ROLE OF THE ENVELOPE GLYCOPROTEINS IN THE INFECTION CYCLE OF TOMATO SPOTTED WILT VIRUS

Promotor:	Dr R.W. Goldbach Hoogleraar in de Virologie Landbouwuniversiteit Wageningen
Co-promotor:	Dr ir R. Kormelink Universitair docent Laboratorium voor Virologie Landbouwuniversiteit Wageningen

ROLE OF THE ENVELOPE GLYCOPROTEINS IN THE INFECTION CYCLE OF TOMATO SPOTTED WILT VIRUS

Marjolein Kikkert

Proefschrift

ter verkrijging van de graad van doctor

op gezag van de rector magnificus

van de Landbouwuniversiteit Wageningen,

Dr C.M. Karssen,

in het openbaar te verdedigen

op vrijdag 18 juni 1999

des namiddags te vier uur in de Aula.

The work described in this thesis was performed at Wageningen Agricultural University, Laboratory for Virology, Binnenhaven 11, 6709 PD Wageningen, The Netherlands.
Financial support by the Stichting "Landbouw Export Bureau 1916/1918", and the Dutch Scientific Organisation (NWO) STIR-fund is gratefully acknowledged.

Role of the envelope glycoproteins in the infection cycle of tomato spotted wilt virus.
M. Kikkert.
Thesis Agricultural University Wageningen, The Netherlands.
With references – With summary in Dutch
ISBN 90-5808-058-7

STELLINGEN

1. De evolutionaire ontwikkeling van een dier-infecterend virus naar een plant-infecterend virus wordt, behalve door overdracht door insecten, vergemakkelijkt door overeenkomsten in intracellulaire eiwitprocessing- en transportmechanismen in dieren- en plantencellen.
   Dit proefschrift.

2. Het "wrapping" mechanisme, waarbij in één stap virusdeeltjes met een dubbele membraan worden gevormd, is niet voorbehouden aan dier-infecterende virussen met een dubbelstrengs DNA genoom.
   Dit proefschrift

3. Het feit dat twee onderzoeksgroepen, gebruikmakend van gelijksoortige technieken, elk een ander aan TSWV bindend tripseiwit hebben gevonden, betekent ofwel dat er een complex mechanisme is aangeboord, ofwel dat de gebruikte technieken niet geschikt zijn voor dit type onderzoek.
   Dit proefschrift,
   Bandla et al., 1998. Interaction of tomato spotted wilt tospovirus (TSWV) glycoproteins with a thrips midgut protein, a potential cellular receptor for TSWV. Phytopathology **88**:98-104.

4. De ontdekking van retrograad eiwittransport van het endoplasmatisch reticulum naar het cytoplasma via het translocon opent geen nieuwe kanalen, maar wel een scala aan nieuwe onderzoekswegen.
   Wiertz et al., 1996. Sec61-mediated transfer of a membrane protein from the endoplasmic reticulum to the proteasome for destruction. Nature **384**:432-438.
   Wiertz et al., 1996. The human cytomegalo virus US11 gene product dislocates MHC class I heavy chains from the endoplasmic reticulum to the cytosol. Cell **84**:769-779.

5. Ondanks de 'allergische' reactie op genetisch gemanipuleerde voedselproducten kunnen deze producten bijdragen aan het voorkómen van voedselallergieën.

6. Hygiëne is de westerse mens zijn opkomst, maar ook zijn ondergang.

7. Het verkorten van de studieduur en het besluit van sommige universiteiten om de stellingen bij proefschriften af te schaffen benadrukt dat men tegenwoordig een verkeerd beeld heeft van wat een wetenschapper zou moeten kunnen.

8. Het feit dat in veel, vooral prestigieuze, onderzoeksinstellingen vrouwen nog steeds worden achtergesteld, vraagt om het expliciet formuleren van de benodigde eigenschappen en voorwaarden voor het bedrijven van goede wetenschap.

9. Integratie van vakgebieden is de toekomst van de wetenschap.

10. Domme blondjes zijn niet blond.

11. Filosofie heeft geen reden, doch wel rede, nodig.

12. Voor veel Crohnpatiënten vormt de ontlasting juist een belasting.

Stellingen behorende bij het proefschrift:
'Role of the envelope glycoproteins in the infection cycle of tomato spotted wilt virus'
door Marjolein Kikkert.
18 juni 1999,
Wageningen.

Niets is zo moeilijk als zich niet te bedriegen.

Ludwig Wittgenstein, 1938

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

Tomato spotted wilt virus (TSWV), member of the virus family *Bunyaviridae*, is able to infect a large variety of different plant species and causes severe crop-losses worldwide in for instance tomato, lettuce, pepper, and many ornamental crops (Goldbach and Peters, 1996; Goldbach and Peters, 1994). The virus is transmitted by thrips (order *Thripidae*), predominantly by *Frankliniella occidentalis*, the Western flower thrips.

TSWV encodes two glycoproteins, designated G1 and G2, which are present on the surface of the virus particle, embedded in a lipid envelope. The work described in this thesis focussed on the role of these viral glycoproteins during the life cycle of TSWV. The thesis provides a model for the maturation of enveloped TSWV particles in plants, and the involvement of the viral glycoproteins therein, furthermore an investigation of the intracellular trafficking and retention behavior of the glycoproteins, and an analysis of their interaction with the thrips vector.

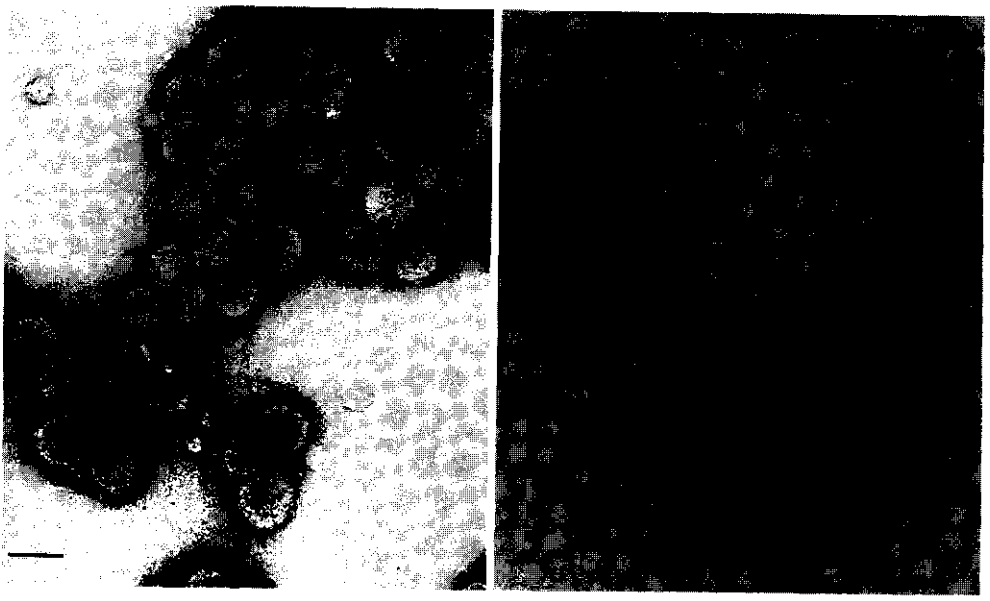
The Bunyaviridae

The family *Bunyaviridae* is congregated of viruses predominantly infecting rodents and other small mammals, primates, birds and ungulates. Most of these viruses are arthropod-borne (arbo-viruses), transmitted by mosquitoes, biting flies or ticks. One of the first viruses identified was Bunyamwera virus, isolated from its *Aedes* mosquito vector in 1943 (Smithburn et al., 1946), causing only mild illness in humans. Many other, serologically related viruses were found over the following decades, put together initially into the *Bunyamwera* Supergroup by Casals (Casals, 1963). After the identification of more viruses that shared size, morphological, biochemical and genetic aspects, the International Committee for Taxonomy of Viruses acknowledged the new virus family *Bunyaviridae* (Bishop et al., 1980). The *Bunyamwera* Supergroup became the genus *Bunyavirus*, and at the same time the genera *Phlebovirus*, *Uukuvirus*, *Nairovirus*, and *Hantavirus* were assigned, each consisting of serologically related viruses resembling the members of the genus *Bunyavirus*. Later, the uukuviruses were combined with the phleboviruses into a single genus *Phlebovirus*, based on their close genetic resemblence (Francki et al., 1991).

Tomato spotted wilt virus. The "spotted wilt" disease in tomatoes was first identified in Australia (Brittlebank, 1919). The virus causing this plant disease was identified ten years later (Samuel et al., 1930), and denoted Tomato spotted wilt virus (TSWV). A few years earlier, the disease was shown to be transmitted by thrips (Pittman, 1927). For almost six decades, the virus was regarded as the single representative of a monotypic plant virus group, the "tomato spotted wilt virus group" (Matthews, 1979). In the 1980's, when the virus became a serious threat to agriculture, intensified investigations on the (molecular) biology of TSWV revealed a strong resemblance with the members of the animal infecting bunyaviruses. Therefore, TSWV was reclassified as the prototype of a newly created genus within the *Bunyaviridae*, the genus *Tospovirus* (Francki et al., 1991). Gradually, more plant infecting viruses that resembled TSWV, but with distinct sequence and serological features, were identified, the first one being Impatiens necrotic spot virus (INSV; Law and Moyer, 1990). During the last meeting on tospoviruses, held in Wageningen, May 1998, tospovirologists agreed on the establishment of thirteen different tospovirus species, and undoubtedly more tospoviruses will be identified in the future.

Molecular biology of bunyaviruses

Bunyaviridae members, including the plant infecting tospoviruses, share a number of features. Their particles are spherical or pleomorphic in shape, 80-120 nm in diameter (Fig. 1.1). A nucleocapsid core, consisting of the genetic elements encapsidated by nucleoprotein (N, see Fig. 1.1), together with a few copies of the L protein (for TSWV 10-20 copies per



(a) *(b)*

Figure 1.1 *Electron micrographs of (a) purified TSWV particles, and (b) ribonucleocapsids. Bars represent 100 nm.*

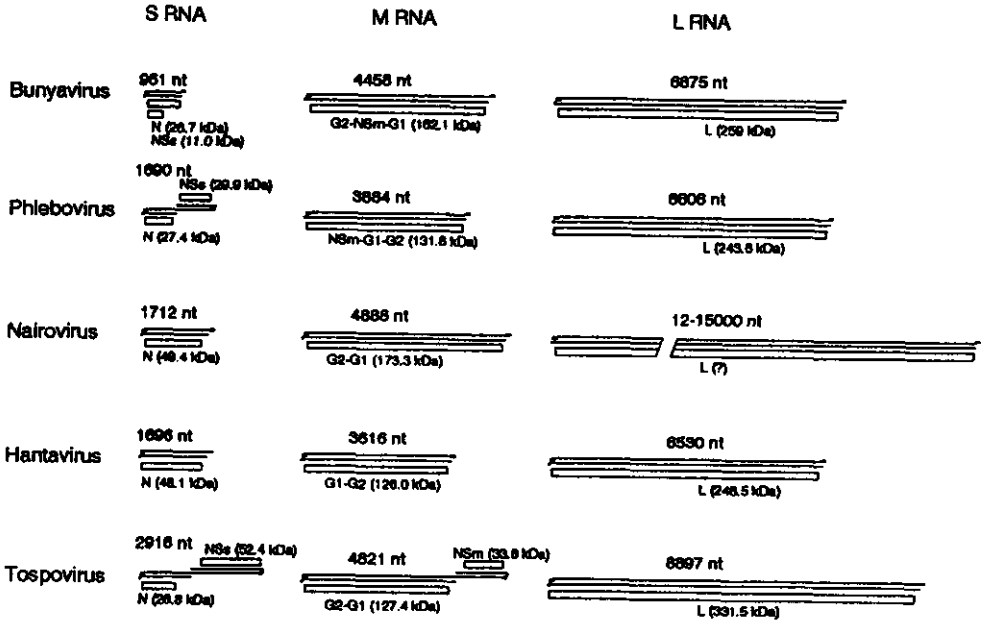


Figure 1.2 *Schematic representation of the genomes and expressed proteins of the genera of the family Bunyaviridae. Single lines represent viral RNA or mRNA (the latter with spheres at the 5' terminus, representing snatched caps). Open boxes represent expressed proteins/precursors.*

particle (van Poelwijk et al., 1993), are surrounded by a lipid bilayer envelope, in which the glycoproteins G1 and G2 are embedded as surface projections, 5-10 nm in length (Fig. 1.1). All bunyaviruses have three single stranded RNA segments of negative or ambisense polarity denoted small (S, between 0.85 and 2.9 kilobases (kb)), medium (M, between 3.6 and 4.8 kb) and large (L, between 6.4 and 12 kb), the total size being between 11 and 19 kb (Fig. 1.2). Each genetic element has complementary terminal sequences, which allow base-pairing to form non-covalently closed panhandle structures. Terminal sequences are conserved among members of the same genus, but are different for members of other genera (Elliott, 1996).

The plant infecting tospoviruses which are completely sequenced up till date, TSWV and INSV, have a negative L (de Haan et al., 1991; van Poelwijk et al., 1997), an ambisense S (de Haan et al., 1990; de Haan et al., 1992) and also an ambisense M segment (Kormelink et al., 1992a; Law et al., 1992) (Fig. 1.2).

Generally, the bunyavirus genome encodes four to six proteins (Fig. 1.2): a nucleocapsid protein (N) on the S segment, two glycoproteins (G1 and G2) on the M segment and a large transcriptase protein (L) on the L segment. Some species also have one or two nonstructural proteins: NSm, encoded by the M segment, and NSs, encoded by the S segment (Fig. 1.2). The function and significance of the NSs protein has remained unknown for any of the bunyaviruses, as is that of the NSm protein encoded by the animal-infecting members. In contrast, the NSm protein encoded by tospoviruses has been shown to represent the "movement protein" involved in intercellular virus movement through plants (Kormelink et al., 1992a; Kormelink et al., 1994 Storms et al., 1995). This cell-to-cell transport function is essential to plant viruses, but unnecessary and consequently absent in animal infecting viruses. It is therefore postulated that the NSm gene was acquired by an assumed animal infecting ancestral (bunya)virus during evolution, to make it suitable for infection of plants, resulting in the plant infecting tospoviruses (Goldbach and Peters, 1996).

Morphogenesis of bunyaviruses

At the onset of this PhD research very little was known about the morphogenesis of the plant-infecting tospovirus members of the *Bunyaviridae*. In contrast, for some of the animal infecting bunyaviruses this process has been studied to certain detail, especially for Uukuniemi phlebovirus.

As early as 1970, von Bonsdorff and others observed the intracellular envelopment of Uukuniemi virus particles in smooth membranes in the perinuclear region of the cell. As was later also observed for other bunyaviruses (reviewed in Elliott, 1996), Kuismanen and others (Kuismanen et al., 1982) showed that the perinuclear region of Uukuniemi glycoprotein accumulation represents the Golgi system, using specific Golgi markers in immunofluorescence experiments, together with electron microscopy of infected cells. The latter studies also revealed that under influence of the Uukuniemi virus infection, the Golgi region progressively becomes vacuolized and considerably extended. In immunofluorescence, nucleocapsids are found throughout the cell in a punctuate pattern during infection, suggesting aggregation in the cytoplasm, but also increasingly in the Golgi region. These and subsequent results (Kuismanen et al., 1984) led to the hypothesis that nucleocapsids interact with the glycoproteins in the Golgi membranes, and that the accumulation of glycoproteins somehow alters the structure of the Golgi system, inducing vacuolization and extension of the organel. Virus particles then bud into the vacuolized lumen of Golgi cisternae, as illustrated in Figure 1.3. They are transported in vesicles to the plasma membrane, presumably through the regular secretory vesicle transport machinery. The accumulation of viral glycoproteins in the Golgi system seems a prerequisite for this maturation pathway, and further studies (Gahmberg et al.,

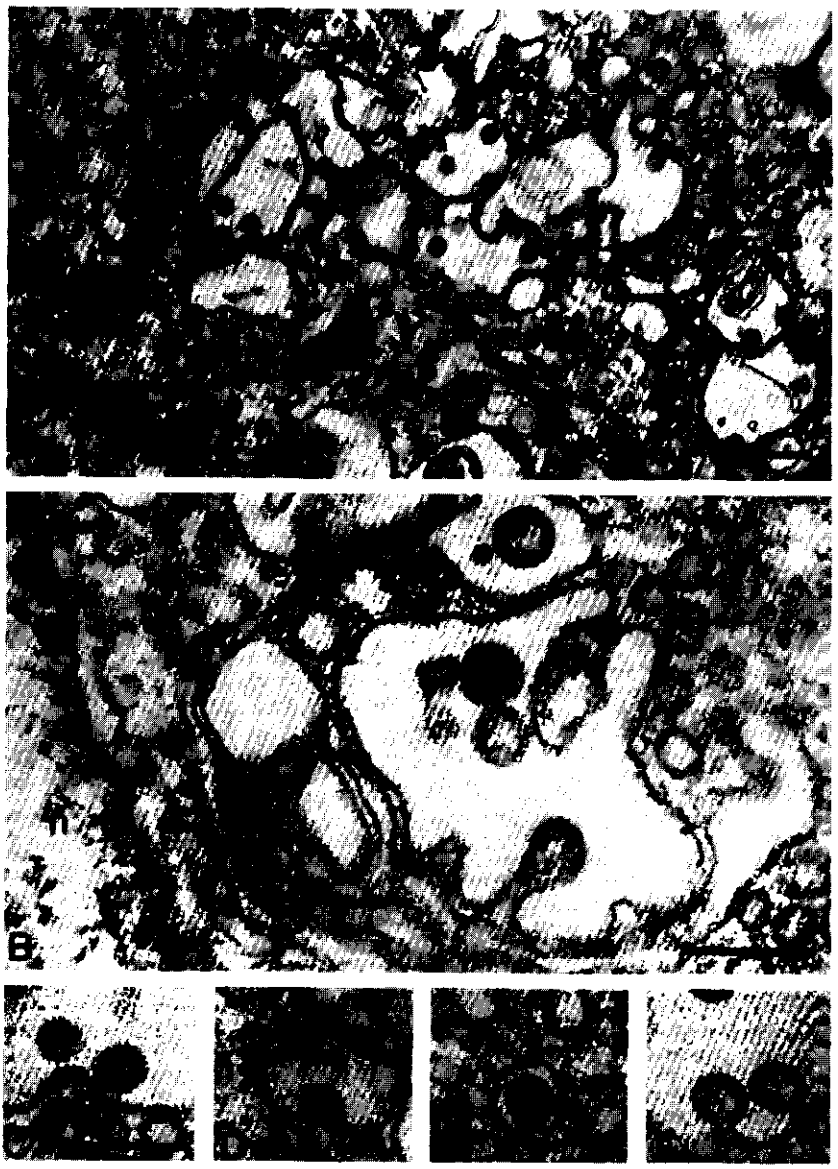


Figure 1.3 *Electron micrographs of thin sections of Uukuniemi virus-infected chicken embryo cells at 9 h p.i. (A and B). Virus particles are seen in the lumen of vacuolized Golgi cisternae, close to the nucleus (n). (C-F) Virus particles at different stages of budding.*
Bars: (A) 400 nm, (B) 200 nm, (C-F) 200 nm. (Figure from Kuismanen et al., 1982, with permission).

1986; Melin et al., 1995; Ronnholm, 1992) showed that the glycoproteins themselves contain necessary and sufficient information for their retention in the Golgi (see also next section). A recent report (Jantti et al., 1997) additionally showed that envelopment of Uukuniemi virus particles already starts in the pre-Golgi intermediate compartment, a relatively recently identified separate organel (Saraste and Svensson, 1991; Schweizer et al., 1990; Schweizer et al., 1990), and additionally takes place in the cis, medial and trans Golgi stacks. When infection was followed in the presence of the drug tunicamycin, which inhibits N-linked glycosylation, the Uukuniemi virus glycoproteins seem to accumulate in the ER region instead of the Golgi, resulting in the association of nucleocapsids also in the ER region (Kuismanen et al., 1982). This was shown for Punta Toro virus as well, for which it was additionally shown that indeed also budding takes place at the ER in situations were glycoproteins accumulate in this organel (Chen et al., 1991a). This suggests that the mere accumulation of the viral glycoproteins is a prerequisite for budding, and association of the nucleocapsids with the cytoplasmic tails of the glycoproteins takes place to initiate the bud.

Morphogenesis of TSWV particles. Although several researchers in the past thirty-five years have looked at the structures associated with TSWV infection in plants and thrips, a clear view of the morphogenesis pathway of particles is not available. Because a prominent observation is the clustering of enveloped particles inside ER-derived membranes (Ie, 1964; Ie, 1971; Kitajima, 1965; Milne, 1970; Francki and Grivell, 1970; Kitajima et al., 1992a; Kitajima et al., 1992b), many authors of reviews and textbooks have

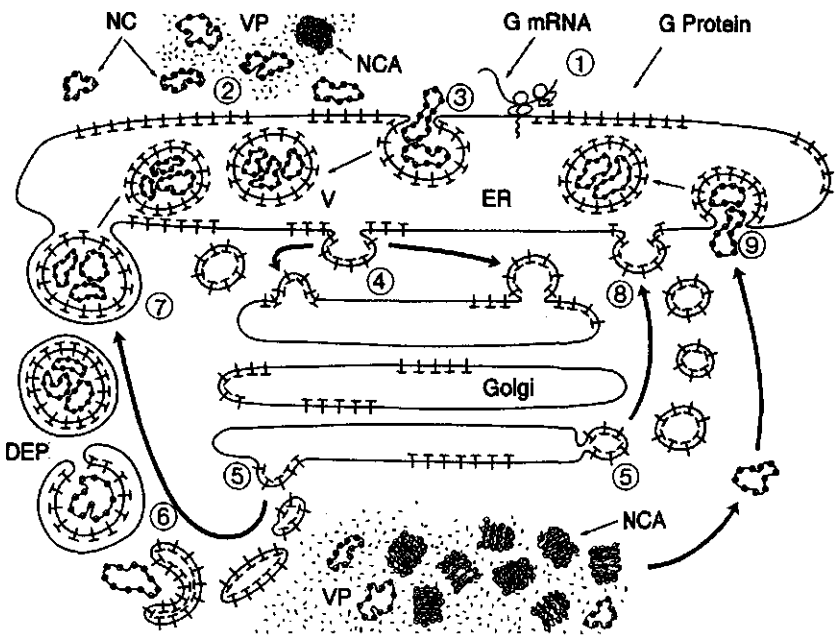


***Figure 1.4** Three possible models for TSWV morphogenesis in plants, as proposed by Kitajima et al., 1992a. At the top of the scheme the direct budding of nucleocapsids into the lumen of the ER is drawn. At the right side of the scheme a pathway is drawn that assumes the transport of the glycoproteins to the Golgi system, followed by retrograde transport back to the ER where budding takes place. The pathway on the left of the scheme proposes the involvement of Golgi membranes in the formation of particles, and explains the presence of doubly enveloped particles, which fuse with the ER.*
NC: nucleocapsid; NCA: nucleocapsid aggregate; V: virus particles; VP: viroplasm; ER: endoplasmic reticulum; DEP: doubly enveloped particle.

filled the knowledge-gap with the assumption that enveloped TSWV particles mature in the ER. The simplest, and probably therefore favorite, model is the budding of particles into the lumen of the plant ER, analogous to the budding of animal-infecting bunyavirus particles into Golgi cisternae. However, apart from the accumulation of mature TSWV particles in the ER, no indications have been found that support this scenario. Moreover, this hypothesis does not explain the doubly enveloped particles that are found in considerable amount during TSWV infections. Interestingly, nearly all reports dealing with TSWV maturation, mention so-called paired parallel membranes, which in many cases are suggested to be associated with the formation of doubly enveloped particles (Milne, 1970), or "mature particles within tight vesicles" (Kitajima et al., 1992a). These structures are however rarely found when examining infections of plants, compared to nucleocapsid aggregates and clustered particles within ER membranes. Therefore, it was thought that these structures may be artifacts, or by-products of the infection (Goldbach and Peters, 1996), although Kitajima and co-workers (1992a) noticed that they are present in every plant infection, albeit in low frequency (5% of examined sections). Discussing their results, some authors speculated about the involvement of the Golgi system in TSWV maturation, in association with the paired parallel membranes and doubly enveloped particles (Kitajima et al., 1992a; Lawson et al., 1996), but no evidence could be presented. The report of Kitajima et al. (1992a) proposed three possible models, involving the ER and the Golgi membranes, as shown in Figure 1.4.

In conclusion, little is known about the morphogenesis of TSWV particles, but from the observations in plant infections it seems clear that there are differences in the morphogenesis pathway between tospoviruses and the animal-infecting bunyaviruses.

Bunyavirus glycoproteins

The viral glycoproteins G1 and G2, in all bunyaviruses encoded by the M segment, and present on the surface of virus particles, play several crucial roles during the animal-infecting bunyavirus life-cycle. They dictate the site of particle morphogenesis by accumulating at particular cellular membranes and interact with the nucleocapsid core. They display hemaglutination activity, and thus are attachment proteins interacting with receptors on the surface of susceptible vertebrate and insect vector cells. They induce and interact with neutralizing antibodies, and they play a great role in the virulence of the virus. They furthermore influence vector transmission of virus, not only by directing initial entry into the cells of the vector, but probably also by guiding the virus during the circulation through the vector.

When translation products of the glycoprotein precursor gene of bunyaviruses were identified, the larger, thus slower migrating glycoprotein in SDS-polyacrylamide electrophoresis was designated G1, and the smaller, faster migrating protein was called G2. This tradition causes some confusion, since it results in alternating relative locations of G1 and G2 in the precursor for members of different genera, and even among the members of the same genus the relative location of G1 and G2 can be different. Research, however, has shown that functional glycoprotein equivalents share an N or C-terminal position in the precursor, which led to the proposal of calling the N-terminal glycoprotein G_N and the C-terminal one G_C (Lappin et al., 1994). In recent publications most researchers adapted to the new nomenclature, the International Committee on the Taxonomy of Viruses however still has to officially approve it. To avoid confusion, in this introduction the G_N/ G_C nomenclature will be used as far as appropriate, instead of G1/G2.

The sequence, translation, cleavage, spatial topology, glycosylation, oligomerization, transport and retention of the glycoproteins of some animal-infecting bunyaviruses have been

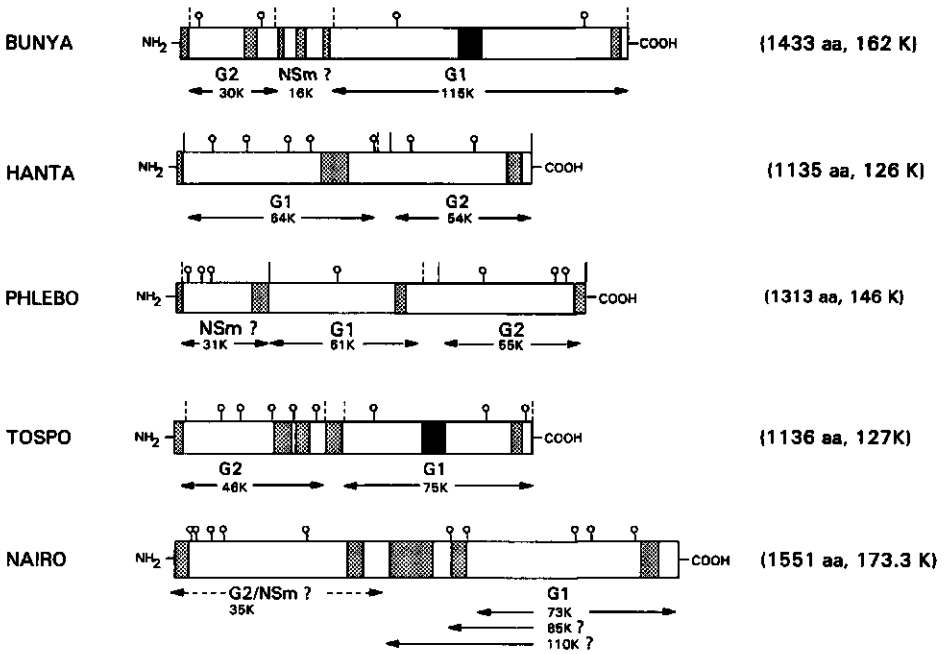


Figure 1.5 *Diagrams of the glycoprotein precursors of representative members of the Bunyaviridae.*

studied in more or less detail (reviewed in Elliott, 1996). Figure 1.5 shows an overview of the coding strategies of members of all of the genera, including the relative positions of G1 and G2. Note, that although the topology of hydrofobic sequences is similar in almost all precursors, the length of the individual glycoproteins can differ quite dramatically among the different genera (Fig. 1.5). The coding strategy of nairovirus glycoproteins seems a lot more complex than that of the other genera (Fig. 1.5). Apart from what are believed the mature G_N and G_C proteins, a number of putative intermediates are found during infection of several members of the genus *Nairovirus*. For Dugbe virus an 85 kDa glycoprotein was identified as the precursor to the mature G_C protein, which surprisingly is not immediately preceded by a hydrophobic signal sequence, as found for other bunyaviruses (Marriott et al., 1992). A 73 kDa protein could be a precursor to the mature G_N protein of Dugbe virus. A 110 kDa product remains unclarified (Fig. 1.5).

Uukuniemi virus glycoproteins. The processing, transport and retention of particularly Uukuniemi phlebovirus glycoproteins were studied in considerable detail. Therefore the following paragraphs will be focussed on this virus, while (contrasting) results for other animal infecting bunyaviruses will be mentioned for completion.

The Uukuniemi virus glycoprotein precursor (p110) is co-translationally cleaved, to produce the separate G_N and G_C proteins, as judged from pulse chase experiments in which the uncleaved precursor was never found (Kuismanen, 1984). For all other bunyaviruses investigated this co-translational cleavage was found as well (Elliott, 1996). G_N and G_C are found to be type I membrane-spanning glycoproteins, implying that they span the membrane once and that their N-terminus is oriented towards the lumen of the ER, after translation. Both G_N and G_C are therefore preceded by a hydrofobic signal sequence. The presence of the internal signal sequence in the precursor, preceding the G_C protein, suggests the separation of G_N from G_C to be achieved by the action of the ER luminal signal peptidase. Indeed, for Uukuniemi phlebovirus, it was shown that a single cleavage takes place behind the internal

signal sequence, and that the latter consequently stays attached to G_N (Andersson et al., 1997b).

Bunyavirus glycoproteins are found to be glycosylated with so-called high-mannose, intermediate, or complex type glycans, referring to the increasing rate of sugar residue processing in the ER and Golgi complex, dependent on the glycoprotein and the virus. Differently processed glycans can be attached to one protein (Elliott, 1996). The Uukuniemi virus G_C protein contains a mixture of high mannose, intermediate, and complex type glycans, while G_N primarily contains complex type glycans (Pesonen et al., 1982).

Both Uukuniemi virus glycoproteins are, besides glycosylated, then folded. G_N folds quickly, within 10 minutes, with help of BiP (ER luminal chaperone) and protein disulfide isomerase (PDI), whereas G_C folds very slowly (45-60 minutes) in a similar way (Persson and Pettersson, 1991; Pettersson et al., 1993). Having folded correctly, G_N and G_C form heterodimers in the ER, and because the folding of G_N is much faster than that of G_C, dimers not necessarily consist of G_N and G_C molecules from the same precursor (Kuismanen, 1984; Persson and Pettersson, 1991; Melin et al., 1995). Subsequent transport to the Golgi is quite slow (30-45 minutes), the reason for which is unclear (Kuismanen, 1984). Experiments in which the Uukuniemi virus glycoproteins were expressed from cDNAs in the absence of other viral proteins have shown that after they are transported out of the ER into the Golgi system, the glycoproteins are then not transported further to the plasma membrane, but are retained in the Golgi system as judged from co-localizations with Golgi markers in indirect immunofluorescence studies (Melin et al., 1995; Ronnholm, 1992). When G_N was expressed separately it was also transported to the Golgi complex and retained there, although with less efficiency, suggesting that this protein is transport competent on its own, and contains a Golgi retention signal. G_C alone could not be transported out of the ER, and was partially degraded there. Co-expression of G_N and G_C from different RNAs rescued the efficient transport of both Uukuniemi virus glycoproteins to the Golgi, confirming that they normally associate with each other during transport, possibly in heterodimers (Melin et al., 1995; Ronnholm, 1992). Similar experiments have also been carried out for other bunyaviruses. Results for Bunyamwera virus glycoproteins are very similar to those of Uukuniemi virus glycoproteins (Lappin et al., 1994). La Crosse virus glycoproteins also behave in a similar way, although no rescue of G_C transport to the Golgi was observed in co-expression experiments (Bupp et al., 1996). This however, according to the authors, could be due to the constructs used, which may not reflect nascent processing of G_N and G_C proteins. Punta Toro virus (PTV) glycoproteins behave as Uukuniemi virus glycoproteins, except that separate expression of G_C of PTV does not result in transport incompetent ER retained protein, but transport of this protein to the plasma membrane. (Chen and Compans, 1991; Chen et al., 1991a; Chen et al., 1991b). In fact, due to this behavior of G_C, PTV is the only bunyavirus for which it could be proven that G_C does not contain a Golgi retention signal of its own. Neither of the Hantaan virus glycoproteins expressed on their own could be transported out of the ER, while co-expression rescued the transport and retention in the Golgi, as seen in expression of the Hantaan virus glycoprotein precursor (Ruusala et al., 1992).

The accumulation of the glycoproteins of animal-infecting bunyaviruses in the Golgi complex is apparently due to a Golgi retention signal, which prevents the proteins to be transported to the plasma membrane as is usual for proteins that have entered the secretory pathway. The location of this putative signal was examined for Uukuniemi virus and Punta Toro virus. For both viruses it was found in a stretch of amino acids in the cytoplasmic tail of G_N, close to the transmembrane domain, which seems to play a supportive role as well (Matsuoka et al., 1996; Andersson et al., 1997a).

Following the budding process, by which the Uukuniemi virus glycoproteins are incorporated in the virus particle, the glycoproteins seem to acquire a different complex

formation. Rönka and co-workers (Ronka et al., 1995) elegantly showed that on the surface of the virus particle, G_N surprisingly forms stable, pH-insensitive homodimers. G_C also exists as homodimers, and partially as monomers at pH 6.4 or above, but completely dissociates into monomers at a pH lower then 6.0. Apparently, the Uukuniemi virus glycoproteins completely abandon their heterodimer transport complex on the surface of the virus particle, possibly to prepare for their function in interaction with receptors. For Punta Toro virus it was shown, that the majority of transport of the glycoproteins of this virus through the secretory pathway, and their accumulation in the Golgi system, takes place as expected G_N - G_C heterodimers. However, part of the G_C proteins travels as G_C – G_C homodimers, which associate with the G_N – G_C heterodimers during transport, in order to be targeted correctly to the Golgi. This can be explained by the fact that the retention signal necessary for accumulation of both glycoproteins in the Golgi is present only in G_N (Chen et al., 1991b; Chen and Compans, 1991).

In summary, bunyavirus glycoproteins harbor many interesting characteristics with respect to signal sequences, folding, oligomerization, transport and retention. Thus, apart from providing fundamental knowledge of the virus infection, results on the behavior and characteristics of bunyavirus glycoproteins also serve a model of basic cellular protein trafficking and Golgi-retention.

TSWV glycoproteins. Whereas most plant viruses possess a rigid protein capsid as their protection against external threats, two plant virus groups instead have a much less structured protein protection of their genetic material, combined with a surrounding membrane, in which viral glycoproteins are inserted. Both of these groups, the plant infecting rhabdoviruses (genera *Cytorhabdovirus* and *Nucleorhabdovirus*) and the tospoviruses (genus *Tospovirus*), are members of virus families that predominantly infect vertebrates and are transmitted by insects: the *Rhabdoviridae* and the *Bunyaviridae* respectively (Elliott, 1996). The animal virus-like glycoprotein containing envelope of the plant-infecting viruses in those families, may reflect their ability to infect the (animal) cells of their insect vectors, and could additionally be an indication towards their putative evolution from animal infecting ancestral viruses into plant infecting viruses.

As mentioned, the tospovirus glycoprotein gene was sequenced (Kormelink et al., 1992a), but little homology was found with other bunyavirus glycoproteins. However, TSWV G_C contains a stretch of amino acids that is homologous with a corresponding stretch in G_C of several members of the genus *Bunyavirus* (Fig. 1.5, black boxes). Furthermore an RGD (Arg-Gly-Asp) motif (Ruoslahti and Pierschbacher, 1987, Pierschbacher and Ruoslahti, 1987) was found at the N-terminus of G_N, analogous to G_N of INSV tospovirus (Law et al., 1992), Germiston bunyavirus (Pardigon et al., 1988) and Snow Shoe Hair bunyavirus (Eshita and Bishop, 1984) and Punta Toro phlebovirus (Ihara et al., 1985), which may be involved in virus attachment to cells. The N-terminal hydrophobic domain was identified as a possible signal sequence and analysis with the Von Heijne (von Heijne, 1986) algorithm revealed a predicted signal peptidase cleavage site at amino acid residue 35 (Fig. 1.6), counted from the N-terminus of the precursor, in front of a lysine residue (Kormelink et al., 1992a). The third hydrophobic region in the TSWV precursor, ranging from residue 428-484 (Fig. 1.6) could also be a signal peptide, directing TSWV G_C into the ER membrane analogous to other bunyavirus glycoproteins, but this is unknown. The second and fourth hydrophobic regions probably represent membrane anchors, by which the glycoproteins span the membrane.
Little research has been done on the expression and processing of TSWV glycoproteins. Expression in the baculovirus expression system resulted in cleavage of the precursor, although much less efficient than in plant infections (Kormelink, 1994; Adkins et al., 1996). The proteins (including the precursor) were also glycosylated in Sf21 cells. Subcellular localization of the TSWV glycoproteins showed staining of the plasma-membrane, which led

Adkins et al. (1996) to believe that the glycoproteins are transported to the cell surface. Staining of the nucleus in such cells, however, revealed that the cytoplasm of the baculovirus infected cell is forced to a small ring around the extremely enlarged nucleus (Kikkert et al., unpublished results), and thus the interpretation of the localization results in baculo-virus infected cells is doubtful.

In summary, the TSWV glycoprotein topology resembles that of animal infecting bunyavirus glycoproteins, although very little is known about the transport, oligomerization and localization during infection of its alternating plant and insect hosts.

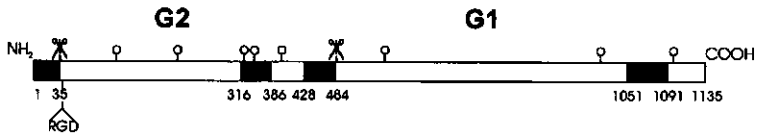


Figure 1.6 *Schematic representation of the topology of the TSWV glycoprotein precursor, showing possible cleavage sites (scissor symbols), hyfrofobic areas (black boxes), amino acid positions, the RGD motif, and predicted N-glycosylation sites (spheres on stick).*

Interaction of bunyaviruses with their insect vectors

Bunyaviruses are generally transmitted by insect vectors, except the members of the genus *Hantavirus*, which have their virus reservoir in rodents transmitting the viral disease to the human host (Elliott, 1996). The vector specificity is, to a certain extend, reflected in the taxonomic classification. Members of the genus *Bunyavirus* are generally transmitted by mosquitoes, and *Nairovirus* members are transmitted by ticks. Phleboviruses are transmitted by biting flies, but some of them by ticks or mosquitoes. Tospoviruses are exclusively transmitted by thrips (Thysanoptera; order Thripidae). As far as determined, all bunyaviruses replicate within the vector, and cause a persistent, life-long infection, which has however very little or no influence on the vectors life functions (reviewed in Elliott, 1996). Some viruses, a.o. La Crosse virus, are transmitted transovarially from one insect generation to the other, ensuring a persistent natural reservoir of virus in nature. Entry of virus into the vector is most probably regulated by interactions of the viral glycoproteins with receptors in the gut of vectoring insects. There are also indications that further circulation of the virus, with the saliva as final destination, requires receptor interactions, to overcome different dissemination barriers. The membrane fusion of bunyavirus particles, as far as investigated, requires low pH values (Gonzalez, 1985; Gonzalez et al., 1984a; Gonzalez et al., 1984b), indicating that the first step of entry into vector or animal host cells is a receptor mediated endocytosis of the virus particle, and only after acidification, the virus envelope fuses with the endosome (lysosome), releasing the nucleocapsids into the cytoplasm. Relatively, little is known about the molecular interactions of bunyaviruses with the cells of their insect vectors. No cellular receptors have been identified, and only for La Crosse virus the involvement of the glycoproteins in attachment to its vector cells (predominantly *Aedes triseriatus* mosquitoes) have been investigated in some detail. Ludwig and co-workers (Ludwig et al., 1989; Ludwig et al., 1991; Ludwig et al., 1991) found that after proteolytic digestion of the sensitive G_C, G_N mediated entry of mosquito cells was enhanced. It was postulated that G_C is the attachment protein for vertebrate cells and G_N serves the attachment to vector cells. Other reports however, claim that La Crosse G_C does play a role in insect cell entry (Hacker and Hardy, 1997; Hacker et al., 1995; Hacker et al., 1995; Sundin et al., 1987), probably indicating that the molecular processes involved in insect cell entry are rather complicated and need more investigation.

Interaction of TSWV with its thrips vector. Like the animal-infecting bunyaviruses, TSWV replicates in its vector (Ullman et al., 1993; Wijkamp et al., 1993), while it is transported from the gut to the saliva during a propagative circulation (Tsuda et al., 1996; Ullman et al., 1995) (Fig. 1.7). Virus must be acquired in larval stages (van de Wetering et al., 1996; Wijkamp et al., 1996), be replicated, and excreted in the thrips saliva, after which it can be transmitted to a susceptible plant. The mean latent period between uptake and transmission is temperature dependent, and takes between 84 and 171 hours for TSWV (Wijkamp and Peters, 1993). This implicates that, at the earliest, thrips can transmit virus while they are still in the second larval stage. Transmission ability continues throughout the further life-time. Seven transmitting thrips species have been reported, either belonging to the genus *Frankliniella* or *Thrips*, and they vary in their ability to transmit different tospoviruses (Wijkamp et al., 1995). The most important vector at present is *Frankliniella occidentalis*, the Western flower thrips.

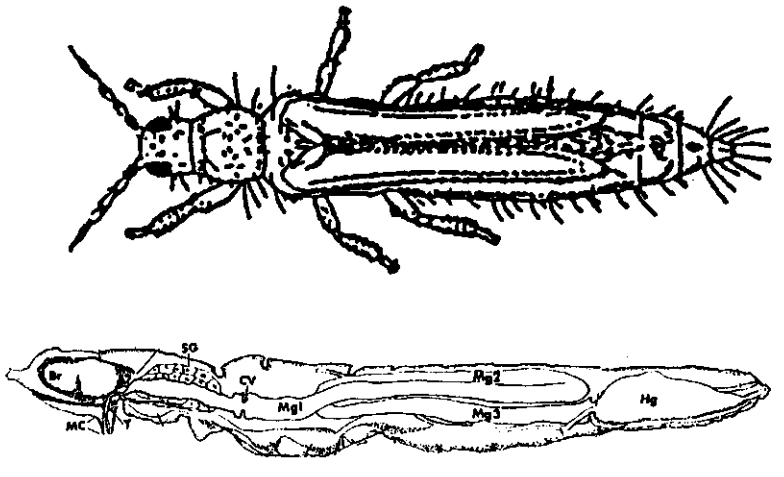


***Figure 1.7** (a) Drawing of adult Frankliniella occidentalis female (top view). (b) Diagrammic side view of adult F. occidentalis, showing relative orientation of internal organs in situ. Br = brain; MC = mouthcone; Y = Y-shaped salivary ducts leading to salivarium; SG = salivary glands; CV = cardiac valve; Mg1 = anterior midgut; Mg2 = central midgut; Mg3 = posterior midgut; Hg = hindgut. (Modified from Ullman et al., 1992).*

It is anticipated that the TSWV glycoproteins G1 and G2 have an important role during the virus life cycle in the thrips vector. In analogy with other enveloped viruses, the glycoproteins may be involved in attachment to the midgut tissue of the thrips vector, and in further replication and transport of the virus through the insect. At the onset of the research described in this thesis, virtually nothing was known about the molecular interaction of TSWV with the cells of its vector. In infections of plants, the viral glycoproteins do not seem to have a crucial attachment function, since one can not think of a reason for carrying surface glycoproteins, typically designed to interact with receptors, during the infection of plant cells. Indeed, when the thrips cycle is deliberately avoided, and virus is repeatedly transmitted mechanically to plants, TSWV tends to loose the ability to express the glycoproteins, which, not surprisingly, also abolishes the ability to form enveloped particles (Ie, 1982; Verkleij and Peters, 1983; Resende et al., 1991). These mutant viruses are perfectly able to induce symptoms in plants and be mechanically transmitted, although at the electron microscopical

level they only produce nucleocapsid aggregates, and no enveloped virus particles. Recently it became clear that these so-called envelope-deficient mutants cannot be transmitted by thrips any longer (Wijkamp, 1996), which gives a strong indication that the TSWV glycoproteins indeed have a crucial role in the thrips cycle, and that they are not essential for the infection of plants. The fact that in nature, TSWV alternatingly replicates in plants and in thrips, ensures the stable presence of the glycoprotein containing envelope of TSWV, which is so uncommon for most other plant viruses. The enveloped deficient mutants will not be addressed in this thesis, but they may provide an important tool for additional research on the functions of the glycoproteins, both in particle formation and in interaction with the thrips vector, as well as on the regulation of the expression of the precursor gene.

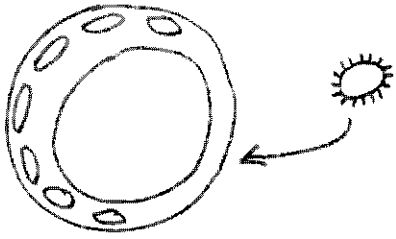
Outline of this thesis

The surface glycoproteins of enveloped animal-infecting viruses play crucial roles during several stages of the virus lifecycle. They form the interface between the virus particle and the host and vector, and additionally regulate the particle-morphogenesis process. The role of the glycoproteins of the plant-infecting tomato spotted wilt bunyavirus, however, is poorly defined, but of particular interest. TSWV replicates in both plants and insects, and the functioning of the glycoproteins will therefore reflect this dual tropism. The anticipated key roles of the TSWV glycoproteins in infection of plant and thrips cells are the major focus of the research described in this thesis.

Our first goal was to investigate the enveloped TSWV particle morphogenesis in plant cells, and the role of the glycoproteins therein. Since the analyses of infections in whole plants, though extensive, have not resulted in a clear view of this process, a protoplast infection system for TSWV was developed. In this system, described in Chapter 2, a synchronous, controllable infection was established, facilitating the temporal analysis of the infection process. Using this system, a data-based model of the morphogenesis of enveloped TSWV particles in plant cells could be designed, presented in Chapter 3. The proposed TSWV morphogenesis is surprisingly distinct from the morphogenesis process of animal-infecting bunyaviruses. The next question was therefore whether this altered morphogenesis would be accompanied by changes in the molecular make-up of the viral glycoproteins, which have an important role in guiding the morphogenesis process, or whether other factors are involved. To address this question the TSWV glycoproteins, and mutants thereof, were expressed in mammalian cells, to check whether they still contain the characteristics of the glycoproteins of their ancestral animal-infecting bunyavirus(es) (Chapter 4). The second major function of surface glycoproteins of enveloped viruses is their association with receptors, to induce entry of virus particles into host or vector cells. For TSWV glycoproteins, this function is reflected in the anticipated molecular interaction with cells of the thrips vector. A first analysis based on ligand blot techniques, using extracts of whole insects, resulted in the identification of a 94 kDa thrips protein which specifically binds to TSWV G2. These experiments are described in Chapter 5. Chapter 6 generally discusses the contents of this thesis, connects the different chapters, and places the results in the context of the assumed evolution of TSWV from an animal-infecting ancestral bunyavirus. Additionally this chapter discusses the use of TSWV glycoproteins as models in cell biology and provides additional, preliminary, insights in the structure and function of TSWV glycoproteins.

CHAPTER 2

A PROTOPLAST SYSTEM FOR STUDYING TOMATO SPOTTED WILT VIRUS INFECTION



Kikkert, M., F. van Poelwijk, M. Storms, H. Bloksma, W. Karsies, R. Kormelink, and R. Goldbach. (1997) A protoplast system for studying tomato spotted wilt virus infection. *Journal of General Virology* **78,** 1755-1763.

Abstract

A plant protoplast system for studying tomato spotted wilt tospovirus (TSWV) infection was established and tested. Using PEG (polyethylene glycol)-mediated inoculation with highly infectious TSWV particles, generally 50% or more of *Nicotiana rustica* protoplasts were infected. In these cells viral RNA and viral protein synthesis became detectable at 16 h post-inoculation (p.i.) and continued at least until 90 h p.i.. Both the structural viral proteins (nucleoprotein (N) and the envelope glycoproteins G1 and G2) and the nonstructural viral proteins NSs and NSm accumulated to amounts suitable for detection and immunocytological analysis. Local lesion tests on petunia leaves and electron microscopical analysis confirmed the production of mature, infectious virus particles, underlining the conclusion that a full infection cycle was completed in this system. Upon inoculation of *Vigna unguiculata* (cowpea) protoplasts with TSWV particles, comparable proportions of infected cells and amounts of NSs, NSm and N protein were obtained, but much lower amounts of viral glycoproteins were detected than in *N. rustica* protoplasts, and progeny virus particles were less abundant.
With the *N. rustica*-based protoplast system, a powerful synchronised single cell infection system has now become available for more precise in vivo studies of the processes occurring during tospovirus infection.

Introduction

Over the past few years accumulating data on the molecular biology of tospoviruses, a genus of plant-infecting bunyaviruses, have been published (reviewed in: German et al., 1992; Goldbach et al., 1992; van Poelwijk et al., 1996; Mumford et al., 1996; Goldbach and Peters, 1996). These studies, which mainly focused on tomato spotted wilt virus (TSWV), type species of the genus, revealed that tospoviruses have a tripartite RNA genome typical of all *Bunyaviridae*. The genomic RNAs are tightly associated with nucleoprotein (N) and enclosed by a lipid envelope from which the viral glycoproteins (G1 and G2) protrude, thus forming virus particles 80-110 nm in diameter. Sequence and translational analyses have shown the large (L) genomic RNA of TSWV to be negative stranded (de Haan et al., 1991) and the other two (S and M) RNAs to be ambisense (de Haan et al., 1990; Kormelink et al., 1992a, respectively). In total six viral proteins appear to be specified by the genome of TSWV. The single translation product of L RNA (referred to as L protein) represents the putative viral polymerase (de Haan et al., 1991; van Poelwijk et al., 1993). The M RNA encodes, in its viral (v) strand, a common precursor to both envelope glycoproteins and, in its viral complementary (vc) strand, a nonstructural protein NSm, implicated in cell-to-cell movement of the virus (Kormelink et al., 1992a; Kormelink et al., 1994; Storms et al., 1995). The (smallest) S RNA has been shown to encode the N protein in the vc strand and an additional nonstructural protein NSs of still unknown function in the v strand (de Haan et al., 1990; Kormelink et al., 1991).

The TSWV infection cycle has so far only been studied in whole plants. An alternative would be to develop a protoplast infection system. Due to better synchronisation of infection and, in general, high proportions of infection, single cell suspensions have been of great value in the study of a considerable number of plant viruses. No such system has been described for tospoviruses, but high proportions of infection for *Vigna unguiculata*, *Nicotiana edwardsonii* and *Nicotiana benthamiana* protoplasts inoculated with sonchus yellow net rhabdovirus (SYNV) have been reported (van Beek et al., 1985; Jones and Jackson, 1990), showing that such an approach is feasible for enveloped plant viruses.

This report describes the development of a protoplast system for TSWV, based on *Nicotiana rustica* and *V. unguiculata*, involving PEG-mediated inoculation with purified virus particles.

Methods

Virus and plants. A Brazilian isolate of TSWV, BR01 (de Avila et al., 1993b), was used, and maintained in *N. rustica* plants by mechanical inoculation and thrips transmission.

Polyclonal antisera. Polyclonal antibodies were raised, as earlier described, against NSs (Kormelink et al., 1991), NSm (Kormelink et al., 1994) and N (de Avila et al., 1993b) proteins. Antibodies against the viral glycoproteins were raised by immunisation of rabbits with purified fragments of G1 and G2, expressed in *E. coli* using the pET11t system (Novagen).

Isolation and storage of TSWV particles. Complete virus particles were isolated at 4 °C from systemically infected *N. rustica* leaves, essentially as described by Gonsalvez & Trujillo (Gonsalves and Trujillo, 1986), with some modifications. Harvested leaves were ground in 3 ml of extraction buffer (0.01 M sodium sulphite; 0.1 M sodium phosphate pH 7.0) per g leaf material in a Philips blender by giving 5 to 10 short pulses at medium speed. The homogenate was filtered through cheesecloth and the extract was centrifuged at 10,000 *g* for 15 min. The pellet obtained was gently homogenised for 30 min in 1 ml 0.01 M sodium sulphite per g initial leaf material using a small pestle and a magnetic stirrer. The suspension was clarified by centrifugation at 8000 *g* for 15 min, the supernatant was collected and centrifuged at 100,000 *g* for 30 min. The resulting pellet was homogenised in 5 ml of 0.01 M sodium sulphite per 100 g of initial leaf material and 2.5 ml was layered per 10-40% sucrose gradient. After centrifugation for 45 min at 70,000 *g* in a Beckmann SW28 rotor, the opalescent zone containing virus particles was collected, diluted 1:1 with 0.01 M sodium sulphite and concentrated by centrifuging for one hour at 100,000 *g*. The pellet, containing virus particles, was resuspended in sterile double distilled water at a concentration of approximately 1 mg/ml. In some experiments the sucrose gradient at the end of the procedure was omitted, and the preparation was homogenised in sterile double distilled water. This also yielded infectious virus suitable for inoculation of protoplasts. The amount of viral protein from each isolation was estimated using a BioRad protein assay kit according to the manufacturers procedure. Virus (-protein) yield was usually between 0.3 and 1 mg per 100 g of leaves. The preparations were kept on ice and used immediately, or promptly frozen in liquid nitrogen and stored at -80 °C. Before use, samples were then thawed slowly on ice.

Preparation of protoplasts. Protocols for isolation of protoplasts were based on those described by Hibi *et al.* (Hibi et al., 1975) and van Beek *et al.* (van Beek et al., 1985), and modified to some extend for *N. rustica*. Protoplasts of *N. rustica* were prepared from both greenhouse and *in vitro* grown plants, although the latter usually gave better results. *In vitro* grown *N. rustica* plants were initiated by sterilisation of seed, using bleach and SDS, and germination on sterile solidified Murashige and Skoog medium including vitamins (Murashige and Skoog, 1962), antibiotics and a fungicide (cefotaxime; 50 mg/l, vancomycin; 50 mg/l, nystatin; 25 mg/l respectively, all from Duchefa biochemicals BV. the Netherlands). Seedlings were first transferred to growth tubes (Sigma), or immediately to "Vitro Vent" growth boxes (Duchefa biochemicals BV, The Netherlands) with solidified medium and grown for two and a half to six weeks. After removal of the midrib, leaves were transversally incised to generate a fine "comb", and floated, lower epidermis down, on an enzyme solution containing 0.6 M Mannitol, 10 mM $CaCl_2$ (pH5.6), and 1% cellulase (Onozuka R-10) and 0.05% Macerozyme (both from Yakult Honsha Co.,LTD. Japan). After ± 3.5 hours of incubation at 30 °C in the dark, with gentle shaking every hour, protoplasts were harvested by

gentle shaking, collected, and put through a 64 mesh sieve. They were washed with 0.6 M mannitol, 10 mM $CaCl_2$ (pH5.6), by centrifuging at 50 *g* for 3 min and subsequent resuspension; this was repeated three times. Preparations with less than 70% of living cells, as judged by FDA (fluorescein di-acetate) fluorescence, were discarded.

V. unguiculata protoplasts were isolated from primary leaves of plants grown on liquid Hoagland medium (van Beek et al., 1985). The lower epidermis was stripped and the leaves were incubated with the stripped side down. The rest of the procedure was as described for *N. rustica*.

Inoculation of protoplasts with purified TSWV. The inoculation was based on protocols described by van Beek *et* al. (1985) and Eggen *et al* (Eggen et al., 1989). Glass tubes containing 1 million living protoplasts (pelleted) were put on ice. Approximately 10 µg of ice-cold virus was added and the mixture was gently shaken. Subsequently, 0.5 ml cold 40% PEG, 10 mM $CaCl_2$ was added and the tube was shaken more vigorously for 15 sec to mix well. To this, 4.5 ml 0.6 M mannitol, 10 mM $CaCl_2$ (pH5.6) (room temperature) was then added and the tube inverted three times. The protoplasts were incubated for 15-20 minutes at room temperature after which they were washed 2-3 times with 0.6 M mannitol, 10 mM $CaCl_2$ (pH5.6). After washing, the cells were resuspended in 2 ml of nutrition medium (0.6 M mannitol (pH5.6), 2.5 mM MES (pH5.6), 0.2 mM KH_2PO_4, 1.0 mM KNO_3, 1.0 mM $MgSO_4$, 10 mM $CaCl_2$, 1 mM KI, 0.01 mM $CuSO_4$, 25 mg/l nystatin, 50 mg/l vancomycin , and 50 mg/l cefotaxime), and transferred to small polystyrene petridishes (3.5 cm diameter, Nunc) in a climate chamber at constant temperature (25 °C), constant light (about 5000 Lx) and constant high humidity (>70%). As a negative control aliquots of one million protoplasts were inoculated with virus that was inactivated with 213 nm UV light for 10 minutes (4.8 J/cm^2) using a UV tray commonly used for analysing ethidium bromide stained gels.

Western immunoblot analysis. Protoplasts were pelleted by centrifugation at 50 *g* for 3 min, the supernatant was removed and 1 vol. of homogenisation buffer (50 mM Tris/acetate pH 8.2, 10 mM KAc, 1 mM EDTA, 5 mM DTT, and 1 mM PMSF) was added. Subsequently, one vol. of 4X Laemmli buffer (Laemmli, 1970) was added and prior to electrophoresis the samples were boiled for three minutes. Five µl of the samples was loaded on a 10 % SDS-polyacrylamide gel. After electrophoresis, proteins were transferred onto PVDF membrane (Millipore) using the Trans-Blot semi-dry blotting procedure of Bio-Rad. Filters were blocked overnight in 3% Elk skim instant milk in PBS, containing 0.1% Nonidet P-40. After washing with 0.3% instant milk, 0.1% Nonidet P-40 in PBS, filters were incubated with 0.5 µg/ml polyclonal antisera against N, NSs, or glycoproteins respectively for 1 h at room temperature. After washing, antigen-antibody complexes were detected using 1 µg/ml alkaline phosphatase conjugated goat-anti-rabbit immunoglobulins (Tago inc., Burlingame, CA, USA) and a mixture of nitroblue tetrazolium (NBT) and bromochloroindolyl phosphate (BCIP) as a substrate.

RNA isolation and Northern blot analyses. Aliquots of one million protoplasts were pelleted, the supernatant removed, and the pellet frozen in liquid nitrogen and stored at -80 °C. Total RNA was isolated using the TRIzol method of GIBCO BRL. In brief, pellets were thawed and resuspended in 200 µl TRIzol reagent. After 5 min. 40 µl chloroform was added, the suspension mixed well and centrifuged for 15 min. at 21,000 *g* in an Eppendorf centrifuge. The aqueous phase was transferred to a fresh tube containing 100 µl isopropanol. The RNA was pelleted, washed, dried and resuspended in 35 µl double distilled water. Five µl samples were resolved in a 1% agarose gel after treatment with methylmercuric hydroxide (Bailey and Davidson, 1976). The RNA was blotted onto Genescreen filter (New England Nuclear, NEN), and hybridised to ^{32}P-labelled riboprobes of TSWV-specific sequences (Kormelink et al., 1992b). As a control, total RNA of TSWV infected *N. rustica* plants was included.

Immunofluorescence of inoculated protoplasts. After incubation, protoplasts were spotted onto poly-L-lysine (0.05%) treated glass plates, blocked with 5% BSA (bovine serum albumin) in PBS pH 7.2 and subsequently incubated with 1-5 µg/ml of antiserum against NSs, NSm, N or the viral glycoproteins in 1% BSA in PBS for up to one hour at room temperature. Antigen-antibody complexes were detected using fluorescein isothiocyanate (FITC) conjugated swine anti rabbit serum (0.02 mg/ml, 45 min.), and examined with a fluorescence microscope. At least 200 cells were judged for specific fluorescence to calculate infection percentages, using an antiserum against NSs.

Local lesion tests on petunia leaves. The infectivity of newly synthesised viral products was analysed on leaves of petunia (cv. Polo Blauw), a local lesion host of TSWV. Petunia plants were put in the dark two days before being used, to increase their susceptibility to infection. Leaves were cut from the plant and subsequently cut through the midvein. After dusting with carborundum 500 powder, one half of each leaf was inoculated with purified virus as an internal control, and the other half with an amount of a pelleted and disrupted (vigorously shaken) protoplast suspension equivalent to 200,000 - 500,000 living cells. Lesions were counted four to five days later.

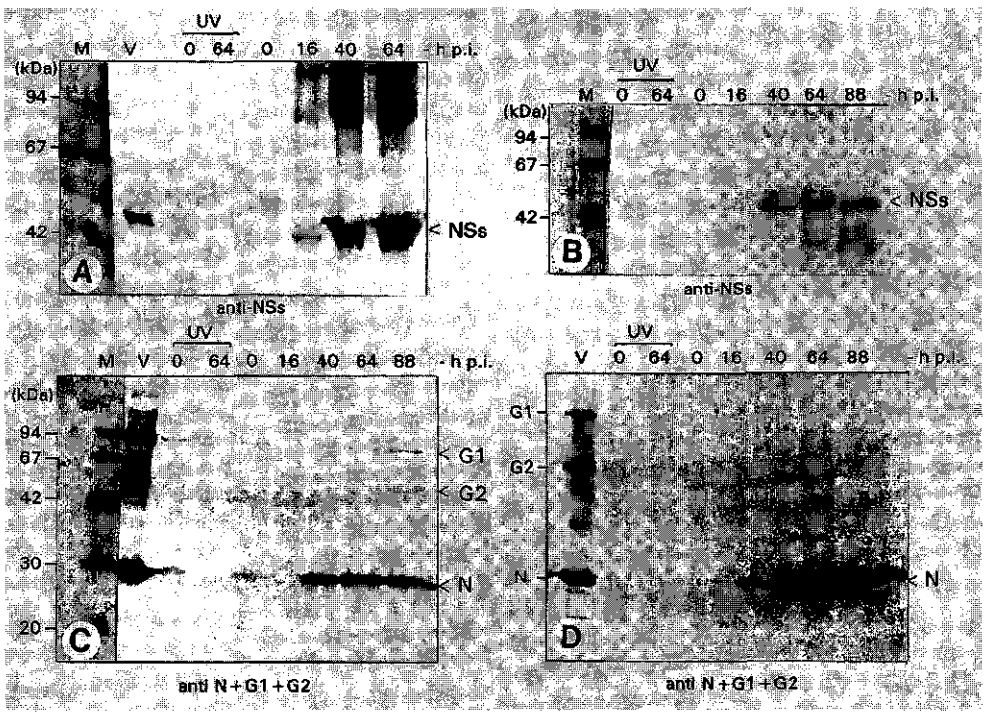


Figure 2.1 *Western blot analysis of TSWV-inoculated V. unguiculata and N. rustica protoplasts at different times (h p.i.) showing the production of NSs protein in N. rustica protoplasts (A) and V. unguiculata protoplasts (B) using anti-NSs serum; (C) production of N, G1 and G2 protein in N. rustica protoplasts and (D) production of N protein, but not G1 and G2 in V. unguiculata protoplasts, using a mixture of antisera against N, G1 and G2. M = molecular weight markers, V = purified TSWV. Protoplast samples in (A) and (C); lanes 3 and 4 and (B) and (D); lanes 2 and 3 (marked "UV") are inoculated with UV-inactivated virus.*

Electron microscopy. Protoplasts were pre-fixed in 0.75% (w/v) glutaraldehyde (GA) in nutrition medium for 30 minutes at room temperature, and then fixed with 3% GA, 2% paraformaldehyde (PA) in phosphate-citrate (PC) buffer (0.1 M $Na_2HPO_4 \cdot 2H_2O$ and 9.7 mM citric acid pH 7.2) for at least one hour. The fixed cells were washed several times with PC buffer, resuspended in 200 µl PC buffer, and layered onto 500 µl 5% (w/v) liquid gelatine in PC buffer. Tubes were centrifuged at 50 *g* and excess gelatine was removed. After solidification pellets were cut in smaller pieces and kept in fixative (3% GA/2% PA) at 4 °C. Samples were dehydrated with ethanol and embedded in LR Gold without additional fixation to produce specimens for immunocytological analysis. General ultrastructural analysis was done on samples fixed additionally in 1% (w/v) osmium tetroxide in PC buffer, stained with 1% (w/v) uranyl acetate, and dehydrated and embedded in LR White.

Results

TSWV inoculation of N. rustica *and* V. unguiculata *protoplasts.* To test their suitability for a single cell infection system, freshly prepared *N. rustica* and *V. unguiculata* (cowpea) protoplasts were inoculated with purified TSWV using PEG. The percentage of protoplasts infected was estimated by immunofluorescence, using an antiserum against NSs, a non-structural protein only produced after replication of the genome (de Haan et al., 1990), and a reliable indicator of infection. The proportion of infected protoplasts was calculated as numbers of NSs-positive cells relative to total numbers of living cells.

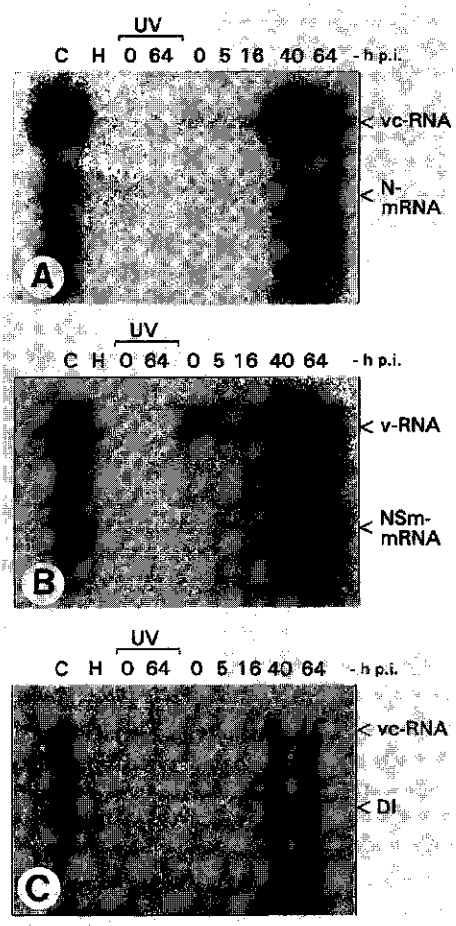


***Figure** 2.2 Production of viral RNA species in N. rustica protoplasts at different times after TSWV inoculation (h p.i.), shown on Northern blots using riboprobes as described by Kormelink et al. (1992b), and specific for (A) full-length S-RNA (vc-sense; 2.9 kb) and N mRNA (0.9 kb), (B) full-length M-RNA (v-sense; 5.0 kb) and NSm mRNA (1.0 kb) and (C) full-length L-RNA (vc-sense; 8.9 kb). C = control, i.e. total RNA from infected N. rustica plants; H = total RNA from healthy N. rustica protoplasts. As a negative control, protoplast samples were inoculated with UV-inactivated virus (lanes marked "UV"). DI = defective interfering RNA.*

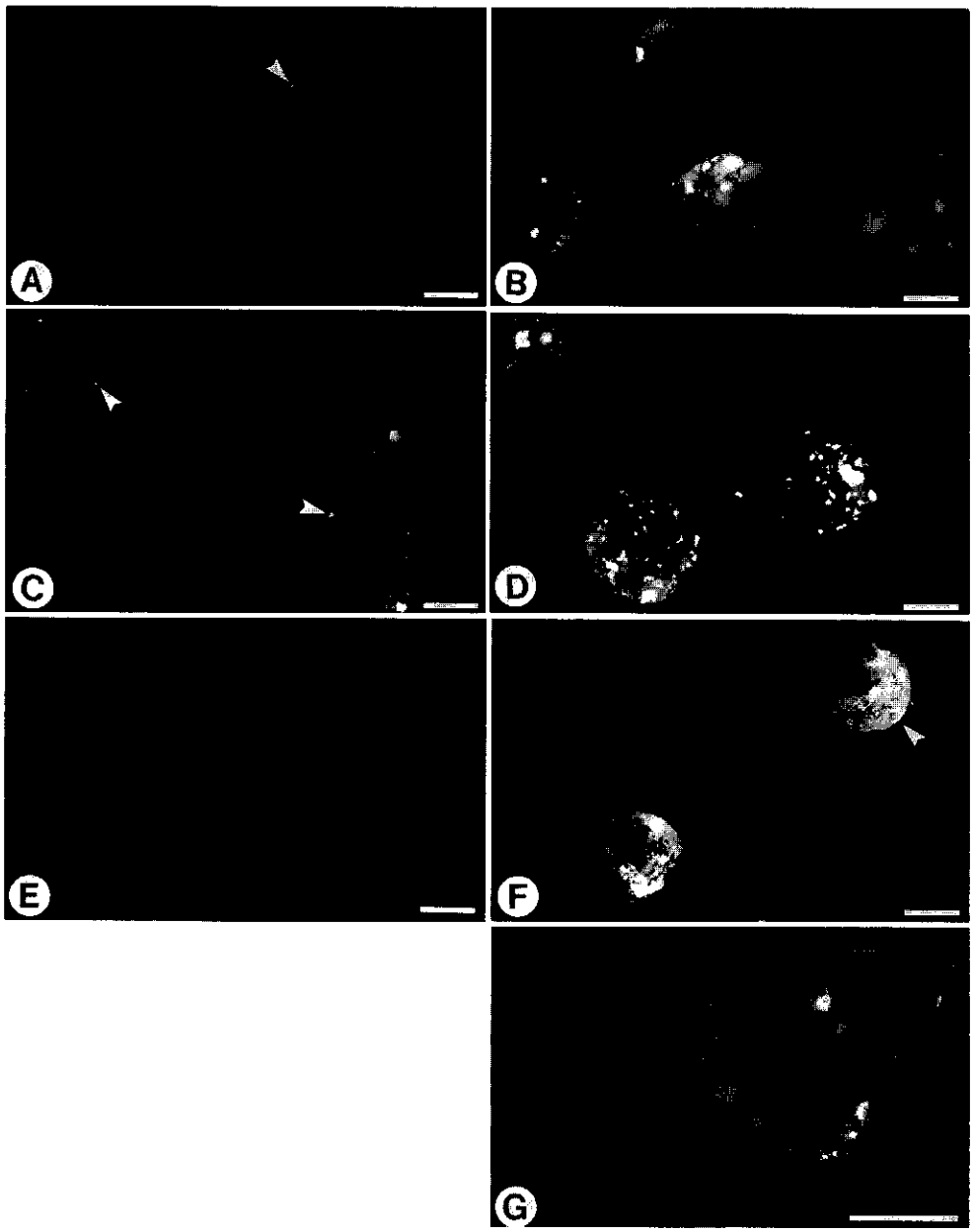


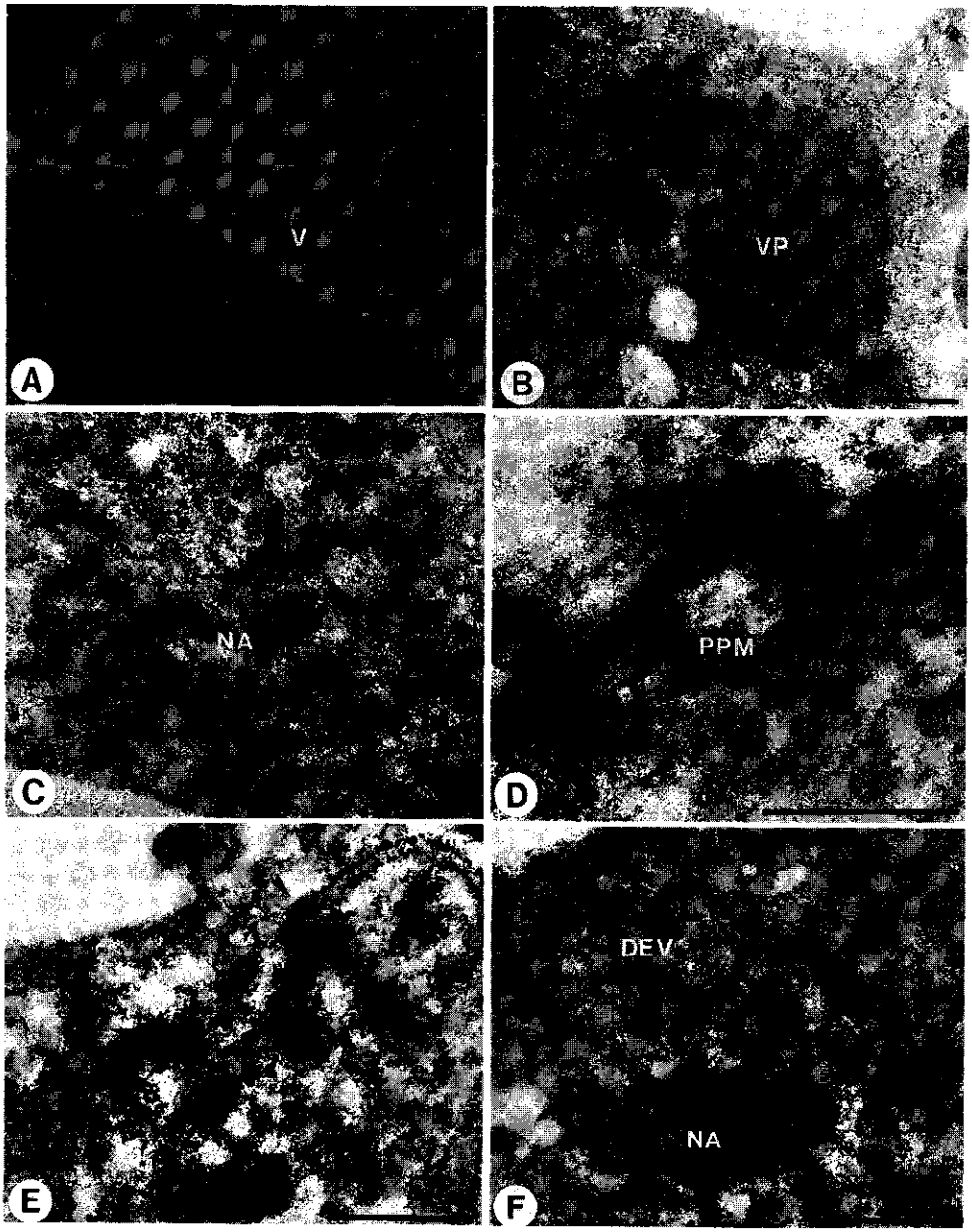
Figure 2.3 *Immunofluorescent images of N. rustica protoplasts at different times after TSWV inoculation. Clusters of inoculum virus adherent to the surface of inoculated protoplasts at 0 h p.i. are visualised (arrowheads) with anti-N serum (A) and anti G1/G2 serum (C). Panel (B) and (D) show intracellular labelling of newly produced structural proteins at 64 h p.i., using anti-N serum and anti-G1/G2 serum respectively. E and F show labelling of protoplasts using anti-NSs serum at 0 h p.i. and 64 h p.i. respectively, showing intracellular and cell-surface location (arrowhead) of newly produced NSs protein at 64 h p.i.. (G) Labelling of protoplasts with anti-NSm serum at 40 h p.i. showing tubular structures protruding from the cell surface. Bars represent 10 μm.*

Survival of TSWV-inoculated cells was similar for *N. rustica* and *V. unguiculata* protoplasts, and decreased from at least 75% at 0 h post-inoculation (p.i.) to a minimum of 50% at 90 h p.i.. In repeated experiments, generally 50% or more of both *N. rustica* and *V. unguiculata* protoplasts were infected, while synthesis of NSs became detectable as early as 16 h p.i. (see also Western blot results below). Highly infective inoculum, needed to reach such values, was best maintained by using virus preparations immediately after isolation. Freezing of purified virus in liquid nitrogen and slow thawing on ice still rendered 80% of the original infectivity, but storage of virus at -80 °C for more than a week led to a significant drop of infectivity, making results of protoplast inoculations less reproducible.

Viral protein and RNA synthesis. Using polyclonal antibodies, the synthesis of viral structural and non-structural proteins was followed in *N. rustica* and *V. unguiculata* protoplasts. The results showed that from about 16 h p.i. on, NSs was clearly detected in both systems (Fig. 2.1 a,b). At about the same time, production of the nucleoprotein (N) took place in both species, after an apparent partial degradation of the proteins of the inoculum during a latency period (Fig. 2.1 c,d). Viral glycoproteins (G1 and G2) were detected in *N. rustica* protoplasts (Fig. 2.1c), but surprisingly, in *V. unguiculata* protoplasts, these proteins did not accumulate appreciably (Fig. 2.1d).

Production of viral RNAs after inoculation of *N. rustica* protoplasts is shown in Fig. 2.2. After a latency period of about 16 h p.i., increasing amounts of full-length viral (v) and viral complementary (vc) RNA were produced, as well as subgenomic sized RNA species, that, according to their size, most probably represent viral mRNAs. The results are in accordance with the production of viral proteins as described above. When probed for L-RNA, specific smaller products were observed (Fig.2 c), indicating the presence of defective interfering (DI) RNAs (Resende et al., 1991). Protoplasts inoculated with UV-inactivated virus did not show any increase in the amount of viral protein (Fig. 2.1 a,c; lanes 3 and 4; Fig. 2.1 b,d; lanes 2 and 3) or viral RNA (Fig.2 a,b,c; lanes 3 and 4).

Immunofluorescence studies of inoculated N. rustica *protoplasts.* Using immunofluorescence microscopy, the intracellular location of viral proteins produced during protoplast infection was analysed. The results for *V. unguiculata* protoplasts were very similar to those for *N. rustica*, except for the lack of viral glycoprotein production; this paragraph therefore focuses on the results with *N. rustica*. Using antisera against N, G1 or G2, the inoculum virus was shown to adhere to the outside of the protoplasts at 0 h p.i. (Fig.3 a,c), whereas at times later than 16 h p.i. internal production of the structural viral proteins became evident (Fig.3 b,d). The structural proteins appeared to be located in patches (Fig.3 b,d) whereas NSs (Fig. 2.3 f) was found more dispersed throughout the cytoplasm. *De novo* synthesised N, G1 and G2 were never seen at the cell surface, whereas NSs did appear at the cell surface later in the infection (Fig.3 f). The disperse occurrence of NSs in the cytoplasm fits earlier studies (Kitajima et al., 1992a) which showed for isolate BR-01 that this nonstructural protein does not form fibrous aggregates, as do some other TSWV isolates.The second nonstructural protein of TSWV, NSm, could also be monitored in infected *N. rustica* protoplasts. Kormelink *et al.* (Kormelink et al., 1994) and Storms *et al.* (Storms et al., 1995) have shown that this protein is involved in cell-to-cell translocation of the virus. In infected tissue NSm is targeted to plasmodesmata and subsequently aggregated into (plasmodesmata-penetrating) tubules (Storms et al., 1995). Although protoplasts do, of course, lack plasmodesmata, NSm appears to be correctly addressed in these cells. Immunofluorescent analysis of *N. rustica* protoplasts 40 h p.i. revealed the presence of long, NSm-containing tubules which extend from the surface of the cell (Fig. 2.3 g).


V
A
VP
B
NA
C
PPM
D
E
DEV
NA
F

Electron microscopy. Immediately after inoculation, only a few clusters of inoculum virus were recognised at the periphery of *N. rustica* (see Fig. 2.3 a,c) and *V. unguiculata* protoplasts (Fig. 2.4 a). Later, viroplasm and nucleocapsid aggregates that could be labelled with antibodies against N were observed in the cytoplasm of inoculated *V. unguiculata* protoplasts (Fig. 2.4 b,c), but only very few enveloped particles were found (data not shown). This low incidence of enveloped particles in *V. unguiculata* protoplasts fits the observation that in this cell system the viral glycoprotein synthesis is hampered (Fig. 2.1 d). In *N. rustica* protoplasts however, enveloped progeny virus was frequently found in the cytoplasm, often associated with ER membranes, starting from 16 h p.i. and increasing with time (Fig. 2.4 e, f). This confirms our earlier conclusion that in this system the TSWV infection cycle is efficiently completed. In addition, other cytopathological structures characteristic of TSWV infection (Kitajima, 1965; Francki and Grivell, 1970; Milne, 1970; Ie, 1971; Kitajima et al., 1992a; Lawson et al., 1996) were also encountered in inoculated *N. rustica* protoplasts such as viroplasm (exemplified for *V. unguiculata* in Fig. 2.4 b), nucleocapsid aggregates (Fig.4 f) and paired parallel membranes (Fig. 2.4 d).

Infectivity of viral products produced in N. rustica *protoplasts.* To obtain further evidence that inoculation of protoplasts with TSWV resulted in newly synthesised infectious virus, petunia leaves were inoculated with samples of TSWV-inoculated *N. rustica* protoplasts. Protoplasts inoculated with TSWV and immediately applied to petunia leaves only occasionally generated a few lesions (1-2 lesions per million protoplasts), due to the inoculum virus, but usually no lesions at all. In three independent experiments (using different batches of virus) the *N. rustica* protoplast suspensions harvested 40-64 h p.i., generated a clear increase in local lesion numbers (5-24 lesions per million protoplasts), with the infection percentages being relatively low in these experiments (30-40%).

Discussion

Development of a versatile analytical infection system, an obvious goal in research on tospoviruses, has not been reported before. Most likely a major problem has been, and still remains, the instability of the virus once isolated from the plant (Brunt et al., 1996). This problem can be partially circumvented by using a fast and efficient protocol for particle isolation, and by immediate use of the preparations. *In vitro* grown *N. rustica* plants gave better and more reproducible results than greenhouse grown plants, probably due to a better susceptibility of plants grown *in vitro*, and smaller seasonal effects.

The results presented in this paper show that a complete infection cycle is accomplished in protoplasts, and the characteristics are very similar to what has previously been observed in infection of whole plants. The occurrence of tubular structures containing NSm on the surface of inoculated protoplasts, similar to those observed in protoplasts from pre-infected leaves (Storms et al., 1995), underscores the utility of the developed protoplast inoculation protocol for studying the tospoviral infection cycle.

Figure 2.4 *Immuno-electron microscopical analysis of TSWV-inoculated N. rustica and V. unguiculata protoplasts. (A) Inoculum virus (V) adherent to V. unguiculata protoplasts at 0 h p.i. and labelled with anti-G1 serum; (B) Viroplasm (VP) labelled with anti-N serum in V. unguiculata protoplasts at 40 h p.i.; (C) Accumulation of nucleocapsid aggregates (NA) in V. unguiculata protoplasts at 52 h p.i. labelled with anti-N serum; (D) Paired parallel membranes (PPM) surrounded by viroplasm in N. rustica protoplasts 40 h p.i. labelled with anti-N serum; (E) Virus particles associated with and surrounded by ER membranes (arrowheads) in N. rustica protoplasts 40 h p.i. labelled with anti-G2 serum; (F) Nucleocapsid aggregates and doubly enveloped virus (DEV) particles in inoculated N. rustica protoplasts 40 h p.i. labelled with anti -G1 serum. Bars represent 1 μm (panel A), or 0.5 μm (panel B, C, D, E and F).*

Additionally, in analogy to the expression of NSs, the synthesis of NSm is an indication for replication of TSWV input material.

It appeared that TSWV infection in *N. rustica* protoplasts is more efficient than in *V. unguiculata* (cowpea) protoplasts. Particularly the production of viral glycoproteins, and subsequently enveloped particles, seemed hampered in *V. unguiculata* protoplasts, whereas the production of mature particles in *N. rustica* protoplasts was abundant. Electron microscopy on thin sections also showed that at whole plant level, the production of enveloped particles was hampered in *V. unguiculata* (data not shown). Apparently, *V. unguiculata* is a less suitable natural host than *N. rustica*, and our results indicate that the use of *N. rustica* protoplasts for studying TSWV infection should be preferred.

The system is still relatively fragile, due to the instability of virus preparations and varying susceptibility of protoplasts, but it does make several interesting experiments possible. Specific agents that influence intracellular processes can now be applied, to gain insight on a number of aspects of the infection cycle. Among those are tunicamycin (which inhibits N-linked glycosylation) and Brefeldin A (which inhibits protein transport from the ER to the Golgi-system in plants; Satiat-Jeunemaitre and Hawes, 1992a; Satiat-Jeunemaitre and Hawes, 1992b). The synchronism of the infection in the protoplast system also provides potential to investigate the switch between transcription and replication that takes place during an infection. More detailed time-course experiments should pin-point this switch, and by using protoplasts from N-protein expressing transgenic plants (Gielen et al., 1991), the role of the level of nucleoprotein in inducing the switch can be investigated. The possibility of using protoplasts from transgenic plants also provides a tool for checking TSWV resistance at the cellular level. Experiments of this kind have recently been successfully performed in our lab by Prins *et al.* (Prins et al., 1997).

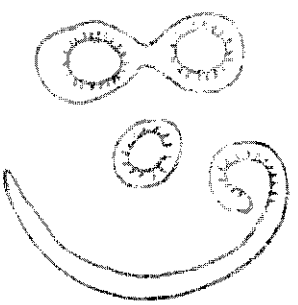
Another obvious application for the developed system is the detailed investigation of TSWV particle maturation. Since the infection in protoplasts is far more synchronous than in whole plants, we may well be able to draw conclusions about the chronology of the events observed in infection. Immunofluorescence studies have given some preliminary indications for certain aspects of maturation. Both nucleocapsids and glycoproteins are found clustered in patches in late stages of the infection (Fig. 2.3 b,d), most probably depicting the accumulation of mature particles within (ER-) membranes grouped within the cytoplasm (Fig. 2.4 e). In earlier stages, when few mature particles were formed, smaller patches were seen in immunofluorescence tests when looking at the glycoproteins, which might indicate that free glycoprotein tends to be concentrated at certain cellular sites. When using a marker for the plant Golgi apparatus, the pattern co-localised with the glycoproteins (data not shown). This leads to the suggestion that by analogy with the animal-infecting bunyaviruses, tospovirus glycoproteins accumulate in the Golgi-system where possibly budding of TSWV particles also may take place. By exploiting the single cell infection system described here, and by using detailed time-course analyses and specific inhibitors, we should be able to further unravel the maturation pathway of TSWV.

Acknowledgements

We thank Cor Meurs for skillful technical assistance, and Claire Pacot-Hiriart for setting up the *in vitro N. rustica* plants. This research was supported in part by the Netherlands Foundation for Chemical research (S.O.N.) and the Netherlands Organisation for Scientific research (N.W.O.).

CHAPTER 3

TOMATO SPOTTED WILT VIRUS PARTICLE MORPHOGENESIS IN PLANT CELLS



Kikkert, M., J. van Lent, M. Storms, P. Bodegom, R. Kormelink, and R. Goldbach. (1999) Tomato spotted wilt virus particle morphogenesis in plant cells. *Journal of Virology* 73, 2288-2297.

Abstract

A model for the maturation of tomato spotted wilt virus (TSWV) particles is proposed, mainly based on results with a protoplast infection system, in which the chronology of different maturation events could be determined. Using specific monoclonal and polyclonal antisera in immunofluorescence and electron microscopy the site of TSWV particle morphogenesis was determined to be the Golgi system. The viral glycoproteins G1 and G2 accumulate in the Golgi prior to a process of wrapping, by which the viral nucleocapsids obtain a double membrane. In a later stage of the maturation, these doubly enveloped particles fuse together and to the ER to form singly enveloped particles clustered in membranes. Similarities and differences between the maturation of animal infecting (bunya)viruses and the plant infecting tospoviruses are discussed.

Introduction

Increased knowledge of the molecular biology of tomato spotted wilt virus (TSWV), type species of the genus *Tospovirus*, has shed light on the replication and gene coding strategy of this plant-infecting bunyavirus (for reviews see German et al., 1992, Goldbach and Peters, 1996, Goldbach et al., 1992, Mumford et al., 1996, van Poelwijk et al., 1996). In contrast, a clear view of the TSWV particle maturation pathway in plant cells has not yet been reported, mainly due to the fact that useful single cell systems to study this process have been lacking. Early electron microscopical studies by Milne (Milne, 1970) and Ie (Ie, 1971) using infected leaf tissues, summarized typical structures associated with tospovirus infections that were observed, such as viroplasm (VP), nucleocapsid aggregates (NCA), paired parallel membranes (PPM) thought to be involved in budding events, doubly enveloped particles (DEV), and singly enveloped particles (SEV) clustered within ER membranes. It was observed that VP, NCA, PPM and DEV were present mostly in early stages of infection, whereas clustered SEV seemed to be a late, or even final state in the maturation. Since TSWV is an enveloped bunyavirus it is anticipated that pre-existing, intracellular membranes are used for enveloping, although it has been reported (Milne, 1970) that the PPM may be produced *de novo* as they did not seem to resemble any of the cellular membrane structures. More recently however, Kitajima and co-workers (Kitajima et al., 1992a) presented three possible models for the morphogenesis of tospovirus particles, which included morphogenesis at the intracellular membranes of the ER or the Golgi-system, although no conclusive choice could be made between the models proposed. While studying defective and non-defective isolates of impatiens necrotic spot virus (INSV), Lawson and co-workers (Lawson et al., 1996) suggested that PPM, showing budding structures, could be Golgi derived, based on their morphology. None of the above studies however presented extensive labeling data that supported either of the different models on the maturation pathway of tospoviruses in plants.

Structures, possibly associated with morphogenesis of TSWV have also been observed in cells of the thrips vector *Frankliniella occidentalis* (Ullman et al., 1995). Both N and G1/G2 proteins were immuno-localized to intracellular membranes, which were suggested to be Golgi derived again based on their morphology, in cells of the midgut epithelium. However, no intermediate structures associated with budding virus were observed in these membranes, and no mature particles were observed in the midgut.

Since the study of non-synchronous infections have hampered the understanding of the morphogenesis process of TSWV and other tospoviruses in plants, we now applied the recently developed protoplast infection system (Kikkert et al., 1997) to study this. Both cowpea (*Vigna unguiculata*) and *Nicotiana rustica* protoplast suspensions have been shown to support TSWV multiplication, reaching at least 50% of infection, provided that freshly prepared, highly infectious virus preparations are used as inoculum (Kikkert et al., 1997). The

TABLE 3.1. Relative appearance of TSWV maturation associated structures and intracellular membranes during TSWV infection in protoplasts[a]

h.p.i.	VP	NCA	PPM	DEV	SEV	ER	Golgi
0	-	-	-	-	-	+	+
18	+	+	++	+	-	+	+
22	+	+	++	+	+	+	+/-
26	+	++	+	++	+	+	+/-
30	+	++	+/-	+	++	+	+/-
40	+	++	-	-	++	+	-

[a]Judgement of relative presence of structures within one timepoint was based on at least three different specimens with 10 to 20 infected cells each.

application of a protoplast system not only has the advantage of a high level of infected cells, but also allows a temporal analysis of the maturation pathway since a high synchrony of infection is obtained. It has been shown that in cowpea protoplasts the infection cycle leads to only low amounts of enveloped particles, whereas in *N. rustica* protoplasts large amounts of mature particles accumulate, and a complete infection is accomplished (Kikkert et al., 1997). Therefore the system based on *N. rustica* protoplasts was used in the present studies to investigate the chronology of the TSWV maturation.

Materials and methods

Virus, plants and protoplasts. Throughout this study, a Brazilian isolate of TSWV, BR01 (de Avila et al., 1993a) was used, and maintained in *Nicotiana rustica* plants by mechanical inoculation and transmission by thrips. For (cryo-)electron microscopical analysis, *N. rustica* plants were harvested 5 to 10 days after inoculation with TSWV, and local lesions were isolated from petunia (cv. Polo Blauw) 3 to 5 days after inoculation with TSWV. *N. rustica* protoplasts were inoculated with freshly isolated TSWV particles as described earlier (Kikkert et al., 1997), and harvested for immunofluorescence microscopy, (immuno-)electron microscopy or Western blots between 0 hours post inoculation (h.p.i.) and 40 h.p.i..

Antisera. Polyclonal antisera against TSWV N protein, and the hydrophylic ectodomains of G1 and G2, were raised as earlier described (Kikkert et al., 1997). The rat monoclonal IgM serum against the plant Golgi-system (JIM84) has been described earlier (Horsley et al., 1993), anti-ßF$_1$ against the plant Golgi-system has been described (Laurière et al., 1989) as well as anti-RGP1 (3).

Immunofluorescence light-microscopy. Immunofluorescence of protoplasts was performed as described earlier (Kikkert et al., 1997). Double labelings were performed using a combination of rabbit polyclonal antiserum and rat monoclonal antiserum, with swine anti-rabbit serum conjugated to tetramethylrhodamine isothiocyanate (TRITC) and goat anti-rat serum conjugated to fluorescein isothiocyanate (FITC) as respective second antibodies.

Figure 3.1 *Overview of TSWV maturation associated structures found in infected N. rustica protoplasts. (A) NCA embedded in VP, immuno-gold labeled with antiserum against N at 26 h.p.i.. (B) Large cluster of VP/NCA as found in late stages of infection at 40 h.p.i.. (C) Detail of (B) (boxed) showing DEV and ER on the edge of the VP/NCA cluster labeled with antiserum against N. (D) SEV clusters surrounded by membrane envelopes (membranes indicated with arrow) and NCA close to PPM structures at 30 h.p.i.. Bars represent 200 nm.*

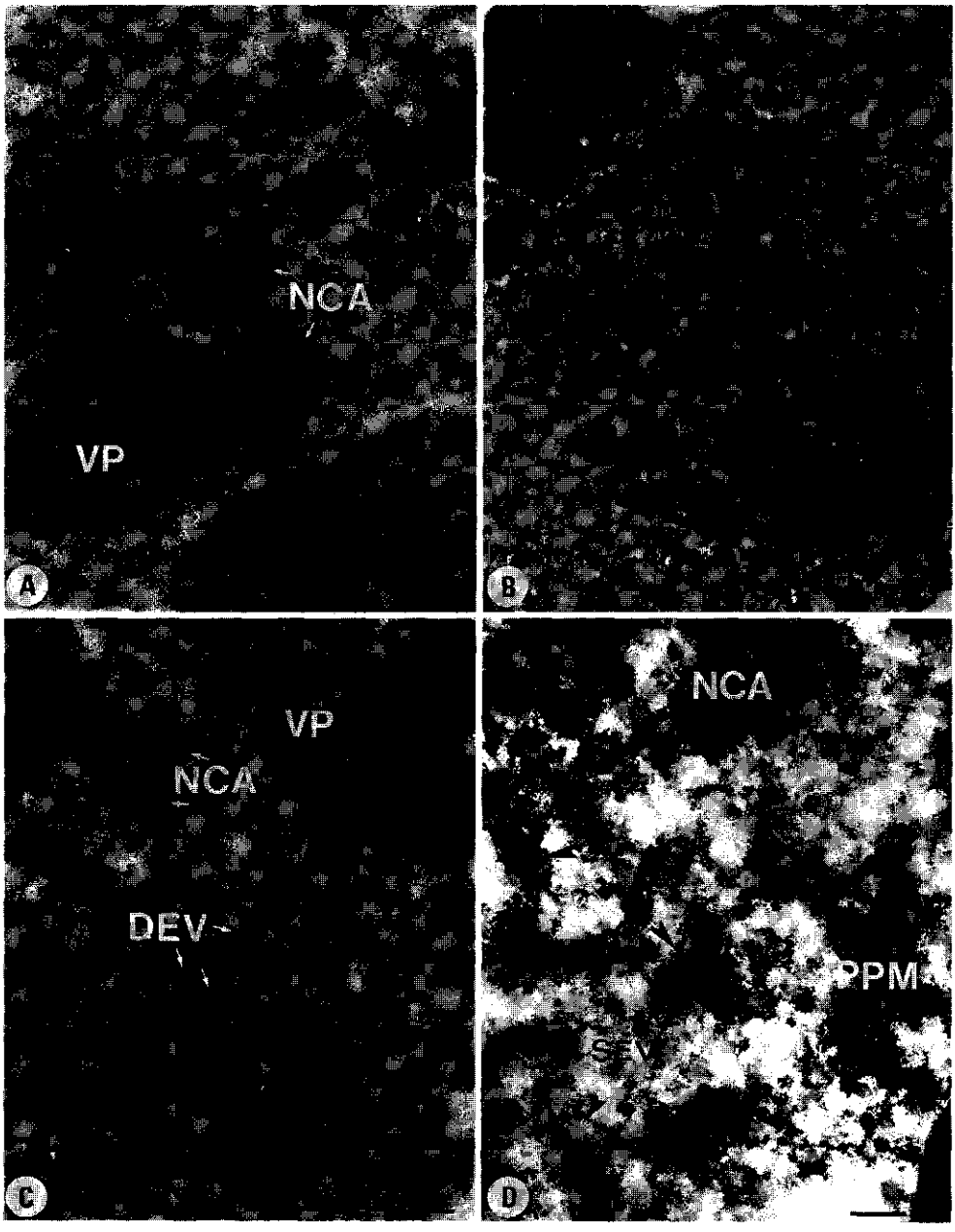

NCA
VP
A
B
VP
NCA
DEV
C
NCA
PPM
SV
D

TABLE 3.2. Reactivity of TSWV maturation associated structures and intracellular membranes with different antisera in immuno-gold electron microscopy, in TSWV infected protoplasts[b]

antiserum	VP	NCA	PPM	DEV	SEV	ER	Golgi
α N	+	++	+	+	+	-	-
α G1/G2	+/-	+/-	+	+	+	-	-
α-ßF1	-	-	+	+	+	-	+
α RGP1	-	-	+	-	-	-	+

[b]Samples were taken between 0 and 40 h.p.i. as indicated in Table 3.1.

(Immuno-) electron-microscopy. Immuno-electron microscopy of protoplasts fixed with 3% glutaraldehyde (GA)/ 2% paraformaldehyde (PF) was performed as described earlier, as well as ultrastructural analysis using osmium tetroxide fixation (Kikkert et al., 1997).

Cryo-electron-microscopy. Aldehyde fixation, infiltration with sucrose, cryosectioning and negative staining of infected leaf material was carried out as described in van der Wel *et al.* (van der Wel et al., 1998).

Western blotting. Sodium dodecyl sulfate-poly acrylamide gel electrophoresis and Western immunoblot analysis using alkaline phosphatase detection was carried out as described in Kikkert *et al.* (Kikkert et al., 1997).

Results

Chronology of appearance, and labeling of structures associated with maturation. N. rustica protoplasts were inoculated with TSWV particles using poly-ethylene glycol, and samples were taken between 0 and 40 h.p.i. for immunofluorescence and electron microscopy analyses. Table 3.1 shows the appearance of different structures that have previously been reported to be associated with TSWV maturation (Ie, 1971, Kitajima et al., 1992a,Milne, 1970) at different time-points during the infection in protoplasts. Table 3.2 summarizes the reactivity of these maturation-associated structures with antisera used in electron microscopy, as will be discussed further throughout the results and discussion sections.

Examples of the different structures are depicted in Figure 3.1. VP (Fig. 3.1A), characterized as amorphous medium-dense material, was seen throughout the infection, very often in association with NCA, which is much more dense (Fig. 3.1A) and which often appears as aggregated nodules of particular size (around 60-80 nm) embedded in VP (Fig. 3.1). Both structures strongly label with antiserum against the nucleocapsid protein N (Fig. 3.1A,C and Table 3.2), suggesting that both contain viral nucleocapsid protein. They only slightly label with serum against the viral glycoproteins G1 and G2 (Table 3.2, data not shown). In early stages of infection mostly small patches of VP and NCA dispersed throughout the cytoplasm were observed (Fig. 3.1A), whereas in later stages often only one or two very large areas of NCA and VP were found (Fig. 3.1B).

PPM are characterized as membrane cisternae that are often strongly curved, and dense material is tightly associated with them (Fig. 3.1D). PPM have earlier been suggested to be derived from Golgi stacks (20) and the modification of Golgi stacks that was observed in protoplast infections can indeed be interpreted as the formation of PPM (Fig. 3.2). In infected cells the cisternae of a Golgi stack seem to drift apart, dense material accumulating in between them (Fig. 3.2B). The cisternae curve and often seem extended (Fig. 3.2C). The formation of PPM was quite obvious in protoplast infections at several time-points, but a clear peak of their appearance was found in early stages of infection around 18-22 h.p.i. (Table

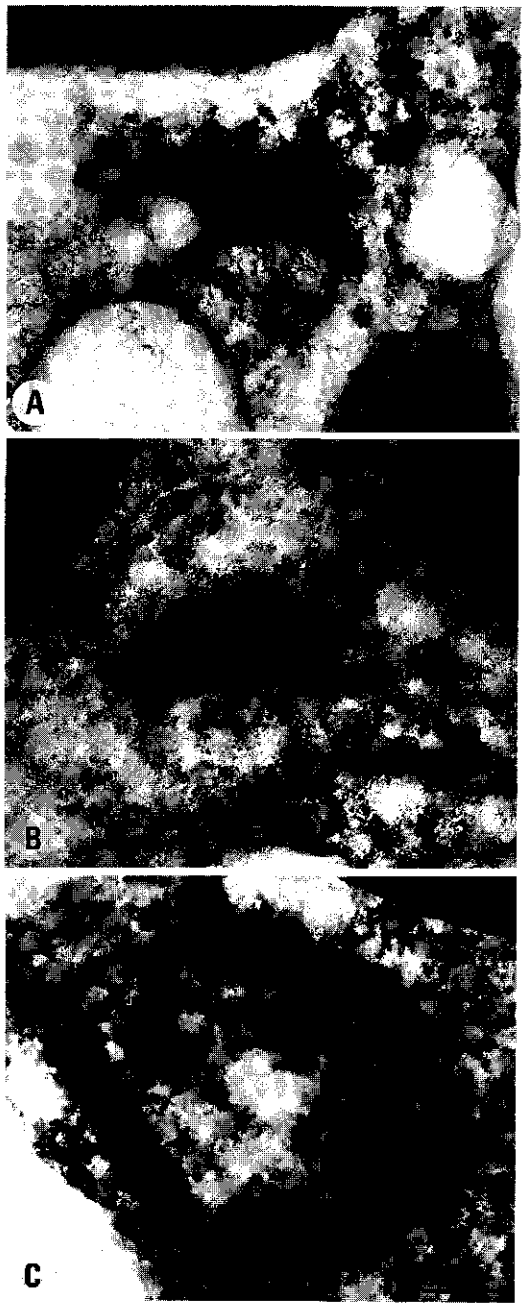


Figure 3.2 *Formation of PPM structures by modification of Golgi stacks. (A) Apparently unmodified Golgi stack at 18 h.p.i.. (B) Putative, modified Golgi stack with cisternae moving away from each other and dense material, presumably nucleocapsids, accumulating in between them at 18 h.p.i.. (C) PPM structure showing extended and curved Golgi cisternae presumably surrounded by nucleocapsid material, 18 h.p.i.. Bars represent 200nm.*

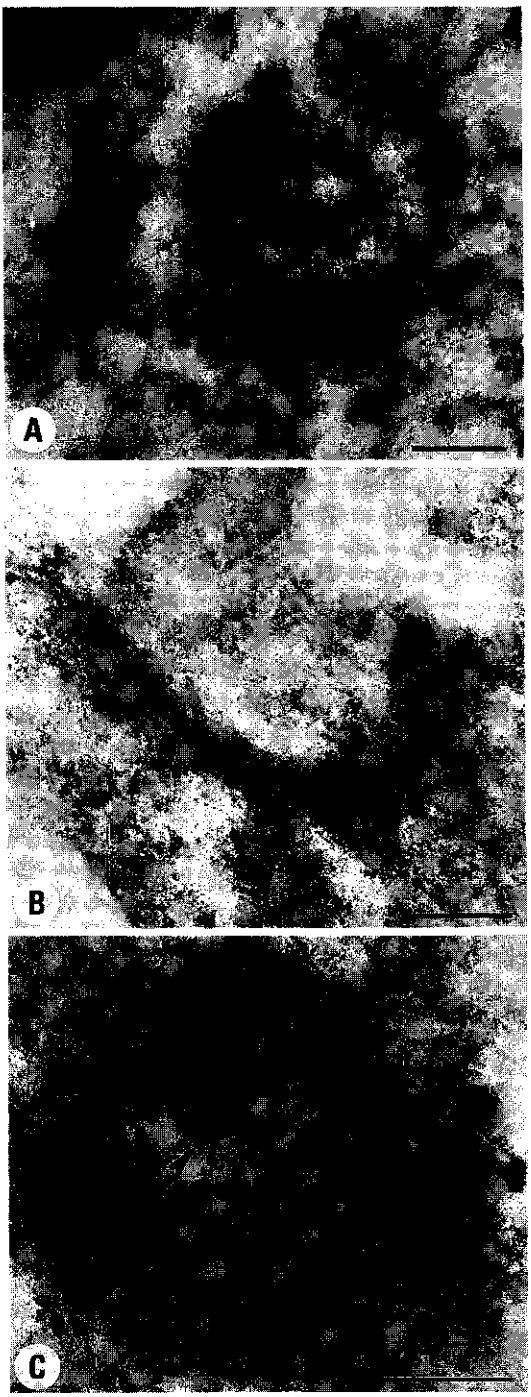


Figure 3.3 *Immuno-gold labeling of PPM structures with antiserum against G1 (A), G2 (B) and N (C), all at 22 h.p.i.. Arrows indicate DEV particles being formed. Bars represent 200 nm.*

3.1). PPM and their surroundings labeled with antiserum against G1 (Fig. 3.3A), G2 (Fig. 3.3B) and N proteins (Fig. 3.3C) (see also Table 3.2) in infected protoplasts, while these antisera gave no background on healthy plant cells (data not shown). Often DEV particles were found in the vicinity, which appear to form at the PPM (Fig. 3.3C arrows). To confirm that these structures found in protoplast infections were not artifacts of the system, early stages of systemic infections in *N. rustica* plants as well as TSWV local lesions in petunia were investigated. Particular clear examples of curving and wrapping PPM were found in these plants as well (Fig. 3.4). The data suggest that DEV are formed by curving and wrapping of modified Golgi cisternae around dense nucleocapsid material in the cytoplasm.

From Table 3.1 it becomes clear that PPM largely precedes the formation of DEV, and that SEV seem to represent a final stage in the infection. At late stages of infection (30-40 h.p.i.) PPM as well as DEV were rarely found any more, whereas SEV surrounded by membranes were abundant, often found near the large NCA clusters (Table 3.1 and Fig. 3.1D). ER membranes were found throughout the infection, often in the vicinity of maturation-associated structures (Fig. 3.1A,B,C). Apparently unmodified Golgi-stacks however, became more scarce during the infection process, and were virtually absent at late stages.

The Golgi-system is the site of TSWV enveloped particle morphogenesis. Since the morphology of PPM structures strongly suggests that they are derived from Golgi-stacks, three independent plant-Golgi markers were used to confirm this. JIM84, a rat monoclonal antiserum raised against carrot Golgi protein epitopes (Horsley et al., 1993), anti-ßF$_1$, a polyclonal antiserum raised in carrot against complex glycans common to plant Golgi glycoproteins (Laurière et al., 1989), and anti-RGP1, a polyclonal antiserum against the peptide fraction of a reversibly glycosylated trans-Golgi protein from pea (Dhugga et al., 1997) were used. As JIM84 is a monoclonal antiserum, it could be used in double-labelings together with polyclonal sera against viral proteins in immunofluorescence microscopy. JIM84 was raised against carrot Golgi epitopes, and thus it was verified whether it can also recognize the Golgi-system of *N. rustica*. Indeed, in healthy *N. rustica* protoplasts, or at 0 h.p.i., JIM84 produced a typical pattern of small spots scattered throughout the cytoplasm representing individual Golgi-stacks (Fig. 3.5A), as also observed in carrot, onion and maize cells using JIM84 (Horsley et al., 1993). As a result of transport of the epitope-containing protein, usually parts of the plasma membrane were labeled by JIM84 as well (Fig. 3.5A) (Horsley et al., 1993, Satiat-Jeunemaitre and Hawes, 1992a). During TSWV infection the Golgi system did not appear dramatically different, although larger clusters were increasingly observed using JIM84, besides the smaller ones (Fig. 3.5B,D). The viral glycoproteins co-localized at least partly with these Golgi structures at different time-points during the protoplast infection (Fig. 3.5B-E). Viral N protein also co-localized with the Golgi during protoplast infections (data not shown).

Both polyclonal sera anti-ßF$_1$ and anti-RGP1 labeled Golgi stacks in healthy *N. rustica* protoplasts, or protoplasts at 0 h.p.i., using immuno-gold electron microscopy (Fig. 3.6A,D). In infected protoplasts anti-ßF$_1$ labeled PPM structures (Fig. 3.6B) as well as virus particles (Fig. 3.6C). Anti-RGP1 labeled PPM very specifically, though not heavily (Fig. 3.6E,F), but did not tag virus particles (Table 3.2). Fig. 3.7 shows that in Western blots anti-ßF$_1$ crossreacts with G1 from virus particles, which most probably explains the labeling of virus particles. The serum however did not react with unglycosylated G1 expressed in *E. coli* (data not shown), indicating that the crossreaction is an interaction with the glycans of G1. Since the anti-ßF$_1$ serum was raised against Golgi-specific glycans (Laurière et al., 1989), the crossreaction may indicate that G1 contains epitopes for anti-ßF$_1$, confirming the Golgi targeting of TSWV glycoproteins.

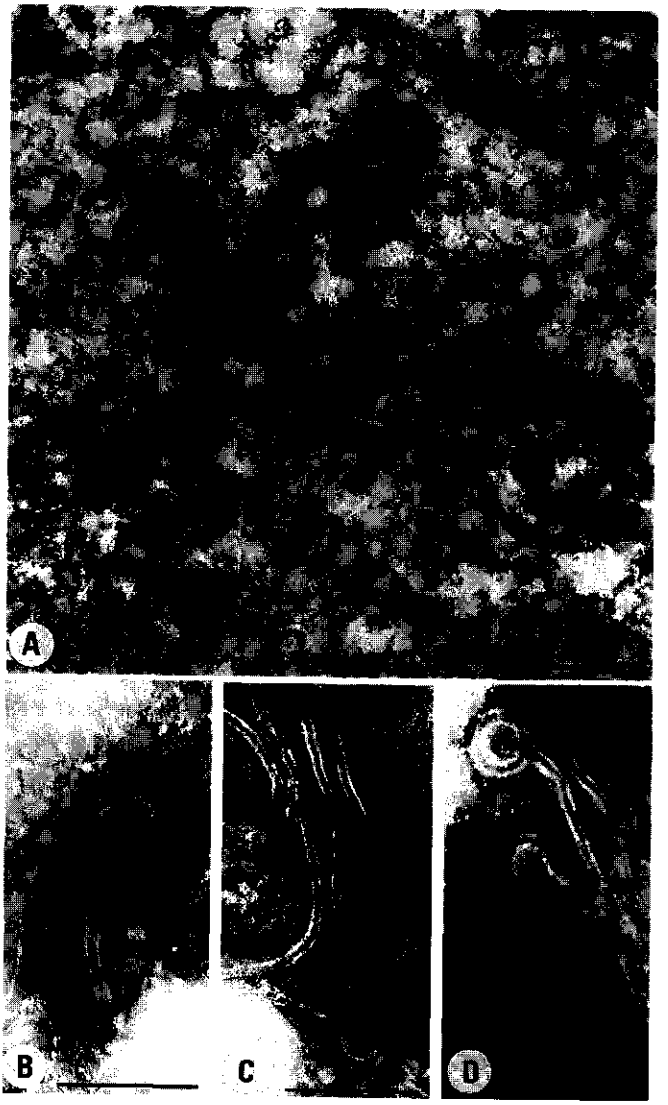


Figure 3.4 *PPM structures found in petunia local lesions (A) at 4 days post inoculation, and systemically infected N. rustica plants (B, C, D) at 5 days post inoculation, clearly showing formation of DEV. Bars represent 200 nm.*

These data together indicate that PPM are in fact derived from the Golgi, and form the site of particle morphogenesis.

SEV are produced by fusion of DEV. From osmium tetroxide fixed specimens of infected protoplasts, in which DEV and SEV can be clearly distinguished, it was observed that at stages around 26 h.p.i. SEV virus particles started to accumulate in clusters surrounded by membranes (see also Table 3.1), while DEV particles could also be observed in these areas (Fig. 3.8A, arrow). These kinds of structures were also found in petunia local lesions (not shown) as well as structures suggesting the formation of these SEV accumulations in

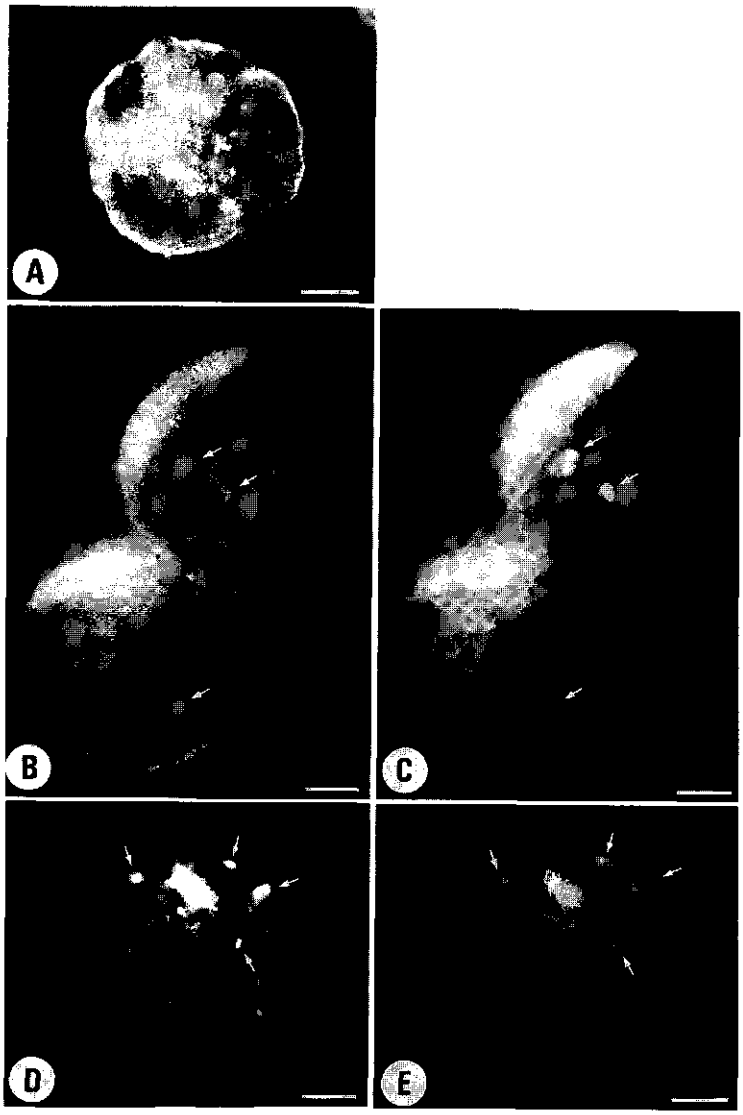
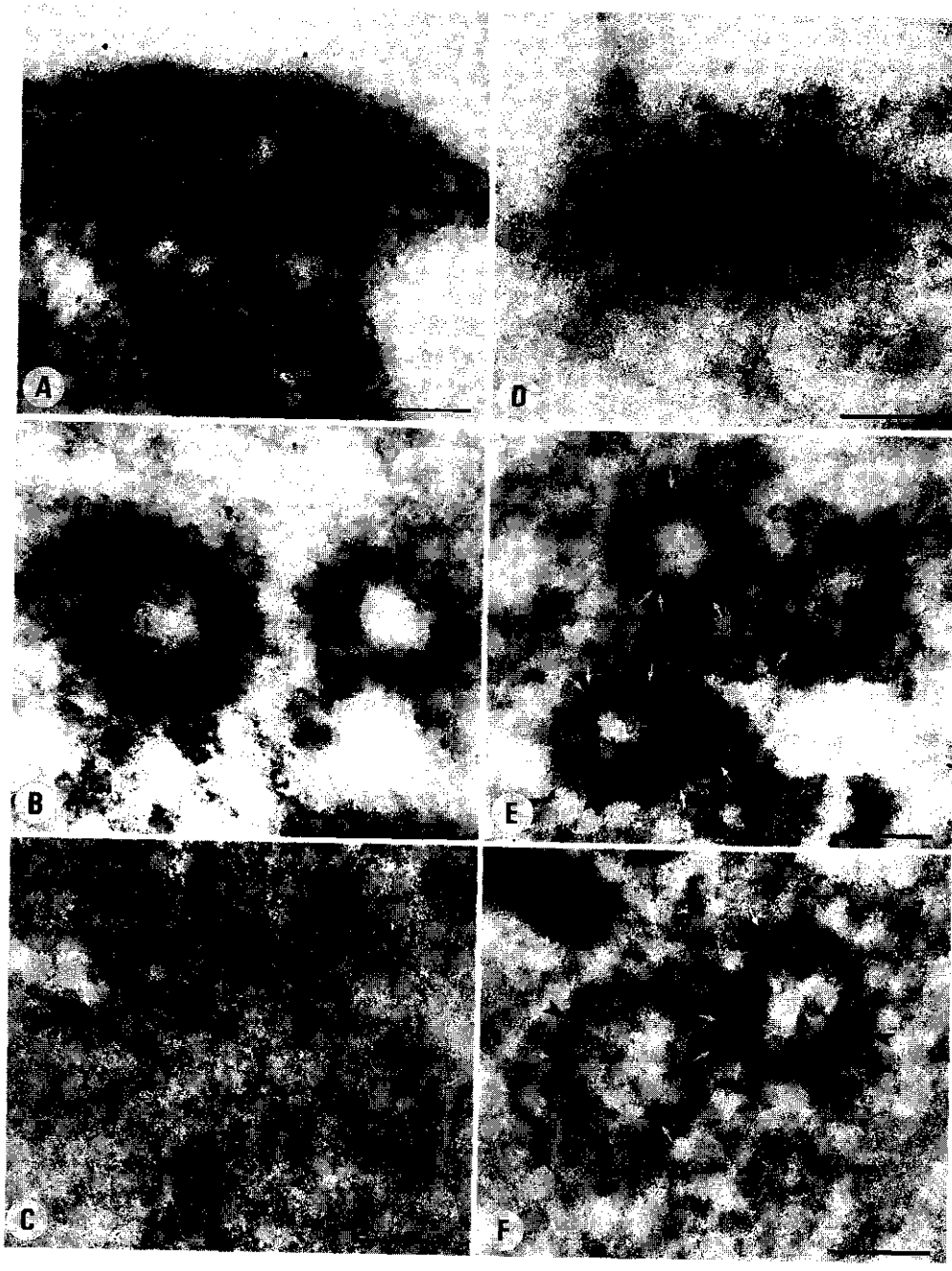


Figure 3.5 *Immunofluorescence images of N. rustica protoplasts. (A) Healthy protoplast labeled with JIM84 antiserum against the plant Golgi-system, showing individual Golgi stacks as small clusters throughout the cytoplasm. The plasma membrane also labels with JIM84 due to transport of the epitope-containing Golgi proteins. (B,D) TSWV infected protoplasts at 30 h.p.i. labeled with JIM84 antiserum. (C,E) The same infected protoplasts labeled with mixed antisera against G1 and G2. Areas of clear co-localization of the viral glycoproteins with the Golgi system are indicated with arrows. Cloudy areas within cells represent autofluorescence background. Bars represent 5 μm.*

membranes (Fig. 3.8B). Considering the chronology of the appearance of DEV and SEV (Table 3.1), these images suggest that SEV is formed by fusion of DEV. In this process, the outer membranes of DEV form a tight collective smooth envelope around single enveloped particles (Fig. 3.8B, small arrows). DEV seem also able, either individually or already fused together, to fuse to (rough) ER membranes (Fig. 3.8B, large arrows).


A
D
B
E
C
F

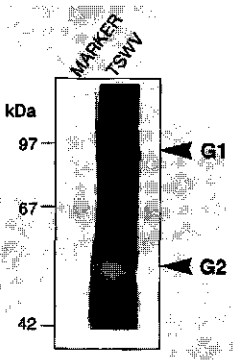


Figure 3.7 (right) *Western blot analysis using anti-βF_1 serum, showing crossreaction of this serum with G1 protein from purified TSWV particles. TSWV = Purified TSWV particles, MARKER = Low molecular weight marker proteins.*

Discussion

Using the recently developed *N. rustica* protoplast infection system, a time-course of TSWV particle maturation could be produced. Analysis of the maturation intermediates in these protoplasts as well as in petunia local lesions and systemically infected *N. rustica* plants leads to a TSWV particle maturation model (Fig. 3.9).

The use of three independent plant-Golgi markers confirmed the suggestion that PPM are derived from Golgi-stacks, and form the site of doubly enveloped particle morphogenesis.

The apparent retention of the viral glycoproteins G1 and G2 in the Golgi system is in agreement with the observations in immunofluorescence, which indicated that these proteins are not found on the surface of the plasma membrane (ref. 13, Fig. 3.5), while they are assumed to enter the secretory pathway due to their N-terminal signal peptide (Kormelink et al., 1992a). It is therefore likely that either one or both of the TSWV glycoproteins contain a Golgi retention signal, as recently documented for Uukuniemi virus (Andersson et al., 1997a).

In the proposed maturation model (Fig. 3.9), DEV is formed by wrapping of modified Golgi membranes (PPM) around nucleocapsids in the cytoplasm, and subsequently SEV is formed by fusion of DEV with each other or with ER membranes. Whether this latter process is based on an existing targeted membrane fusion mechanism in the cell, or whether it is a virally induced phenomenon remains to be investigated.

Apart from the structures that now have been identified as being involved in the particle morphogenesis, other virus-associated structures were also observed, but not clarified. VP in association with NCA, consisting of nucleocapsid protein, does not seem to be directly involved in particle morphogenesis. However, PPM and DEV are often found in the vicinity of VP and NCA (Fig. 3.1B,C), or are even embedded in them, as suggested by the labeling of NCA and VP with anti-G1/G2 sera (Table 3.2). NCA and VP thus could be a source of nucleocapsids meant to be wrapped with Golgi-membranes containing viral glycoproteins.

Still, a large part of the nucleocapsid protein that is produced during an infection is not used for producing particles, but seems to be stored in large NCA clusters (Fig. 3.1B,C). This is also evident from Western blots of crude extracts of infected plants and purified TSWV particles, where the ratio of nucleocapsid protein versus glycoproteins is much greater in crude extracts than in purified particles (data not shown). It is thought that TSWV moves

Figure 3.6 (left) *Immuno-gold labeling of maturation-associated structures with antisera against the plant Golgi-system. (A) Unmodified Golgi stack in healthy cell, labeled with anti-βF_1 serum. (B) PPM structure labeled with anti-βF_1 serum at 22 h.p.i.. (C) Virus particles labeled with anti-βF_1 serum at 40 h.p.i.. (D) Unmodified Golgi stack at 0 h.p.i., labeled with anti-RGP1 serum. (E,F) PPM structures labeled with anti-RGP1 serum at 26 h.p.i.. Small arrows indicate gold particles, larger arrows indicate PPM membranes. Bars represent 200 nm.*

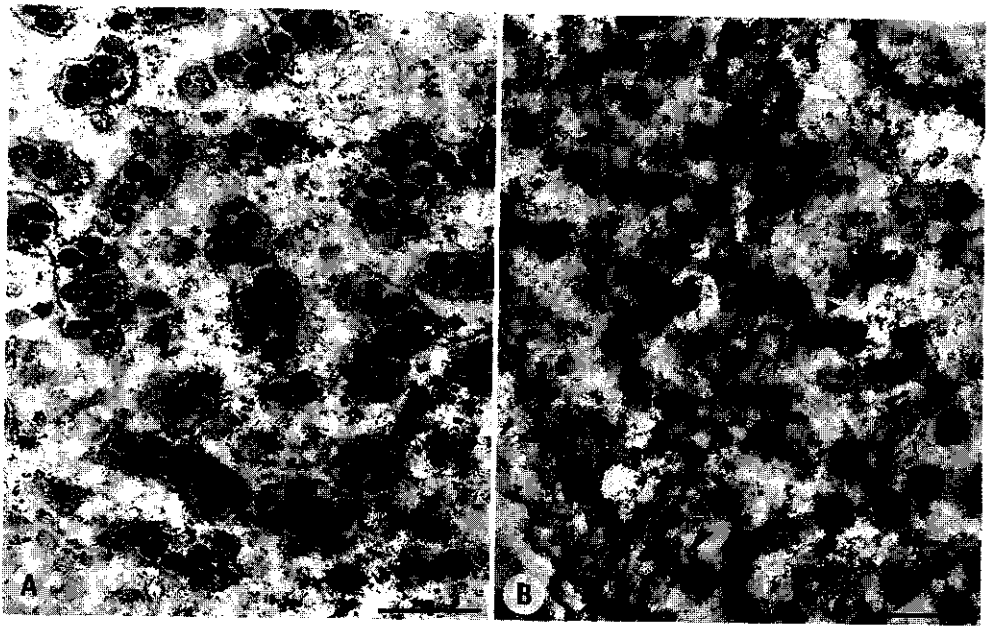


Figure 3.8 *The formation of SEV by fusion of DEV. (A) Clustered SEV inside smooth and rough membranes, as found in late stages of TSWV infections of N. rustica protoplasts at 40 h.p.i.. Note the DEV particle in the bottom of the image (arrow). (B) Image from TSWV local lesion in petunia showing DEV particles fusing together (small arrows) and with ER membranes identified by ribosomes on the surface (larger arrows). Bars represent 200 nm.*

from cell to cell by the action of NSm (non-structural protein encoded by the M-segment) which modifies the plasmodesmata so that infectious nucleocapsid units can pass the cell-barrier (Kormelink et al., 1994, Storms et al., 1995). This counts for a part of the overproduction of nucleocapsids, but most probably not for all.

The production of NSs protein has not been investigated here, but was observed in earlier studies (Ie, 1971, Kitajima et al., 1992a, Kormelink et al., 1991, Milne, 1970). So far, there is no evidence that this protein has a role in particle morphogenesis.

Although the TSWV maturation pathway as proposed here is very distinct from that of the animal infecting bunyaviruses (reviewed in Elliott, 1990, Griffiths and Rottier, 1992, Matsuoka et al., 1991, Pettersson, 1991,Stephens and Compans, 1988), the assembly of particles in the Golgi-system appears to be a common feature of both plant- and animal-infecting bunyaviruses. Modification of the Golgi-system, as observed in TSWV infections, has also been reported for Uukuniemi virus and Nairobi sheep virus (Gahmberg et al., 1986, Kuismanen et al., 1982,Rwambo et al., 1996), however instead of curling and wrapping, the Golgi cisterna vacuolize extensively during these animal bunyavirus infections, thus increasing the volume of the cisterna and allowing budding of singly enveloped particles into the lumen. The formation of doubly enveloped particles by wrapping of cisternae has never been reported for animal infecting bunyaviruses, however, it is not an unknown phenomenon in enveloped animal infecting dsDNA viruses. Vaccinia poxvirus is wrapped by membranes of the intermediate compartment (IC) between the ER and the Golgi, and in a later stage of the maturation by trans-Golgi-network membranes (Schmelz et al., 1994a,Sodeik et al., 1993). Varicella-zoster herpesvirus makes use of the trans-Golgi-network membranes for wrapping

its particles as well (Gershon et al., 1994,Whealy et al., 1991), and African swine fever virus is wrapped by ER membranes (Rouiller et al., 1998). The wrapping phenomenon has not been reported for any other plant-infecting virus, and it is not yet precisely clear which part of the Golgi is involved in TSWV morphogenesis.

The final stages of the particle maturation also seem to be different. Animal infecting bunyaviruses produce groups of particles inside vesicles pinched off from the Golgi (Matsuoka et al., 1991), and the ER has no role in particle morphogenesis. TSWV singly enveloped particles end up in large membrane envelopes as a result of self-fusion of DEV particles (which have two Golgi derived membranes), or fusion with ER membranes. These envelopes surrounding SEV clusters must consequently consist of both Golgi- and ER derived membranes.

In order to infect new cells, animal infecting bunyaviruses are transported to the plasma membrane and released into the cell's exterior via the vesicular transport pathway of the cell, whereas TSWV particles retain and accumulate in the plant cell until feeding thrips vectors ingest them for transport to other host plants.

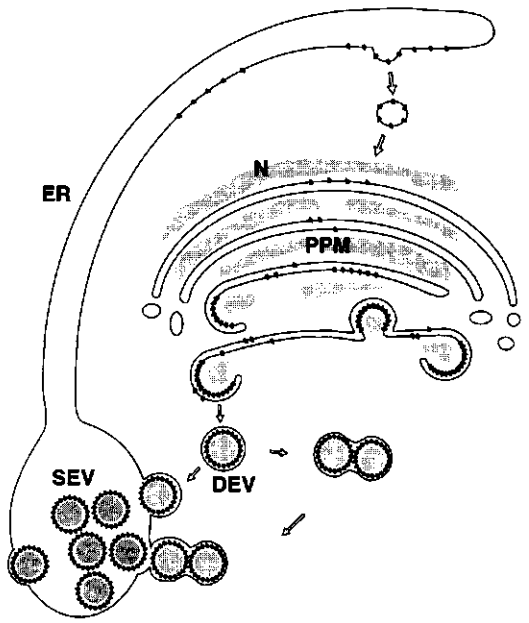


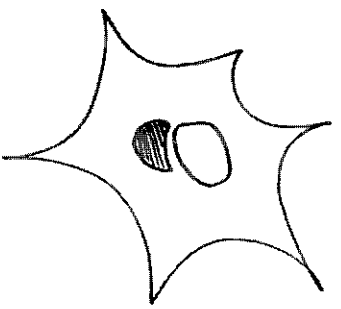
***Figure 3.9** Model of TSWV particle morphogenesis. ER = endoplasmic reticulum, N = nucleocapsid protein, PPM = Golgi derived paired parallel membranes, DEV = doubly enveloped particles, SEV = singly enveloped particles.*

Acknowledgments

We thank Dr Chris Hawes of the Oxford School of Biological and Molecular Sciences for kindly providing the rat monoclonal JIM84 and Dr Maarten Chrispeels of the University of California in La Jolla for kindly providing the polyclonal antiserum anti-ßF$_1$. We also thank Dr Kanwarpal Dhugga of Stanford University California for sending us the anti-RGP1 antiserum. We are grateful to Wiesje Kassies for excellent technical assistance, and to Dr Beatrice Satiat-Jeunemaitre and Dr Dick Peters for helpful discussions.

CHAPTER 4

TOMATO SPOTTED WILT VIRUS GLYCOPROTEINS EXHIBIT TRAFFICKING AND LOCALIZATION SIGNALS THAT ARE FUNCTIONAL IN MAMMALIAN CELLS



Marjolein Kikkert, Ad Verschoor, Richard Kormelink, Peter Rottier and Rob Goldbach. (1999). Tomato spotted wilt virus glycoproteins exhibit trafficking and localization signals that are functional in mammalian cells. Submitted for publication.

Abstract

The glycoprotein precursor (G1/G2) gene of tomato spotted wilt virus (TSWV) was successfully expressed in BHK cells using the Semliki Forest virus expression system. The results reveal that in this cell system the precursor is efficiently cleaved and that the resulting G1 and G2 glycoproteins are transported from the endoplasmic reticulum (ER) to the Golgi complex where they are retained, a process that could be blocked by tunicamycin. Expression of G2 alone resulted in transport to and retention in the Golgi complex, albeit less efficient, suggesting that G2 contains a Golgi retention signal. G1 alone was retained in the ER, irrespective of whether it contained the precursor's signal sequence or its own N-terminal hydrophobic sequence. Co-expression of G1 and G2 from separate gene constructs resulted in rescue of efficient G1 transport, as the proteins co-accumulated in the Golgi complex, indicating that their interaction is essential for proper targeting to this organel.

The results demonstrate that transport and targeting of the plant TSWV glycoproteins in mammalian BHK cells is strikingly similar to that of animal-infecting bunyavirus glycoproteins in mammalian cells. The observations are likely to reflect the dual tropism of TSWV, which replicates both in its plant host and in its animal (thrips) vector.

Introduction

Among the *Bunyaviridae* tomato spotted wilt virus (TSWV) takes a distinct position by its ability to infect plants rather than animals (Elliott, 1990, Francki et al., 1991, German et al., 1992, Goldbach et al., 1992, Goldbach and Peters, 1996,Mumford et al., 1996). Like the other bunyaviruses, TSWV particles carry a membrane envelope with viral spike proteins, a feature quite uncommon for plant infecting viruses, but rather typical among animal viruses. This led to the suggestion that an ancestral animal-infecting bunyavirus may have evolved into the plant-infecting tospoviruses, of which TSWV is the type species (Goldbach and Peters, 1996). TSWV also replicates in its animal (thrips) vector (Ullman et al., 1993,Wijkamp et al., 1993), indicating the dual tropism of this "shuttle" virus, which has to be able to produce virus particles in both plant and animal cells. The presence of the membrane glycoproteins is essential for the virus' ability to alternately replicate in its plant host and its thrips vector (Wijkamp, 1996). This is illustrated by the observation that when the thrips transmission cycle is bypassed by repeated mechanical inoculation of plants, mutants are generated that infect plants, but can no longer be transmitted by thrips (Wijkamp, 1996). This feature correlates with the loss of the viral glycoprotein containing envelope in these mutants (Ie, 1982, Resende et al., 1991,Verkleij and Peters, 1983). Apparently, the insect transmission cycle guarantees the maintenance of an intact envelope, because infection of the thrips vector is dependent on it and thus selective for it, while for infection of plants the envelope is dispensable.

The formation of the enveloped virus particles is strongly regulated by the viral glycoproteins. They generally accumulate independently at a particular cellular membrane by targeted transport through the secretory pathway, to facilitate the interaction with the viral nucleocapsids and the initiation of budding (reviewed in Griffiths and Rottier, 1992, Pettersson, 1991,Stephens and Compans, 1988). For animal infecting bunyaviruses this accumulation site was determined to be the Golgi system (reviewed inMatsuoka et al., 1991,Pettersson and Melin, 1996).

The morphogenesis of enveloped TSWV particles has recently been studied in a plant cell system, i.e. *Nicotiana rustica* protoplasts (Kikkert et al., 1997), and appeared to be a unique process, very distinct from the morphogenesis of animal infecting bunyaviruses (Kikkert et al., 1999). During infection of plant cells the TSWV structural proteins, including the glycoproteins, accumulate at the Golgi system, a feature also observed during animal

bunyavirus maturation (for Uukuniemi virus: Kuismanen et al., 1984; Kuismanen et al., 1982). Subsequently, however, doubly enveloped virus particles are formed as a result of wrapping of glycoprotein-containing Golgi cisternae around nucleocapsids in the cytoplasm. In a later stage, these doubly enveloped particles fuse with each other and with ER membranes giving rise to mature, singly enveloped particles, clustered inside large membrane sacks, where they accumulate and await the uptake by thrips for the transmission to other plants (Kikkert et al., 1999). In contrast, animal infecting bunyaviruses produce singly enveloped particles by direct budding of nucleocapsids into the lumen of glycoprotein-containing Golgi cisternae, without the formation of doubly enveloped particles as intermediates (reviewed in Elliott, 1996, Griffiths and Rottier, 1992, Matsuoka et al., 1991,Stephens and Compans, 1988). These particles are then excreted in order to infect neighbouring cells.

Obviously, the rigid cell wall of plants prevents the excretion of plant viruses from the cell, which explains the need to regulate the cell-to-cell transport alternatively, via the transport of infectious nucleocapsid units through the plasmodesmata (Kormelink et al., 1994,Storms et al., 1995). The cell wall also dictates the accumulation of virus particles within the plant cell, as opposed to the excretion of animal-infecting viruses into the bloodstream, illustrating an adapted vector transmission mechanism for plant-infecting TSWV. Assuming that TSWV has evolved from an ancestral animal bunyavirus, an intruiging question is then: in what way have TSWV glycoproteins changed to adapt to the distinct morphogenesis pathway in the plant host, while the virus additionally has to be able to replicate and produce particles in the animal vector?

In this report we addressed this question by expressing the TSWV glycoproteins in mammalian cells, using the Semliki Forest virus expression system, and studying their intracellular trafficking and accumulation behavior. In this way it could be verified whether the TSWV glycoproteins still contain the general transport and targeting signals characteristic of the glycoproteins of the animal-infecting bunyavirus ancestor, or whether the molecular features of TSWV glycoproteins have changed to meet the specific prerequisites for infection of its plant host.

Materials and methods

Cell culture. Baby hamster kidney cells (BHK-21) were maintained at 37 °C with 5% CO_2 in Glasgow MEM culture medium (Life Technology) supplemented with 10% foetal calf serum, tryptose phosphate broth, pencillin (100 U/ml) and streptomycin (100 µg/ml).

Antisera. Antibodies against the TSWV glycoproteins G1 and G2 were raised by immunization of rabbits with purified fragments of G1 and G2, expressed in *E. coli* using the pET11t system (Novagen) as described earlier (Kikkert et al., 1997). The monoclonal antibody 2B6 against G1 was a gift of Guenter Adam, and was described by Adam *et al.* (Adam et al., 1996). Antiserum against enveloped TSWV virus particles and against N were produced as described by de Ávila *et al.* (de Avila et al., 1993a). Monoclonal antibodies against the Intermediate Compartment (IC) p58 protein were kindly provided by J. Saraste, and described by Saraste *et al.* (Saraste et al., 1987) and Saraste and Svensson (Saraste and Svensson, 1991). The Golgi-stack marker anti-p58 was produced by Sigma. The chemical ER marker DiOC6 was purchased from Molecular Probes inc.

Construction of recombinants. The pSFV1 vector (GIBCO-BRL, Life Technology inc.), containing a NruI linearization site (designated pSFV1-N), was used for cloning and expression in BHK cells. The BamHI site of the multiple cloning site was used to insert cDNA of the G1/G2 precursor gene of TSWV. Mutants of the precursor were produced by

PCR, using specific primers containing a start-codon at the 5' end and a stop-codon at the 3' end of the gene fragment, flanked by a BamHI restriction site for feasible cloning into the SFV1-N vector. The mutant fragments were first cloned into a pGEM-T vector (Promega) or pSK(-) (Stratagene) and verified by sequencing prior to subcloning into the BamHI site of the SFV1-N vector.

Recombinants in which the signal sequence of the N-terminus (amino acid 1-35) of the precursor (Kormelink et al., 1992a) was linked in frame to the G1 coding sequence (from amino acid 486, see Fig. 4.1), were produced using a modified ExSite ™ (Stratagene) mutagenesis procedure as follows: cDNA encompassing the glycoprotein precursor ORF was cloned into the BamHI site of pSKδ, a pSK(-) vector which lacked the stretch of restriction sites from Sma I until HincII of the polylinker, including the ClaI site. This construct was digested with ClaI, which cuts the precursor once, in the G2 sequence. Two PCR primers were engineered, one annealing to the 3' end of the N-terminal signal sequence of the precursor, and extending upstream, and one annealing at the beginning of the G1 coding sequence, extending dowstream. With these primers a PCR was performed on the ClaI cut pSKδGP template, using the proofreading PCR polymerase Elongase (Gibco BRL), resulting in a product in which the G2 sequence is deleted from the precursor. The PCR product was ligated in the presence of T4 polymerase and dNTPs and then cut with ClaI again to linearize any remaining wild-type GP sequences. After transformation, several clones were selected and sequenced. Positive clones were identified, and mutated ORFs were excised from pSKδ using BamHI, and cloned into a BamHI cut pSFV-N vector. Fig. 4.1 schematically shows all investigated recombinants schematically.

Semliki Forest virus expression system. The system was first described by Liljeström and Garoff (Liljestrom and Garoff, 1991). We here utilized transfection of *in vitro* capped RNA transcripts of the constructs. To this end, the recombinant vectors were linearized using NruI, cleaned of RNase activity by treatment with proteinase K, and subsequently transcribed in the presence of SP6 RNA polymerase and Cap-analogs. RNA products were checked by electroforesis in a 1% agarose gel.

BHK cells were seeded in tissue culture flasks of 80 cm^2. Subconfluent cell monolayers obtained in one to two days were detached using a trypsin-EDTA solution (Life Tech.), centrifuged 5 min in a table centrifuge at 900 r.p.m., and washed once with PBS-0 (138 mM NaCl, 2.7 mM KCl, 8 mM Na2HPO4, 1.7 mM KH2PO4, pH 7.3). The cell pellet was resuspended carefully in 800 µl of PBS-Ca/Mg (PBS-0, 0.89 mM CaCl2 · 2H2O, 0.5 mM MgCl2 · 6H2O) and 10 – 50 µg of RNA transcript was added. Electroporation was performed in a BioRad electroporator by two consecutive pulses at 850 Volt, 25 µF, and 200 Ω. This resulted in a time constant of about 0.8 ms at each pulse. Transfected cells were added to 15-20 ml of culture medium in 80 cm^2 culture flasks, and from this, samples were taken for immunofluorescence, which were seeded on thin microscopic coverslips in six-well plates. Cells were incubated at 37°C and 5% CO_2 for 6 to 21 hours. In some experiments 5 µg/ml tunicamycin was added 1 hour after transfection, after which cells were incubated as mentioned above. Cycloheximide was added in some experiments to a concentration of 50 µg/ml 6 hours after incubation, after which the incubation was continued for 2-3 hours before harvesting.

Immunofluorescence microscopy. Coverslips with attached cells were washed with PBS-0, fixed with ice-cold methanol for 5-10 minutes or with 4% paraformaldehyde for 20-30 minutes. In the latter case cells were permeabilized with 0.1% Triton-X100 for 5 minutes when proteins were to be detected within the cell. Permeabilization was omitted when surface expressed proteins were to be detected. After fixation cells were washed with PBS-0, and blocked for at least 30 minutes with 5% BSA in PBS-0. Poly- or monoclonal antisera were

diluted in 1% BSA in PBS-0, and incubated for 1 hour at room temperature (RT). After several washes with PBS-0, goat- or swine anti rabbit or mouse second antibodies conjugated to fluorochromes fluorescein isothiocyanate (FITC), tetramethylrhodamine isothiocyanate (TRITC) or amino methylcoumarin acid (AMCA) were incubated on the cells for 45 minutes to an hour at RT. Procedures were repeated for double labelings with a different antiserum and fluorescent probe, and at the end of the procedure slides were washed with PBS-0 overnight. Direct labeling of ER and Golgi membranes was performed using the lectins concanavalinA and wheat germ agglutinin respectively, coupled to AMCA or FITC fluorochromes (both Molecular Probes). ER membranes were also stained using the chemical DiOC6 (Molecular Probes). Preparations were examined and photographed in a Leitz fluorescence microscope.

Western blotting. Transfected BHK cells were harvested by trypsin treatment from 80 cm^2 tissue culture flasks, mostly at 21 hours after transfection. Cells were pelletted by centrifugation at 900 r.p.m. for 5 minutes in a table centrifuge, and washed once or twice with PBS-0 to remove traces of medium. The pellet was resuspended in PBS-0 with a protease inhibitor cocktail (Complete ™, Boehringer Mannheim Germany), SDS-PAGE sample buffer (Laemmli, 1970) was added and the samples were boiled for 3 to 10 minutes. Samples were frozen at –20 °C for later use, or twenty microliter was immediately applied to a 10% poly acrylamide gel. After electrophoresis, the gel was blotted onto Immobilon PVDF membrane (Millipore) using a semi dry blotter (BioRad).

Immunodetection. Immunoblot analysis using alkaline phosphatase detection was carried out as described by Kikkert *et al.* (Kikkert et al., 1997).

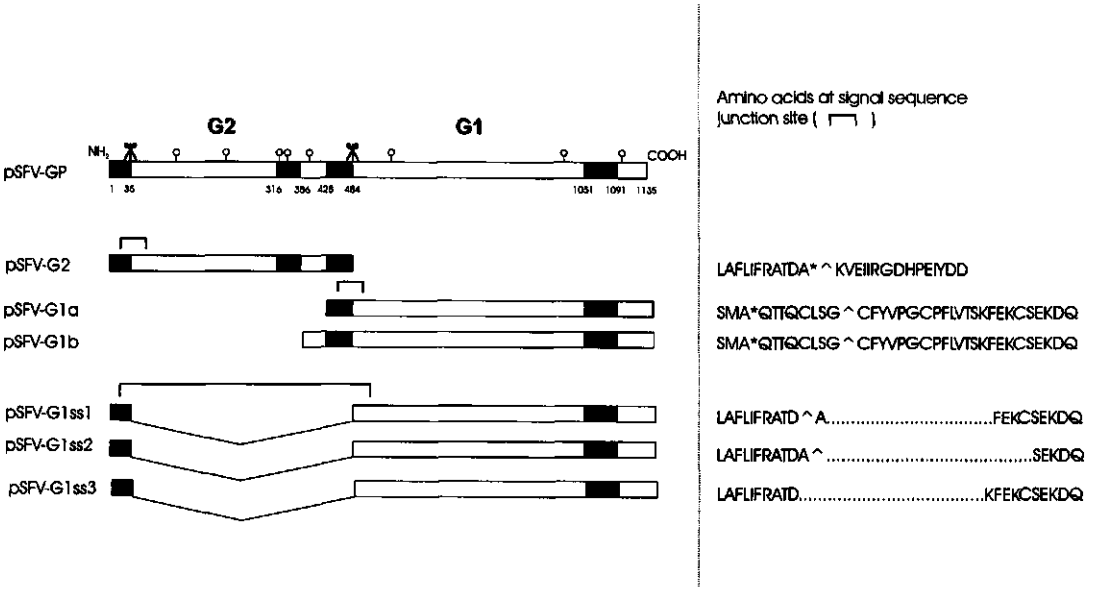


Figure 4.1 *Schematic representation of SFV constructs described in this chapter. Scissor symbols indicate possible (signal peptidase) cleavages in the precursor. Amino acid sequences around the signal sequence junction sites are indicated, where an asteriks (*) indicates a cleavage site predicted by the von Heijne (1986) algorithm and an accent circonflex (^) a cleavage site predicted by the Jagla (unpublished) algorithm.*

Results

The expressed wild-type TSWV glycoprotein precursor is processed, transported to and retained in the Golgi system. Expression, processing, and intracellular targeting of G1 and G2 expressed from the precursor ORF in pSFV-GP (Fig. 4.1) in BHK cells was analyzed by Western blotting and by indirect immunofluorescence. Western blots (Fig. 4.2a,b) indicated efficient cleavage between G1 and G2, since no precursor molecules (~127 kDa) were detected (Fig. 4.2a lane 4). SFV expressed G1 and G2 co-migrated with glycoproteins from purified TSWV particles isolated from N. rustica plants (Fig. 4.2a lanes 2 and 4, and Fig. 4.2b lanes 2 and 3). This suggests that the precursor is cleaved in a similar way in animal and plant cells, and also that the glycosylation patterns of the TSWV glycoproteins are similar in either cell type.

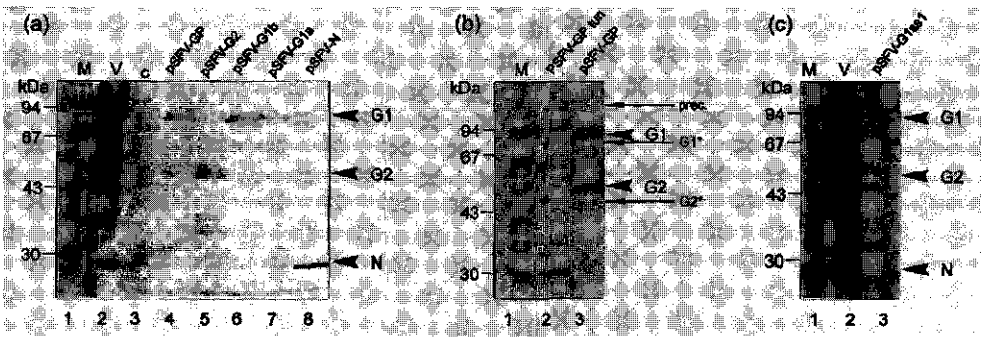


***Figure 4.2** Western blots using antiserum against TSWV particles, showing (a) the expression of the TSWV glycoprotein precursor (pSFV-GP), G2 alone (pSFV-G2), G1 alone (pSFV-G1a and pSFV-G1b), and N (pSFV-N) in BHK cells (b) the expression of GP in the presence of tunicamycin (G1* and G2* indicate unglycosylated forms of G1 and G2, prec = glycoprotein precursor protein) and (c) the expression of pSFV-G1ss1. M = Low molecular weight markers, V = Isolated TSWV particles.*

The subcellular location of G1 and G2 in BHK cells was determined using polyclonal antisera raised against intact TSWV particles (containing G1/G2 glycoproteins) or against the separately *E.coli*-expressed glycoproteins, by means of indirect immunofluorescence microscopy. Identical results were obtained using the three different sera, indicating that G1 and G2 co-localize and are probably closely associated during transport. At 6 hours after transfection, a typical picture was observed predominanated by an extensive reticular pattern virually covering the whole cell, combined with a significant perinuclear signal (Fig. 4.3a). Upon treatment with cycloheximide, or after prolonged incubation times (16-24 hours), the reticular signal largely vanished and only the perinuclear signal remained (Fig. 4.3b). Using the ER markers concanavalin A (not shown) and DiOC6 (Fig. 4.3c,d,e), the reticular signal could be identified as ER, while the Golgi marker wheat germ agglutinin (WGA) identified the perinuclear signal to represent the Golgi system (Fig. 4.3f,g,h). The anti-p58 Golgi-stack marker confirmed this localization (Fig. 4.3i,j,k). The intermediate compartment (IC) marker anti-p58 primarily gave a punctuate pattern throughout the cell, which showed no clear co-localization with perinuclear G1 and G2, expressed from the precursor (data not shown). These results strongly suggest that the TSWV glycoproteins are transported from the ER to the Golgi system and are then retained in the Golgi stacks, with a low rate of turn-over. Upon labeling of the surface of G1/G2 expressing cells, no signal was detected (data not shown),

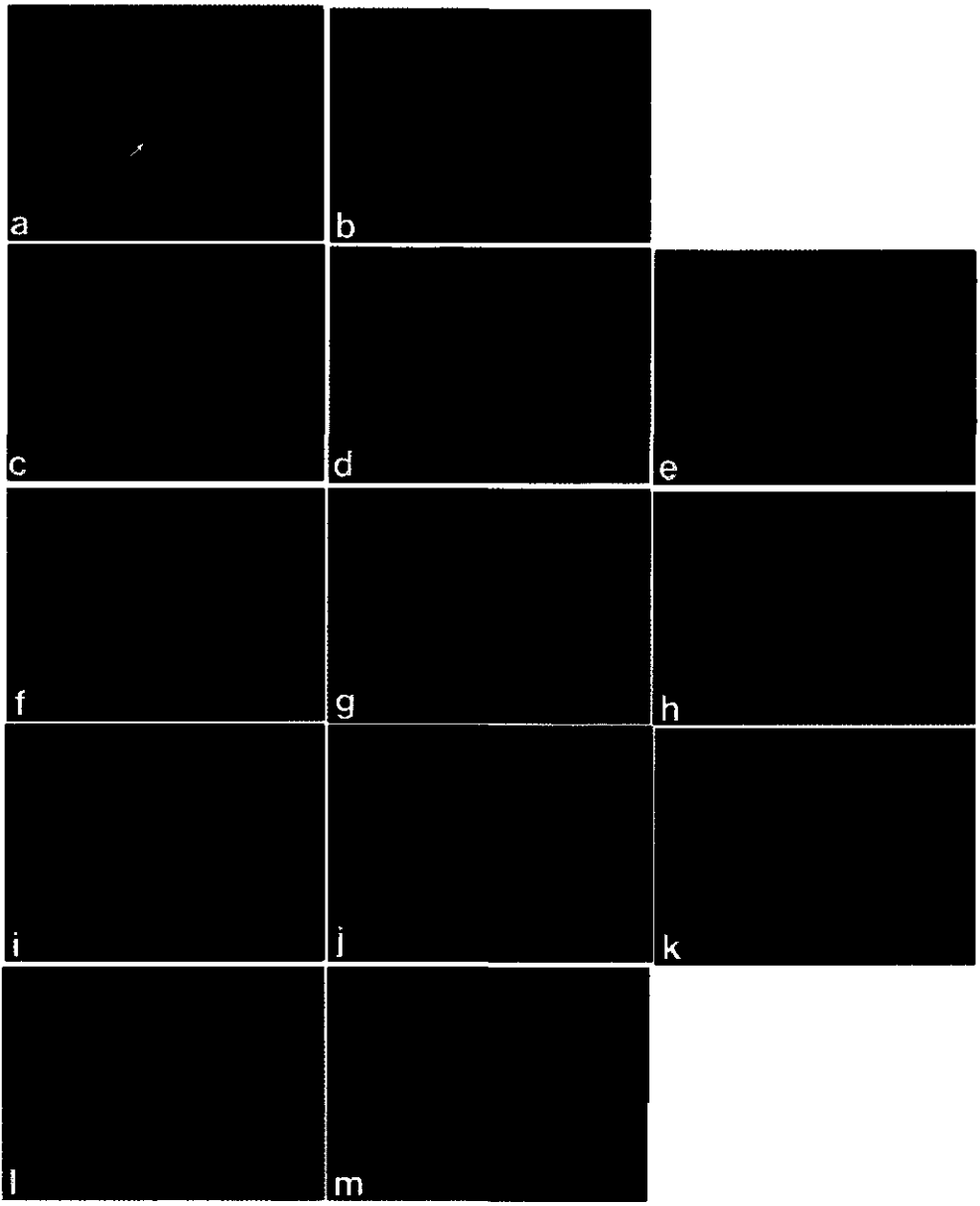


Figure 4.3 *Immunofluorescence analysis of expression of pSFV-GP (a-l) and pSFV-N (m). (a:) t= 6 hours post transfection (h.p.t.), anti-TSWV, arrow indicates perinuclear signal (b:) t = 21 h.p.t., anti TSWV. (c:) t = 6 h.p.t., anti-TSWV (d:) same cell treated with ER marker DiOC6 (e:) merge (double exposure) of (c) and (d). (f:) t = 21 h.p.t., anti-TSWV (g:) same cell treated with Golgi marker WGA-FITC (h:) merge of (f) and (g). (i:) t = 21 h.p.t., anti-TSWV (j:) same cell treated with Golgi-stack marker anti-p58 (k:) merge of (i) and (j). (l:) pSFV-GP expression in the presence of glycosylation inhibitor tunicamycin, t = 21 h.p.t, anti-TSWV. (m:) pSFV-N at t = 21 h.p.t., anti-N. Yellow areas in (c), (f) and (i) indicate overexposure of the film due to high intensity of red signals.*

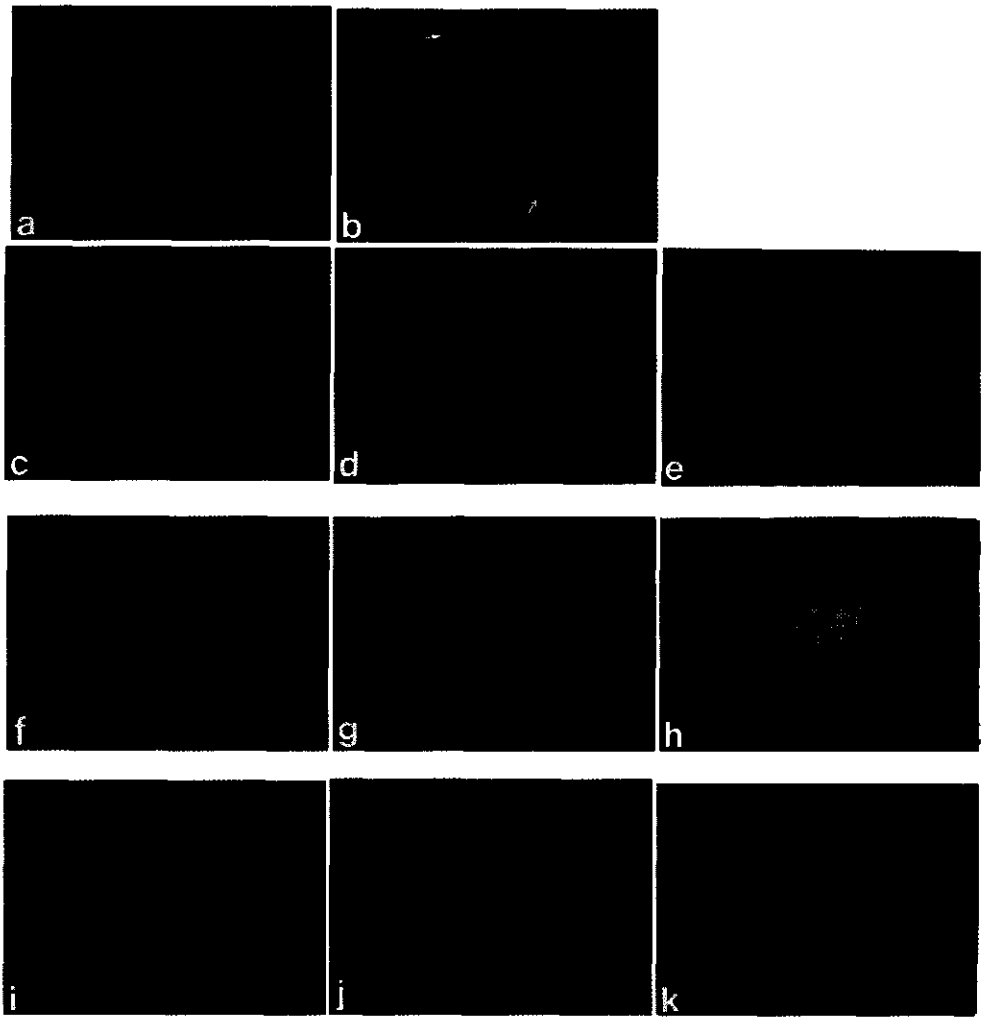


Figure 4.4 *Immunofluorescence analysis of (co-)expression of G2 and G1 constructs.*
(a:) pSFV-G2 at t = 6 hours post transfection (h.p.t.), anti-G2 (b:) pSFV-G2 at t = 21 h.p.t., anti-G2, arrows indicate perinuclear signal. (c:) pSFV-G2 at t = 6 h.p.t., anti-G2 (d:) same cells treated with Golgi marker WGA-FITC (e:) merge (double exposure) of (c) and (d).
(f:) pSFV-G1b at t = 21 h.p.t. (g:) same cells treated with ER marker DiOC6 (h:) merge (double exposure) of (f) and (g).
(i,j,k:) Immunofluorescence analysis of co-expression of pSFV-G2 with pSFV-G1ss1 at 21 h.p.t.. (i:) 2B6 monoclonal against G1 (j:) anti-G2 (k:) merge double exposures of panels (i) and (j).Yellow areas in (c) indicate overexposure of the film, due to high intensity of red signals.

suggesting that in BHK cells the retention of G1/G2 in the Golgi complex is strong and without detectable leakage.

When the N-glycosylation inhibitor tunicamycin (Takatsuki et al., 1971,Tkacz and Lampen, 1975) was added during expression of the precursor, the staining pattern of the G1/G2 proteins was reticular only (Fig. 4.3l), indicating that tunicamycin affects the exit of G1 and G2 from the ER. As shown in Western blots (Fig. 4.2b), this was not caused by complete inhibition of precursor cleavage. Faster migrating G1 and G2 proteins were produced in the presence of tunicamycin, corresponding to the unglycosylated forms of the proteins (Fig. 4.2b); in addition, some uncleaved precursor protein was observed, as well as some smaller immuno-reactive polypeptides presumably representing degradation products (Fig. 4.2b lane2). These findings may indicate that the glycosylation of G1/G2 is important for proper transport out of the ER, for stability of the proteins, and apparently also for efficient cleavage of the precursor in BHK cells.

As a control, a construct containing a cDNA of the nucleoprotein (N) gene was also expressed, which resulted in the expected 29 kDa protein (Fig. 4.2a lane 8). Immunofluorescence showed a clustered staining pattern of nucleoprotein in the cytoplasm (Fig. 4.3m), as earlier observed in infection of protoplasts (Kikkert et al., 1997).

G2 can reach the Golgi complex on its own. To investigate the trafficking and location of G1 and G2 separately, different deletion mutants of the precursor were produced encompassing either G2 or G1 sequences alone. As the precursor cleavage sites have not been mapped precisely, the C-terminus of G2 and the N-terminus of G1 are not known for TSWV. The pSFV-G2 construct (Fig. 4.1), meant to produce mature G2, therefore included the hydrophobic domain contained within amino acids 428 and 484 in analogy with the known topology of mature Uukuniemi G_N (Andersson et al., 1997b).

Transfection of mRNA from construct pSFV-G2 produced a protein comigrating with the G2 species from virus particles and reacting with antiserum against G2 (not shown) and against purified virus (Fig. 4.2a, lane 5). Also, some faster migrating products were found in Western blot analysis, most probably representing degradation products of the protein. Six hours after transfection, immunofluorescence analysis showed a reticular staining pattern in about 50% of the transfected cells (Fig. 4.4a) while in addition to this a clear perinuclear fluorescence was also seen in the rest of the cells (Fig. 4.4b). The percentage of cells with perinuclear signal increased after prolonged incubation times. Co-staining with ER and Golgi markers showed that the reticular signal represented again the ER (data not shown), and the perinuclear signal the Golgi complex (Fig. 4.4c-e). The results suggest that transport of G2 alone to the Golgi apparatus is possible, albeit less efficient than when co-expressed with G1 from the precursor, since a considerable part of G2 stayed in the ER. Addition of cycloheximide to the cells showed that much of the signal of G2 was gradually lost during this treatment (not shown), suggesting degradation of G2 protein in the ER and the Golgi complex. Cell-surface staining for G2 did not show an immunofluorescence signal in any of the G2 expression experiments (data not shown), indicating that G2 alone is not transported beyond the Golgi apparatus.

G1 can be inserted into the ER membrane by its own signal sequence or that of the precursor, but is not transported any further. Several different mutants encompassing G1 were produced, since the native N-terminus of G1 is not precisely known, and because it is also not known whether G1 carries its own functional signal sequence, or whether the signal sequence of the precursor is used to direct both G2 and G1 into the ER.

SFV-G1a and SFV-G1b (Fig. 4.1) contained the hydrophobic region between amino acid 428-484, which has been suggested to act as an internal signal sequence (Kormelink et al., 1992a), while another set of constructs, pSFV-G1ss1, pSFV-G1ss2, and pSFV-G1ss3,

contained the sequence for the signal peptide of the N-terminus of the precursor (amino acids 1-35) attached in frame to the G1 open reading frame i.e. lacking the sequence encoding the hydrophobic region of amino acid 428-484 (Fig. 4.1). These three constructs differed slightly in the junction area, and were tested to obtain insight into the prerequisites for signal peptidase cleavage of these chimeras (Fig. 4.1).

Construct pSFV-G1a, which contained the putative internal signal sequence at its N-terminus, produced a protein co-migrating with G1 from virus particles, although expression was not very high (Fig. 4.2a, lane 7). Construct pSFV-G1b produced a protein of exactly the same size as well (Fig. 4.2a, lane 6) although it contained an extra hydrophylic sequence (residues 386-428), N terminal of the putative signal sequence. Expression of construct pSFV-G1ss1 also resulted in a protein of the same size (Fig. 4.2c lane 3), as did pSFV-G1ss2 (data not shown). Immunofluorescence analysis showed an ER staining for pSFV-G1a, pSFV-G1b, pSFV-G1ss1, and pSFV-G1ss2 (results exemplified for pSFV-G1b in Fig. 4.4f-h). Even after prolonged incubation times, when ER staining could still be detected, no Golgi signal was observed for either of the G1 constructs. The pSFV-G1ss3 construct did not result in detectable protein levels in Western blots or immunofluorescence analysis.

Co-expression of G1 and G2 from separate constructs results in rescue of efficient transport to the Golgi system. With the purpose to test whether the separately translated G1 and G2 proteins were able to complement each other in such a way that efficient trafficking to the Golgi complex would be restored, construct pSFV-G2 was co-transfected together with pSFV-G1b or with pSFV-G1ss1. For immmunofluorescence analysis of these co-transfections a polyclonal antiserum against G2 was used, in combination with a monoclonal antiserum against G1 (Mab 2B6, kindly provided by G. Adam, University of Hamburg, Germany). This anti-G1 monoclonal serum only detects Golgi-localized G1, and it does not react with G1 localized in the ER. Cells that expressed both G1 (from either pSFV-G1b or pSFV-G1ss1) and G2 (from pSFV-G2) at 21 h post transfection, gave a signal using the monoclonal against G1 (Fig. 4.4i), whereas cells only expressing G1 (which is then ER localized) did not (not shown). This indicates that G1 is transported to the Golgi when co-expressed with G2 (Fig. 4.4i-k). Detection of G1 in co-expressing cells using the polyclonal serum against G1 (which also detects ER localized G1) showed only little G1 staining associated with ER in these cells (data not shown), confirming an efficient transport of G1 in the presence of G2.

Co-transfection of G1 and G2 also rescued the impaired transport of G2 when expressed alone, as concluded from the reduced ER signal of G2 in co-expressing cells (compare Fig. 4.4j with Fig. 4.4a-c).

Discussion

As a first step towards the detailed understanding of the molecular processes underlying the maturation of TSWV glycoproteins and the subsequent assembly of virus particles in both plant and animal cells, we used the Semliki Forest virus expression system to study these glycoproteins in animal cells.

The results show, that like for the animal infecting bunyaviruses, the TSWV glycoproteins themselves contain information necessary and sufficient for their transport to and retention in the Golgi system of mammalian cells, when expressed as the precursor. Strikingly, as was found for the animal infecting bunyaviruses Uukuniemi virus (Melin et al., 1995,Ronnholm, 1992) and Bunyamwera virus (Lappin et al., 1994), the N-terminal protein G2 (G_N) of the TSWV glycoprotein precursor could be transported to the Golgi system on its own, though with decreased efficiently, and apparently contains a Golgi retention signal. The C-terminal protein G1 (G_C) expressed on its own was unable to leave the ER, and thus seems

to require the interaction with G2 (G_N) in order to be transported out of the ER. This was illustrated in co-transfections where the interaction of G2 with G1 from separate constructs rescued the efficient transport of both glycoproteins to the Golgi system.

G1 and G2 are glycoproteins, presumably acquiring two N-linked oligosaccharide side chains at the predicted sites in their luminal (i.e. N-terminal) domains (see Fig. 4.1). When the glycosylation was inhibited by treatment with tunicamycin, precursor cleavage still occurred, though less efficiently (Fig. 4.2b lane 2), but the proteins were unable to leave the ER. The absence of N-linked glycans probably results in aberrant folding of the proteins, which generally leads to a hampered transport from the ER to the Golgi complex, as observed. The same was also found for Uukuniemi virus proteins lacking their N-linked glycans (Kuismanen et al., 1984).

The TSWV glycoproteins apparently do not reach the plasma membrane, since they were undetectable by cell surface immunofluorescence staining. This indicates that the proteins are tightly retained in the Golgi complex. We can, however, not rule out that a fraction of the proteins, too small to be detectable, escapes to the plasma membrane but is continually retrieved to the Golgi complex, as has been demonstrated for some resident Golgi membrane proteins.

Expression of different TSWV G1 constructs in BHK cells indicated that the hydrophobic sequence encompassing amino acid 428-484 can function as a separate, internal, signal sequence for G1, since it is able to guide G1 into the ER. The same was found for the G_C glycoprotein of other bunyaviruses (reviewed in Pettersson and Melin, 1996), and also for the glycoprotein E1 of Semliki Forest virus (Hashimoto et al., 1981). TSWV G1 can be targeted to the ER by attachment of the precursor signal peptide to its sequence as well. A number of such constructs were produced (Fig. 4.1), since we were interested in the prerequisites needed for efficient cleavage of these chimeric molecules. Apparently, omitting a few of the N-terminal residues of G1, as in pSFV-G1ss2 (Fig. 4.1), did not affect the ER targeting. However, when the putative last residue of the signal sequence itself was missing, as in pSFV-G1ss3 (Fig. 4.1), no protein could be detected. Using the von Heijne algorithm (von Heijne, 1986), and a new algorithm, based on a computer neural network (Jagla et al., personal communication of unpublished results), cleavage sites could indeed be predicted for all constructs produced, except for pSFV-G1ss3 (Fig. 4.1). This may account for abberant targeting of G1 from this construct, and result in an unstable protein product, as observed.

Further research is needed to map the region(s) in the TSWV G2 sequence that are necessary for its Golgi retention in mammalian cells. This issue was already investigated for Punta Toro virus (Matsuoka et al., 1996) and Uukuniemi virus (Andersson et al., 1997a). For these viruses the signal was found to be located in the cytoplasmic tail of G_N, close to the transmembrane anchor. There was, however, no sequence homology found for these and other Golgi retention signals, suggesting that signals like these are based on the conformation of the protein rather than on a primary sequence motif.

Our results surprisingly indicate that the TSWV glycoproteins contain transport and retention characteristics that are functional in mammalian cells, and which resemble those of animal-infecting bunyavirus glycoproteins very closely. These observations could be interpreted as a strong confirmation of the putative evolution of an animal-infecting ancestral bunyavirus into the plant-infecting tospoviruses. However, it is unlikely that such detailed molecular features are conserved, if they would not have a function in the infection cycle of TSWV. Therefore, these features probably reflect the ability of TSWV to replicate in its animal thrips vector, in which the formation of particles may be very homologous to the process observed for other bunyaviruses in mammalian and insect vector cells. Furthermore, literature increasingly indicates that cellular transport and retention signals are not only conserved among closely related organisms, but are similar, if not identical for all eucaryotes

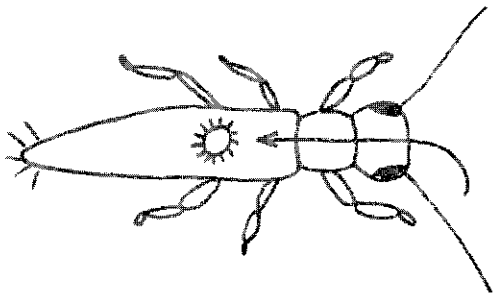
(reviewed in Bar et al., 1996, Kermode, 1996). This would suggest, that the molecular features underlying the behavior of TSWV glycoproteins in mammalian cells may also be functional during the maturation in plant cells. In particular, the accumulation in the Golgi system was also observed during the TSWV infection of plant cells (Kikkert et al., 1999), and may thus be regulated by the same molecular signals as in mammalian cells. The differences in the subsequent formation of particles in plant and animal cells could be the result of extra regulatory signals acquired by the TSWV (glyco)proteins to meet the prerequisites of the plant host.

Acknowledgments

We are extremely grateful to Gert-Jan Godeke of Utrecht University in the Netherlands for assistance with the SFV expression system. Christina Spiropoulou is thanked for sending us her Sigma Golgi marker anti 58K, and Agneta Andersson for sending us the IC marker anti 58K. Guenter Adam of the University of Hamburg sent us the monoclonal 2B6 against G1, for which we are very grateful. We furthermore thank Cecile van Woensel and Bart Hesselink for excellent technical assistance and Claire Pacot-Hiriart for preparing useful constructs in preparation of this work.

CHAPTER 5

BINDING OF TOMATO SPOTTED WILT VIRUS TO A 94 kDa THRIPS PROTEIN



Kikkert, M., C. Meurs, F. van de Wetering, R. Kormelink, and R. Goldbach. (1998) Binding of tomato spotted wilt virus to a 94 kDa thrips protein. *Phytopathology* **88**, 63-69.

Abstract

Using protein blot assays, a 94 kDa thrips protein was identified which exhibited specific binding to tomato spotted wilt virus (TSWV) particles. Renaturation of the 94 kDa protein, which is conserved among the two major vector species of TSWV, *Frankliniella occidentalis* and *Thrips tabaci*, turned out to be crucial for its virus binding properties while under the same conditions no specific binding was observed with aphid (*Myzus persicae*) proteins. The 94 kDa protein species was present in all developmental stages of both vectoring thrips, whereas it was mainly present in the adult stage of a non-vectoring thrips species *Parthenothrips dracenae*. Using antibodies against the different TSWV structural proteins, the G2 envelope glycoprotein could be identified as the viral determinant involved. As the virus-binding protein is present throughout the thrips body but not in the gut, it may represent a receptor protein involved during circulation of the virus through its vector but most likely not during viral uptake in the midgut.

Introduction

Tomato spotted wilt virus (TSWV), the type species of the *Tospovirus* genus within the arthropod born family of *Bunyaviridae*, is transmitted by thrips (*Thysanoptera*) in a propagative manner (Ullman et al., 1993, Wijkamp et al., 1993). In addition to a polymerase (L protein), nucleoprotein (N) and two non-structural proteins (NSs and NSm), the tripartite ambisense RNA genome of TSWV encodes two envelope glycoproteins, G1 and G2, which are expressed from a common precursor gene (German et al., 1992, Goldbach and Peters, 1996). These glycoproteins are thought to be involved mainly in uptake by and replication within the thrips vector, but are not essential for the systemic infection of plants (Resende et al., 1991).

The most common vector of TSWV, the Western flower thrips (*Frankliniella occidentalis)*, is increasingly distributed throughout the world, and this spread of the vector is the main cause of a dramatic emergence of TSWV, and other tospoviruses, in vegetable and ornamental crops worldwide (Goldbach and Peters, 1996).

Since the interaction between viruses and their vectors is crucial in the natural infection cycle, many researchers have focused on epidemiological as well as molecular aspects of this interaction. For an increasing number of plant viruses, more information has become available about the intrinsic factors affecting vector specificity and competence, and for some propagatively transmitted viruses, molecular data have been obtained describing different steps of the interaction between virus and vector in detail (Ammar, 1994). For different virus-vector combinations it has been shown or suggested that several dissemination barriers must be overcome during the circulation of the virus through its vector. Sequentially, these could be the mesenteron or midgut infection barrier (MI barrier), the midgut escape barrier (ME barrier), the salivary gland infection barrier and the salivary gland escape barrier (Ammar, 1994, Hardy et al., 1983). All of these barriers could involve receptor mediated processes, directing regulated circulation of the virus through the vector, coupled to several rounds of replication at specific sites.

For TSWV, two rounds of viral replication have been identified during circulation. The first one takes place in the cells of the midgut and the second, major one, in the salivary glands (Tsuda et al., 1996, Ullman et al., 1992; Ullman et al., 1995, Wijkamp et al., 1993). Ullman *et al.* (Ullman et al., 1992) suggested a ME barrier in *F. occidentalis* that may be blocked in the adult stage, thus explaining why adult thrips can transmit TSWV only after they have ingested the virus in their larval stage.

The molecular processes that take place after ingestion of the virus remain to be resolved. However, the glycoproteins G1 and G2, which are exposed at the surface of the

enveloped particle, are the obvious candidates for being viral attachment proteins, directing the entry into thrips tissues.

In this paper, possible specific molecular interactions between TSWV proteins and proteins of the thrips are investigated, using virus overlay assays as previously used to characterize luteovirus-aphid interactions (van den Heuvel et al., 1994). Our results, described in this report, suggest a specific binding of the viral G2 protein to a protein of approximately 94 kDa, which is conserved between *F. occidentalis* and *Thrips tabaci*, two major vectors of TSWV.

Materials and Methods

Isolation of TSWV particles. All binding studies were performed with the Brazilian TSWV isolate BR-01 (de Avila et al., 1993), which was maintained in *Nicotiana rustica* plants by mechanical inoculation and thrips transmission. Intact enveloped virus particles were isolated from infected *N. rustica* plants as described by Kikkert *et al.* (Kikkert et al., 1997). Preparations were kept on ice and used immediately, or promptly frozen in liquid nitrogen and stored at -80°C. Before use, samples were thawed slowly on ice.

Thrips cultures and aphids. Cultures of virus-free *F. occidentalis* were reared on bean pods (*Phaseolus vulgaris* L. 'Prelude') and *T. tabaci* cultures on leek at 27(±0.5)°C and 16 h photoperiod (light/dark: 16/8 h). Colonies were started with adults collected from a greenhouse infestation in the Netherlands. To obtain uniformly aged larvae, fresh bean pods were placed in the thrips colonies for one day to achieve egg oviposition. The thrips *Parthenothrips dracenae*, which have never been reported to be virus transmitting, were reared on *Hedera helix* plants. Aphids (*Myzus persicae)* were reared on *Brassica napus* L. ssp. *oleifera* (oilseed rape) plants. Larvae and adults of the different thrips species and aphids were collected, placed in Eppendorf tubes, frozen in liquid nitrogen and stored at -80°C, prior to further manipulations.

Polyclonal antibodies. Polyclonal antisera against the viral glycoproteins G1 and G2, N and complete TSWV particles were produced as described previously (de Avila et al., 1993, Kikkert et al., 1997). Anti-BMVcp serum was supplied by Dr. B.J.M. Verduin.

Protein electrophoresis and Western blotting. Frozen or fresh insects were ground in PBS-T (0.14 M NaCl, 1 mM KH_2PO_4, 8 mM Na_2HPO_4, 2.5 mM KCl, 0.05% Tween-20) to a final concentration of approximately 3 µg of protein per microliter (estimated using a Biorad protein assay). Protein sample buffer (Laemmli, 1970) was added and the samples boiled for 5 min. A total of 30 µg of insect protein per lane was loaded on a SDS-polyacrylamide gel. After electrophoresis, proteins were blotted onto PVDF membrane (Millipore) using a semi-dry blotting procedure of Biorad. To verify equal amounts of protein in each lane, gels were also CBB stained.

Renaturation of blots. Western blots of insect proteins were renatured essentially as described by de Jong *et al.* (de Jong et al., 1992) with some modifications. Blots were washed three times 10 min in binding buffer (25 mM Tris/HCl pH 7.5, 50 mM NaCl, 2 mM DTT, 2 mM EDTA, 0.3% BSA, 0.025% Nonidet P-40) at room temperature (RT). Subsequently, the blots were incubated 45 min in denaturing buffer (7 M guanidine hydrochloride, 50 mM Tris/HCl pH 8.0, 2 mM DTT, 2 mM EDTA, 0.3% BSA) at RT. Blots were then washed four times 5 min in renaturation buffer (25 mM Tris/HCl pH 7.5, 50 mM NaCl, 2 mM DTT, 2 mM EDTA) followed by an overnight incubation in the same buffer at RT.

Virus overlay assays. The protocol used was basically as described by van den Heuvel *et al.* (van den Heuvel et al., 1994) with some modifications. Western blots were washed twice for 5 min in blocking buffer (PBS-T, 5% Elk (skim instant milk)) and subsequently incubated 30 min in overlay buffer (PBS-T, 5% Elk, 2% polyvinylpyrrolidone). Blots were

then incubated 1-2 h in overlay buffer containing 5 µg virus particles per milliliter. After washing three times 10 min in diluted blocking buffer (PBS-T, 0.5% Elk), blots were incubated with antiserum against complete TSWV particles or against one or more of the structural proteins (N, G1 and G2) at 1 µg/ml in diluted blocking buffer for 1 h, at RT.

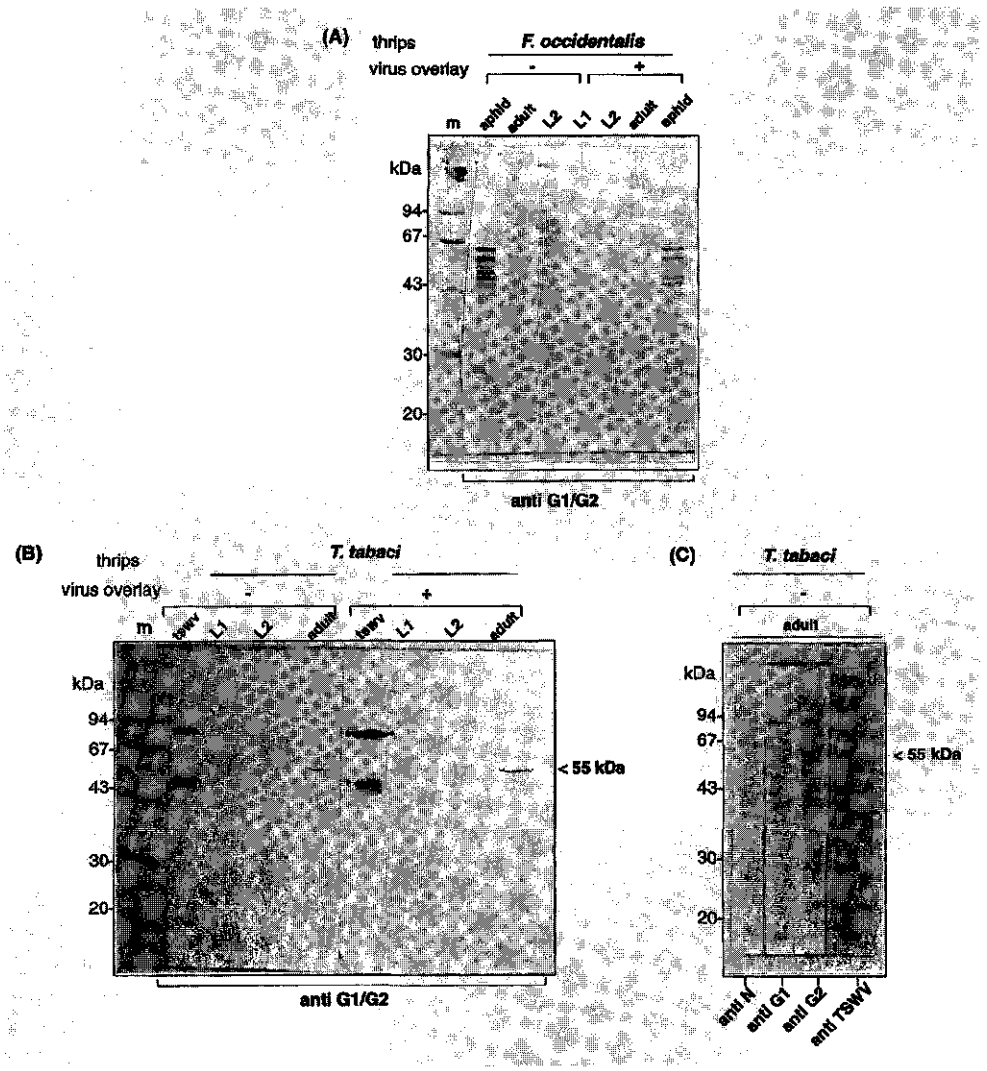


Figure 5.1 *Tomato spotted wilt virus (TSWV) overlay studies on* ***denatured*** *thrips and aphid proteins (30 µg insect protein per gel lane, blotted onto PVDF membrane). (A) TSWV overlay on proteins of Frankliniella occidentalis L1 and L2 larvae and adults, as well as aphid (Myzus persicae) adult proteins, using mixed anti-G1 and anti- G2 sera after overlay with overlay buffer ("-") or overlay buffer containing TSWV particles ("+"). m= molecular mass marker. (B) Like (A), but using Thrips tabaci larvae and adults. TSWV= purified TSWV particles. (C) Denatured T. tabaci adult proteins overlaid with overlay buffer without virus and then treated with several different antisera.*

After washing with diluted blocking buffer, antigen-antibody complexes were detected using 0.5 µg/ml alkaline phosphatase-conjugated goat anti-rabbit immunoglobulins (Tago Inc., Burlingame, CA, USA), and 0.33 mg/ml nitroblue tetrazolium and 0.165 mg/ml bromochloroindolyl phosphate as a substrate.

Protease treatment of virus. TSWV particles were isolated from 100 g of infected *N. rustica* leaves as described earlier (Kikkert et al., 1997), but the sucrose gradient at the end of the procedure was omitted. Virus particles were treated with bromelain (Sigma) based on the protocol described by Mohamed (Mohamed, 1981). The virus suspension was divided into four portions, which were treated as follows: 20 min on ice in double distilled water (portion 1); 20 min at 37°C in protease buffer (1 mM K_2HPO_4 pH 7.0, 0.1% ß-mercaptoethanol) (portion 2); 10 min at 37°C and 10 min on ice in protease buffer containing 2.5 mg bromelain per milliliter (portion 3); 20 min at 37°C in protease buffer containing 2.5 mg bromelain per milliliter (portion 4). The four portions were then loaded separately onto 10-40% sucrose gradients. After centrifugation, the opalescent band was collected from each gradient, diluted with sterile double distilled water and pelleted by ultracentrifugation. Resulting virus pellets were resuspended in double distilled water. The samples were used in overlay assays at 5 µg/ml. The integrity of the particles after the different treatments was checked by electron microscopy using a simple PTA staining (van Lent et al., 1991).

Dissection of thrips larvae. L2 larvae of *F. occidentalis* were dissected by cutting off the head and transferring the body to a drop of water, resulting in release of the gut from the body. Heads, guts and remaining body parts were pooled separately. In another experiment, L2 larvae were cut in two behind the thorax. Front and back parts were pooled separately. The samples were prepared for SDS-PAGE as described for whole insects, except that they were not adjusted for equal protein content, but for equal number of animals from which the body parts (or whole bodies) originated. Per lane, a protein sample prepared from body parts of approximately 10 animals was loaded.

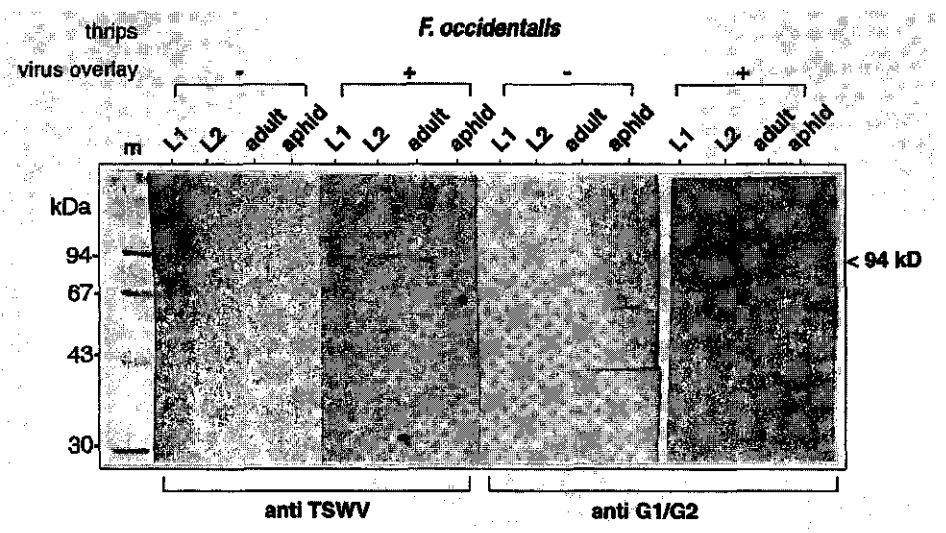


Figure 5.2 *Tomato spotted wilt virus (TSWV) overlay studies on* ***renatured*** *Frankliniella occidentalis (L1, L2 and adult stages) and aphid (Myzus persicae) adult proteins (30 µg insect protein per gel lane, blotted onto PVDF membrane), with or without virus present in the overlay buffer ("+" or "-" respectively), using anti-TSWV serum and mixed anti-G1 and anti-G2 sera. m = molecular mass marker.*

Results

TSWV specifically binds to a thrips protein of 94 kDa. Recently, a virus overlay assay allowing the identification of proteins of vectoring aphids which specifically bind to luteoviruses was described (van den Heuvel et al., 1994). In the present study a similar overlay assay was applied, including a protein renaturation step, to monitor specific binding of TSWV to thrips proteins. When the renaturation step was omitted from the protocol, non-specific binding to *F. occidentalis* as well as to aphid proteins was observed (Fig. 5.1A). Moreover, in adult *T. tabaci* a clear signal was obtained with a 55 kDa protein species using anti G1/G2 serum after overlay, but this protein was also detected when TSWV particle overlay was omitted from the assay (Fig. 5.1B). Further analysis revealed a direct binding of anti-G2 (and anti TSWV-) serum to this 55 kDa thrips protein (Fig. 5.1C). Addition of Tween-20 to the binding buffer resulted in the loss of this non-specific binding in further assays.

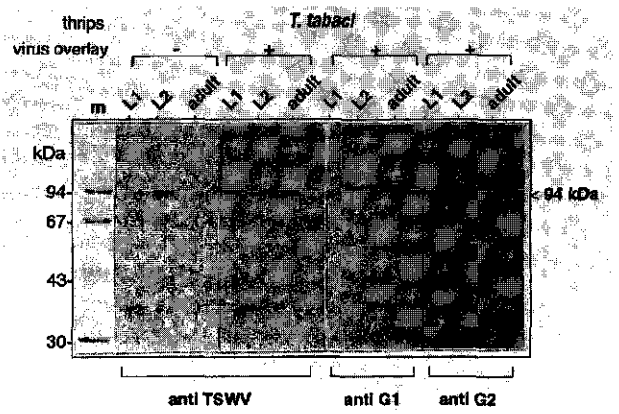


Figure 5.3 Tomato spotted wilt virus (TSWV) overlay studies on renatured Thrips tabaci L1, L2 and adult proteins (30 µg per gel lane, blotted onto PVDF membrane), with or without virus present in the overlay buffer ("+" or "-" respectively), using anti-TSWV serum, anti-G1 and anti-G2 sera. m = molecular mass marker.

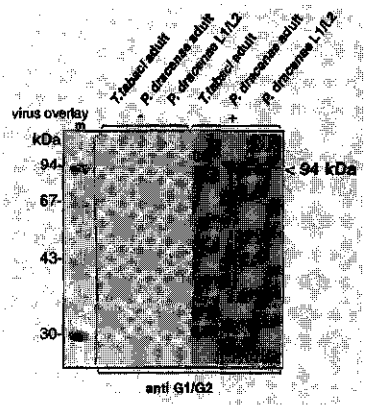


Figure 5.4 Tomato spotted wilt virus (TSWV) overlay on renatured Parthenothrips dracenae proteins of adults and pooled L1 and L2 larvae (30 µg per gel lane, blotted onto PVDF membrane), with or without virus present in the overlay buffer ("+" or "-" respectively). m = molecular mass marker.

After renaturation of the proteins on the blot, a discrete protein band, co-migrating with the 94 kDa molecular-mass marker protein, was reproducibly found after overlay with TSWV particles in both *F. occidentalis* and *T. tabaci* (Figs. 2 and 3, respectively). This protein species was detected in all developmental stages examined of both vectoring thrips species. In control experiments in which virus was omitted from the overlay assay, no reaction with this 94 kDa thrips protein was found (Fig. 5.2 lanes 2-5; 10-13 and Fig. 5.3 lanes 2-4). No specific binding of TSWV particles to aphid proteins was observed (Fig. 5.2, lanes 5, 9, 13 and 17). The 94 kDa protein was also found in a non-vectoring thrips species, *P. dracenae*, though predominantly in the adult stage and to a much lesser extent in the larval (L1 and L2) stages (Fig. 5.4).

To study whether the 94 kDa protein is present in a complex or in multimeric forms, virus overlays were done on non-reduced, renatured proteins of *F. occidentalis* and *T. tabaci*. No other reacting protein bands were detected in these experiments, suggesting that the 94 kDa protein is present as a monomer (data not shown). Overlay with purified TSWV nucleocapsids devoid of envelopes, or with purified brome mosaic virus (BMV) particles on renatured proteins of *T. tabaci* larvae or adults did not result in a specific signal (data not shown). To investigate whether the 94 kDa protein may originate from intestinal bacteria of thrips, three different strains of the major gut bacteria of *F. occidentalis*, *Enterobacter agglomerans*, were subjected to SDS-PAGE, blotted and renatured, and overlaid with TSWV particles (data not shown). As no binding was observed to proteins of any of these bacteria, the 94 kDa protein is most probably a genuine thrips protein.

Glycoprotein G2 binds to the 94 kDa thrips protein. To investigate which of the proteins of the virus particle bound to the 94 kDa thrips protein, several different antisera raised against the separate structural TSWV proteins were used after overlay. The 94 kDa protein was detected only with anti-G2 and anti-TSWV sera (Figs. 2 and 3) but not with

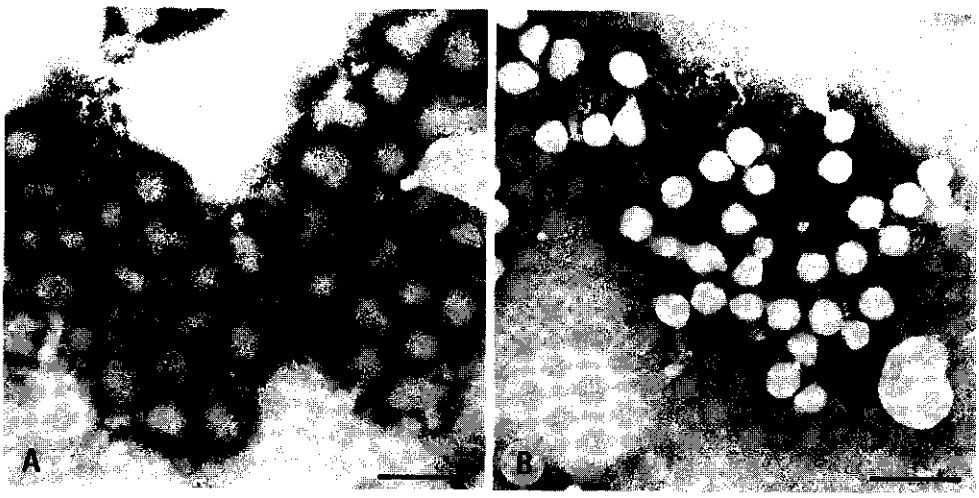


***Figure 5.5** Electron micrographs of PTA stained (A) Buffer treated tomato spotted wilt virus (TSWV) particles (20 min at 37°C). (B) Bromelain treated TSWV particles (10 min at 37°C). Bars represent 200 nm.*

antibodies against G1 alone (Fig. 5.3) or N (data not shown). These results suggest that during the overlay incubation with virus, only G2 became firmly bound to the 94 kDa thrips protein, the other structural proteins apparently being washed away, most probably due to collapse of

the enveloped virus particles during the procedure, in combination with an apparent lack of binding affinity of these other viral proteins to thrips in these assays.

Binding of virus through one of the glycoproteins was further confirmed by assays with virus particles pre-treated with bromelain, which cleaves both glycoproteins but does not affect the nucleoprotein inside the particle (Mohamed, 1981). EM pictures of bromelain treated and buffer treated TSWV particles showed that bromelain leaves the spherical integrity of the particles intact, but removes the "fuzzy" surface of untreated particles (Fig. 5.5), as has earlier been shown by Mohamed (Mohamed, 1981) for TSWV and by Brand and Skehel (Brand and Skehel, 1972) for influenza virus particles. Using renatured blots with *T. tabaci* adult stage proteins it was demonstrated that virus samples treated with buffer only (20 min at 37°C) could still bind to the 94 kDa thrips protein (Fig. 5.6C, lane 3) albeit somewhat less profound than in the untreated control (Fig. 5.6C, lane 2). However, samples treated with bromelain for 10 min at 37°C had lost their binding capacity completely (Fig. 5.6C, lane 4). From Western blot analysis of such bromelain-treated samples (Fig. 5.6B, lanes 3 and 7) it could be concluded that these virus particles contained some intact G1 but lacked G2, suggesting that the 94 kDa protein is normally recognized by G2 rather than G1. Twenty min of bromelain treatment cleaved both G1 and G2 almost completely (Fig. 5.6B, lanes 4 and 8), and resulted, as expected, in virus particles unable to bind to thrips protein (Fig. 5.6C, lane 5). Taken together these studies confirm that the 94 kDa thrips protein is recognized by the G2 glycoprotein of TSWV.

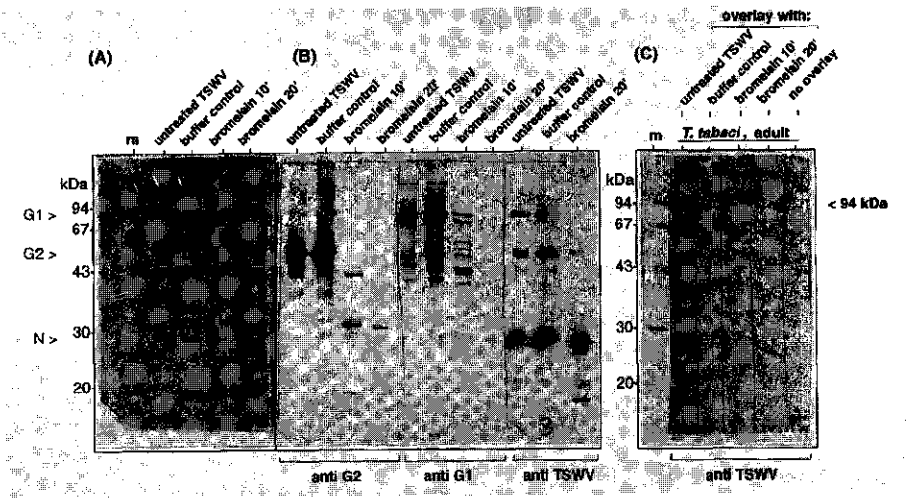


Figure 5.6 *(A) Coomassie brilliant blue stained gel with untreated and bromelain treated tomato spotted wilt virus (TSWV) particles. (B) Western blot study of untreated and bromelain treated TSWV particles. (C) Overlay on Thrips tabaci adult proteins with untreated and bromelain treated TSWV particles. m = molecular mass marker; buffer control = TSWV treated for 20 min at 37°C in protease buffer without enzyme; bromelain 10' = TSWV treated for 10 min at 37°C with 2.5 mg bromelain per milliliter in protease buffer; bromelain 20' = TSWV treated for 20 min at 37°C with 2.5 mg bromelain per milliliter in protease buffer.*

The 94kDa thrips protein is not a midgut-related protein. The 94 kDa protein of *F. occidentalis* and *T. tabaci* binding to the TSWV G2 protein may be a receptor protein, playing a key role in the interaction between TSWV and its vectors at one of the dissemination barriers during circulation. To obtain a first indication of where the 94 kDa protein is

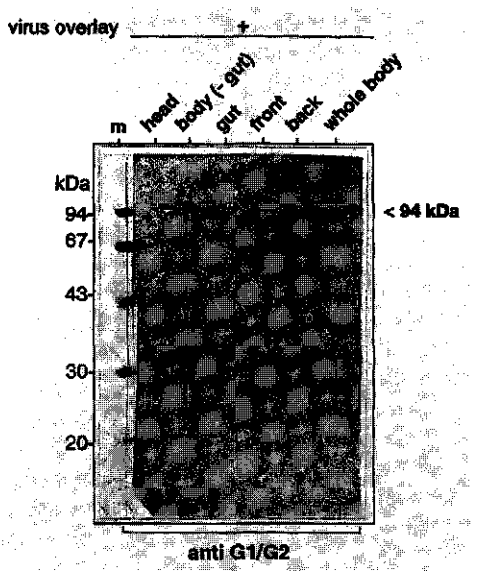


Figure 5.7 *Tomato spotted wilt virus (TSWV) overlay on renatured proteins of body parts of Frankliniella occidentalis. Each gel lane contained 10 body parts or whole bodies, proteins were blotted onto PVDF membrane. m = molecular mass marker.*

localized within the thrips body, L2 larvae of *F. occidentalis* were separated into three parts (head, gut and remaining body parts), or two parts (front and back, cut behind thorax). Overlay assays on proteins from these separated body parts showed that the 94 kDa thrips protein was found in the head, the remaining body part lacking the gut, and in the front and back parts of L2 larvae, but was clearly absent from the gut system of the insect (Fig. 5.7). As the back part of the larvae did contain the 94 kDa protein, it can also safely be concluded that the binding is not, or at least not conclusively, associated with the salivary glands either. Instead, our results indicate that the 94 kDa protein is present throughout the body of the L2 larvae, and could for example be associated with a specific organ or tissue like the hemolymph, or fat body.

Discussion

The use of overlay studies to investigate the binding of virus to proteins of the vector is a technically feasible, straight-forward method, that has yielded important results in luteovirus research (van den Heuvel et al., 1994). Using this method, we could however not detect specific binding of TSWV to any protein of either *F. occidentalis* or *T. tabaci*.

Only after renaturation of the proteins on the blots, by which presumably the secondary structure of the thrips proteins is somewhat restored, a clear binding of TSWV particles to a single, conserved, thrips protein of around 94 kDa was observed. The protein was present in all stages examined of the two vectoring thrips species *F. occidentalis* and *T. tabaci*. The slightly faster migration of the binding band in adults of *F. occidentalis* and *T. tabaci* (Figs. 2 and 3), is most probably due to interference of an abundant protein that is only present in adults, and which migrates slightly slower than the 94 kDa protein, as observed in CBB stained gels (data not shown).

The virus binding 94 kDa protein was also found in the non-vectoring thrips species *P. dracenae* (Fig. 5.4), albeit not in its larval stages. Transmission studies have demonstrated

that virus acquisition must take place in the larval stage in order for the thrips to become viruliferous in older larval and adult stages (Sakimura, 1962, van de Wetering et al., 1996, Wijkamp and Peters, 1993), suggesting that at least the majority of the replication and circulation events of the virus take place before the adult stage is reached. A possible role of the identified 94 kDa thrips protein could be somewhere along this circulation process in the larval stages, thus possibly explaining the inability of *P. dracenae* to transmit TSWV.

Further experiments showed that TSWV-G2 is the viral protein binding to the 94 kDa thrips protein. The presence of an RGD-motif near the N-terminus of G2 (Kormelink et al., 1992a) might be an important determinant in this attachment to thrips tissue, since for example foot and mouth disease virus (Fox et al., 1989) and coxsackievirus (Berinstein et al., 1995; Rieder et al., 1996; Roivainen et al., 1991; Roivainen et al., 1994) RGD-motifs were shown to be of importance for cell attachment. Further experiments have to show whether the RGD-motif is involved in the binding of G2 to the 94 kDa thrips protein.

Dissection of *F. occidentalis* L2 larvae showed that the 94 kDa protein seems not present in the gut. In this experiment we used body parts of ten animals in each lane, which resulted in an unequal amount of protein per lane, but would give a similar binding signal as in whole bodies if the 94 kDa protein were concentrated in one of the parts. Particularly the head and the gut fractions contained relatively low amounts of protein due to the lower volume of these body parts (data not shown) which could have masked a signal in the gut fraction in overlay experiments. However, since we did find a clear 94 kDa protein signal in the head fraction, we argue that if there would be a reasonable amount of 94 kDa protein in the gut fraction, we would have found a signal there as well. The results of this experiment indicate that the 94 kDa-G2 interaction is unlikely to be involved in a MI or ME barrier, but rather in other stages of the circulation.

This conclusion is also indirectly supported by experiments with protease digestion of virus particles using bromelain (Fig. 5.6) and trypsin (data not shown) showing that G2 seems more sensitive to proteases than G1. It is tempting to assume that the viral protein that is the most protease sensitive does not have a major role in receptor binding in the (mid)gut, since the gut lumen of insects usually contains very high concentrations of proteases (Ludwig et al., 1989; Ludwig et al., 1991). In this view, and consistent with our results, G2 would not be an obvious candidate for establishing binding to midgut cells.

Bandla *et al.* (Bandla et al., 1998) recently identified a 50 kDa *F. occidentalis* protein, able to bind to the TSWV glycoproteins, and apparently present in the midgut of the insect, using similar overlay assays. It is remarkable that we do not find binding of TSWV proteins to gut-related thrips proteins, whereas such an interaction is certainly expected to enable entry of virus into gut cells. One explanation is based on the methodology used in our binding studies, which may have excluded certain interactions that are actually present *in vivo*, dependent on environmental aspects such as pH and salt concentration. Small differences in experimental circumstances may thus have accounted for the identification of a different thrips protein by Bandla *et al.* Binding assays using yet different circumstances and experimental set-up may elucidate additional interactions of importance between TSWV and thrips proteins.

Acknowledgments

We thank Dr. J.J. Fransen of the Research Station for Floriculture and Glasshouse Vegetables, Aalsmeer, for providing *Parthenothrips dracenae* thrips, Drs. E. de Vries, University of Amsterdam, for different strains of *Enterobacter agglomerans*, and Dr. B.J.M. Verduin, Department of Virology at Wageningen Agricultural University, for purified brome mosaic virus and anti-BMV serum preparations.

CHAPTER 6

GENERAL DISCUSSION

Introduction

This thesis describes the roles of the two virally encoded glycoproteins G1 and G2 in the life cycle of TSWV. A rather unique feature of TSWV is that it replicates in two very distinct types of hosts, i.e. plants and insects, which are fundamentally different in many aspects. This dual tropism of TSWV suggests an even larger variety of functional features in the TSWV (glyco)proteins in order to meet the prerequisites of its different hosts.

The results described in this thesis show that TSWV particle morphogenesis in plant cells, in which the glycoproteins have a prominent role, is very distinct from that of animal infecting bunyaviruses in animal cells and presumably also from TSWV morphogenesis in insect cells. Apparently, TSWV has adapted its morphogenesis to the replication in plants, while it still has to be able to infect insect cells, presumably requiring a different morphogenesis strategy. Analysis of the expression of the TSWV glycoproteins in animal cells suggests that the early steps of maturation and targeting of the viral glycoproteins probably do not require different strategies in plant and animal cells, implicating parallelism in protein trafficking and retention mechanisms between plants and animals exploited by the virus. Later stages in the morphogenesis must require specialized adaptations in either host, which have to be characterized as yet.

Apart from the role of the glycoproteins in morphogenesis, an investigation of the role in the thrips cycle is also presented in this thesis. A candidate thrips receptor interacting with the TSWV glycoproteins was thus identified, which opens the way to further explore the molecular processes underlying TSWV entry and circulation in the thrips vector.

Tomato spotted wilt virus envelope glycoproteins

Particle morphogenesis and the role of the glycoproteins. The particle morphogenesis process of TSWV in plants has been an enigma for a long period. The development of a protoplast infection system, as described in chapter 2, provided a crucial means to unravel the chronology of the process, and allowed the reconstruction of the model now available (chapter 3). Additionally, specific antisera against the TSWV structural proteins, and the availability of specific markers to identify the Golgi system as the organel involved in particle formation, were as important. The virus associated structures, as identified in the past, could be assigned a chronological position in the morphogenesis pathway, thereby providing the key to the solution of the puzzle. The puzzle-piece that was most cryptic in the past, was the putative involvement of the Golgi system in the rather unique wrapping event resulting in doubly enveloped particles. Researchers investigating the infection in whole plants, had concluded that the actual process of formation of particles must take place with a relative high speed, since intermediate structures were very scarcely encountered. In fact, the only candidates that could possibly serve as intermediate structures in the morphogenesis were the paired parallel membranes, which, however, were looked upon with suspicion and their significance was doubted (Lawson et al., 1996; Kitajima et al., 1992a). The most common process by which enveloped virus particles are formed is budding, a mechanism also used by the animal-infecting bunyaviruses (reviewed by Griffiths and Rottier, 1992, see also Fig. 6.1 which was taken from this review), and thus it was assumed this would also be the most obvious way for TSWV to form particles in plants. Wrapping of double membranes is a much less obvious event involved in particle formation, and above all, not analogous to a known common cellular process. It is probably therefore that the paired parallel membranes have long been interpreted as artefacts or by-products of the infection. Analyses of the synchronous infections in protoplasts, however, presently strongly suggest that they form a crucial step in the morphogenesis pathway of TSWV in plant cells. Figure 6.2 compares the budding and wrapping of animal-infecting and plant-infecting bunyaviruses respectively.

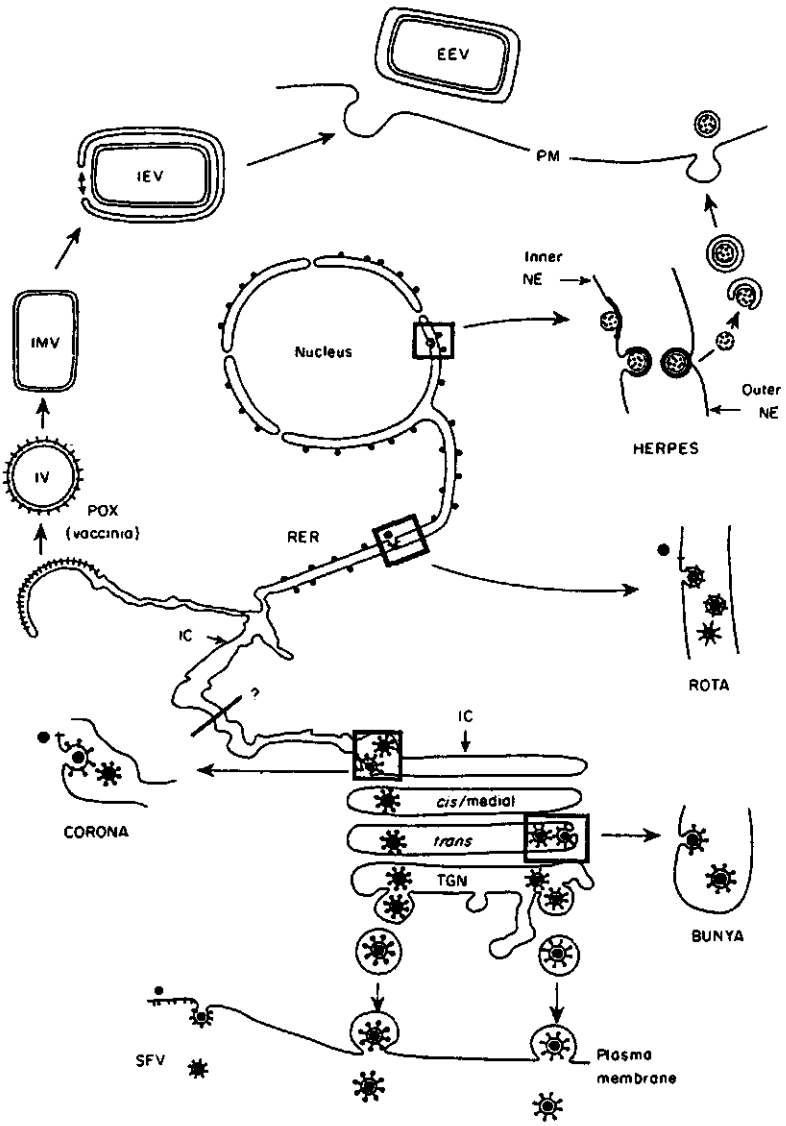


Figure 6.1 *Schematic diagram of the assembly of five enveloped viruses. Budding of animal-infecting bunyavirus into the lumen of Golgi cisternae is depicted in the lower right corner of the diagram. (From Griffiths and Rottier, 1992, with permission).*

There are only a few other virus taxa known to use a similar wrapping mechanism to form their particles, i.e. herpesviruses (Gershon et al., 1994; Whealy et al., 1991;Granzow et al., 1997), poxviruses (Schmelz et al., 1994b; Sodeik et al., 1993) (see also Fig. 6.1) and African swine fever like viruses (Rouiller et al., 1998). All of those are large double stranded DNA viruses infecting animals, making the addition of TSWV to this list remarkable. An intruiging question is therefore, why a relatively simple, plant-infecting virus like TSWV would make use of such an exceptional mechanism to form its particles. Why does the virus not bud directly into the lumen of the ER, to reach its apparent final destination? One aspect is

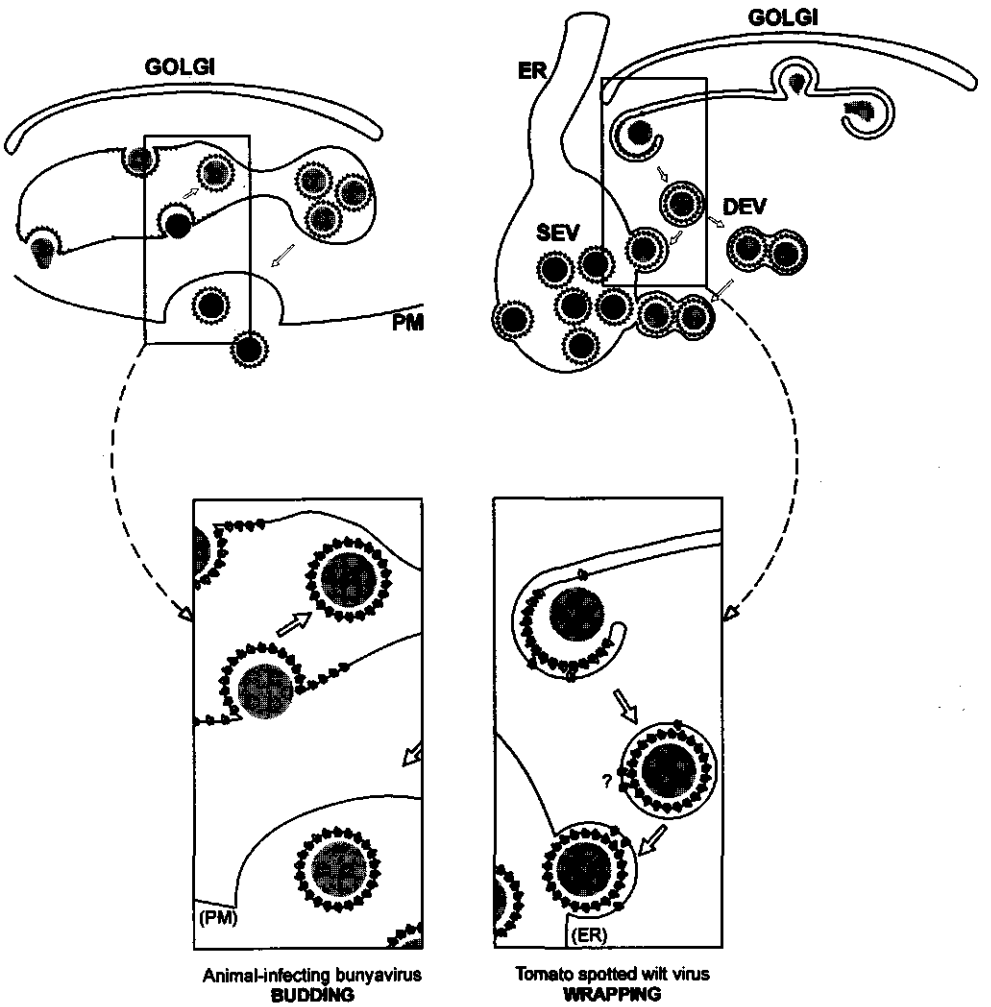


Figure 6.2 *Comparison of the morphogenesis pathway of animal-infecting bunyaviruses (budding of particles into the lumen of vacuolized Golgi cisterna) with TSWV morphogenesis (wrapping of Golgi membranes around nucleocapsids). GOLGI = Golgi complex; ER = endoplasmic reticulum; SEV = singly enveloped particles; DEV = doubly enveloped particles; PM = plasma membrane.*
For the formation of doubly enveloped TSWV particles it is unclear whether glycoproteins are incorporated into the outer membrane. This is indicated with a question mark.

that the viral glycoproteins probably need modification of their glycans, which take place in the Golgi system (Kornfeld and Kornfeld, 1985), thus making the passage through this organel necessary. However, transport of proteins from the Golgi apparatus back to the ER exists (Cole et al., 1998; Lewis and Pelham, 1996; Lippincott et al., 1990; Townsley et al., 1993), although it has not been conclusively shown in plants (B. Satiat-Jeunemaitre, personal communication), so that budding into the ER would seem to be easier, after retrograde

transport of the modified glycoproteins (see also Fig. 1.4 in the Introduction section). Perhaps the assumption that tospoviruses have evolved relatively recently from an ancestral bunyavirus provides a point of explanation. The retention and accumulation of the viral glycoproteins in the Golgi system may be a stably inherited feature, which may account for the formation of TSWV particles at this organel. Additionally, the virus needs a means to transport its particles back to the ER for a secure intracellular accumulation instead of excretion, an adaptation needed in the plant cell. In this view, the wrapping event may be interpreted as a combined budding into the lumen of the Golgi cisternae (as inherited from the ancestor) and a simultaneous pinching off of vesicles for retrograde transport (for which an added ER retrieval signal could be responsible). At second glance, the wrapping may then not be such a strikingly different mechanism.

An intriguing idea is that the TSWV particle morphogenesis may be different in plants and insects, whereby the morphogenesis in thrips may resemble that of the animal-infecting bunyaviruses more closely. This implicates that the glycoproteins cause curling and extension of the Golgi cisternae in plant cells as seen in preparation of the wrapping (this thesis), while their accumulation in insect cells may result in vacuolization of Golgi cisternae to facilitate budding, as seen in animal-infecting bunyavirus morphogenesis (Gahmberg et al., 1986). Subsequently, particles formed in the insect have to be excreted into the saliva, while particles in plant cells have to accumulate intracellularly. This would mean that the TSWV glycoproteins must carry several different characteristics, which functions have to be alternatingly regulated dependent on the host organism.

However, as mentioned, the retention and accumulation of the TSWV glycoproteins in the Golgi system seems to be a stable aspect, that functions in bunyavirus particle morphogenesis in both plant and animal cells. The results of chapter 4 show, that the TSWV glycoproteins carry a signal (most probably in G2) to target them to the Golgi system in animal cells, as was shown for several other, animal-infecting bunyaviruses (Andersson et al., 1997a; Chen et al., 1991b; Chen and Compans, 1991; Lappin et al., 1994; Matsuoka et al., 1994; Matsuoka et al., 1996; Melin et al., 1995; Ronnholm, 1992; Ruusala et al., 1992). Also in the infection of plant cells, circumstantial evidence suggests that the TSWV glycoproteins accumulate in the Golgi system (chapter 3). They accumulate at the (modified) Golgi-membranes on their anticipated way through the secretory pathway as indicated by their immunogold-labeling at the Golgi system. This accumulation must be achieved by an active retention, otherwise the glycoproteins would have also be found on the plasma membrane, which is not the case. Since literature indicates that many aspects of the protein trafficking and targeting machinery is homologous among all eucaryotes and even prokaryotes (Kermode, 1996), the same retention signal, most probably present in G2, will probably have a function during infection of both plant and animal cells.

In this respect, it is interesting to note that during infections in plant tissues and protoplasts (chapter 1), two forms of TSWV G2 are found in isolated virus particles, of 58 kDa and 55 kDa. It has been postulated that they may represent different glycosylated forms of the protein, but another possibility would be that the smaller form is generated after additional cleavage, by which the internal signal sequence is removed from the C-terminus of G2 (depicted with a question mark in Figure 6.3). It is remarkable that expression of TSWV G2 alone, or of the precursor, in heterologous animal cell systems like the baculovirus expression system (Kormelink, 1994; Adkins et al., 1996) or the SFV expression system (chapter 4), results in only one form of the G2 protein co-migrating with the larger 58 kDa G2 protein found in infection of plants. Also, all animal-infecting bunyaviruses produce a single form of G2. These observations together suggest that the putative additional cleavage is a specific feature of particle morphogenesis in plants. A tempting hypothesis would be that the cleavage takes place after, or during, the formation of doubly enveloped particles in plant

cells. This would have to be achieved by a cytoplasmic enzyme, or an enzyme that is anchored at the cytoplasmic face of the ER membrane, since the proposed cleavage is in the cytoplasmic tail of G2 (see Fig. 6.3). Only G2 molecules that are present in the outer membrane of the doubly enveloped particle would be accessible to the cleavage (see Fig. 6.2). Intruigingly, such a cleavage would reveal a so-called C-terminal double lysine motif, which is present in that area: KHKWTS at amino acid position 420-425 (see Fig. 6.1 in chapter 4). A motif like this at the cytoplasmic C-terminus of type I membrane proteins implicates an ER retention signal (Kermode, 1996; Jackson et al., 1990; Jackson et al., 1990). Such a signal ensures retrieval of membrane proteins from the Golgi complex back to the ER in mammalian cells (Jackson et al., 1993). This then possibly explains the specific fusion of doubly enveloped particles, derived from Golgi membranes and exposing the cytoplasmic tails of G2 to the cytoplasm (see Fig. 6.2, question mark), back to the ER during infections of plant cells. Of course this hypothesis is quite speculative, but may form an interesting basis for further research.

Membrane insertion and complex formation of the TSWV glycoproteins. It has not been investigated in what way exactly the TSWV glycoproteins are cleaved, and how they are inserted into the ER membrane. However, based on bunyavirus literature and the results described in chapter 4, a model for the translation and insertion of the TSWV glycoprotein precursor can be proposed as depicted in Figure 6.3. The G1 sequence is preceded by a separate, internal, signal sequence, as was shown in chapter 4, and this feature is apparently used by the virus, not only to achieve a correct targeting and insertion of the G1 protein, but it is also a means to separate G2 from G1 via the signal peptidase cleavage site as depicted in the Figure 6.3.

During subsequent transport of the glycoproteins through the secretory pathway, G1 and G2 are associated, as concluded from the experiments described in chapter 4. Unanswered questions are, however, what the constitution of the transport competent complex is exactly, and whether the complex changes after incorporation in the virus particle. Also for the animal-infecting bunyaviruses these questions have not been answered satisfactory. For a long time, it was assumed that the bunyavirus glycoproteins form heterodimers, during transport as well as on the surface of the virus particle. However, as mentioned already in the Introduction section, Rönka and co-workers showed that the Uukuniemi virus glycoproteins surprisingly do not form stable heterodimers on the particle membrane, but instead G1 homodimers were found, as well as G2 homodimers (Ronka et al., 1995). The G1 homodimers are stable, and pH insensitive, but the G2 homodimers completely dissociate into monomers at pH values of 6.0 and lower. This pH dependent change of conformation may obviously be associated with viral entry into the cell, by means of fusion with the lysosomal membrane, in the acidic environment of the lysosome.

For TSWV, the complex formation of the glycoproteins has not been further investigated yet. The results of Chapter 4 show that transport competent complexes contain both G1 and G2, possibly as heterodimers. Additionally, preliminary results of electrophoresis of glycoproteins from isolated virus particles in reducing and non-reducing gels, suggest that G1 homodimers as well as G2 homodimers exist on the surface of virus particles, but also G1/G2 heterodimers could be found (unpublished results). Pilot co-immunoprecipitation experiments of the proteins in the virus particle, treated with Triton-X100 to remove the envelope, also suggest an association of G1 with G2, since G1 is co-immunoprecipitated with anti-G2, and vice versa. Anti-N serum also co-immunoprecipitates a little G2, but almost no G1 (unpublished results), suggesting that G2 may be the "matrix protein" which normally interacts with N in the core of the particle. Interestingly, the G2b form is only immunoprecipitated with anti-G2 serum, suggesting that G2b does not form a complex with G1 or N. The smaller G2b could exist as a monomer or homomeric complex in the particle,

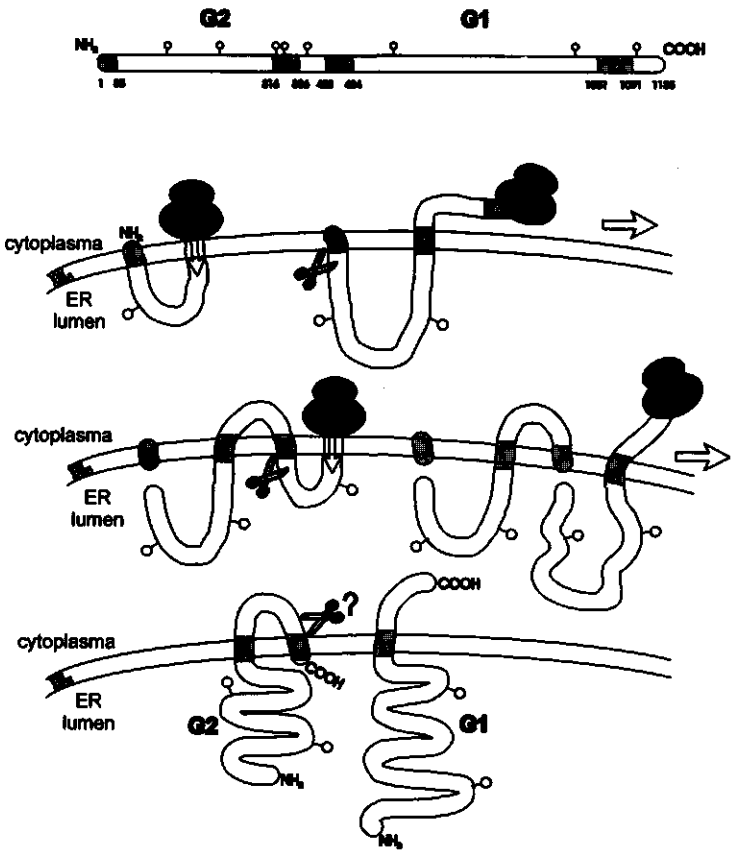


Figure 6.3 Schematic view of proposed translation and membrane insertion of the TSWV glycoproteins. Amino acid numbers are indicated in the representation of the complete precursor at the top of the figure. Anticipated N-linked glycosylation is indicated with small spheres on sticks. Scissor symbols indicate (signal peptidase) cleavages. The cleavage of G2, releasing the internal signal sequence of amino acids 428-484 from the protein, is uncertain, and therefore indicated with a question mark.

or, consistent with the hypothesis proposed earlier in this section, represent cleaved G2 that is present only in the outer membrane of doubly enveloped particles, which directs those particles back to the ER, but is not a component of mature, singly enveloped particles.

Although the results on complex formation of TSWV glycoproteins are still preliminary, and further research is needed, the current depiction of glycoprotein monomers on the surface of schematic representations of TSWV particles (see thesis Kormelink, 1994) does not seem appropriate any more. Considering the bunyavirus literature and the results mentioned above, the presence of homo- and/or heterodimers, besides possible monomers, on the particle membrane surface is anticipated to be more likely. The complex form of the glycoproteins probably represents a dynamic constitution that varies throughout the time-span of existence of a virus particle, dependent on the environment. Figure 6.4 depicts a proposed representation of the TSWV particle, showing a variety of glycoprotein complexes on the surface, and in this way probably better harboring the reality.

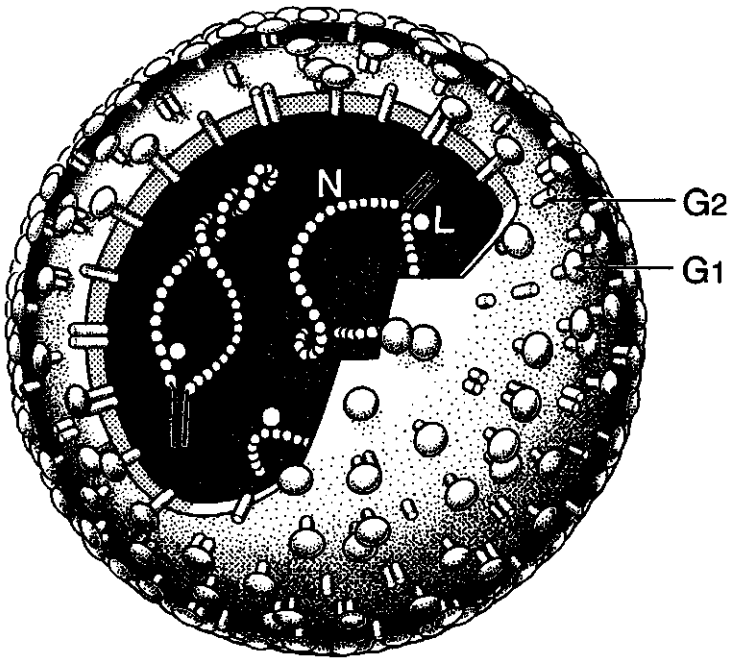


Figure 6.4 *Schematic representation of the TSWV enveloped particle. The glycoproteins G1 and G2 are shown in different complexes, emphasizing the anticipated dynamic nature of the complex formation of the glycoproteins on the surface of the particle, under influence of the pH differences in the encountered environments during infection.*

Role of TSWV glycoproteins in thrips. At the onset of the research described in this thesis, nothing was known about the molecular interaction of the glycoproteins with its vector. Also for other, animal-infecting, bunyaviruses the molecular interactions between virus and vector are still largely unclear. Chapter 5 describes a specific interaction between TSWV glycoproteins and a thrips protein, using relatively simple techniques. It is the first time that a putative receptor is identified that is involved in infection of any bunyavirus with its vector. The significance of the results however needs to be confirmed, since further experiments have to show whether the binding that was found under the experimental circumstances can also be found *in vivo*. An important aspect will be to show that the 94 kDa protein is indeed expressed on the surface of thrips cells. If not, the protein may be a chaperone that is involved in folding or processing of the G2 protein within the cell, rather than a receptor to allow entry.

Shortly after chapter 5 was published, Bandla and co-workers (Bandla et al., 1998) described another, 50 kDa thrips protein, interacting with the TSWV glycoproteins, using similar techniques. It is still an enigma as to why two research groups have found different proteins using virtually the same technique. Obviously the experimental circumstances were different, which may have accounted for the different results. Additionally, it seems likely that the molecular interactions between the TSWV glycoproteins and the thrips vector are quite complex, and involve different steps of binding throughout the circulation. As was shown or suggested for many virus-vector relations, up to three or four dissemination barriers have to be overcome during circulation of the virus through the insect vector, all of which

may require receptor mediated passage. The two identified thrips proteins may each represent a receptor at a different barrier, and the TSWV-thrips relation therefore deserves extended investigations to unravel this relatively neglected subject in tospovirus research.

Nagata et al. (Nagata et al., 1997a) recently reported on the development of primary thrips (*F. occidentalis* and *Thrips tabaci*) cell cultures, and their infection with purified TSWV particles (Nagata et al., 1997b). The production of NSs and N protein could be shown in these cells 24 h.p.i., indicating that the virus is able to enter the cells and is subsequently replicated. Furthermore, the authors showed that the culture medium of infected cells contained infectious virus, strongly suggesting that newly produced TSWV particles are excreted from those thrips cells. These are in fact the first data suggesting that TSWV particle morphogenesis is different in plant and in thrips cells, since in plant cells enveloped particles accumulate within the cell instead of being excreted. Concerning the 94 kDa thrips protein, recent results suggest that also these primary thrips cell lines, contain this protein that binds to G2, as shown in overlay assays (unpublished results, Nagata et al.). This may open the way to further unraveling of the significance of this binding for entry of TSWV into insect cells.

Another point that needs further investigation is the significance of the RGD motif (the amino acid sequence Arg-Gly-Asp) at the N-terminus of the TSWV G2 protein. The RGD tri-peptide is a well-known ligand for specific cell-surface integrins (Pierschbacher and Ruoslahti, 1987; Ruoslahti and Pierschbacher, 1987) normally involved in attachment of cells to extracellular glycoprotein matrix. A number of viruses contain an RGD motif in their surface attachment proteins, which can mediate entry of the virus through binding with the integrin receptors on the surface of target cells. For foot-and-mouth disease virus (FMDV), it was shown that the RGD motif in the N-terminus of the VP1 protein mediates entry into for example BHK cells, by binding to the vitronectin integrin receptor, or to a lower extend, to the fibronectin integrin receptor (Fox et al., 1989; Villaverde et al., 1996). Similarly, the RGD containing VP1 protein of coxsackievirus is also involved in cell entry through the vitronectin receptor into several different cell-lines (Roivainen et al., 1991). For the latter virus, however, it was shown that binding to the gut of the host exposes the virus to proteolytic enzymes, which cleave off the N-terminal RGD motif. The virus remains infectious, suggesting a bypass entry route that circumvents the RGD interaction *in vivo* (Roivainen et al., 1996).

For TSWV, the role of the RGD motif at the N-terminus of G2 is unknown. The results of chapter 5 and those of earlier experiments (Mohamed, 1981) suggest that particularly G2 is sensitive to proteolytic cleavage. It is therefore not unlikely that *in vivo*, attachment of TSWV to the midgut of thrips is accompanied by the cleavage of G2 by proteolytic enzymes abundantly present in the insect's gut. Since the RGD motif is almost N-terminal in G2, and thus the chance for removal of the motif upon any cleavage is very high, this may implicate that the entry into thrips gut cells is not mediated by the RGD motif, but rather by other domains in G2, or by the more protease insensitive G1 protein. This suggests that like for coxsackievirus, the entry mechanism of TSWV into cells may involve bypasses upon destroyal of the RGD entry mechanism. Cleavage of G2 abolishes the binding to the 94 kDa thrips protein (chapter 5). It has to be investigated whether this binding is mediated by the RGD motif of G2 or not. The fact that the 94 kDa protein was not found in the midgut of thrips may implicate that the RGD motif is involved in other parts of the cycling of the virus through the thrips, in compartments that contain less proteolytic activity.

BHK cells, as used in chapter 4, contain the integrin cell-surface receptors vitronectin and fibronectin, which are able to interact with RGD motifs from FMDV and coxsackievirus (Fox et al., 1989; Roivainen et al., 1991). Preliminary experiments using freshly purified TSWV virus particles from plants that were added to these mammalian cells, however, have not clearly shown the entry and subsequent replication of TSWV in BHK cells.

Immunofluorescence experiments showed the intracellular localization of small amounts of N protein, but none of the other viral proteins were found (unpublished results). This may demonstrate that TSWV is able to enter the BHK cells to a certain extend, possibly by means of its RGD motif, but that subsequent replication is not possible.

TSWV envelope glycoproteins as probes for the unraveling of the cellular protein processing machinery

Many basic cellular mechanisms have been discovered in the past by using viruses or viral proteins as probes. The viral membrane protein that is used most often in studies of cellular processing and trafficking mechanisms is the G (glyco)protein of vesicular stomatitis virus (VSV), a rhabdovirus. Future discovery of novel cellular processes will undoubtedly continue to be connected in many cases with the interaction of viruses with cells. Since the TSWV glycoproteins have functions in both plants and thrips, their study potentially provides information about cellular processes in both organisms, which may be more widely applicable in other organisms and cell-types. The dual tropism of TSWV implicates that the TSWV glycoproteins harbour different characteristics, functional in either or both of the host and vector organisms. These may give information about potential overlap, parallelisms and discrepansies between cellular processes in plants and insects, providing possibilities for particularly interesting integrating research.

The identification of an internal signal sequence in the TSWV glycoprotein precursor, preceding the G1 ORF (chapter 4), implicates that signal sequences do not necessarily have to be located N-terminal in the protein to be functional. This has also been demonstrated in mammalian cells for other bunyaviruses and Semliki Forest alphavirus (Andersson et al., 1997b; Hashimoto et al., 1981), and our results suggest that this is most probably also true in plant cells.

Transport competence of membrane proteins, which need to leave the ER, is thought to be regulated by correct folding and oligomerization assisted by chaperones, and is stimulated by glycosylation (reviewed in: Boston et al., 1996; Craig et al., 1994; Tatu and Helenius, 1997; Trombetta and Helenius, 1998; Imperiali and Rickert, 1995; O'Connor and Imperiali, 1996; Craig et al., 1994; Tatu and Helenius, 1997; Trombetta and Helenius, 1998; Imperiali and Rickert, 1995; O'Connor and Imperiali, 1996). The results of chapter 4 are consistant with this, and the prerequisites for export from the ER may be further investigated using the TSWV glycoproteins as a model. The study of the oligomerization of TSWV glycoproteins may provide more information on the cellular factors involved in this process, and the oligomerization may be related to transport competence, and to masking and demasking of trafficking signals on either of the glycoproteins. The partial retention of G2 on its own, as observed in chapter 4, may be caused by misfolding, but there could also be an ER retention signal present in this protein which additionally hampers the export from the ER, in order to let G1 be translated and folded before too much of the G2 protein has escaped to the Golgi complex. This signal may then be masked by the interaction with G1, thereby promoting the efficient joined export of G1 and G2 from the ER.

Apart from the regulatory factors mentioned, the transport of (membrane) proteins through the secretory pathway from the ER via the Golgi system to the plasmamembrane was believed to be a default process, not further regulated by positive signals (review: Bar et al., 1996). However, in contrast, a report of Nishimura and Balch (Nishimura and Balch, 1997) suggests that the export from the ER of VSV-G membrane protein may be a selective mechanism, regulated (among possible other signals) by a di-acidic motif (DXE), which is present in the cytoplasmic tail of the protein: TM(transmembrane domain)-18aa-YTDIE-6aa. The Yxxø motif (underlined), representing a tyrosine (Y), two other amino acids of any kind

(xx) and a hydrofobic residue (ø), in the vicinity of the di-acidic signal seems necessary for basolateral sorting of VSV-G (Thomas and Roth, 1994), and they together ensure efficient recruitment of VSV-G into COPII vesicles (Nishimura and Balch, 1997). The latter are generally accepted to be responsable for protein transport from the ER to the Golgi complex (Schekman and Orci, 1996). Many animal and animal-infecting virus membrane proteins were shown to possess such a signal, whereby there was always a spacer region between the transmembrane domain and the signal, and the Yxxø signal was also present, N-terminal of the DXE motif. However, not all transmembrane cargo molecules leaving the ER contain these signals, and most probably they rather are a stimulus and not a prerequisite for exit from the ER (Nishimura and Balch, 1997). Furthermore, they can be overruled by other signals, for example retention signals. When examining the sequence of the TSWV glycoprotein precursor, indeed the TSWV G1 ORF also contains the di-acidic signal, in combination with the Yxxø motif. The sequence is: TM-YVKN-11 aa's-**DDE**-26 aa's. In G2 there is one in the oposite direction: TM-26aa's-**EAD**-12aa's, which may not be functional. Signals like these may account for varying transport kinetics from the ER that have been observed for different proteins, such as the very slow release of Uukuniemi glycoproteins from the ER (Kuismanen, 1984) to the Golgi complex. Bunyavirus glycoproteins, including TSWV G1 and G2 may be suitable models for research in this direction.

The subsequent event after transport from the ER to the Golgi complex, is the retention of the TSWV glycoproteins in the Golgi stacks. This feature was identified in BHK cells (chapter 4), but as mentioned, it is likely that this signal works in plant cells as well, to achieve a similar accumulation of glycoproteins in the Golgi system in infections of plants and protoplasts as was observed. The location of the retention signal that is probably present only in G2 of TSWV (chapter 4) can be further investigated using truncated G2 molecules. The mechanism of Golgi-retention itself is generally very unclear. Whereas for ER retention a number of different short sequence motifs like KDEL and the double lysine motif were identified (reviewed in Kermode, 1996), the Golgi retention signals found up till date do not contain such conserved sequence motifs and have no homology with each other. Furthermore, ER retention involves the retrieval of the signal carrying proteins from the Golgi back to the ER, directed by specific receptors present on the cytoplasmic face of Golgi membranes (reviewed by Bar et al., 1996). In contrast, nothing is known about the mechanism of Golgi retention, though currently there are two hypotheses (reviewed by Bar et al., 1996; Nilsson et al., 1993; Nilsson and Warren, 1994; Nilsson et al., 1993; Nilsson and Warren, 1994). Firstly, the "kin-recognition" hypothesis, which assumes the interaction of Golgi-resident proteins with each other, forming large aggregates that are thereby prevented from transport to the plasma membrane. Secondly, a theory involving the length of transmembrane domains. It is thought that Golgi membranes are thinner than the plasmamembrane, and therefore proteins with short transmembrane domains will prefer to remain or be retrieved to Golgi-membranes, since their transmembrane domains may be too short to fit into the plasmamembrane. A third hypothesis may be postulated that assumes that specific proteins are present on the cytoplasmic face of the Golgi stacks, which may interact with the cytoplasmic tails of Golgi-resident proteins, thereby preventing further transport. TSWV glycoproteins, which retain in the Golgi complex of mammalian and plant cells, are excellent models for investigation of this cellular mechanism, and they may be the tools for showing that Golgi-retention signals are conserved among mammalian and plant cells.

The specifically targeted fusion of Golgi-derived doubly enveloped particles back to the ER (chapter 3) is an intruiging observation described in this thesis. In the past years, the retrograde (backward) transport of proteins in the secretory pathway has been described to some detail in mammalian and yeast cell systems (Cole et al., 1998; Lewis and Pelham, 1996; Lippincott et al., 1990; Townsley et al., 1993; Lewis and Pelham, 1996; Lippincott et al.,

1990; Townsley et al., 1993). The research in plants is proceeding slower, and up till date, there is no clear proof of the existence of retrograde transport in plant cells. The lack of this mechanism in plants would possibly explain why TSWV glycoproteins, after modifications of their glycans in the Golgi system, are not immediately transported back to the Golgi complex to facilitate direct budding of nucleocapsids into the ER. On the other hand, the specific fusion of Golgi-derived doubly enveloped particles with the ER may be an indication for an existing retrograde transport machinery in plants, of which TSWV makes use in this stage of its morphogenesis. As proposed earlier in this discussion, the retrograde transport process may be regulated by a di-lysine motif present in the cytoplasmic tail of G2. Such ER retrieval motifs were shown to interact with COPI (reviewed in Pelham, 1995), which is involved in the formation of vesicles planned for retrograde transport of proteins in mammalian cells. Thus, further study of the morphogenesis pathway of TSWV may reveal important novel cellular mechanisms, not earlier identified in plants.

Future prospects

The results descibed in this thesis provide the first steps towards the detailed unraveling of the structure and function of the TSWV glycoproteins. More discoveries are to be done on these crucial TSWV proteins and their role in different aspects of the virus life cycle. As emphasized in the general discussion, they may also be suitable models for the study of basic cellular processes such as antero- and retrograde trafficking, oligomerization, glycosylation, and retention, in different organisms.

The morphogenesis of TSWV in thrips cells is one of the first subjects to be investigated, in order to verify whether this process is different in plant and insect cells. If this is indeed the case, further investigations will have to show how these distinct morphogenesis pathways are regulated in the different cells. This will require the individual expression of the TSWV glycoproteins in plant and thrips cells, to confirm that their retention in the Golgi system is caused by the intrinsic Golgi retention signal in those organisms as well, and to be able to analyse the modification of Golgi membranes. The further unraveling of the entry and circulation of TSWV in the thrips is another subject that deserves attention. Together, the results of those experiments will give more insight in the intruiging dual tropism of TSWV in plants and insects, potentially providing possibilities for new means to control the virus.

SUMMARY

Tomato spotted wilt virus (TSWV) forms the type member of the genus *Tospovirus*, which today harbors more than twelve different species. TSWV is able to infect an enormous variety of plants species, to which it often causes devastating effects, resulting in severe economical losses. Among the plant viruses, TSWV and the other tospoviruses form a distinct group. Taxonomically, they surprisingly do not belong to a plant virus family, but to a virus family which further consists of animal-infecting viruses, the *Bunyaviridae*. Consequently, they harbor features that are more common to animal-infecting viruses than to plant viruses. The most eye-catching animal-infecting virus-like feature of the tospoviruses is their envelope, in which two viral surface glycoproteins are embedded, denoted G1 and G2. These surface glycoproteins are designed for interaction with receptors, an important step in the infection of animals, but useless in the infection of plants. The plant-infecting tospoviruses are transmitted by thrips, in which they also replicate, and for the entry and circulation of the virus through this insect the glycoproteins are essential. This ensures their continues presence despite their lack of function during the infection of plants. The structure and function of the TSWV glycoproteins during infection in plants and insects form the subject of this thesis, of which the contents will be summarized in the next paragraphs, and also visually represented in Figure 7.1.

First develop the tools...

Although the TSWV glycoproteins may not have a crucial function in the plant, they do play an essential role in the formation of virus particles. At the onset of these studies, however, no clear view of this morphogenesis process was available, so it was the obvious first goal to unravel this process for TSWV in plants. In the past, virus associated structures observed during the infection of whole plants were reported, but thirty five years of observations had not resulted in a clear model of the particle morphogenesis. Useful antibodies against the separate TSWV proteins had not been available, and most importantly, a system in which a synchronous infection could be investigated lacked, so that interpretation of the chrology of the events in the morphogenesis had been difficult. *Chapter 2* of this thesis describes the development of a protoplast infection system for TSWV, which enabled the study of a synchronous TSWV infection in plant cells. Using newly produced antibodies against the viral glycoproteins together with antibodies against the nucleoprotein, it could be shown that a full, synchronous, TSWV infection is achieved, by the PEG-mediated inoculation of freshly isolated *Nicotiana rustica* protoplasts with freshly (and quickly) isolated TSWV particles. Similar inoculation of *Vigna unguiculata* protoplasts did not result in a full infection, since the production of enveloped particles was hampered due to low expression of the viral glycoproteins.

...to unravel the essence of TSWV particle making...

Using the system, based on *N. rustica* protoplasts, the different virus associated structures could be assigned a chronological position in the morphogenesis process. Specific antibodies against the TSWV structural proteins, as well as antibodies against plant cell organels were used, and the model could thus be completed (*Chapter 3*). TSWV structural components, nucleocapsids as well as glycoproteins, accumulate at Golgi membranes, which are consequently modified to form the paired parrallel membranes. Doubly enveloped particles are formed by the so-called "wrapping" of these viral glycoprotein containing membranes around nucleocapsid cores, a process unique among plant viruses. The subsequent step is the fusion of these doubly enveloped particles with each other and specifically with ER membranes. This results in the formation of singly enveloped particles that accumulate within the ER. The proposed model is surprisingly quite distinct from that of the animal-infecting bunyaviruses.

...and then look what regulates this process...

After the complete model of the morphogenesis became available, the next step was to investigate what (molecular) features of the glycoproteins regulate the process. An important observation in the morphogenesis process is the apparent accumulation of viral glycoproteins in the Golgi system. This may, analogous to other enveloped viruses, be caused by the specific targeting of the glycoproteins to this organel due to a retention signal. The trafficking and retention behavior of TSWV glycoproteins was investigated in mammalian cells, the results of which are described in *Chapter 4*. TSWV G1 and G2 accumulate in the Golgi system when expressed together, which indeed implicates that at least one of the proteins must harbor a Golgi retention signal. Separate expression of G1 and G2 revealed that a retention signal is present in G2. G1 on its own is transport incompetent, but this can be rescued by the co-expression with G2, which suggests that G1 is dependent on, and interacts with G2 during transport and retention. These molecular features, identified in mammalian cells, are most probably also functional in plant cells, causing the observed accumulation of glycoproteins in the plant Golgi system during infection. The TSWV glycoproteins thereby show their crucial role in directing the particle morphogenesis process.

...and how these particles interact with thrips.

Once the particles are formed and accumulated inside ER membranes, they await the uptake by the thrips vector to be transferred to another plant. Earlier research has shown that between this uptake and the release of virus there is replication and circulation of TSWV in the thrips. However, nothing was known about the molecular interactions between TSWV proteins and proteins of the thrips during this process. In *Chapter 5* an overlay blot technique was used to investigate the possible binding of TSWV structural proteins with thrips proteins, that could be potential receptors involved in entry or circulation of TSWV. A 94 kDa thrips protein was identified, displaying specific binding to TSWV G2 protein. This 94 kDa protein was found in known vectors of TSWV, and also in a non-vector thrips species, albeit it not in the larval stages of the latter. Although a receptor is anticipated in the gut of the vectoring insects, the 94 kDa protein is not found there, but appears present in all other parts of the thrips body. This suggests that it may have a role elsewhere during the replicative circulation of the virus.

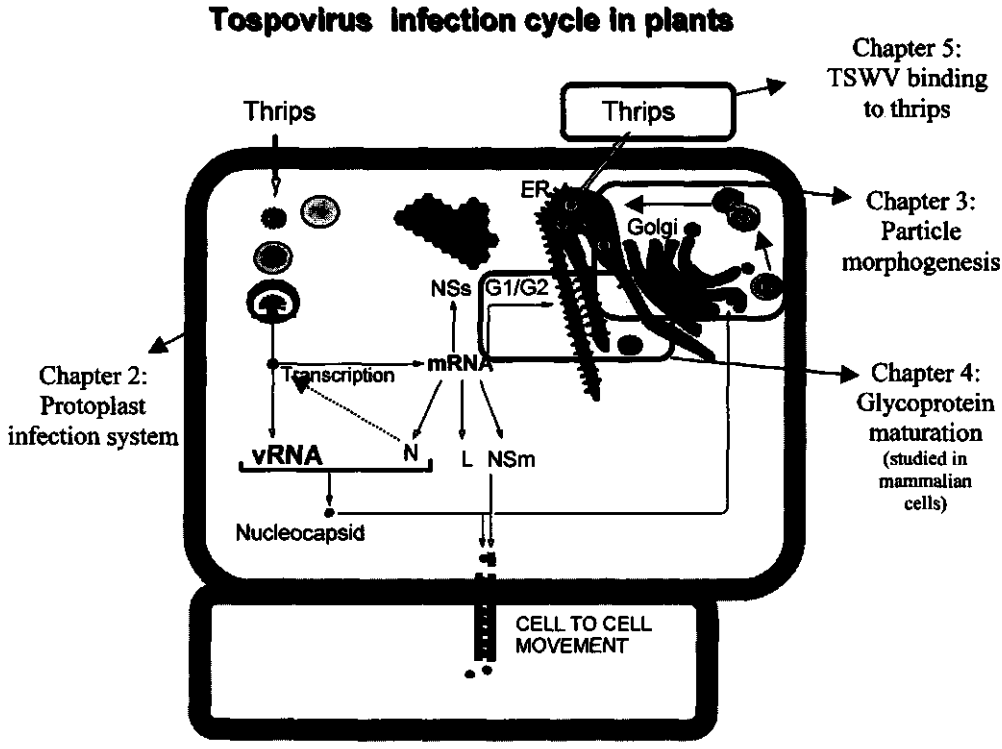


Figure 7.1 *Diagram of TSWV infection in a plant cell, indicating the scope of the experimental chapters of this thesis.*

SAMENVATTING

Het tomatenbronsvlekkenvirus (Engels: tomato spotted wilt virus, afgekort TSWV) is in staat een enorme variëteit aan plantensoorten te infecteren, waaronder een aantal belangrijke kasgroenten (bijv. sla, paprika's en tomaten) en siergewassen (bijv. chrysanten, irissen en petunia's). Taxonomisch behoort TSWV tot de tospovirussen, een geslacht binnen de familie der *Bunyaviridae*. Een tospovirusdeeltje komt de plant binnen doordat tripsen het met zich meedragen, en via het voeden het virus in de plant brengen. Het onderzoek aan tospovirussen richt zich uiteraard op de beheersing en bestrijding van de veroorzaakte ziekten, maar er vindt ook fundamenteel onderzoek plaats om meer over deze bijzondere groep virussen te weten te komen.

Virussen bestaan uit genetisch materiaal, in de vorm van enkele of dubbele strengen DNA of RNA, die coderen voor een aantal voor het virus noodzakelijke eiwitten. Een infectie is bedoeld om viruseiwitten en genetisch materiaal, en daarmee nieuwe virusdeeltjes, te produceren. Daarbij wordt, op een egoïstische manier, geprofiteerd van de diverse cellulaire processen en energiebronnen van de gastheer.

Bij de meeste plantenvirussen is het genetische materiaal in het virusdeeltje beschermd tegen invloeden van buitenaf door een stevige, viraal gecodeerde, eiwitmantel. De architectuur van tospovirussen is echter geheel anders. Het genetische materiaal, in dit geval drie verschillende stukken enkelstrengs RNA, is slechts losjes ingepakt in eiwit, dit complex wordt nucleocapside genoemd. Daaromheen bevindt zich een membraan, en daaruit steken twee soorten viraal gecodeerde glycoproteinen, genaamd G1 en G2 (zie ook figuur 4 uit hoofdstuk 6). Veel virussen die mensen of dieren infecteren hebben eenzelfde opbouw, en het idee is dan ook dat de tospovirussen waarschijnlijk ooit zijn ontstaan uit een mens- of dier-infecterend virus.

De glycoproteinen op het oppervlak van een virusdeeltje zijn ondermeer ontworpen om te binden aan receptoren op diercellen, en deze binding zorgt ervoor dat het virusdeeltje wordt binnengelaten in de cel, waarna het infectieproces kan beginnen. Plantencellen hebben niet van dergelijke receptoren, en de TSWV glycoproteinen lijken inderdaad geen cruciale functie te hebben bij het infecteren van planten. Het virus infecteert echter ook de tripsen (die het virus overbrengen naar andere planten), waarbij de glycoproteinen wel een belangrijke rol spelen.

De structuur en de functie van de TSWV glycoproteinen, en hun verwachte rol tijdens de productie van nieuwe virusdeeltjes, vormen het onderwerp van dit proefschrift, waarvan de inhoud in onderstaande paragrafen, en in Figuur 8.1, wordt samengevat.

Ontwikkel eerst het gereedschap....

Toen met deze studies werd begonnen was niet bekend hoe het TSWV deeltje precies in elkaar wordt gezet tijdens de infectie van plantencellen, en wat de rol van de glycoproteinen hierbij was. Het eerste doel was dus de formatie van de virusdeeltjes te ontrafelen. Gedurende de afgelopen vijfendertig jaar zijn er vele observaties gedaan aan de infectie in de plant, voornamelijk met behulp van de elektronenmicroscoop, het enige instrument waarmee de miniscule virusdeeltjes en andere virusgerelateerde structuren zichtbaar kunnen worden gemaakt. Dit heeft echter niet geleid tot een helder beeld van de formatie van virusdeeltjes, doordat er geen bruikbare antilichamen tegen de virale eiwitten beschikbaar waren, waarmee de plaats van deze eiwitten tijdens het infectie proces bepaald kan worden. Nog belangrijker was echter, dat er geen systeem bestond waarin een synchrone TSWV infectie bestudeerd kan worden. In planten verloopt de infectie namelijk niet synchroon, en worden alle stadia van infectie door elkaar gevonden, waardoor de chronologie van het proces daarin moeilijk te bestuderen is. *Hoofdstuk 2* van dit proefschrift beschrijft de ontwikkeling van bruikbare specifieke antilichamen tegen met name de glycoproteinen, en

een systeem waarin een synchrone infectie kan worden bestudeerd. Dit systeem maakt gebruik van protoplasten, dit zijn plantencellen waarvan de celwand is verwijderd, en allemaal tegelijk kunnen worden geïnfecteerd met geïsoleerde TSWV deeltjes. Wanneer tabakscellen (*Nicotiana rustica*) worden gebruikt levert dit een complete infectie op waarbij nieuwe, infectieuze, virusdeeltjes worden geproduceerd. Bij gebruik van cellen uit de bladeren van kouseband (*Vigna unguiculata*) is de productie van viral glycoproteinen erg laag, waardoor er zeer weinig nieuwe deeltjes ontstaan.

.... om de essentie van het maken van TSWV deeltjes te ontrafelen.....

Gebruikmakend van tabaksprotoplasten konden de eerder geïdentificeerde virusgerelateerde structuren in een chronologisch verband geplaatst worden. Door gebruik te maken van nieuwe, specifieke antilichamen tegen virale eiwitten, en tegen organellen in de cel, kon uiteindelijk een compleet model van de deeltjesformatie worden opgesteld (*hoofdstuk 3*). De structurele componenten van het virusdeeltje; de nucleocapsiden en de glycoproteinen, hopen zich op in en bij Golgi membranen (het Golgi apparaat is een celorganel dat bestaat uit membranen). Hierdoor worden deze membranen van vorm veranderd en dan "gepaarde parallelle membranen" genoemd. Uit deze structuren onstaan virusdeeltjes met een dubbele membraan doordat de gepaarde parallelle membranen, die virale glycoproteinen bevatten, zich om nucleocapsiden heen vouwen (zie ook figuur 9 uit hoofdstuk 3). Vervolgens fuseren de buitenste menbranen van deze deeltjes met elkaar, of fuseren ze met het ER. Zodoende ontstaan uiteindelijk virusdeeltjes die slechts één membraan over hebben, en die zich ophopen als de inhoud van ER membranen. Het verrassende aan dit model is, dat het erg verschilt van dat van de morfogenese van de dier-infecterende virussen uit de *Bunyaviridae* familie.

...en bekijk dan hoe dit proces wordt gereguleerd...

De volgende vraag was hoe de morphogenese wordt gereguleerd door met name de glycoproteinen. Deze lijken zich op te hopen in de membranen van het Golgi apparaat, en dit zou, zoals bij andere membraanvirussen het geval is, veroorzaakt kunnen worden doordat ze worden vastgehouden onder invloed van een zogenaamd retentiesignaal. Dit signaal zou aanwezig kunnen zijn in beide of in slechts één van de glycoproteinen zelf, en dit kon onderzocht worden door ze in afwezigheid van een volledige infectie-achtergrond te produceren. De aanmaak, het transport en de retentie van de TSWV glycoproteinen is bestudeerd in zoogdiercellen (*hoofdstuk 4*). Cellulaire glycoproteinen worden normaal gesproken geproduceerd in het ER, waarna ze via het Golgi apparaat naar het plasmamembraan worden getransporteerd. De productie van virale glycoproteinen maakt gebruik van ditzelfde cellulaire mechanisme. De resultaten van de proeven in hoofstuk 4 geven aan dat de TSWV glycoproteinen zich tijdens het transport door de cel in het Golgi apparaat ophopen, wat erop wijst dat tenminste één van de twee een Golgi retentiesignaal bevat. Aparte expressie van G1 en G2 laat zien dat er een rententie signaal aanwezig is in G2. G1 kan niet uit zichzelf het ER verlaten, tenzij het samen met G2 wordt geproduceerd. Dit suggereert dat G1 interacteert met, en afhankelijk is van G2 tijdens transport en retentie. Deze eigenschappen, bepaald in zoogdiercellen, zijn hoogstwaarschijnlijk ook functioneel tijdens de infectie in planten, want ook daarin hopen de glycoproteinen zich tijdens de infectie op in het Golgi apparaat. Zodoende bepalen de glycoproteinen de plaats van de morfogenese, en rekruteert het virus daarbij Golgi membranen van de gastheer.

....en hoe de virusdeeltjes interacteren met tripsen.

Als de deeltjes eenmaal accumuleren in de membranen van het ER zijn ze klaar voor de opname door tripsen, die ze tijdens het voeden binnenkrijgen, en uiteindelijk weer afgeven aan andere planten. Eerder onderzoek heeft aangetoond dat tussen de opname en afgifte van virus, replicatie en circulatie van het virus plaatsvindt in de trips. Er was echter niets bekend over de moleculaire interacties tussen het virus en eiwitten van de trips, die ervoor zorgen dat het virus door het insect wordt opgenomen, en kan worden getransloceerd van het

maag/darmkanaal naar de speekselklieren, via welke het virus het insect weer verlaat. In *hoofdstuk 5* werden zogenaamde overlay blots gebruikt om specifieke binding van viruseiwitten met eiwitten van de trips aan te tonen. Via deze techniek werd inderdaad een trips eiwit gevonden van 94 kilodalton (kDa), dat specifiek bindt aan G2 van TSWV. Dit eiwit werd gevonden in de trips soorten die TSWV overbrengen, en ook in een soort die het virus niet kan overbrengen, maar dan alleen in het volwassen stadium. Hoewel men verwacht dat er een receptor nodig is die in de darm van de trips het binnendringen van het virus bewerkstelligt, werd het 94 kDa eiwit niet hier gevonden. Het komt juist voor in andere delen van het insect, en zou derhalve betrokken kunnen zijn bij het replicatieve circulatieproces van het virus.

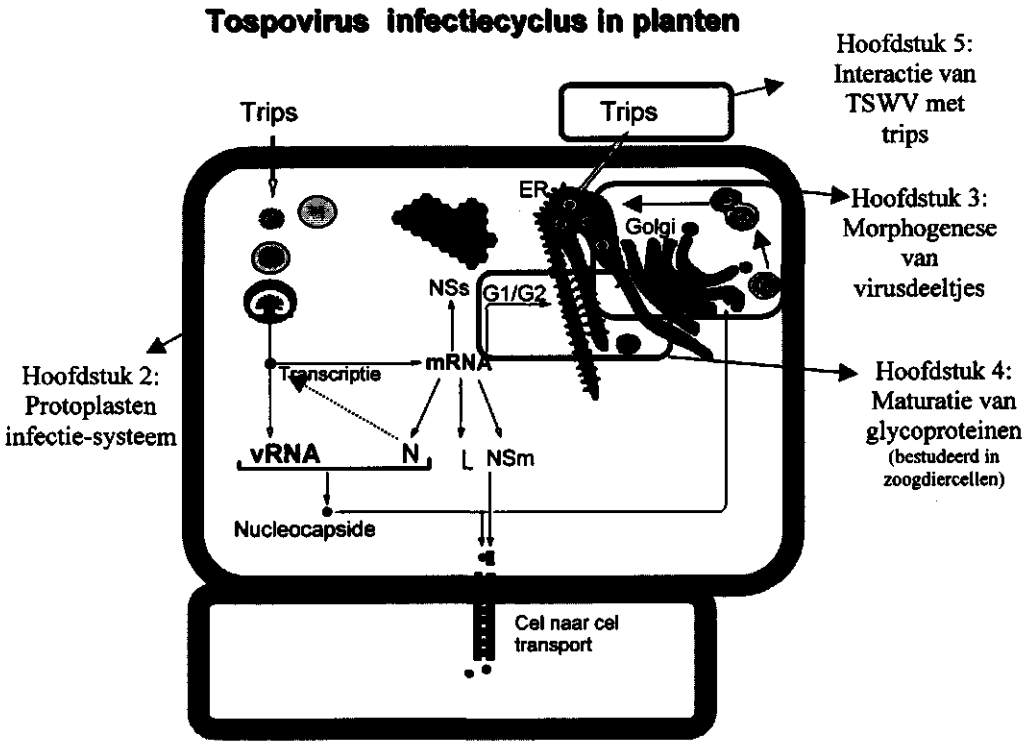


***Figure 8.1** Diagram van de TSWV infectie in een plantencel, waarin de onderwerpen van de experimentele hoofdstukken van dit proefschrift zijn aangegeven.*

References

Adam, G., Peters, D., and Goldbach, R.W. (1996). Serological comparison of tospovirus isolates using polyclonal and monoclonal antibodies. Proceedings of the International Symposiom on Tospoviruses and Thrips of Floral and Vegetable Crops. Acta Hortic *431*, 135-185.

Adkins, S. A., Choi, T-J, Israel, B. A., Bandla, M. D., Richmond, K. E., Schultz, K. T., Sherwood, J. L., and German, T. L. (1996). Baculovirus expression and processing of tomato spotted wilt tospovirus glycoproteins. Phytopathology *86*, 849-855. 1996.

Ammar, E. D. (1994). Propagative transmission of plant and animal viruses by insects: factors affecting vector specificity and competence. Adv Dis Vect Res *10*, 289-333.

Andersson, A.M., Melin, L., Bean, A., and Pettersson, R.F. (1997a). A retention signal necessary and sufficient for Golgi localization maps to the cytoplasmic tail of a Bunyaviridae (Uukuniemi virus) membrane glycoprotein. J Virol *71*, 4717-4727.

Andersson, A.M., Melin, L., Persson, R., Raschperger, E., Wikstrom, L., and Pettersson, R.F. (1997b). Processing and membrane topology of the spike proteins G1 and G2 of Uukuniemi virus. J Virol *71*, 218-225.

Bailey, J.M. and Davidson, N. (1976). Methylmercury as a reversible denaturing agent for agarose gel electrophoresis. Anal Biochem *70*, 75-85.

Bandla, M.D., Campbell, L.R., Ullman, D.E., and Sherwood, J.L. (1998). Interaction of tomato spotted wilt tospovirus (TSWV) glycoproteins with a thrips midgut protein, a potential cellular receptor for TSWV. Phytopathology *88*, 98-104.

Bar, P.M., Bassham, D.C., and Raikhel, N.V. (1996). Transport of proteins in eukaryotic cells: more questions ahead. Plant Mol Biol *32*, 223-249.

Berinstein, A., Roivainen, M., Hovi, T., Mason, P.W., and Baxt, B. (1995). Antibodies to the vitronectin receptor (integrin alpha V beta 3) inhibit binding and infection of foot-and-mouth disease virus to cultured cells. J Virol *69*, 2664-2666.

Bishop, D.H., Calisher, C.H., Casals, J., Chumakov, M.P., Gaidamovich, S.Y., Hannoun, C., Lvov, D.K., Marshall, I.D., Oker, B.N., Pettersson, R.F., Porterfield, J.S., Russell, P.K., Shope, R.E., and Westaway, E.G. (1980). Bunyaviridae. Intervirology *14*, 125-143.

Boston, R.S., Viitanen, P.V., and Vierling, E. (1996). Molecular chaperones and protein folding in plants. Plant Mol Biol *32*, 191-222.

Brand, C.M. and Skehel, J.J. (1972). Crystalline antigen from the influenza virus envelope. Nat New Biol *238*, 145-147.

Brittlebank, C.C. (1919). Tomato diseases. J Agric Victoria *27*, 231-235.

Brunt, A.A., Crabtree, K., Dallwitz, M.J., Gibbs, A.J., and Watson, L. (1996). Viruses of Plants; description and lists from the VIDE database (Oxon, UK: CAB International).

Bupp, K., Stillmock, K., and Gonzalez, S.F. (1996). Analysis of the intracellular transport properties of recombinant La Crosse virus glycoproteins. Virology *220*, 485-490.

Casals, J. (1963). New developments in the classification of arthropod-borne viruses. Anal Microbiol *11*, 13-34.

Chen, S.Y. and Compans, R.W. (1991). Oligomerization, transport, and Golgi retention of Punta Toro virus glycoproteins. J Virol *65*, 5902-5909.

Chen, S.Y., Matsuoka, Y., and Compans, R.W. (1991a). Assembly and polarized release of Punta Toro virus and effects of brefeldin A. J Virol *65*, 1427-1439.

Chen, S.Y., Matsuoka, Y., and Compans, R.W. (1991b). Golgi complex localization of the Punta Toro virus G2 protein requires its association with the G1 protein. Virology *183*, 351-365.

Cole, N.B., Ellenberg, J., Song, J., DiEuliis, D., and Lippincott, S.J. (1998). Retrograde transport of Golgi-localized proteins to the ER. J Cell Biol *140*, 1-15.

Craig, E.A., Weissman, J.S., and Horwich, A.L. (1994). Heat shock proteins and molecular chaperones: mediators of protein conformation and turnover in the cell. Cell *78*, 365-372.

de Ávila, A.C., de Haan, P., Smeets, M.L., Resende, R.d., Kormelink, R., Kitajima, E.W., Goldbach, R.W., and Peters, D. (1993). Distinct levels of relationships between tospovirus isolates. Arch Virol *128*, 211-227.

de Haan, P., de Avila, A.C., Kormelink, R., Westerbroek, A., Gielen, J.J., Peters, D., and Goldbach, R. (1992). The nucleotide sequence of the S RNA of Impatiens necrotic spot virus, a novel tospovirus. FEBS Lett. *306*, 27-32.

de Haan, P., Kormelink, R., de Oliveira Resende, R., van Poelwijk, F., Peters, D., and Goldbach, R. (1991). Tomato spotted wilt virus L RNA encodes a putative RNA polymerase. J Gen Virol *72*, 2207-2216.

de Haan, P., Wagemakers, L., Peters, D., and Goldbach, R. (1990). The S RNA segment of tomato spotted wilt virus has an ambisense character. J Gen Virol *71*, 1001-1007.

de Jong, R., de Laaf, L., Vennema, H., and Meijlink, F. (1992). DNA-binding activity of the murine homeodomain protein Hox-2.3 produced by a hybrid phage T7/vaccinia virus system. Gene *116*, 195-203.

Dhugga, K.S., Tiwari, S.C., and Ray, P.M. (1997). A reversibly glycosylated polypeptide (RGP1) possibly involved in plant cell wall synthesis: purification, gene cloning, and trans-Golgi localization. Proc Natl Acad Sci U S A *94*, 7679-7684.

Eggen, R., Verver, J., Wellink, J., De-Jong, A., Goldbach, R., and van Kammen, A. (1989). Improvements of the infectivity of in vitro transcripts from cloned cowpea mosaic virus cDNA: impact of terminal nucleotide sequences. Virology *173*, 447-455.

Elliott, R.M. (1990). Molecular biology of the Bunyaviridae. J Gen Virol *71*, 501-522.

Elliott, R.M. (1996). The *Bunyaviridae* (New York: Plenum Press).

Eshita, Y. and Bishop, D.H. (1984). The complete sequence of the M RNA of snowshoe hare bunyavirus reveals the presence of internal hydrophobic domains in the viral glycoprotein. Virology *137*, 227-240.

Fox, G., Parry, N.R., Barnett, P.V., McGinn, B., Rowlands, D.J., and Brown, F. (1989). The cell attachment site on foot-and-mouth disease virus includes the amino acid sequence RGD (arginine-glycine-aspartic acid). J Gen Virol *70*, 625-637.

Francki, R. I., Fauquet, C. M., Knudson, D. L., and Brown, F. (1991). Classification and nomenclature of viruses: fifth report of the International Committee on Taxonomy of Viruses. Arch.Virol supplementum 2.

Francki, R.I. and Grivell, C.J. (1970). An electron microscope study of the distribution of tomato spotted wilt virus in systemically infected Datura stramonium leaves. Virology *42*, 969-978.

Gahmberg, N., Kuismanen, E., Keranen, S., and Pettersson, R.F. (1986). Uukuniemi virus glycoproteins accumulate in and cause morphological changes of the Golgi complex in the absence of virus maturation. J Virol *57*, 899-906.

German, T.L., Ullman, D.E., and Moyer, J.W. (1992). Tospoviruses: diagnosis, molecular biology, phylogeny, and vector relationships. Annu Rev Phytopath *30*, 315-348.

Gershon, A.A., Sherman, D.L., Zhu, Z., Gabel, C.A., Ambron, R.T., and Gershon, M.D. (1994). Intracellular transport of newly synthesized varicella-zoster virus: final envelopment in the trans-Golgi network. J Virol *68*, 6372-6390.

Gielen, J.J., de Haan, P., Kool, A.J., Peters, D., van Grinsven, M.Q., and Goldbach, R.W. (1991). Engineered resistance to tomato spotted wilt virus, a negative strand RNA virus. Bio/Technology *9*, 1363-1367.

Goldbach, R.W., Kormelink, R., de Haan, P., Resende, R.d.O., de Avila, A.C., van Poelwijk, F., van Lent, J.W., Wijkamp, I., Prins, M., and Peters, D. (1992). Tomato spotted wilt virus: genome organization, transmission and symptom induction. Proceedings of the fifth international symposium on Biotechnology and plant protection at College park, Marylan, U.S.A.

Goldbach, R.W. and Peters, D. (1994). Possible causes of the emergence of tospoviruses diseases. Semin Virol *5*, 113-120.

Goldbach, R.W. and Peters, D. (1996). Molecular and biological aspects of tospoviruses. In The *Bunyaviridae*. R.M. Elliott, ed. (New York: Plenum Press), pp. 129-157.

Gonsalves, D. and Trujillo, E.E. (1986). Tomato spotted wilt virus in papaya and detection of the virus by ELISA. Plant Disease *70*, 501-506.

Gonzalez, S.F. (1985). La Crosse virus G1 glycoprotein undergoes a conformational change at the pH of fusion. Virology *140*, 209-216.

Gonzalez, S.F., Pobjecky, N., and Nathanson, N. (1984). La Crosse bunyavirus can mediate pH-dependent fusion from without. Virology *132*, 222-225.

Granzow, H., Weiland, F., Jons, A., Klupp, B.G., Karger, A., and Mettenleiter, T.C. (1997). Ultrastructural analysis of the replication cycle of pseudorabies virus in cell culture: a reassessment. J Virol *71*, 2072-2082.

Griffiths, G. and Rottier, P. (1992). Cell biology of viruses that assemble along the biosynthetic pathway. Semin Cell Biol *3*, 367-381.

Hacker, J.K. and Hardy, J.L. (1997). Adsorptive endocytosis of California encephalitis virus into mosquito and mammalian cells: a role for G1. Virology *235*, 40-47.

Hacker, J.K., Volkman, L.E., and Hardy, J.L. (1995). Requirement for the G1 protein of California encephalitis virus in infection in vitro and in vivo. Virology *206*, 945-953.

Hardy, J.L., Houk, E.J., Kramer, L.D., and Reeves, W.C. (1983). Intrinsic factors affecting vector competence of mosquitoes for arboviruses. Annu Rev Entomol. *28*, 229-262.

Hashimoto, K., Erdei, S., Keranen, S., Saraste, J., and Kaariainen, L. (1981). Evidence for a separate signal sequence for the carboxy-terminal envelope glycoprotein E1 of Semliki forest virus. J Virol *38*, 34-40.

Hibi, T., Rezelman, G., and van Kammen, A. (1975). Infection of cowpea mesophyll protoplasts with cowpea mosaic virus. Virology *64*, 308-318.

Horsley, D., Coleman, J., Evans, D., Crooks, K., Peart, J., Satiat-Jeunemaitre, B., and Hawes, C. (1993). A monoclonal antibody, JIM 84, recognizes the Golgi apparatus and plasna membrane in plant cells. Journal of Experimental Botany 44 supplement, 223-229.

Ie, T.S. (1964). An electron microscope study of tomato spotted wilt virus in the plant cell. Netherlands J Plant pathol *70*, 114-115.

Ie, T.S. (1971). Electron microscopy of developmental stages of tomato spotted wilt virus in plant cells. Virology *43*, 468-479.

Ie, T.S. (1982). A sap transmissible, defective form of tomato spotted wilt virus. J Gen Virol *59*, 387-392.

Ihara, T., Smith, J., Dalrymple, J.M., and Bishop, D.H. (1985). Complete sequences of the glycoproteins and M RNA of Punta Toro phlebovirus compared to those of Rift Valley fever virus. Virology *144*, 246-259.

Imperiali, B. and Rickert, K.W. (1995). Conformational implications of asparagine-linked glycosylation. Proc Natl Acad Sci U S A *92*, 97-101.

Jackson, M.R., Nilsson, T., and Peterson, P.A. (1990). Identification of a consensus motif for retention of transmembrane proteins in the endoplasmic reticulum. EMBO J *9*, 3153-3162.

Jackson, M.R., Nilsson, T., and Peterson, P.A. (1993). Retrieval of transmembrane proteins to the endoplasmic reticulum. J Cell Biol *121*, 317-333.

Jantti, J., Hilden, P., Ronka, H., Makiranta, V., Keranen, S., and Kuismanen, E. (1997). Immunocytochemical analysis of Uukuniemi virus budding compartments: role of the intermediate compartment and the Golgi stack in virus maturation. J Virol *71*, 1162-1172.

Jones, R.W. and Jackson, A.O. (1990). Replication of sonchus yellow net virus in infected protoplasts. Virology *179*, 815-820.

Kermode, A. R. (1996). Mechanisms of intracellular protein transport and targetting in plant cells. Critical Reviews in Plant Sciences 15(4), 285-423.

Kikkert, M., van Lent, J.W., Storms, M., Bodegom, P., Kormelink, R., and Goldbach, R.W. (1999). Tomato spotted wilt virus particle morphogenesis in plant cells. J Virol *73*, 2288-2297.

Kikkert, M., van Poelwijk, F., Storms, M., Kassies, W., Bloksma, H., van Lent, J., Kormelink, R., and Goldbach, R. (1997). A protoplast system for studying tomato spotted wilt virus infection. J Gen Virol *78*, 1755-1763.

Kitajima, E.W. (1965). Electron microscopy of vira-cabecca virus (Brazilian tomato spotted wilt virus) within the host cell. Virology *26*, 89-99.

Kitajima, E., Àvila de, A. C., Resende, R. de O., Goldbach, R. W., and Peters, D. (1992a). Comparative cytological and immunogold labelling studies on different isolates of tomato spotted wilt virus. J Submicrosc Cytol Pathol 24, 1-14.

Kitajima, E.W., Resende, R.D., de Avila, A.C., Goldbach, R.W., and Peters, D. (1992b). Immuno-electron microscopical detection of tomato spotted wilt virus and its nucleocapsids in crude plant extracts. J Virol Methods *38*, 313-322.

Kormelink, R. (1994). Structure and expression of the tomato spotted wilt virus genome, a plant-infecting bunyavirus. Thesis Agricultural University of Wageningen.

Kormelink, R., de Haan, P., Meurs, C., Peters, D., and Goldbach, R. (1992a). The nucleotide sequence of the M RNA segment of tomato spotted wilt virus, a bunyavirus with two ambisense RNA segments [published erratum appears in J Gen Virol 1993 Apr;74(Pt 4):790]. J Gen Virol *73*, 2795-2804.

Kormelink, R., de Haan, P., Peters, D., and Goldbach, R. (1992b). Viral RNA synthesis in tomato spotted wilt virus-infected *Nicotiana rustica* plants. J Gen Virol *73*, 687-693.

Kormelink, R., Kitajima, E.W., de Haan, P., Zuidema, D., Peters, D., and Goldbach, R. (1991). The nonstructural protein (NSs) encoded by the ambisense S RNA segment of tomato spotted wilt virus is associated with fibrous structures in infected plant cells. Virology *181*, 459-468.

Kormelink, R., Storms, M., van Lent, J., Peters, D., and Goldbach, R. (1994). Expression and subcellular location of the NSM protein of tomato spotted wilt virus (TSWV), a putative viral movement protein. Virology *200*, 56-65.

Kornfeld, R. and Kornfeld, S. (1985). Assembly of asparagine-linked oligosaccharides. Annu Rev Biochem *54*, 631-664.

Kuismanen, E. (1984). Posttranslational processing of Uukuniemi virus glycoproteins G1 and G2. J Virol *51*, 806-812.

Kuismanen, E., Bang, B., Hurme, M., and Pettersson, R.F. (1984). Uukuniemi virus maturation: immunofluorescence microscopy with monoclonal glycoprotein-specific antibodies. J Virol *51*, 137-146.

Kuismanen, E., Hedman, K., Saraste, J., and Pettersson, R.F. (1982). Uukuniemi virus maturation: accumulation of virus particles and viral antigens in the Golgi complex. Mol Cell Biol *2*, 1444-1458.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*, 680-685.

Lappin, D.F., Nakitare, G.W., Palfreyman, J.W., and Elliott, R.M. (1994). Localization of Bunyamwera bunyavirus G1 glycoprotein to the Golgi requires association with G2 but not with NSm. J Gen Virol *75*, 3441-3451.

Laurière, M., Laurière, C., Chrispeels, M. J., Johnson, K. D., and Sturm, A. (1989). Characterization of a xylose-specific antiserum that reacts with the complex asparagine-linked glycans of extracellular and vacuolar glycoproteins. Plant Physiol *90*, 1182-1188.

Law, M.D. and Moyer, J.W. (1990). A tomato spotted wilt virus with a serologically distinct N protein. J Gen Virol *71*, 933-938.

Law, M.D., Speck, J., and Moyer, J.W. (1992). The M RNA of impatiens necrotic spot Tospovirus (Bunyaviridae) has an ambisense genomic organization. Virology *188*, 732-741.

Lawson, R. H., Dienelt, M. M., and Hsu, H. T. (1996). Ultrastructural comparisons of defective, partially defective, and nondefective isolates of Impatiens necrotic spot virus. Phytopathology *86*, 650-661.

Lewis, M.J. and Pelham, H.R. (1996). SNARE-mediated retrograde traffic from the Golgi complex to the endoplasmic reticulum. Cell *85*, 205-215.

Liljestrom, P. and Garoff, H. (1991). A new generation of animal cell expression vectors based on the Semliki Forest virus replicon. Biotechnology (N Y) *9*, 1356-1361.

Lippincott, S.J., Donaldson, J.G., Schweizer, A., Berger, E.G., Hauri, H.P., Yuan, L.C., and Klausner, R.D. (1990). Microtubule-dependent retrograde transport of proteins into the ER in the presence of brefeldin A suggests an ER recycling pathway. Cell *60*, 821-836.

Ludwig, G.V., Christensen, B.M., Yuill, T.M., and Schultz, K.T. (1989). Enzyme processing of La Crosse virus glycoprotein G1: a bunyavirus-vector infection model. Virology *171*, 108-113.

Ludwig, G.V., Israel, B.A., Christensen, B.M., Yuill, T.M., and Schultz, K.T. (1991). Role of La Crosse virus glycoproteins in attachment of virus to host cells. Virology *181*, 564-571.

Marriott, A.C., el-Ghorr, A.A., and Nuttall, P.A. (1992). Dugbe Nairovirus M RNA: nucleotide sequence and coding strategy. Virology *190*, 606-615.

Matsuoka, Y., Chen, S.Y., and Compans, R.W. (1991). Bunyavirus protein transport and assembly. Curr Top Microbiol Immunol *169*, 161-179.

Matsuoka, Y., Chen, S.Y., and Compans, R.W. (1994). A signal for Golgi retention in the bunyavirus G1 glycoprotein. J Biol Chem. *269*, 22565-22573.

Matsuoka, Y., Chen, S.Y., Holland, C.E., and Compans, R.W. (1996). Molecular determinants of Golgi retention in the Punta Toro virus G1 protein. Arch.Biochem.Biophys. *336*, 184-189.

Matthews, R.E. (1979). Third report of the International Committee on Taxonomy of Viruses. Classification and nomenclature of viruses. Intervirology *12*, 129-296.

Melin, L., Persson, R., Andersson, A., Bergstrom, A., Ronnholm, R., and Pettersson, R.F. (1995). The membrane glycoprotein G1 of Uukuniemi virus contains a signal for localization to the Golgi complex. Virus Res. *36*, 49-66.

Milne, R. G. (1970). An electron microscope study of tomato spotted wilt virus in sections of infected cells and in negative stain preparations. J Gen Virol 6, 267-276.

Mohamed, N.A. (1981). Isolation and characterization of subviral structures from tomato spotted wilt virus. J Gen Virol *53*, 197-206.

Mumford, R.A., Barker, I., and Wood, K.R. (1996). The biology of the tospoviruses. Annals Of Applied Biology *128*, 159-183.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bio-assays with tobacco tissue. Physiology of plants *15*, 473-497.

Nagata, T., Goldbach, R., and Peters, D. (1997a). Establishment of cell cultures of two thrips species and their application in studies on tospovirus-vector interactions. Proceedings Of The Section Experimental And Applied Entomology Of The Netherlands Entomological Society Nev Vol 8 1997 139-144.

Nagata, T., Storms, M.M., Goldbach, R., and Peters, D. (1997b). Multiplication of tomato spotted wilt virus in primary cell cultures derived from two thrips species. Virus Res *49*, 59-66.

Nilsson, T., Slusarewicz, P., Hoe, M.H., and Warren, G. (1993). Kin recognition. A model for the retention of Golgi enzymes. FEBS Lett. *330*, 1-4.

Nilsson, T. and Warren, G. (1994). Retention and retrieval in the endoplasmic reticulum and the Golgi apparatus. Curr Opin Cell Biol *6*, 517-521.

Nishimura, N. and Balch, W.E. (1997). A di-acidic signal required for selective export from the endoplasmic reticulum. Science *277*, 556-558.

O'Connor, S.E. and Imperiali, B. (1996). Modulation of protein structure and function by asparagine-linked glycosylation. Chem Biol *3*, 803-812.

Pardigon, N., Vialat, P., Gerbaud, S., Girard, M., and Bouloy, M. (1988). Nucleotide sequence of the M segment of Germiston virus: comparison of the M gene product of several bunyaviruses. Virus Res *11*, 73-85.

Pelham, H.R. (1995). Sorting and retrieval between the endoplasmic reticulum and Golgi apparatus. Curr Opin Cell Biol *7*, 530-535.

Persson, R. and Pettersson, R.F. (1991). Formation and intracellular transport of a heterodimeric viral spike protein complex. J Cell Biol *112*, 257-266.

Pesonen, M., Kuismanen, E., and Pettersson, R.F. (1982). Monosaccharide sequence of protein-bound glycans of Uukuniemi virus. J Virol *41*, 390-400.

Pettersson, R., Simons, J.F., Melin, L., Persson, K., and Ronnholm, R. (1993). Uukuniemi virus as a model for elucidating the molecular biology of bunyaviruses. *In* Concepts in Virology from Ivanovsky to the present. B.W. Mahy and D.K. Lvov, eds. (Harwood Academic), pp. 309-319.

Pettersson, R.F. (1991). Protein localization and virus assembly at intracellular membranes. Curr Top Microbiol Immunol *170* , 67-106.

Pettersson, R.F. and Melin, L. (1996). Synthesis, assembly, and intacellular transport of *Bunyaviridae* membrane proteins. In The *Bunyaviridae*. R.M. Elliott, ed. (New York: Plenum Press), pp. 159-188.

Pierschbacher, M.D. and Ruoslahti, E. (1987). Influence of stereochemistry of the sequence Arg-Gly-Asp-Xaa on binding specificity in cell adhesion. J Biol Chem *262*, 17294-17298.

Pittman, H.A. (1927). Spotted wilt of tomatoes. Preliminary note concerning the transmission of the "spotted wilt" of tomatoes by an insect vector (*Thrips tabaci* Lind.). J Aust Counc Sci Ind Res Bull *1*, 74-77.

Prins, M., Kikkert, M., Ismayadi, C., de Graauw, W., de Haan, P., and Goldbach, R. (1997). Characterization of RNA-mediated resistance to tomato spotted wilt virus in transgenic tobacco plants expressing NSm gene sequences. Plant Mol Biol *33*, 235-243.

Resende, R. de O., de Haan, P., de Avila, A.C., Kitajima, E.W., Kormelink, R., Goldbach, R., and Peters, D. (1991). Generation of envelope and defective interfering RNA mutants of tomato spotted wilt virus by mechanical passage. J Gen Virol *72*, 2375-2383.

Rieder, E., Berinstein, A., Baxt, B., Kang, A., and Mason, P.W. (1996). Propagation of an attenuated virus by design: engineering a novel receptor for a noninfectious foot-and-mouth disease virus. Proc Natl Acad Sci U S A *93*, 10428-10433.

Roivainen, M., Hyypia, T., Piirainen, L., Kalkkinen, N., Stanway, G., and Hovi, T. (1991). RGD-dependent entry of coxsackievirus A9 into host cells and its bypass after cleavage of VP1 protein by intestinal proteases. J Virol *65*, 4735-4740.

Roivainen, M., Piirainen, L., and Hovi, T. (1996). Efficient RGD-independent entry process of coxsackievirus A9. Arch Virol *141*, 1909-1919.

Roivainen, M., Piirainen, L., Hovi, T., Virtanen, I., Riikonen, T., Heino, J., and Hyypia, T. (1994). Entry of coxsackievirus A9 into host cells: specific interactions with alpha v beta 3 integrin, the vitronectin receptor. Virology *203*, 357-365.

Rönka, H., Hilden, P., von Bonsdorff, C.H., and Kuismanen, E. (1995). Homodimeric association of the spike glycoproteins G1 and G2 of Uukuniemi virus. Virology *211*, 241-250.

Rönnholm, R. (1992). Localization to the Golgi complex of Uukuniemi virus glycoproteins G1 and G2 expressed from cloned cDNAs. J Virol *66*, 4525-4531.

Rouiller, I., Brookes, S.M., Hyatt, A.D., Windsor, M., and Wileman, T. (1998). African swine fever virus is wrapped by the endoplasmic reticulum. J Virol *72*, 2373-2387.

Ruoslahti, E. and Pierschbacher, M.D. (1987). New perspectives in cell adhesion: RGD and integrins. Science *238*, 491-497.

Ruusala, A., Persson, R., Schmaljohn, C.S., and Pettersson, R.F. (1992). Coexpression of the membrane glycoproteins G1 and G2 of Hantaan virus is required for targeting to the Golgi complex. Virology *186* , 53-64.

Rwambo, P.M., Shaw, M.K., Rurangirwa, F.R., and DeMartini, J.C. (1996). Ultrastructural studies on the replication and morphogenesis of Nairobi sheep disease virus, a Nairovirus. Arch Virol *141*, 1479-1492.

Sakimura, K. (1962). The present status of thrips-borne viruses. In Biological transmission of disease agents. K. Maramorosh, ed. (New York: Academic Press), pp. 30-40.

Samuel, G., Bald, J.G., and Pittman, H.A. (1930). Investigations on "spotted wilt" of tomatoes. Aust Commonw Counc Sci Ind Res Bull *44*,

Saraste, J., Palade, G.E., and Farquhar, M.G. (1987). Antibodies to rat pancreas Golgi subfractions: identification of a 58-kD cis-Golgi protein. J Cell Biol *105*, 2021-2029.

Saraste, J. and Svensson, K. (1991). Distribution of the intermediate elements operating in ER to Golgi transport. J Cell Sci *100*, 415-430.

Satiat-Jeunemaitre, B. and Hawes, C. (1992a). Resdistribution of a Golgi glycoprotein in plant cells treated with Brefeldin A. J Cell Sci 103, 1153-1166.

Satiat-Jeunemaitre, B. and Hawes, C. (1992b). Reversible dissociation of the plant Golgi apparatus by Brefeldin A. Biol Cell *74*, 325-328.

Schekman, R. and Orci, L. (1996). Coat proteins and vesicle budding. Science *271*, 1526-1533.

Schmelz, M., Sodeik, B., Ericsson, M., Wolffe, E.J., Shida, H., Hiller, G., and Griffiths, G. (1994). Assembly of vaccinia virus: the second wrapping cisterna is derived from the trans Golgi network. J Virol *68*, 130-147.

Schweizer, A., Fransen, J.A., Matter, K., Kreis, T.E., Ginsel, L., and Hauri, H.P. (1990). Identification of an intermediate compartment involved in protein transport from endoplasmic reticulum to Golgi apparatus. Eur J Cell Biol *53*, 185-196.

Smithburn, K.C., Haddow, A.J., and Mahaffy, A.F. (1946). Neurotropic virus isolated from Aedes mosquitoes caught in Semliki Forest. Am J Trop Med Hyg *26*, 189-208.

Sodeik, B., Doms, R.W., Ericsson, M., Hiller, G., Machamer, C.E., van 't Hof, W., van Meer, G., Moss, B., and Griffiths, G. (1993). Assembly of vaccinia virus: role of the intermediate compartment between the endoplasmic reticulum and the Golgi stacks. J Cell Biol *121*, 521-541.

Stephens, E.B. and Compans, R.W. (1988). Assembly of animal viruses at cellular membranes. Annu Rev Microbiol *42*, 489-516.

Storms, M.M., Kormelink, R., Peters, D., van Lent, J.W., and Goldbach, R.W. (1995). The nonstructural NSm protein of tomato spotted wilt virus induces tubular structures in plant and insect cells. Virology *214*, 485-493.

Sundin, D.R., Beaty, B.J., Nathanson, N., and Gonzalez, S.F. (1987). A G1 glycoprotein epitope of La Crosse virus: a determinant of infection of Aedes triseriatus. Science *235*, 591-593.

Takatsuki, A.K., Arima, K., and Tamura, G. (1971). Tunicamycin, a new antibiotic. I. Isolation and characterization of tunicamycin. J Antibiot *24*, 215-223.

Tatu, U. and Helenius, A. (1997). Interactions between newly synthesized glycoproteins, calnexin and a network of resident chaperones in the endoplasmic reticulum. J Cell Biol *136*, 555-565.

Thomas, D.C. and Roth, M.G. (1994). The basolateral targeting signal in the cytoplasmic domain of glycoprotein G from vesicular stomatitis virus resembles a variety of intracellular targeting motifs related by primary sequence but having diverse targeting activities. J Biol Chem *269* , 15732-15739.

Tkacz, J.S. and Lampen, J.O. (1975). Tunicamycin inhibition of polyisoprenol N-acetylglucosaminyl pyrophosphate formation in calf liver microsomes. Biochem Biophys Res Commun. *65*, 248-257.

Townsley, F.M., Wilson, D.W., and Pelham, H.R. (1993). Mutational analysis of the human KDEL receptor: distinct structural requirements for Golgi retention, ligand binding and retrograde transport. EMBO J *12*, 2821-2829.

Trombetta, E.S. and Helenius, A. (1998). Lectins as chaperones in glycoprotein folding. Curr Opin Struct Biol *8*, 587-592.

Tsuda, S., Fujisawa, I., Ohnishi, J., Hosokawa, D., and Tomaru, K. (1996). Localization of tomato spotted wilt tospovirus in larvae and pupae of the insect vector Thrips setosus. Phytopathology *86*, 1199-1203.

Ullman, D. E., Cho, J. J., Mau, R. F., Westcot, D. M., and Custer, D. M. (1992). A midgut barrier to tomato spotted wilt virus acquisition by adult Western flower thrips. Phytopathology 82, 1333-1342.

Ullman, D.E., German, T.L., Sherwood, J.L., Westcot, D.M., and Cantone, F.A. (1993). *Tospovirus* replication in insect vector cells:immunocytochemical evidence that the non-structural protein encoded by the S RNA of tomato spotted wilt tospovirus is present in thrips vector cells. Phytopathology *83*, 456-463.

Ullman, D. E., Westcot, D. M., Chenault, K. D., Sherwood, J. L., German, T. L., Bandla, M. D., Cantone, F. A., and Duer, H. L. (1995). Compartmentalization, intracellular transport, and autophagy of tomato spotted wilt tospovirus proteins in infected thrips cells. Phytopathology 85, 644-654.

van Beek, N.A., Derksen, A.C., and Dijkstra, J.M. (1985). Polyethylene glycol-mediated infection of cowpea protoplasts with sonchus yellow net virus. J Gen Virol *66*, 551-557.

van den Heuvel, J.F., Verbeek, M., and van der Wilk, F. (1994). Endosymbiotic bacteria associated with circulative transmission of potato leafroll virus by Myzus persicae. J Gen Virol *75*, 2559-2565.

van der Wel, N.N., Goldbach, R.W., and van Lent, J.W. (1998). The movement protein and coat protein of alfalfa mosaic virus accumulate in structurally modified plasmodesmata. Virology *244*, 322-329.

van de Wetering, F., Goldbach, R. W., and Peters, D. (1996). Tomato spotted wilt tospovirus ingestion by first instar larvae of *Frankliniella occidentalis* is a prerequisite for transmission. Phytopathology 86, 900-905.

van Lent, J., Storms, M., van der Meer, F., Wellink, J., and Goldbach, R. (1991). Tubular structures involved in movement of cowpea mosaic virus are also formed in infected cowpea protoplasts. J Gen Virol *72*, 2615-2623.

van Poelwijk, F., Boye, K., Oosterling, R., Peters, D., and Goldbach, R. (1993). Detection of the L protein of tomato spotted wilt virus. Virology *197*, 468-470.

van Poelwijk, F., Prins, M., and Goldbach, R. (1997). Completion of the impatiens necrotic spot virus genome sequence and genetic comparison of the L proteins within the family Bunyaviridae. J Gen Virol *78*, 543-546.

van Poelwijk, F., Boye, K., Oosterling, R., Peters, D., and Goldbach, R. (1996). Replication and expression of the tospoviral genome. Proceedings of the International Symposium on Tospoviruses and Thrips of Floral and Vegetable Crops. Acta Hortic *431*, 201-208.

Verkleij, F.N. and Peters, D. (1983). Characterization of a defective form of tomato spotted wilt virus. J Gen Virol *64*, 677-682.

Villaverde, A., Feliu, J.X., Harbottle, R.P., Benito, A., and Coutelle, C. (1996). A recombinant, arginine-glycine-aspartic acid (RGD) motif from foot-and-mouth disease virus binds mammalian cells through vitronectin and, to a lower extent, fibronectin receptors. Gene *180*, 101-106.

von Bonsdorff, C.H., Saikku, P., and Oker, B.N. (1970). Electron microscope study on the development of Uukuniemi virus. Acta Virol *14*, 109-114.

von Heijne, G. (1986). A new method for predicting signal sequence cleavage sites. Nucleic.Acids.Res *14*, 4683-4690.

Whealy, M.E., Card, J.P., Meade, R.P., Robbins, A.K., and Enquist, L.W. (1991). Effect of brefeldin A on alphaherpesvirus membrane protein glycosylation and virus egress. J Virol *65*, 1066-1081.

Wijkamp, I. (1996). Studies on thrips transmission of defective tospovirus mutants. *In* Virus-vector relationships in the transmission of tospoviruses. Thesis Agricultural University Wageningen, pp. 107-119.

Wijkamp, I., Almarza, N., Goldbach, R., and Peters, D. (1995). Distinct levels of specificity in thrips transmission of tospoviruses. Phytopathology 85, 1069-1074.

Wijkamp, I. and Peters, D. (1993). Determination of the median latent period of two tospoviruses in *Frankliniella occidentalis*, using a novel leaf disk assay. Phytopathology 83, 986-991.

Wijkamp, I., van Lent, J., Kormelink, R., Goldbach, R., and Peters, D. (1993). Multiplication of tomato spotted wilt virus in its insect vector, Frankliniella occidentalis. J Gen Virol *74*, 341-349.

Wijkamp, I., van de Wetering, F., Goldbach, R., and Peters, D. (1996). Transmission of tomato spotted wilt virus by Frankliniella occidentalis; Median acquisition and inoculation access period. Ann Appl Biol *129*, 303-313.

NAWOORD

En uiteindelijk heb je een boek geschreven. Daar moest een boel voorwerk voor gebeuren. Soms pipetteerde je weken, maanden, dingen bij elkaar zonder dat er überhaupt iets bruikbaars gebeurde. Maar voorzichtig en vooral langzaam, soms zelfs ongemerkt, bedreef je opeens wetenschap. Erachter komen hoe de dingen inelkaar steken, of er voorzichtig een vinger achter zetten. Speculeren, hypotheses opstellen en toetsen, ideeën generen en omverwerpen. Mislukte doch briljante proeven, bijna gelukte proeven, (eindelijk) een gelukte proef.

Het beheerst je, het boeit je, je vergeet dat je ook nog een leven hebt. Nee wacht, promoveren *is* je manier van leven geworden. Jarenlang staat er "proefschrift" op je voorhoofd geschreven, en eer je echt aan het schrijven bent geraakt staat het er zelfs met koeieletters.

Daar moeten de mensen om je heen al die tijd mee leven. Voor sommigen sprak het vanzelf, die deden er vrolijk aan mee: Rob Goldbach, mijn immer geïnteresseerde, soms bezorgde, vogelende promotor, Richard Kormelink, zijn eigen promotie nog vers in het geheugen, dus perfecte vraagbaak en tipgever, mijn co-promotor en bovenal prettig mens. Ook Dick Peters zag het met een glimlach aan en voorzag me van nuttige tips. Anderen zaten in hetzelfde schuitje of waren reeds post-doc, en behalve dat ze goede-sfeerbepalend waren in het lab ging je daarmee soms naar donkere kroegen, zodat dat woordje "proefschrift" op de voorhoofden bijna niet meer te lezen viel, en de drank ons de rest even liet vergeten: Ineke, Frank, Bep, John, Monique, Marcel K., Axel, en Kit in de eerste jaren, en soms in dezelfde tijd maar vooral daarna: Cor, René, Jacco, Isabel, Liesbeth, Flix, Lilian, Mariëlle, Wilfred, Alice, Tatsuya, André, Marcel P., Erwin, Rolf, Danny, Kitty, Marc, Daniëlla, Nicole, Yuh Kun and Steve. Met Fennet deelde ik een kantoor, en daar kletsten we soms lekker wat af en bij.

Er waren nog meer mensen waar je wat aan had, voor een praatje en voor onmisbare hulp van allerlei kaliber: Jan en Joop brachten mij het E.M.-en bij, Magda leerde me verantwoord omgaan met insectencellen, en Gert-Jan Godeke van diergeneeskunde in Utrecht deed me voor hoe het moest met baby hamster cellen. Hanke hielp ook en Cor was en is op vele fronten nog steeds onmisbaar. Wout Rozeboom voerde een strak doch rechtvaardig chemicaliënbeleid waarvan het nut onschatbaar is, zo ervaar ik vooral achteraf. Ook vakgroepsecretaresses snapten waar je mee bezig was: Annemieke, Henny, Liesbeth en Thea in mijn tijd. De mannen van de fotolocatie en tekenkamer waren handig met bandjes, en die van de kassen met plantjes.

Er kwamen ook (veel) studenten, die je hielpen of ophielden en vervolgens weer, hopelijk op één of andere manier wijzer geworden, gingen: Jindra, Glenn, Hilda, Aletta, Pentcho, Geert, Cécile, Ad, Simone, Asztéria, Mariëtte, Wilko-Jan, en Bart.

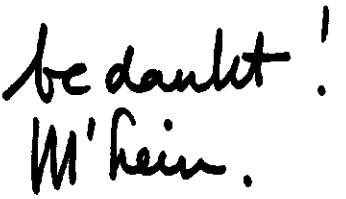
Voor de mensen buiten het lab viel het soms bijna niet op dat je met dat proefschrift in/op je hoofd rondliep. Een verademing, die frisse lucht. Eten en kletsen met het eetgroepje, eten en kletsen met het filosofiegroepje, eten en zingen en kletsen met het BKK (Blauwe Kamer Koor), eten en zingen en kletsen en optreden met het bandje, eten en kletsen met Marjo, of Teun, of José, of Peter, of Marcel van K., of Liesbeth, of Cor, of..wat is het leven toch geweldig met jullie erbij!

Ergens vanuit de achterban, en dat vergeet je soms bijna, zitten pa en ma en broer Michiel (Kermit?) de zaak eens aan te kijken, en vormen de onmisbare basis.

En dan, na een lange werkdag, kwam je thuis.

Charlie was er altijd, en het klinkt cliché, maar ik hoop werkelijk dat we nog heel lang vrienden zullen blijven. Voor Freek was ik vaak één en al proefschrift. Maar door hem kon ik er beter tegen. Jij mag me helpen op 18 juni mijn voorhoofd te wassen, zodat ik weer met scherpe blik in je ogen kijk.

Iedereen,



CURRICULUM VITAE

Marjolein Kikkert werd op 1 mei 1969 geboren in Vlaardingen. In 1987 behaalde zij haar VWO diploma aan het Twickel College in Hengelo (ov), en begon haar studie Moleculaire Wetenschappen aan de Landbouwuniversiteit in Wageningen (LUW). Tijdens deze studie deed zij onderzoek aan de replicatie van het tabaks mozaiek virus bij de vakgroepen Moleculaire Biologie en Virologie van de LUW (begeleidt door Dr Hans van Bokhoven, Prof. Ab van Kammen en Prof. Rob Goldbach), en onderzocht zij gist homologen van componenten van synaptonemale complexen van de rat bij de vakgroep Erfelijkheidsleer (begeleidt door Dr Ralph Meuwissen, en Prof. Christa Heyting). Vervolgens ging zij een half jaar naar de University of California in Berkeley in de Verenigde Staten, en werkte zij aan het cel-naar-cel transport van tomato bushy stunt virus onder begeleiding van Dr Herman Scholthof en Prof. Andrew Jackson. In 1993 studeerde zij af en begon met promotieonderzoek in de "tospogroep" van de vakgroep Virologie van de LUW, bij Dr Richard Kormelink en Prof. Rob Goldbach. De resultaten van dit onderzoek staan beschreven in dit proefschrift.

Intussen werkt zij als post-doc in de groep van Prof. Emmanuel Wiertz aan de "immune-escape" van herpesvirussen bij het Leids Universitair Medisch Centrum, en is vooralsnog gedetacheerd op het Rijks Instituut voor Volksgezondheid en Milieu (RIVM) te Bilthoven.

Scholthof, H.B., K.B.Goldberg-Scholthof, M. Kikkert and A.O. Jackson. (1995) Tomato bushy stunt virus spread is regulated by two nested genes that function in cell-to-cell movement and host-dependent systemic invasion. *Virology* **213** 425-438.

Poelwijk van, F., M. Kikkert, M. Prins, R. Kormelink, M. Storms, J. van Lent, P. de Haan, D. Peters and R. Goldbach. (1996) Replication and expression of the tospoviral genome. Proceedings of the international symposium on tospoviruses and thrips of floral and vegetable crops. *Acta Horticultura* **431**, 201-208.

Prins, M., M. Kikkert, C. Ismayadi, W. de Graauw, P. de Haan, and R. Goldbach. (1997) Characterization of RNA mediated resistance to tomato spotted wilt virus in transgenic tobacco plants expressing NSm-gene sequences. *Plant. Mol. Biol.* **33**, 235-243.

Feldhoff, A., M. Kikkert, R. Kormelink, G. Krczal, R. Goldbach and D. Peters. (1997) Serological comparison of tospoviruses with polyclonal antibodies against the main structural proteins of tomato spotted wilt virus. *Archives of Virology* **142**, 781-793.

Kikkert, M., F. van Poelwijk, M. Storms, H. Bloksma, W. Karsies, R. Kormelink, and R. Goldbach. (1997) A protoplast system for studying tomato spotted wilt virus infection. *Journal of General Virology* **78**, 1755-1763.

Kikkert, M., C. Meurs, F. Van de Wetering, R. Kormelink, and R. Goldbach. (1998) Binding of tomato spotted wilt virus to a 94 kDa thrips protein. *Phytopathology* **88**, 63-69.

Kikkert, M., J. van Lent, M. Storms, P. Bodegom, R. Kormelink, and R. Goldbach. (1999) Tomato spotted wilt virus particle morphogenesis in plant cells. *Journal of Virology* **73**, 2288-2297.

Kikkert, M., A. Verschoor, C. van Woensel, R. Kormelink and R. Goldbach. (1998) Tomato spotted wilt virus glycoproteins exhibit trafficking and localization signals that are functional in mammalian cells. Submitted for publication.

Selection of presentations at international meetings

Kikkert, M., R. Kormelink, M. Storms, A. Feldhoff, D. Peters and R. Goldbach. (1995) "Characterization of the glycoproteins G1 and G2 of tomato spotted wilt virus." American Society for Virology (ASV) - 14th annual meeting at the University of Texas, Austin, USA (poster presentation).

Kikkert, M., F. van Poelwijk, M. Storms, H. Bloksma, R. Kormelink and R. Goldbach. (1996) "A protoplast system for studying tomato spotted wilt virus infection." Xth International Congress of Virology (ICV), Jerusalem, Israel (oral presentation).

Kikkert, M., P. Bodegom, M. Storms, J. van Lent, R. Kormelink, en R. Goldbach. (1996) "Maturation of tomato spotted wilt virus in plant cells." Tenth International Conference on Negative Strand Viruses (INSV), Dublin, Ireland (oral presentation).

Kikkert, M, F. van Poelwijk, M. Storms, P. Bodegom, C. van Woensel, J. van Lent, R. Kormelink and R. Goldbach. (1997) "Studying tomato spotted wilt virus maturation: exploitation of plant and animal single-cell systems." EMBO workshop "Molecular mechanisms in the replicative cycle of viruses in plants.", Las Navas del Marqués, Spain (oral presentation).

Kikkert, M, J. van Lent, M. Storms, R. Kormelink and R. Goldbach. (1998) "trafficking and localization of tomato spotted wilt virus glycoproteins; implications for particle morphogenesis." Fourth International Symposium on Tospoviruses and Thrips in Floral and Vegetable Crops, Wageningen, Netherlands (oral presentation).