morphological aberrations, indicating that accumulation of this viral protein has a negative effect on the growth of the plant, resulting in the deviating phenotype. The modified plants might be either the homozygous $NS_M$ expressor plants (e.g. in line A23), expressing the protein to such a level that it influenced the morphology of the plant, or also hemizygous plants with a single, but more actively translated $NS_M$ transgene (e.g. in line A25). In all cases tested, expression levels of the transgenic protein were low and protein was only observed in fractions enriched for cell wall material derived from stunted plants. Possibly, higher expression levels than those observed are lethal to plants, and therefore no such plants were recovered during the transformation and regeneration process. Immunocytological analyses of transgenic tissues revealed that the $NS_M$ protein was specifically associated with plasmodesmata. Random distribution among plasmodesmata of leaf tissue was observed, illustrating that the $NS_M$ protein was expressed in all leaf cell-types. This is not surprising considering the use of the constitutive CaMV 35S promoter. In addition, it can be concluded that $NS_M$ protein

expressed in various tissues behaves in similar manner with regard to association with plasmodesmata. In contrast to the constitutive expression in transgenic plants, the level of $NS_M$ expression during viral infection seems to be regulated. Expression levels of $NS_M$ are low during the early stages of infection, in which the $NS_M$ protein is probably associated with both viral nucleocapsids and plasmodesmata, and can be observed in both P1 and P30 fractions (Kormelink *et al.*, 1994). Higher expression levels are reached later in the infection process, coincidental with the formation of tubular structures in the plasmodesmata (Storms *et al.*, 1995). Since the $NS_M$ protein in transgenic plants can only be detected in Western blot analysis after concentrating the cell wall fractions, the level of protein expression seems significantly lower than during virus infection. This could be the reason why in transgenic plants no tube-like extensions are observed in tissue. However, association of the $NS_M$ protein with plasmodesmata as such, even without forming tubular structures, most likely underlies the inability of these plants to develop normally. Indeed, it is well recognized that plasmodesmata play an important role in development and supracellular organisation of plants (Lucas *et al.*, 1993).

In contrast to what has been found in transgenic tobacco plants expressing the 30K movement protein of TMV, the $NS_M$ protein was observed along the entire length of the plasmodesma and not specifically associated with the central cavity of structurally modified secondary plasmodesmata (Ding *et al.*, 1992). Morphologic changes have not been reported for plants expressing the TMV movement protein, although these plants are susceptible to drought and wilt readily while they have less root mass. Moreover, under high light and temperature conditions these plants are "spindly" and tend to fall over (R.N. Beachy, personal communication). However, an increase of the size exclusion limit was demonstrated, which is a clear indication of the modification of the plasmodesmata. Also transgenic plants expressing the 3a movement protein of CMV showed an increased size exclusion limit, while no clear change was observed in the morphology of the plant (Vaquero *et al.*, 1994). However, transgenic tobacco plants expressing the BL1 movement protein of the geminivirus SqLCV, like TSWV $NS_M$ expressors, display a clearly visible change in the morphology of the plant. Plants expressing this viral protein exhibited symptoms comparable to viral infection, potentially caused by association of the expressed protein with plasmodesmata, because the protein was demonstrated to be associated with cell wall fractions (Pascal *et al.*, 1993). Since also the cell-to-cell movement of geminiviruses has been suggested to involve tubules (Kim and Lee, 1992), the aberrant development of transgenic plants might be characteristic of tubule-forming movement proteins.

The occurrence of aberrations in the morphology of plants expressing the TSWV $NS_M$ protein, together with a clear association of this protein with plasmodesmata, present further evidence of the involvement of this protein in cell-to-cell movement of tomato spotted wilt tospovirus by modifying plasmodesmata. Additional studies have been carried

out to reveal the effect of this transgenically expressed protein on the size exclusion limit of plasmodesmata and are discussed in Chapter 6.

**Acknowledgements**

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# 6

# Modification of plasmodesmal size exclusion limit by the $NS_M$ movement protein of tomato spotted wilt virus.

## 6.1. SUMMARY

Based on cytological evidence the non-structural protein $NS_M$ of TSWV has previously been implicated in viral cell-to-cell movement during systemic infection of plants (Kormelink *et al.*, 1994; Storms *et al.*, 1995). To confirm that $NS_M$ represents the movement protein (MP) of TSWV, this protein was transgenically expressed in *Nicotiana tabacum* and its effect on the plasmodesmal size exclusion limit (SEL) determined. Pressure injection experiments with fluorescent probes of different molecular mass showed a plasmodesmal SEL that was increased to a similar extent as in tobacco plants that transgenically expressed the TMV MP, thus demonstrating that $NS_M$ is also a MP. Strikingly, when iontophoretic injection was performed, the plasmodesmal SEL showed an apparent decrease in transgenic tissues expressing either plant viral MP. As iontophoresis is a less invasive technique than pressure injection, these different results may have major implications for our understanding of viral MP function and of protein trafficking between plant cells in general.

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## 6.2. INTRODUCTION

Compared to animal viruses plant-infecting viruses have one extra barrier to overcome for establishing a systemic host infection, i.e. the passage of the rigid plant cell walls. It is now generally accepted that movement of plant viruses from cell-to-cell during systemic invasion of plant tissue is achieved by active modification of the plasmodesmata, which in their normal state have a size exclusion limit (SEL) which does not allow the passage of macromolecules with a molecular mass larger than approximately 850-900 Da (Lucas *et al.*, 1993). For a growing number of plant viruses it has been shown that a virus encoded protein, the movement protein (MP), is responsible for the modification of the plasmodesmal SEL and intercellular movement of either the viral genome or mature particles, depending on the virus species (Deom *et al.*, 1987, 1990; Wolf *et al.*, 1989, 1991; Fujiwara *et al.*, 1993; Waigmann *et al.*, 1994; Vaquero *et al.*, 1994; Noueiry *et al.*, 1994; Ding *et al.*, 1992, 1995; Waigmann and Zambryski, 1995). Several models have been proposed describing how plant viral MPs are able to modify plasmodesmata during the infection process and how they target an infectious entity to neighbouring cells (e.g. Van Lent *et al.*, 1990, 1991; Citovsky and Zambryski, 1991; Deom *et al.*, 1992; Lucas *et al.*, 1993; Lucas and Gilbertson, 1994). The identification of viral MPs has been based on genetic studies (involving either natural or engineered mutant viruses which are blocked in their systemic spread), on cytological studies, and on microinjection studies, in which potential, MP-induced differences in plasmodesmal SEL are monitored by injecting plant cells with fluorescent probes of different molecular mass. We have been interested in the role of the $NS_M$ protein in cell-to-cell movement of tomato spotted wilt virus (TSWV). Immunocytological studies of the TSWV infection process has provided evidence that $NS_M$ indeed has a function during cell-to-cell transport of TSWV (Chapters 2-4; Kormelink *et al.*, 1994; Storms *et al.*, 1995). For TSWV it has remained impossible to obtain direct proof for a MP function by mutational analysis of an infectious cDNA clone, due to the fact that its genome is negative stranded. Therefore we have employed, as alternative approach, microinjection studies on transgenic plant tissues expressing the TSWV $NS_M$ protein, in order to determine possible effects of this protein on the plasmodesmal SEL. Such an approach has been successfully applied for study of the TMV MP, which upregulates the SEL as determined by pressure microinjection of fluorescent probes (Deom *et al.*, 1990, 1991; Wolf *et al.*, 1989, 1991; Ding *et al.*, 1992). Here we show that transgenic expression of the TSWV $NS_M$ protein leads to a significant increase of the plasmodesmal SEL for pressure injected dyes, to a similar extent as for TMV MP. In addition to pressure driven injection, iontophoretic injection of the same fluorescent probes was employed to investigate how the TSWV $NS_M$ protein modifies plasmodesmal functioning, using again transgenic plants expressing the TMV MP as control. So far iontophoresis has not been

applied in studies aiming to analyse the effect of viral MPs on plasmodesmata, in spite of the fact that it represents a sensitive injection technique, which avoids possible disturbance of cellular functions by pneumatic pressure (Duckett *et al.*, 1994; Van der Schoot and Lucas, 1995). It is shown in this paper that in transgenic plants expressing the TMV or TSWV MP, the plasmodesmal SEL is recorded as decreased rather than increased. This finding has major implications for our understanding of viral MP functioning and demonstrates that results obtained with the commonly applied pressure injection systems should be interpreted with more reservation.

## 6.3. MATERIAL AND METHODS

### 6.3.1. Plant material

A cDNA construct containing the $NS_M$ gene of TSWV under the control of the 35S CaMV promoter, was cloned into the binary vector pBIN19 and subsequently transferred into the genome of *Nicotiana tabacum* var. SR1 plants by *Agrobacterium tumefaciens* leaf disk transformation (chapter 5; Prins *et al.*, 1997). Nine S1 progeny lines derived from self-pollination of the original transformants, displayed detectable levels of the $NS_M$ protein. Three $NS_M$ expressors, i.e. A25-13, A25-26 and A25-31, were selected for the present investigations. Microinjection studies were carried out for these three plants and compared with the original non-transformed SR1 tobacco plants. In addition, dye-coupling studies were carried out with other transgenic plants in order to facilitate interpretation and comparison with the established literature. Transgenic tobacco plant line 12 expressing the nucleoprotein (N) of TSWV (Gielen *et al.*, 1991) was used to investigate the possibility that continuous production of any viral protein may interfere with plasmodesmal functioning. In addition, the previously investigated (Deom *et al.*, 1987, 1990; Wolf *et al.*, 1989, 1991) transgenic tobacco line 277, which accumulates the 30 kDa movement protein of TMV, was used for comparison with the $NS_M$ plants. For all studies plant material was taken from mature source leaves of 4-7 week old plants. For microinjection studies, representative greenhouse plants were selected and kept in a climate chamber under standardised conditions, i.e. a 16h photoperiod at 24°C and an 8h dark period at 18°C prior to microinjection.

### 6.3.2. Dyes, pipettes and electrodes

Leaf tissue selected for microinjection was removed from the plants and locally stripped of its abaxial epidermis to uncover spongy mesophyll cells. The leaf tissue was placed,

stripped side up, fixed on a microscope slide with transparent tape and kept in a 200 mM mannitol solution in distilled water for a 2 hour recovery period (Van der Schoot and Van Bel, 1990; Van der Schoot and Lucas, 1995). Glass micropipettes were fabricated from borosilicate capillaries with an inner filament (World Precision Instruments Inc [WPI], Sarasota, FL, USA) on a horizontal pipette puller (Narishige PN-3, Narishige Scientific Instrument Lab, Tokyo, Japan). The tip diameters, approximately 0.5 µm, were capillary back filled with 1% dye solutions (in distilled water). All dye solutions were freshly made, filtered through 0.2 µm syringe filters (Whatman Int. Ltd, UK) and stored at 4°C. The standard dye, Lucifer Yellow CH (LYCH: 457 Da), was employed free or coupled to 10 or 40 kDa dextrans (Molecular Probes Inc, Eugene, OR, USA). The micropipettes were either used for pressure driven injection (Wolf *et al.*, 1989, 1991; Deom *et al.*, 1990; Ding *et al.*, 1992) or, after filling with an electrolyte, for current driven injection (iontophoresis) in combination with electrophysiological measurements (e.g. Van der Schoot and Van Bel, 1990; Van der Schoot and Lucas, 1995; Van der Schoot *et al.*, 1995).

### 6.3.3. Pressure injection

For pressure injection studies the pipettes were loaded with a dye solution and fixed in a pipette holder. The holder was attached to the headstage of a course movement manipulator which was hydraulically connected to a 3-D controller (MN-2 and MO-203 respectively, Narishige Scientific Instrument Lab, Tokyo, Japan). The headstage was fixed to the stage of the standing microscope (Nikon Optiphot II, Nikon, Japan), while the pipette holder was connected by plastic tubing to the outlet of a $N_2$ driven pressure injection system (Narishige IM-200, Narishige-USA Inc, Greenvale, New York, USA). Leaf tissue was placed in a bathing chamber on the stage of the microscope and was kept submerged during the entire experiment in order to prevent drying and to avoid reflections of fluorescence from the injected cell on surrounding cells which could be mistaken for cell-cell transport. After selecting an appropriate mesophyll cell in layer one or two, the micropipette was positioned and lowered into the target cell. A series of pressure pulses (1-2 seconds at 0.40 - 0.75 bar) was then applied to the pipette. Dye inflow and potential dye movement to other cells (dye coupling) was visually monitored and photographically documented. LYCH, LYCH 10 kDa-dextran as well as LYCH 40 kDa-dextran was injected using this method.

### 6.3.4. Iontophoretic microinjection

After filling the tip with a dye solution, the shanks and the upper part of the micropipette were filled with 100 mM KCl, allowing the pipette to function as a microelectrode. The stem of the electrode was subsequently pushed into a half cell microelectrode holder

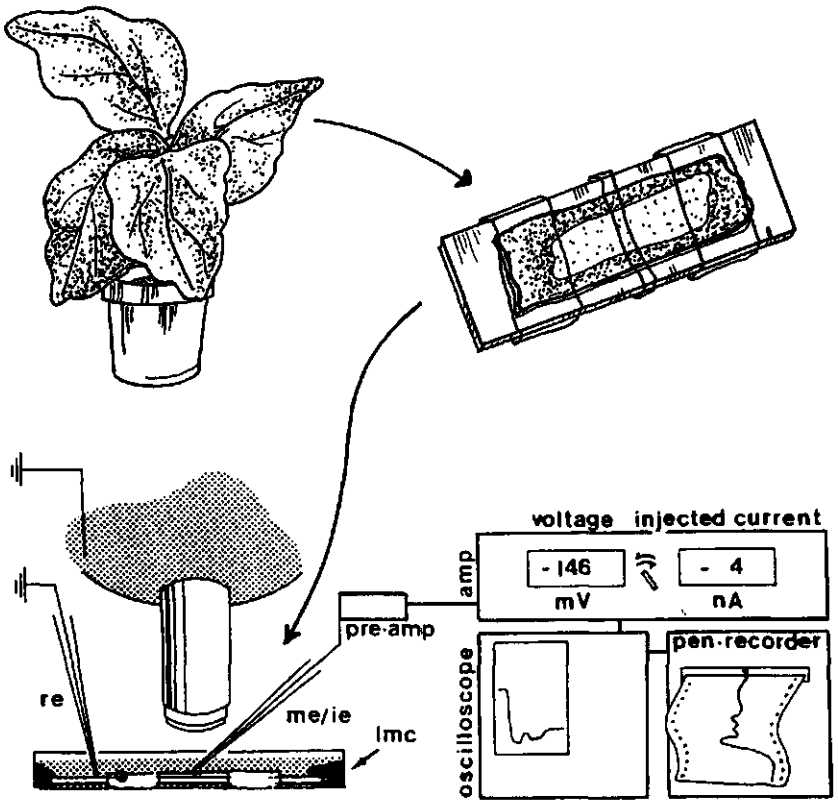


**Figure 6.1.** Preparation and iontophoresis of leaf material. The abaxial epidermis of a tobacco leaf was partly removed, the leaf fixed on a microscope slide and mounted in a bathing chamber containing 200 mM of mannitol in double distilled water. The chamber was fixed on the stage of an epifluorescent microscope and was grounded by a reference electrode. Membrane potentials (voltages) were measured by the the tip of a microelectrode and, after amplification, visualised by an oscilloscope or pen recorder. Outflow of the dye into the impaled cell occurred by application of an intermittent current on the injection electrode. re: reference electrode; me/ie: measuring/injection electrode; pre.amp: pre-amplifier; lmc: laboratory modelling clay.

(MEH3SF10, WPI, Sarasota, FL, USA) and filled with 3 M KCl (Van der Schoot and Van Bel, 1990; Van der Schoot and Lucas, 1995; Van der Schoot *et al.*, 1995). The holder was directly connected to a preamplifier probe (WPI-712P; WPI. Sarasota, FL, USA) which was clamped to the headstage of the micromanipulator and electrically connected to the $10^{12}$ Ω input of the electrometer (WPI Duo 773, WPI, Sarasota, FL, USA)(Figure 6.1.). Tip resistance of the selected microelectrodes varied between 20 and 50 mΩ. For iontophoretic microinjection a piece of leaf tissue was transferred to a transparent bathing chamber on the stage of the standing microscope (Figure 6.1.). After visual inspection and selection of an appropriate cell, the electrode was lowered and impaled into a target cell. Entry of the

microelectrode into the bathing medium, grounded with a reference electrode, gave a voltage reading that was set to zero. Entry of the microelectrode into a cell was recorded (Kenwood CS 1021 dual trace oscilloscope, Kenwood Corp., Tokyo, Japan) and documented (single channel flatbed recorder BDIII, Kip & Zonen Delft BV, Delft, The Netherlands) as an initial small drop in the measured voltage due to contact with the wall and a subsequent sharp fall due to entry through the plasmamembrane into the cell (Figure 6.1). After recording a stable membrane potential ($E_m$ usually between -80 mV and -150 mV) indicating an adequately sealed cell membrane, a low (intermittent) current was applied to the microelectrode (-1 nA to -10 nA) in order to drive out the negatively charged dye. This was difficult to achieve when the measured $E_m$ was small (less than 40 or 50 mV potential difference). Therefore, we injected only cells with an $E_m$ in range indicated in the text. In these cells it was commonly possible to trigger light/dark (L/D) transients of the $E_m$ (Figure 6.3) indicating an immediate response of membrane mechanisms to the acidification of the cytoplasm by the chloroplasts (Pallagahy and Lüttge, 1970; Bentrup, 1974; Felle and Bertl, 1986). In control plants, dye coupling of LYCH correlated with these L/D transients. We have used this feature of L/D transition to avoid injecting vacuoles, which would contaminate the dye coupling score. The population of cells in the pressure injection experiments may deviate to some extent from those used in the iontophoresis experiments since in the former no selection could be made on the basis of $E_m$ or cell viability (Van der Schoot and Lucas, 1995). Iontophoresis was employed for microinjection of LYCH and LYCH 10 kDa-dextran.

### 6.3.5. Illumination, observation and photography

The incandescent light of the microscope obtained illumination with white light. Additionally, epi-illumination with white light via fibre optics was used to examine leaf tissues (Schott KL 1500 Electronic: Schott Glaswerke, Wiesbaden, Germany), whereas epi-illumination for fluorescence microscopy was revealed by a super high-pressure mercury arc lamp (HBO 100W/2, Osram, Germany). Excitation and barrier filters were standard BV and B filtersets of Nikon (BV: excitation 400-440/ barrier 470/ dichroic mirror 455; B: excitation 470-490/ barrier 515/ dichroic mirror 510). Monitoring of dye injections and dye coupling was done by direct observation through the microscope. To avoid photobleaching due to continuous illumination of tissue with BV or B light, we established the time frames of dye outflow and dye coupling in a series of preliminary experiments with the help of a stopwatch. In the final series of experiments, the appropriate stages of dye coupling were photographed with blue light illumination on 320 tungsten Ektachrome slide film and the photographs were reproduced from the slides.

## 6.4. RESULTS

### 6.4.1. TSWV and TMV MP's increase plasmodesmal SEL in transgenic plants as established by pressure injection of fluorescent probes.

To confirm previous indications (Kormelink *et al.*, 1994; Storms *et al.*, 1995) that the $NS_M$ protein of TSWV represents the viral MP, its effect on plasmodesmal SEL was studied in transgenic tobacco lines by pressure injection of the fluorescent probe Lucifer Yellow CH (LYCH) linked to dextran molecules of increasing molecular mass. Three previously described (Prins *et al.*, 1997) transgenic $NS_M$ expressors were selected for these investigations. These plants (A25-13, A25-26 and A25-31) express $NS_M$ in detectable amounts, resulting in aberrations in plant growth and appearance (chapter 5; Prins *et al.*, 1997). As positive control a transgenic tobacco line was used that expressed the TMV 30 kDa MP (line 277; Deom *et al.*, 1987), while non-transgenic tobacco lines and a transgenic tobacco line that expressed the TSWV nucleoprotein (line 12) were used as negative controls. Three mature leaves were sampled of each transgenic line, the epidermis removed and the first layer of mesophyll cells microinjected with a fluorescent dye (LYCH, LYCH-10 kDa dextran or LYCH-40 kDa dextran). Confinement of the probe to the target cell or diffusion to neighbouring cells was observed and documented. As expected, in tissue of both transgenic lines and non-transgenic control lines, pressure injected free dye moved readily to neighbouring cells, resulting in diminishing fluorescence in the injected cells within 1 to 5 minutes (Figure 6.2.A-D). This was anticipated since the SEL of normal, functional plasmodesmata is reported to be twice the molecular mass of LYCH (approximately 0.9 kDa versus 0.457 kDa; Lucas *et al.*, 1993). When LYCH linked to 10 kDa dextran (LYCH-10 kDa dextran) was injected, diffusion to neighbouring cells was observed only in tissue of the three $NS_M$ transgenic plants (Figure 6.2.E and 6.2.F; Table 6.1). Speed and efficiency (80% of the injections or more) was similar to that observed for the injection of this probe in the TMV MP transgenic plant. For both the $NS_M$ transgenic plants and the TMV MP transgenic line the fluorescent signal vanished within 10 to 30 minutes after injection. In all non-transgenic control lines, LYCH-10 kDa dextran did not diffuse to neighbouring cells, the fluorescent signal being retained in the injected cell throughout the recording period (30 minutes; Figure 6.2.G and 6.2.H). This also held true for transgenic control line 12 (Table 6.1.); this plant line accumulates the TSWV nucleoprotein (N). When LYCH-40 kDa dextran was used, no intercellular diffusion was observed in the three $NS_M$ expressors or the TMV MP expressor plants (Table 6.1), supporting the conclusion that the TSWV $NS_M$ protein increases the plasmodesmal SEL to a similar extent as the TMV movement protein. Together with previously reported evidence on the $NS_M$ protein (Kormelink *et al.*, 1994; Storms *et al.*, 1995) and the informa-

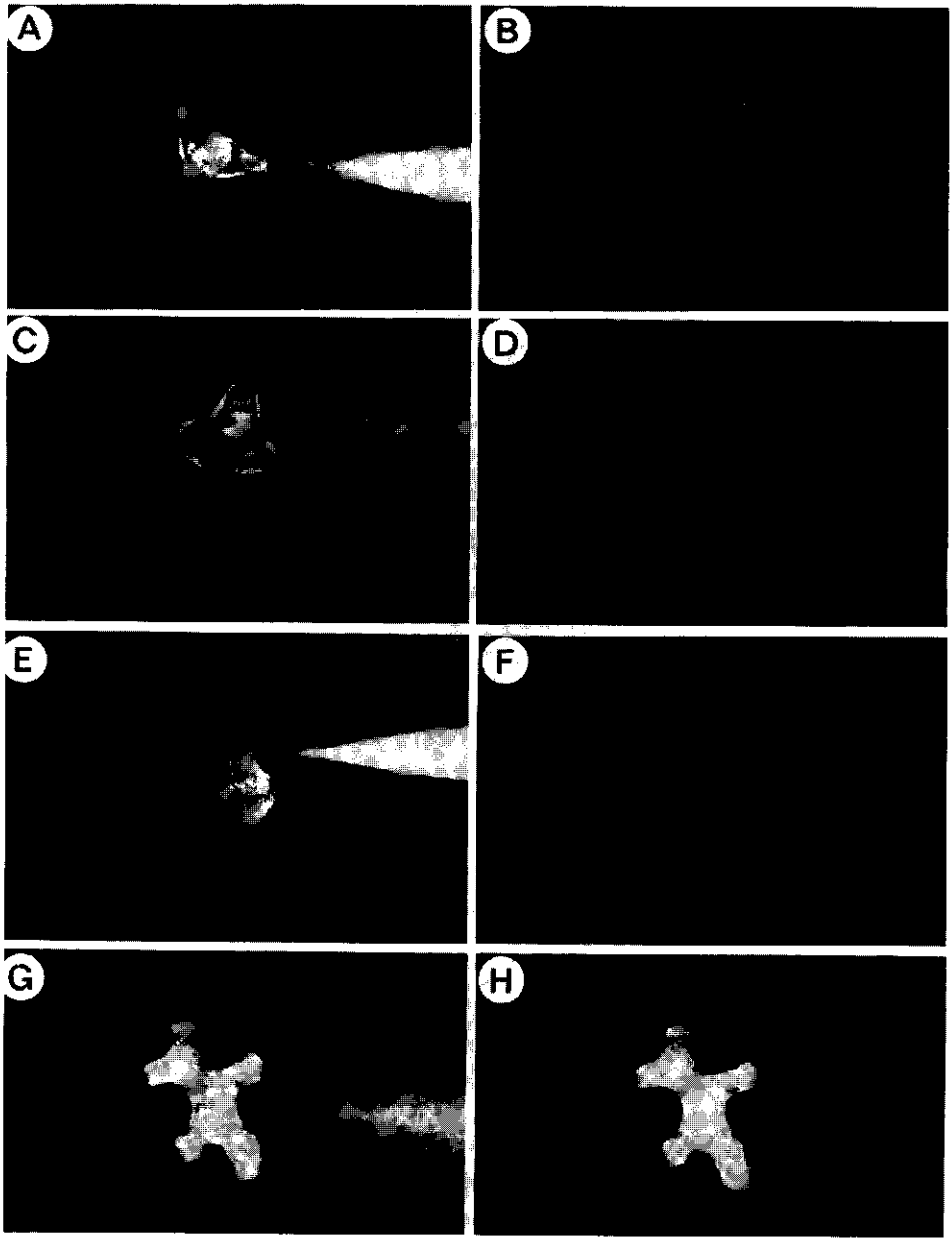


**Figure 6.2.** Pressure driven microinjection of fluorescent probes in TSWV $NS_M$ transgenic mesophyll tissue. Injection of free LYCH in a mesophyll cell of **(A,B)** $NS_M$ expressing transgenic plant line A25-13 and **(C,D)** in non transgenic control line SR1 at 0 (left panels) and 5 min. (right panels) post injection, respectively. **(E,F)** Injection of LYCH-10 kDa in a mesophyll cell of transgenic line A25-13 at 0 and 30 min. post injection. **(G,H)** Injection of LYCH-10 kDa dextran injected in mesophyll cells of non-transgenic control line SR1, at respectively 0 and 30 minutes post inoculation.

tion presented in the previous paragraph, these data provide conclusive evidence that $NS_M$ indeed represents the tospoviral MP, and that one of its activities is to modify the plasmodesmal SEL of the host.

**Table 6.1.** Dye coupling of pressure-delivered fluorescent probes between mesophyll cells of transgenic *Nicotiana tabacum* plants

| | | Fluorescent probes | | |
|---|---|---|---|---|
| Plant lines | Transgene | LYCH | LYCH-10k dextran | LYCH-40k dextran |
| SR1 | - | 28 (30) | 1 (30) | 0 (20) |
| 12 | TSWV-N | 19 (20) | 2 (20) | 0 (20) |
| A 25-26 | TSWV-$NS_M$ | 19 (20) | 17 (20) | 1 (20) |
| A 25-13 | TSWV-$NS_M$ | 18 (20) | 16 (20) | 0 (20) |
| A 25-31 | TSWV-$NS_M$ | 19 (20) | 16 (20) | 0 (20) |
| Xanthi nn | - | 18 (20) | 1 (20) | 0 (20) |
| 277 | TMV-30 kDa | 18 (20) | 17 (20) | 0 (20) |

Data represent the number of injections that resulted in movement at t=30 min after injection. Values in parentheses represent the total number of injections for each transgenic plant line.

### 6.4.2. TSWV and TMV MP's decrease plasmodesmal SEL in transgenic plants as determined by iontophoretic injection of fluorescent probes.

Having established the MP function of TSWV $NS_M$, we next analysed possible differences in plasmodesmal SEL in transgenic plant lines expressing this MP or the TMV MP, but now employing iontophoretic microinjection of the probes. During iontophoresis, probes are injected into a target cell by application of a small current to the microelectrode tip (Van der Schoot and Van Bel, 1990; Van der Schoot and Lucas, 1995; Damen and Dictus, 1996; Bossinger and Schierenberg, 1996). Iontophoresis has several advantages over the conventional pressure injection in which a pressure-pulse in excess of the cell turgor forces a probe into the cytoplasm (Van der Schoot and Lucas, 1995) with the danger of cytoplasmic disruption (Duckett *et al.*, 1994). One advantage of the iontophoretic approach is the ability to select physiologically active cells by measuring the membrane potential ($E_m$). In addition, the prolonged viability of the impaled cell during the experiment can be assessed by monitoring the $E_m$ over time (Van der Schoot and Lucas, 1995). As a general

rule, a strongly negative potential of the cell membrane reflects an active physiological state of the cell, whereas a weakly negative potential indicates a relatively inactive state (Van Bel and Van der Schoot, 1990). With the tip of the electrode in the cytoplasm transient fluctuations in $E_m$ can be recorded by switching the light on and off (Figure 6.3.). These so called light-dark (L/D) transients result from changes in the activity of the proton pumps at the cell membrane in response to sudden cytoplasmic acidification, and reflect L/D-triggered proton movements over the chloroplast membranes (Pallaghy and Lüttge, 1970; Bentrup, 1974; Felle and Bertl, 1986). In the practise of microinjection the recording of L/D transients requires an adequate membrane seal around the tip of the microelectrode and localisation of the tip in the cytoplasm. Based on the capability to determine L/D transients and the membrane potential of both the cell membrane and the tonoplast, the position of the needle in the cell could be assessed.

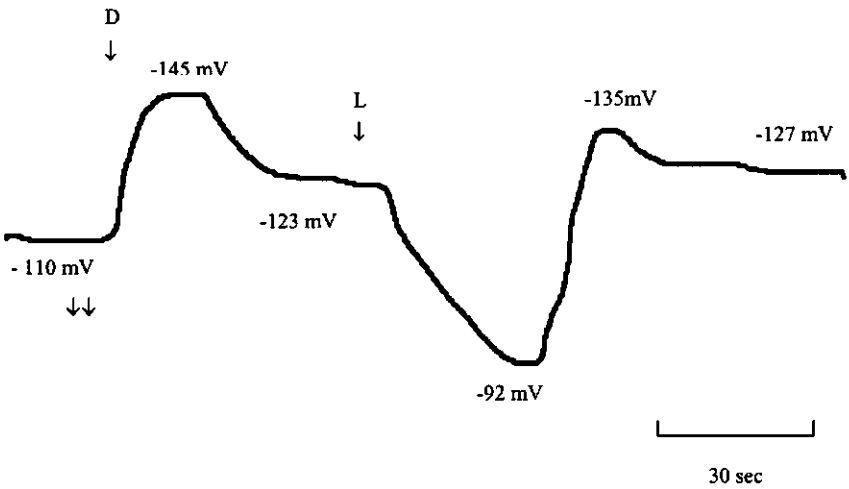


**Figure 6.3.** Typical sequential response of the membrane potential of a spongy mesophyll *Nicotiana tabacum* cell to light-dark and dark-light transitions, as measured with the microelectrode tip located in the cytoplasm during iontophoresis. These $E_m$ transients reflect changes in proton pump activities at the cell membrane in response to acidification of the cytoplasm by chloroplasts. Recordings were performed with the leaf tissue in standard incubation medium. D: dark period; L: light period.

Figure 6.4. illustrates the behaviour of free LYCH in mesophyll cells of the various transgenic and non-transgenic lines in time. From the moment the current is switched off (t=0) dye diffusion was monitored up to 30 minutes after injection. In striking contrast to the results with pressure injection, iontophoretic supply of free LYCH to mesophyll cells of all three $NS_M$ expressors consistently resulted in retention of the fluores-

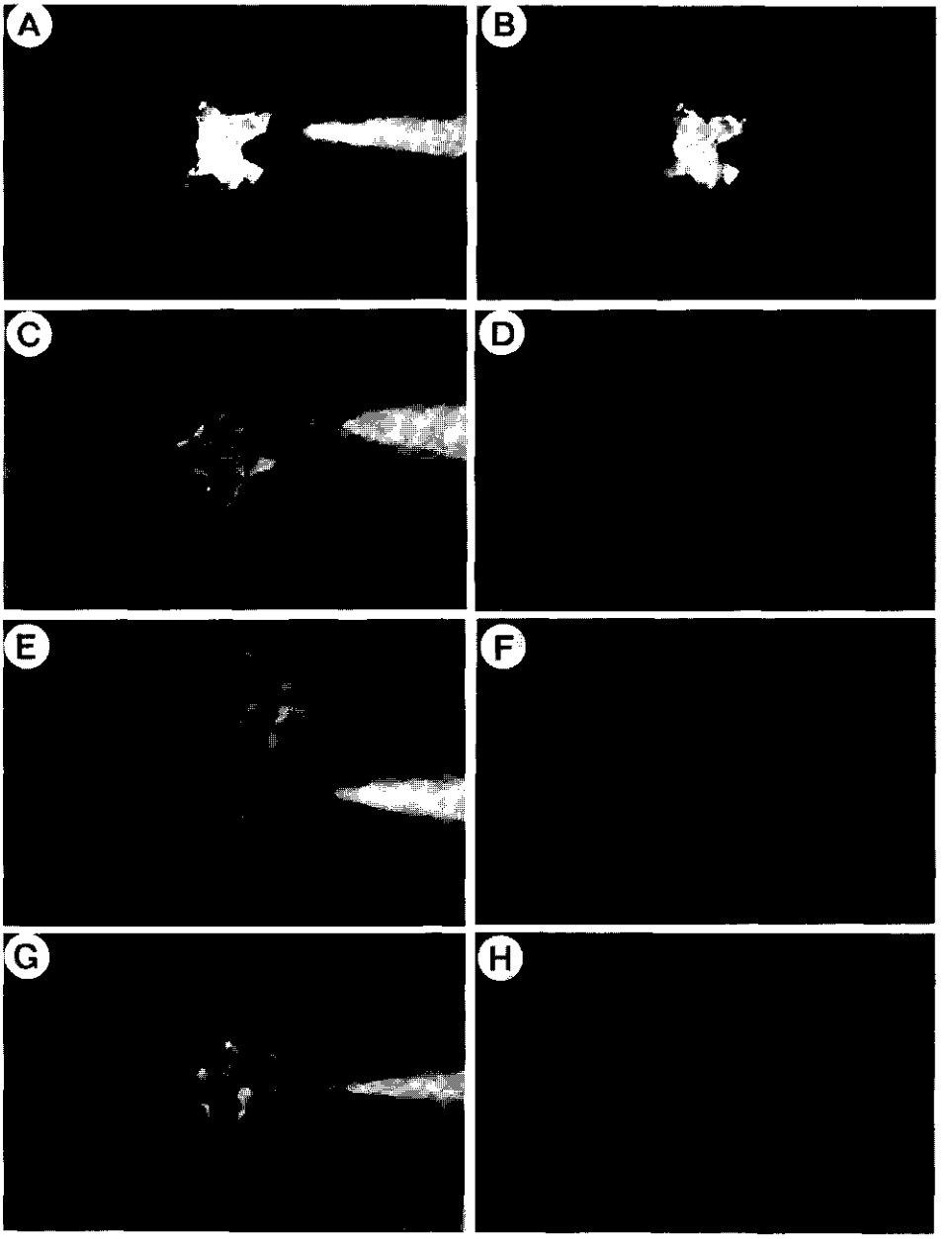


**Figure 6.4.** Current driven microinjection (iontophoresis) of fluorescent probes in TSWV $NS_M$ transgenic mesophyll tissue. Injection of free LYCH in a mesophyll cell of $NS_M$ transgenic line A25-13 at 0 **(A)** and 30 min. **(B)** post injection. In the non-transgenic control line SR1 LYCH rapidly diffuses (**C**: t=0, **D**: 1 min. post injection). **(E,F)** Injection of free LYCH in a mesophyll cell of TMV MP transgenic plant line 277 at 0 and 30 min. p.i., respectively **(G,H)** Injection of LYCH in the control line Xanthi nn at 0 and 1 min. post injection, respectively.

cent signal within the injected cell (Figure 6.4.A and 6.4.B). This contrasted with the rapid diffusion of free LYCH to neighbouring cells in the non-transgenic controls (Figure 6.4.C and 6.4.D) and in the TSWV N protein transgenic plants (Table 6.2.). Apparently, in TSWV MP ($NS_M$) transgenic plants, the SEL of the plasmodesmata was decreased rather than increased for iontophoretically injected LYCH (0.457 kDa). To verify whether this unexpected effect was due to certain properties of the TSWV $NS_M$ protein or due to its activity as MP, the TMV MP-expressing transgenic line 277 was tested using the same iontophoretic method. In this line, again a decrease of the plasmodesmal SEL was recorded, since free LYCH diffused less effective (12 times out of 20 observations respectively; Table 6.2.) in the TMV MP transgenic line (Figure 6.4.E and 6.4.F) compared to the control line Xanthi nn (Figure 6.4.G an 6.4.H). LYCH-10 kDa dextran, moreover, did not move at all (Table 6.2.). Hence, the decrease of the plasmodesmal SEL as observed after iontophoretic supply of fluorescent probes appeared to be specifically induced by viral MPs.

**Table 6.2.** Dye coupling of iontophoretically injected fluorescent probes between mesophyll cells Of transgenic *Nicotiana tabacum* plants

| | | Fluorescent probes | |
|---|---|---|---|
| Plant lines | Transgene | LYCH | LYCH-10k dextran |
| SR1 | - | 18 (20) | 0 (20) |
| 12 | TSWV-N | 19 (20) | 2 (20) |
| A 25-26 | TSWV-$NS_M$ | 1 (20) | 0 (20) |
| A 25-13 | TSWV-$NS_M$ | 1 (20) | 1 (20) |
| A 25-31 | TSWV-$NS_M$ | 0 (20) | 0 (20) |
| Xanthi nn | - | 18 (20) | 0 (20) |
| 277 | TMV-30 kDa | 12 (20) | 0 (20) |

Data represent the number of injections that resulted in movement at t=30 minutes after injection. Values in parentheses represent the total number of injections for each transgenic plant line.

## 6.5. DISCUSSION

To further assess the previously proposed cell-to-cell movement function of the TSWV $NS_M$ protein (Kormelink *et al.*, 1994; Storms *et al.*, 1995), a microinjection approach was

chosen to determine the effect of this protein on macromolecular trafficking through plasmodesmata. For a number of plant viral MPs it is well established by pressure injection that they can trigger an upregulation of the plasmodesmal SEL allowing larger molecules (e.g. fluorescent probes >1000 Da and viral RNA) to pass from one cell to another (Fujiwara *et al.*, 1993; Waigmann *et al.*, 1994, Waigmann and Zambryski, 1995; Noueiry *et al.*, 1994; Ding *et al.*, 1995). This observation on the increase of SEL was extended to transgenic plants expressing the TMV MP (Deom *et al.*, 1987, 1990; Wolf *et al.*, 1989, 1991; Ding *et al.*, 1992), the 3a protein of cucumber mosaic virus (CMV; Vaquero *et al.*, 1994) and the P3 protein of alfalfa mosaic virus (AlMV; Poirson *et al.*, 1993). In analogy to these studies, transgenic plants expressing the $NS_M$ protein were tested for alterations in intercellular macromolecular trafficking.

Strikingly, by using two fundamentally different microinjection delivery techniques, opposite results were obtained with respect to the alteration of the plasmodesmal SEL in transgenic plant tissue that accumulates viral MP. Whereas a significant increase of SEL was measured for both TSWV MP and TMV MP-expressing tobacco lines when pressure driven microinjection of fluorescent probes was employed, a downregulation was observed upon iontophoretic microinjection of the same probes. Iontophoresis is unlikely to close plasmodesmata or interfere otherwise with plasmodesmal functioning as a decrease of plasmodesmal SEL was restricted to transgenic plant tissue that accumulated TSWV or TMV MP and was not found in non-transgenic controls or in a plant line that accumulated a viral protein not directly involved in cell-to-cell movement (TSWV N protein). Hence, the effect appears to occur specifically in the presence of viral MPs, which are targeted, to the plasmodesmata. The question now arises which microinjection method is most valid for assessing MP function and provides the most reliable information of how plasmodesmata are modified *in situ* by transgenically expressed viral MP. Sofar iontophoretic microinjection of probes has not yet been employed for determining viral MP functions and the considerable number of papers reporting on such functions have all used pressure injection (Deom *et al.*, 1987, 1990; Wolf *et al.*, 1989, 1991; Fujiwara *et al.*, 1993; Waigmann *et al.*, 1994; Vaquero *et al.*, 1994; Noueiry *et al.*, 1994; Ding *et al.*, 1992, 1995; Waigmann and Zambryski, 1995). We feel that the outcome of iontophoresis better reflects the actual situation at the modified plasmodesmata. Iontophoresis has previously been applied in many cases as a more sensitive microinjection technique which can be used in combination with the monitoring of cell viability and physiological activity of plant cells (e.g. Tucker *et al.*, 1989; Tucker, 1990; Van der Schoot en Van Bel, 1990; Van der Schoot en Lucas, 1995) as well as animal cells (Damen and Dictus, 1996; Bossinger and Schierenberg, 1996). When applied with caution, i.e. keeping the applied current low, iontophoresis is unlikely to damage cells (Van der Schoot and Lucas, 1995), whereas plant cells are prone to cytoplasmic disruption by the sudden pressure differences caused by

pressure injection (Oparka *et al.*, 1991; Duckett *et al.*, 1994). For the injection of small charged molecules, iontophoresis is therefore the preferred technique (Tucker *et al.*, 1989; Tucker, 1990; Van der Schoot and Lucas, 1995). It is important to note that in our experiments the outcome of pressure injection and iontophoresis was identical for the non-transgenic control plants, and neither of the two techniques affected cell-cell transport in these plants. This strongly suggests that accumulation of viral MP makes the cells respond differently to the microinjection techniques. In summary, iontophoresis reveals that targeting of both the TSWV and TMV MPs to the plasmodesmata inhibits or partly blocks the communication pathway for macromolecular trafficking between plant cells in stead of facilitating this process. This observation has major implications for our understanding of viral MP function and of macromolecular trafficking between plant cells in general. It is clear that further studies are needed to validate microinjection approaches for studying intercellular communication in plant tissues.

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# Expression and subcellular localisation of the $NS_M$ plant viral movement protein of tomato spotted wilt virus (TSWV) in insect and mammalian cells.

## 7.1. SUMMARY

The $NS_M$ movement protein of tomato spotted wilt virus forms tubular structures in infected plant leaf tissue and protoplasts. To investigate whether plant specific host components are involved in the formation of the tubular structures, the $NS_M$ gene was expressed in two different heterologous animal cell systems i.e. insect (*Spodoptera frugiperda* and *Trichoplusia ni*) cells and mammalian (baby hamster kidney, BHK21) cells and its intracellular behaviour studied. $NS_M$ expression in insect cells revealed specific targeting of the protein to cellular membranes while tubular structures were formed at the cell surface in a similar fashion as observed in plant protoplasts. However, in mammalian cells no plasmamembrane targeting and tubule formation was found. The results indicate that the $NS_M$ protein has the capacity to form tubular structures in the absence of plant specific components, although conserved host components may be essential.

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## 7.2. INTRODUCTION

Tomato spotted wilt virus (TSWV) achieves cell-to-cell movement by employing a tubule-guided movement mechanism. This mechanism comprises the modification of plasmodesmata, the formation of a tubule herein (Chapter 3; Kormelink *et al.*, 1994; Storms *et al.*, 1995) and the translocation of the virus probably as nucleocapsids (Chapter 4). We have previously shown that the TSWV $NS_M$ protein is the actual tubule forming protein (Chapter 3; Storms *et al.*, 1995) and that tubule formation is a transient phenomenon during TSWV infection of plants (Storms *et al.*, 1995). Tubules are also formed at the surface of protoplasts infected with TSWV (Kikkert *et al.*, 1997). Besides plants, TSWV also replicates and spreads in tissues of its thrips vector (Wijkamp *et al.*, 1993; Ullman *et al.*, 1993). As all studies support the idea that $NS_M$ is the plant viral MP (Chapters 2,3,4,5,6), the question remains whether the protein is expressed in insect tissue and has a function during the infection of the thrips. To establish the phenotype of $NS_M$ upon expression in insect cells, the $NS_M$ gene was cloned into a recombinant baculovirus (AcNPV/$NS_M$; Kormelink *et al.*, 1994; Chapter 2) and expressed in *Spodoptera frugiperda* and *Trichoplusia ni* cultured insect cells. The insect cell types were chosen in the absence of cultured cell lines of the true thrips host. Chapter 8 reports on the analysis of $NS_M$ in intact tissues of infected thrips. To compare the phenotype of the TSWV $NS_M$ protein in a cell system that is unrelated to its natural hosts (plants and insects), $NS_M$ was also expressed and its behaviour studied in mammalian (baby hamster kidney, BHK21) cells by immunocytology.

## 7.3. MATERIALS AND METHODS

### 7.3.1. Construction of AcNPV recombinant virus

The baculovirus *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) was used for expression of $NS_M$ in *Spodoptera frugiperda* and *Trichoplusia ni* insect cells. Complementary DNA clone pTSWV28 containing the complete open reading frame (ORF) of $NS_M$ was digested with *Bam*HI and cloned in the *Bam*HI site of pAc33DZ1 (Kormelink *et al.*, 1992; Zuidema *et al.*, 1990). The resulting transfer vector, pAc33DZ1/$NS_M$+L, contained the complete ORF of $NS_M$ including the TSWV viral 5'-untranslated sequence (+L). For convenient cloning of the $NS_M$ gene without the viral 5'-untranslated sequence, the gene was amplified by polymerase chain reaction (PCR) using oligonucleotides Zup 51 (dGGGAATTCTTTTCGGTAACAAGAGGCC), containing 27 nucleotides of which 21 are identical to nucleotides 109 to 129 of the viral (v) strand of M RNA, and Zup 14

(dCCCTGCAGGATCCGAAATTTAAGCTTAAATAAGTG), having 22 nucleotides complementary to nucleotides 1022 to 1043 of the vRNA (Figure 7.1.A). After PCR-amplification, the DNA was digested with *Eco*RI, a *Bam*HI-*Eco*RI adaptor (containing the nucleotide sequence GGATCCGGCAACGAAGGTACCATGGGAATTC), with an internal start codon, ligated in order to restore the $NS_M$ ORF, and subsequently digested with *Bam*HI to generate the $NS_M$ gene as a *Bam*HI fragment. This fragment was purified from an agarose gel, cloned in the *Bam*HI site of plasmid pAc33DZ1 resulting in transfer vector pAc33DZ1/$NS_M$ (Figure 7.1.B). Recombinant baculoviruses were produced by co-transfection of *S. frugiperda* (Sf) cells with a mixture of BSu36I digested AcMNPV PAK6 DNA and pAc33DZ1/$NS_M$+L or pAc33DZ1/$NS_M$ DNA according to Kitts and Possee (1993). The recombinant baculovirus (AcMNPV/$NS_M$) in which the incorporated gene was expressed from the polyhedrin promoter, was plaque purified (Brown and Faulkner, 1977) and subsequently grown in high titre stocks. *Spodoptera frugiperda* (Sf21) and *Trichoplusia ni* (T. ni 368) cells were infected with wild-type (control) or recombinant baculovirus AcMNPV with a multiplicity of 20 $TCID_{50}$ units per cell and incubated at 27°C for 16-48 hours (Kormelink *et al.*, 1991).

### 7.3.2. Construction of pSFV1/$NS_M$ and recombinant *in vitro* transcripts

The complete ORF of the $NS_M$ gene as present in vector pAc33DZ1/$NS_M$ (Kormelink *et al.*, 1994) was digested with *Bam*HI and cloned into the *Bam*HI multiple cloning site of the semliki forest virus vector pSFV1 (Liljeström and Garoff, 1991). The fragment was ligated and subsequently transformed by electroporation into the electrocompetent *E. coli* strain DH5alfa. The transformed bacteria were grown on agar disk containing 100µg/ml ampicilin overnight at 37°C, single colonies were transferred to ampicilin selective Lurai Broth medium overnight at 37°C and prepared for DNA plasmid isolation by the alkaline lysis method (Birnboim and Doly, 1979). The SFV plasmid was linearised by *Spe*I and mRNA was transcribed in the presence of GIBCO BRL SP6 RNA polymerase, nucleotides and 5'mG RNA capping analogue. The *in vitro* transcript was according to the expected size as checked by Northern blotting.

### 7.3.3. Cell culture and transfection of BHK cells

Baby Hamster Kidney (BHK)21 type cells were cultured in Glasgow Minimum Essential Medium (G-MEM) containing 5% (w/v) fetal bovine serum (FBS), 10% (w/v) tryptose phosphate broth, 10 mM HEPES (pH 7.3), 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin). The cultures were placed at 37°C and 5% $CO_2$. Prior to transfection, an average of $10^7$ cells were washed with PBS-O (0.13 M NaCl, 2.7 mM KCl, 8 mM

$Na_2HPO_4$, 1.7 mM $KH_2PO_4$), trypsinised for 30 seconds with 2 ml trypsin solution (0.5 g/l trypsine (Difco 1:250), 0.13 M NaCl, 5.4 mM KCl, 7 mM $NaHCO_3$, 0.5 mM $Na_2EDTA$, 1% (w/v) phenol red, 1 g/l dextrose) and resuspended in 10 ml G-MEM medium. After centrifuging at 1000 rpm for 5 min, the cells were rinsed with 10 ml PBS-O, again centrifuged at the same speed and time and resuspended in 800 µl PBS-O containing 9 mM $CaCl_2$ and 7.5 mM $MgCl_2$. In addition, 10 µg of recombinant or non-recombinant (control) mRNA was added and two short electroporation pulses were performed at 850 V/cm, 25 mF and an infinite resistance. The cells were subsequently incubated in 20 ml BHK G-MEM medium at 37°C and 5% $CO_2$ .

### 7.3.4. Immunofluorescence microscopy

For immunofluorescent localisation of $NS_M$ in *S. frugiperda* / *T. ni* insect cells and BHK mammalian cells, polyclonal anti-$NS_M$ serum was used as prepared by Kormelink *et al.*, 1994 (Chapter 2).

*S. frugiperda* 21 and *T. ni* 368 insect cells, cells were grown on sterile glass cover slips and infected with buffer (mock infection), wild-type or recombinant AcMNPV/$NS_M$ and incubated in Hinks medium (Hink *et al.*, 1970) at 27°C. At 16, 24, 36 and 48 hours p.i., the attached cells were fixed by immersion in aceton at -70°C for 15-20 minutes. 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 hour at room temperature and subsequently incubated with 1 µg/ml anti-$NS_M$ serum for 1 hour. After washing three times 20 minutes in PBS, the cells were incubated with FITC-conjugated goat anti-rabbit secondary antibodies for 1 hour in the dark. After washing again three times 20 minutes in PBS, a drop of citifluor was added to the cells and the cells (attached on a cover slip) were put on a glass slide. The samples were examined by a Leitz laborlux S fluorescent microscope and a Biorad MRC 600 confocal scanning laser microscope with an argon ion laser and a BHS 488 nm excitation filter (Van Lent *et al.*, 1991). Alternatively, mammalian BHK21 cells, transfected with buffer (mock transfection), wild type and recombinant mRNA were treated in a similar way after taking samples at 6, 22 and 40 hours after transfection.

### 7.3.5. Electron microscopy

For *in situ* immuno-electron microscopical analysis, Sf 21 and T. ni 368 cell were infected with buffer, wild-type or recombinant virus while BHK cells were transfected with *in vitro* $NS_M$ transcripts. At 6, 22 and 40 hours post transfection (BHK cells) and 48 hours post infection (insect cells) cells were fixed in 0.75% (w/v) glutaraldehyde in nutrition medium,

rinsed in phosphate citrate buffer and additionally fixed in 3% (w/v) glutaraldehyde/2% (w/v) paraformaldehyde in phosphate citrate (PC) buffer for 1 hour at room temperature. After rinsing in phosphate citrate buffer, the cells were pelleted in 5% (w/v) gelatine in PC buffer and the gelatine was fixed with 3% (w/v) glutaraldehyde/2% (w/v) paraformaldehyde in PC buffer. The cells were dehydrated in increasing ethanol concentrations and low temperature embedded in London Resin Gold at -25°C. Immunogold localisation using pre-immune serum (control), anti-$NS_M$ serum and protein A-gold probes with a diameter of 5 nm followed by silver enhancement was carried out on ultrathin sections of insect cells as described by Van Lent *et al.* (1990).

Additionally, insect cells were fixed and embedded in gelatine and infiltrated with 2.3 M sucrose in PC buffer at 4°C for 16 hours. Small blocks of gelatine embedded insect cells were cryo-fixed by immersion in liquid nitrogen and sectioned with a Reichert FCS ultracryotome. Cryosections were immunolabelled 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, cells were sampled at various hours after infection and stained with 2% PTA, pH 5.5 as described by Van Lent *et al.* (1991).

## 7.4. RESULTS

### 7.4.1. Expression of the $NS_M$ protein in insect cells

To investigate whether TSWV $NS_M$ is able to form tubular structures in cultured insect cells, *S. frugiperda* 21 and *T. ni* 368 insect cells were infected with baculovirus recombinant AcMNPV/$NS_M$ that contains the $NS_M$ gene behind the polyhedrin promoter. As already shown in Chapter 2, Western blot analyses of AcNPV/$NS_M$ infected *S. frugiperda* cells revealed a protein band at the expected position of the $NS_M$ protein. Immunofluorescent staining of $NS_M$ showed an accumulation of the protein in the cytoplasm and more specifically at the periphery of the cell at 16 and 24 hours p.i (Figure 7.1.A). At 36 and 48 hours 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 and around the nucleus (Figure 7.1.B). Using immunogold electron microscopy the $NS_M$ protein could be specifically localised to the plasmamembrane (Figure 7.2.A), to membranes of cytoplasmic vesicles (Figure 7.2.B) and to fibrillar aggregates in the cytoplasm (Figure 7.2.C). Tubule-like structures, easily observed on the surface of immunofluorescent stained cells, were also found in thin cryo sections of infected cells. These structures appeared as bundles of filaments with various

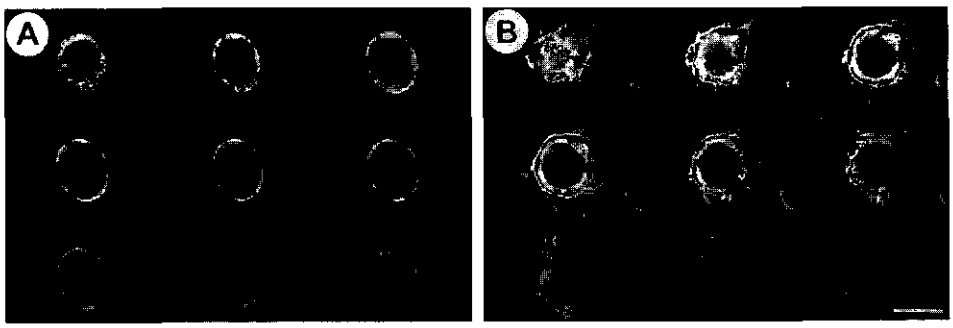


**Figure 7.1.** Immunofluorescent CSLM-images showing the $NS_M$ protein in *T. ni* insect cells. **(A)** At 16 hour p.i. $NS_M$ is specifically present at the cell periphery, while **(B)** at 36 hours p.i. tubule-like extensions occur. Scans are made form top (upperleft corner) to bottom (bottom right corner) with an optical section thickness of 1.1 µm in sequential focal planes. Scale bar represents 10 µm.

diameters, protruding from the cell surface and surrounded by an extension of the plasmamembrane. The $NS_M$ protein was specifically associated with these tubules, confirming their viral origin (Figure 7.2.D). Negative staining electron microscopy of infected Sf 21 and T. ni 368 cells also showed numerous tubule-like structures (Figure 7.3.A), containing the $NS_M$ protein (Figure 7.1.B,C). 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 (Figure 7.3.). It is plausible that the plasmamembrane detached from the tubules during preparation of the samples for electron microscopy.

### 7.4.2. Expression of the $NS_M$ protein in mammalian cells

To investigate the cellular behaviour of $NS_M$ in a cell system that has no direct relation with the natural host cell types (plant and insect cells), mammalian BHK21 cells were transfected with *in vitro* transcripts encoding the non-structural proteins of SFV and the $NS_M$ protein. $NS_M$ appeared highly expressed at all time points investigated (6, 22 and 40 hours p.t.) as was shown by Western immunoblot analyses (data not shown). Immunofluorescence microscopy revealed numerous small cytoplasmic aggregates at 6 hours post transfection (Figure 7.4.A). From 22 hours and on, similar aggregates were discerned although often in an increasing size and number (Figure 7.4.B). Moreover, thread-like structures were frequently present in a wired configuration in the cytoplasm around the nucleus (Figure 7.4.C,D). Electron microscopical examination revealed the presence of $NS_M$ at the membranes of cytoplasmic vesicles and in fibrils found scattered in the cytoplasm at 6 and 22 hours post transfection. At later time points large aggregates of

these fibrils could be observed which varied in size and appearance with the time of expression (22 and 40 hours p.t.) (Figure 7.5.). In contrast to plant and insect cells neither tubules were observed at the cell surface nor was $NS_M$ found at the plasmamembrane.

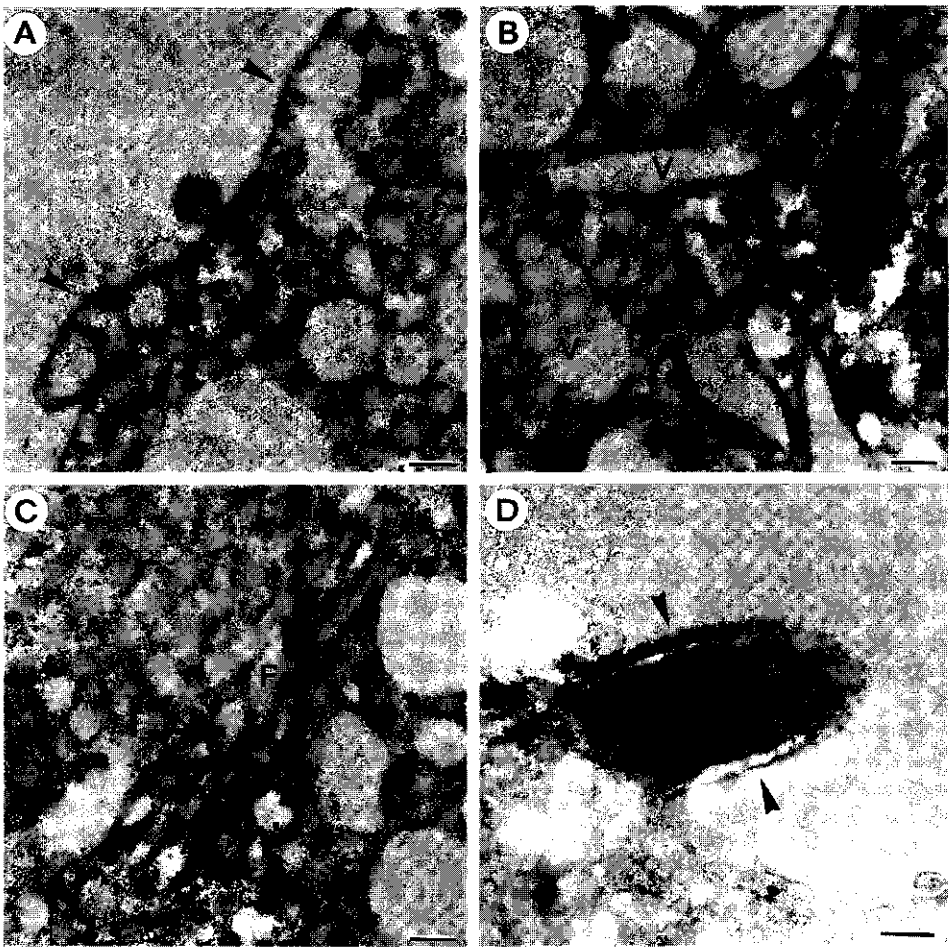


**Figure 7.2.** Immunogold localisation of $NS_M$ in *T. ni* **(A,B,C)** and *S. frugiperda* **(D)** insect cells at 48 hours p.i. **(A)** $NS_M$ specifically localised to the plasmamembrane, **(B)** membranes of cytoplasmic vesicles but also to **(C)** fibrillar aggregates in the cytoplasm. **(D)** Cryo-section of a tubule-like structure. Immunogoldlabelling shows the presence of $NS_M$ in this structure emerging form 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.

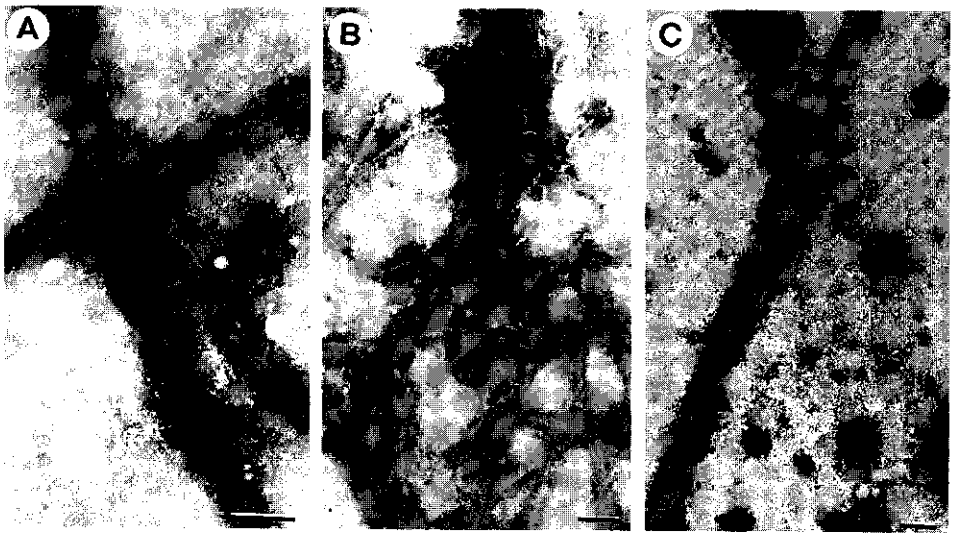


**Figure 7.3.** Electron micrographs of negative stained *T. ni* cells infected with the recombinant AcNPV/$NS_M$ at 48 hours p.i. **(A)** Tubule-like structures **(B,C)** specifically labelled with anti-$NS_M$ serum conjugated with protein A-gold (10 nm). Scale bars represent 200 nm **(A,B)** and 100 nm **(C)**, respectively.

## 7.5. DISCUSSION

The results of this study show that the TSWV $NS_M$ protein is able to form tubular structures, similar to those observed in plant cells, at the surface of insect cells (*S. frugiperda* and *T. ni*) but not of mammalian (BHK) cells. In *S. frugiperda* and *T. ni* insect cells, the $NS_M$ protein was specifically localised to the plasmamembrane, to membranes of cytoplasmic vesicles, to fibrillar aggregates in the cytoplasm and to tubule-like structures emerging from the cell surface. The similarity in behaviour of $NS_M$ in both plant and insect cells suggests that $NS_M$ intracellular targeting and assembly into tubules does not require plant specific components, unless such components are of a very conserved nature (conserved among plant and insect cells). This is in line with similar observations of tubule formation by the MPs of cowpea mosaic virus (CPMV) and cauliflower mosaic virus (CaMV) in insect cells (Kasteel *et al.*, 1996). Possible candidates for such conserved proteins would be those involved in macromolecular transport (e.g. cytoskeletal elements) and proteins in the plasmamembrane. The latter are thought to be essential for anchoring of the MP prior to assembly of the outward directed tubule. However, no such proteins have been identified up till now.

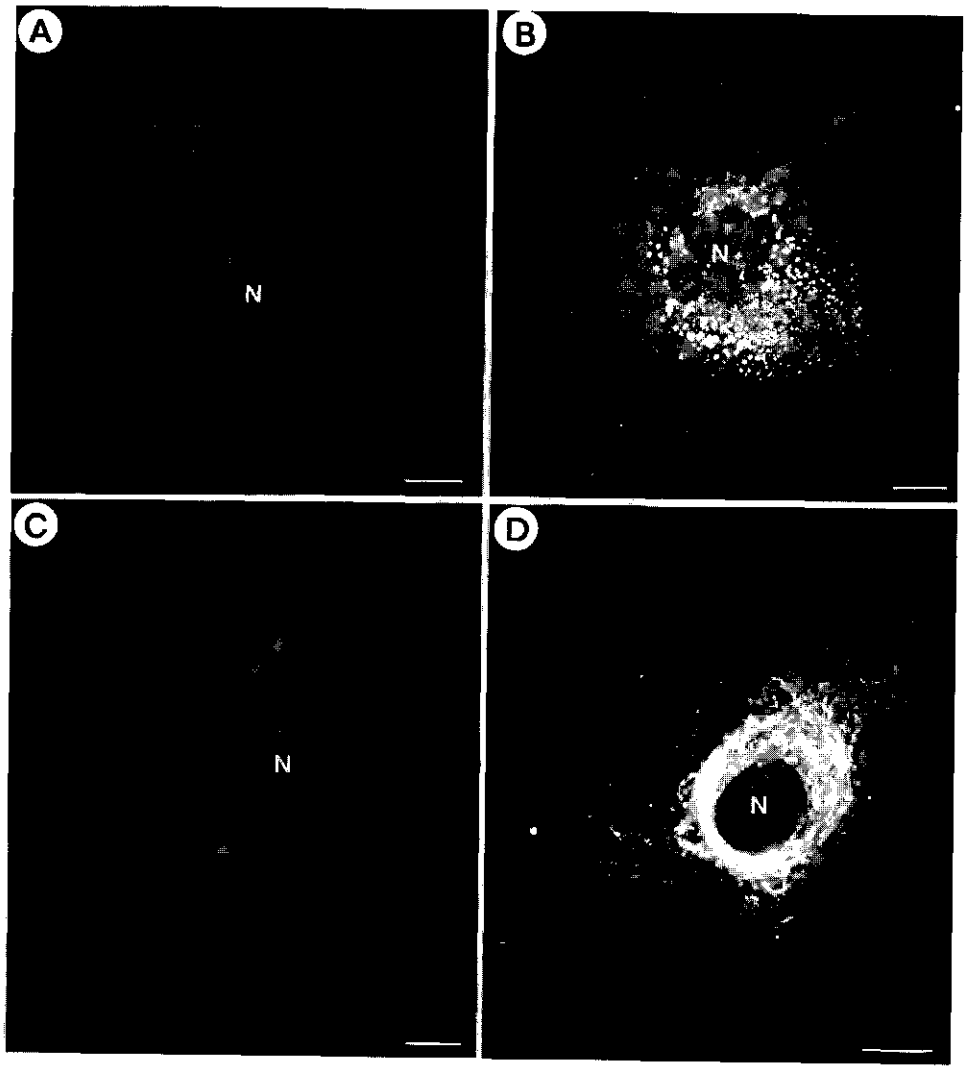


**Figure 7.4.** Immunofluorescent images of $NS_M$ in BHK21 cells at 6 **(A)** and 22 hours **(B,C,D)** post transfection. **(A, B)** The $NS_M$ protein is present in the cytoplasm as aggregates and **(C,D)** fibrous-like structures. Scale bars represent 5 μm **(B)**, 10 μm **(A,D)**, and 15 μm **(C)**. *N: nucleus.*

When $NS_M$ is expressed in mammalian BHK21 cells, the intracellular location is similar to that in insect cells. In BHK cells, $NS_M$ also localises to membranes of cytoplasmic vesicles and to fibrillar aggregates. However, no targeting of the protein to the plasmamembrane and no tubular structures were found extending from the cell surface. It remains speculative why $NS_M$ expression in BHK cells does not lead to the formation of tubules, despite the

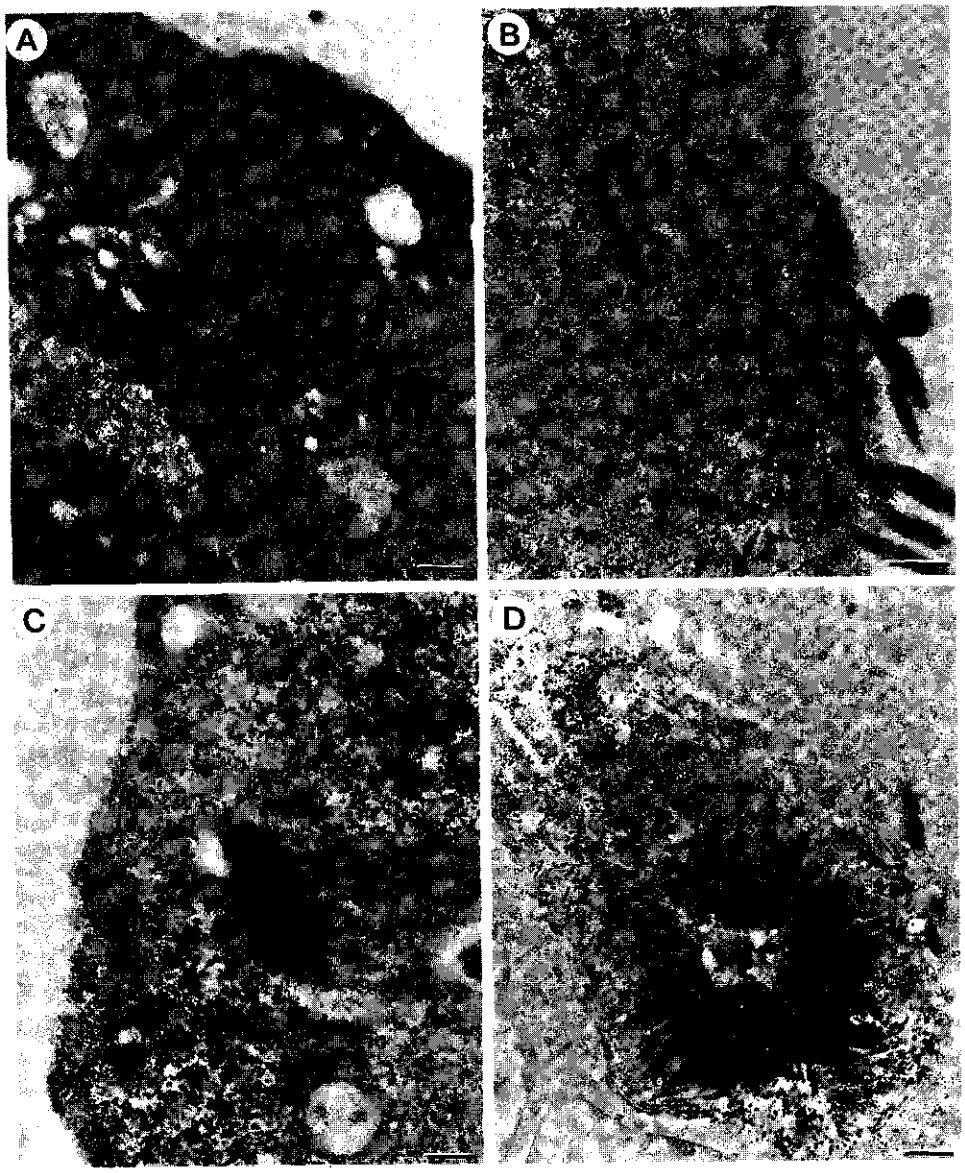


**Figure 7.5.** Electron micrographs of $NS_M$-expressed BHK21 cells immunogold labelled with anti-$NS_M$ serum conjugated with 10 nm protein-A gold. **(A,B)** At 6 and 22 hours p.t. $NS_M$ is specifically associated with membranous vesicles in the cytoplasm, while at 22 hours **(C,D)** $NS_M$ is also tightly associated with fibre-like structures and dense fibrillar aggregates in the cytoplasm. Scale bars represent 300 nm **(A,B)**, 200 nm **(C)** and 400 nm **(D)**.

significant level of the $NS_M$ protein in BHK cells. One possibility could be that $NS_M$ fails to target and anchor at the plasmamembrane of BHK cells, due to the absence of the required host factors. However, also the different experimental conditions of the insect and mammalian cell expression systems, which may reflect on the difference in recording phenotype, have to be taken into account.

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# 8

# Expression of the movement protein of tomato spotted wilt virus in its insect vector *Frankliniella occidentalis*.

## 8.1. SUMMARY

The $NS_M$ protein of tomato spotted wilt virus (TSWV) functions as the viral movement protein to achieve systemic infection in plants. As the virus also replicates in its thrips vectors, it was of interest to study the expression and intercellular manifestation of this protein in infected thrips tissue. Immunoblot analyses of all developmental stages of viruliferous thrips revealed that $NS_M$ was present in detectable amounts only in the L2 and the adult stages. Immuno-electron microscopical analysis of infected tissues in individuals from these two developmental stages showed the presence of $NS_M$ only in small aggregates in the midgut epithelium of L2-larvae and in electron-dense inclusion bodies in the salivary glands and midgut muscle cells of adult thrips. No further clues were obtained with respect to a possible function of this protein in the TSWV infection cycle in the thrips.

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## 8.2. INTRODUCTION

In the previous chapters it was convincingly demonstrated that $NS_M$ represents the tospoviral MP involved in cell-to-cell movement of TSWV in plant hosts (Kormelink *et al.*, 1994; Storms *et al.*, 1995, 1998). As shown for the MPs of other plant viruses (Kasteel *et al.*, 1996), $NS_M$ is also able to form tubules when expressed in insect cells. This indicates that host components, if at all involved in tubule formation, should be conserved in plant and animal cells. However, unlike the other plant viruses TSWV also replicates in its thrips vector (Wijkamp *et al.*, 1993; Ullman *et al.*, 1993). Therefore, a function of $NS_M$ in the infection of insect tissue can not be excluded. To investigate this, we have studied the expression and location of the $NS_M$ protein in all developmental stages of TSWV-infected thrips by Western immunoblot ECL analysis and immuno-electron microscopy. It has been reported that $NS_M$ is expressed in midgut epithelial cells of larvae where it is present in amorphous, electron dense areas (Ullman *et al.*, 1995a). Here we show that $NS_M$ is indeed present in low amounts in midgut epithelial cells of larvae but also in salivary glands and midgut muscle cells of adult thrips. Moreover, the intracellular behaviour of $NS_M$ differs significantly from that in single insect cells.

## 8.3. MATERIALS AND METHODS

### 8.3.1. Virus isolate and thrips

First instar larvae of *F. occidentalis,* up to 4 hours old, were allowed to acquire TSWV isolate BR-01 from infected *Datura stramonium* leaves for a period of 24 hours. Control larvae were placed on healthy *D. stramonium* leaf tissue for the same period. Larvae were then collected at 1 day (L1 larvae) and at 3 and 5 days (L2 larvae) after the start of acquisition. Subsequently, samples were also collected from later developmental stages i.e. from pre-pupal, pupal and one- and six-day old adult thrips, respectively. One sample consisted of adult viruliferous thrips, with a sex ratio of 1:1, of which infection with TSWV was confirmed for each individual with a local lesion test on *Petunia hybridum* leaf disks as described by Wijkamp and Peters (1993). Each sample consisted of 65 individuals, of which 50 were homogenised and analysed for presence of viral proteins by Western ECL immunoblot analyses (Figure 8.1. paragraph 8.4.1.) and 15 were fixed for microscopical examination (paragraph 8.4.2.).

### 8.3.2. Isolation of bacteria

Isolation of bacteria from larval and adult thrips occurred as described by De Vries *et al.*, 1995. After external sterilisation with 70% ethanol and 5% hypochlorite for 60 seconds, the thrips were rinsed in distilled water, transferred to 50µl 10mM Tris and 1mM EDTA (pH 7.6) and macerated. The suspension was incubated on Luria Broth medium for 24 hours at 25°C and subsequently used for Western immunoblot analyses.

### 8.3.3. Antisera

Polyclonal antiserum raised against the TSWV nucleoprotein of isolate BR-01 was used through all experiments (De Avila *et al.*, 1992). Polyclonal antiserum against the $NS_M$ protein was produced as by Kormelink *et al.*, 1994. The $NS_M$ serum was cross-absorbed with extracts from larval and adult stages of *Frankliniella occidentalis*, *Thrips tabaci* and *Spodoptera exigua* and partially purified by ammonium acetate immunoprecipitation as essentially described by Nagata *et al.*, 1997.

### 8.3.4. Western immunoblot ECL analyses of TSWV proteins in thrips

For each developmental stage, samples of 50 randomly selected *F. occidentalis* species were probed by homogenising in 40 µl PBS containing 0.05% Tween-20. An equal volume of 2x protein loading buffer (10mM Tris-HCl, pH 6.8; 2% (w/v) SDS; 10% (v/v) glycerol; 0.001% (w/v) bromophenolblue) and 8 µl ß-mercaptoethanol (4% w/v) was added and 15µl of the total volume (the equivalent of 9 individual thrips) was applied on a 10% SDS-polyacrylamide gel. After SDS-PAGE, the proteins were transferred to Immobilon membranes (Millipore) by semi-dry blotting using a semi-dry transfer cell (Trans-blot SD, Biorad). The membranes were subsequently blocked overnight in 3% (w/v) milkpowder (ELK) and 0.05% nonidet NP40 in PBS. After several washing steps in 10 fold diluted blocking buffer, the membranes were incubated in the same buffer containing 1µg/ml cross-absorbed $NS_M$ antiserum and/or 1µg/ml antiserum against the BR-01 nucleoprotein for 1 hour at room temperature. After washing, the blots were treated with blocking buffer containing 1:3000 diluted horseradish peroxidase linked secondary antibodies for light emitting chemiluminescent ECL detection (Amersham Life Science Inc.). After rinsing the blot in PBS containing 0.05% Tween-20 for 35 minutes, it was used for exposure of X-Omat AR autoradiographic paper.

### 8.3.5. Immunogold detection of the $NS_M$ protein in thrips

Developmental stages which express the $NS_M$ protein as determined by Western immunoblot analyses and virus-free insects were probed for immunogold labelling at the level of electron microscopy. Herefore, thrips was immersed in 2% (w/v) paraformaldehyde and 3% (w/v) glutaraldehyde in phosphate citrate buffer (0.1 M $NA_2HPO_4.2H_2O$ 9.7 mM citric acid, pH 7.2, 1.5 mM $CaCl_2$). In order to optimise the infiltration of the fixative, a small piece of the posterior part of the abdomen as well as the antennae and legs were carefully removed by microsurgery. The fixative was infiltrated for 2 hours under vacuum conditions (7000 Pa) at room temperature, subsequently postfixed in a water cooled microwave for 1.5 hours at an outstream temperature of the water of 25°C and additionally fixed for another 48 hours under atmospheric conditions at 4°C. The tissue was then dehydrated in increasing ethanol concentrations, embedded in London Resin Gold and polymerised under UV-light conditions (Van Lent *et al.*, 1990; Wijkamp *et al.*, 1993). After sectioning on a Reichert Ultracut S ultramicrotome, the tissue was prepared for immunocytochemistry and examined with a Philips CM12 transmission electron microscope.

## 8.4. RESULTS

### 8.4.1. Immunoblot detection of $NS_M$ in TSWV-infected *F. occidentalis*.

Blots were first treated with antiserum against the TSWV N protein to confirm infection of the thrips. The N protein appeared to be present in all TSWV-infected developmental stages (Figure 8.1.). To detect the $NS_M$ protein, the same blots were also treated with anti-$NS_M$ serum. Two additional protein bands appeared in these blots. One band corresponding to a protein of approximately 36 kDa, which was also present in non-infected control, samples, excluding a viral origin of this protein. The second, slightly faster migrating and less prominent protein species (approx. 34 kDa) was detected only in samples of infected L2 and adult stages (Figure 8.1.). It was most abundant in the sample consisting of 100% infected adult thrips (Figure 8.1. lane 15) and therefore represents the $NS_M$ protein.

The non-viral 36 kDa protein, that apparently cross-reacted with the anti-$NS_M$ serum, varied in quantity in different the samples and was predominantly present in the L2, pupa and adult stages. The origin of this cross-reacting 36 kDa protein was further investigated. Control incubations, omitting the primary $NS_M$ antibody or using anti-$NS_M$ serum cross absorbed with extracts from uninfected larvae and adult thrips (Nagata *et al.*, 1997), excluded an endogenous peroxidase or a cross-reactive insect protein. Incubation of

similar blots with another antiserum raised against an *E. coli* derived protein revealed the same 36 kDa protein, but failed to detect the $NS_M$ protein. This result would point to a bacterial origin of the cross-reactive protein. The presence of symbiotic bacteria in the hindgut and Malpigian tubules of thrips has been previously reported (Ullman *et al.*, 1989; Dalai *et al.*, 1991), and recently these bacteria, designated as *Enterobacter agglomerans*, have been isolated from the gut of *F. occidentalis* (De Vries *et al.*, 1995). Quantitative studies revealed the presence of bacteria in L2 larvae and, to a lesser extend, in adult stages and their virtual absence in pupal and L1 larval stages (De Vries *et al.*, in preparation).

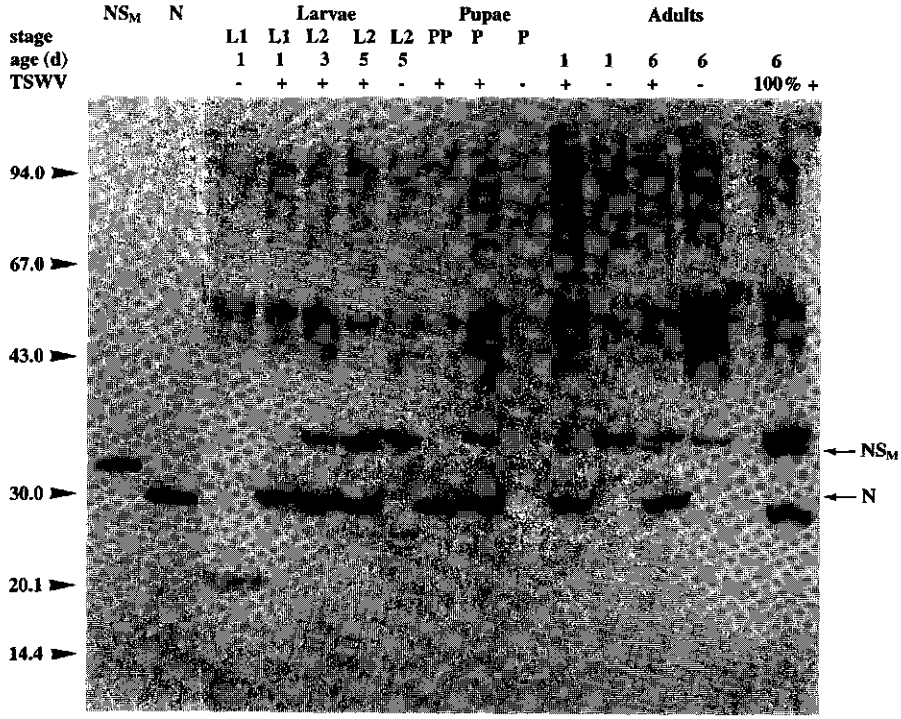


**Figure 8.1.** ECL immunoblot of protein extracts from viruliferous larvae, pupae or adults (animals per sample) taken at subsequent time points after an AAP of 24 hours (+). Larval stages were harvested immediately at the end of the AAP (1 day; L1 larvae) and at 3 and 5 days (L2 larvae) respectively. Virus free thrips of every developmental stage were included as control (-). A sample of 9 viruliferous thrips at 6 days was also tested (100% +). As controls, baculovirus expressed $NS_M$ and N protein was included (lanes: $NS_M$ and N). All samples were applied on a 10% SDS polyacrylamidegel. After electrophoresis, the proteins were transferred to an Immobilon membrane and the blot treated with a mixture of 1 µg/ml anti-N and anti-$NS_M$ serum each. The blot was further processed with horseradish peroxidase linked secondary antibodies for light emitting chemiluminescent ECL detection (Amersham Life Sciences Inc) and exposed to X-Omat AR autoradiographic paper.

Hence, the appearance of the 36 kDa protein in the different developmental stages (Figure 8.1.) coincides with the presence of the bacterial symbionts. Indeed, immunoblots with extracts of cultured bacterial symbionts (De Vries *et al.*, 1995) treated with anti-$NS_M$ serum, show indeed the presence of high quantities of a similar 36 kDa protein in extracts of these bacterial symbionts (Figure 8.2.).

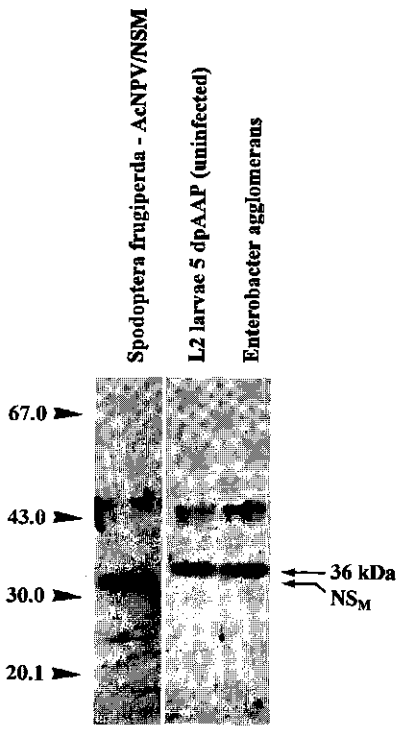


**Figure 8.2.** ECL immunoblot comparison of extracts from symbionts (*Enterobacter agglomerans*) isolated from *F. occidentalis*, uninfected L2 larvae and AcNPV/$NS_M$ infected *S. frugiperda* cells. Isolation occurred after sterilization of thrips in 70% ethanol and 5% hypochlorite, maceration in 50 µl 10mM Tris and 1 mM EDTA (pH 7.6) and subsequent incubation in Luria Broth medium for 24 hours at 25°C. Samples were applied on a 10% SDS polyacrylamidegel and transferred to Immobilon membrane which was after treatment of the blot with 1 µg/ml anti-$NS_M$ serum further processed for ECL detection.

### 8.4.2. Localisation of $NS_M$ in TSWV-infected *F. occidentalis*

During TSWV infection of thrips, the $NS_M$ protein is apparently present in low quantities, only detectable in the L2 and adult stages. To localise $NS_M$ in tissues of these stages, specimen were prepared for *in situ* immunolocalisation as described by Wijkamp *et al.*

(1993). Scrutinous analyses of 5 days-old infected L2 larvae only revealed the presence of large nucleocapsid aggregates in epithelium cells of the midgut. These dense aggregates, which were absent from the midgut of non-infected larvae, specifically labelled with anti-N serum (Figure 8.3.A) as previously reported (Ullman *et al.*, 1993). In these cells the $NS_M$ protein was only occasionally observed in small aggregates in the cytoplasm (Figure 8.3.B), in the vicinity of so called residual bodies. Such residual bodies are part of an autophagic pathway and TSWV G1 and G2 protein have been localised to them previously by Ullman *et al.* (1995a,b). In other tissues of the infected L2 larvae no significant amounts of N or $NS_M$ protein were detected. In contrast, six day old TSWV-positive adult thrips contained large nucleocapsid aggregates in the salivary glands (Figure 8.4.A) as reported before (Wijkamp *et al.*, 1993; Ullman *et al.*, 1993). Here, $NS_M$ was occasionally detected in amorphous electron dense inclusions encircled by endoplasmatic reticulum (Figure 8.4.B), which were never observed in the glands of non-infected individuals. Also in smooth muscle cells surrounding the midgut epithelium, nucleocapsid aggregates were observed (Figure 8.4.C; Wijkamp *et al.*, 1993; Ullman *et al.*, 1993) and $NS_M$ was again occasionally localised in electron-dense inclusions (Figure 8.4.D) which were absent in non-infected individuals. No infection of midgut epithelium was encountered in these adult thrips.

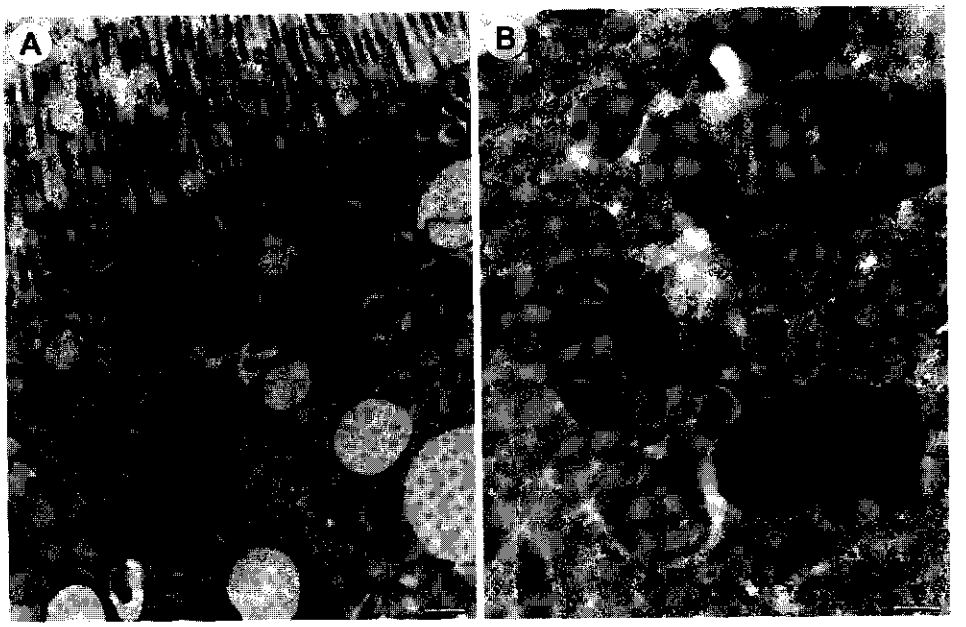


**Figure 8.3.** Immunogold localisation of **(A)** the nucleoprotein (N) and **(B)** the $NS_M$ protein in the cytoplasm of midgut epithelium cells in TSWV-infected L2 larvae at 5 days after start of the acquisition. ME: midgut epithelium; MV: microvilli; R: residual bodies. Scale bar represents 500 nm **(A)** and 200 nm **(B)** respectively.

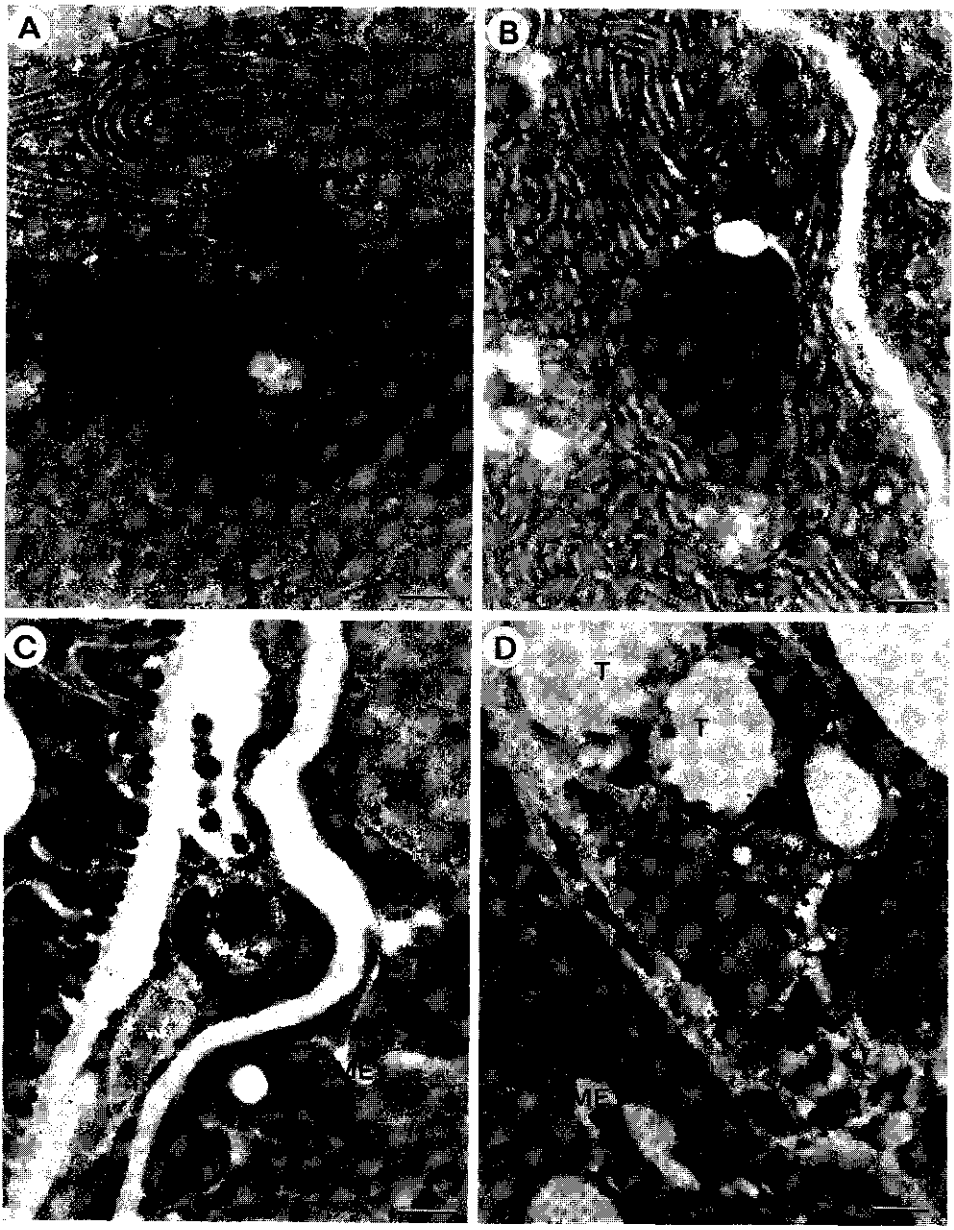


**Figure 8.4.** Immunogold detection of the N and $NS_M$ protein in TSWV-infected 6 days old adult thrips. **(A)** The nucleoprotein is present as large nucleocapsid aggregates in the cytoplasm of salivary gland cells while **(B)** the $NS_M$ protein associates with amorphous electron dense bodies in the cytoplasm. In the midgut region of adults, **(C)** the N-protein appears as electron dense aggregates in smooth muscle tissue of the gut while **(D)** the $NS_M$ protein was only found in association with electron dense inclusions. ME: midgut epithelium; T: trachea. Scale bars represent 200 nm **(A,B,C)** and 400 nm **(D)** respectively.

## 8.5. DISCUSSION

Our results indicate that the $NS_M$ protein of TSWV is expressed at detectable levels only in L2 and adult developmental stages of the Western flower thrips *F. occidentalis*. Immuno-electron microscopical analysis of individuals in these developmental stages revealed infection only of the midgut epithelium (L2 larvae) or salivary glands and midgut muscle cells (adults). In these infected cells the $NS_M$ protein was associated to small residual bodies or electron dense inclusions thought to be part of an autophagic pathway for degradation or recycling of proteins (Fawcett, 1981; Ullman *et al.*, 1995b). No further intracellular manifestation of $NS_M$ was found that could give a clue of a possible function in the insect infection cycle. As all other, non-phytopathogenic members of the Bunyaviridae lack this gene product, it is tempting to assume that the tospoviral $NS_M$ exclusively functions as cell-to-cell movement protein during infection of plant tissue, in which it aggregates into well recognisable plasmodesmata-associated tubules (Kormelink *et al.*, 1994; Storms *et al.*, 1995). Despite the observation that $NS_M$ is able to form tubules in insect cells (*S. frugiperda* and *T. ni*) upon heterologous expression from a baculovirus vector (Storms *et al.*, 1995), the current studies on all developmental stages and infected tissues indicate that this does not occur in thrips vector tissues. Moreover, $NS_M$ does not associate to viral nucleocapsids and neither to gap junctions, the functional equivalent of plasmodesmata. Although an additional function of the expressed $NS_M$ in thrips tissue can not be ruled out, our studies indicate that such function would then be unrelated to its activity as tubule-forming movement protein in plant tissues.

**Acknowledgements**

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# 9

## Summary and Concluding Remarks

During the past ten years the genome organisation of tomato spotted wilt virus (TSWV) has been intensively studied in our laboratory. Complete genome sequence data revealed that this enveloped plant virus belongs to the Bunyaviridae, a virus family further restricted to animals.

Hence, TSWV is a splendid model to investigate which viral encoded factors are needed for a virus to successfully infect a plant. Comparison of the genome of TSWV with those of the animal-infecting members of the Bunyaviridae reveals the presence of an extra cistron in the TSWV genome. This cistron encodes a non-structural protein of 33.6 kDa, denoted $NS_M$. Although some animal infecting Bunyaviridae also specify a protein referred to as $NS_M$, this polypeptide does not represent a separate gene product. Hence, the extra $NS_M$ gene may very well represent the key function for genetic adaptation of Bunyaviruses to plant hosts. For a successful infection of plants, viruses have to pass the plant specific cell wall barrier through plasmodesmata. As the size of most viruses, and even their genomes, exceeds the physical space provided by plasmodesmata for intercellular transport of macromolecules (the size exclusion limit or SEL), cell-cell movement requires a structural modification of the plasmodesmata. For a number of plant viruses it has been shown that they encode one or more movement proteins (MPs) which achieve viral cell-cell movement, among others by modification of the plasmodesmata, most likely in co-operation with host factors and/or other viral proteins.

At the onset of this PhD research, the working hypothesis was that $NS_M$ could very well represent the MP of TSWV. This was also based on the presence of a conserved sequence in the $NS_M$ protein, the so-called "D-motif", characteristic for several other plant viral MPs.

To obtain experimental data for the role of $NS_M$ in the TSWV infection cycle, first the expression kinetics and intracellular behaviour of the protein were studied in systemically infected *Nicotiana rustica* leaf tissue. The $NS_M$ gene was cloned and expressed in *E. coli*, the protein was purified and a specific antiserum made. Time course analyses of systemically infected *N. rustica* leaf tissue, using this anti-$NS_M$ serum, revealed an early and transient presence of the protein in infected cells, a manifestation that is unique for $NS_M$ and not found for other viral proteins of TSWV (Chapter 2). The early and temporary appearance of $NS_M$ suggested that the protein has an early function in the infection cycle in concert with the idea that it is involved in cell-cell movement. During infection of a cell, $NS_M$ was first localised to newly formed viral nucleocapsids in the cytoplasm and plasmodesmata. Moreover, $NS_M$ was found to assemble into tubular

structures that penetrate the plasmodesmata in a unidirectional way. These tubules were also formed upon expression of the cloned $NS_M$ gene in protoplasts (Chapter 3). The formation of similar tubular structures has been shown to be an essential step during cell-to-cell movement of a number of RNA (e.g. como- and nepoviruses) and DNA (caulimoviruses) plant viruses. For these latter viruses, it was shown that mature virions are transported from cell-to-cell through MP-induced tubular structures. Investigations on the contents of the TSWV tubules, formed on synchronously infected protoplasts, revealed the presence of only the nucleoprotein inside the tubular structures (Chapter 4). Based on this, it is tempting to assume that TSWV is translocated into the adjacent non-infected cell as non-enveloped, viral nucleocapsids.

An important characteristic of several plant virus MPs is that they evoke a modification of the macromolecular diffusion limits (SEL) of plasmodesmata. To investigate whether $NS_M$ has a similar function, the $NS_M$ gene was transgenically expressed in *Nicotiana tabacum* SR1 plants (Chapter 5). The resulting transgenic plants were examined for $NS_M$ expression, subcellular localisation of this protein, and changes in the diffusion properties of the plasmodesmata. The $NS_M$ expression levels obtained were invariably low. However, the $NS_M$ protein could be localised to over 80% of the plasmodesmata in various transgenic tissues, demonstrating its specific targeting to and association with these organelles. Probably as a result of $NS_M$ accumulation at the plasmodesmata, the plants showed severe aberrations in growth and development (Chapter 5). Possible alterations in plasmodesmal functioning were further analysed by microinjection of fluorescent probes of various sizes into mesophyll cells of the $NS_M$ transgenic plants. Two different methods of microinjection were used and compared, one by which the influx of probes was achieved by a pressure pulse and a second, by diffusion (iontophoresis). Pressure injection studies on $NS_M$ transgenic plants with fluorescent probes of different molecular mass showed an increase in plasmodesmal SEL similar to that of control transgenic plants expressing the TMV MP. As, in this respect, $NS_M$ modifies the plasmodesmal function in a similar manner as the TMV MP, $NS_M$ was concluded to be the tospoviral MP. Strikingly, when iontophoresis was performed on $NS_M$- or TMV-MP transgenic plants, the plasmodesmata showed a decrease rather than an increase in SEL. In control (non-transgenic) plants, the outcome of pressure injection and iontophoresis was identical. This strongly suggested that accumulation of viral MP made the cells respond differently to the microinjection methods used. A possible interpretation for these remarkable differences is that TSWV and TMV MP, inhibits or partly blocks, the communication pathway for macromolecular trafficking, as recorded by the less invasive iontophoresis method, which also explains the disturbed morphology and physiology of the MP transgenic plants. The protein barrier at the plasmodesmata may be overcome by a

pressure pulse, thereby revealing an increase of SEL induced by the plant viral MP (Chapter 6).

As TSWV also replicates in its thrips vectors, it was of interest to explore whether $NS_M$ also had a function during the infection cycle in the insect. The expression kinetics and localisation of the $NS_M$ protein was studied in all developmental stages of the major viral vector, the thrips *Frankliniella occidentalis* (Chapter 8). Besides the *in situ* analyses of $NS_M$ in the vector itself, the behaviour of the protein was also investigated in heterologous *Spodoptera frugiperda* and *Trichoplusia ni* insect cell cultures and in baby hamster kidney (BHK21) cells, a mammalian cell system unrelated to the natural hosts of TSWV (plants and insects)(Chapter 7). Upon expression of $NS_M$ in cultured insect cells, the protein specifically targeted to the cell periphery and formed tubular structures at the cell surface in a similar fashion as found in plant protoplasts. However, in mammalian cells no plasmamembrane targeting or tubular structures were found. The results obtained for insect cells indicate that the $NS_M$ protein has the potential to form tubular structures in the absence of any plant specific component.

In the vector *F. occidentalis*, $NS_M$ was present at only low levels in mostly midgut epithelium cells of L2 larvae and in salivary gland cells and midgut muscle cells of adult thrips. Characteristic structures or associations as found in infected plants (e.g. tubule formation, association with viral nucleocapsids) could not be discerned in any of the developmental stages of the thrips. Although this does not exclude a function for $NS_M$ in the TSWV vector, this function would then be unrelated to its activity as MP.

In summary, the investigations described in this thesis all demonstrate that $NS_M$ represents the MP of TSWV, and that it mediates the cell-cell movement of presumably non-enveloped viral nucleocapsids through transiently produced tubular structures that penetrate plasmodesmata. As such, the extra $NS_M$ gene in the genome of TSWV indeed encodes the key protein required for pathogenicity of Bunyaviridae towards plants.

# Samenvatting

Het genoom van het tomatenbronsvlekkenvirus (TSWV) is het afgelopen decennium intensief bestudeerd en de genetische organisatie en de nucleotidenvolgorde ervan zijn opgehelderd. Uit deze informatie kwam ondermeer naar voren dat dit plant-infecterende virus behoort tot de familie der Bunyaviridae, een familie van dier-infecterende virussen. Bij vergelijking van het genoom van TSWV met dat van andere Bunyavirussen blijkt dat TSWV een extra gen bevat dat codeert voor een eiwit van 33,6 kDa, aangeduid als het $NS_M$-eiwit. Acquisitie van dit $NS_M$-gen tijdens de evolutie van het virus zou een belangrijke genetische aanpassing van bunyavirussen geweest kunnen zijn, verantwoordelijk voor uitbreiding van het gastheerbereik van dieren naar planten. Gezien het unieke vermogen van TSWV om zowel planten als dieren (tripsen) te infecteren, is dit virus uitermate geschikt voor identificatie en analyse van de specifieke virale factoren die het virus pathogeen voor planten maakt.

Plantenweefsels onderscheiden zich van dierlijke weefsels o.a. doordat de cellen omgeven zijn door een rigide celwand. Verspreiding van de infectie vanuit de initieel geïnfecteerde cel naar omliggende cellen en andere weefsels vereist derhalve dat het virus in staat moet zijn om deze celwand te passeren. Inmiddels is voor een aantal plantenvirussen vastgesteld dat transport van virus van cel naar cel via de plasmodesmata geschied. Plasmodesmata zijn complexe kanaaltjes in de celwand die normaal zorgen voor de intercellulaire communicatie door het reguleren van het transport van macromoleculen. In het algemeen wordt aangenomen dat moleculen die groter zijn dan 1000 Dalton (Da) de plasmodesma niet zondermeer kunnen passeren. De doorlaatbaarheid (in het Engels "size exclusion limit" of SEL) van plasmodesmata is dus grofweg 1 kDa. Virusdeeltjes, en zelfs hun vrije genoomdelen, zijn echter vele malen groter dan de SEL van plasmodesmata, en deze dienen dus een aanmerkelijke modificatie te ondergaan om viraal transport mogelijk te maken. Voor een toenemend aantal plantenvirussen is vastgesteld dat zij de genetische informatie bezitten voor één of meerdere zgn. transporteiwitten. Deze eiwitten bewerkstelligen het transport van virussen ondermeer door plasmodesmata zodanig te modificeren dat grotere macromoleculen kunnen passeren.

De werkhypothese aan het begin van het hier beschreven promotie-onderzoek was dat het $NS_M$-eiwit het transporteiwit van TSWV is. Dit op basis van het feit dat het $NS_M$-gen niet voorkomt in dierinfecterende Bunyavirussen, maar ook het voorkomen van een "D-motief" dat kenmerkend is voor diverse andere plantenvirale transporteiwitten. Derhalve is de rol van het $NS_M$-eiwit in de infectiecyclus van TSWV gedetailleerd bestudeerd, met name m.b.t. de eventuele betrokkenheid bij het intercellulaire transport.

Allereerst is de expressie en het intracellulaire gedrag van het eiwit onderzocht in systemisch geïnfecteerde bladeren van *Nicotiana rustica* (tabak). Hiertoe werd eerst een specifiek antiserum gemaakt door het $NS_M$-gen te kloneren en tot expressie te brengen in *Escherichia coli*. Door analyse van systemisch geïnfecteerde *N. rustica* bladeren als functie van de infectietijd, gebruik makend van dit antiserum, bleek dat het eiwit reeds vroeg in de infectie gemaakt werd en slechts gedurende een korte tijd detecteerbaar aanwezig bleef (Hoofdstuk 2). De vroege en transiënte aanwezigheid van het $NS_M$-eiwit veronderstelt dat dit eiwit alleen vroeg in infectie een functie vervult. In de geïnfecteerde cel werd $NS_M$ allereerst aangetroffen in associatie met virale nucleocapsiden in het cytoplasma en met plasmodesmata. Voorts werden tubulaire structuren aangetroffen in plasmodesmata. Deze tubuli bleken gevormd uit het $NS_M$-eiwit en werden zelfs, onafhankelijk van een volledige virusinfectie, gevormd indien het gekloneerde $NS_M$-gen in plantencellen (protoplasten) tot expressie gebracht wordt (Hoofdstuk 3). Van soortgelijke tubulaire structuren is bij een aantal RNA (bijv. como-, nepovirussen) en DNA (caulimovirussen) virussen vastgesteld dat ze een essentiële rol spelen bij systemisch transport van deze virussen. Voor deze virussen is vastgesteld dat via tubulaire structuren complete deeltjes worden getransporteerd door de plasmodesmata. Gezien de afmetingen van volwassen TSWV-deeltjes (80–100 nm) en de diameter van de in plantencellen en protoplasten gevormde buisvormige structuren (40-50 nm) is het onwaarschijnlijk dat ook TSWV als compleet virusdeeltje van cel naar cel wordt getransporteerd. Bij elektronenmicroscopische analyse van de tubulaire structuren werden bovendien nog nooit virusdeeltjes in de buizen waargenomen. Wel werd, middels immunogoudlabeling, aangetoond dat het nucleocapside-eiwit (N-eiwit) als enig structureel viruseiwit in de buis aantoonbaar is (Hoofdstuk 4), hetgeen het zeer aannemelijk maakt dat TSWV getransporteerd wordt in de vorm van nucleocapsiden.

Een belangrijk kenmerk van diverse plantenvirale transporteiwitten is hun vermogen om de SEL van de plasmodesma ten behoeve van het transport van macromoleculen te veranderen. Om vast te stellen of het $NS_M$-eiwit wellicht een soortgelijke functie vervult, werden *Nicotiana tabacum* SR1 planten getransformeerd met het $NS_M$-gen (Hoofdstuk 5). De transgene planten werden onderzocht op expressie van het $NS_M$-eiwit, de subcellulaire locatie van het eiwit en op veranderingen in de doorlaatbaarheid van plasmodesmata. Ofschoon het niveau van $NS_M$-expressie in de verkregen transgene planten laag was, werd het eiwit d.m.v. immuno-elektronenmicroscopie aangetoond in meer dan 80% van de plasmodesmata in diverse weefsels. De transgene planten waren sterk gestoord in groei en ontwikkeling, wat mogelijk een gevolg is van ophoping van het $NS_M$-eiwit in de plasmodesmata resulterend in verstoorde intercellulaire communicatie in de plant. Veranderingen in de SEL van plasmodesmata werden onderzocht d.m.v. microinjectie van, in grootte variërende,

fluorescente markers. Deze probes werden geïnjecteerd in mesophylcellen van transgene en niet-transgene planten. Tevens werden bij dit onderzoek twee verschillende methoden van microinjectie gebruikt en met elkaar vergeleken. Eén methode was gebaseerd op het inbrengen van de fluorescente probes door middel van een drukpuls, (drukinjectie), een techniek die ook door anderen gebruikt is bij het onderzoeken van plasmodesmale doorlaatbaarheid. De tweede methode was gebaseerd op het inbrengen van de probes d.m.v. elektro-diffusie (iontoforese) in het cytoplasma van de cel. Bij deze methode kan d.m.v. het meten van membraanpotentialen worden vastgesteld of de injectienaald in het cytoplasma zit en of er mogelijk een lekkage is tussen de naald en de celmembraan. Met drukinjectie van probes van diverse afmetingen werd aangetoond dat de SEL van plasmodesmata in transgene $NS_M$-planten inderdaad groter was dan die in niet-transgene controle planten. De mate waarin deze vergroting optrad was vergelijkbaar met die in transgene planten waarin het transporteiwit van tabaksmozaïekvirus (TMV) tot expressie is gebracht. Hieruit kan geconcludeerd worden dat $NS_M$ daadwerkelijk het transporteiwit van TSWV is. Indien iontoforese werd gebruikt voor introductie van de markers bleek de doorlaatbaarheid van de plasmodesmata in planten die transgeen het $NS_M$ of TMV transporteiwit tot expressie brengen juist verminderd te zijn. In niet-transgene controleplanten gaven beide injectietechnieken echter hetzelfde resultaat, nl. geen vergroting van de doorlaatbaarheid van plasmodesmata. Een mogelijke verklaring voor dit opmerkelijke verschil tussen drukinjectie en iontophorese is dat het transporteiwit van zowel TSWV als TMV door ophoping aan de plasmodesma deze gedeeltelijk afsluit voor transport van macromoleculen en metabolieten, hetgeen tevens de afwijkende morfologie en ontwikkeling van de transgene planten verklaart. Deze gedeeltelijke blokkade blijft intact bij microinjectie d.m.v. iontoforese, maar wordt wellicht opgeheven door drukinjectie, waarbij dan een toename in de doorlaatbaarheid van de plasmodesmata wordt waargenomen (Hoofdstuk 6).

Omdat TSWV ook in staat is om haar vector (tripsen) te infecteren was het uiteraard interessant om te onderzoeken of het $NS_M$-eiwit ook tijdens infectie van het insect een functie vervult. De expressie en lokalisatie van het $NS_M$-eiwit werd geanalyseerd in alle ontwikkelingsstadia van de voornaamste vector van het virus, de trips *Frankliniella occidentalis* (Hoofdstuk 8). Naast de *in situ* analyse van het infectieproces en met name de functie van $NS_M$ in de geïnfecteerde trips, werd eveneens het gedrag van het eiwit in heterologe celsystemen zoals die van *Spodoptera frugiperda* en *Trichoplusia ni* en in zoogdier (hamster)cellen onderzocht (Hoofdstuk 7). Bij expressie van $NS_M$ in insectencellen werd het eiwit met name gevonden in de periferie van de cel. In deze cellen werden eveneens, geheel overeenkomstig de eerdere waarnemingen in protoplasten, tubulaire structuren gevormd aan het oppervlak van de cel. In hamstercellen werd het eiwit eveneens goed tot expressie gebracht, maar werd geen buisvorming waargenomen. De

locatie van het $NS_M$-eiwit in insectencellen en de vorming van tubulaire structuren in deze cellen tonen het vermogen van $NS_M$ om dergelijke structuren te vormen in afwezigheid van plantspecifieke factoren. Indien er al gastheerfactoren betrokken zijn bij dit proces, zijn deze dus geconserveerd tussen plant en insect.

In de vector *F. occidentalis* werd het $NS_M$-eiwit slechts in geringe hoeveelheden gevonden, hoofdzakelijk in geïnfecteerde cellen van het darmepitheel van L2-larven en in speekselklier- en darmspiercellen van volwassen tripsen. Karakteristieke structuren en specifieke associaties met organellen, zoals gevonden in geïnfecteerd plantenweefsel (o.a. associatie van $NS_M$ met nucleocapsiden en de vorming van tubulaire structuren), werden niet waargenomen. Ofschoon deze waarnemingen een actieve functie van $NS_M$ tijdens de infectie van de vector niet uitsluiten, is het onwaarschijnlijk dat dit eiwit eveneens een transportfunctie in het insect verzorgt.

Op basis van de in dit proefschrift beschreven resultaten kan geconcludeerd worden dat $NS_M$ het transporteiwit van TSWV is, verantwoordelijk voor de modificatie van plasmodesmata en de vorming van tubulaire buizen daarin. Verspreiding van het virus vindt dan waarschijnlijk plaats in de vorm van nucleocapsiden die door deze buizen via plasmodesmata van cel naar cel worden getransporteerd. Derhalve vervult het $NS_M$-gen een sleutelrol bij het totstandkomen van de pathogeniciteit van Bunyavirussen voor planten.

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# Curriculum Vitae

Marc Storms zag als Marcus Maria Hendricus Storms het levenslicht in Heerlen op 18 april 1966. Na de school voor Hoger Algemeen Voortgezet Onderwijs en het Voorbereidend Wetenschappelijk Onderwijs doorlopen te hebben aan het toenmalige Coriovallum College te Heerlen werd in 1986 begonnen met de studie Plantenziektenkunde aan de Landbouwuniversiteit in Wageningen. Binnen de ecologische en epidemiologische oriëntatie van deze studie werden een drietal afstudeervakken gevolgd op respectievelijk het vakgebied van de nematologie, entomologie en virologie. Voor dit laatste vakgebied is gedurende 8 maanden stage gelopen bij de Division of Biological Sciences van de University of California in Davis. Na het behalen van het doctoraaldiploma in 1992 begon hij middels een zelf geïnitieerd projectvoorstel op het Laboratorium voor Virologie van de Landbouwuniversiteit aan zijn promotie-onderzoek waarvan de resultaten staan beschreven in dit proefschrift. Vanaf juli 1998 is hij als applicatiespecialist transmissie elektronenmicroscopie werkzaam bij FEI/Philips Electron Optics B.V. in Eindhoven.