STRUCTURE AND EXPRESSION OF THE TOMATO SPOTTED WILT VIRUS GENOME, A PLANT-INFECTING BUNYAVIRUS

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Stellingen

1. De expressie-strategie van het TSWV M RNA segment ondersteunt de hypothese dat de evolutionaire ontwikkeling van ambisense RNA segmenten terug te voeren is op een fout in de RNA replicatie.

   Dit proefschrift.
   D.H.L. Bishop (1986). Ambisense RNA viruses: Positive and negative polarities combined in RNA virus genomes. *Microbiological Sciences* 3, 183-187.

2. De aanname van Lucas *et al.* (1993) voor het bestaan van een "plant protein I" en een "plant protein II" in het TMV transport model is voorbarig en maakt dit model onnodig ingewikkeld.

   Lucas *et al.* (1993). Plasmodesmata and the supracellular nature of plants. *New Phytol.* **125**, 435-476.

3. Het feit dat het genoom van tenuivirussen uit 4 of 5 RNA segmenten bestaat, waarvan tenminste 3 een ambisense structuur bezitten, rechtvaardigt een classificatie van deze virussen in een aparte virusfamilie.

4. De conclusie dat AVR9$^-$ stammen naast het avr9 gen een aanzienlijk stuk flankerende sequenties van dit gen missen, is prematuur.

   Marmeisse *et al.* (1993). Disruption of the avirulence gene avr9 in *Cladosporium fulvum* causes virulence on tomato genotypes with the complementary resistance gene Cf9. *Molecular Plant-Microbe Interactions* 6.

5. De term *in-planta-induced* betekent nog niet dat expressie van deze genen wordt aangeschakeld door een interactie met de plant gastheer.

   C.M.J. Pieterse (1993). Differential gene expression in *Phytophthera infestans* during pathogenesis on potato. Thesis, Wageningen.

6. Door zich sinds 1947 slechts op papier uit te spreken voor een opdeling van (de Westelijke helft van) Palestina is de VN mede verantwoordelijk voor de huidige toestand op de bezette Westelijke Jordaanoever en de Gaza-strook.

7. Vanwege het hoge kwaliteitsgehalte van Grolsch bier kan een "grolse kater" maar voor één uitleg vatbaar zijn.

8. Het is de vraag of in artikelen betreffende intercellulair plantevirustransport de openingszin "It is generally accepted that plant viruses move from cell-to-cell through plasmodesmata" geoorloofd is.

   Wellink *et al.* (1993). The cowpea mosaic virus M RNA-encoded 48-kilodalton protein is responsible for induction of tubular structures in protoplasts. *Journal of General Virology* **67**, 3660-3664.
   M.-C. Perbal (1994). A functional analysis of the cauliflower mosaic virus movement protein. Ph.D. thesis, University of East Anglia (UK).

9. De dagen van het A.I.O.-schap zijn geteld.

10. De latente aanwezigheid van een derde groep virussen, phylogenetisch niet verwant aan plante- en diervirussen, kan funest zijn voor de afronding van een proefschrift.

Stellingen behorend bij het proefschrift:

Structure and expression of the tomato spotted wilt virus genome, a plant-infecting bunyavirus

Amersfoort, 29 april 1994 Richard Kormelink

Voorwoord

Nu is het dan zover, voor u ligt resultaat van enkele jaren onderzoek, niet alleen van mijzelf maar ook van vele anderen die direkt of indirekt aan het onderzoek hebben bijgedragen. Bij deze wil ik dan ook vele mensen bedanken voor zijn of haar aandeel in dit proefschrift. Ten eerste Rob Goldbach en Dick Peters die met het schrijven van het projectvoorstel het startsein hebben gegeven voor dit onderzoek en vervolgens door hun begeleiding en kritische houding een grote bijdrage hebben geleverd aan het tot stand komen van dit proefschrift. Vervolgens wil ik de mensen van de TSWV-groep bedanken voor de plezierige samenwerking en fijne tijd: Antonio de Avila, Peter de Haan, Renato O. de Resende, Lia Wagemakers, Claire Huguenot, Elliot Kitajima, Cor Meurs, Ineke Wijkamp, Frank van Poelwijk, Wies Smeets, Kit Boye, Marcel Prins, Marjolein Kikkert, Marc Storms, en de (ex)studenten Cécile Bergmans, Annemarie Philipsen, Maarten Penning, Johan Leveau, Jeroen Corver, Arie Smolders, Heleen Frings en Wendy Clark. Daarnaast wil ik Jan van Lent en Joop Groenewegen van de EM-afdeling, Magda Usmany, Just Vlak en Douwe Zuidema van de baculovirus groep, de Fotolocatie Binnenhaven, de tekenafdeling en de mensen van het kassencomplex bedanken voor hun ondersteuning. Naast bovengenoemde personen bedank ik ook alle (ex-)medewerkers en (ex-)studenten van de vakgroep Virologie voor de prettige samenwerking. Het mag wel duidelijk zijn dat zonder deze mensen dit proefschrift niet tot stand zou zijn gekomen.

Hodie mihi, cras tibi

CONTENTS

Chapter 1

Tomato spotted wilt virus

Introduction

The history of the "spotted wilt" disease goes back to 1919, when Brittlebank reported the first observations of this severe disease of tomatoes in Australia. It was not until 1930 that Samuel *et al.* (1930) identified the causal agent of this disease as a virus, for which the name tomato spotted wilt virus (TSWV) was coined. Ever since, TSWV has been reported from most of the other continents and to date it is recognized as a cosmopolitic virus, widespread in (sub)tropical as well as in temperate climate zones. The virus causes great yield losses in a large number of economically important crops, e.g. tomato, lettuce, pepper, potato, tobacco, groundnut, pea, papaya and in ornamental crops, such as chrysanthemum, begonia, alstroemeria, cyclamen, gerbera, gloxinia and impatiens. To date more than 650 different plant species belonging to 72 botanical families, both monocotyledons and dicotyledons, have been reported to be susceptible to TSWV (Cho *et al.*, 1987; Matthews, 1982; D. Peters, unpublished data). Disease symptoms vary from chlorosis, mottling, stunting and wilting to severe necrosis on leaf and stem tissues. They even may vary within the same host species due to environmental conditions, as well as to the conditions and age of the plant itself (Best, 1968; Francki and Hatta, 1981).

TSWV is the only plant virus shown to be biologically transmitted by thrips (*Thysanoptera*: *Thripidae*) (Sakimura, 1962a). Thus far, eight thrips species have been described as a vector of TSWV (Table 1; Pittman, 1927; Samuel *et al.*, 1930; Gardner *et al.*, 1935; Sakimura, 1962a, b; Kobatake *et al.*, 1984; Paliwal, 1974; Bournier, A. and Bournier, J.P., 1987; Zitter *et al.*, 1989). Since 1980, the occurrence of TSWV revived due to the global expansion of one of its major vectors, *Frankliniella occidentalis* Pergande. It is generally accepted that adult thrips do not acquire the virus. Thrips can only

Frankliniella occidentalis Pergande	western flower thrips
Frankliniella schultzei Trybom	common blossom or cotton bud thrips
Frankliniella fusca Hinds	tobacco thrips
Frankliniella tenuicornis Uzel	
Thrips tabaci Lindeman	onion thrips
Thrips palmi Karny	melon thrips
Thrips setosus Moulton	
Scirtothrips dorsalis Hood	chilli thrips

Table 1: Thrips species reported as vectors of TSWV.

transmit the virus after acquisition in their larval stage (Linford, 1932; Smith,1932; Bald and Samuel, 1931; Bailey, 1935). Recent investigations have revealed that up to 80% of the larvae can become infective and transmit the virus before they pupate (Wijkamp and Peters, 1993). The minimal acquisition period is 15 to 30 minutes. After acquisition and before transmission, i.e. during the latent period, the thrips is not infectious. Depending on the thrips species, different lengths of this period have been reported, varying from 4 to 18 days (Sakimura 1962). The virus is transstadially passed and in most cases retained for life, although irregularly transmitted. Weed plants play an important role in the spread and survival of virus. They form a virus reservoir from where infective thrips migrate to crop fields that thereafter become heavily infected (Bond *et al.*, 1983; Cho *et al.*, 1986; Kobatake *et al.*, 1984).

For decades, TSWV isolates have been discriminated mainly on the basis of disease symptoms (Best and Gallus, 1953; Norris, 1946; Best, 1968). In some cases the phenomena of cross-protection, and genetic recombination (reassortants) have been brought up as an explanation for some observations on distinct isolates (Best and Gallus, 1955; Best, 1968). During the last decade not only thrips-specificity but also host range and serology formed the basis for discrimination between isolates (Amin *et al.*, 1981; Cho *et al.*, 1988; Ghanekar *et al.*, 1979; Gonsalves and Trujilo, 1986; Kameyi-Iwaki *et al.*, 1988;

Sherwood *et al.*, 1989; Hayati *et al.*, 1990; Avila *et al.*, 1990; Law and Moyer, 1990; Wang and Gonsalves, 1990; Avila *et al.*, 1992; Yeh *et al.*, 1992; Reddy *et al.*, 1992). In the past, however, the preparation of specific polyclonal antisera has often been hampered due to host protein contaminations in virus preparations, resulting in high background reactions with healthy control material (Tas *et al.*, 1977b; Gonsalves and Trujillo, 1986).

Currently, three distinct serogroups can be distinguished using polyclonal and monoclonal antisera directed to the structural nucleoprotein of TSWV (Avila *et al.*, 1990, 1992). Based on the absence of any serological cross-reactivity between viruses of serogroups I and III, they are considered as different species. For serogroup I, the name tomato spotted wilt virus has been reserved, and for serogroup III the name *Impatiens* necrotic spot virus (INSV) has been coined (Avila *et al.*, 1992). Based on different reactivities with monoclonal antibodies, two serotypes, named tomato chlorotic spot virus (TCSV; serotype I) and groundnut ringspot virus (GRSV; serotype II), are recognized within sergroup II (Avila *et al.*, 1990). In near future, other isolates currently reported as TSWV may turn out to be further new species, among those are groundnut bud necrosis (Reddy *et al.*, 1992), watermelon silver mottle virus (Kameya-Iwaki *et al.*, 1988; Yeh *et al.*, 1992), an isolate of *Verbesina alternifolia* (Hayati *et al.*, 1990), and peanut yellow spot virus (Reddy *et al.*, 1991).

Cytopathology and virus structure

Extensive electron microscopical analyses demonstrated that TSWV is found in almost all tissues and organs following systemic infection of plants (Kitajima, 1965; Francki and Grivell, 1970; Ie, 1973). Mature virus particles are mainly found clustered in the cisternae of the rough endoplasmatic reticulum (RER) and consist of spherical lipid-bound particles, 80-120 nm in diameter, covered with spike projections (Fig.1a and b; Best and Palk, 1964; Best and Katekar, 1964; Martin, 1964; Ie, 1964; Kitajima, 1965; Van Kammen *et al.*, 1966; Francki and Grivell, 1970 Francki *et al.*, 1984; Francki and Hatta, 1981). The core of the particles consists of ribonucleocapsid structures ("nucleocapsids"), in which the three genomic RNAs are wrapped with nucleoprotein

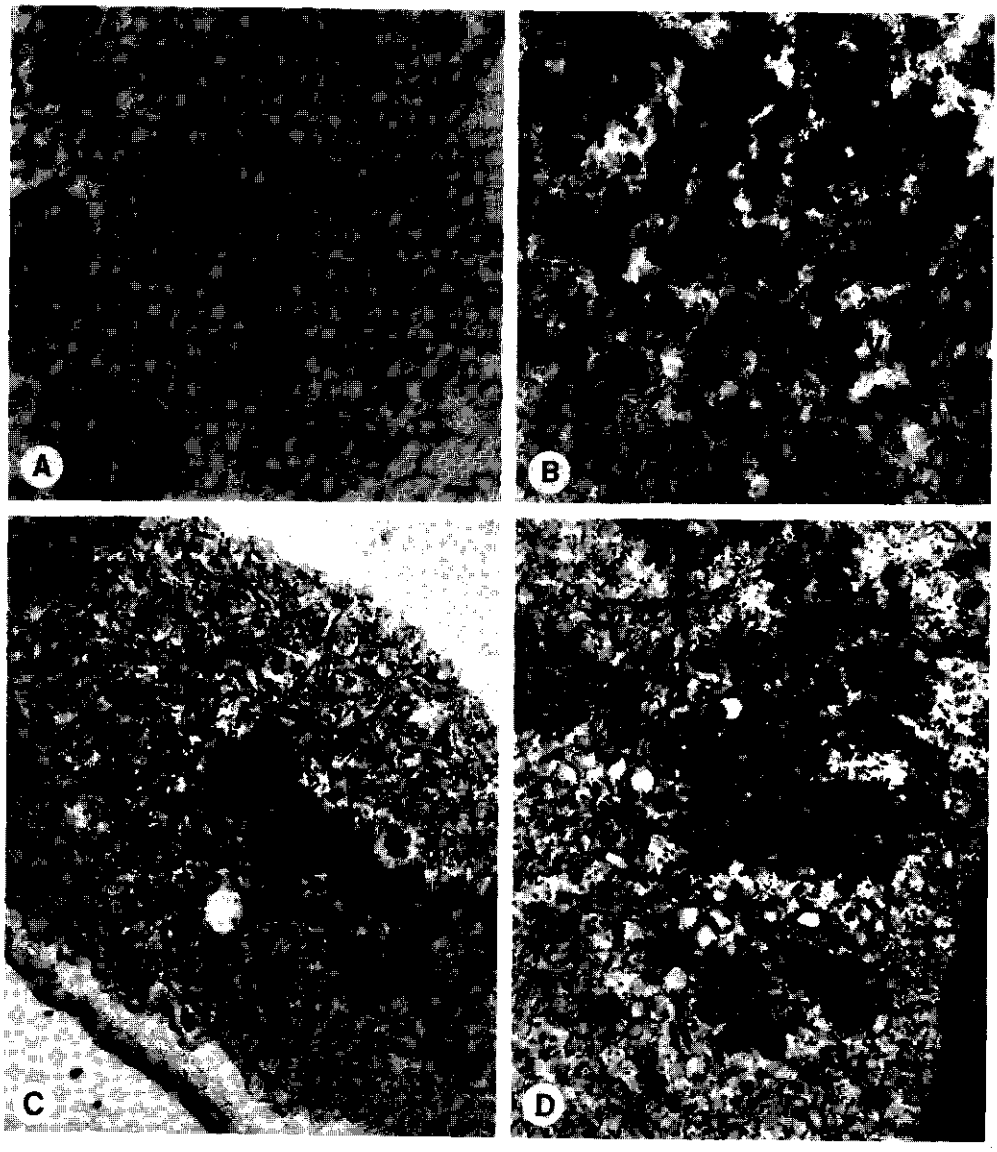

Figure 1: Cytopathology of TSWV. (A) presence of TSWV virions in the lumen of the endoplasmatic riticulum (ER); (B) electron dense massess consisting of non-enveloped nucleocapsid aggregates in the cytoplasm; (C) fibrous structures in the cytoplasm of a TSWV-infected cell; (D) virus particles bound by two membranes. Scale bars represent 200 nm. V: Virus; NA: Nucleocapsid aggregates; F: Fibrous structures.

units. The virus particles most likely mature by budding of nucleocapsids through the ER membrane (Milne, 1970).

In addition to mature virus particles, specific cytopathic structures associated with TSWV infection are found. One type is characterized by dark diffuse amorphous masses, also denoted viroplasm, with locally electron dense striated spots, located freely dispersed in the cytoplasm (Fig.1b; Ie, 1971; Kitajima, 1965; Milne, 1970; Francki and Grivell, 1970; Francki *et al.*, 1984; Francki and Hatta, 1981). These dense spots have a diameter slightly smaller than mature virus particles and are never found in older systemically infected mesophyll cells (Ie, 1971). They have a proteinaceous nature, suggested to consist of ribonucleoprotein and to form a normal developmental stage in the formation of TSWV particles (Milne, 1970; Ie, 1971). The nature of these dense masses as non-enveloped nucleocapsid aggregates is founded by studies of morphological defective TSWV isolates (Ie, 1982, Verkleij and Peters, 1983). In these studies purified infectious fractions of TSWV resemble the amorphous masses, and therefore are suggested to present aggregates of nucleocapsids of morphologically defective TSWV that for some reason can not produce enveloped particles (Ie, 1982; Verkleij and Peters, 1983). The second type of cytopathic structures associated with TSWV infections consists of fibrous structures (Fig.1c; Francki *et al.*, 1984; Francki and Grivell, 1970), the nature and function of these, however, are unknown.

Sometimes, though only at an early stage of infection, doubly-enveloped particles are found in the cytoplasm (Fig.1d; Milne, 1970; Francki *et al.*, 1984). They are proposed to arise as a result from budding of the electron dense aggregates, i.e. nucleocapsids, in parallel membranes to form enveloped particles (Milne, 1970; Francki *et al.*, 1984). Subsequent joining of several doubly-enveloped particles then leads to a cluster of enveloped particles in the cisternae of RER (Milne, 1970). Sofar, virus particles are never observed in the Golgi complex or vacuoles (Kitajima *et al.*, 1965), suggesting that these are not involved in the maturation or transport of the virus. Still, only limited information is available on the intermediate stages of particle budding, probably because these are passed through rapidly.

Purified virus particles contain at least 4 structural proteins with a molecular weight

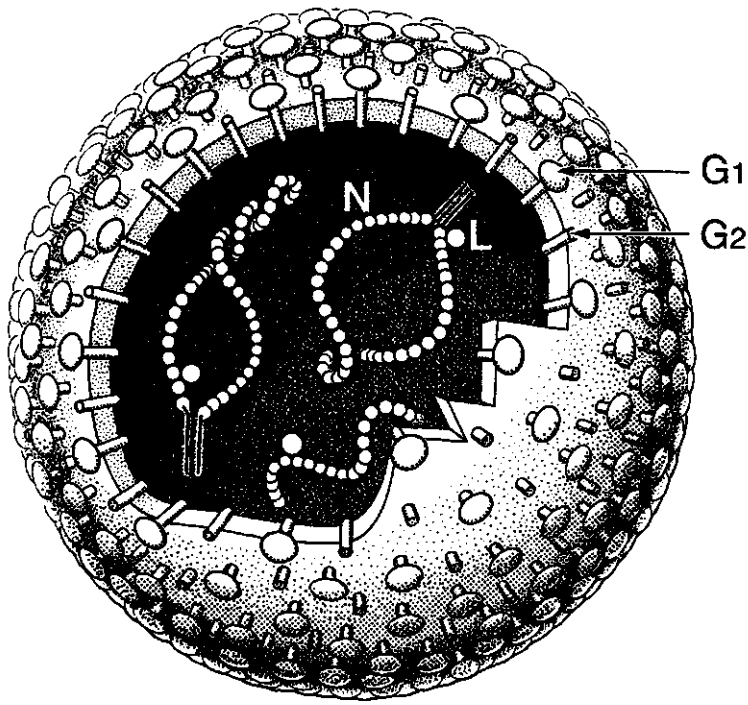

Figure 2: Schematic representation of a TSWV particle.

of 29 kilodalton (kDa), 58 kDa, 78 kDa and a large (L) protein of >200 kDa present in minor amounts (Mohamed *et al.*, 1973; Tas *et al.*, 1977a). The 78 kDa and 58 kDa proteins are glycosylated and represent the envelope glycoproteins often referred to as G1 (78 kDa) and G2 (58 kDa) (Mohamed *et al.*, 1973; Tas *et al.*, 1977a). Often a fifth structural protein of 52 kDa is observed (Tas *et al.*, 1977), however this product is believed to represent a stable degradation product of the 58 kDa G2 protein. The 29 kDa protein represents the nucleoprotein (N) (Tas *et al.*, 1977a; Mohamed, 1981). Further, the large protein has recently been found encoded by the viral genome and its function elucidated as the putative viral RNA polymerase (De Haan, thesis 1991). A schematic representation of the particle morphology of TSWV is depicted in Figure 2.

The genome of TSWV consists of three single-stranded linear RNA segments

denoted S RNA (small), M RNA (medium) and L RNA (large) with reported sizes of 2900 bases, 5000 bases and 9000 bases, respectively (Van den Hurk *et al.*, 1977; Verkleij *et al.*, 1982). Isolated TSWV RNA is non-infectious and does not contain poly(A)-sequences.

Taxonomy

It is clear from the preceding paragraph that tomato spotted wilt virus is very distinct from other plant viruses. It has a unique particle morphology, a very broad host range and is biologically transmitted by thrips. Hence, this virus has originally been classified as the representative of the monotypic tomato spotted wilt virus group (Ie, 1970; Matthews, 1982). Though, Milne and Francki (1984) already suggested that TSWV may be considered as a member of the arthropod-borne *Bunyaviridae*, a virus family exclusively consisting of animal-infecting viruses (Matthews, 1982; Bishop, 1980), the data presented in the thesis of De Haan (1991) and in this thesis provide the conclusive molecular evidence that this classification would be correct.

Scope of investigation

The aim of the research described in this thesis was to elucidate the genetic organisation, the coding functions and the expression strategy of the TSWV genome.

At the onset of this research, only preliminary sequence data were available mainly on the TSWV L and S RNA segments, but the availability of cDNA clones derived from all three genomic RNA segments made it possible to study the viral RNA synthesis in plants and to gain insight in the transcription/replication strategy of TSWV (Chapter 2). The initiation of transcription of the viral genome was subsequently analysed in more detail and showed analogies with that of other segmented negative strand RNA viruses, i.e. *Bunyaviridae*, *Orthomyxoviridae* and *Arenaviridae* (Chapter 3). Meanwhile the nucleotide sequence of the TSWV S and L RNA segments was elucidated (De Haan, thesis 1991). With the determination of the nucleotide sequence of the M RNA segment,

as described in Chapter 4, the complete nucleotide sequence of the TSWV genome became available. Whereas the M RNAs of the animal-infecting *Bunyaviridae* only contain one ORF encoding the precursor to the glycoproteins and, in some cases, a nonstructural protein, the TSWV M RNA, in addition to the glycoprotein precursor gene, was found to contain an extra ORF encoding a putative nonstructural protein (NS_M) of 33.6 kDa. In order to analyse the functions of this and a second nonstructural protein (NS_S) of TSWV, both proteins were expressed in heterologous expression systems to obtain specific antibodies. Using these antibodies, the intracellular locations of both NS_S and NS_M were determined (Chapter 5 and 6). Additionally, the gene encoding the precursor to the glycoproteins (G1 and G2) was expressed in a heterologous expression system as a first step towards understanding the biosynthesis and maturation of these envelope glycoproteins (Chapter 7).

REFERENCES

Amin, P.W., Reddy, D.V.R. and Ghanekar, A.M. (1981). Transmission of TSWV, the causal agent of bud necrosis of peanut, by *Scirtothrips dorsalis* and *Frankliniella schultzei*. *Plant Disease* **65**, 663-665.

Avila, A.C. de, Huguenot, C., Resende, R. de O., Kitajima, E.W., Goldbach, R. and Peters, D. (1990). Serological differentiation of 20 isolates of tomato spotted wilt virus. *Journal of General Virology* **71**, 2801-2807.

Avila, A.C. de, De Haan, P., Kitajima, E.W., Kormelink, R., Resende, R. de O., Goldbach, R.W. and Peters, D. (1992). Characterization of a distinct isolate of tomato spotted wilt virus from *Impatiens* sp. in the Netherlands. *Journal of Phytopathology* **134**, 133-151.

Bailey, S.F. (1935). Thrips as vectors of plant diseases. *Journal of Economical Entomology* **28**, 856-863.

Bald, J.G. and Samuel, G. (1931). Investigations on spotted wilt of tomatoes - II. *Commonwealth of Australia, Council for Scientific and Industrial Research* **Bull.no.54**.

Best, R.J. (1968). Tomato spotted wilt virus. *Advances in Virus Research* **vol.13**, 65-146.

Best, R.J. and Gallus, H.P.C. (1953). Strains of tomato spotted wilt virus. *Australian Journal of Science* **15**, 212-214.

Best, R.J. and Gallus, H.P.C. (1955). Further evidence for the transfer of character determinants (recombination) between strains of tomato spotted wilt virus. *Enzymologia* **17**, 207-221.

Best, R.J. and Katekar, G.F. (1964). Lipid in a purified preparation of tomato spotted wilt virus. *Nature* **203**, 671-672.

Best, R.J. and Palk, B.A. (1964). Electron microscopy of strain E of tomato spotted wilt virus and comments on its probable biosynthesis. *Virology* **23**, 445-460.

Bond, W.P., Whitman, H.K. and Black, L.L. (1983). Indigenous weeds as reservoirs of tomato spotted wilt virus. *Phytopathology* **73**, 499.

Bournier, A. and Bournier, J.P. (1987). L'introduction en France d'un nouveau ravageur: *Frankliniella*

occidentalis. *Phytoma* **388**, 14-17.
Brittlebank, C.C. (1919). A new tomato disease Spotted Wilt. *Journal of Agriculture Victoria* **27**, 231-235.
Cho, J.J., Mau, R.F.L., Gonsalves, D. and Mitchell, W.C. (1986). Reservoir weed hosts of TSWV. *Plant Disease* **70**, 1014-1017.
Cho, J.J., Mau, R.F.L., Hamasaki, R.T. and Gonsalves, D. (1988). Detection of TSWV in individual thrips by ELISA. *Phytopathology* **78**, 1348-1352.
Cho, J.J., Mau, R.F.L., Mitchell, W.C., Gonsalves, D. and Yudin, L.S. (1987). Host list of plants susceptible to tomato spotted wilt virus (TSWV). *Research Ext.* Series 078.
Francki, R.I.B. and Grivell, C.J. (1970). An electron microscopy study of the distribution of tomato spotted wilt virus in systemically infected *Datura stramonium* leaves. *Virology* **42**, 969-978.
Francki, R.I.B. and Hatta, T. (1981). Tomato spotted wilt virus. In: "*Plant virus infections and comparative diagnosis*", 491-512, E. Kurstak ed., Elsevier/North-Holland Biomedical Press.
Francki, R.I.B., Milne, R.G. and Hatta, T. (1984). Tomato spotted wilt virus group. In: "*Atlas of Plant Viruses*" vol.1, 101-110, Boca Raton, CRC Press.
Gardner, M.W., Tompkins, C.M. and Whipple, O.C. (1935). Spotted wilt of truck crops and ornamental plants. *Phytopathology* **25**, 17.
Ghanekar, A.M., Reddy, D.V.R., Iizuka, N., Amin, P.W. and Gibbons, R.W. (1979). Bud necrosis of groundnut (*Arachis hypogaea*) in India caused by TSWV. *Annals of Applied Biology* **93**, 173-179.
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.
Hayati, I., Kline, A.S., Kim, K.S. and Gergerich, R.C. (1990). A tomato spotted wilt virus from *Verbesina alternifolia*. *Phytopathology* **80**, 1060-1061 (Abstract).
Ie, T.S. (1964). An electron microscope study of tomato spotted wilt virus in the plant cell. *Netherlands Journal of Plant Pathology* **70**, 114-115.
Ie, T.S. (1970). Tomato spotted wilt virus. *CMI/AAB Description of Plant Viruses* **39**, 4.
Ie, T.S. (1971). Electron microscopy of developmental stages of tomato spotted wilt virus in plant cells. *Virology* **2**, 468-479.
Ie, T.S. (1973). Tomato spotted wilt virus in the anthers of *Tropaeolum majus*. *Netherlands Journal of Plant Pathology* **79**, 387-391.
Ie, T.S. (1982). A sap-transmissible, defective form of tomato spotted wilt virus. *Journal of General Virology* **59**, 387-391.
Kameyi-Iwaki, M., Hanada, K., Honda, Y. and Tochihara, H. (1988). A watermelon strain of tomato spotted wilt virus (TSWV-W) and some properties of its nucleocapsid (Abstracts of Papers). Fifth International Congress of Plant Pathology, Japan, p.46.
Kitajima, E.W. (1965). Electron microscopy of vira cabeça virus (Brazilian tomato spotted wilt virus) within the host cell. *Virology* **26**, 89-99.
Kobatake, H., Osaki, T. and Inouye, T. (1984). The vector and reservoirs of tomato spotted wilt virus in Nara prefecture (Japan). *Annals of the Phytopathological Society of Japan* **50**, 541-544.
Law, M.D. and Moyer, J.W. (1990). A tomato spotted wilt-like virus with a serologically distinct N protein. *Journal of General Virology* **71**, 933-938.
Linford, M.B. (1932). Transmission of the pineapple yellow-spot virus by thrips tabaci. *Phytopathology* **22**, 301-324.
Martin, M.M. (1964). Purification and electron microscope studies of tomato spotted wilt virus (TSWV) from tomato roots. *Virology* **22**, 645-649.
Matthews, R.E.F. (1982). Classification and nomenclature of viruses. *Intervirology* **17**, 1-199.
Milne, R.G. (1970). An electron microscope study of TSWV in sections of infected cells and in negative stain preparations. *Journal of General Virology* **6**, 267-276.
Milne, R.G. and Francki, R.I.B. (1984). Should TSWV be considered as a possible member of the family *Bunyaviridae* ? *Intervirology* **22**, 72-76.
Mohamed, N.A. (1981). Isolation and characterization of subviral structures of tomato spotted wilt virus.

Journal of General Virology **53**, 197-206.

Mohamed, N.A., Randles, J.W. and Francki, R.I.B. (1973). Protein composition of tomato spotted wilt virus. *Virology* **56**, 12-21.

Norris, D.O. (1946). The strain complex and symptom development of tomato spotted wilt virus. *Commonwealth of Australia, Council for Scientific and Industrial Research* **Bull.no.202.**

Paliwal, Y.C. (1974). Some properties and thrips transmission of tomato spotted wilt virus in Canada. *Canadian Journal of Botany* **52**, 1177-1182.

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.). *Commonwealth of Australia, Council for Scientific and Industrial Research* **Bull.no.1**, 74-77.

Reddy, D.V.R., Sudarshana, M.R., Ratna, A.S., Amin, P.W., Kumar, I.K. and Murphy, A.K. (1991). The occurrence of yellow spot virus, a member of the tomato spotted wilt virus group, on peanut (*Arachis hypogaea* L.) in India. In: *Virus-Thrips-Plant Interactions of TSWV, Proceedings USDA Workshop, Beltsville USA* (H.-T Hsu & R.H. Lawson Eds.), National Technology Information Service, Springfield, 77-78.

Reddy, D.V.R., Ratna, A.S., Sudarshana, M.R., Poul, F. and Kumar, I.K. (1992). Serological relationships and purification of bud necrosis virus, a tospovirus occurring in peanut (*Arachis hypogaea* L.) in India. *Annals of Applied Biology* **120**, 279-286.

Sakimura, K. (1962a). The present status of thrips-borne viruses. In: "*Biological transmission of disease agents*", 33-40, K. Maramorosch ed., Academic Press, New York.

Sakimura, K. (1962b). *Frankliniella occidentalis* (*Thysanoptera: Thripidae*), a vector of tomato spotted wilt virus, with special reference to the color forms. *Annals of the Entomological Society of America* **vol.55**, 387-389.

Samuel, G., Bald, J.G. and Pittman, H.A (1930). Investigations on spotted wilt of tomatoes. *Commonwealth of Australia, Council for Scientific and Industrial Research* **Bull.no.44.**

Sherwood, J.L., Sanborn, M.R., Keyser, G.C. and Meyers, L.D. (1989). Use of monoclonal antibodies in detection of tomato spotted wilt virus. *Phytopathology* **79**, 61-64.

Smith, K.M. (1932). Studies on plant virus diseases. XI. Further experiments with a ringspot virus: Its identification with spotted wilt of tomato. *Annals of Applied Biology* **19**, 305-330.

Tas, P.W.L., Boerjan, M.L. and Peters, D. (1977a). The structural proteins of tomato spotted wilt virus. *Journal of General Virology* **36**, 267-279.

Tas, P.W.L., Boerjan, M.L. and Peters, D. (1977b). Purification and serological analysis of tomato spotted wilt virus. *Netherlands Journal of Plant Pathology* **83**, 61-72.

Van den Hurk, J., Tas, P.W.L. and Peters, D. (1977). The ribonucleic acid of tomato spotted wilt virus. *Journal of General Virology* **36**, 81-91.

Van Kammen, A., Henstra, S. and Ie, T.S. (1966). Morphology of tomato spotted wilt virus. *Virology* **30**, 574-577.

Verkleij, F.N., De Vries, P. and Peters, D. (1982). Evidence that tomato spotted wilt virus RNA is a positive strand. *Journal of General Virology* **58**, 329-338.

Verkleij, F.N. and Peters, D. (1983). Characterization of a defective form of tomato spotted wilt virus. *Journal of General Virology* **64**, 677-686.

Wang, M. and Gonsalves, D. (1990). ELISA detection of various tomato spotted wilt virus isolates using specific antisera to structural proteins of the virus. *Plant Disease* **74**, 154-158.

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.

Yeh, S.-D., Lin, Y.-C., Cheng, Y.-H., Jih, C.-L. and Chen, M.-J. (1992). Identification of tomato spotted wilt virus on watermelon in Taiwan. *Plant Disease* **76**, 835-840.

Zitter, T.A., Daughtrey, M.L. and Sanderson, J.P. (1989). Tomato spotted wilt virus. *Cornell Cooperative Extension* Fact sheet 735.90: 6p.

Chapter 2

Viral RNA synthesis in tomato spotted wilt virus-infected *Nicotiana rustica* plants[1]

SUMMARY

The synthesis of viral RNA species in tomato spotted wilt virus (TSWV)-infected *Nicotiana rustica* plants was followed in terms of time and relative abundancy. While systemic symptoms were visible after 4 days post-infection (p.i.), viral (v) and viral-complementary (vc) strands of all three genomic RNA segments (L RNA, M RNA and S RNA) were detected from 2 days p.i. on. In addition, two subgenomic mRNAs, derived from S RNA, were detected. For the L RNA segment no subgenomic mRNAs were detected, suggesting that this segment is expressed via the synthesis of a genome-sized vc mRNA. A possible M-specific subgenomic mRNA was detected, showing a similar time course of appearance as the subgenomic mRNAs derived from the S RNA segment. Analysis of cytoplasmic RNA fractions revealed that both v- and vc strands of all three genomic segments associate with the nucleoprotein (N) into nucleocapsid structures, the vc RNA species being present in lower amounts. Intact, enveloped virus particles showed the presence of the v strand of the L RNA segment only and surprisingly, both v-and vc strands of the M and S RNA segments, though in different ratios.

[1]This chapter has been published in a slightly modified version as : Richard Kormelink, Peter de Haan, Dick Peters and Rob Goldbach (1992). Viral RNA synthesis in tomato spotted wilt virus-infected *Nicotiana rustica* plants. *Journal of General Virology* 73, 687-693.

INTRODUCTION

Tomato spotted wilt virus (TSWV) is a representative of the arthropod-borne *Bunyaviridae*, unique in its property to infect plants (De Haan *et al.*, 1989a). At the ICTV meeting in Berlin (1990) TSWV has been accepted as the sole member of a newly created genus within the *Bunyaviridae*, denoted *Tospovirus*. Typical for members of the *Bunyaviridae* (Francki *et al.*, 1991), TSWV has enveloped particles that contain three different segments of single stranded RNA, denoted S RNA (2.9 kb), M RNA (5.0 kb) and L RNA (8.9 kb) (Van Den Hurk *et al.*, 1977; Mohamed *et al.*, 1981; De Haan *et al.*, 1990, 1991). The genomic RNAs form pseudo-circular structures tightly associated with the nucleoprotein (N) of 29 kDa, and a few copies of a large L protein (>200 kDa), proposed to represent the viral transcriptase. Two membrane glycoproteins of 78 kDa (G1) and 58 kDa (G2) form the spikes on the viral envelope (Mohamed *et al.*, 1973; Tas *et al.*, 1977; De Haan *et al.*, 1989a).

Recently, the nucleotide sequences of the S- and L RNA segments have been determined (De Haan *et al.*, 1990 and 1991). The L RNA is of negative polarity, containing a single large open reading frame (ORF) in the viral complementary (vc) strand. This ORF encodes a protein of expected size 331.5 kDa which, based on sequence homology with the Bunyamwera L protein and Influenza PB1, is proposed to represent the viral transcriptase. The S RNA segment of TSWV has an ambisense gene arrangement, as also found for the S RNAs of phleboviruses, another genus within the *Bunyaviridae* (Ihara *et al.*, 1984; Marriot *et al.*, 1989; Simons *et al.*, 1990; Giorgi *et al.* 1991). The gene encoding the N protein is located on the vc strand, while a second gene, encoding a nonstructural protein (NS_S) of 52.4 kDa, is found on the viral strand (De Haan *et al.*, 1990).

To further study the expression and replication of the TSWV genome the synthesis of viral RNA species has now been followed during systemic infection of *Nicotiana rustica*. To this end total RNA extracts, prepared from infected leaf material at different times after inoculation, were analysed using strand specific probes for each of the three

genomic RNA segments. The results obtained demonstrate that TSWV follows the replication strategy characteristic for negative strand RNA viruses, by which L, M, and S viral (v) RNA, and M and S viral complementary (vc) RNA become encapsidated in enveloped virus particles. In addition to S-RNA-derived subgenomic mRNAs, a possible M-specific subgenomic mRNA has been detected in TSWV-infected *N. rustica*.

MATERIALS AND METHODS

Plants, virus and cDNA clones

The Brazilian isolate BR-01 (CNPH1) of TSWV was maintained in *Nicotiana rustica* 'America' by mechanical inoculation. Complementary DNA clones representing the different RNAs of TSWV BR-01 have been described previously (De Haan *et al.*, 1989b, 1990, 1991).

Purification of TSWV and total cellular nucleocapsid material

Intact virus particles were purified according to Tas *et al.* (1977) with one modification as described by Avila *et al.* (1990). Viral particles were banded in 10-40% sucrose gradients and subsequently used for RNA extraction. Virus yield was usually in the range of 0.8-1.2 mg virus/100 g infected leaf material. Viral nucleocapsids were isolated from infected leaf tissue as described by Avila *et al.* (1990), omitting the sucrose gradient step. RNA from intact virus particles or free nucleocapsids was isolated by treatment with 1% SDS and phenol extraction. After ethanol precipitation, the RNA pellet was washed with 70% ethanol, dried and resuspended in H_2O.

Total RNA extraction and Northern blot analysis

Young seedlings of *N. rustica* 'America' (three leaves stage) were mechanically inoculated with extracts of TSWV BR-01-infected leaves. After inoculation, systemically infected leaf samples were taken at different times until 14 days post inoculation (p.i.). Total RNA was extracted from healthy and infected *N. rustica* according to De Vries *et*

al. (1982). Total RNA samples of 7 μgwere resolved by electrophoresis in 0.6-1.2% agarose gels after treatment with methylmercuric hydroxide (Bailey and Davidson, 1976). The RNA was blotted onto Genescreen (New England Nuclear, NEN), and hybridised to ^{32}P-labelled riboprobes of TSWV specific sequences. As a control, 0.5 μgTSWV BR-01 nucleocapsid RNA was included.

Preparation of strand-specific probes

Complementary DNA clones of TSWV BR-01 representing the 3' and 5' terminal regions of the L, M and S RNAs were cloned in transcription vector pSK+ (Stratagene) according to standard procedures (Maniatis *et al.*, 1982). The cDNA clones used for this purpose are described in Fig.1. The pSK+ plasmids were linearized immediately downstream of the viral inserts and radioactive RNA probes were prepared by *in vitro* transcription using T7 or T3 RNA polymerase (BRL) in the presence of [α-^{32}P]UTP (3000 Ci/mmol, Amersham) as described by Melton *et al.* (1984). The run-off transcripts were checked for strand specificity by hybridisation on spotted, template-free RNA transcripts.

RESULTS

RNA species present in virions and nucleocapsid fractions

Nucleotide sequence determinations (De Haan *et al.*, 1989b, 1990 and 1991) have revealed that the genomic L RNA of TSWV is of negative polarity, while the S RNA is ambisense, containing one ORF in the v strand (encoding the NS_S protein) and a second ORF in the vc strand (the N protein gene, Fig.1). For the M RNA, the limited sequence data available (De Haan *et al.*, 1989 and unpublished results) indicate that this genomic segment, is at least partly of negative polarity. To investigate which viral RNA species are present in enveloped viral particles and which RNA species are found encapsidated by N protein in infected cells, RNA extracts from purified virions and from cellular nucleocapsid fractions were denatured with methylmercuric hydroxide and

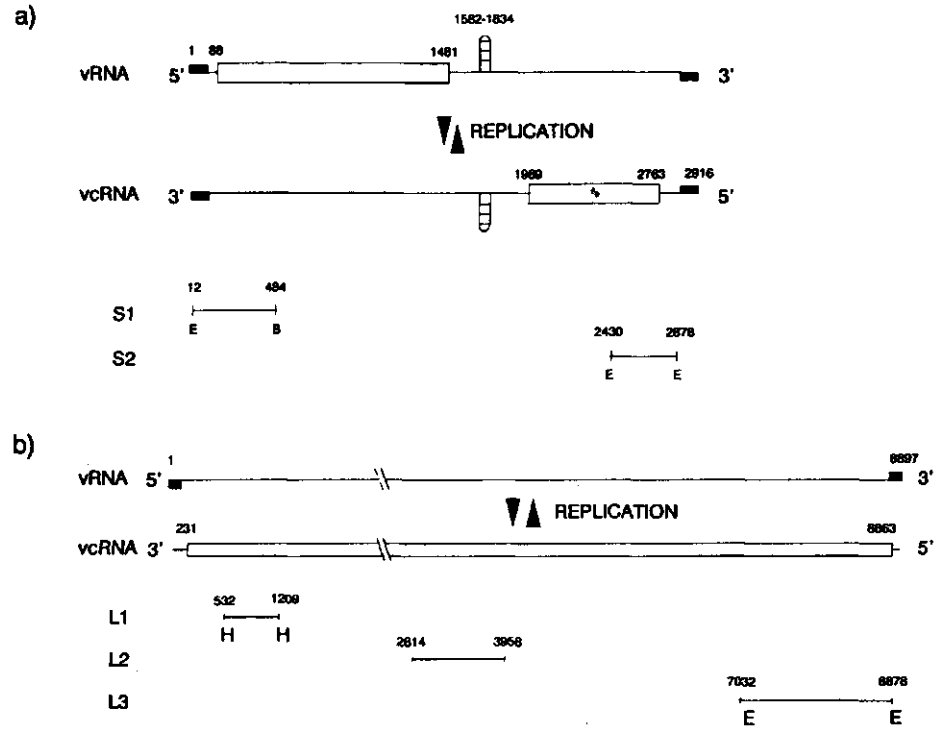

Figure 1: Schematic representation of the genomic structure of the S and L RNA (A,B). The location of cDNA fragments used for the synthesis of riboprobes is shown in alignment with the L and S RNA segments. The replication of the vRNA strand into the vcRNA strand is indicated by the arrows. The black boxes at the termini of the RNA strands represent complementary sequences. Due to the lack of sequence data, no genomic map for the M RNA is shown. E, H and B are *Eco*RI, *Hind*III and *Bgl*II restriction sites, respectively. L2 is a L-specific blunt-end cDNA fragment.

resolved by agarose gel electrophoresis (Fig.2a; Bailey and Davidson, 1976). The RNA was transferred to genescreen membranes and hybridised to ^{32}P-labelled strand specific probes corresponding to the S, M or L RNA segment of TSWV (Fig.1). Northern analysis showed that cellular nucleocapsid fractions contained both v- as well as vc strands of all 3 genomic segments, the vc strands mostly occurring in lower quantities (Fig.2b, lanes indicated with N). Intact, enveloped virus particles, however, only contained the viral strand of the L RNA segment and surprisingly, both v- and vc strands of the M and S RNA segments, though in different amounts (Fig.2b, lanes indicated with

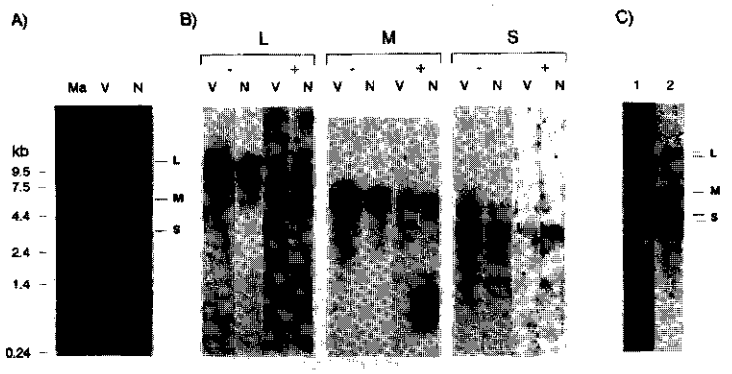

Figure 2: Analyses of purified intact virus particles and cytoplasmic nucleocapsid fractions for content of vRNA and vcRNA. A) Ethidium bromide stained agarose gel containing RNA markers (Ma), RNA from purified virus particles (V) and RNA from cytoplasmic nucleocapsids (N). The sizes of the RNA markers (in kilobases) are shown on the left side. B) RNA from purified virus and nucleocapsids was resolved on agarose gels and subsequently transferred to gene screen membrane. The Northern blots were hybridised with riboprobes, specific for the L, M and S RNA segment. V and N represent the RNA fractions of purified intact virus particles and cytoplasmic nucleocapsids, respectively. - and + indicate vRNA and vcRNA, respectively. Northern blots of (+) RNA strands have been exposed ten times as long as the (-) RNA strands. C) Ethidium stained pattern of total RNA of TSWV-infected *N. rustica* (1) and Northern blot containing total RNA of TSWV-infected *N. rustica* hybridised first to a dsDNA probe of the L RNA (L1), and subsequently also to probes of the M (M1) and S (S1) RNA segments (2).

V). The reason for this phenomenon is not clear and was further investigated. As a first step, total RNA of TSWV-infected *N. rustica* 8 days p.i. was analysed on agarose gels (Fig.2c, lane 1). The pattern of TSWV RNA species visible, did not differ from the RNA pattern of purified virus or nucleocapsids (Fig.2a), but Northern blot analyses using ^{32}P-labelled dsDNA probes of all 3 TSWV genomic RNA species demonstrated the presence of six distinct viral RNA species, ranging in size between 1.7 kb and 9 kb (Fig.2c, lane 2). The RNA bands appeared as 2 doublets, on the position of L and S RNA respectively, a single band at the position of M RNA and one additional minor RNA species. In order to resolve the identity and polarity of these bands, time courses of total RNA extracts of TSWV-infected *N. rustica* were analysed using strand specific riboprobes corresponding to the 3' and 5' terminal regions of the S, M, and L RNA. Fig.1 shows the location of most of the probes used for these analyses in alignment with the original viral S and L RNA segments. Due to the lack of sequence data, the genomic

structure of the M RNA and, as a consequence the location of the M RNA riboprobe used, is not represented.

Synthesis of TSWV S RNA species

Total RNA extracted from systemically infected *N. rustica* leaves at different times p.i. were resolved by agarose gel electrophoresis, transferred to genescreen membrane and hybridized to ^{32}P-UTP labelled riboprobes from pSK+/S1 (Fig. 3a) and pSK+/S2 (Fig. 3b) of either polarity. As controls, total RNA from healthy *N. rustica* and purified TSWV nucleocapsids were included. The riboprobes clearly distinguished two genome length S RNA species, i.e. the vRNA and vcRNA strands of this genome segment (Fig.3). In case time courses were analysed with respect to the synthesis of vRNA strands, probe S1-vc always showed, although to a lesser extent, some hybridisation with the vcRNA strand (Fig.3a, upper panel). This was caused by the fact that this probe never showed a 100% strand specificity (see Materials and Methods). After comparison with the S RNA doublet observed during analysis of a total RNA extract of TSWV-infected *N. rustica* (Fig. 2c), it was concluded that the vc S RNA strand migrated ahead of the v S RNA strand. Whereas the amount of vRNA increased with time, the production of vcRNA appeared to reach a steady state level at 4 days p.i.. Densitometric analysis of several autoradiograms indeed showed that, whereas the vRNA strand clearly accumulated, the vcRNA strand did hardly or not accumulate from 4 days p.i. on (data not shown). Both strands became detectable at 2 days p.i.. At the same time two subgenomic RNA molecules with the size of 1.7 kb (Fig. 3a, probe pSK+/S1-vc) and 1.2 kb (Fig. 3b, probe pSK+/S2-v) respectively, were detected. In view of the location of the probes within the physical map of the S RNA, and the sizes of the NS_S and N gene encoded by this RNA segment (Fig.1), the subgenomic RNA molecules are likely to correspond to these two genes and represent subgenomic messengers. Both mRNAs are transcribed from complementary strands as they are detected by probes of different polarities, which is in agreement with the ambisense coding arrangement of the S RNA. These data furthermore indicate that the genome-length vc S RNA is not a mRNA but represents a replicative intermediate.

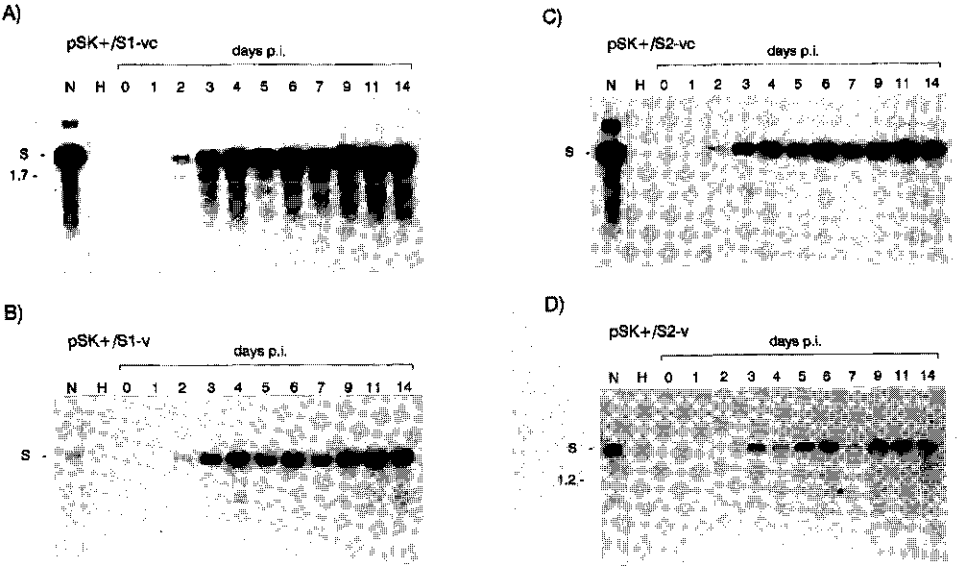

Figure 3: Time course of synthesis of S RNA species in TSWV-infected *N. rustica*. Total RNA extracts from the times indicated at the top of the figure (days p.i.) were analysed using riboprobes of cDNA fragments S1 (A, B) and S2 (C, D). As controls, total RNA of healthy *N. rustica* (H) and nucleocapsid RNA (N) were included. The sizes of the RNA strands detected (in kb) and the position of the S RNA doublet, observed in total RNA extracts of TSWV-infected *N. rustica*, are indicated. The notation pSK+/S1-v (B) and pSK+/S1-vc (A) indicates that riboprobe S1, detecting vcRNA and vRNA strands respectively, was used in the hybridisation experiment. The Northern blots were hybridised with equal amounts of cpm to detect v- or vcRNA strands. Exposure of pSK+/S1-v and pSK+/S2-v was three times longer than for S1-vc and S2-vc, respectively.

Synthesis of TSWV M RNA species

To analyse the synthesis of M-specific RNA species during TSWV infection cDNA clone pSK+/M1, containing 600 basepairs of viral insert, was used to prepare riboprobes. According to the restriction map and partial sequence data of the M RNA segment, the position of clone M1 was located within the 3' terminal region of the M RNA. Time courses similar to those produced for TSWV S RNA species were analysed using these M RNA-derived riboprobes (Fig.4). As found for the TSWV S RNA segment, both genome-length strands of the M RNA were detected from 2 days p.i. on. The vRNA, was synthesised in increasing amounts during the course of infection,

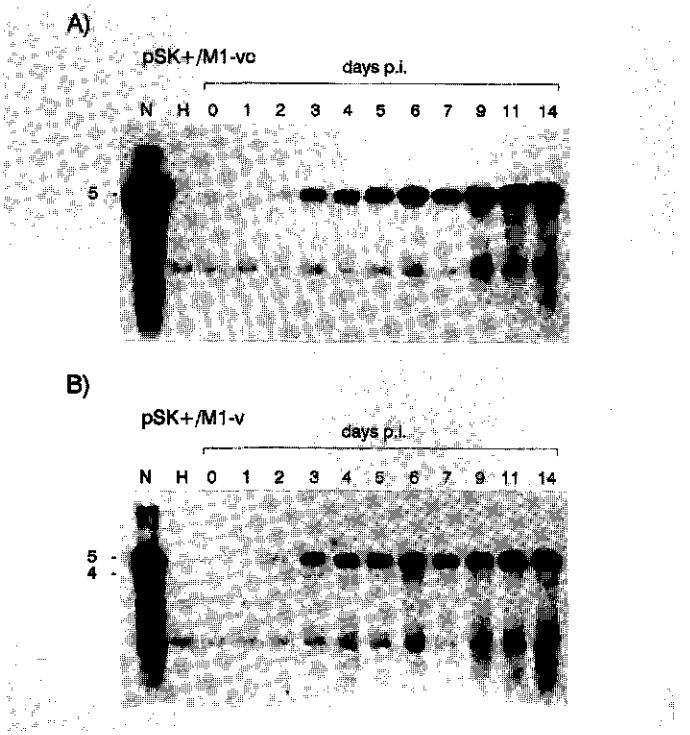

Figure 4: Time course of synthesis of M RNA species in TSWV-infected *N. rustica*. Total RNA extracts from the times indicated at the top of the figure (days p.i.) were analysed using riboprobes of cDNA fragment M1: (A) pSK+/M1-vc and (B) pSK+/M1-v. Controls were as for Fig.3. Exposure of pSK+/M1-v was three times longer than for pSK+/M1-vc.

indicating that newly synthesised M RNA was present in the extracts. The antigenomic strand accumulated much less, and from 4 days p.i. on hardly or not at all. This was confirmed by densitometric analysis of several autoradiograms (data not shown). Furthermore, also an M-specific vc sense RNA molecule with a size of approximately 4.0 kb was detected (Fig.4). Since this RNA molecule has a plus polarity it may represent a subgenomic mRNA, which would indicate the presence of two distinct cistrons in M RNA. Indeed, this RNA species showed a similar course of appearance as the two subgenomic mRNAs of the TSWV S RNA segment.

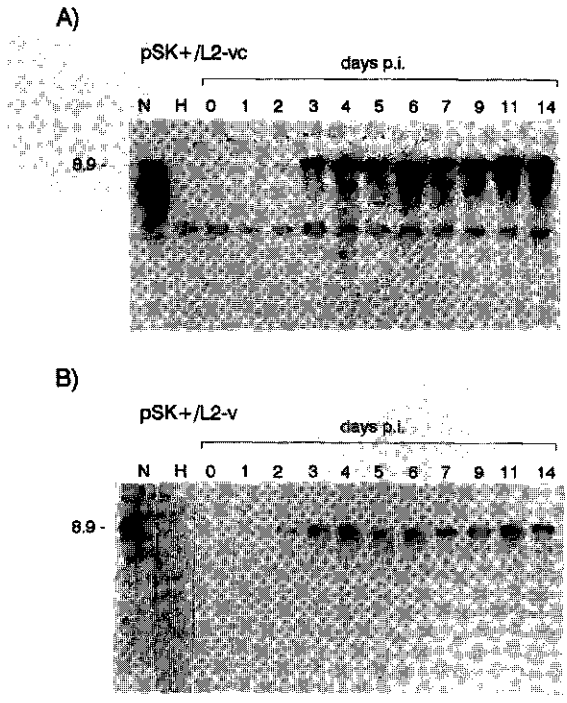

Figure 5: Time course of synthesis of L RNA species in TSWV-infected *N. rustica*. Total RNA extracts from the times indicated at the top of the figure (days p.i.) were analysed using riboprobes of cDNA fragment L2; (A) pSK+/L2-vc and (B) pSK+/L2-v. Controls were as for Fig.3.

Synthesis of TSWV L RNA species

The L RNA segment of TSWV is 8.9 kb long, containing one large ORF in the vcRNA (De Haan *et al.*, 1991). Analyses of TSWV-infected *N. rustica* revealed that various smaller L specific RNA species may be generated from L RNA, by the introduction of deletions of variable sizes (Resende, R. de O. *et al.*, 1991). These smaller defective L RNA species seem to interfere with the replication of wild type L RNA and may play a role in symptom attenuation in the host. For this reason, precautions had to be taken when analysing time courses on the appearance of functional TSWV L RNA species. Therefore, riboprobes of three different cDNA clones, two corresponding to the 5' and 3' terminal regions (pSK+/L1 and pSK+/L3) and one located internally

(pSK+/L2) were used (Fig.1b). As no different L RNA species were detected with all of the three probes, only the results with probe pSK+/L2 (Fig. 5) are shown. Two full-length L RNA molecules were detected, a vRNA molecule being found in increasing amounts during the course of infection and a vcRNA molecule reaching a steady state level (Fig.5). The accumulation of the v strand and the lower, steady-state amount of the vc-strand is characteristic for (-) RNA viruses. Comparison of these results with the pattern of viral RNA species in a total RNA extract of TSWV-infected *N. rustica* demonstrated that, similar to the doublet of the S RNA, the L RNA doublet (Fig. 2c) consisted of a vc strand migrating ahead of the v strand. In accordance to the sequence data that revealed that L RNA contains one large ORF in vc sense, comprising the whole RNA segment, no subgenomic mRNA for the L RNA segment was detected, indicating that the ORF in the vc strand of the L RNA is expressed from a genome-sized mRNA.

DISCUSSION

Analyses of viral RNA synthesis in TSWV-infected *N. rustica* revealed the presence of both the v- and vc strand of all three genomic RNA segments, and three subgenomic RNA species. Using strand specific riboprobes it was demonstrated that the doublets observed in the RNA patterns from infected leaf extracts (Fig.2c) consisted of full-length v- and vcRNA strands of the genomic RNA segments, the vcRNA strands migrating faster than the v strands. For L RNA, the vcRNA strand was present in smaller amounts (Fig.2c and 5), whereas for M RNA the v- and vcRNA strands could not be observed separately (Fig. 2c). Similar doublets have been shown within other Bunyavirus-infected animal cells (Cunningham and Szilagyi, 1987). The full-length RNA strands of both polarities were shown to be present in nucleocapsids, purified from infected cells, indicating that vcRNA is also associated with N protein. The vc strands, however, are encapsidated in smaller amounts, possibly reflecting the difference in amounts present in TSWV-infected *N. rustica*.

From 2 days p.i. on, full-length v- and vcRNA strands of all three genomic segments (L, M and S RNA) could be detected. The replication of TSWV as a (-) strand RNA virus is clearly demonstrated in the time course analyses of the L RNA, where the positive strand did hardly or not accumulate, and increasing amounts of the negative strand were observed during the course of infection. Similar results were obtained for the M and S RNA segments. In addition, for the S RNA, two subgenomic mRNAs were detected, transcribed from opposite strands. No subgenomic mRNAs of the L RNA could be detected, indicating that the single ORF in the vc strand of the L RNA (De Haan *et al.*, 1991) is expressed from a genome-sized mRNA.

Although analyses on M RNA species demonstrated the presence of a subgenomic RNA molecule with the size of approximately 4.0 kb, the identity of this RNA molecule is still unknown. According to the nucleotide sequence of clone 201, used for the synthesis of riboprobes, this subgenomic RNA species is of plus polarity, indicating that it may function as a mRNA. If this is true, then M RNA of TSWV must contain at least two different translational units, which may be arranged in one strand, or, similar to S RNA, possibly in an ambisense way. In that case, a subgenomic mRNA with the size of approximately 4.0 kb could potentially encode a precursor to both G1 (78 kDa) and G2 (58 kDa) glycoproteins. Future elucidation of the nucleotide sequence will provide the definite determination of the gene arrangement in this segment.

The RNA synthesis of TSWV specific RNA species during the time course of infection resembles that of the animal *Bunyaviridae* and other segmented negative-strand RNA viruses like the *Orthomyxoviridae* and *Arenaviridae* where RNA species of viral sense occur in excess over those of viral complementary sense (Fuller-Pace and Southern, 1988; Ihara, T. *et al.*, 1985; Eshita, Y. *et al.*, 1985; Smith and Hay, 1982).

After infection, negative-strand viruses usually start RNA synthesis with primary transcription of the infecting genome by the virus associated RNA polymerase. By using cycloheximide or other protein synthesis inhibitors, it has been shown for several negative strand RNA viruses, that ongoing protein synthesis is required for replication of the viral genome, indicating that a virally encoded protein is necessary for the synthesis of vc- and vRNA. Primary transcription of the infecting genome is, however, often not or only

partly inhibited (Hay *et al.*, 1977; Barrett *et al.*, 1979; Abraham and Pattnaik, 1983; Pattnaik and Abraham, 1983; Patterson and Kolakofsky, 1984; Eshita, Y. *et al.*, 1985; Ihara, T., 1985; Bellocq and Kolakofsky, 1987; Bellocq *et al.*, 1987; Franze-Fernandez *et al.*, 1987; Raju and Kolakofsky, 1987; Shapiro *et al.*, 1987). In case of influenzavirus, a member of the *Orthomyxoviridae*, the nucleocapsid protein has been shown to antiterminate transcription resulting in the synthesis of full-length vc- and vRNA, thus causing a switch from transcription to replication (Beaton and Krug, 1984, 1986). A temporal separation of TSWV primary transcription and replication *in vivo* was not observed. This might be due to the asynchronous infection of cells during systemic infection of plant tissue. This is best illustrated by the time course analyses of the S RNA species where the N mRNA and NS_S mRNA both appear at 2 days p.i.. According to the ambisense coding arrangement of the S RNA, the NS_S mRNA, however, can only appear after viral replication has taken place (Fig.1). A protoplast system may enable to distinguish between primary transcription and replication, and to investigate a possible involvement of virally encoded proteins in the switch from transcription to replication, in analogy to animal-infecting negative strand viruses.

Analysis of RNA from purified, enveloped virions showed the encapsidation of both v- and vc strands of the S and M RNA segment. Encapsidation of the subgenomic RNAs, however, appeared not to take place. The reason for encapsidation of both full-length strands is unknown but a similar situation has previously been shown to occur with the ambisense S RNA segment of Uukuniemi virus (Simons *et al.*, 1990). The presence of v- and vc S RNA in virus particles implies that both the N and NS_S subgenomic mRNAs may be produced through primary transcription. Along this line, involvement of NS_S in the replication of the viral genome, is plausible. A similar situation also appears with the M RNA, where vc M RNA becomes encapsidated in enveloped virions, even in relatively larger amounts than in the case of S RNA. Ethidium bromide stained RNA patterns of purified, enveloped virions and free nucleocapsids, however, do not show an excess of M RNA over S RNA indicating that encapsidation of relatively larger amounts of vc M RNA is not merely a concentration effect. In this case, the absence of vc L RNA in purified intact virions is plausible, since the L RNA always

appears in nucleocapsids and purified intact virions in similar amounts as the M RNA.

The reason for encapsidation of vc M RNA in purified enveloped virions is not clear but the encapsidation of both v and vc RNA strands of the ambisense S RNA segments of TSWV and Uukuniemi virus respectively (this Chapter and Simons *et al.*, 1990) indicate that the encapsidation of vc M RNA may be explained by an ambisense gene arrangement within the M RNA segment. The detection of a possible M-specific subgenomic mRNA supports this hypothesis.

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REFERENCES

Abraham, G. and Pattnaik, A.K. (1983). Early RNA synthesis in Bunyamwera virus-infected cells. *Journal of General Virology* **64**, 1277-1290.

Avila, A.C. De, Huguenot, C., Resende, R. de O., Kitajima, E.W., Goldbach, R.W. and Peters, D. (1990). *Journal of General Virology* **71**, 2801-2807.

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

Barrett, T., Wolstenholme, A.J. and Mahy, B.W.J. (1979). Transcription and replication of Influenza virus RNA. *Virology* **98**, 211-225.

Beaton, A.R. and Krug, R.M. (1984). Synthesis of the templates for influenza virion RNA replication *in vitro*. *Proceedings of the National Academy of Sciences, USA* **81**, 4682-4686.

Beaton, A.R. and Krug, R.M. (1986). Transcription antitermination during influenza viral template RNA synthesis requires the nucleocapsid protein and the absence of a 5' capped end. *Proceedings of the National Academy of Sciences, USA* **83**, 6282-6286.

Bellocq, C. and Kolakofsky, D. (1987). Translational requirement for La Crosse virus S-mRNA synthesis: A possible mechanism. *Journal of Virology* **61**, 3960-3967.

Bellocq, C., Raju, R., Patterson, J.L. and Kolakofsky, D. (1987). Translational requirement of La Crosse virus S-mRNA synthesis: In vitro studies. *Journal of Virology* **61**, 87-95.

Cunningham, C. and Szilagyi, J.F. (1987). Viral RNAs synthesized in cells infected with Germiston Bunyavirus. *Virology* **157**, 431-439.

De Vries, S.C., Springer, J. and Wessels, J.G.H. (1982). Diversity of abundant mRNA sequences and patterns of protein synthesis in etiolated and greened pea seedlings. *Planta* **156**, 129-135.

De Haan, P., Wagemakers, L., Goldbach, R. and Peters, D. (1989a). Tomato spotted wilt virus, a new member of the *Bunyaviridae*?. In *Genetics and pathogenicity of negative strand viruses*, 287-290. Edited by D. Kolakofsky & B.W.J. Mahy, Elsevier Science Publishers B.V., Amsterdam.

De Haan, P., Wagemakers, L., Peters, D. and Goldbach, R.W. (1989b). Molecular cloning and terminal sequence determination of the S and M RNAs of tomato spotted wilt virus. *Journal of General Virology*

70, 3469-3473.

De Haan, P., Wagemakers, L., Peters, D. and Goldbach, R.W. (1990). The S RNA of tomato spotted wilt virus has an ambisense character. *Journal of General Virology* 71, 1001-1007.

De Haan, P., Kormelink, R., Resende, R. de O., Van Poelwijk, F., Peters, D. and Goldbach, R. (1991). Tomato spotted wilt virus L RNA encodes a putative RNA polymerase. *Journal of General Virology* 72, 2207-2216.

Eshita, Y., Ericson, B., Romanowski, V. and Bishop, D.H.L. (1985). Analyses of the mRNA transcription processes of Snowshoe Hare bunyavirus S and M RNA species. *Journal of Virology* 55, 681-689.

Francki, R.I.B., 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. *Archives of Virology* suppl. 2.

Franze-Fernandez, M.-T., Zetina, C., Iapalucci, S., Lucero, M.A., Bouissou, C., Lopez, R., Rey, O., Daheli, M., Cohen, G.N. and Zakin, M.M. (1987). Molecular structure and early events in the replication of Tacaribe arenavirus S RNA. *Virus Research* 7, 309-324.

Fuller-Pace, F.V. and Southern, P.J. (1988). Temporal analyses of transcription and replication during acute infection with lymphocytic choriomeningitis virus. *Virology* 162, 260-263.

Giorgi, C., Accardi, L., Nicoletti, L., Gro, M.C., Takehara, K., Hilditch, C., Morikawa, S. and Bishop, D.H.L. (1991). Sequences and coding strategies of the S RNAs of Toscana and Rift Valley fever viruses compared to those of Punta Toro, Sicilian Sandfly fever, and Uukuniemi viruses. *Virology* 180, 738-753.

Hay, A.J., Lomniczi, B., Bellamy, A.R. and Shekel, J.J. (1977). Transcription of the Influenza virus genome. *Virology* 83, 337-355.

Ihara, T., Akashi, H. and Bishop, D.H.L. (1984). Novel coding strategy (ambisense genomic RNA) revealed by sequence analyses of Punta Toro phlebovirus S RNA. *Virology* 136, 293-306.

Ihara, T., Matsuura, Y. and Bishop, D.H.L. (1985). Analyses of the mRNA transcription processes of Punta Toro phlebovirus (*Bunyaviridae*). *Virology* 147, 317-325.

Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982). *Molecular Cloning: A Laboratory Manual.* New York: Cold Spring Harbor Laboratory.

Marriott, A.C., Ward, V.K. and Nuttall, P.A. (1989). The S RNA segment of Sandfly fever Sicilian virus: Evidence for an ambisense genome. *Virology* 169, 341-345.

Melton, D.A., Krieg, P.A., Rebagliati, M.R., Maniatis, T., Zinn, K. and Green, M.R. (1984). Efficient *in vitro* synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucleic Acids Research* 12, 7035-7055.

Mohamed, N.A., Randles, J.W. and Francki, R.I.B. (1973). Protein composition of tomato spotted wilt virus. *Virology* 56, 12-21.

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

Patterson, J.L. and Kolakofsky, D. (1984). Characterization of La Crosse virus small-genome transcripts. *Journal of Virology* 49, 680-685.

Pattnaik, A.K. and Abraham, G. (1983). Identification of four complementary RNA species in Akabane virus-infected cells. *Journal of Virology* 47, 452-462.

Raju, R. and Kolakofsky, D. (1987). Translational requirement of La Crosse virus S-mRNA synthesis: In vivo studies. *Journal of Virology* 61, 96-103.

Resende, R. de O., De Haan, P., Avila, A.C. de, 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. *Journal of General Virology* 72, 2375-2383.

Shapiro, G.I., Gurney JR., T and Krug, R.M. (1987). Influenza virus gene expression: Control mechanisms at early and late times of infection and nuclear-cytoplasmic transport of virus-specific RNAs. *Journal of Virology* 61, 764-773.

Simons, J.F., Hellman, U. and Pettersson, R.F. (1990). Uukuniemi virus S RNA segment: Ambisense coding strategy, packaging of complementary strands into virions, and homology to members of the genus *Phlebovirus*. *Journal of Virology* 64, 247-255.

Smith, G.L. and Hay, A.J. (1982). Replication of the Influenza virus genome. *Virology* **118**, 96-108.

Tas, P.W.L., Boerjan, M.L. and Peters, D. (1977). The structural proteins of tomato spotted wilt virus. *Journal of General Virology* **36**, 267-279.

Van Den Hurk, J., Tas, P.W.L. and Peters, D. (1977). The ribonucleic acid of tomato spotted wilt virus. *Journal of General Virology* **36**, 81-91.

Chapter 3

Non-viral heterogeneous sequences at the 5' ends of tomato spotted wilt virus mRNAs[2]

SUMMARY

Subgenomic messenger RNAs transcribed from the tomato spotted wilt virus (TSWV) S RNA segment were partially purified from total RNA extracts of TSWV-infected *Nicotiana rustica* and analysed by primer extension analysis. The data obtained show the presence of non-viral sequences, 12-20 nucleotides in length, at the 5' ends of the N and NS_S mRNAs, indicating a cap-snatching mechanism for the initiation of transcription. This is the first report of a plant virus using such a mechanism for transcription of the viral genome.

[2]This chapter has been published in a slightly modified version as : Richard Kormelink, Frank van Poelwijk, Dick Peters and Rob Goldbach (1992). Non-viral heterogeneous sequences at the 5' ends of tomato spotted wilt virus mRNAs. *Journal of General Virology* 73, 2125-2128.

INTRODUCTION

Recent molecular studies on the genome of tomato spotted wilt virus (TSWV) have revealed that this virus represents a member of the *Bunyaviridae*, being unique in its property to infect plants. Mainly based on its distinct host range TSWV has been classified into the newly created genus *Tospovirus*, within this large family of arthropod-borne viruses (Francki *et al.*, 1991). For both the S RNA and L RNA genomic segments, complete nucleotide sequences have become available. The L RNA segment (8897 nucleotides long) is completely of negative polarity, encoding the putative (331.5 kDa) viral transcriptase (De Haan *et al.*, 1991). The S RNA segment (2916 nucleotides long), like that of phleboviruses (Ihara *et al.*, 1984; Marriott *et al.*, 1989; Simons *et al.*, 1990; Giorgi *et al.*, 1991), is ambisense and encodes the N protein of 28.8 kDa and a nonstructural protein (NS_S) of 52.4 kDa (De Haan *et al.*, 1990).

Expression of the L RNA has been demonstrated to occur via the synthesis of a genome-sized mRNA (Chapter 2). The two open reading frames in the ambisense S RNA are expressed from two subgenomic mRNAs that are transcribed from opposite strands and terminate at the central, intercistronic region, most probably in a long A-U rich hairpin (De Haan *et al.*, 1990; Chapter 2).

For several animal-infecting members of the *Bunyaviridae* the process of initiation- and termination of transcription has been further studied by characterizing the 5' and 3' ends of viral messenger RNAs. These studies have demonstrated the presence of short heterogeneous nonviral sequences at the 5' ends of the mRNAs, indicating that the viral transcriptase utilizes RNA primers to initiate transcription (Bishop *et al.*, 1983; Patterson and Kolakofsky, 1984; Eshita *et al.*, 1985; Ihara *et al.*, 1985; Collett, 1986; Gerbaud *et al.*, 1987; Bouloy *et al.*, 1990; Simons and Pettersson, 1991; Gro *et al.*, 1992; Jin and Elliott, 1993a and b). These primers are generated from capped host messenger RNA species by a process referred to as "cap-snatching", i.e. the 5' terminal sequence of a cellular messenger RNA is cleaved off by an endonuclease and subsequently used to initiate transcription on the viral genome (Braam *et al.*, 1983; Ulmanen *et al.*, 1981a). Less is

known about the termination of transcription, but some typical structural features, i.e. palindromic sequences or hairpin structures, have been found close to the sites where termination occurs (Bouloy *et al.*, 1990; Simons and Pettersson, 1991). How these features are involved in transcription termination has remained unknown sofar.

To determine whether TSWV, differing from all other bunyaviruses in being completely adapted to multiplication in plant cells, also utilizes cap-snatching to initiate transcription, the 5' ends of the N and NS_s subgenomic mRNAs were analysed. Primer extension analysis revealed the presence of a heterogeneous sequence at the 5' end of both mRNAs ranging in size from 12 to 20 nucleotides.

MATERIALS AND METHODS

Plants, virus and cDNA clones

The Brazilian isolate BR-01 (CNPH1) of TSWV was maintained in *Nicotiana rustica* 'America' by mechanical inoculation. Complementary DNA clones representing the different RNAs of TSWV BR-01 have been described previously (De Haan *et al.*, 1989b, 1990, 1991).

Total RNA extraction

Young seedlings of *N. rustica* 'America' (three leaves stage) were mechanically inoculated with extracts of TSWV BR-01-infected leaves. After inoculation, systemically infected leaf samples were taken at 8 days post inoculation (p.i.). Total RNA was extracted from infected *N. rustica* according to De Vries *et al.* (1982).

Sucrose gradient centrifugation

Total RNA extracted from TSWV-infected *N. rustica* was resolved by centrifugation through 15-22.5% sucrose gradients in 50 mM Tris pH8.0, 1 mM EDTA and 0.5% SDS. Prior to loading, the RNA was denatured with methyl mercuric hydroxide at a final concentration of 25 mM. Centrifugation was for 17 hr at 24,000 rpm at 20°C in a SW41

rotor. RNA was recovered from individual fractions of the gradient and subsequently resolved on 1% agarose gels (Bailey and Davidson, 1976). The RNA was blotted onto Genescreen (New England Nuclear, NEN), and hybridised to ^{32}P-labelled strand specific riboprobes corresponding to the 3' or 5' terminal region of the S RNA (Chapter 2). Relevant fractions were pooled and the RNA was ethanol precipitated.

CsCl preparation of RNA

RNA samples from sucrose gradients were further prepared by centrifugation through a CsCl cushion according to Davis *et al.* (1986). To this end, RNA samples were resuspended in 2 ml GIT buffer (4M guanidine isothiocyanate (GIT); 25 mM NaAc, pH6). After the addition of 1.67 µl 14.4 M β-mercaptoethanol, the RNA samples were loaded on top of a 1.7 ml CsCl foot (5.7 M CsCl; 25 mM NaAc, pH6) and centrifuged for 21 hr at 35,000 rpm at 20°C in a SW55 rotor. The RNA pellet obtained, was resuspended in 200 µl 0.3 M NaAc, pH6, transferred to a microfuge tube and the SW55 tube rinsed with an additional 100 µl 0.3 M NaAc pH6. The RNA was precipitated by the addition of 750 µl ethanol and incubated for 1-2 hr at -70°C. The RNA pellet was washed with 80% ethanol, dried and resuspended in H_2O.

Primer extension analysis

The 5' ends of the N and NS_S mRNAs were analysed using two oligonucleotides representing the viral sense cDNA hybridising with nucleotides 2834 to 2852 (SV) and the viral complementary sense cDNA hybridising with nucleotides 32 to 51 (SIV) of the S RNA, respectively (Fig.2). The oligonucleotides were labeled at their 5' end using [gamma-^{32}P]ATP and T4 polynucleotide kinase and subsequently purified from an 8% sequencing gel. The primers were mixed with the RNA in 10 µl annealing buffer (250 mM KCl; 10 mM Tris-HCl pH8.3), heated at 90°C for 2 min and subsequently incubated at 37°C for 5 min. Ten µl reverse transcriptase mix (100 mM Tris-HCl pH8.3; 10 mM MgCl2; 10 mM DTT; 50 U MuMLV reverse transcriptase; 20 U RNasin; 1 mM dNTP) was added and the reaction incubated at 37°C for 30 min to synthesize run-off copies of the upstream sequences in the S-specific RNAs.

RESULTS

Enrichment for subgenomic TSWV S RNA-specific mRNAs

Recent studies on viral RNA synthesis in TSWV-infected *Nicotiana rustica* revealed the presence of low amounts of the S-specific mRNAs and viral complementary (vc) S RNA strands, relative to full-length viral (v) S RNA (Chapter 2). To allow unequivocal analysis of the 5' ends of the N and NS_S mRNAs, and of the 5' end of the S vcRNA, it was necessary to purify these RNA species to a certain extent. To this end, total RNA from TSWV-infected *N. rustica* was extracted 8 days post inoculation (p.i.) according to de Vries *et al.* (1982), and resolved on 15-22.5% sucrose gradients (Ulmanen *et al.*, 1981b; Bishop *et al.*, 1983). The fractions collected were analysed for their absorbance at 254 nm (Fig.1a), and for their RNA content. For the latter, RNA samples were resolved on a 1% agarose gel (Bailey and Davidson, 1976), transferred to Genescreen membrane and hybridised to strand-specific probes corresponding to the N (Fig.1a) and NS_S (data not shown) coding regions in S RNA. The relevant fractions, enriched for the N and NS_S mRNA (fractions 3 and 4), and enriched for the S vcRNA strands (fractions 7 and 8), were pooled and the RNA precipitated after the addition of 0.1 vol. 3 M NaAc and 2.5 vol. ethanol.

CsCl preparation of enriched RNA fractions

The RNA fractions enriched for N mRNAs, NS_S mRNAs and S vcRNAs respectively, were resuspended in GIT buffer and subsequently pelleted through a 5.7 M CsCl cushion as described in Materials and Methods. The RNA pellets obtained were analysed on Northern blots for the contents of full-length S vcRNA, and the subgenomic mRNAs (Fig.1b). It is clear from the results that a significant enrichment has been obtained for both S RNA-specific subgenomic mRNAs (Fig.1b; Chapter 2, Fig.3).

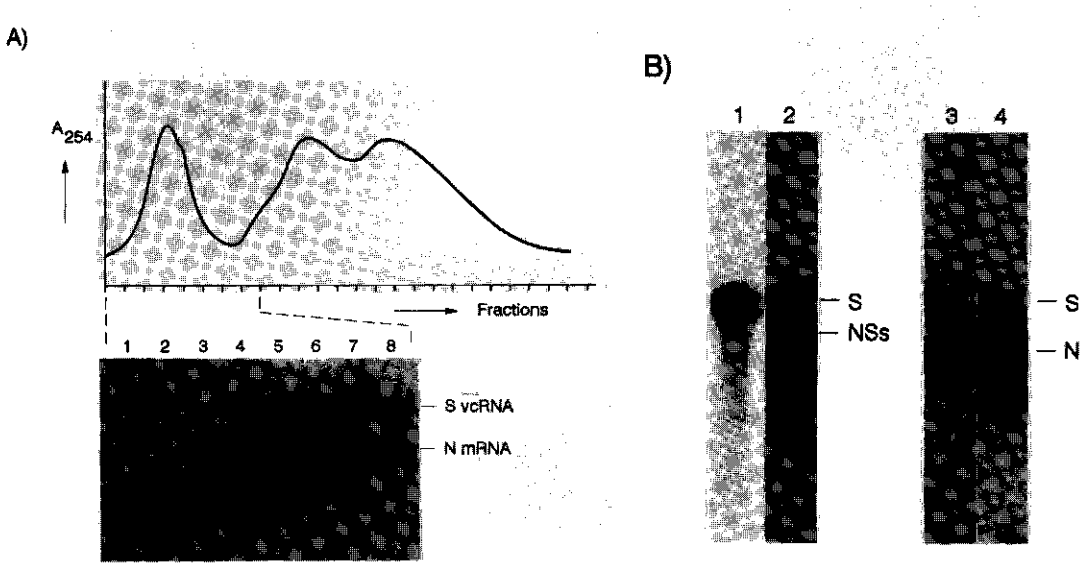
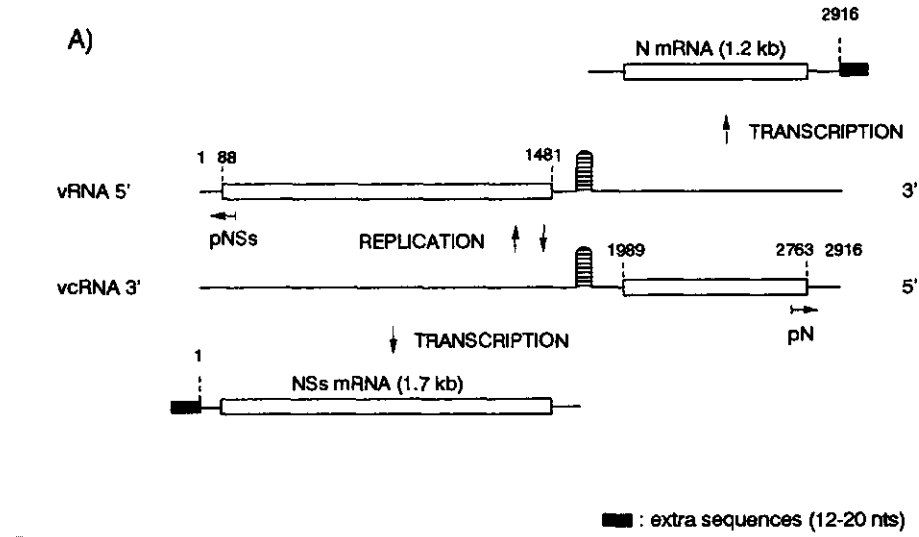


Figure 1: Sedimentation analysis and separation of RNA species from TSWV-infected *N. rustica* plants, 8 days post-inoculation. Five hundred μg of total RNA from infected tissues was layered on a 15-22.5% sucrose gradient. A) Absorbance profile of RNA fractions collected from the gradient. Sedimentation was from left to the right. Two μg RNA from each fraction was resolved on a 1% agarose gel, transferred to Genescreen membrane and hybridised to riboprobe S2-v, specific for the N gene. B) RNA pellets obtained after centrifugation through a CsCl cushion (as described in the text), enriched for the NS_S mRNA (lane 2), for N mRNA (lane 4), and for the S vcRNA (lane 3), analysed on a Northern blot using riboprobes S1-vc (lanes 1 and 2) and S2-v (lanes 3 and 4). Lane 1 contains purified nucleocapsid RNA which was used for primer extension analysis of the 5' end of the S vRNA strands. The riboprobes were prepared as described in Chapter 2.

Primer extension analyses on the N and NS_S mRNAs

Ten μg of the selected RNA samples, and two μg of purified nucleocapsid RNA were used for primer extension experiments. For this purpose, two oligonucleotides, one complementary to the vc sense RNA at positions 2834 to 2852 (primer pN) and one complementary to the v sense RNA at positions 32 to 51 (primer pNS_S) of the S RNA (Fig.2a), were used. From the sequence data of the S RNA segment (De Haan *et al.*, 1990) it was predicted that primer pNS_S should be extended for 31 nucleotides (nt) on the v sense S RNA template, resulting in a run-off product with the size of 51 nt. Primer extension synthesis on nucleocapsid RNA using pNS_S, and subsequent analysis of the



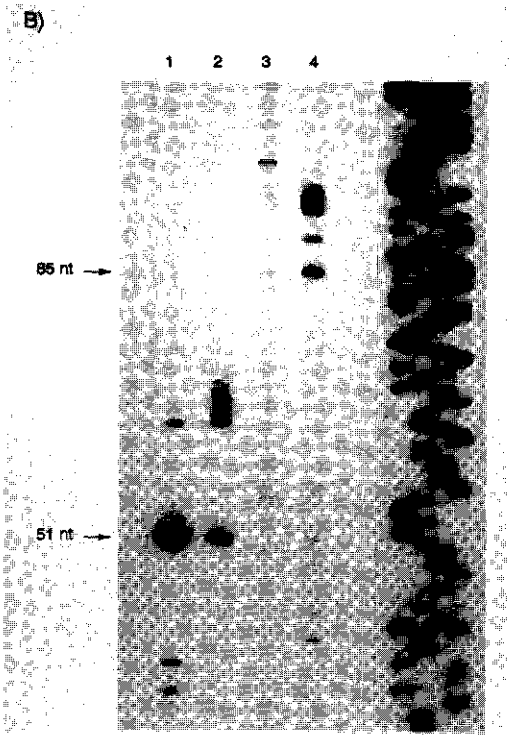


Figure 2: (A) Genetic organisation and expression of the TSWV S RNA segment. Nucleotides are numbered from the 5' end of the v strand, and open reading frames presented as open bars. The locations of the primers (pNS_S and pN) used for primer extension analysis, are indicated. (B) Primer extension analyses on unfractionated nucleocapsid RNA (lane 1), NS_S mRNA-enriched RNA fraction (lane 2), S vcRNA-enriched RNA fraction (lane 3), and the N mRNA-enriched RNA fraction (lane 4) using primers pNS_S or pN as described in the text. Reaction products were analysed on an 8% sequencing gel. A sequence ladder is included, as a standard to determine sizes of the various primer extension products.

run-off products on an 8% sequencing gel, indeed showed the presence of the expected band (Fig.2b, lane 1). Similarly, when primer pN was extended on vc sense S RNA, a run-off product with an expected size of 85 nt was found (Fig.2b, lane 3). Analyses of NS_S- and N mRNA enriched fractions revealed an additional ladder of products 12-20 nt larger in size than the 51 and 85 nt products, respectively (Fig.2b lanes 2 and 4). This indicates the presence of extra sequences, heterogeneous in length, at the 5' ends of the TSWV-specific mRNAs. As these additional terminal sequences do not appear at the 5' end of the nucleocapsid S RNA, except for a distinct extension product of 63 nt, it is unlikely that the extra sequences in both the N and NS_S mRNA are templated by the viral RNA. They probably originate from host messenger RNAs, and are utilized by the viral RNA transcription machinery to initiate transcription. An additional single extension product of 63 nt was found in minor amounts when pNS_S was extended on nucleocapsid RNA (Fig.2b, lane 1). The origin of this band has not been further investigated, but could be caused by spurious amounts of viral mRNA co-purified (or even co-encapsidated) with the genomic RNA.

DISCUSSION

The presence of non-viral sequences in mRNAs has previously been found for other members of the *Bunyaviridae* (Bishop *et al.*, 1983; Patterson and Kolakofsky, 1984a; Eshita *et al.*, 1985; Ihara *et al.*, 1985; Collett, 1986; Gerbaud *et al.*, 1987; Bouloy *et al.*, 1990; Simons and Pettersson, 1991; Gro *et al.*, 1992; Jin and Elliott, 1993a and b), and also for members of the *Orthomyxoviridae* (Caton and Robertson, 1980; Dhar *et al.*, 1980) and *Arenaviridae* (Garcin and Kolakofsky, 1990; Raju *et al.*, 1990) (Table 1). In most cases, the added sequences ranged in size between 15 and 18 nt in length, in the case of the arenavirus Tacaribe however, only 1 to 4 extra nucleotides have been reported (Garcin and Kolakofsky, 1990; Raju *et al.*, 1990). Here it is demonstrated that TSWV, a bunyavirus with a very distinct host range, also uses cap-snatching to initiate

Table 1:Presence of cellular leader sequences in mRNAs of multipartite negative strand RNA viruses.

Family	Genus	Species	mRNA	Non-viral leader sequence	3'-terminal base of leader
Bunyaviridae	Tospo	TSWV	N, NSs	12 - 20 nts	
	Phlebo	Rift Valley fever	G	12 - 14	
		Punta Toro	N, NSs	11 - 18	
		Uukuniemi	N, NSs	7 - 25	no consensus
		Toscana	N, NSs	9 - 15	
	Bunya	Bunyamwera	N	12 - 17	
		Germiston	N, G	12 - 18	U, C
		Snowshoe hare	N, G, NSs	12 - 17	A
		La Crosse	N, NSs	10 - 14	
	Nairo	Dugbe	N	5 - 16	
Arenaviridae		Tacaribe	N	1 - 4	
Orthomyxoviridae	Orthomyxo	Influenza A	M (RNA7) NS (RNA8) H (RNA4)	10 - 18	A, G

transcription. This indicates that all members of the *Bunyaviridae*, irrespective whether they infect animal or plant cells, use the same mechanism to initiate transcription of their genome. The process of cap-snatching, in particular the involvement of a host- or virally encoded endonuclease activity, needs to be further investigated. For Influenza virus (Plotch *et al.*, 1981), La Crosse virus (Patterson *et al.*, 1984b), Germiston virus (Vialat and Bouloy, 1992), and Bunyamwera virus (Jin and Elliott, 1993a) the responsible endonuclease activity has already been demonstrated to be virus encoded. During influenza virus replication three different viral proteins are involved in genome transcription and replication, i.e. proteins PA, PB1, and PB2, of which PB1 represents the core RNA polymerase and PB2 probably the endonuclease activity required for cap-snatching (Plotch *et al.*, 1981; Ulmanen *et al.*, 1981a). In view of the size of the L protein of TSWV (331.5 kDa), which exceeds the sum of the sizes of the 3 replication proteins of influenza, it is tempting to assume that this L-RNA-encoded product encompasses, in addition to a core polymerase domain (De Haan *et al.*, 1991), an endonuclease domain charged with the cap-snatching.

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REFERENCES

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

Bishop, D.H.L., Gay, M.E. and Matsuoko, Y. (1983). Nonviral heterogeneous sequences are present at the 5' ends of one species of snowshoe hare bunyavirus S complementary RNA. *Nucleic Acids Research* **11**, 6409-6418.

Bouloy, M., Pardigon, N., Vialat, P., Gerbaud, S. and Girard, M. (1990). Characterization of the 5' and 3' ends of viral messenger RNAs isolated from BHK21 cells infected with Germiston virus (*Bunyavirus*). *Virology* **175**, 50-58.

Braam, J., Ulmanen, I. and Krug, R.M. (1983). Molecular model of a eucaryotic transcription complex:

Functions and movements of Influenza P proteins during capped RNA-primed transcription. *Cell* **34**, 609-618.

Caton, A.J. and Robertson, J.S. (1980). Structure of the host-derived sequences present at the 5' ends of influenza virus mRNA. *Nucleic Acids Research* **8**, 2591-2603.

Collett, M.S. (1986). Messenger RNA of the M segment RNA of Rift Valley Fever virus. *Virology* **151**, 151-156.

Davis, L.G., Dibner, M.D. and Battey, J.F. (1986). *Basic methods in molecular biology.* Amsterdam: Elsevier Science Publishers.

Dhar, R., Chanock, R.M. and Lai, C.-J. (1980). Nonviral oligonucleotides at the 5' terminus of cytoplasmic influenza viral mRNA deduced from cloned complete genomic sequences. *Cell* **21**, 495-500.

De Haan, P., Wagemakers, L., Peters, D. and Goldbach, R.W. (1990). The S RNA of tomato spotted wilt virus has an ambisense character. *Journal of General Virology* **71**, 1001-1007.

De Haan, P., Kormelink, R., Resende, R. de O., Van Poelwijk, F., Peters, D. and Goldbach, R. (1991). Tomato spotted wilt virus L RNA encodes a putative RNA polymerase. *Journal of General Virology* **72**, 2207-2216.

De Vries, S.C., Springer, J. and Wessels, J.G.H. (1982). Diversity of abundant mRNA sequences and patterns of protein synthesis in etiolated and greened pea seedlings. *Planta* **156**, 129-135.

Eshita, Y., Ericson, B., Romanowski, V. and Bishop, D.H.L. (1985). Analyses of the mRNA transcription processes of Snowshoe Hare bunyavirus S and M RNA species. *Journal of Virology* **55**, 681-689.

Francki, R.I.B., 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. *Archives of Virology* suppl. **2**.

Garcin, D. and Kolakofsky, D. (1990). A novel mechanism for the initiation of Tacaribe Arenavirus genome replication. *Journal of Virology* **64**, 6196-6203.

Gerbaud, S., Pardigon, N., Vialat, P. and Bouloy, M. (1987). The S segment of the Germiston bunyavirus genome: Coding strategy and transcription. In *The biology of negative strand viruses*, pp.191-198. Edited by B. Mahy & D. Kolakofsky. Elsevier, Amsterdam.

Giorgi, C., Accardi, L., Nicoletti, L., Gro, M.C., Takehara, K., Hilditch, C., Morikawa, S., and Bishop, D.H.L. (1991). Sequences and coding strategies of the S RNAs of Toscana and Rift Valley fever viruses compared to those of Punta Toro, Sicilian sandfly fever, and Uukuniemi viruses. *Virology* **180**, 738-753.

Gro, M.C., Di Bonito, P., Accardi, L. and Giorgi, C. (1992). Analysis of 3' and 5' ends of N and NS_S messenger RNAs of Toscana phlebovirus. *Virology* **191**, 435-438.

Ihara, T., Akashi, H., and Bishop, D.H.L. (1984). Novel coding strategy (ambisense genomic RNA) revealed by sequence analyses of Punta Toro phlebovirus S RNA. *Virology* **136**, 293-306.

Ihara, T., Matsuura, Y. and Bishop, D.H.L. (1985). Analyses of the mRNA transcription processes of Punta Toro phlebovirus (*Bunyaviridae*). *Virology* **147**, 317-325.

Jin, H. and Elliott, R.M. (1993a). Characterization of Bunyamwera virus S RNA that is transcribed and replicated by the L protein expressed from recombinant vaccinia virus. *Journal of Virology* **67**, 1396-1404.

Jin, H. and Elliott, R.M. (1993b). Non-viral sequences at the 5' ends of Dugbe nairovirus S mRNAs. *Journal of General Virology* **74**, 2293-2297.

Marriott, A.C., Ward, V.K. and Nuttall, P.A. (1989). The S RNA segment of sandfly fever Sicilian virus: Evidence for an ambisense genome. *Virology* **169**, 341-345.

Patterson, J.L. and Kolakofsky, D. (1984a). Characterization of La Crosse virus small-genome transcripts. *Journal of Virology* **49**, 680-685.

Patterson, J.L., Holloway, B. and Kolakofsky, D. (1984b). La Crosse virions contain a primer-stimulated RNA polymerase and a methylated cap-dependent endonuclease. *Journal of Virology* **52**, 215-222.

Plotch, S.J., Bouloy, M., Ulmanen, I. and Krug, R.M. (1981). A unique cap(m^7GpppXm)-dependent Influenza virion endonuclease cleaves capped RNAs to generate the primers that initiate viral RNA transcription. *Cell* **23**, 847-858.

Raju, R., Raju, L., Hacker, D., Garcin, D., Compans, R. and Kolakofsky, D. (1990). Nontemplated bases at the 5' ends of Tacaribe virus mRNAs. *Virology* **174**, 53-59.

Simons, J.F., Hellman, U., and Pettersson, R.F. (1990). Uukuniemi virus S RNA segment: Ambisense coding strategy, packaging of complementary strands into virions, and homology to members of the genus *Phlebovirus*. *Journal of Virology* **64**, 247-255.

Simons, J.F. and Pettersson, R.F. (1991). Host-derived ends and overlapping complementary 3' ends of the two mRNAs transcribed from the ambisense S segment of Uukuniemi virus. *Journal of Virology* **65**, 4741-4748.

Ulmanen, I., Broni, B.A. and Krug, R.M. (1981a). Role of two of the influenza virus core P proteins in recognizing cap 1 structures (m^7GpppNm) on RNAs and in initiating viral RNA transcription. *Proceedings of the National Academy of Sciences, USA* **78**, 7355-7359.

Ulmanen, I., Seppala, P. and Pettersson, R.F. (1981b). In vitro translation of Uukuniemi virus-specific RNAs: Identification of a nonstructural protein and a precursor to the membrane glycoproteins. *Journal of Virology* **37**, 72-79.

Vialat, P. and Bouloy, M. (1992). Germiston virus transcriptase requires active 40S ribosomal subunits and utilizes capped cellular RNAs. *Journal of Virology* **66**, 685-693.

Chapter 4

The nucleotide sequence of the M RNA segment of tomato spotted wilt virus, a bunyavirus with two ambisense RNA segments[3]

SUMMARY

The complete sequence of the tomato spotted wilt virus (TSWV) M RNA segment has been determined. The RNA is 4821 nucleotides long and has an ambisense coding strategy, similar to the S RNA segment. The M RNA segment contains two open reading frames (ORFs), one in viral sense which encodes a protein with a predicted size of 33.6 kDa, and one in viral complementary sense which encodes the precursor to the G1 and G2 glycoproteins, with a predicted size of 127.4 kDa. Both ORFs are expressed via the synthesis of subgenomic messenger RNAs that possibly terminate at a stable hairpin structure, located at the intergenic region. The precursor for the glycoproteins contains a sequence motif (RGD) which is characteristic for cellular attachment domains. Significant sequence homology was found between the G1 glycoprotein of members of the genus *Bunyavirus* and a corresponding region in the glycoprotein precursor of TSWV, indicating a close evolutionary relationship between these viruses. With the elucidation of the M RNA sequence, the complete nucleotide sequence of TSWV has been determined. The sequence data obtained show that TSWV represents the first member of the *Bunyaviridae* that contains two ambisense RNA segments.

[3]This chapter has been published in a slightly modified version as: Richard Kormelink, Peter de Haan, Cor Meurs, Dick Peters and Rob Goldbach (1992). The nucleotide sequence of the M RNA segment of tomato spotted wilt virus, a bunyavirus with two ambisense RNA segments. *Journal of General Virology* 73, 2795-2804.

INTRODUCTION

Tomato spotted wilt virus (TSWV) is an enveloped plant virus that causes great yield losses in many economically important crops in (sub)tropical and temperate regions. More than 400 species in 50 plant families, both mono- and dicotyledons, are susceptible to TSWV (Cho *et al.*, 1987; Peters, *et al.*, 1991). The virus is exclusively transmitted by thrips in a persistent manner (Sakimura, 1962; Paliwal, 1974). Based on morphological and molecular data, TSWV has recently been classified into a newly created genus *Tospovirus*, within the large family of arthropod-borne *Bunyaviridae* (Francki *et al.*, 1991; Elliott, 1990).

Typical for members of the *Bunyaviridae*, TSWV consists of a spherical membrane-bound particle, 80-110 nm in diameter, covered with surface projections that consist of two glycoproteins denoted G1 (78 kDa) and G2 (58 kDa; Mohamed *et al.*, 1973; Tas *et al.*, 1977). The virus contains three single-stranded RNA segments called S RNA (2.9 kb), M RNA (5.0 kb), and L RNA (8.9 kb). Each RNA segment is associated with nucleoproteins (N) to form pseudo-circular nucleocapsid structures (Van den Hurk *et al.*, 1977; Mohamed, 1981; De Haan *et al.*, 1989).

Recently, the nucleotide sequences of the S and L RNA segments have been determined (De Haan *et al.*, 1990, 1991). The L RNA (8897 nucleotides long) is completely of negative polarity, encoding a single large protein of 331.5 kDa which represents the putative viral transcriptase. Expression of this protein has been demonstrated to occur via the synthesis of a full-length mRNA (Chapter 2). The S RNA (2916 nucleotides long) has an ambisense gene arrangement and encodes the N protein of 29 kDa in viral complementary sense and a nonstructural protein (NS_S) of 52.4 kDa in viral sense. Both proteins are expressed by subgenomic mRNAs, transcribed from complementary strands via a process of cap-snatching (Chapter 3). The mRNA molecules terminate at the central, intercistronic region, most probably in a long A-U rich hairpin (De Haan *et al.*, 1990; Chapter 2).

Here we report the complete nucleotide sequence of the genomic M RNA segment

of TSWV. It is shown that this RNA segment, like the S RNA segment, has an ambisense gene arrangement, encoding a putative nonstructural protein in viral sense and a precursor to G1 and G2 in viral complementary sense. With the sequence of the M RNA segment the complete nucleotide sequence of the TSWV genome has become available, allowing precise comparison with the animal-infecting members of the *Bunyaviridae*.

METHODS

Virus and plants

The Brazilian isolate BR-01 (CNPH1) of TSWV was maintained in *Nicotiana rustica* 'America' by thrips transmission and mechanical inoculation. Virus was purified according to Tas *et al.* (1977). Viral nucleocapsids were isolated from infected leaf tissue as described by De Avila *et al.* (1990). RNA from virus particles or nucleocapsids was isolated by treatment with 1% SDS followed by phenol extraction, and ethanol precipitation.

Molecular cloning and sequence determination

First-strand cDNA to the M RNA segment of TSWV was synthesized using a specific oligonucleotide, complementary to the 3' end of the M RNA (M1; de Haan *et al.*, 1989). Second-strand synthesis was performed according to Gubler and Hoffman (1983). Double-stranded (ds) cDNA was made blunt-ended using T4 DNA polymerase and subsequently cloned in pUC19 after addition of *Eco*RI adaptors (Amersham). To verify the sequences at the 5' and 3' end of the M RNA, an additional cloning experiment was done. To obtain cDNA clones containing the 3' end of the M RNA, 5 μg of genomic RNA was polyadenylated at the 3' end, using poly-A-polymerase (Bethesda Research Laboratories) according to Devos *et al.* (1976). First-strand cDNA synthesis was primed with oligo(dT) and subsequently used in a polymerase chain reaction to amplify the 3' end sequences of the M RNA. For this purpose, oligo$(dT)_{16\text{-}18}$ and J16, identical to

nucleotides 4548-4562 of the vRNA strand, were used. For cloning the 5' terminal sequence, an oligonucleotide, complementary to the M RNA sequence at nucleotide position 584-600 from the 5' end (Z10; dCCCTTCAGGATGACTTG), was used to prime first-strand cDNA, followed by second strand synthesis (Gubler and Hoffman, 1983). DNA sequencing was performed by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977) on alkaline denatured dsDNA templates (Zhang *et al.*, 1988). Nucleotide and amino acid sequences were compiled and analysed using programs developed by the University of Wisconsin Genetics Computer Group (Devereaux *et al.*, 1984).

RNA extraction and Northern blot analysis

Total cellular RNA was extracted from TSWV-infected *N. rustica* according to de Vries *et al.* (1982). Total RNA samples of 7 μg were resolved by electrophoresis through 1% agarose gels after treatment with methylmercuric hydroxide (Bailey and Davidson, 1976). The RNA was blotted onto GeneScreen (New England Nuclear) and hybridised to ^{32}P-labelled riboprobes of TSWV M-specific sequences corresponding to the small ORF (probe M-2; transcribed from a 600 bp *Eco*RI/*Hind*III cDNA fragment from clone M16) or the large ORF (probe M-3; transcribed from a 800 bp *Eco*RI/*Hind*III cDNA fragment from clone M43) as described in Chapter 2.

RESULTS

Cloning and sequencing of the TSWV M RNA

The nucleotide sequence of 30 nucleotides at the 3' end of the M RNA was determined after end-labelling of the RNA, followed by partial degradation with base-specific ribonucleases (de Haan *et al.*, 1989). From the deduced sequence, a synthetic oligonucleotide (M1; dAGAGCAATCAGTGCAAA) complementary to the 20 3' terminal nucleotides was synthesized and used to prime first-strand synthesis, followed by second-strand synthesis and cloning in pUC19. A set of overlapping cDNA clones to

M RNA was obtained after repeated screening of the cDNA library, which yielded a restriction map covering approximately 4800 nucleotides (Fig.1). The nucleotide sequence of the TSWV M RNA segment was determined from cDNA clones M201, M10, M11, M43, M12, M13, M14, M16, M17, M18, M24, M28, M31 (Fig.1).

The 3' terminal sequence of the M RNA was verified in an independent experiment using RNA from a separate virus purification. Genomic RNA was polyadenylated at the 3' end and first-strand cDNA was synthesized by priming with oligo(dT). Single-stranded cDNA containing the 3' terminal sequence of the M RNA was amplified by PCR using oligo(dT) and oligonucleotide J16 (dGTTGAATCGATGCAG) as primers (Fig.1). The DNA was subsequently cloned in pUC19 and clones were selected using a 250 bp *Kpn*I restriction fragment of clone M11 as probe in a hybridisation experiment. The conserved 5'-terminal sequence of the M RNA was present in clones M24, M28, and M31 as they contained the sequence 5' AGAGCAATCAGTGCA.., which is complementary to the sequence at the 3' end of the TSWV M RNA.

The 5'-terminal sequence was also verified in an independent cDNA synthesis experiment. For this purpose primer Z10 (Fig.1) was used to prime first-strand cDNA synthesis, followed by second-strand synthesis according to Gubler and Hoffman (1983).

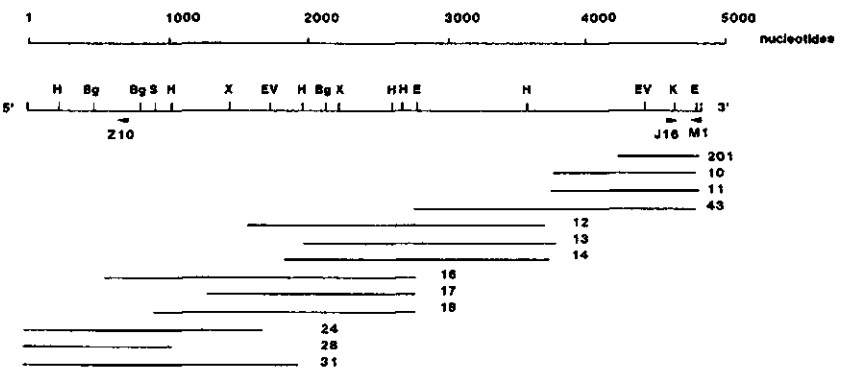


Figure 1: Cloning strategy for the TSWV M RNA segment. M1, J16, and Z10 represent the synthetic oligonucleotides used for cDNA synthesis. The numbers correspond to the cDNA clones used. Restriction enzymes are abbreviated as follows: Bg, *Bgl*I; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; S, *Sst*I; X, *Xba*I.

The cDNA was made blunt-end with T4 DNA polymerase and subsequently cloned into the *Sma*I site of pUC19. A 400 bp *Eco*RI/*Bgl*II restriction fragment from clone M28 was used as a probe to select positive clones in a colony hybridisation experiment. Their nucleotide sequences were identical to that of clone M24, M28 and M31, except for the last 16 5' terminal nucleotides, which were only present in M24, M28 and M31.

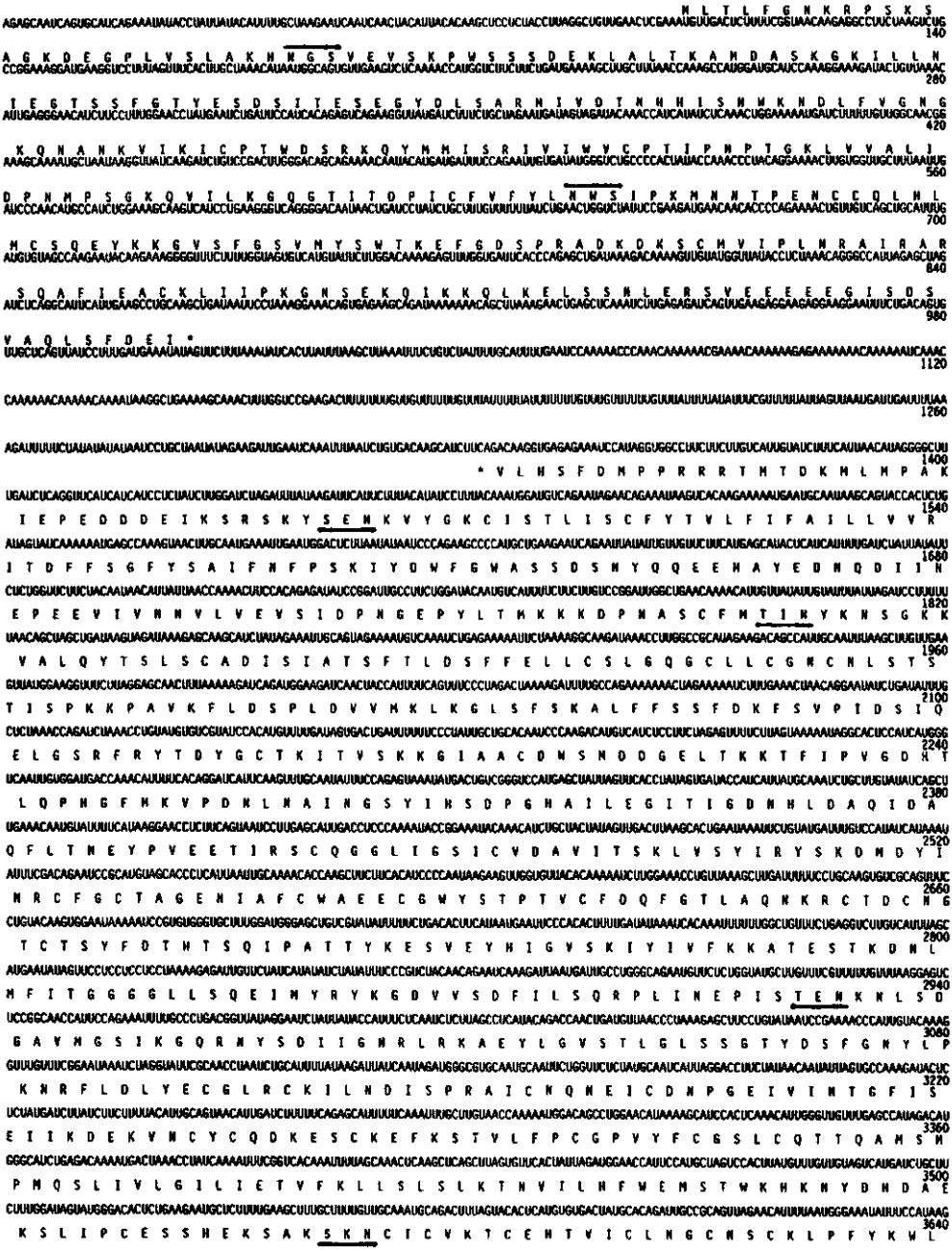
Characteristics of the TSWV M RNA sequence

The complete nucleotide sequence of the TSWV M RNA (Fig. 2) is 4821 nucleotides long, and has a base composition of 31.8% A, 32.5% U, 18.0% C, and 17.6% G. The termini of the M RNA show a complementarity between the 5' and 3' ends for about 64 nucleotides, and can be folded into a stable 'panhandle' structure with a free energy of G = -187.3 kj/mol (Fig. 3a). The length of the panhandle is similar as found for those of the L and S RNA segments, which also involve basepairing of approximately 65-70 nt (De Haan *et al.*, 1990, 1991).

An internal inverted sequence of A-rich stretches followed by U-rich stretches is located between positions 1075 and 1245 (numbered from the 5' end of the viral RNA strand), and can be folded into a hairpin structure with a G = -151.2 kj/mol. The stem of the hairpin structure is slightly shorter (85 nt in length) than that found in the TSWV S RNA (126 nt). A sequence located at the top of the hairpin (CAAACUUUGG; Fig.3b) is conserved among the top of the hairpins in the S RNA segments of TSWV and a second tospovirus, *Impatiens* necrotic spot virus (INSV) (CAAUUUGG) (Maiss *et al.*, 1991; De Haan *et al.*, 1992). This nucleotide sequence motif may therefore have a possible role as a signal for transcription termination of the ORFs within the ambisense RNA segments of tospoviruses.

Predicted gene products of TSWV M RNA

Analysis of the six different reading frames of the viral and viral complementary RNA revealed the presence of two open reading frames (ORFs) located in opposite strands (Fig.4). One smaller ORF is located in the viral strand, starting with an AUG codon at position 101, and terminating with a UAG stop codon at position 1007. This ORF



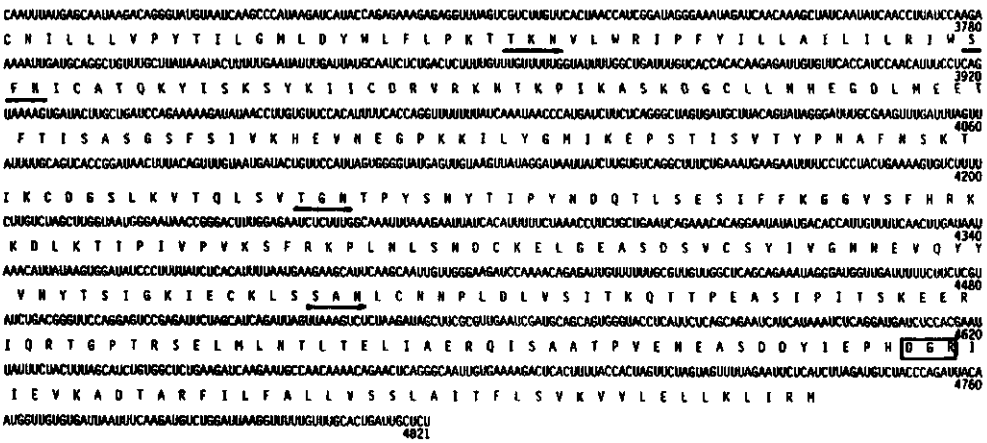
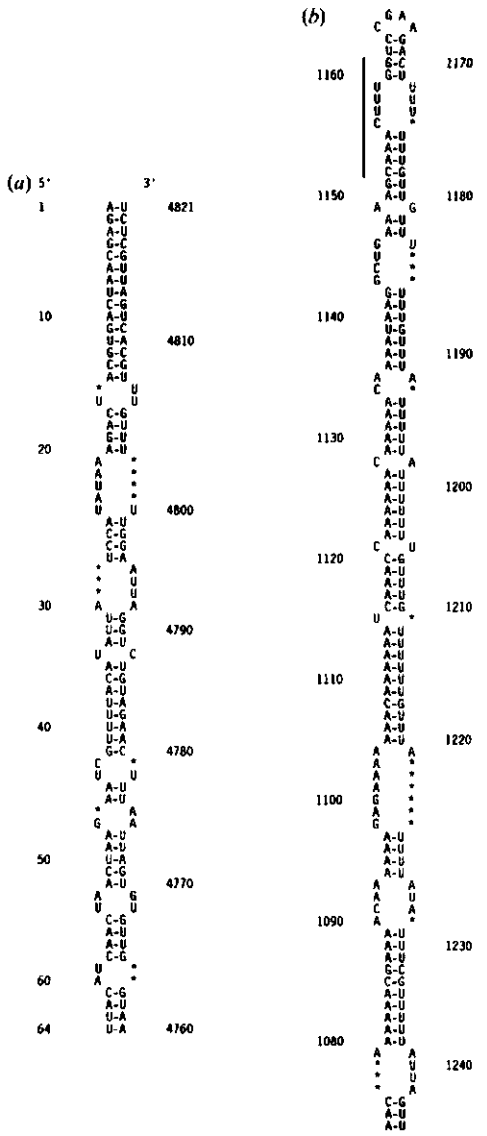


Figure 2: The complete nucleotide sequence of the TSWV M RNA (numbered from the 5' end of the viral RNA strand) and the predicted gene products. The deduced amino acid sequence of the protein encoded by the viral RNA is shown above the RNA sequence. The sequence of the protein encoded by the viral complementary strand is shown below the RNA sequence. Potential N-glycosylation sites are underlined. The asterisks (*) indicate termination codons. The cell-attachment site is boxed.

encodes a protein of 302 amino acids (Fig.2) with a predicted M_r of 33.6 kDa. Analysis of this amino acid sequence did not reveal any hydrophobic domains that could function as signal sequences or transmembrane domains, according to the hydropathy algorithms of Hopp and Woods (1981) and Kyte and Doolittle (1982) (data not shown). Instead, a rather acidic carboxy terminus was found, as can be seen by the number of aspartic (D) and glutamic (E) acid residues (Fig.2). An acidic carboxy terminus is also present in the large protein encoded by the L RNA segment (De Haan *et al.*, 1991). The amino acid sequence of the small ORF contains 2 potential N-glycosylation sites, but it is not known whether these are indeed used *in vivo*. A search in the EMBL protein and nucleotide sequence database did not reveal significant homology to any other published sequence.



Since a protein with the size of 33.6 kDa is not found in purified virus particles on SDS-PAGE, the predicted protein is probably nonstructural, and therefore tentatively named NS_M.

A second, larger ORF is located in the viral complementary strand, starting with an AUG codon at position 4737 (numbered from the 5' end of the vRNA) and terminating with a UGA codon at position 1332. This ORF has the capacity to code for a protein of 1136 amino acids and a M_r of 127.4 kDa. Previous studies on morphological defective isolates of TSWV linked the genetic information for the glycoproteins to the M RNA (Verkleij and Peters, 1983). Analysis of the amino acid sequence of the predicted translation product from the vcORF indeed demonstrated the presence of 8 potential N-glycosylation sites (Fig.2) and several hydrophobic regions (Fig.5), as expected for a precursor to the glycoproteins.

Figure 3: (A) The complementary sequences at the 5' and 3' ends, and (B) the secondary structure in the intergenic region of the TSWV M RNA. The nucleotide positions are numbered from the 5' end of the viral RNA. Asterisks (*) represent gaps corresponding to unpaired nucleotides in the sequence. The conserved sequence motif, a putative transcription termination signal, is indicated with a bar.

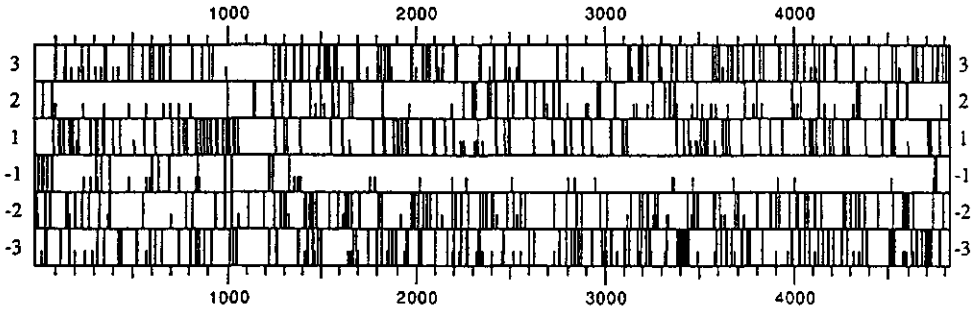


Figure 4: Distribution of translation initiation (short vertical bars) and termination (long vertical bars) codons in the three possible reading frames of the viral (1,2, and 3) and viral complementary (-1, -2, and -3) M RNA strands.

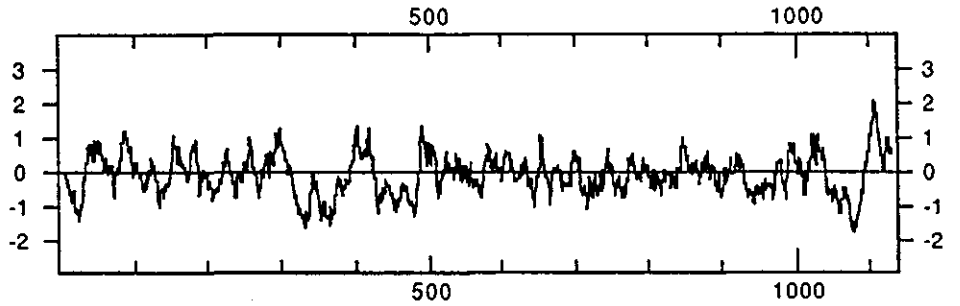


Figure 5: Hydropathy plot of the large gene product encoded by the vcORF of the M RNA. The plot was obtained as described by Hopp and Woods (1981). Regions above the line represent sequences of net hydrophilicity, and below the line that of hydrophobicity.

Furthermore a search in the EMBL protein database revealed sequence homology with the glycoprotein precursor encoded by the M RNA of Bunyamwera and snowshoe hare, members of the genus *Bunyavirus*. This homology was mainly restricted to G1 with 45% homology, and 22% identity, in a 485 amino acid overlap. No homology, however, was found with members from other genera of the *Bunyaviridae* (Fig. 6a; Lees *et al.*, 1986; Collett, *et al.*, 1985; Ihara *et al.*, 1985; Ronnholm and Pettersson, 1987; Schmaljohn *et al.*, 1987). Alignment of the amino acid sequences from G1 of SSH, Bunyamwera, La Crosse,

and Germiston virus (Eshita and Bishop, 1984; Lees *et al.*, 1986; Grady *et al.*, 1987; Pardigon *et al.*, 1988), and the carboxy-terminal part of the protein encoded by the vcORF of the TSWV M RNA demonstrated the presence of a homologous stretch of amino acids, indicating motifs in glycoprotein G1 conserved among members of the genera *Bunyavirus* and *Tospovirus* (Fig.6b).

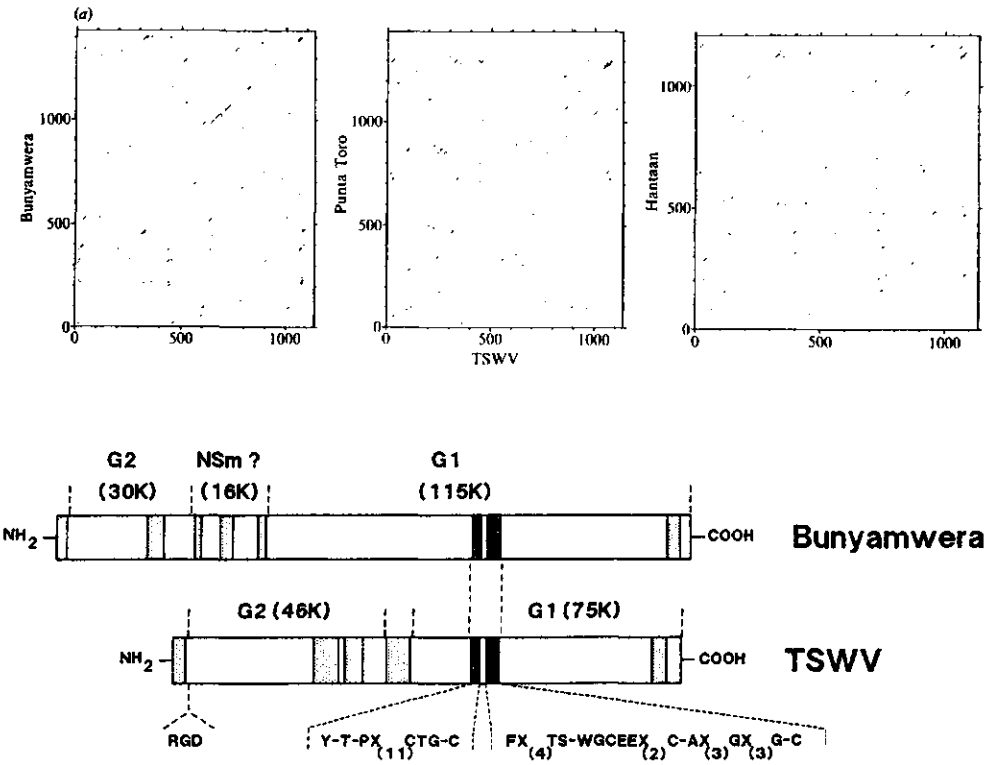


Figure 6: (A) Dot plot comparison of the glycoprotein precursors of TSWV, Bunyamwera, Punta Toro, and Hantaan viruses, using the Compare (window = 30, stringency = 16) and Dotplot programs of the GCG package. Sequence data were obtained from Lees *et al.* (1986) (Bunyamwera virus), Ihara *et al.* (1985) (Punta Toro virus), and Schmaljohn *et al.* (1987) (Hantaan virus). Homologous sequences are indicated by diagonal lines. (B) The conserved amino acid sequence within the G1 glycoprotein of TSWV with that of members of the genus *Bunyavirus* is shown. Hydrophobic domains are indicated by hatched areas. X and hyphens refer to non-identical amino acids. The position of the RGD-motif is indicated.

Based on these data, and since the two envelope glycoproteins of TSWV were previously found not to be encoded by either S or L RNA (De Haan *et al.*, 1990, 1991), it is concluded that the vcORF in the M RNA segment, similar to the M RNAs of animal-infecting bunyaviruses, encodes the precursor to the glycoproteins (G1 and G2). Taking into account that the sizes of glycoproteins G1 (78 kDa) and G2 (58 kDa) are overestimated due to the glycosylation, the size of the predicted protein of the large ORF fits with this conclusion. From the homology between G1 of the bunyaviruses and the glycoprotein precursor of TSWV it is assumed that the carboxy-terminal portion of the glycoprotein precursor encodes the G1 glycoprotein. The hydropathy plot of the precursor to the glycoproteins is in agreement with this, indicating the larger glycoprotein (G1; 78 kDa) to be located at the C-terminus, and the smaller glycoprotein (G2; 58 kDa) at the N-terminus (Fig.5, Fig.6b). Analysis of the hydropathy plot of the glycoprotein precursor furthermore shows a hydrophobic N-terminus for a stretch of 30 residues that probably corresponds with a signal sequence for translocation across the endoplasmatic reticulum (ER) membrane. This sequence will be cleaved from the glycoprotein precursor at amino acid residue 35 from the N-terminus (VLLAFLIFRATDA^KV) according to the algorithms of Von Heijne (1986). Similar to this, the hydrophobic domain found between amino acid residues 400 and 500 probably functions as a signal sequence for the G1 protein which is located at the C-terminus of the precursor. Hydrophobic domains found between amino acid residues 300-400, and 1000-1100 may represent anchoring domains of the two glycoproteins in the ER membrane (Fig.5). Based on these assumptions, the M_r of G2 and G1 can be estimated at about 46 and 75 kDa, respectively. The latter is in good agreement with the previously estimated size of G1 (78 kDa). However, the calculated size of G2 is considerably smaller than the previously estimated size from SDS-PAGE gels (58 kDa), which could be due to glycosylation.

Furthermore, analysis of the amino acid sequence of the glycoprotein precursor revealed the presence of an RGD-motif (Fig.2, Fig.6B; Ruoslahti and Pierschbacher, 1986, 1987) at the amino terminus, immediately downstream of the hydrophobic signal sequence. As this latter sequence is proposed to be cleaved off the precursor protein,

it is anticipated that the RGD motif is not found close to a membrane anchoring site, but rather exposed from the viral envelope membrane.

Transcriptional expression of the M RNA segment

Analysis of M RNA-derived RNA species in infected *N. rustica* plants recently demonstrated the presence of a possible (4 kb) subgenomic mRNA transcribed from the 3' part of the vRNA (Chapter 2). This result was the first indication for the presence of two distinct cistrons in the M RNA. The nucleotide sequence of this RNA now indeed demonstrates the presence of two cistrons in M RNA, arranged in an ambisense manner.

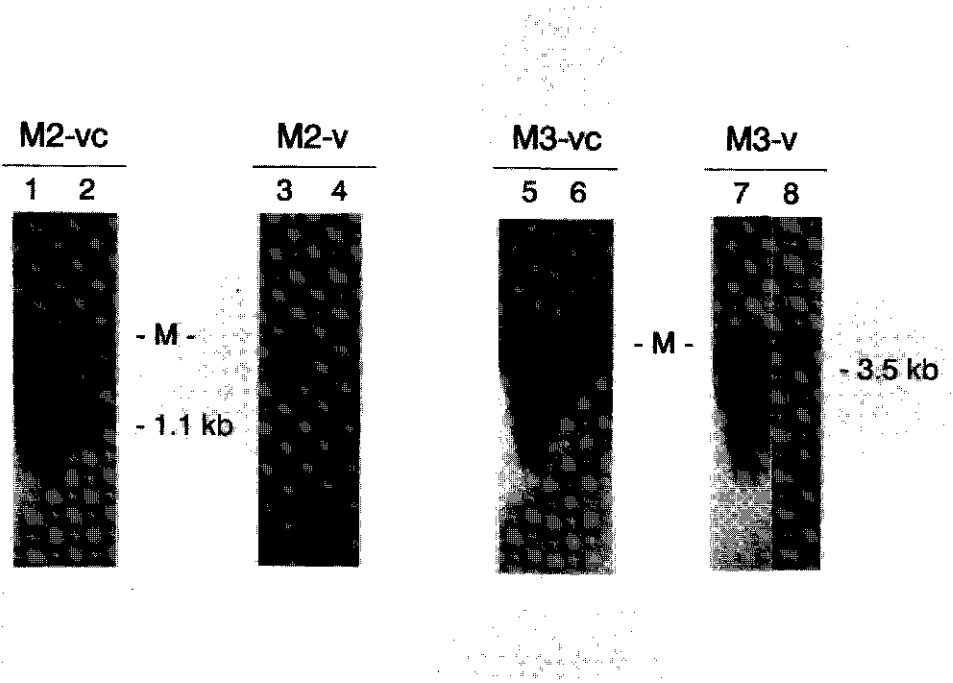


Figure 7: Identification of M-specific subgenomic RNA species in TSWV-infected *N. rustica*. Northern blots were prepared and analysed according to procedures given in Methods. Total RNA was hybridised with strand-specific riboprobes corresponding to the small (lanes 1 to 4) or large (lanes 5 to 8) ORF to detect M-specific vRNA species (lanes 1, 2, 5, and 6) and M-specific vcRNA species (lanes 3, 4, 7, and 8). The subgenomic RNA species are indicated. Lanes 1, 3, 5, and 7 contain 0.5 µg of purified nucleocapsid RNA, and lanes 2, 4, 6, 8 contain 7 µg of total RNA from TSWV-infected *N. rustica* plants purified 8 days post-inoculation. The notation M2-v and M2-vc indicates that riboprobe M2, detecting vcRNA and vRNA strands respectively, was used in the hybridisation experiment. Northern blot M3-v was exposed three times as long as blot M-3vc.

To demonstrate that these cistrons in M RNA are expressed via subgenomic messenger RNAs transcribed from opposite strands, RNA extracts of TSWV-infected *N. rustica* were analyzed on Northern blots using strand-specific riboprobes. Previous analyses of cytoplasmic nucleocapsid RNA fractions demonstrated the presence of M RNA strands of both polarities (Fig.7, lanes 1, 3, 5, and 7), the viral complementary M RNA species being present in smaller amounts (Fig.7, lanes 3 and 7; Chapter 2). In addition to genome-length M RNA species, a viral-sense subgenomic RNA species of approximately 1.1 kb (Fig. 7, lane 1 and 2) was detected with riboprobe M2-vc, corresponding to the small ORF. A riboprobe (probe M3-v) corresponding to the glycoprotein precursor gene also hybridised with genome-length M RNA species and revealed a second subgenomic RNA species of approximately 3.5 kb (Fig.7, lane 7 and 8). A fast migrating RNA species (Fig.7, lane 8) was detected with probe M3-v, but appeared to be a-specific as it was also detected in a total RNA extract of healthy plants. No subgenomic M-specific RNA species were found when probes M2-v, and M3-vc were used in a hybridisation experiment (Fig.7, lanes 3 to 6). In view of the location and polarity of both probes on the physical map of the M RNA, and in view of the sizes of the NS_M gene and glycoprotein precursor gene encoded by this RNA segment, it is concluded that the M RNA-derived species probably represent subgenomic mRNAs corresponding to both cistrons in M RNA. As these mRNAs are transcribed from opposite strands, the ambisense coding strategy of the TSWV M RNA could thus be confirmed.

DISCUSSION

With the determination of the M RNA sequence the genetic organisation of the complete genome of TSWV has been unraveled, the features of which are shown in figure 8. The sequence data on the M RNA show that this genomic segment is 4821 nucleotides long. Like the S and L RNA segments, the M RNA has complementary termini for about 64 nucleotides which are involved in the formation of a stable panhandle structure, causing the RNA segments to appear as pseudo-circular structures

in virus particles (Peters *et al.*, 1991). The terminal sequences of the M RNA are identical for the first eight nucleotides to the 5' and 3' termini of the S and L RNA, and probably have an important function in genome transcription and replication. From the nucleotide sequence it can be deduced that the M RNA has an ambisense coding strategy. One small ORF encoding a putative protein of 33.6 kDa is located in viral sense. Based on its absence in purified virus particles, this protein is tentatively called NS_M. A second, but larger, ORF is located in viral complementary sense and encodes

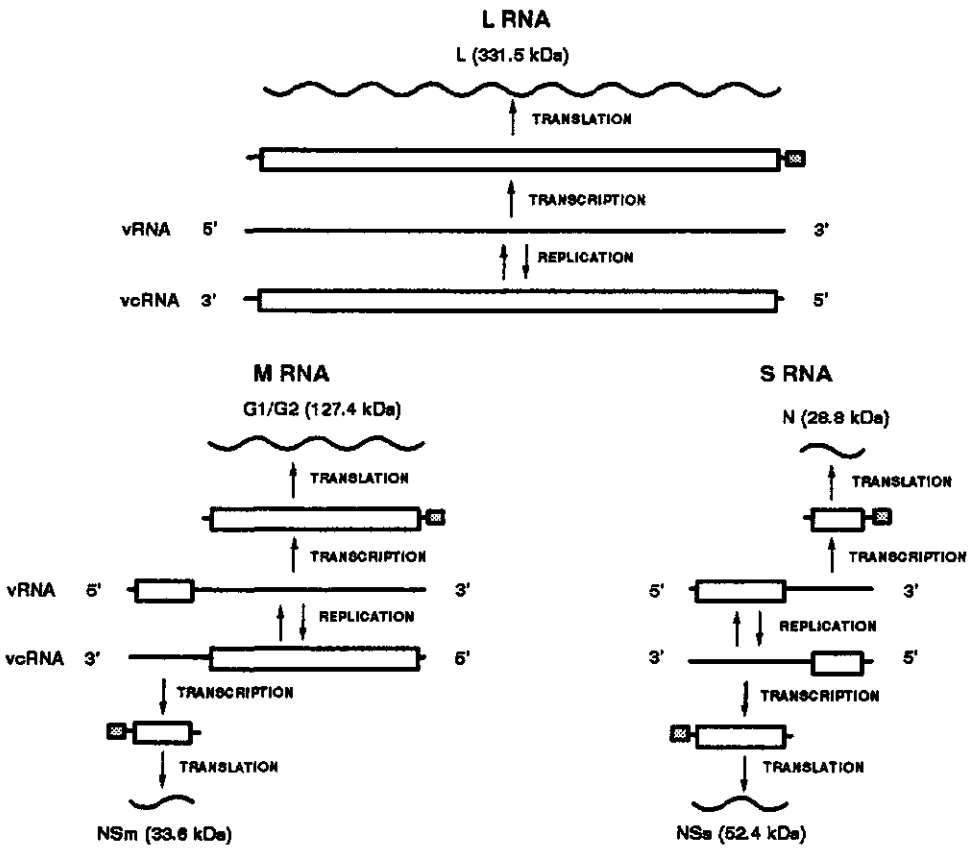


Figure 8: The genome structure and expression strategy of the tripartite genome of TSWV. The hatched areas indicate non-viral sequences used to initiate transcription of the viral mRNAs (Chapter 3).

a protein with a predicted size of 127.4 kDa. From sequence homology with glycoprotein G1 of the bunyaviruses and the hydropathy plot of the predicted protein, it is concluded that this ORF encodes the precursor to both glycoproteins, in the order NH_2-G2-G1-COOH. The ambisense gene arrangement in the M RNA has been confirmed with northern blot analyses, revealing the presence of a 1.1 kb (v-sense) and a 3.5 kb (vc-sense) subgenomic RNA species in infected plant cells. Analysis of the nucleotide sequence of the intergenic region showed the presence of internal inverted A- and U-rich stretches capable of forming a stable hairpin structure. As previously discussed for the TSWV S RNA, this structure could be involved in termination of transcription of the subgenomic mRNAs. A conserved sequence found at the top of the hairpins of the TSWV and INSV S RNA was also found at the top of the TSWV M RNA hairpin, pointing towards a possible function in transcription termination. Whether both structural features are a requirement for termination of transcription or only one of them, remains to be investigated. Ambisense coding strategies have recently been demonstrated for the S RNA segments of two different tospoviruses, TSWV and INSV (De Haan *et al.*, 1990, 1992), and is also found for the S RNA segments of viruses of the genus *Phlebovirus* (Ihara *et al.*, 1984; Marriot *et al.*, 1989; Simons *et al.*, 1990; Giorgi *et al.*, 1991). However, TSWV is the first member of the *Bunyaviridae* with an ambisense M RNA, thus having a genome with two ambisense RNA segments.

Sofar, computer alignments between L and S RNA encoded gene products of TSWV with those of their animal-infecting counterparts of the *Bunyaviridae* revealed significant homology only among the L RNA- encoded RNA polymerases of TSWV and Bunyamwera, the type species of the genus *Bunyavirus* (De Haan *et al.*, 1991). No significant homology was found between the other proteins of TSWV and other members of the *Bunyaviridae* (De Haan *et al.*, 1990). The data presented now reveal significant homology between the G1 glycoproteins of TSWV and members of the genus *Bunyavirus*. This finding seems to confirm that TSWV is most closely related to the genus *Bunyavirus*, although they do not share an ambisense S RNA. This could suggest that the creation of ambisense RNA molecules is a relative late event in bunyavirus evolution.

No significant homology was found between the G2 glycoproteins of TSWV and

members of the genus *Bunyavirus*, but analysis of the putative G2 glycoprotein of TSWV revealed the presence of a putative cell attachment site (RGD) in the amino terminus. Sofar, this motif has only been found in glycoproteins that are found in the extracellular matrix of animal and plant cells, and are thought to be involved in adhesion of cells to the extracellular matrix (Pierschbacher and Ruoslahti, 1984; Ruoslahti and Pierschbacher, 1986, 1987; D'Souza *et al.*, 1988; Schindler *et al.*, 1989; Sanders *et al.*, 1991). The RGD sequence in such adhesive proteins is crucial for the recognition by cell surface receptors (referred to as integrins). For TSWV it is tempting to assume that this sequence is only involved in the insect part of the virus multiplication cycle, possibly in binding of virus particles to cell receptors in the midgut of the thrips vectors. Indeed, morphological defective isolates, i.e. virus mutants lacking a lipid membrane, are still able to infect plants but are deficient in thrips transmission (Ie, 1982; Verkleij and Peters, 1983; Resende *et al.*, 1991). The relevance of the RGD motif in the glycoprotein precursor of TSWV remains to be tested, also since analysis of the glycoprotein precursor proteins of other members of the *Bunyaviridae* only revealed this motif in Germiston and SSH bunyavirus, and Punta Toro phlebovirus.

The TSWV M RNA encodes, in addition to the glycoproteins, a putative nonstructural protein (NS_M). A search in the EMBL protein database did not reveal significant homology with other known proteins. Alignment with the glycoprotein precursor (containing potential NS_M sequences) encoded by the M RNAs of different members of the *Bunyaviridae* also did not reveal any homology. The protein has not yet been detected in TSWV-infected plant cells and appears to be absent in virus preparations.

With the determination of the M RNA sequence the genetic organisation of the complete genome of TSWV has been elucidated. This will be a good starting point for future research aimed to unravel the adaptation of this bunyavirus to plants. Comparison of the genome of TSWV with those of other members of the animal-infecting *Bunyaviridae* reveals that one extra ORF is present in the genome of TSWV, i.e. the gene in the M RNA, encoding the putative nonstructural protein of 33.6 kDa. Therefore, it is likely to assume that this protein is involved in adaptation of TSWV to plants as host.

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REFERENCES

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

Cho, J.J., Mau, R.F.L., Mitchell, W.C., Gonsalves, D. and Yudin, L.S. (1987). Host list of plants susceptible to tomato spotted wilt virus (TSWV). *Research extension series*, Honolulu University of Hawaii.

Collett, M.S., Purchio, A.F., Keegan, K., Frazier, S., Hays, W., Anderson, D.K., Parker, M.D., Schmaljohn, C., Schmidt, J. and Dalrymple, J.M. (1985). Complete nucleotide sequence of the M RNA segment of Rift Valley fever virus. *Virology* **144**, 228-245.

De Avila, A.C., Huguenot, C., Resende, R. de O., Kitajima, E.W., Goldbach, R.W. and Peters, D. (1990). Serological differentiation of 20 isolates of tomato spotted wilt virus. *Journal of General Virology* **71**, 2801-2807.

De Haan, P., Wagemakers, L., Peters, D. and Goldbach, R. (1989). Molecular cloning and terminal sequence determination of the S and M RNAs of tomato spotted wilt virus. *Journal of General Virology* **70**, 3469-3473.

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

De Haan, P., Kormelink, R., Resende, R. de O., Van Poelwijk, F., Peters, D. and Goldbach, R. (1991). Tomato spotted wilt virus L RNA encodes a putative RNA polymerase. *Journal of General Virology* **71**, 2207-2216.

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

Deveraux, J., Haeberli, P. and Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Research* **12**, 387-395.

Devos, R.G.E., Gillis, E. and Fiers, W. (1976). The enzymatic addition of poly(A) to the 3' end of RNA using bacteriophage MS2 RNA as a model system. *European Journal of Biochemistry* **62**, 401-410.

D'Souza, S.E., Ginsberg, M.H., Burke, T.A., Lam, S.C.-T. and Plow, E.F. (1988). Localization of an Arg-Gly-Asp recognition site within an integrin adhesion receptor. *Science* **242**, 91-93.

De Vries, S.C., Springer, J. and Wessels, J.G.H. (1982). Diversity of abundant mRNA sequences and patterns of protein synthesis in etiolated and greened pea seedlings. *Planta* **156**, 129-135.

Elliott, R.M. (1990). Molecular biology of the *Bunyaviridae*. *Journal of General Virology* **73**, 501-522.

Eshita, Y. and Bishop, D.H.L. (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.

Francki, R.I.B., 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. *Archives of Virology* suppl. **2**.

Giorgi, C., Accardi, L., Nicoletti, L., Gro, M.C., Takehara, K., Hilditch, C., Morikawa, S. and Bishop, D.H.L.

(1991). Sequences and coding strategies of the S RNAs of Toscana and Rift Valley fever viruses compared to those of Punta Toro, Sicilian sandfly fever, and Uukuniemi viruses. *Virology* **180**, 738-753.

Grady, L.J., Sanders, M.L. and Campbell, W.P. (1987). The sequence of the M RNA of an isolate of La Crosse Virus. *Journal of General Virology* **68**, 3057-3071.

Gubler, U. and Hoffman, B.J. (1983). A simple and very efficient method for generating cDNA libraries. *Gene* **25**, 263-269.

Hopp, T.P. and Woods, K.R. (1981). Prediction of protein antigenic determinants from amino acid sequences. *Proceedings of the National Academy of Sciences, USA* **78**, 3824-3828.

Ie, T.S. (1982). A sap-transmissible, defective form of tomato spotted wilt virus in plant cells. *Virology* **59**, 387-391.

Ihara, T., Akashi, H. and Bishop, D.H.L. (1984). Novel coding strategy (ambisense genomic RNA) revealed by sequence analyses of Punta Toro phlebovirus S RNA. *Virology* **136**, 293-306.

Ihara, T., Schmidt, J., Dalrymple, J.M. and Bishop, D.H.L. (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.

Kyte, J. and Doolittle, R.F. (1982). A simple method for displaying the hydropathic character of a protein. *Journal of Molecular Biology* **157**, 105-132.

Lees, J.F., Pringle, C.R. and Elliott, R.M. (1986). Nucleotide sequence of the Bunyamwera virus M RNA segment: Conservation of structural features in the bunyavirus glycoprotein gene product. *Virology* **148**, 1-14.

Maiss, E., Ivanova, L., Breyel, E. and Adam, G. (1991). Cloning and sequencing of the S RNA from a Bulgarian isolate of tomato spotted wilt virus. *Journal of General Virology* **72**, 461-464.

Marriott, A.C., Ward, V.K. and Nuttall, P.A. (1989). The S RNA segment of sandfly fever Sicilian virus: Evidence for an ambisense genome. *Virology* **169**, 341-345.

Mohamed, N.A., Randles, J.W. and Francki, R.I.B. (1973). Protein composition of tomato spotted wilt virus. *Virology* **56**, 12-21.

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

Paliwal, Y.C. (1974). Some properties and thrips transmission of tomato spotted wilt virus in Canada. *Canadian Journal of Botany* **52**, 1177-1182.

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 Research* **11**, 73-85.

Peters, D., De Avila, A.C., Kitajima, E.W., Resende, R. de O., De Haan, P. and Goldbach, R. (1991). An overview of tomato spotted wilt virus. In *Virus-Thrips-Plant Interactions of TSWV, Proceedings of USDA workshop*, Beltsville, U.S.A., pp.1-14. Edited by H.-T. Hsu and R.H. Lawson. Springfield: National Technology Information Service.

Pierschbacher, M.D. and Ruoslahti, E. (1984). Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. *Nature* **309**, 30-33.

Resende, R. de O., De Haan, P., Avila, A.C.de, 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. *Journal of General Virology* **72**, 2375-2383.

RonnHolm, R. and Pettersson, R.F. (1987). Complete nucleotide sequence of the M RNA segment of Uukuniemi virus encoding the membrane glycoproteins G1 and G2. *Virology* **160**, 191-202.

Ruoslahti, E. and Pierschbacher, M.D. (1986). Arg-Gly-Asp: A versatile cell recognition signal. *Cell* **44**, 517-518.

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

Sakimura, K (1962). The present status of thrips-borne viruses. In: "*Biological transmission of disease agents*", 33-40, K. Maramorosch ed., Academic Press, New York.

Sanders, L.C., Wang, C.-S., Walling, L.L. and Lord, E.M. (1991). A homolog of the substrate adhesion molecule vitronectin occurs in four species of flowering plants. *Plant Cell* **3**, 629-635.

Sanger, F., Nicklen, S. and Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences, USA* **74**, 5463-5467.

Schindler, M., Meiners, S. and Cheresh, D.A. (1989). RGD-dependent linkage between plant cell wall and plasma membrane: Consequences for growth. *Journal of Cellular Biology* **108**, 1955-1965.

Schmaljohn, C.S., Schmaljohn, A.L. and Dalrymple, J.M. (1987). Hantaan virus M RNA: Coding strategy, nucleotide sequence, and gene order. *Virology* **157**, 31-39.

Simons, J.F., Hellman, U. and Pettersson, R.F. (1990). Uukuniemi virus S RNA segment: Ambisense coding strategy, packaging of complementary strands into virions, and homology to members of the genus *Phlebovirus*. *Journal of Virology* **64**, 247-255.

Tas, P.W.L., Boerjan, M.L. and Peters, D. (1977). The structural proteins of tomato spotted wilt virus. *Journal of General Virology* **36**, 267-279.

Van Den Hurk, J., Tas, P.W.L. and Peters, D. (1977). The ribonucleic acid of tomato spotted wilt virus. *Journal of General Virology* **36**, 81-91.

Verkleij, F.N. and Peters, D. (1983). Characterization of a defective form of tomato spotted wilt virus. *Journal of General Virology* **64**, 677-686.

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

Zhang, H., Scholl, R., Browse, J. and Somerville, C. (1988). Double stranded DNA sequencing as a choice for DNA sequencing. *Nucleic Acids Research* **16**, 1220.

Chapter 5

The non-structural protein (NS_S) encoded by the ambisense S RNA segment of tomato spotted wilt virus is associated with fibrous structures in infected plant cells[4]

SUMMARY

The open reading frame located in the viral strand of the ambisense S RNA of tomato spotted wilt virus (TSWV), was cloned into transfer vector pAc33DZ1 and inserted downstream of the polyhedrin promoter in the *Autographa californica* nuclear polyhedrosis virus genome. A recombinant baculovirus was obtained that showed a high level expression of a 52.4 kDa protein corresponding to the inserted TSWV gene. The viral protein thus produced, was purified and injected into rabbits to raise antibodies. Western immunoblot analyses of extracts from TSWV-infected plants demonstrated that the 52.4 kDa TSWV-specific polypeptide represents a non-structural protein (denoted NS_S), being absent in purified virus particles. Immunogold labelling of tissue sections of TSWV-infected *Nicotiana rustica* plants showed that this protein was, depending on the virus isolate, either found dispersed throughout the cytoplasm or associated with fibers which appeared as elongated flexible filaments or paracrystalline rods.

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INTRODUCTION

Tomato spotted wilt virus (TSWV) causes great yield losses worldwide in a large number of economically important crops. The host range of TSWV includes at least 400 plant species in 50 botanical families, both dicotyledons and monocotyledons (Matthews, 1982; Cho *et al.*, 1987; Peters, pers. commun.). TSWV is the only plant virus shown thus far to be transmitted by thrips (Sakimura, 1962). TSWV virions are spherical, enveloped particles (80-110 nm) containing 4 structural proteins: the nucleoprotein (N) of 29 kDa, two membrane glycoproteins of 78 kDa (G1) and 58 kDa (G2) which form spikes on the viral envelope, and a large protein (L) of >200 kDa present in minor amounts associated with the nucleocapsids (Mohamed *et al.*, 1973; Tas *et al.*, 1977). The viral genome consists of 3 single stranded RNA molecules, denoted S RNA (2.9 kilobases), M RNA (4.8 kb) and L RNA (8.9 kb) (De Haan *et al.*, 1990, 1991; Chapter 4). The genomic RNAs are tightly associated with the nucleoprotein and form circular nucleocapsids (Van Den Hurk *et al.*, 1977; Mohamed, 1981; De Haan *et al.*, 1989a).

Recently, the nucleotide sequence of the S RNA has been determined (De Haan *et al.*, 1990). The genetic organisation of this genome segment is very similar to that of the S RNAs of phleboviruses, another genus within the family of arthropod-borne *Bunyaviridae* (Bishop *et al.*, 1980). This, and other similarities has led to the conclusion that TSWV is a bunyavirus, unique in its property to infect plants (De Haan *et al.*, 1989a, 1990; Milne and Francki, 1984). TSWV S RNA is 2916 nucleotides long and has, like phleboviral S RNA (Ihara *et al.*, 1984; Marriott *et al.*, 1989; Simons *et al.*, 1990), an ambisense coding strategy (Fig. 1a). One open reading frame (ORF), located on the viral complementary strand (vcORF), has been shown to encode the nucleoprotein. The second ORF, in the viral strand (vORF), corresponds with a putative protein of 52.4 kDa, which has not yet been detected. This hypothetical protein has been suggested to represent a non-structural protein which we, in analogy to the bunyaviral nomenclature, propose to refer to as NS_S protein (De Haan *et al.*, 1990). Both ORFs are expressed by two subgenomic messenger RNAs, transcribed from complementary strands via a process

of cap-snatching (Chapter 3). Termination of transcription occurs at the central, intercistronic region, most probably in a long A-U rich hairpin (De Haan *et al.*, 1989b, 1990; Chapter 2).

Although the nucleoproteins of TSWV and phleboviruses share a similar molecular weight of about 25-29 kDa, the NS_S protein of TSWV is much larger than the corresponding proteins of the animal *Bunyaviridae* (TSWV, 52.4 kDa; phleboviruses, 30 kDa). To characterize the gene product corresponding to the vORF, a cDNA fragment containing this ORF was expressed using a novel baculovirus vector (Zuidema *et al.*, 1990) and the resultant TSWV protein used for the production of specific antibodies. In this report it is shown by immunogold labelling techniques that these antibodies react specifically with a TSWV-specific protein that is found either associated with fibrous structures or dispersed throughout the cytoplasm of infected plant cells, depending on the virus isolate.

MATERIALS AND METHODS

Viruses, cells and cDNA clones

TSWV isolates were maintained in *Nicotiana rustica* 'America' plants by mechanical inoculation. Complementary DNA clones representing the S RNA of the Brazilian TSWV isolate BR-01 (CNPH1) were described previously (De Haan *et al.*, 1989b, 1990). Wild type (wt) and recombinant *Autographa californica* nuclear polyhedrosis viruses (AcNPV) were grown in monolayers of *Spodoptera frugiperda* 21 cells (Vaughn *et al.*, 1977) in TNMFH medium (Hink, 1970) containing 10% foetal calf serum.

Construction of the AcNPV recombinant transfer vector

Plasmid vector pAc33DZ1 (Zuidema *et al.*, 1990) was used to construct a transfer vector containing the TSWV NS_S gene. A bacterial plasmid containing the complete open reading frame of NS_S (pTSWV514), was digested with *Eco*RI, and supplied with *Bam*HI linkers after treatment with T4 DNA polymerase (Maniatis *et al.*, 1982). After

purification by LMP agarose gel electrophoresis the cDNA fragment was cloned into *Bam*HI-digested pAc33DZ1. A recombinant plasmid, pAc33DZ1/NS_S, was recovered and its composition verified by restriction enzyme analysis. Nucleotide sequences at insertion sites were verified (Fig.1c) by the dideoxy chain termination method (Sanger *et al.*, 1977) using oligonucleotide S IV as a primer (De Haan *et al.*, 1990).

Construction of recombinant virus

Recombinant baculoviruses expressing the NS_S gene were produced by co-transfection of *S. frugiperda* cells with a mixture of AcNPV DNA and pAc33DZ1/NS_S DNA (Smith *et al.*, 1983). After 3 days of incubation at 27°C, nonoccluded virus (NOV) was collected from the medium and titrated in dilutions to render separated plaques in a plaque assay (Brown and Faulkner, 1977). Recombinant viruses were plaque purified 4 times and grown in high titer stocks.

Southern blot analyses of recombinant AcNPV DNA

S. frugiperda cells were infected with wt or recombinant AcNPV with a multiplicity of 20 $TCID_{50}$ units per cell and incubated at 27°C for 4 days. NOV was collected and DNA was purified according to Summers and Smith (1987). Viral DNA samples were digested with *Bam*HI and the restriction fragments were resolved by electrophoresis in 1% agarose gels and transferred to Genescreen (New England Nuclear, NEN). The immobilised DNA samples were hybridised (Southern, 1975) to an 350 bp *Eco*RI-*Bgl*II fragment of pTSWV520 (De Haan *et al.*, 1989b), labelled with ^{32}P using the 'Prime-a-Gene' Labelling System (Promega) according to the manufacturers procedure. The membranes were washed and autoradiographed.

SDS-PAGE of proteins from infected *S. frugiperda* cells

S. frugiperda cells were infected in portions of $5x10^6$ cells, with a multiplicity of 20 $TCID_{50}$ units per cell, and incubated at 27°C for 48-52 hr. The cells were collected, washed twice with PBS (phosphate buffered saline) and resuspended in 200 µl PBS. For SDS-PAGE analyses of proteins, the cells were boiled in 10 mM Tris-HCl pH 8.0, 1 mM

EDTA, 2% (w/v) SDS, 10% (v/v) glycerol, 0.001% (w/v) bromophenol-blue, 5% (v/v) ß-mercaptoethanol (protein loading buffer). Samples were analyzed on a 12.5% SDS-polyacrylamide gel (Laemmli, 1970).

Purification of NS_S protein

For large scale preparation of the TSWV NS_S protein, proteins from *S. frugiperda* cells infected with Ac33DZ1/NS_S-1 were resolved on 0.75 mm thick 10% preparative SDS-polyacrylamide gels (Protean II system, Bio-Rad). The proteins were stained with $CuCl_2$ (Lee *et al.*, 1987) and the NS_S protein band excised from the gel. Gel slices were destained for 1.5 hr in 3 changes of 0.25 M EDTA, 0.25 M Tris-HCl, pH 9.0, and the protein subsequently electro-eluted from the gel in 20 mM Tris, 150 mM glycine, 0.01% SDS for 5 hr using an ISCO electrophoretic concentrator applying 100 V at 4°C. The polarity was reversed for 1 min prior to protein sample collection. Eluted proteins were analysed on a SDS-polyacrylamide gel and its yield determined by the Bio-Rad protein assay according to the manufacturers procedure.

Preparation of antibodies to the NS_S protein

Portions of 50 to 100 µg purified NS_S protein were emulsified in Freund's incomplete adjuvant (Difco Laboratories) and injected into the hind legs of a rabbit at days 1, 8 and 22. From day 36 on, the rabbit was bled several times and gamma-globulin fractions isolated according to Clark and Adams (1977) and tested with protein blots.

Immunoblot analyses

TSWV BR-01 virions were purified according to Tas *et al.* (1977). Samples from TSWV-infected *N. rustica* were prepared by homogenizing 0.1 g of systemically infected leaves (12 days p.i.) in 0.5 ml PBS containing 0.05% Tween-20. After combining the extract with 4x protein loading buffer, 15 µl of healthy- and TSWV-infected *N.rustica* extracts were applied on a SDS-polyacrylamide gel. After SDS-PAGE, proteins were transferred to Immobilon membrane (Millipore) by electroblotting in an LKB Transphor electroblotting unit at 60 V overnight in 20 mM Tris-HCl pH 8.3, 150 mM glycine, 20%

(v/v) methanol at 4°C. Membranes were dried, washed in PBS containing 0.3% BSA (PBS-BSA) and blocked for 3 hr in 3% BSA (in PBS) at room temperature. After several washings with PBS-BSA, membranes were incubated in the same buffer containing 1 μg/ml NS_s antiserum for 1 hr. After washing, antigen-antibody complexes were detected using 1 μg/ml alkaline phosphatase conjugated goat-anti-rabbit immunoglobulins (Tago Inc., Burlingame, CA, USA), using 0.33 mg/ml nitroblue tetrazolium (NBT) and 0.165 mg/ml bromochloroindolyl phosphate (BCIP) as a substrate.

Immunogold labelling of NS_s protein in tissue sections of TSWV-infected *N.rustica* plants

In situ detection of NS_s protein was performed in tissue sections of TSWV-infected plants, fixed in glutaraldehyde and embedded in LRGold, using essentially the protocol described by Van Lent *et al.* (1990). Tissue sections were prepared from systemically infected leaves, 12 days post infection (p.i.). For immunolabelling, sections were treated with NS_s antiserum at a 1:400 dilution in PBS-BSA for 2 hr.

RESULTS

Construction and analyses of recombinant baculoviruses

A cDNA fragment containing the complete open reading frame of the TSWV NS_s protein (vORF, Fig.1a) was cloned into transfer vector pAc33DZ1 (Zuidema *et al.*, 1990), according to Materials and Methods. This resulted in a construct, pAc33DZ1/NS_s, in which the NS_s gene was inserted downstream of the polyhedrin promoter (Fig.1b). After verification of the nucleotide sequences at the insertion sites (Fig.1c), the NS_s gene was transferred to AcNPV by co-transfection of *S. frugiperda* cells with a mixture of wild type AcNPV and pAc33DZ1/NS_s DNA. NOV-DNA from these recombinants was isolated and its composition analysed. To this end, the viral DNA was digested with *Bam*HI and analysed by agarose gel electrophoresis and Southern blotting. Using TSWV

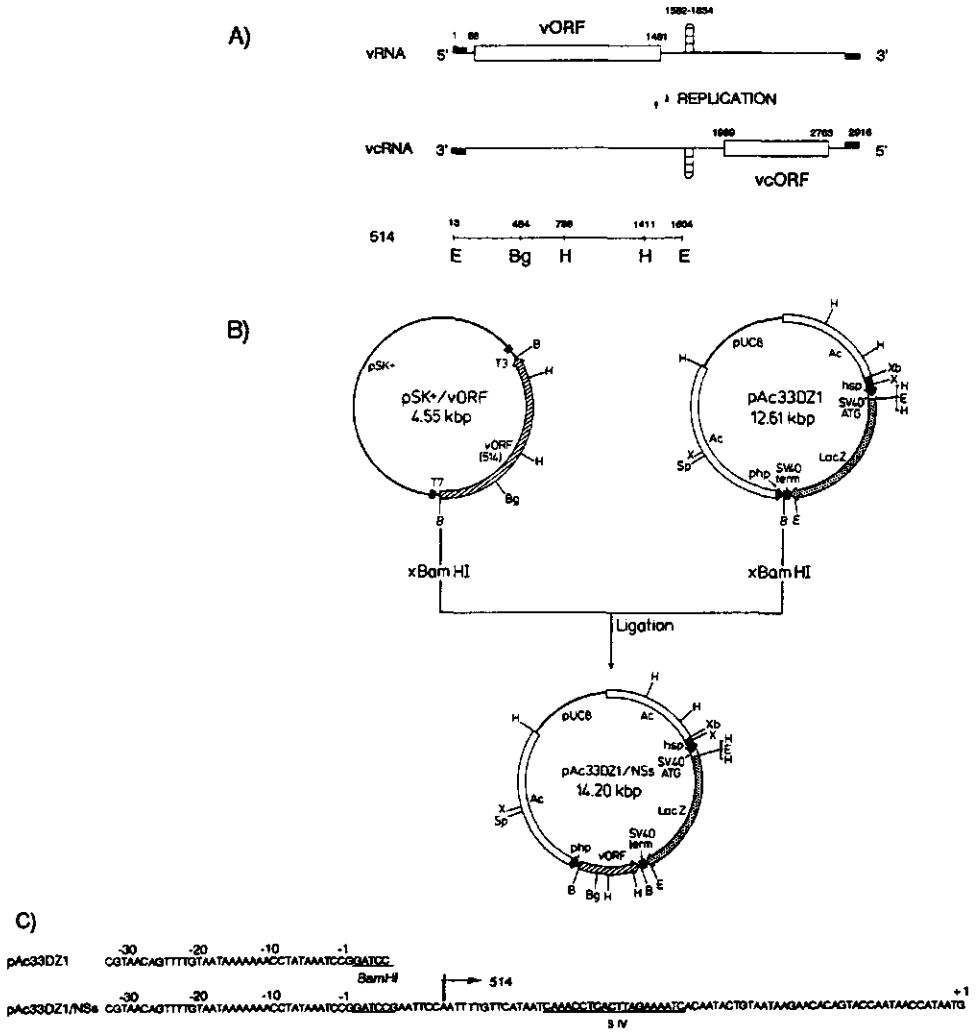

Figure 1: Genetic organisation of TSWV S RNA (A) and construction of the transfer vector pAc33DZ1/NS_S (B,C). The position of cDNA clone 514, containing the vORF encoding protein NS_S, is depicted in alignment with the sequence of S RNA. The replication of the vRNA strand into the vcRNA strand illustrates the ambisense coding strategy of the TSWV S RNA segment (A). The insert of clone 514 was transferred into transcription vector SK+ and subsequently cloned into the *Bam*HI site of transfer vector pAc33DZ1 (Zuidema *et al.*, 1990), resulting in pAc33DZ1/NS_S (B). The nucleotide sequence surrounding the insertion site in pAc33DZ1/NS_S was verified using oligonucleotide S IV, of which the position is indicated. The sequence is shown in comparison to pAc33DZ1 (C).
Arrows indicate the direction of transcription from the polyhedrin (php) and heat shock (hsp) promoters. H, Xb, X, E, B, Sp and Bg indicate the position of *Hind*III, *Xba*I, *Xho*I, *Eco*RI, *Bam*HI, *Sph*I and *Bgl*I cleavage sites, respectively. *Autographa californica* NPV polyhedrin flanking sequences are denoted Ac.

S RNA specific clone pTSWV520, positive recombinants were obtained, containing the NS_S gene in the right position (Fig.2). The level and correctness of transcription of TSWV specific sequences from the polyhedrin promoter were verified by Northern blot analyses (data not shown). In this way, correct recombinant baculoviruses were identified of which Ac33DZ1/NS_S-1 was selected for further studies.

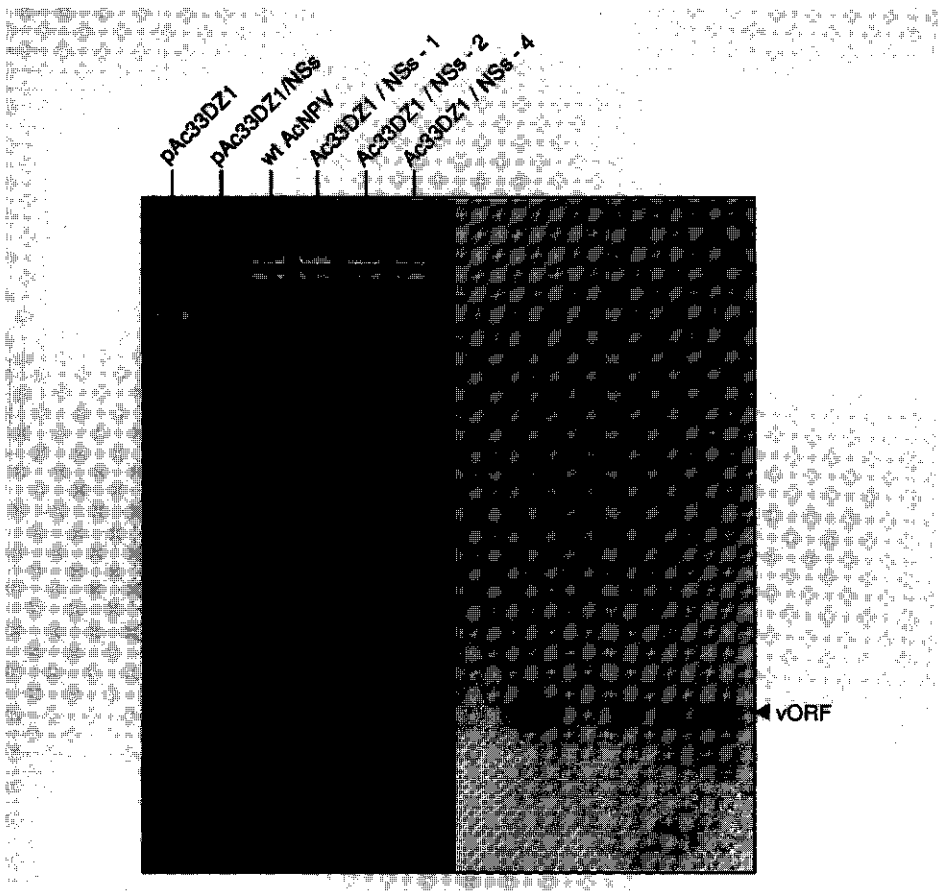

Figure 2: Southern blot analyses of baculovirus recombinants Ac33DZ1/NS_S-1, -2, and -4. NOV DNA was purified from the recombinant viruses and digested with *Bam*HI. The resulting restriction fragments were resolved in a 1% agarose gel (left), blotted to Genescreen and hybridised to a ^{32}P-labelled NS_S probe (right). As controls pAc33DZ1, pAc33DZ1/NS_S and wt AcNPV were analysed. The *Bam*HI fragment containing the ORF encoding NS_S is indicated vORF. The *Bam*HI restriction fragments of wt AcNPV DNA are sized 86.5 kb, 23.3 kb, 8.50 kb, 3.45 kb, 3.33 kb, 1.92 kb and 0.96 kb respectively.

Expression and purification of TSWV NS_S protein

Production of the NS_S protein was analysed by comparing the protein pattern of Ac33DZ1/NS_S-1 infected *S. frugiperda* cells with that of wt AcNPV infected *S. frugiperda* cells. A major protein band corresponding with the expected size of the TSWV NS_S protein (52.4 kDa; De Haan *et al.*, 1990) was identified for Ac33DZ1/NS_S-1 (Fig.3). This protein was produced at levels comparable to that of the 33 kDa polyhedrin protein in wt AcNPV infected *S. frugiperda* cells (Fig.3, lane wt AcNPV). Similar results were obtained in case the other, separately plaque purified Ac33DZ1/NS_S recombinants were analysed. NS_S protein from Ac33DZ1/NS_S-1 infected *S. frugiperda* cells was purified and used for the production of antibodies. The purity and quality of the isolated protein was verified by electrophoresis (Fig.3, lane NS_S purified). Yields of NS_S protein were always in the range of 75-100 µg per $1.5x10^7$ infected *S. frugiperda* cells. The NS_S protein appeared to be slightly contaminated with proteins from the insect cells. It was anticipated, however, that these contaminations would not interfere with further immunological studies in plant systems.

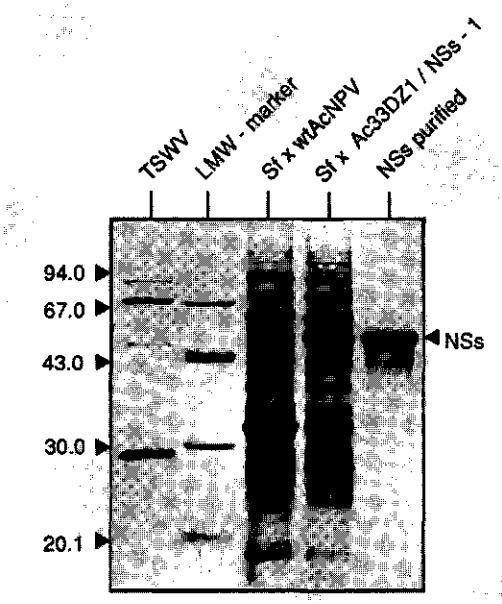

Figure 3: Production of TSWV NS_S protein in Ac33DZ1/NS_S-1 infected *S. frugiperda* cells. Proteins from recombinant baculovirus infected Sf cells were resolved on a 12.5% SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue. Purified NS_S protein (1.5 µg) used for the production of antibodies is shown in the last lane. As a control wt AcNPV-infected *S. frugiperda* cells were included in the analyses. Low Molecular Weight (LMW) size markers (Pharmacia) are indicated at the left. The NS_S protein expressed by the recombinant baculovirus is indicated by the arrow. The 33 kDa band present in wt AcNPV-infected *S. frugiperda cells* cells represents the polyhedrin protein, which was substituted by the NS_S protein in case of the recombinant.

Antiserum to NS_S protein

Purified NS_S protein was injected into rabbits three times at intervals of 1-2 weeks. After the third injection, blood was collected and the immunoglobulin fraction isolated. To test the serum for the presence of antibodies against NS_S protein, a sample of Ac33DZ1/NS_S-infected *S. frugiperda* cells was subjected to electrophoresis, transferred to an Immobilon membrane and subjected to Western immunoblot analysis using 1 μg/ml of NS_S antiserum. The resulting immunoblot confirmed the presence of antibodies against denatured NS_S (Fig.4, lanes SfxAc33DZ1/NS_S-1 and NS_S purified). By dot-immunoblot analyses purified NS_S could be detected in amounts as low as 10 ηg (data not shown). The titer of the antiserum was concluded to be high enough to allow further studies on the presence of NS_S in TSWV-infected plant samples.

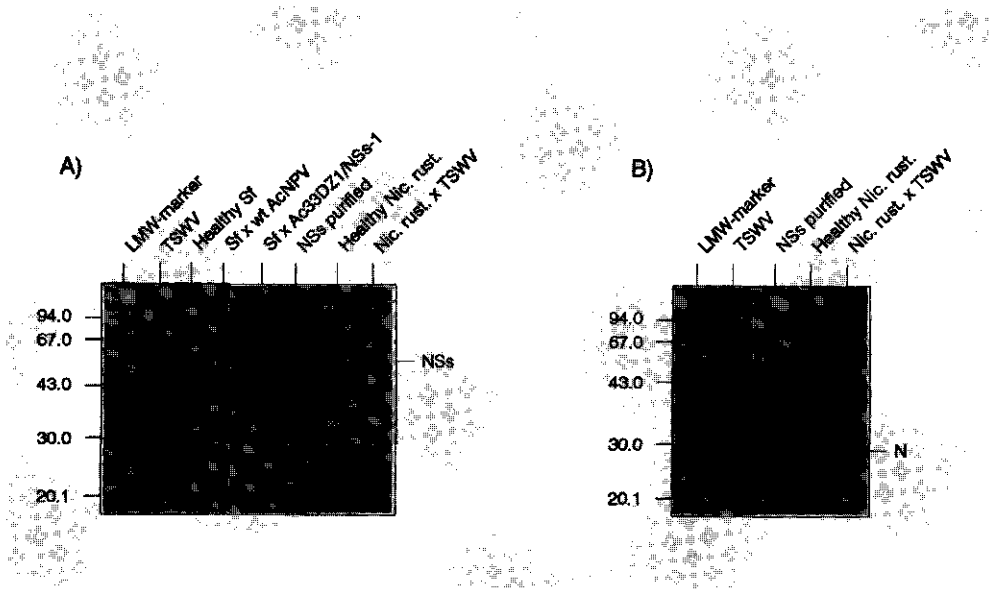

Figure 4: Specificity of antibodies raised against TSWV BR-01 NS_S protein. (A) Proteins from healthy, wt AcNPV and Ac33DZ1/NS_S-1 infected *S. frugiperda* cells were resolved by 12.5% SDS-PAGE and electroblotted on Immobilon. A sample of 250 ηg purified TSWV virus and 15 μl portions of extracts from healthy *N. rustica* and TSWV BR-01 infected *N. rustica* plants (prepared according to Materials and Methods) were included. The Western blot was analyzed using 1 μg/ml NS_S antiserum. As a control a similar Western blot (B) was screened with antiserum against N protein. LMW size markers are indicated on the left and were stained with amidoblack.

Detection of NS_S protein in TSWV-infected plant material

In order to establish the actual production of NS_S during the TSWV infection cycle, purified TSWV preparations and extracts from healthy and infected plants were analysed by immunoblotting. The results obtained indicate that purified TSWV virions appear not to contain NS_S (Fig.4a, lane TSWV) but that a protein with a size expected for NS_S can be specifically detected in TSWV-infected plants (Fig.4a, lane Nic.rust.xTSWV). This result provides evidence that the vORF of TSWV S RNA indeed encodes a non-structural protein. The control blot, shown in Fig.4B, verifies that the absence of any signal with anti-NS_S serum in case of purified virus, is not due to the lack of viral antigen. The conclusion that NS_S protein is not present in virus particles is also supported by immunogold analysis (see below).

Intracellular localization of the NS_S protein

To localize the intracellular position of the NS_S protein and to gain insight in the function of this protein during the TSWV infection cycle, ultrathin sections of TSWV-infected *N. rustica* were prepared for electron microscopy and immunogold analysis. Also, in order to test whether NS_S is conserved among various TSWV isolates *N. rustica* plants infected with different isolates were screened. As described in detail elsewhere (Kitajima *et al.*, 1992), TSWV-infected cells usually contain virus particles (80-110 nm in diameter) within the cisternae of the endoplasmatic reticulum system, viroplasm of medium density and masses with a high density ("dense masses"). The dense masses represent accumulations of nucleocapsids, as shown by their specific labelling with antibodies to nucleocapsid protein. In addition, for approximately half of the twenty TSWV isolates studied, fibrous structures were found in infected cells which consisted of either elongated flexible filaments (Fig.5a) or more rigid rods (Fig.5b), about 10 nm in diameter and of variable length . The filaments formed a loose parallel mass, while the rigid rods were organized in a paracrystalline array. These fibrous structures, which could form clusters as large as the nucleus, did not react with antisera against TSWV structural proteins (Kitajima *et al.*, 1992), but are now found to be specifically labelled with NS_S antiserum (Fig.6a and b). The rigid rods were consistently found to

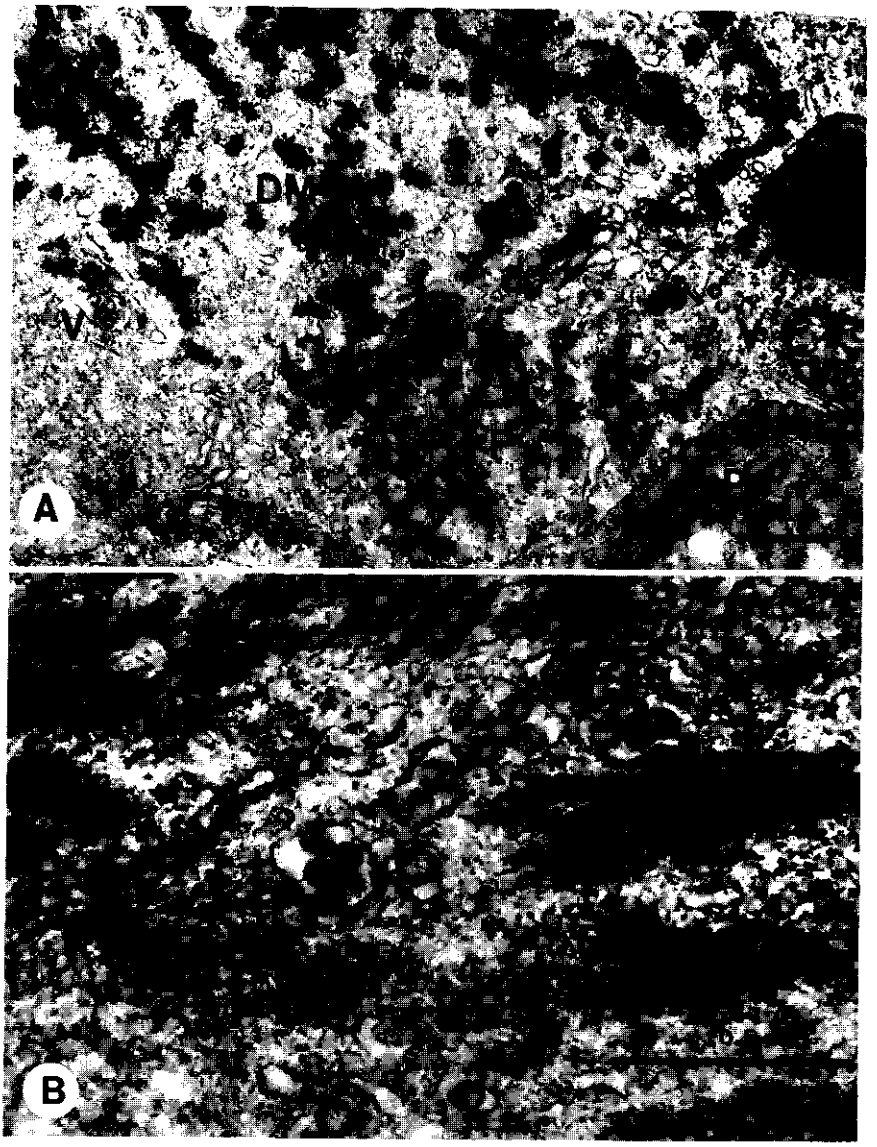

Figure 5: Electron micrographs of the intracellular location of fibrous structures in tissue sections of *N. clevelandii* infected with TSWV isolate S1 (A) and *D. stramonium* infected with isolate INSV NL-07 (B), respectively. Fibrous structures consisting of either flexible filaments (A) or paracrystalline rods (B) are shown. Tissue sections were from osmium fixed preparations. F, fibrous structures; V, virus particles; DM, dense masses; M, mitochondrion; P, chloroplast. The bars in both electron micrographs represent 0.5 μm.

immunostain less intensively than the flexible filaments. On the other hand, virion particles were not specifically labelled when using anti-NS_S serum (data not shown), confirming the conclusion drawn from the Western blot analysis that the NS_S protein indeed represents a non-structural protein. The specificity of the labelling with the NS_S antiserum was clearly demonstrated by double labelling experiments in which sections were first incubated with NS_S antiserum labelled with gold particles of 7 nm, followed by a treatment with nucleocapsid antiserum labelled with gold particles of 15 nm. The 15 nm gold particles were mainly found on virus particles, viroplasm and dense masses, while the small 7 nm gold particles consistently tagged the fibrous material (Fig. 6c). Some isolates did not induce the formation of flexible filaments or paracrystalline rods. With such isolates the NS_S antiserum reacted dispersed throughout the cytoplasm (Fig. 6d). Time course experiments furthermore showed that specific labelling of the cytoplasm with NS_S antibodies started 6 days post infection, i.e. when the first symptoms of TSWV infection became visible.

DISCUSSION

The results presented in this paper demonstrate that TSWV S RNA specifies, in addition to the nucleoprotein, a non-structural protein (NS_S) of 52.4 kDa, that may form long, filamentous masses in the cytoplasm of infected cells. From sequence data and comparison with the genetic organisation of the S RNAs of the related animal *Bunyaviridae*, we already suggested previously that such protein would be expressed from S RNA (De Haan *et al.*, 1990). For the definite detection of this protein the production of specific antibodies was crucial. These were obtained after producing the NS_S protein in a heterologous expression system, i.e. the baculovirus/insect cell system.

Using a novel transfer vector (Zuidema *et al.*, 1990), a recombinant baculovirus was obtained that expressed the TSWV NS_S protein to similar levels as the polyhedrin protein in wt baculovirus infected insect cells (Fig.3). Antibodies raised against NS_S protein thus produced reacted with a protein in TSWV-infected *N. rustica* similar in size

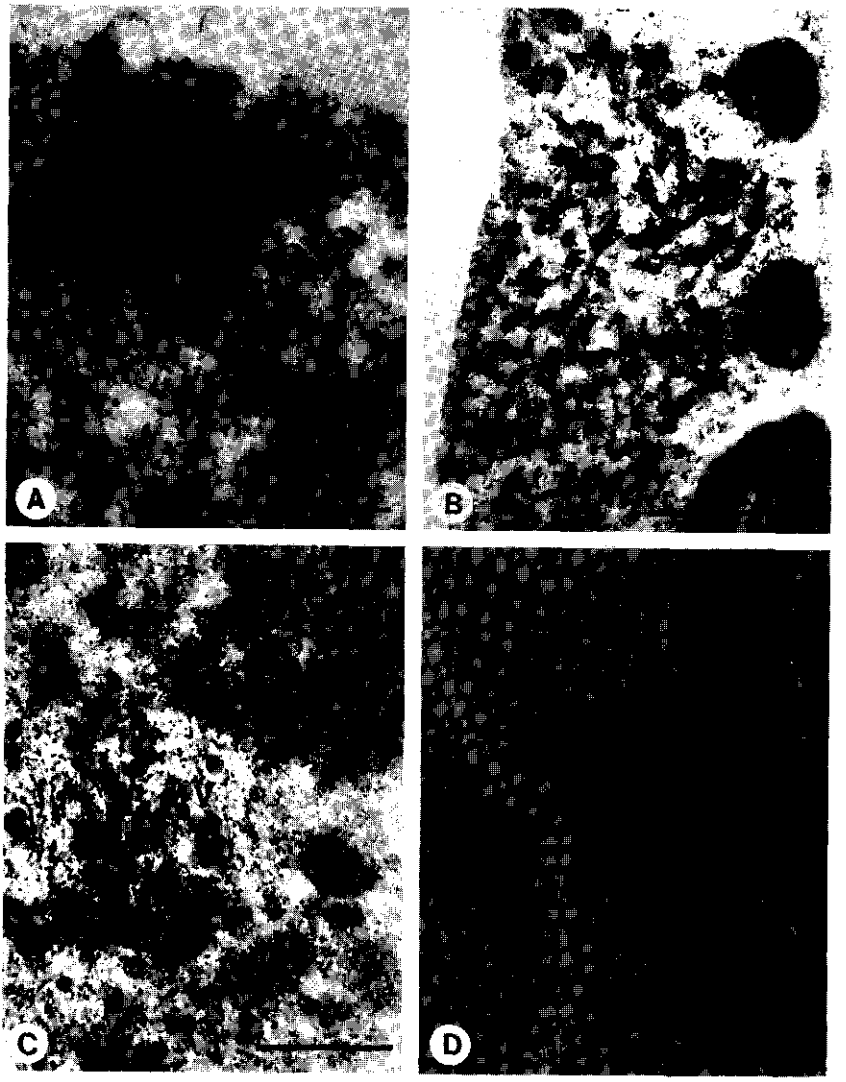

Figure 6: Sections of TSWV-infected *N. rustica* leaf tissue, treated with antiserum against NS_S and complexed with protein A-gold. Fibrous structures (F) consisting of flexible filaments (panel A) or rigid rods (panel B) types, present in cells infected by TSWV isolate NL-04 and INSV NL-07 respectively, were labelled with antiserum against NS_S. Gold particles were enhanced by silver. C: A double labelling experiment in a cell infected by TSWV isolate S1. The section was incubated with NS_S antiserum, labelled with 7 nm pAg, and then treated with nucleocapsid (isolate BR-01) antiserum labelled with 15 nm pAg. D: Cytoplasm of a leaf parenchyma cell of *N. rustica* infected by isolate BR-01, treated with antiserum against NS_S and complexed with protein A-gold. DM, M and P refer to dense masses, mitochondrion and chloroplast, respectively. The bars in all electron micrographs correspond to 0.5 μm.

as the NS_S protein produced by the recombinant baculovirus. This protein appeared to be present in the cytoplasm of infected cells and (virtually) absent in purified virions, as demonstrated both by Western blot analysis and immunogold electron microscopy.

As a first step to resolve the function of NS_S in the TSWV infection cycle, ultrathin sections of *N. rustica* plants infected with different isolates of TSWV were screened for the presence of NS_S using immunogold labelling techniques. It appeared that for approximately 50% of the TSWV isolates tested, fibrous structures were found that reacted with the antiserum against NS_S. The amount and morphology of these fibrous structures differed from isolate to isolate. For some isolates only small amounts of fibrous material, consisting of flexible filaments, were found while for other isolates large amounts were discernible or appeared in well organised paracrystalline arrays (Fig.6A and B; Kitajima *et al.*, 1992).

Similar fibrous structures, associated with TSWV infections, were previously described (Francki and Grivell, 1970; De Avila *et al.*, 1990; Law and Moyer, 1990) but never analysed in terms of the presence of virus-encoded proteins. Although Law and Moyer (1990) suggested that the paracrystalline arrays of filamentous structures consist of nucleocapsids, the double immunolabelling experiments presented in this paper (Fig.6C) demonstrate that these cytopathological structures do hardly or not contain N protein.

Although the immunogold labelling experiments unequivocally demonstrate the presence of the NS_S protein within the fibrous material, the question whether these fibrous structures are solely made up of NS_S protein remains to be answered. In relation to this it is interesting to note that recombinant baculovirus Ac33DZ1/NS_S-1 infected *S. frugiperda* cells, containing high amounts of NS_S protein, do not reveal fibrous structures (data not shown). This observation supports the hypothesis that the appearance of fibrous structures in TSWV infected plants is not a matter of concentration only. It is also not clear why for some TSWV isolates the NS_S protein is organised in fibrous structures while for others it is found dispersed throughout the cytoplasma. A more detailed and extensive description on the cytopathology will be published elsewhere.

As mentioned before, both the structural organisation and ambisense coding strategy

of TSWV S RNA show striking similarities to those of the S RNAs of phleboviruses, another genus of the *Bunyaviridae*. In fact these similarities have been important reasons to propose TSWV as a member of the *Bunyaviridae*, being unique in its property to infect plants (De Haan *et al.*, 1990). For phleboviruses, the S RNA also contains two ORFs in ambisense arrangement, one encoding the viral nucleoprotein and the other a non-structural protein. Although the nucleoproteins of TSWV and phleboviruses share a similar molecular weight (approximately 25-29 kDa), the NS_S protein of TSWV is significantly larger (52.4 kDa) than those of the phleboviruses (approximately 30 kDa). This raises the question whether the TSWV NS_S protein contains an extra domain, that could be involved in plant infection-related processes, and thus represents an adaptation of bunyaviruses to plants. So far, only the NS_S protein of Punta Toro phlebovirus has been analysed (Overton *et al.*, 1987). In contrast to the results with TSWV, the NS_S protein of this phlebovirus was found associated with virions and nucleocapsids. The amounts of the phleboviral NS_S protein detected were low and its function during the infection cycle has remained unclear.

Also the function of the TSWV NS_S protein in infection, though specifically localized in infected plant cells, still remains to be elucidated. The similarities in genome structure and expression strategy of the S RNAs of TSWV and phleboviruses may indicate similar functions for the NS_S protein. Along this line, it is worthwhile mentioning that intranuclear filaments, observed in Rift Valley fever phlebovirus infected cells, have been proposed to correspond to a virally encoded non-structural protein (Swanepoel and Blackburn, 1977; Struthers and Swanepoel, 1982). Whatever the primary function of the NS_S protein may be, preliminary results seem to indicate a correlation between the amount of NS_S protein produced and the severity of disease symptoms induced (De Haan and Kormelink, unpublished results). Analyses of TSWV-infected plants by dot blot immunobinding assay suggest that severe TSWV isolates express, in general, higher amounts of NS_S protein than mild isolates. Also, time course analyses of total RNA from TSWV BR-01 infected *N. rustica* point in that direction since the synthesis of NS_S mRNA starts just before symptoms are apparent (Chapter 2).

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REFERENCES

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

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

Brown, M. and Faulkner, P. (1977). A plaque assay for nuclear polyhedrosis viruses using a solid overlay. *Journal of General Virology* **36**, 361-364.

Cho, J.J., Mau, R.F.L., Mitchell, W.C., Gonsalves, D. and Yudin, L.S. (1987). Host list of plants susceptible to tomato spotted wilt virus (TSWV). *Research extension series*, Honolulu University of Hawaii.

Clark, M.F. and Adams, A.N. (1977). Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *Journal of General Virology* **34**, 475-483.

De Avila, A.C., Pena, L., Kitajima, E.W., De O. Resende, R., Mugica, M.V.D., Ruiz, J.R.D. and Peters, D. (1990). Characterization of a tomato spotted wilt virus (TSWV), isolated from *Capsicum annuum L.* in the Canary Islands. *Phytopathologia Mediterranea* **30**, 23-28.

De Haan, P., Wagemakers, L., Goldbach, R. and Peters, D. (1989a). Tomato spotted wilt virus, a new member of the *Bunyaviridae*?. In *Genetics and pathogenicity of negative strand viruses*, 287-290. Edited by D. Kolakofsky & B.W.J. Mahy, Elsevier Science Publishers B.V. , Amsterdam.

De Haan, P., Wagemakers, L., Peters, D. and Goldbach, R.W. (1989b). Molecular cloning and terminal sequence determination of the S and M RNAs of tomato spotted wilt virus. *Journal of General Virology* **70**, 3469-3473.

De Haan, P., Wagemakers, L., Peters, D. and Goldbach, R.W. (1990). The S RNA of tomato spotted wilt virus has an ambisense character. *Journal of General Virology* **71**, 1001-1007.

De Haan, P., Kormelink, R., Resende, R. de O., Van Poelwijk, F., Peters, D. and Goldbach, R. (1991). Tomato spotted wilt virus L RNA encodes a putative RNA polymerase. *Journal of General Virology* **71**, 2207-2216.

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

Hink, W.F. (1970). Established insect cell line from the cabbage looper, *Trichoplusia ni. Nature* (London) **226**, 466-467.

Ihara, T, Akashi, H. and Bishop, D.H.L. (1984). Novel coding strategy (ambisense genomic RNA) revealed by sequence analyses of Punta Toro phlebovirus S RNA. *Virology* **136**, 293-306.

Kitajima, E.W., Avila, A.C., Resende, R.O., Goldbach, R.W. and Peters, D. (1990). Comparative cytological and immunogold labelling studies on different isolates of tomato spotted wilt virus. *Journal of Submicroscopical Cytology and Pathology* **24**, 1-14.

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

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

Lee, C., Levin, A. and Branton, D. (1987). Copper staining: A five-minute protein stain for sodium dodecyl sulfate-polyacrylamide gels. *Analytical Biochemistry* **166**, 308-312.

Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982). *Molecular Cloning: A Laboratory Manual.* New York: Cold Spring Harbor Laboratory.

Marriott, A.C., Ward, V.K. and Nuttall, P.A. (1989). The S RNA segment of sandfly fever Sicilian virus: Evidence for an ambisense genome. *Virology* **169**, 341-345.

Matthews, R.E.F. (1982). Classification and nomenclature of viruses: Fourth report of the International Committee on Taxonomy of Viruses. *Intervirology* **17**, 1-199.

Milne, R.G. and Francki, R.I.B. (1984). Should tomato spotted wilt virus be considered as a possible member of the family *Bunyaviridae*? *Intervirology* **22**, 72-76.

Mohamed, N.A., Randles, J.W. and Francki, R.I.B. (1973). Protein composition of tomato spotted wilt virus. *Virology* **56**, 12-21.

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

Overton, H.A., Ihara, T. and Bishop, D.H.L. (1987). Identification of the N and NS_S proteins coded by the ambisense S RNA of Punta Toro phlebovirus using monospecific antisera raised to baculovirus expressed N and NS_S proteins. *Virology* **157**, 338-350.

Sakimura, K (1962). The present status of thrips-borne viruses. *Maramorosch, Biological transmission of disease agents*, 33-40, Academic Press, New York.

Sanger, F., Nicklen, S. and Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences, USA* **74**, 5463-5467.

Simons, J.F., Hellman, U. and Pettersson, R.F. (1990). Uukuniemi virus S RNA segment: Ambisense coding strategy, packaging of complementary strands into virions, and homology to members of the genus *Phlebovirus*. *Journal of Virology* **64**, 247-255.

Smith, G.E., Summers, M.D. and Fraser, M.J. (1983). Production of human beta interferon in insect cells infected with a baculovirus expression vector. *Molecular and Cellular Biology* **3**, 2156-2165.

Southern, E. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of Molecular Biology* **98**, 503-518.

Struthers, J.K. and Swanepoel, R. (1982). Identification of a major non-structural protein in the nuclei of Rift Valley fever virus-infected cells. *Journal of General Virology* **60**, 381-384.

Summers, M.D. and Smith, G.E. (1987). *A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures.* College Station: Texas A & M University.

Swanepoel, R. and Blackburn, N.K. (1977). Demonstration of nuclear immunofluorescence in Rift Valley fever infected cells. *Journal of General Virology* **34**, 557-561.

Tas, P.W.L., Boerjan, M.L. and Peters, D. (1977). The structural proteins of tomato spotted wilt virus. *Journal of General Virology* **36**, 267-279.

Van Den Hurk, J., Tas, P.W.L. and Peters, D. (1977). The ribonucleic acid of tomato spotted wilt virus. *Journal of General Virology* **36**, 81-91.

Van Lent, J.W.M., Groenen, J.T.M., Klinge-Roode, E.C., Rohrmann, R., Zuidema, D. and Vlak, J.M. (1990). Localization of the 34 kd polyhedral envelope protein in *Spodoptera frugiperda* cells infected with *Autographa californica* nuclear polyhedrosis virus. *Archives of Virology* **111**, 103-114.

Vaughn, J.L., Goodwin, R.H., Tompkins, G.J. and McCawley (1977). The establishment of two cell lines from the insect *Spodoptera frugiperda*. *In Vitro* **13**, 213-217.

Zuidema, D., Schouten, A., Usmany, M, Maule, A.J., Belsham, G.J., Klinge-Roode, E.C., Van Lent, J.W.M. and Vlak, J.M. (1990). Expression of cauliflower mosaic virus gene I using a novel polyhedrin-based baculovirus expression vector. *Journal of General Virology* **71**, 2201-2209.

Chapter 6

Expression and subcellular location of the NS_M protein of tomato spotted wilt virus, a putative viral movement protein[5]

SUMMARY

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

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INTRODUCTION

Tomato spotted wilt virus (TSWV) is the type species of the genus *Tospovirus*, in which a number of plant-infecting bunyaviruses have been classified (De Haan *et al.*, 1989; Elliott, 1990; Milne and Francki, 1984; Francki *et al.*, 1991). Whereas animal-infecting members of the *Bunyaviridae* are mainly transmitted by ticks, mosquitoes and sandflies (Elliott, 1990), TSWV is exclusively transmitted by thrips in a persistent manner (Sakimura, 1962).

Recently, the complete nucleotide sequence of the genome of TSWV has become available (De Haan *et al.*, 1990, 1991; Chapter 4). The TSWV L RNA is 8897 nucleotides (nt) and completely of negative polarity, encoding the putative viral RNA polymerase (De Haan *et al.*, 1991). The M RNA is 4821 nt and has an ambisense gene arrangement. It encodes a putative nonstructural protein (NS_M) of 33.6 kDa in viral (v) sense, and the precursor to the glycoproteins (G1 and G2) of 127.4 kDa in viral complementary (vc) sense (Chapter 4). The S RNA is 2916 nt and has, similar to the M RNA, an ambisense gene arrangement. This genome segment encodes a nonstructural protein (NS_S) of 52.4 kDa in v sense and the nucleocapsid (N) protein of 28.8 kDa in vc sense (De Haan *et al.*, 1990; Chapter 5).

Comparison of the TSWV genome with those of animal-infecting bunyaviruses (Elliot, 1990; Francki *et al.*, 1991) reveals the presence of one extra gene in the former, i.e. the NS_M gene located on the M RNA segment (Chapter 4). This extra gene, therefore, may reflect an adaptation of bunyaviruses to botanical hosts.

In order to investigate the function of the NS_M protein in the infection cycle of TSWV, this protein was expressed in the *E. coli* pET-11t system to enable the production of a specific polyclonal antiserum. Using this antiserum both the synthesis and the intracellular location of NS_M during TSWV multiplication was analysed.

MATERIALS AND METHODS

Virus, plants and cDNA clones

TSWV isolate BR-01 was maintained in *Nicotiana rustica* "America" plants by thrips transmission and mechanical inoculation. Virus was purified according to the method of Tas *et al.* (1977). Nucleocapsids were isolated from infected leaf tissue as described by de Avila *et al.* (1990), omitting the sucrose gradient step. Complementary DNA clones representing the M RNA of TSWV BR-01 have been described previously (Chapter 4). Wild type (wt) and recombinant *Autographa californica* nuclear polyhedrosis viruses (AcNPV) were grown in monolayers of *Spodoptera frugiperda* 21 cells (Vaughn *et al.*, 1977) in TNMFH medium (Hink, 1970) containing 10% fetal calf serum.

Construction of AcNPV recombinant virus

The baculovirus *Autographa californica* Multiple Nuclear Polyhedrosis Virus (AcNPV) was used for eukaryotic expression of NS_M in *Spodoptera frugiperda* insect cells. Complementary DNA clone pTSWV28 containing the complete open reading frame (ORF) of NS_M was digested with *Bam*HI and cloned in the *Bam*HI site of pAc33DZ1 (Chapter 4; Zuidema *et al.*, 1990). The resulting transfer vector, pAc33DZ1/NS_M+L (+L denotes the presence of the TSWV-specific 5'-untranslated sequence), contained the complete ORF of NS_M including the TSWV viral 5'-untranslated sequence.

For convenient cloning of the NS_M gene without the viral 5'-untranslated sequence, the gene was amplified by polymerase chain reaction (PCR) using oligonucleotides Zup51 (dGGGAATTCTTTTCGGTAACAAGAGGCC), containing 27 nucleotides of which 21 are identical to nucleotides 109 to 129 of the viral (v) strand of M RNA, and Zup14 (dCCCTGCAGGATCCGAAATTTAAGCTTAAATAAGTG), having 22 nucleotides complementary to nucleotides 1022 to 1043 of the vRNA strand (Fig.1A). After PCR-amplification, the DNA was digested with *Eco*RI, a *Bam*HI-*Eco*RI adaptor (containing the nucleotide sequence GGATCCGGCAACGAAGGTACCATGGGAATTC), with an internal start codon, ligated in order to restore the NS_M ORF, and subsequently digested with *Bam*HI to

generate the NS_M gene as a *Bam*HI fragment. This fragment was purified from an agarose gel, cloned in the *Bam*HI site of plasmid pAc33DZ1 resulting in transfer vector pAc33DZ1/NS_M. Recombinant baculoviruses were produced by co-transfection of *S. frugiperda* (Sf) cells with a mixture of *BSu*36 I digested AcNPV PAK6 DNA and pAc33DZ1/NS_M + L or pAc33DZ1/NS_M DNA according to Kitts and Possee (1993).

Recombinant baculoviruses were plaque purified (Brown and Faulkner, 1977) and subsequently grown in high titer stocks. Analysis of proteins from infected *S. frugiperda* on SDS-PAGE were as described previously (Chapter 5).

Construction of pET-11t/NS_M

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

Expression in *Escherichia coli*

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

For SDS-PAGE analyses of the proteins, the cells were subsequently boiled in 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 2% (w/v) SDS, 10% (v/v) glycerol, 0.001% bromophenol

blue, 5% (v/v) beta-mercaptoethanol (protein loading buffer). Samples were analysed on a 12.5% polyacrylamide gel containing SDS (Laemmli, 1970).

Purification of NS_M protein

For large-scale preparation of the TSWV NS_M protein, proteins from 4 ml IPTG-induced *E.coli* cells transformed with pET-11t/NS_M were resolved on 0.75-mm-thick preparative 12.5% polyacrylamide gels containing SDS (Protean II system, Bio-Rad). The NS_M protein was purified as described previously for NS_S (Chapter 5).

Preparation of antibodies to the NS_M protein

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

Western immunoblot analyses

Samples from TSWV-infected *N. rustica* were prepared by homogenizing 0.1 g of systemically infected leaves in 0.1 ml PBS containing 0.05% Tween-20. After combining the extract with 4x protein loading buffer, 10 μl of healthy- and TSWV-infected *N. rustica* extracts were applied on a 12.5% SDS-polyacrylamide gel. After SDS-PAGE, proteins were transferred to Immobilon membrane (Millipore) and screened with polyclonal antisera as described previously (Chapter 5).

Immunogold labelling of NS_M protein in tissue sections of TSWV-infected *N. rustica* plants

In situ detection of NS_M protein was performed in tissue sections of TSWV-infected plants, fixed in paraformaldehyde/glutaraldehyde, dehydrated and embedded in LRGold at low temperature. Tissue sections were prepared from systemically infected leaves from 5 days post infection (p.i.) on. For immunolabelling, sections were treated with NS_M antiserum at a 1:500 dilution in PBS-BSA for 2 hr, using essentially the protocols

described by Van Lent *et al.* (1990).

Preparation of subcellular extracts from TSWV-infected leaves

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

RESULTS

Construction of recombinant baculovirus AcNPV/NS_M

To express the NS_M gene in the baculovirus/insect cell system a cDNA fragment from clone pTSWV28 (Chapter 4) containing the complete ORF and most of the 5'-untranslated sequence of the TSWV NS_M gene (nucleotides 10 to 1065 of the vRNA strand of TSWV M RNA) was cloned into plasmid pAc33DZ1 (Zuidema *et al.*, 1990), and transferred to baculovirus AcNPV by co-transfection of *S. frugiperda* cells with a

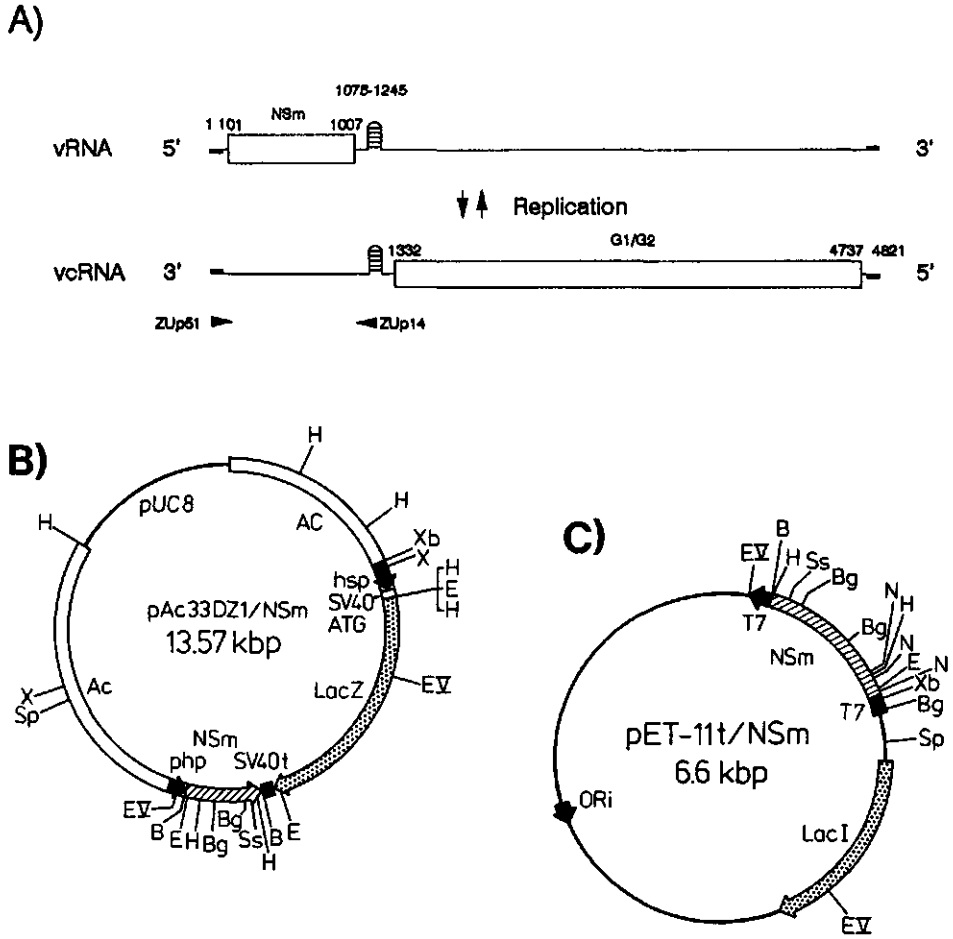

Figure 1: Genetic organisation of TSWV M RNA (A) and vectors used for the expression of NS_M in eucaryotic (B) and procaryotic (C) systems. The position of primers ZUp51 and ZUp14 used for PCR-amplification of NS_M are indicated. The replication of the vRNA strand into the vcRNA strand is indicated by the arrowheads. The black boxes at the termini of the RNA strands represent conserved TSWV complementary sequences (A). The NS_M gene was PCR-amplified with primers ZUp51 and ZUp14, and cloned into pAc33DZ1 as described in Materials and Methods. The resulting transfervector pAc33DZ1/NS_M was used for the production of recombinant baculoviruses (B). For cloning into the pET-11t expression vector, PCR-amplified NS_M was digested with *Eco*RI and *Bam*HI. The resulting vector pET-11t/NS_M was used for expression in *E.coli* (C). Restriction enzymes are abbreviated as follows: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; EV, *Eco*RV; H, *Hind*III; N, *Nco*I; Sp, *Sspl*; Ss, *Sstl*; Xb, *Xba*I; X, *Xho*I.

mixture of *BSu*36 I digested AcNPV PAK6 DNA (Kitts and Possee, 1993) and pAc33DZ1/NS_M + L (+ L stands for the presence of the TSWV-specific 5'-untranslated sequence). In addition, an NS_M construct lacking the viral 5'-untranslated sequence was PCR-amplified using oligonucleotides Zup51 and Zup14 (Fig.1A; Materials and Methods), cloned into pAc33DZ1 (Fig.1B) to form transfer vector pAc33DZ1/NS_M, and transferred to AcNPV. Recombinants of AcNPV containing the NS_M gene with (AcNPV/NS_M + L) or without (AcNPV/NS_M) the TSWV 5'-untranslated sequence were isolated and the production of the NS_M protein was analysed by comparing the protein patterns of the recombinant baculovirus infected *S. frugiperda* cell extracts with that of

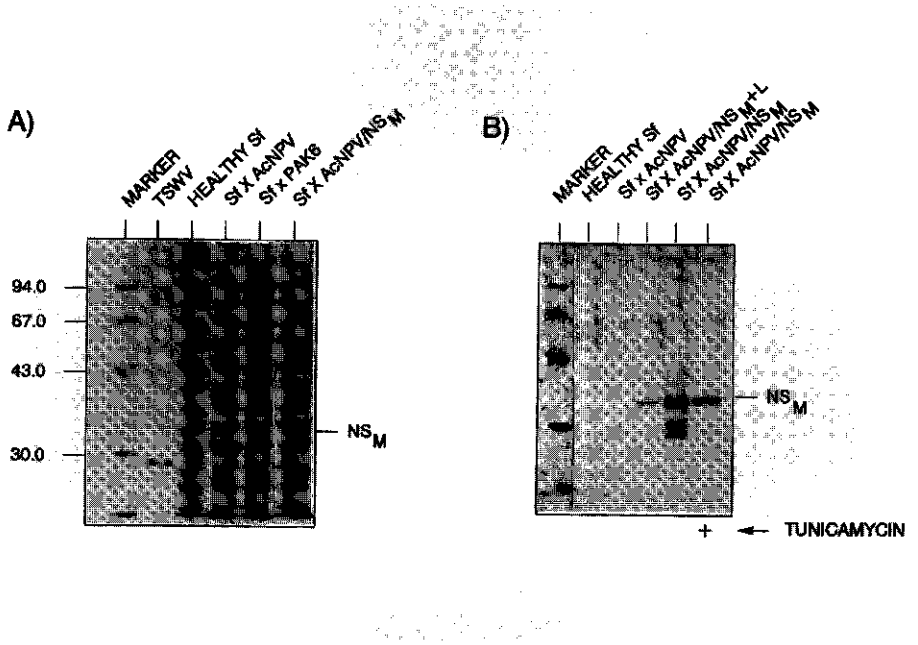

Figure 2: Production of TSWV NS_M protein in AcNPV/NS_M + L and AcNPV/NS_M -infected *S. frugiperda* (Sf) cells. Proteins from infected Sf cells were resolved on a SDS-polyacrylamide gel and stained with Coomassie brilliant blue (A). As controls healthy, wild type AcNPV-infected and AcNPV PAK6 infected Sf cells were included in the analyses. Low-molecular-weight size markers (Pharmacia) are indicated at the left. The NS_M protein expressed by the recombinant baculovirus is indicated. Western blot analysis of NS_M protein produced in recombinant baculovirus-infected Sf cells using antiserum against *E.coli* expressed NS_M (B). Also included is a protein sample of Sf cells infected with the AcNPV/NS_M + L construct, which included the TSWV NS_M 5'-untranslated sequence. The last lane contains a protein sample of AcNPV/NS_M-infected Sf cells grown under 25 µg/ml tunicamycin pressure, indicated with a "+".

wild type AcNPV-infected *S. frugiperda* cell extract. No protein band corresponding to the expected size of the TSWV NS_M protein (33.6 kDa; Chapter 4) was identified for the AcNPV/NS_M + L recombinant (data not shown), but a minor protein band at the expected position was identified for the AcNPV/NS_M recombinant (Fig.2B, lane SfxAcNPV/NS_M). However, the amounts of NS_M produced were too low to be purified for the production of a polyclonal antiserum.

Expression of the NS_M gene in *E.coli*

In order to produce larger quantities of the NS_M protein for the production of a polyclonal antiserum, it was decided to express the NS_M protein in *E.coli* using expression vector pET-11t. To this end, the PCR-amplified NS_M construct previously cloned into pAc33DZ1, was digested with *Eco*RI and *Bam*HI and cloned in frame with the ATG start codon in pET-11t. Clones containing the NS_M gene were selected and analysed by restriction enzyme analyses. The nucleotide sequence in the resulting pET-11t/NS_M construct (Fig.1C) was verified in order to confirm the intactness of the open reading frame.

The production of NS_M protein in pET-11t/NS_M transformed BL21 cells was induced with IPTG, and analysed by SDS-PAGE. A protein band corresponding to the expected size of the NS_M protein was clearly visible (Fig.3A, lane pET-11t/NS_M). The NS_M protein produced in this way was purified (Fig.3A, lane NS_M) and subsequently used for the production of antibodies.

Antiserum to NS_M protein

Purified NS_M protein was injected into rabbits four times at intervals of 1-2 weeks. After the third injection, blood was collected, the immunoglobulin fraction isolated, and the antiserum tested for the presence of antibodies against NS_M protein. A sample of pET-11t/NS_M transformed BL21 cells, induced with IPTG, was subjected to electrophoresis, transferred to Immobilon membrane, and analysed by immunoblot analysis using 1 μg/ml of NS_M antiserum. The results demonstrate the presence of antibodies against denatured NS_M (Fig.3B, lane pET-11t/NS_M), although also

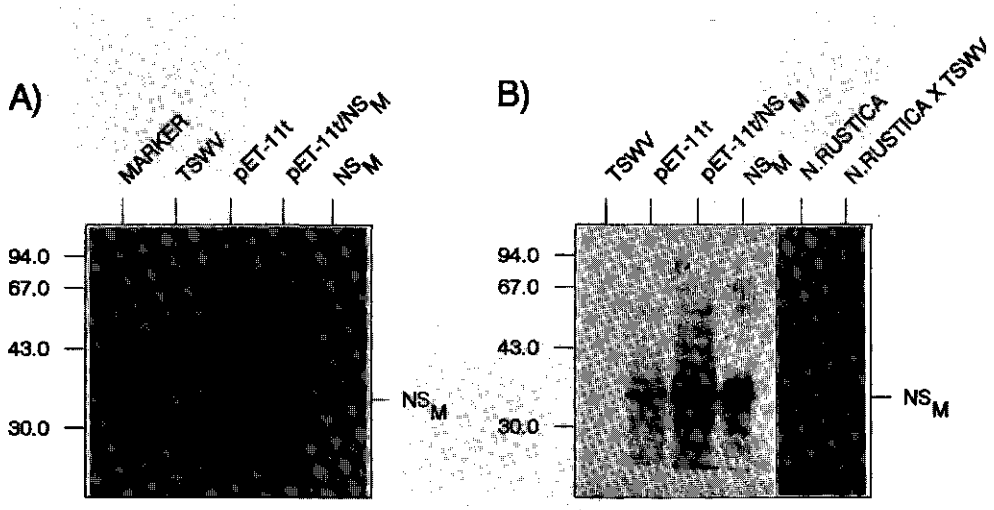

Figure 3: Production of TSWV NS_M protein in *E.coli* (A) and specificity of the antibodies raised against TSWV NS_M protein (B). The production of NS_M in *E.coli* was induced with IPTG, and analysed on Coomassie brilliant blue stained SDS-polyacrylamide gel (A). Purified NS_M protein used for the production of antibodies is shown in the fifth lane. As a control pET-11t transformed BL21 cells was included. Low-molecular-weight size markers (Pharmacia) are indicated at the left. The NS_M protein expressed in *E.coli* is indicated. Similar protein samples were analyzed on a Western blot to test the specificity of antibodies raised against the NS_M protein (B). A sample of 250 ng purified TSWV BR-01 virus and 10 µl portions of extracts from healthy- and TSWV-infected *N. rustica* plants were included. The Western blot was analyzed using 1 µg/ml NS_M antiserum.

immunoglobulins against proteins co-purified from BL21 cells were present (Fig.3B, lane pET-11t). Moreover, the antiserum detected specifically very low amounts of a protein with the expected size of NS_M in insect cells infected with the AcNPV/NS_M+L recombinant baculovirus (Fig.2B, lane SfxAcNPV/NS_M+L), which was clearly absent from healthy or wt AcNPV-infected insect cells. A higher level of expression of this protein was found when the TSWV NS_M 5'-untranslated sequence was absent from the baculovirus construct (Fig.2B, lane SfxAcNPV/NS_M). These results showed that the protein detected originated from the TSWV NS_M coding sequences in AcNPV/NS_M, and demonstrated the specificity of the anti-NS_M serum. Also, the absence of any cross reaction with proteins from healthy or wt baculovirus-infected insect cells indicated that the contamination of immunoglobulins against proteins co-purified from BL21 cells did

not interfere with immunological analyses. Hence it was anticipated that these contaminations would not interfere with further immunological analyses in plant systems. Some smaller protein bands, probably stable degradation products, were often seen in AcNPV/NS_M-infected insect cells that were reacting with anti-NS_M serum (Fig.2B, lane SfxAcNPV/NS_M; Fig.6A, lane SfxAcNPV/NS_M).

Analysis of the nucleotide sequence of the NS_M gene demonstrated the presence of two potential glycosylation sites in the NS_M protein (Chapter 4). Western immunoblot analysis of NS_M protein produced from the AcNPV/NS_M recombinant in the presence of tunicamycin (25 μg/ml) revealed no detectable shift in migration of this protein indicating that, at least in insect cells, this viral protein is not glycosylated (Fig.2B, lane SfxAcNPV/NS_M "+").

Detection of NS_M protein in TSWV-infected plant material

In order to establish the actual production of NS_M during the TSWV infection cycle, plant extracts from healthy and systemically infected *Nicotiana rustica* leaves were analysed on Western blots using the antiserum raised against *E.coli* expressed NS_M. In extracts from infected plants a protein could be detected with the expected size of NS_M (Fig.3B, lane *N. rustica* x TSWV) which was absent from healthy plant extracts (Fig.3B, lane *N. rustica*), confirming the viral origin of the protein detected in TSWV-infected plants. Additionally, a protein of about 67 kDa specifically reacted with antiserum against NS_M in TSWV-infected *N. rustica*, most likely representing a dimer of NS_M. No reaction was obtained with purified TSWV particles (Fig.3B, lane TSWV), demonstrating that this viral protein indeed represents a nonstructural protein.

To follow the synthesis of NS_M during infection of *N. rustica*, plant extracts, prepared from systemically infected leaves at different times after inoculation, were analysed on Western immunoblots. The results revealed that the production of NS_M, and the putative NS_M dimer (NS_M"), was maximal at days 6 and 7 p.i., coinciding with the appearance of systemic symptoms, and drastically decreased at 8 days p.i. (Fig.4A). The kinetics of NS_M accumulation is therefore clearly distinct from that of N protein and NS_S, the amounts of which accumulated throughout later stages of infection (Fig.4B). These data indicate

a transient character of NS_M and the involvement in an early process during TSWV infection. During the course of infection, another protein of about 40 kDa weakly cross-reacted with the anti-NS_M serum. The identity of this band is not clear, however, its presence in extracts of healthy *N. rustica* (Fig.3B, lane N.rustica) suggests that this protein is of host origin.

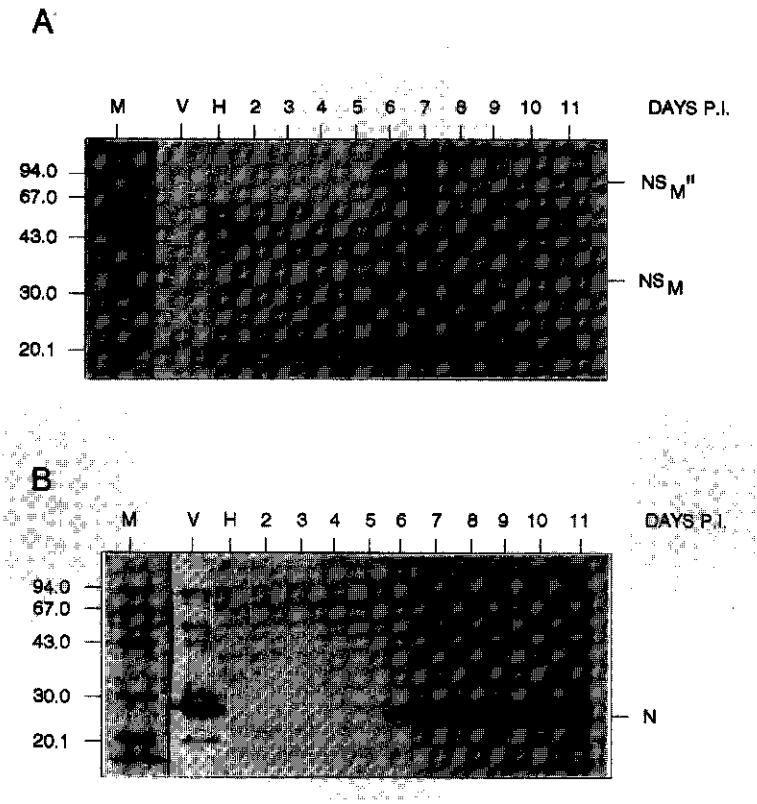

Figure 4: Time course of synthesis of NS_M (A) and N (B) protein in TSWV-infected *N. rustica*. The plant extracts were prepared from systemically infected leaves at different times after inoculation, indicated at the top of the figure, as described in Materials and Methods. Ten µl of plant extract was applied on a 12.5% SDS-polyacrylamide gel. As controls, 250 ng purified TSWV virus (V), and an extract of healthy *N. rustica* (H) were included. After Western blotting on Immobilon membranes, the filters were screened with 1 µg/ml anti-NS_M (A) or anti-TSWV serum (B). Low-molecular-weight size markers (Pharmacia) are indicated at the left. The positions of N, NS_M and a potential dimer of NS_M (denoted as NS_M") are indicated.

Intracellular localization of the NS_M protein

To determine the intracellular position of the NS_M protein and to get a clue about its function during the TSWV infection cycle, ultrathin sections of TSWV-infected *N. rustica* were prepared for electron microscopy and analysed with immunogold labelling. NS_M was found associated with electron dense aggregates (Fig.5A), previously identified as nonenveloped, viral nucleocapsid aggregates (Kitajima *et al.*, 1992). Labelling with antiserum against the N protein confirmed the composition of this electron dense material (Fig.5B). On the other hand, the NS_M protein could not be detected in mature virus particles (data not shown) indicating that during further assembly, the NS_M protein is absent from the viral nucleocapsids.

Additionally, the NS_M protein was found to be associated with plasmodesmata (Fig.5C and D). Within the plasmodesmata the NS_M label was frequently arranged along electron dense extensions (Fig.5D). No labelling as such was ever found in healthy *N. rustica* demonstrating its viral-specific character. The association of NS_M with plasmodesmata suggests a possible involvement in cell-to-cell movement, a function that has not been assigned to any of the proteins encoded by the genome of TSWV yet. Analyses of TSWV-infected cells also showed that in cells where relatively high amounts of NS_M were detected, no enveloped virus particles were detected, while in cells that contained enveloped particles, hardly or no NS_M protein could be detected, again indicating that NS_M has a transient function early in the infection of the cell.

Subcellular localization of NS_M

To confirm the intracellular location of the NS_M protein, different fractions from infected leaf material were isolated and analysed for the presence of the NS_M protein. To this end, cytoplasmic nucleocapsid (TSWV-nu) fractions were prepared from TSWV-infected *N. rustica* at day 6 and day 9 p.i.. Additionally, by another method, subcellular

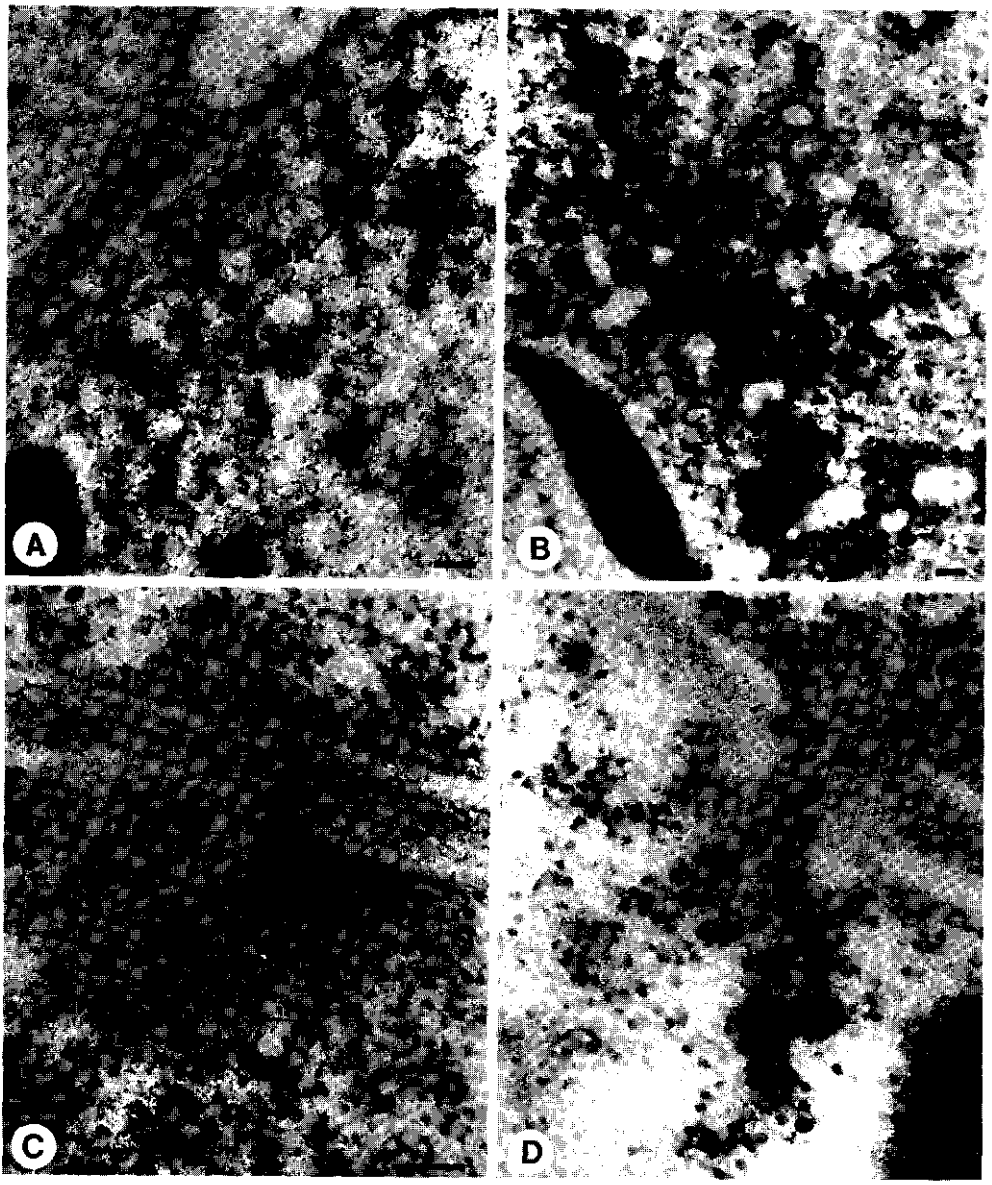

Figure 5: Electronmicrographs of ultrathin sections of TSWV-infected *N. rustica* leaf tissue, immunogold labelled with antiserum against NS_M (A, C and D), and antiserum against N (B). Scale bars represent 200 nm (A and B) and 100 nm (C and D), respectively. PD: Plasmodesma; NA: Nucleocapsid aggregates; CW: Cell wall.

fractions from TSWV-infected leaves were prepared according to Deom *et al.* (1990), and the resulting fractions (Pe-1, Pe-30 and S-30) applied on an SDS-polyacrylamide gel and transferred to Immobilon membranes. Western immunoblot analysis revealed that NS_M co-purified with nucleocapsids, and that it disappeared from those structures in later stages during the infection cycle (Fig.6A, lane TSWV-NU 6 days p.i. and 9 days p.i.). A control Western immunoblot was analysed with anti-N serum to confirm the presence of equal amounts of nucleocapsids in both samples (data not shown). The NS_M protein present in nucleocapsid extracts, though, migrated slower than NS_M produced in the baculovirus system (Fig.6A, lane SfxAcNPV/NS_M). This migration difference was not genuine but rather due to a difference in protein content or ionic strength of the samples.

Using a different fractionation protocol, it could be demonstrated that the NS_M protein was mainly present in the Pe-1 and Pe-30 fractions (Fig.6B). A similar Western blot treated with antiserum against the TSWV N protein revealed highest amounts of N protein in the S-30 fraction (data not shown), indicating that the NS_M protein detected in the Pe-1 and Pe-30 samples was specifically associated with these subcellular fractions, which contained enriched amounts of cell wall and cytoplasmic membrane material (Deom *et al.*, 1990). The presence of high amounts of nucleocapsids in the S-30 fraction suggests that the low amounts of NS_M protein detected in this fraction was probably due to its association with nucleocapsids. In order to test this, the S-30 fraction was further fractionated, via a sucrose-cushion, into a pellet (S-30P) that was enriched for nucleocapsids, and a supernatant (S-30S) fraction. Subsequent Western immunoblot analysis showed the presence of NS_M in the S-30P fraction and the absence in the S-30S fraction (Fig.6B, lane S-30P and S-30S). The subcellular fraction data, therefore, are in agreement with the electron microscopical data that showed a close association of NS_M with plasmodesmata and nucleocapsid aggregates.

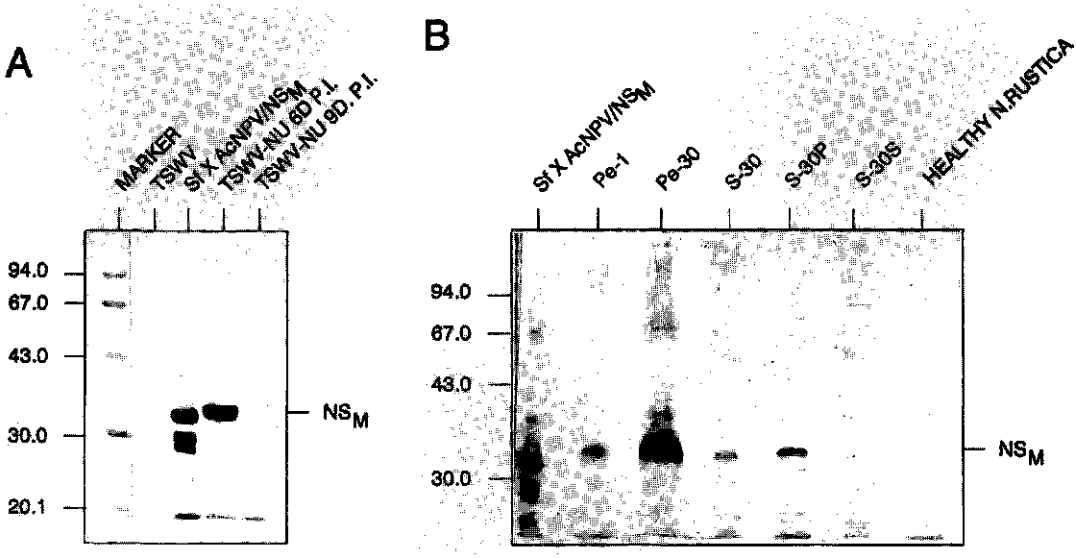

Figure 6: Presence of NS_M in nucleocapsid fractions (A) and subcellular fractions of TSWV-infected *N. rustica* (B). Purification of nucleocapsids and preparation of different fractions of TSWV-infected *N. rustica* leaf material were as described in Materials and Methods. Ten µl of nucleocapsid preparations (TSWV-NU) and 5 µl of the subcellular fractions (Pe-1, Pe-30, S-30, S-30P and S-30S) were applied on a 12.5% SDS-polyacrylamide gel. The proteins were transferred to Immobilon membrane and the filter subsequently screened with 1 µg/ml antiserum against NS_M. As controls were included, 250 ng purified TSWV virus, a protein extract of AcNPV/NS_M-infected *S. frugiperda* (Sf) cells and an extract of healthy *N. rustica*. The position of NS_M is indicated.

DISCUSSION

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

their mode of cell-to-cell movement or about the possible involvement of viral gene products therein.

Comparison of the genome of TSWV with those of animal-infecting members of the *Bunyaviridae* already suggested that NS_M represents a possible candidate for a viral movement protein, as it is specified by an extra open reading frame, not present in the genome of animal-infecting bunyaviruses. In order to gain insight into the function of this protein, an antiserum was produced against NS_M expressed in *E. coli* and its actual synthesis demonstrated in infected *N. rustica*. Time course analyses showed the presence of NS_M only during a short period early in the infection cycle (Fig.4A), always coinciding with the first appearance of systemic symptoms, in contrast to other TSWV specific proteins that further accumulated from that moment on (Fig.4B). This timing may be a first indication that the NS_M protein could actually represent the putative viral movement protein, in agreement with the suggestion by Atabekov and Dorokhov (1984) that virus movement is an early function which is eventually switched off. Similar transient expression has been demonstrated for several other (putative) movement proteins, e.g. the TMV 30 K protein (Watanabe *et al.*, 1984; Lehto *et al.*, 1990) and the AlMV P3 protein (Berna *et al.*, 1986).

Immunogold labelling experiments of ultrathin sections of TSWV-infected *N. rustica* revealed the presence of NS_M in nucleocapsid aggregates and in close association with plasmodesmata. In the latter case, NS_M was often found arranged along electron dense extensions connected to the plasmodesmata. Furthermore, the NS_M protein was only found in those cells where mature, enveloped particles were not yet assembled. In case the latter were present, no NS_M could be detected, providing another indication that NS_M is associated with a process early in the infection cycle of a cell. Similar electron microscopic observations of transient expression were found in case of the (putative) movement proteins of AlMV (Stussi-Garaud *et al.*, 1987) and TMV (Tomenius *et al.*, 1987).

Subcellular localization studies on TSWV-infected *N. rustica* 6 to 7 days p.i. confirmed the intracellular location of NS_M, i.e. the protein was especially found located in the fractions relatively enriched for cytoplasmic membranes (Fig.6B, lane Pe-30) and

cell wall residues (Fig.6B, lane Pe-1), respectively. The presence of NS_M in these fractions suggests that the electron dense extensions, found to contain NS_M in immunogold labelling studies, are somehow specifically associated with the cytoplasmic membranes and/or cell walls. To be more conclusive on this assoiation, the exact nature of these structures has to be analysed. The NS_M protein was also found in low amounts in the S-30 fraction. This localization could be explained by its association to nucleocapsids in this fraction. Hence, the time course analysis, the immunogold studies and the fractionation studies provide strong indications for the involvement of the NS_M protein, as the putative viral movement protein, in the cell-to-cell movement of TSWV.

It is doubtful whether TSWV is transported in the form of enveloped particles through the plasmodesmata as these particles have a diameter of 80-120 nm, requiring drastic modification of plasmodesmata (effective diameter 3 nm, Terry and Robards, 1987). Instead it is more likely that the TSWV genome is transported as infectious ribonucleocapsid structures. This idea is further supported by several observations. Firstly, the NS_M protein is found associated with ribonucleocapsid structures from which it apparently detaches during later stages of the infection cycle (Fig.6A, cf. lanes TSWV-NU 6 and 9 days p.i) resulting in its absence from mature virus particles (Fig.6A, lane TSWV). In this context it is worthwhile mentioning that the NS_M protein contains an acidic carboxy-terminus which could establish the binding of NS_M to the rather basic N protein within the nucleocapsid structures (Chapter 4). Secondly, morphologically defective isolates of TSWV, i.e. isolates which are deficient in glycoprotein synthesis and lack the lipid envelop (Ie, 1982; Verkleij and Peters, 1983; Resende *et al.*, 1991), are able to spread through plant tissues at wild type rate.

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REFERENCES

Atabekov, J.G. and Dorokhov, Y.L. (1984). Plant virus-specific transport function and resistance of plants to viruses. *Advances in Virus Research* **29**, 313-364.

Atabekov, J.G. and Talianski, M.E. (1990). Expression of a plant virus-encoded transport function by different viral genomes. *Advances in Virus Research* **38**, 201-248.

Avila, A.C. de, Huguenot, C., Resende, R. de O., Kitajima, E.W., Goldbach, R.W. and Peters, D. (1990). Serological differentiation of 20 isolates of tomato spotted wilt virus. *Journal of General Virology* **71**, 2801-2807.

Berna, A., Briand, J.-P., Stussi-Garaud, C. and Godefroy-Colburn, T. (1986). Kinetics of accumulation of the three non-structural proteins of Alfalfa mosaic virus in tobacco plants. *Journal of General Virology* **67**, 1135-1147.

Brown, M. and Faulkner, P. (1977). A plaque assay for nuclear polyhedrosis viruses using a solid overlay. *Journal of General Virology* **36**, 361-364.

Citovsky, V., Knorr, D., Schuster, G. and Zambryski, P. (1990). The p30 movement protein of tobacco mosaic virus is a single-strand nucleic acid binding protein. *Cell* **60**, 637-647.

Clark, M.F. and Adams, A.N. (1977). Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *Journal of General Virology* **34**, 475-483.

De Haan, P., Wagemakers, L., Goldbach, R. and Peters, D. (1989). Tomato spotted wilt virus (TSWV), a new member of the *Bunyaviridae* ? In: "*Genetics and pathogenicity of negative-strand viruses*", p.287-291, Kolakofsky, D. and Mahy, B. eds, Elsevier Science Publishers.

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

De Haan, P., Kormelink, R., Resende, R. de O., Van Poelwijk, F., Peters, D. and Goldbach, R. (1991). Tomato spotted wilt virus L RNA encodes a putative RNA polymerase. *Journal of General Virology* **72**, 2207-2216.

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

Deom, C.M., Schubert, K., Wolf, S., Holt, C.A., Lucas, W.J. and Beachy, R.N. (1990). Molecular characterization and biological function of the movement protein of tobacco mosaic virus in transgenic plants. *Proceedings of the National Academy of Sciences USA* **87**, 3284-3288.

Deom, C.M., Lapidot, M. and Beachy, R.N. (1992). Plant virus movement proteins. *Cell* **69**, 221-224.

Elliott, R.M. (1990). Molecular biology of the *Bunyaviridae*. *Journal of General Virology* **73**, 501-522.

Francki, R.I.B., 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. *Archives of Virology* suppl. **2**.

Goldbach, R., Eggen, R., de Jager, C., van Kammen, A., van Lent, J., Rezelman, G. and Wellink, J. (1990). Genetic organization, evolution and expression of plant viral RNA genomes. In: *Recognition and response in plant-virus interactions* (R.S.S. Fraser, ed.). NATO ASI series H, vol. 41, p. 147-162, Springer Verlag, Berlin, Heidelberg.

Gorbalenya, A.E., Blinov, V.M., Donchenko, A.P. and Koonin, E.V. (1989). An NTP-binding motif is the most conserved sequence in a highly diverged monophyletic group of proteins involved in positive strand RNA viral replication. *Journal of Molecular Evolution* **28**, 256-268.

Hink, W.F. (1970). Established insect cell line from the cabbage looper, *Trichoplusia ni*. *Nature* (London) **226**, 466-467.

Hull, R. (1991). The movement of viruses within plants. *Seminars in Virology* **2**, 89-95.

Ie, T.S. (1982). A sap-transmissible, defective form of tomato spotted wilt virus in plant cells. *Virology* **59**, 387-391.

Kitajima, E.W., Avila, A.C. de, Resende, R. de O., Goldbach, R.W. and Peters, D. (1992). Comparitive cytological and immunogold labeling studies on different isolates of tomato spotted wilt virus. *Journal of submicroscopical cytology and pathology* **24**, 1-14.

Kitts, P.A. and Possee, R.D. (1993). A method for producing recombinant baculovirus expression vectors at high frequency. *Biotechniques* **14**, 810-817.

Koonin, E.V., Mushegian, A.R., Ryabov, E.V. and Dolja, V.V. (1991). Diverse groups of plant RNA and DNA viruses share related movement proteins that may possess chaperone-like activity. *Journal of General Virology* **72**, 2895-2903.

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

Lehto, K., Bubrick, P. and Dawson, W.O. (1990). Time course of TMV 30K protein accumulation in intact leaves. *Virology* **174**, 290-293.

Maule, A.J. (1991). Virus movement in infected plants. In: *Critical Reviews in Plant Sciences* **6**, p.457-473.

Milne, R.G. and Francki, R.I.B. (1984). Should tomato spotted wilt virus (TSWV) be considered as a possible member of the family *Bunyaviridae* ? *Intervirology* **22**, 72-76.

Resende, R. de O., De Haan, P., Avila, A.C.de, 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. *Journal of General Virology* **72**, 2375-2383.

Sakimura, K. (1962). The present status of thrips-borne viruses. In: "*Biological transmission of disease agents*", p.33-40, Maramorosch, K. ed., Academic Press, New York.

Stussi-Garaud, C., Garaud, J.-C., Berna, A. and Godefroy-Colburn, T. (1987). *In situ* location of an alfalfa mosaic virus non-structural protein in plant cell walls: Correlation with virus transport. *Journal of General Virology* **68**, 1779-1784.

Tas, P.W.L., Boerjan, M.L. and Peters, D. (1977). The structural proteins of tomato spotted wilt virus. *Journal of General Virology* **36**, 267-279.

Terry, B.R. and Robards, A.W. (1987). Hydrodynamic radius alone governs the mobility of molecules through plasmodesmata. *Planta* **171**, 145-157.

Tomenius, K., Clapham, D. and Meshi, T. (1987). Localization by immunogold cytochemistry of the virus-coded 30K protein in plasmodesmata of leaves infected with tobacco mosaic virus. *Virology* **160**, 363-371.

Van Lent, J.W.M., Groenen, J.T.M., Klinge-Roode, E.C., Rohrmann, R., Zuidema, D. and Vlak, J.M. (1990). Localization of the 34kd polyhedral envelope protein in *Spodoptera frugiperda* cells infected with *Autographa californica* nuclear polyhedrosis virus. *Archives of Virology* **111**, 103-114.

Verkleij, F.N. and Peters, D. (1983). Characterization of a defective form of tomato spotted wilt virus. *Journal of General Virology* **64**, 677-686.

Vaughn, J.L., Goodwin, R.H., Tompkins, G.J. and McCawley (1977). The establishment of two cell lines from the insect *Spodoptera frugiperda*. *In Vitro* **13**, 213-217.

Watanabe, Y., Emori, Y., Ooshika, I., Meshi, T., Ohno, T. and Okada, Y. (1984). Synthesis of TMV-specific RNAs and proteins at the early stage of infection in tobacco protoplasts: Transient expression of the 30K protein and its mRNA. *Virology* **133**, 18-24.

Zuidema, D., Schouten, A., Usmany, M, Maule, A.J., Belsham, G.J., Klinge-Roode, E.C., Van Lent, J.W.M. and Vlak, J.M. (1990). Expression of cauliflower mosaic virus gene I using a novel polyhedrin-based baculovirus expression vector. *Journal of General Virology* **71**, 2201-2209.

Chapter 7

Heterologous expression of the glycoprotein precursor (G1/G2) and nucleoprotein (N) genes of tomato spotted wilt virus

SUMMARY

The tomato spotted wilt virus S RNA-encoded nucleoprotein gene and M RNA-encoded glycoprotein precursor gene were cloned into transfer vector pAc33DZ1 and inserted dowstream of the polyhedrin promoter in the *Autographa californica* nuclear polyhedrosis virus genome. Recombinant baculoviruses were obtained that showed a high-level expression of the 28.8 kDa nucleoprotein. Recombinant baculoviruses containing the glycoprotein precursor gene were found to express the glycoproteins G1 (78 kDa) and G2 (58 kDa), though at lower levels, only detectable on immunoblots. Using tunicamycin it was established that both G1 and G2 undergo N-linked glycosylation. This glycosylation takes place at the stage of the non-processed G1/G2 precursor protein.

INTRODUCTION

Like all members of the *Bunyaviridae* (Elliott, 1991), tomato spotted wilt virus (TSWV), type species of the genus *Tospovirus* (Francki *et al.*, 1991), has enveloped particles that contain two surface glycoproteins G1 (78 kDa) and G2 (58 kDa) (Tas *et al.*, 1977; Mohamed *et al.*, 1973). The core consists of ribonucleocapsid structures, each composed of a single stranded RNA segment tightly associated with the nucleoprotein (N) of 28.8 kDa and minor amounts of the L protein (331.5 kDa), the putative viral polymerase (Van Poelwijk *et al.*, 1993). The TSWV genome consists of 3 single-stranded linear RNA segments denoted S RNA (small), M RNA (medium) and L RNA (large). The L RNA encodes the putative viral RNA polymerase (De Haan *et al.*, 1991). The M RNA encodes, in an ambisense arrangement, the nonstructural protein NS_M, implicated in viral movement and the (G1/G2) precursor to the glycoproteins (Chapter 4 and 6). The S RNA encodes, also in ambisense arrangement, a second nonstructural protein (NS_S) and the N protein (De Haan *et al.*, 1990; Chapter 5).

A characteristic feature of the animal-infecting members of the *Bunyaviridae* is their intracellular maturation by budding of the nucleocapsids at smooth-surfaced membranes of the Golgi complex (Murphy *et al.*, 1973; Lyons and Heyduk, 1973; Smith and Pifat, 1982; Booth *et al.*, 1991). For several of these viruses, studies demonstrated that the viral glycoproteins are synthesized and cleaved from a large precursor at the ER and subsequently targeted to the Golgi complex where they are retained (Ulmanen *et al.*, 1981; Kuismanen, 1984; Kuismanen *et al.*, 1984). This process occurs even in the absence of virus maturation (Gahmberg *et al.*, 1986), as well as when the glycoproteins are expressed by recombinant vaccinia viruses (Wasmoen *et al.*, 1988; Matsuoka *et al.*, 1988; Chen *et al.*, 1991; Pensiero and Hay, 1992; Ruusala *et al.*, 1992), or by SV40 vectors in COS cells (Ronnholm, 1992). Following the accumulation of viral glycoproteins at certain loci in the Golgi complex, viral ribonucleoproteins (RNPs) are found to condense only at the cytoplasmic face of these areas prior to budding (Smith and Pifat, 1982; Kuismanen *et al.*, 1984). This phenomenon has not been observed when the viral

glycoproteins are absent (Smith and Pifat, 1982). Hence, an interaction between the viral glycoproteins, e.g. a cytoplasmic domain, and the nucleocapsid protein could be involved as a trigger for budding into the Golgi vesicles.

During the infection cycle of TSWV, virus particles accumulate in the lumen of the rough endoplasmatic reticulum (RER) (Kitajima, 1965; Francki and Grivell, 1970; Milne, 1970; Ie, 1971; Kitajima *et al.*, 1992). However, further information on the processes leading to maturation of TSWV particles is lacking, neither it is known whether the Golgi complex is involved. As a first step towards understanding the biosynthesis and maturation of the viral glycoproteins, and the function of the N protein in the process of budding, these structural proteins of TSWV were expressed in the heterologous baculovirus/insect cell expression system.

MATERIALS AND METHODS

Viruses, cells and cDNA clones

TSWV isolate BR-01 was maintained in *Nicotiana rustica* "America" plants by thrips transmission and mechanical inoculation. Complementary DNA clones representing the S and M RNA clones of isolate BR-01 were described previously (De Haan *et al.*, 1990; Chapter 4). Wild type (wt) and recombinant *Autographa californica* nuclear polyhedrosis viruses (AcNPV) were grown in monolayers of *Spodoptera frugiperda* 21 cells (Vaughn *et al.*, 1977) in TNMFH medium (Hink, 1970) containing 10% fetal calf serum.

Construction of the AcNPV recombinant transfer vectors

Plasmid vector pAc33DZ1 (Zuidema *et al.*, 1990) was used to construct the transfer vector containing the TSWV N or glycoprotein precursor (GP) gene. A bacterial plasmid containing the complete open reading frame (ORF) of N (pTSWV-vcORF; De Haan *et al.*, 1990) was digested with *Kpn*I and *Hin*dIII in order to completely excise the N-ORF from pSK+. After repair with T4 DNA polymerase, *Bam*HI linkers were ligated and the N-ORF subsequently cloned in the *Bam*HI site of pAc33DZ1 according to Maniatis *et*

al. (1982). The complete ORF of the GP gene was reconstructed via a three-points ligation of an *Eco*RI fragment from clone pTSWV43, containing the coding sequences for the N-terminal part of the precursor to the glycoproteins, and of pTSWV17-12, containing the coding sequences for the C-terminal (Chapter 4), into the *Eco*RI site of pET-11t. As pTSWV43 contained one *Eco*RI site located just 5 nucleotides downstream the ATG start codon sequence (ATGAGAATTC...) of the GP ORF, digestion with *Eco*RI lead to the loss of the ATG start codon sequence from TSWV43. The reading frame for the GP gene, however, was restored again by an "in frame" ligation with an ATG start codon sequence provided by the *Nco*I site, located just 6 nucleotides upstream of the *Eco*RI site (sequence CCATGGGAATTC...) in the multiple cloning linker of pET-11t. Clone pTSWV17-12 provided the genuine TGA stop codon for the GP ORF. The resulting pET-11t/GP construct contained a complete GP-ORF that could be excised from pET-11t as an *Nco*I fragment. After repair with T4 DNA polymerase, *Bam*HI linkers were added and the fragment cloned into the *Bam*HI site of pAc33DZ1. The resulting recombinant plasmids, pAc33DZ1/N and pAc33DZ1/GP, were verified for their constitution by restriction enzyme analysis and sequence analysis at the insertion sites.

Construction of AcNPV recombinant virus

Recombinant baculoviruses expressing the N gene were produced by cotransfection of *S. frugiperda* cells with a mixture of AcNPV DNA and pAc33DZ1/N DNA (Smith *et al.*, 1983). Recombinants containing the GP gene were obtained after cotransfection with a mixture of *Bsu*36 I digested AcNPV PAK6 DNA and pAc33DZ1/GP (Kitts and Possee, 1993). After 3 days of incubation at 27°C, nonoccluded virus (NOV) was collected from the medium and titrated in dilutions to render separated plaques in a plaque assay (Brown and Faulkner, 1977). Recombinant plaques were plaque purified and grown in high-titer stocks.

Analyses of recombinant AcNPV DNA

S. frugiperda cells were infected with wt or recombinant AcNPV with a multiplicity

of 20 $TCID_{50}$ units per cell and incubated at 27°C for 4 days. NOV was collected and DNA was purified according to Summers and Smith (1987). Viral DNA samples were digested with *Bam*HI and the restriction fragments were resolved by electrophoresis in 1% agarose gels.

SDS-PAGE of proteins from infected *S. frugiperda* cells

S. frugiperda cells were infected in portions of $5x10^6$ cells, with a multiplicity of 20 $TCID_{50}$ units per cell, and incubated at 27°C for 48-52 hr. The cells were collected, washed twice with PBS and resuspended in 200 µl PBS. For SDS-PAGE analyses of proteins, the cells were boiled in 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 2% (w/v) SDS, 10% (v/v) glycerol, 0.001% (w/v) bromophenol-blue, 5% (v/v) ß-mercaptoethanol (protein loading buffer). Samples were analysed on a 12.5% SDS-polyacrylamide gel (Laemmli, 1970).

Immunoblot analyses

TSWV-BR01 virions were purified according to Tas *et al.* (1977). After SDS-PAGE, proteins were transferred to Immobilon membrane (Millipore) by electroblotting in an LKB Transphor electroblotting unit at 60 V overnight in 20 mM Tris-HCl, pH 8.3, 150 mM glycine, 20% (v/v) methanol at 4°C. Membranes were dried, washed in PBS containing 0.3% BSA (PBS-BSA) and blocked for 3 hr in 3% BSA (in PBS) at room temperature. After several washings with PBS-BSA, membranes were incubated in the same buffer containing 1 µg/ml antiserum for 1 hr. After washing, antigen-antibody complexes were detected using 1 µg/ml alkaline phosphatase conjugated goat-anti-rabbit immunoglobulins (Tago Inc., Burlingame, CA, USA), using 0.33 mg/ml nitroblue tetrazolium (NBT) and 0.165 mg/ml bromochloroindolyl phosphate (BCIP) as a substrate.

Tunicamycin treatment of infected *S. frugiperda* cells

S. frugiperda cells were infected with wt or recombinant AcNPV with a multiplicity of 20 $TCID_{50}$ units per cell. Tunicamycin was added to the infected cells from a stock

solution, to a final concentration of 25 μg/ml in the tissue culture medium. The drug was added the same time the cells were infected and was maintained until the cells were lysed at 60 hr post infection. Cellular extracts were prepared for SDS-PAGE and Western blot analysis as described above.

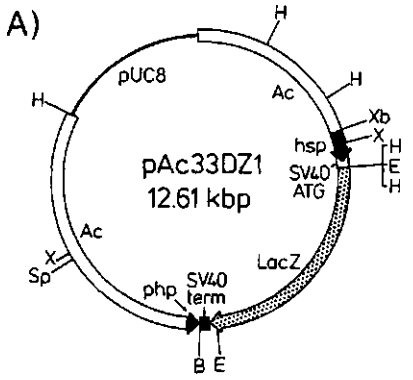
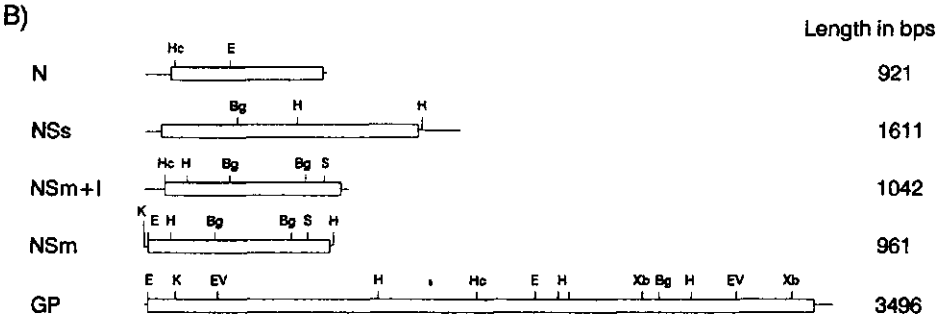


Figure 1: Constructs used for baculovirus expression of the TSWV M and S RNA encoded genes. Schematical representation of pAc33DZ1 (A), and the TSWV cDNA constructs (B) to be expressed in the baculovirus system. The nucleotide sequences of the TSWV cDNA constructs from the *Bam*HI site in pAc33DZ1 until the ATG-start codon is shown (C).



C)

N GGATCCCCCCCTTAACACTCAGTCTTACAAATCATCACATTAAGAACCTAAGAAACGACTGCGGGATACAGAGTTGCACTTT
NS_S GGATCCGAATTCAATTTTGTTCATAATCAAACC
NS_H+L GGATCCGGGTACCATGGCAGAAATATACCTATTATACATTTTGCTAAGAA
NS_H
GP

N CGCACCTTGAGTTACATACGGTCAAAGCATATAACAACTTTTACGATCATCATGTCTAAGGTTAAGCT
NS_S TCACTTAGAAAATCACAATACTGTAATAAGAACACAGTACCAATAACCATAATGTCAACAAGTGTTTA
NS_H+L TCAATCAACTACATTACACAAGCTCCTCTACCTTAGGCTGTTGAACTCGAAATGTTGACTCTTTTCGG
NS_H GGATCCGGCAACGAAGGTACCATGGGAATTCTTTTCGG
GP GGATCCGGCATGGGAATTCTAAAACT

RESULTS

Construction and analyses of recombinant baculoviruses

Complementary DNA fragments containing the complete ORFs of the TSWV N and GP gene were reconstructed as described in Materials and Methods, and subsequently cloned into the *Bam*HI site of pAc33DZ1. The resulting transfer vectors, i.e. pAc33DZ1/N and pAc33DZ1/GP, were analysed by restriction enzyme analysis and nucleotide sequence determination (Fig.1). The N and GP genes were transferred to AcNPV by co-transfection of *S. frugiperda* cells with a mixture of wt AcNPV or AcNPV linearized PAK6 DNA and the transfer vector containing the respective genes. NOV-DNA from these recombinants, i.e. AcNPV/N and AcNPV/GP, was isolated and its composition analyzed by *Bam*HI restriction enzyme analysis (Fig.2). For comparison, previously made recombinants containing the TSWV NS_S and NS_M genes (Chapter 5 and 6) were included. Insertion of the N and GP genes was confirmed by Southern blot analyses (data not shown). The level and correctness of transcription of TSWV specific sequences from the polyhedrin promoter were verified by Northern blot analyses and showed a similar level in all recombinants (data not shown). In this way recombinant AcNPV/N and AcNPV/GP were selected for protein expression analyses.

Expression of the N and GP protein

Production of the N and GP protein was analysed by comparing the protein patterns of AcNPV/N and AcNPV/GP-infected *S. frugiperda* cells with that of wt AcNPV-infected *S. frugiperda* cells. A major protein band corresponding to the size of the N protein (29 kDa; De Haan *et al.*, 1990) was identified for the AcNPV/N recombinant (Fig.3, lane AcNPV/N). The level of expression was similar to that of the TSWV NS_S (52.4 kDa; Fig.3, lane AcNPV/NS_S) or AcNPV polyhedrin protein (Fig.3, lane AcNPV). However, no protein bands that could correspond to the glycoprotein precursor (expected size 127.4 kDa) or the processed G1 (78 kDa) and G2 (58 kDa) glycoproteins were visible in the protein extract AcNPV/GP-infected *S. frugiperda* cells (Fig.3, lane AcNPV/GP). The same results were obtained with three other separately plaque-purified AcNPV/GP

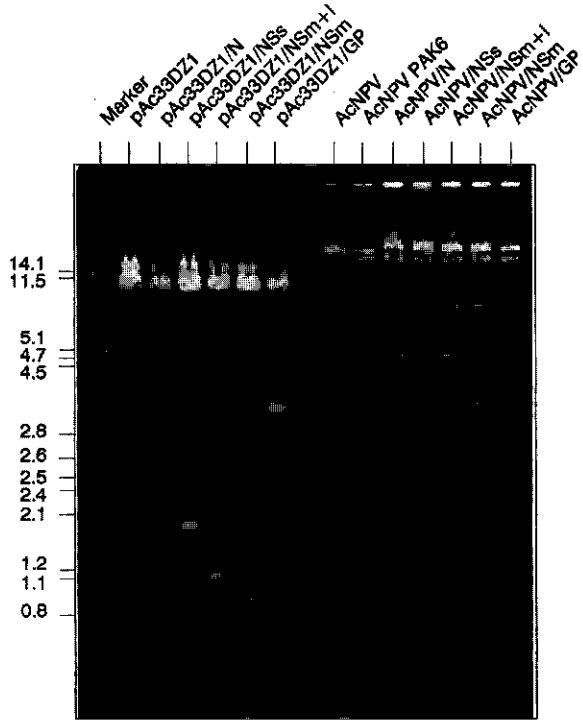


Figure 2: *Bam*HI restriction pattern of baculovirus recombinants AcNPV/N and AcNPV/GP. NOV DNA was purified from the recombinant viruses and digested with *Bam*HI. The resulting restriction fragments were resolved on a 1% agarose gel. Wild type (wt) AcNPV, AcNPV PAK6 and baculovirus recombinants containing the TSWV NS_S and NS_M genes, i.e. AcNPV/NS_S, AcNPV/NS_M+1 and AcNPV/NS_M, were included for analyses. The *Bam*HI restriction fragments of wt AcNPV DNA are sized 86.5, 23.3, 8.50, 3.45, 3.33, 1.92 and 0.96 kb, respectively. Lambda DNA digested with *Pst*I was included as a size marker.

recombinants. As the level of transcription of the GP gene from the polyhedrin promoter was similar to that of the N gene within the AcNPV/N recombinant (data not shown), the low amount of GP expression was regulated at the level of translation.

Immunoblot analyses of TSWV N and GP protein synthesized in insect cells

In order to analyse the presence of low levels of the TSWV GP, G1 or G2 protein produced in *S. frugiperda* cells, Western immunoblot analyses were performed on

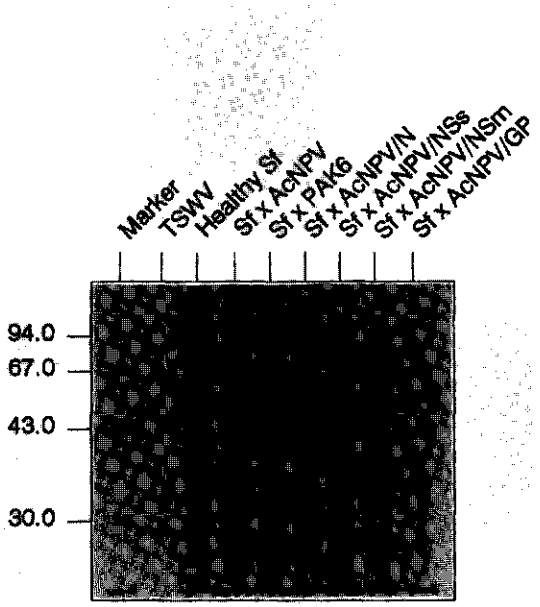
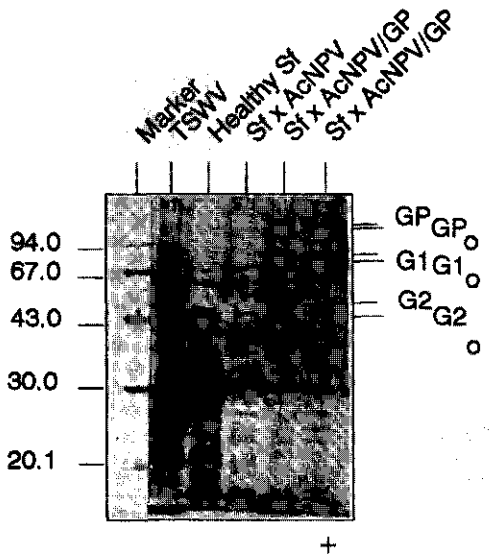


Figure 3: Production of TSWV N and GP-specific proteins in recombinant AcNPV-infected Sf insect cells. Proteins from recombinant baculovirus-infected Sf cells were resolved on a SDS-polyacrylamide gel and stained with Coomassie brilliant blue. *Spodoptera frugiperda* cells infected with wt AcNPV, AcNPV PAK6, AcNPV/NS_S and AcNPV/NS_M were included in the analyses. Low- molecular-weight (LMW) size markers (Pharmacia) are indicated at the left.

recombinant AcNPV infected cell extracts. Protein blots of AcNPV/GP-infected *S. frugiperda* extracts probed with antiserum raised against whole TSWV particles demonstrated the presence, at low levels, of two specific proteins (Fig.4, lane AcNPV/GP) that were absent in wt AcNPV-infected insect cells (Fig.4, lane AcNPV). These two protein products co-migrated with G1 and G2 of purified TSWV (Fig.4, lane TSWV), and therefore most likely represent the glycoproteins. This indicates that the glycoprotein precursor gene is expressed and correctly processed in insect cells, as expected for this eukaryotic system. Additionally, a larger protein with an estimated size of about 140 kDa specifically reacted with the antiserum against whole TSWV particles. The size of this protein is approximately in agreement with the sum of G1 and G2 (glycosylated), and its absence from wt AcNPV-infected insect cells (Fig.4, lane AcNPV) moreover suggests that this protein most likely represents the precursor to the glycoproteins.

Western immunoblot analyses of AcNPV/N-infected *S. frugiperda* cell extracts demonstrated that the major protein produced indeed was the TSWV N protein. It co-migrated with the TSWV N protein (Fig.5, lane TSWV) and reacted with antiserum raised against purified TSWV particles, as well as with antiserum raised against purified TSWV nucleocapsids (Fig.5, lane AcNPV/N; Avila *et al.*, 1993).

Figure 4: Western immunoblot analysis of TSWV GP-specific proteins produced in Sf insect cells. Proteins from AcNPV/GP-infected Sf cells were resolved on a 12.5% SDS-polyacrylamide gel, transferred to Immobilon membrane and subsequently screened with 1 µg/ml anti-TSWV serum. As controls healthy Sf cells, wt AcNPV-infected Sf cells and 250 ng purified TSWV BR-01 virus were included. Low-molecular-weight markers (Pharmacia) are indicated at the left. Expression of the glycoproteins in the presence of 25 µg/ml tunicamycin is indicated by a "+".



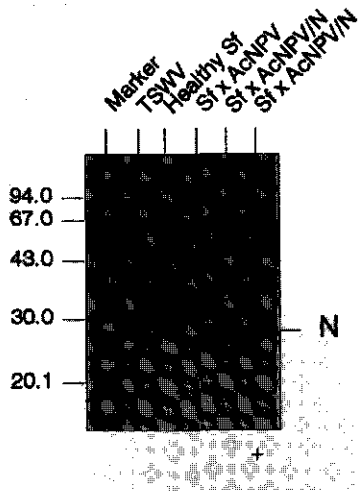


Figure 5: Western immunoblot analysis of TSWV N protein produced in Sf insect cells. Proteins from AcNPV/N-infected Sf cells were resolved on a 12.5% SDS-polyacrylamide gel, transferred to Immobilon membrane and subsequently screened with 1 µg/ml anti-TSWV serum. Controls were as for Fig.4. Low-molecular-weight (LMW) markers (Pharmacia) are indicated at the left. Expression of the N protein in the presence of 25 µg/ml tunicamycin is indicated by a "+".

Post translational modification of TSWV proteins produced in insect cells

Previous studies regarding glycosylation of the structural proteins of TSWV always showed some discrepancy. Two different groups had previously shown that the TSWV 78 kDa and 58 kDa proteins are glycosylated (Tas *et al.*, 1977; Mohamed *et al.*, 1973), but Mohamed *et al.* (1973) also suggested that the 29 kDa N protein was glycosylated. Moreover, a 52 kDa protein band was frequently found in virus purifications. This protein was shown to be G2-specific and therefore may represent a stable degradation product of the 58 kDa G2 protein. Experiments indicated that this protein was also glycosylated (Tas *et al.*, 1977; Mohamed, 1981).

To verify whether the structural proteins of TSWV are glycosylated, the N-linked glycosylation was investigated. To this end, AcNPV/N- and AcNPV/GP-infected *S. frugiperda* cells were grown in the presence of tunicamycin, an inhibitor of N-linked glycosylation. Subsequent SDS-PAGE and Western immunoblot analyses of the proteins synthesized revealed no shift in migration of the N protein compared to N protein produced in insect cells without tunicamycin (Fig.5, lane AcNPV/N and AcNPV/N "+"). Although the baculovirus expression system does not reflect the situation in plant cells, based on the absence of glycosylation of the N protein in insect cells it now seems most unlikely that the N protein is glycosylated in plant cells, opposed to what Mohamed *et al.* (1973) suggested.

Similar analyses for the G1 and G2 proteins showed a shift in migration when the proteins were expressed in the presence of tunicamycin (Fig.4, lane AcNPV/GP and lane AcNPV/GP "+"), indicating that these proteins are indeed glycosylated. For G1, a difference of approximately 5 kDa in migration was observed between the glycosylated and unglycosylated form. The migration shift for G2 was even larger, corresponding with an apparent size of 10 kDa. Inspection of the primary sequence of the TSWV glycoprotein precursor (Chapter 4) reveals the presence of 8 potential N-linked glycosylation sites, i.e 5 sites in G2 and 3 in G1 (Fig.6). The larger shift in migration for G2 compared to G1 may therefore indicate that most, if not all, sites are actually glycosylated. In addition to G1 and G2, the putative glycoprotein precursor also showed a shift in migration when produced in the presence of tunicamycin. However, due to

poor resolution, an exact difference in migration of the unglycosylated- compared to the glycosylated form could not be determined, but could roughly be estimated around 10-15 kDa.

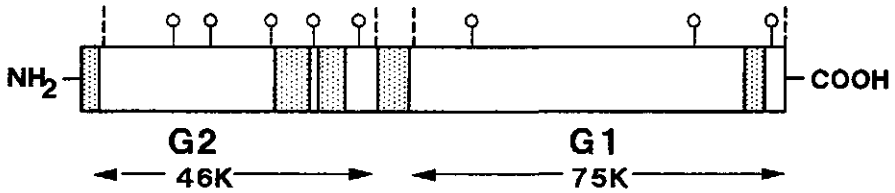


Figure 6: Schematical representation of the TSWV glycoprotein precursor. The potential glycosylation sites (N-X-T/S) are indicated by the mark "ϕ".

DISCUSSION

In some of the previous chapters of this thesis, the baculovirus expression system has already been demonstrated to be a valuable tool in the study of the TSWV nonstructural proteins, i.e. NS_S and NS_M. Therefore, a similar approach was chosen as a first step to analyse the synthesis, processing and structural functions of the TSWV N, G1 and G2 proteins in the viral life cycle.

A recombinant AcNPV, containing the N protein gene, was constructed and showed a high level expression of a protein that comigrated with the TSWV N protein. Western immunoblot analysis confirmed its identity as the N protein.

In addition, recombinant AcNPV containing the TSWV GP gene was constructed. However, expression levels of G1, G2, or their precursor remained low and could not be detected directly in Coomassie stained polyacrylamide gels. Western immunoblots, though, revealed the expression of G1 and G2, comigrating with the authentic viral G1 and G2 glycoproteins. Furthermore, a large protein was found reacting specifically with TSWV antiserum in extracts of AcNPV/GP-infected insect cells. This protein had the

size of G1 and G2 together, and therefore most likely represented the precursor to both glycoproteins. This finding indicates that, at least in insect cells, cleavage does not necessarily occur cotranslationally. Whether the biosynthesis and maturation of the TSWV glycoproteins occurs likewise in plant cells remains to be investigated.

Expression of the glycoproteins in the presence of an inhibitor of N-linked glycosylation, demonstrated the actual glycosylation of G1 and G2, as previously suggested by several authors (Tas *et al.*, 1977; Mohamed *et al.*, 1973). Besides the mature glycoproteins, also the putative precursor to the glycoproteins was found to be glycosylated. Interestingly, the unglycosylated form of G2 comigrated with the 52 kDa protein species often detected in purified virus preparations. This supports the idea that the 52 kDa protein band represents unglycosylated G2, rather than a stable degradation product thereof, as previously suggested (Tas *et al.*, 1977; Mohamed *et al.*, 1973). Along this line, the frequent observation of multiple polypeptide bands of G2 may be explained by differential usage of the multiple (five) sites of glycosylation of this viral glycoprotein (Fig.6).

The large difference in expression levels of the TSWV nucleoprotein and glycoproteins could not be explained, but, in view of similar transcription levels, has to be on the level of translation. It has been suggested that the presence of extra leader sequences in the chimaeric mRNA might impede expression in the baculovirus/insect cell system, but this does not seem the case for the high expressor AcNPV/N recombinant which contains a 127 nucleotides TSWV leader sequence, whereas this sequence is absent from the AcNPV/GP construct. The low level expression of the glycoproteins can also not be explained by an unfavourable AUG context (Kozak, 1986; optimal consensus A/G-CCAUGG), as it is rather optimal in the AcNPV/GP recombinant (G at -3, and G at +4; Table 1), nor does the N-end rule (Bachmair *et al.*, 1989; Tobia *et al.*, 1991), which predicts the turnover speed of proteins due to the presence of rather unfavourable N-end amino acid residues downstream the methionine codon. In addition to the favourable N-end serine, another reason for the high expression level of the N protein could be the stabilization of the N protein when complexed to RNA. This normally occurs within TSWV ribonucleocapsid structures where the N protein is tightly

N	TCATC*ATG*TCTAA
NS_S	CCATA*ATG*TCAAC
NS_M+1	TCGAA*ATG*TTGAC
NS_M	GTACC*ATG*GGAAT
GP	CCGGC*ATG*GGAAT
Consensus	$CXG_ACCATGG$

Table 1: ATG-context within the different recombinant baculovirus constructs.

associated with the viral RNA, but a similar situation may also occur in insect cells where the N protein could be complexed to its own mRNA or to other RNAs. This does not apply for the glycoproteins and sofar, the reason for the low expression level of the glycoproteins remains unknown. A combination of factors, i.e. AUG context, absence of viral leader sequences and N-end amino acid, may all have contributed to the difference in expression levels as found for the two NS_M recombinants (Chapter 6, Fig.2; this chapter Fig.1c and Table 1).

For the animal-infecting bunyaviruses electron microscopical analyses revealed that, prior to maturation, viral RNPs condense at the cytoplasmic face of the Golgi complex only where the bunyaviral glycoproteins are found to accumulate. This indicates that an interaction between the nucleocapsid protein and a cytoplasmic domain of the glycoproteins triggers the process of budding of RNPs into the lumen of the Golgi. For TSWV no information is available on the process of morphogenesis. However, if an interaction of the N protein with either of the two glycoproteins occurs, than it is most likely to be G2, as this protein has a large cytoplasmic domain (Fig.6; Chapter 4). As part of a study on the morphogenesis of TSWV, and to investigate the role of the N protein in this, the interaction of N with G1 and/or G2 was investigated by expressing both the N protein and the glycoproteins in insect cells. Immunoprecipitation of the N protein from these doubly infected-cell extracts, and subsequent analyses of this

precipitate on Western immunoblots for the presence of G1 and/or G2 sofar failed to demonstrate an interaction between the N protein and G1 and/or G2. This could be due to the low level of glycoprotein expression in the insect cells, making these analyses difficult. On the other hand, it is also possible that the interaction between the N protein and the glycoproteins occurs only when the N protein is present in the right conformation, i.e. in viral ribonucleocapsid structures. Co-transfection of insect cells or plant protoplasts, expressing the viral glycoproteins, with purified nucleocapsids might be worthwhile to test this hypothesis.

REFERENCES

Booth, T.F., Gould, E.A. and Nuttall, P.A. (1991). Structure and morphogenesis of Dugbe virus (*Bunyaviridae, Nairovirus*) studied by immunogold electron microscopy of ultrathin sections. *Virus Research* **21**, 199-212.

Brown, M. and Faulkner, P. (1977). A plaque assay for nuclear polyhedrosis viruses using a solid overlay. *Journal of General Virology* **36**, 361-364.

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

De Haan, P., Wagemakers, L., Peters, D. and Goldbach, R. (1991). The S RNA segment of tomato spotted wilt virus has an ambisense character. *Journal of General Virology* **71**, 1001-1007.

De Haan, P., Kormelink, R., Resende, R. de O.,, Van Poelwijk, F., Peters, D. and Goldbach, R. (1991). Tomato spotted wilt virus L RNA encodes a putative RNA polymerase. *Journal of General Virology* **72**, 2207-2216.

Elliott, R.M. (1990). Molecular biology of the *Bunyaviridae*. *Journal of General Virology* **71**, 501-522.

Francki, R.I.B., 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. *Archives of Virology* suppl. **2**.

Francki, R.I.B. 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 in the Golgi complex in the absence of virus maturation. *Journal of Virology* **57**, 899-906.

Hink, W.F. (1970). Established insect cell line from the cabbage looper, *Trichoplusia ni*. *Nature* (London) **226**, 466-467.

Ie, T.S. (1971). Electron microscopy of developmental stages of tomato spotted wilt virus in plant cells. *Virology* **2**, 468-479.

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

Kitajima, E.W., Avila, A.C. de, Resende, R. de O., Goldbach, R. and Peters, D. (1992). Comparative cytological and immunogold labelling studies on different isolates of tomato spotted wilt virus. *Journal of Submicroscopical Cytology and Pathology* **24**, 1-14.

Kitts, P.A. and Possee, R.D. (1993). A method for producing recombinant baculovirus expression vectors at

high frequency. *Biotechniques* **14**, 810-817.

Kuismanen, E. (1984). Post-translational processing of Uukuniemi virus glycoproteins G1 and G2. *Journal of Virology* **51**, 806-812.

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

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

Lyons, M.J. and Heyduk, J. (1973). Aspects of the developmental morphology of California encephalitis virus in cultured vertebrate and arthropod cells and in mouse brain. *Virology* **54**, 37-52.

Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982). *Molecular Cloning: A Laboratory Manual.* New York: Cold Spring Harbor Laboratory.

Matsuoka, Y., Ihara, T., Bishop, D.H.L. and Compans, R.W. (1988). Intracellular accumulation of Punta toro virus glycoproteins expressed from cloned cDNAs. *Virology* **167**, 251-260.

Milne, R.G. (1970). An electron microscopic study of tomato spotted wilt virus in sections of infected cells and in negative stain preparations. *Journal of General Virology* **6**, 267-276.

Mohamed, N.A. (1981). Isolation and characterization of subviral structures of tomato spotted wilt virus. *Journal of General Virology* **53**, 197-206.

Mohamed, N.A., Randles, J.W. and Francki, R.I.B. (1973). Protein composition of tomato spotted wilt virus. *Virology* **56**, 12-21.

Murphy, F.A., Harrison, A.K. and Whitfield, S.G. (1973). *Bunyaviridae*: Morphologic and morphogenetic similarities of Bunyamwera serologic supergroup viruses and several other arthropod-borne viruses. *Intervirology* **1**, 297-316.

Pensiero, M.N. and Hay, J. (1992). The Hantaan virus M segment glycoproteins G1 and G2 can be expressed independently. *Journal of Virology* **66**, 1907-1914.

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

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.

Smith, J.F. and Pifat, D.Y. (1982). Morphogenesis of Sandfly fever viruses (*Bunyaviridae* family). *Virology* **121**,61-81.

Smith, G.E., Summers, M.D. and Fraser, M.J. (1983). Production of human beta interferon in insect cells infected with a baculovirus expression vector. *Molecular and Cellular Biology* **3**, 2156-2165.

Summers, M.D. and Smith, G.E. (1987). *A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures.* College Station: Texas A & M University.

Tas, P.W.L., Boerjan, M.L. and Peters, D. (1977). The structural proteins of tomato spotted wilt virus. *Journal of General Virology* **36**, 267-279.

Ulmanen, I., Seppala, P. and Pettersson, R.F. (1981). *In vitro* translation of Uukuniemi virus-specific RNAs: Identification of a nonstructural protein and a precursor to the membrane glycoproteins. *Journal of Virology* **37**, 72-79.

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

Vaughn, J.L., Goodwin, R.H., Tompkins, G.J. and McCawley (1977). The establishment of two cell lines from the insect *Spodoptera frugiperda*. *In Vitro* **13**, 213-217.

Wasmoen, T.L., Torborg Kakach, L. and Collett, M.S. (1988). Rift valley fever virus M segment: Cellular localization of M segment encoded proteins. *Virology* **166**, 275-280.

Zuidema, D., Schouten, A., Usmany, M, Maule, A.J., Belsham, G.J., Klinge-Roode, E.C., Van Lent, J.W.M. and Vlak, J.M. (1990). Expression of cauliflower mosaic virus gene I using a novel polyhedrin-based baculovirus expression vector. *Journal of General Virology* **71**, 2201-2209.

Chapter 8

General discussion and concluding remarks

Tomato spotted wilt virus (TSWV) is a relatively new and emerging virus, having become of particular economic importance during the past ten years. Currently, more than 650 different plant species within 72 families, monocots as well as dicots, are known to be susceptible to the virus. With respect to particle morphology and genomic structure, TSWV is quite distinct from all other plant viruses and was therefore originally classified as the sole member of a distinct group, the tomato spotted wilt virus group (Matthews, 1982). In 1984 though, Milne and Francki proposed to consider TSWV as a possible member of the *Bunyaviridae*, a family of arthrophod-borne, animal-infecting viruses (Elliott, 1990). Only during the past seven years attention has been given to the elucidation of the genetic organisation, the coding functions and the expression strategy of the TSWV genome. Parts of the data resulting from these analyses have been described in the thesis of De Haan (1991) and in the current thesis. Based on these molecular data TSWV could be definitely identified as a bunyavirus, and has therefore been classified within the *Bunyaviridae* as the representative of a newly created genus, the genus *Tospovirus* (Francki *et al.*, 1991).

As a consequence, the family of *Bunyaviridae* now encompasses 5 genera, i.e. *Bunyavirus*, *Phlebovirus*, *Hantavirus*, *Nairovirus* and *Tospovirus*, the latter containing the viruses which infect plants. For the representatives of most genera, except for *Nairovirus*, the nucleotide sequence of the complete genome has been elucidated. Some features derived from these data are summarized in Figure 1.

In all genera the L RNA is of complete negative polarity and codes, in the viral complementary (vc) strand, for the putative viral RNA dependent RNA polymerase. In case of Uukuniemi virus, Lumbovirus, Hantaan virus, La Crosse and Germiston an RNA polymerase activity has been demonstrated to be associated with purified virion particles

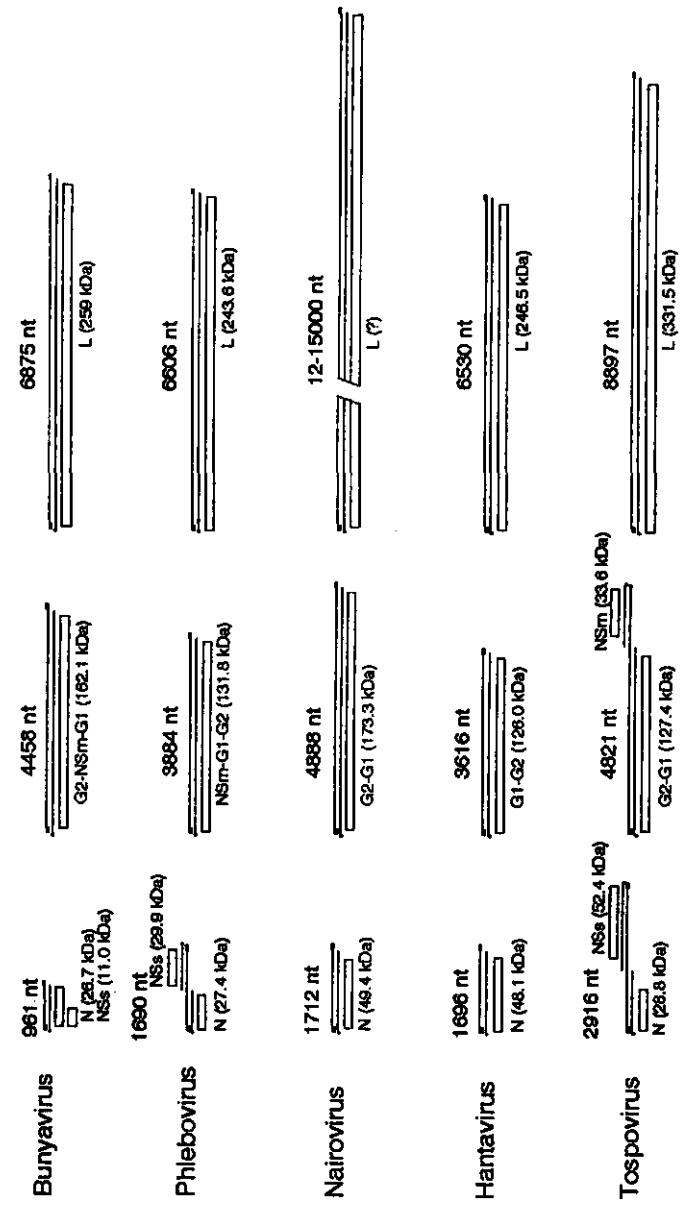


Figure 1: Genetic organisation of the type species of all genera within the family *Bunyaviridae*.

(Ranki and Pettersson, 1975; Bouloy and Hannoun, 1976; Schmaljohn and Dalrymple, 1983; Patterson *et al.*, 1984; Vialat and Bouloy, 1992). However, only in the case of Bunyamwera bunyavirus, the RNA polymerase activity has been demonstrated to be encoded by the L RNA (Jin and Elliott, 1992 and 1993). Additionally, it was shown that the Bunyamwera L RNA encoded protein contains an endonuclease activity (Jin and Elliott, 1993), necessary for the cleavage of 5' capped leader sequences from host cell messenger RNAs. These sequences, 12 to 17 nucleotides in length, are subsequently used as primer for the initiation of transcription of the viral genome. This phenomenon, generally referred to as "cap-snatching", was described first for influenza virus (*Orthomyxoviridae*), where from the three viral proteins encompassing the polymerase complex, PB2 has been shown to recognize and bind the methylated cap-structure and PB1 to initiate transcription by adding the first residue onto the 3' end of the capped primer-fragment (Plotch *et al.*, 1981; Ulmanen *et al.*, 1981; Braam *et al.*, 1983). For La Crosse and Germiston bunyaviruses, *in vitro* transcription experiments have demonstrated the presence of an endonuclease activity in purified virions which, at least for La Crosse, was shown to be methylated cap-dependent (Patterson *et al.*, 1984; Vialat and Bouloy, 1992). Indirect evidence for a virus-encoded cap-dependent endonuclease activity has been found for other members, encompassing all animal-infecting genera of the *Bunyaviridae,* by the presence of heterogeneous non-viral sequences at the 5' end of the viral mRNAs. TSWV was the first tospovirus for which these sequences were shown at the 5' end of the S RNA-specific subgenomic mRNAs (Chapter 3), indicating that a similar strategy for the initiation of transcription occurs in the plant cell system. Recently, similar results were reported for maize stripe tenuivirus (Huiet *et al.*, 1993), another group of segmented, negative-strand RNA plant viruses, and distinct from the *Bunyaviridae* in having 4 or 5 RNA segments, and in lacking a viral envelope (Gingery, 1987). Altogether, the picture emerges that all segmented, negative-strand RNA viruses use a similar mechanism to initiate transcription of the viral genome, irrespective of infecting either a plant or an animal host.

Unique for the genus *Tospovirus*, among the other *Bunyaviridae*, is the ambisense character of the M RNA, encoding a nonstructural protein of 33.6 kDa in the viral (v)

strand, and the precursor to the glycoproteins, G1 and G2, in the vc-strand (Chapter 4, Fig.1). For all the animal-infecting members of the *Bunyaviridae*, the M RNA is of complete negative polarity and codes for the glycoproteins in the vc strand (Fig.1). In these viruses, the glycoproteins are translationally expressed as a large precursor protein which is most likely processed via Golgi- or endoplasmatic reticulum (ER)-associated proteases, as no viral encoded proteases have been found. However, nothing is known on the processing of the TSWV glycoproteins, and whether the Golgi complex is involved herein.

For some animal-infecting species of the *Bunyaviridae* family, the order and mode of processing of the G1 and G2 glycoproteins is known (Fig.2). In some cases, the processing of the glycoprotein precursor at the ER and Golgi has been studied via heterologous expression systems, and these analyses revealed the presence of Golgi retention signals in either or both of the glycoproteins (Wasmoen *et al.*, 1988; Matsuoka *et al.*, 1988; Chen *et al.*, 1991; Pensiero and Hay, 1992; Ruusala *et al.*, 1992; Ronnholm, 1992). However, the picture for the glycoproteins of Dugbe nairovirus is still not clear (Marriott *et al.*, 1992) and apparently involves a complex pattern of processing (Fig.2). As a first step towards elucidation of the processing of the TSWV glycoproteins, a cDNA construct containing the complete ORF of the glycoproteins was cloned and expressed in the baculovirus/insect cell expression system (Chapter 7). Although the expression level was low, both glycoproteins could be detected and were demonstrated to be glycosylated. Moreover, the precursor to both glycoproteins could be detected, and was also shown to be glycosylated. As a next step towards resolving questions on targeting and retention signals within the glycoproteins of this plant-infecting bunyavirus, certain domains of the glycoprotein precursor gene can be deleted and the constructs subsequently analysed.

It is generally believed that for members of the *Bunyaviridae* the glycoproteins are involved in the attachment to and subsequent infection of the midgut cells of their vectors. Only in case of La Crosse virus experimental data are available which demonstrate an interaction of viral glycoproteins with specific cellular sites (Ludwig *et al.*, 1989, 1991). For TSWV several observations suggest that the glycoproteins play a role

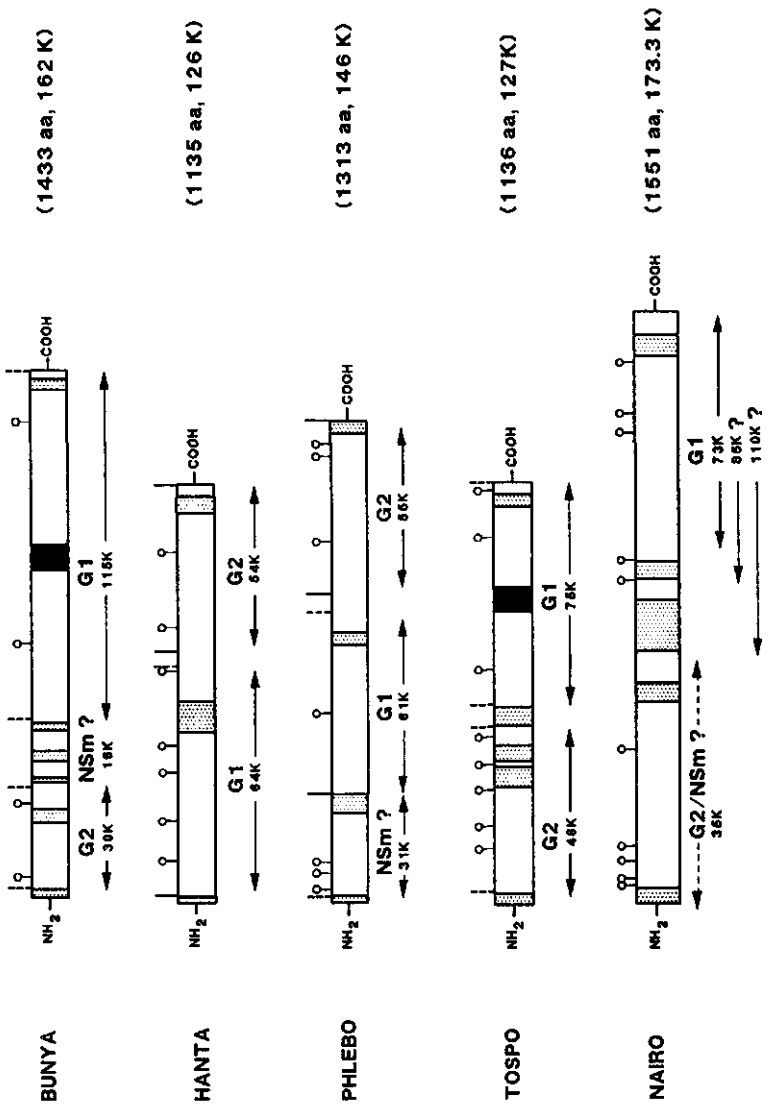


Figure 2: Glycoprotein precursors of the type species of all genera within the family *Bunyaviridae*. The location and sizes of the cleavage products are indicated. Hydrophobic domains are indicated by hatched areas. The conserved amino acid sequence within the G1 glycoprotein of TSWV with that of members of the genus *Bunyavirus* is shown with a black box. "Ψ" represents potential glycosylation sites (N-X-T/S). Vertical dotted and continuous lines represent the putative resp. proven N or C-termini of the maturated glycoproteins.

in the transmission by its vector, the thrips. Studies on morphologically defective TSWV isolates, i.e. virus mutants which are deficient in glycoprotein synthesis and lack the lipid envelope, showed that these mutants are still capable to spread through plant tissues at wild type rate (Resende *et al.*, 1991). However, these mutants fail to be transmitted by thrips (I. Wijkamp, pers. commun.). In this context, it is important to report that within the N-terminal region of the G2 glycoprotein of TSWV an RGD-sequence was found (Chapter 4), a motif which has been previously reported as a cellular attachment domain of extracellular matrix proteins of animal- and plant cells (Pierschbacher and Ruoslahti, 1984; Ruoslahti and Pierschbacher, 1986). In these proteins the RGD-motif was found to be crucial for the recognition of cell surface receptors. The RGD motif has also been found within structural proteins of footh-and-mouth disease virus (FMDV; Fox *et al.*, 1989), Coxsackie virus (Roivainen *et al.*, 1993) and adenovirus type 2 (Bai *et al.*, 1993, Belin and Boulanger, 1993), and shown to be involved in receptor binding, either as a primary target site or mediating the receptor binding at another site. In all of these cases, the RGD sequence is located within a hypervariable region of the structural protein. For TSWV it is tempting to assume that the RGD sequence is the putative binding site to cellular receptors of thrips midgut cells. It can not be excluded, however, that actual binding to cellular receptors takes place at another domain, like for instance the sequence conserved among the G1 glycoproteins of the genera *Bunyavirus* and *Tospovirus* (Fig.2). Sofar, virtually no information is available on binding of the animal-infecting members of the *Bunyaviridae* to cellular receptors in their vectors. Moreover, only for snowshoe hare and Germiston bunyavirus, and Punta Toro phlebovirus an RGD motif is present in the G2 glycoprotein. Whether the RGD sequence in these viruses is of biological significance remains to be established.

The second, smaller ORF in the ambisense M RNA of TSWV is absent from the animal-infecting bunyaviruses and, therefore, most likely reflects an adaptation of this virus to plants as hosts (Chapter 4; Fig.1). In Chapter 6, it has been shown, by immunogold labelling and subcellular fractionation studies, that the product of this gene, the NS_M protein, is found associated with nucleocapsid aggregates and with

plasmodesmata of virus-infected cells. At the plasmodesmata, the protein was found arranged in tubular extensions, suggesting that NS_M most likely represents the putative viral "movement" protein involved in the cell-to-cell transport of TSWV nucleocapsid structures. Furthermore, computer comparisons using the OPTAL program (Gorbalenya *et al.*, 1989) demonstrated the presence of a conserved sequence in the NS_M proteins of TSWV and INSV that resembled the so-called "D-motif" of other plant viral movement proteins (Koonin *et al.*, 1991; Mushegian and Koonin, 1993). Currently, there are two reported mechanisms of plant viral cell-to-cell movement: (1) movement as unencapsidated RNA as exemplified by tobamoviruses, and (2) movement as virus-like particles as exemplified by comoviruses (Goldbach *et al.*, 1990; Hull, 1991; Maule, 1991; Deom *et al*, 1992). In case of the first mechanism, studied in most detail for TMV, viral RNA is proposed to associate with the movement protein resulting in an unfolded protein-RNA complex. Subsequent cell-to-cell movement of this complex does not require the coat protein and occurs through plasmodesmata which are not significantly modified (Tomenius *et al.*, 1987; Wolf *et al.*, 1989). For plant viruses that use this concept of cell-to-cell transport, the viral movement protein has been shown to contain an RNA binding domain, which in some cases is single-stranded RNA-specific (Schoumacher *et al.*, 1992) and in other cases, e.g. TMV, single-stranded DNA and RNA specific (Citovsky *et al.*, 1990). The second concept of cell-to-cell movement, mainly studied for comoviruses, involves movement of virus particles through tubular structures extending from plasmodesmata (Van Lent *et al.*, 1990, 1991; Perbal *et al.*, 1993; Wieczorek and Sanfaçon, 1993). The mechanism of intercellular movement of TSWV is not clear yet, as it is neither transported as whole virions, nor (probably) as naked RNA (Chapter 6). Western immunoblot analysis demonstrated the association of NS_M with nucleocapsid structures, which may suggest that tospoviruses move as (infectious) nucleocapsid structures. Inspection of the amino acid sequence of the NS_M protein reveals the presence of an acidic carboxyl terminus, due to the presence of several aspartic acid (D) and glutamic acid (E) residues. It is, therefore, anticipated that this domain is involved in binding of NS_M to the (basic) N proteins within the nucleocapsid structures. In addition the presence of a second domain can be hypothesized, i.e. a

domain involved in targeting and/or binding to plasmodesmata.

The coding strategy of the S RNA differs among the various genera (Fig.1). Whereas the tospoviral and phleboviral S RNAs encode a nucleoprotein (N) and a nonstructural (NS_S) protein in ambisense arrangement, members of the genus *Bunyavirus* possess an S RNA of complete negative polarity, encoding these two proteins in overlapping reading frames. The S RNAs of members of the genera *Hantavirus* and *Nairovirus* are also of complete negative polarity, but encode only an N protein which, moreover, is considerable larger than that of the other genera (Fig.1). Information about the function or even intracellular location of the nonstructural protein encoded by the phleboviral S RNA is hardly available. Some protein expression studies and immunogold labelling analyses have been performed, but no hints about its possible function have been obtained. Recently it has been demonstrated that the Uukuniemi virus and Punta Toro virus NS_S proteins associate with the 40S ribosomal subunit (Simons *et al.*, 1992; Watkins *et al.*, 1993). Whether this interaction is significant remains to be elucidated. Immunogold labelling studies on the NS_S protein of TSWV, as described in Chapter 5, showed the presence of this protein in fibrous structures, sometimes arranged in paracrystalline arrays, depending on the isolate. The BR-01 isolate of TSWV, of which the complete genomic sequence has been determined, showed the presence of NS_S dispersed throughout the cytoplasma. These localization studies, however, did not give a clue on the function of the NS_S protein. Moreover, it can be doubted whether the tospoviral NS_S protein and phleboviral or bunyaviral NS_S protein share a similar function, since the tospoviral NS_S protein is much larger than those of its animal viral counterparts.

Sequence analyses also revealed some primary and structural features of bunyaviral RNAs that might play important functions in replication, transcription and packaging. As shown in Figure 1, and demonstrated for TSWV in Chapter 4 and in the thesis of De Haan (1991), within each species the viral RNA segments contain a terminal, inverted repeat of about 8 nucleotides. Moreover, within each viral RNA segment, the terminal sequences are complementary to a larger extend than these 8 conserved nucleotides, enabling the formation of a stable panhandle structure. The terminal, inverted repeats are characteristic for segmented, negative strand RNA viruses and, for

members of the *Orthomyxoviridae*, have been shown to contain sequences required for transcription, replication and packaging (Luytjes *et al.*, 1989; Parvin *et al.*, 1989; Li and Palese, 1992; Seong and Brownlee, 1992a, 1992b; Piccone *et al.*, 1993). The studies performed on the replication and transcription of the TSWV genome, as followed in systemically-infected *N. rustica* plants (Chapter 2), did confirm the genetic organisation of the TSWV genome, and showed that TSWV replicates as a negative stranded RNA virus. However, these data did not give any insight into the function of the conserved sequences in these processes, nor in the packaging of the viral RNA strands into mature virus particles. With respect to the latter process, these studies surprisingly revealed the presence of exclusively the negative strand of the L RNA in mature virus particles, while for the other two genomic segments both strands are packaged. This phenomena might perhaps be due to the ambisense character of the M and S RNA segments. A similar observation has been reported for the Uukuniemi phleboviral S RNA (Simons *et al.*, 1991), which also has an ambisense polarity.

Recent investigations with hantaviruses demonstrated that the binding of the nucleoprotein to RNA is not sequence-specific and shows a preference for dsRNA (Gott *et al.*, 1993), suggesting that the panhandle structure, resulting from hybridisation of the complementary termini, might play an essential role in packaging. This idea is supported by the observation for TSWV, and other bunyaviruses, that viral mRNAs are not contained in mature virus particles (Chapter 2).

A second specific feature is the presence of an intergenic region (IR) within the ambisense RNA segments of all *Bunyaviridae*, which is either AU-rich (TSWV, INSV, Punta Toro, Uukuniemi) or GC-rich (Sicilian sandfly fever, Toscana, Rift Valley fever). It is speculated that this region contains transcription termination signals, i.e. the AU-rich IR by formation of a stable hairpin structure and the GC-rich IR by the presence of conserved sequence motifs. For members of the genus *Tospovirus*, an additional sequence at the top of the hairpin within the S and M RNA was found conserved between the species sequenced sofar, i.e TSWV and INSV (Chapter 4). Termination of transcription within all the other, negative stranded RNA segments of the *Bunyaviridae* has been suggested to occur via conserved sequence motifs (Bouloy *et al.*, 1990).

Whether these primary or structural features indeed represent the signals for termination still needs to be determined.

Finally, the availability of the complete nucleotide sequence of TSWV (Chapter 4; De Haan, thesis 1991) enables a thorough comparison with the animal-infecting members of the *Bunyaviridae* and to speculate on possible evolutionary relationships. Interviral sequence comparisons of the glycoprotein precursor revealed a sequence motif conserved between the G1 glycoprotein of TSWV and those of members of the genus *Bunyavirus* (Chapter 4; Fig.2). This, together with the homology between the TSWV and Bunyamwera viral L proteins (De Haan *et al.*, 1991), may indicate that tospoviruses are evolutionary most closely related to the genus *Bunyavirus*, even more closely than the genus *Bunyavirus* is to e.g. the genus *Hantavirus*. It is, therefore, tempting to assume that TSWV has recently descended from an animal-infecting bunyavirus. This idea is further strengthened by two additional observations. Firstly, while the number of vertebrate-infecting bunyaviruses is high, the number of bunyaviruses (tospoviruses) capable of infecting plants is limited. Secondly, the virtual absence of efficient natural resistance genes in host plants may indicate that TSWV and the other tospoviruses have invaded the plant kingdom recently, and that co-evolution of bunyaviruses and plants has yet to start. Hence, it is most likely that TSWV has evolutionary departed from an animal bunyavirus, that became able to infect thrips species and, due to the very close ecological interactions between this insect group and plants, subsequently became adapted to plants instead of vertebrates. Although tospoviruses seem evolutionarily most closely related to members of the genus *Bunyavirus*, they have an ambisense S RNA and share this feature only with members of the genus *Phlebovirus*. This raises the question how the TSWV ambisense S RNA, apparently not encoding proteins required for host adaptation, could evolve from a fully negative strand bunyaviral S RNA. Possible the NS_S protein of both genera are genetically unrelated and is the generation of an ambisense S RNA a relatively late event during bunyaviral evolution. This option is supported by the observation that TSWV has been adapted to plant hosts by inclusion of the NS_M gene, in an ambisense arrangement, within the M RNA segment.

REFERENCES

Bai, M., Harfe, B. and Freimuth, P. (1993). Mutations that alter an Arg-Gly-Asp (RGD) sequence in the Adenovirus type 2 penton base protein abolish its cell-rounding activity and delay virus reproduction in flat cells. *Journal of Virology* **67**, 5198-5205.

Belin, M.-T. and Boulanger, P. (1993). Involvement of cellular adhesion sequences in the attachment of adenovirus to the HeLa cell surface. *Journal of General Virology* **74**, 1485-1497.

Bouloy, M. and Hannoun, C. (1976). Studies on Lumbovirus replication: I RNA-dependent RNA polymerase associated with virions. *Virology* **69**, 258-264.

Bouloy, M., Pardigon, N., Vialat, P., Gerbaud, S. and Girard, M. (1990). Characterizationof the 5' and 3' ends of viral messenger RNAs isolated from BHK21 cells infected with Germiston virus (*Bunyavirus*). *Virology* **175**, 50-58.

Braam, J., Ulmanen, I. and Krug, R.M. (1983). Molecular model of a eucaryotic transcription complex: Functions and movements of Influenza P proteins during capped RNA-primed transcription. *Cell* **34**, 609-618.

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

Citovsky, V., Knorr, D., Schuster, G. and Zambryski, P. (1990). The p30 movement protein of tobacco mosaic virus is a single-strand nucleic acid binding protein. *Cell* **60**, 637-647.

Collett, M.C., Purchio, A.F., Keegan, K., Frazier, S., Hays, W., Anderson, D.K., Parker, M.D., Schmaljohn, C., Schmidt, J. and Dalrymple, J.M. (1985). Complete nucleotide sequence of the M RNA segment of Rift valley fever virus. *Virology* **144**, 228-245.

De Haan, P. (1991). Exploring and exploiting the RNA genome of tomato spotted wilt virus. Thesis, Agricultural University Wageningen.

De Haan, P., Kormelink, R., Resende, R. de O., Van Poelwijk, F., Peters, D. and Goldbach, R. (1991). Tomato spotted wilt virus L RNA encodes a putative RNA polymerase. *Journal of General Virology* **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. *Journal of General Virology* **71**, 1001-1007.

Deom, C.M., Lapidot, M. and Beachy, R.N. (1992). Plant virus movement proteins. *Cell* **69**, 221-224.

Elliott, R.M. (1989). Nucleotide sequence analysis of the Small (S) RNA segment of Bunyamwera virus, the prototype of the family *Bunyaviridae*. *Journal of General Virology* **70**, 1281-1285.

Elliott, R.M. (1989). Nucleotide sequence analysis of the Large (L) genomic RNA segment of Bunyamwera virus, the prototype of the family *Bunyaviridae*. *Virology* **173**, 426-436.

Elliott, R.M. (1990). Molecular biology of the *Bunyaviridae*. *Journal of General Virology* **73**, 501-522.

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). *Journal of General Virology* **70**, 625-637.

Francki, R.I.B., 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. *Archives of Virology* suppl. 2.

Gingery, R.E. (1987). The rice stripe virus group. In: "*The plant viruses*" **vol.4**, 297-329, R.G. Milne ed., Plenum Press, New York and London.

Giorgi, C., Accardi, L., Nicoletti, L., Gro, M.C., Takehara, K., Hilditch, C., Morikawa, S. and Bishop, D.H.L. (1991). Sequences and coding strategies of the S RNAs of Toscana and Rift valley fever viruses compared to those of Punta toro, Sicilian sandfly fever, and Uukuniemi viruses. *Virology* **180**, 738-753.

Goldbach, R., Eggen, R., de Jager, C., van Kammen, A., van Lent, J., Rezelman, G. and Wellink, J. (1990). Genetic organization, evolution and expression of plant viral RNA genomes. In: *Recognition and response in plant-virus interactions* (R.S.S. Fraser, ed.). NATO ASI series H, vol. 41, p. 147-162, Springer Verlag,

Berlin, Heidelberg.
Gorbalenya, A.E., Blinov, V.M., Donchenko, A.P. and Koonin, E.V. (1989). An NTP-binding motif is the most conserved sequence in a highly diverged monophyletic group of proteins involved in positive strand RNA viral replication. *Journal of Molecular Evolution* **28**, 256-268.
Gott, P., Stohwasser, R., Schnitzler, P., Darai, G. and Bautz, E.K.F. (1993). RNA binding of recombinant nucleocapsid proteins of Hantaviruses. *Virology* **194**, 332-337.
Huiet, L., Estabrook, E., Feldstein, P. and Falk, B.W. (1993). Characterization of the 5' termini of the maize stripe tenuivirus (MStV) genomic RNAs and the subgenomic mRNA for the major noncapsid protein (NCP). In: *Abstracts of the IXth International Congress of Virology*, Glasgow, p61-25.
Hull, R. (1991). The movement of viruses within plants. *Seminars in Virology* **2**, 89-95.
Jin, H. and Elliott, R.M. (1991). Expression of functional Bunyamwera virus L protein by recombinant vaccinia viruses. *Journal of Virology* **65**, 4182-4189.
Jin, H. and Elliott, R.M. (1993). Characterization of Bunyamwera virus S RNA that is transcribed and replicated by the L protein expressed from recombinant vaccinia virus. *Journal of Virology* **67**, 1396-1404.
Koonin, E.V., Mushegian, A.R., Ryabov, E.V. and Dolja, V.V. (1991). Diverse groups of plant RNA and DNA viruses share related movement proteins that may possess chaperone-like activity. *Journal of General Virology* **72**, 2895-2903.
Lees, J.F., Pringle, C.R. and Elliott, R.M. (1986). Nucleotide sequence of the Bunyamwera virus M RNA segment: Conservation of structural features in the Bunyavirus glycoprotein gene product. *Virology* **148**, 1-14.
Li, X. and Palese, P. (1992). Mutational analysis of the promoter required for influenza virus virion RNA synthesis. *Journal of Virology* **66**, 4331-4338.
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., Tuill, T.M. and Schultz, K.T. (1991). Role of La Crosse virus glycoproteins in attachment of virus to host cells. *Virology* **181**, 564-571.
Luytjes, W., Krystal, M., Enami, M., Parvin, J.D. and Palese, P. (1989). Amplification, expression, and packaging of a foreign gene by influenza virus. *Cell* **59**, 1107-1113.
Marriott, A.C., El-Ghor, A.A. and Nuttall, P.A. (1992). Dugbe Nairovirus M RNA: Nucleotide sequence and coding strategy. *Virology* **190**, 606-615.
Matsuoka, Y., Ihara, T., Bishop, D.H.L. and Compans, R.W. (1988). Intracellular accumulation of punta toro virus glycoproteins expressed from cloned cDNAs. *Virology* **167**, 251-260.
Matthews, R.E.F. (1982). Classification and nomenclature of viruses: Fourth report on the International Committee on Taxonomy of Viruses. *Intervirology* **17**, 1-199.
Maule, A.J. (1991). Virus movement in infected plants. In: *Critical Reviews in Plant Sciences* **6**, 457-473.
Muller, R., Argentini, C., Bouloy, M., Prehaud, C. and Bishop, D.H.L. (1991). Completion of the genome sequence of Rift Valley fever phlebovirus indicates that the L RNA is negative sense or ambisense and codes for a putative transcriptase-replicase. *Nucleic Acids Research* **19**, 5433.
Mushegian, A.R. and Koonin, E.V. (1993). Cell-to-cell movement of plant viruses. *Archives of Virology* **133**, 239-257.
Parvin, J.D., Palese, P., Honda, A., Ishihama, A. and Krystal, M. (1989). Promoter analysis of influenza virus RNA polymerase. *Journal of Virology* **63**, 5142-5152.
Patterson, J.L., Holloway, B. and Kolakofsky, D. (1984). La Crosse virions contain a primer-stimulated RNA polymerase and a methylated cap-dependent endonuclease. *Journal of Virology* **52**, 215-222.
Pensiero, M.N. and Hay, J. (1992). The Hantaan virus M segment glycoproteins G1 and G2 can be expressed independently. *Journal of Virology* **66**, 1907-1914.
Perbal, M.-C., Thomas, C.L. and Maule, A.J. (1993). Cauliflower mosaic virus gene I product (P1) forms tubular structures which extend from the surface of infected protoplasts. *Virology* **195**, 281-285.
Piccone, M.E., Fernandez-Sesma, A. and Palese, P. (1993). Mutational analysis of the influenza virus vRNA promoter. *Virus Research* **28**, 99-112.

Pierschbacher, M.D. and Ruoslahti, E. (1984). Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. *Nature* **309**, 30-33.

Plotch, S.J., Bouloy, M., Ulmanen, I. and Krug, R.M. (1981). A unique cap(m^7GpppXm)-dependent Influenza virion endonuclease cleaves capped RNAs to generate the primers that initiate viral RNA transcription. *Cell* **23**, 847-858.

Ranki, M. and Pettersson, R.F. (1975). Uukuniemi virus contains an RNA polymerase. *Journal in Virology* **16**, 1420-1425.

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. *Journal of General Virology* **72**, 2375-2383.

Roivainen, M., Piirainen, L., Heino, J., Hyypia, T. and Hovi, T. (1993). Receptor for coxsackievirus A9. In: *Abstracts of the IXth International Congress of Virology*, Glasgow, p.41-11.

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

Ruoslahti, E. and Pierschbacher, M.D. (1986). Arg-Gly-Asp: A versatile cell recognition signal. *Cell* **44**, 517-518.

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.

Schmaljohn, C.S. (1990). Nucleotide sequence of the L genome segment of Hantaan virus. *Nucleic Acids Research* **18**, 6728.

Schmaljohn, C.S. and Dalrymple, J.M. (1983). Analysis of Hantaan virus RNA: Evidence for a new genus of *Bunyaviridae*. *Virology* **131**, 482-491.

Schmaljohn, C.S., Jennings, G.B., Hay, J. and Dalrymple, J.M. (1986). Coding strategy of the S genome segment of Hantaan virus. *Virology* **155**, 633-643.

Schmaljohn, C.S., Schmaljohn, A.L. and Dalrymple, J.M. (1987). Hantaan virus M RNA: Coding strategy, nucleotide sequence, and gene order. *Virology* **157**, 31-39.

Schoumacher, F., Erny, C., Berna, A., Godefroy-Colburn, T. and Stussi-Garaud, C. (1992). Nucleic acid-binding properties of the alfalfa mosaic virus movement protein produced in yeast. *Virology* **188**, 896-899.

Seong, B.L. and Brownlee, G.G. (1992a). A new method for reconstituting influenza polymerase and RNA *in vitro*: A study of the promoter elements for cRNA and vRNA synthesis *in vitro* and viral rescue *in vivo*. *Virology* **186**, 247-260.

Seong, B.L. and Brownlee, G.G. (1992b). Nucleotides 9 to 11 of the influenza A virion RNA promoter are crucial for activity *in vitro*. *Journal of General Virology* **73**, 3115-3124.

Simons, J.F., Hellman, U. and Pettersson, R.F. (1990). Uukuniemi virus S RNA segment: Ambisense coding strategy, packaging of complementary strands into virions, and homology to members of the genus *Phlebovirus*. *Journal of Virology* **64**, 247-255.

Tomenius, K., Clapham, D. and Meshi, T. (1987). Localization by immunogold cytochemistry of the virus-coded 30K protein in plasmodesmata of leaves infected with tobacco mosaic virus. *Virology* **160**, 363-371.

Ulmanen, I., Broni, B. and Krug, R.M. (1981). Role of two of the influenza virus core P proteins in recognizing cap 1 structures (m^7GpppNm) on RNAs and in initiating viral RNA transcription. *Proceedings of the National Academy of Sciences USA* **12**, 7355-7359.

Vialat, P. and Bouloy, M. (1992). Germiston virus transcriptase requires active 40S ribosomal subunits and utilizes capped cellular RNAs. *Journal of Virology* **66**, 685-693.

Van Lent, J., Wellink, J. and Van Kammen, A. (1990). Evidence for the involvement of the 58K and 48K proteins in the intercellular movement of cowpea mosaic virus. *Journal of General Virology* **71**, 219-223.

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. *Journal of General Virology* **72**, 2615-2623.

Ward, V.K., Marriott, A.C., El-Ghorr, A.A. and Nuttall, P.A. (1990). Coding strategy of the S RNA segment

of Dugbe virus (*Nairovirus*; *Bunyaviridae*). *Virology* **175**, 518-524.
Wasmoen, T.L., Torborg-Kakach, L. and Collett, M.S.(1988). Rift Valley fever virus M segment: Cellular localization of M-segment encoded proteins. *Virology* **166**, 275-280.
Wieczorek, A. and Sanfacon, H. (1993). Characterization and subcellular localization of tomato ringspot nepovirus putative movement protein. *Virology* **194**, 734-742.
Wolf, S., Deom, C.M., Beachy, R.N. and Lucas, W.J. (1989). Movement protein of tobacco mosaic virus modifies plasmodesmatal size exclusion limit. *Science* **246**, 377-379.

Summary

This thesis describes studies which are aimed at the elucidation of the genetic organisation and expression strategy of the tomato spotted wilt virus (TSWV) RNA genome.

Using specific cDNA clones, corresponding to all three genomic RNA segments, the synthesis of virus specific RNA species in systemically infected *Nicotiana rustica* plants was followed (Chapter 2). These analyses revealed the presence of low (steady state) levels of vc strands and accumulating amounts of v strands, and confirmed that TSWV replicates as a negative strand RNA virus. Moreover, it could be demonstrated that the two genes contained in the S RNA are transcribed from complementary strands, confirming the ambisense nature of this genome segment.

To gain more information on the first steps in the transcription process of the TSWV genome, the subgenomic mRNAs, transcribed from the S RNA, were partially purified from infected plant material and their 5' ends analysed by primer extension. These analyses revealed the presence of non-viral, heterogeneous sequences, 12-20 nucleotides in length, at the 5' end of the mRNAs (Chapter 3). These observations indicated that the initiation of transcription of the viral genome most likely occurs via a mechanism referred to as "cap-snatching", a process that was first described for influenza virus.

The elucidation of the nucleotide sequence of the M RNA, as described in Chapter 4, revealed an ambisense coding strategy for this RNA segment. Meanwhile, TSWV had been classified as the representative of a newly created genus, denoted *Tospovirus*, within the *Bunyaviridae*, a family that until then only consisted of animal-infecting viruses. Thus it was concluded that TSWV represents a bunyavirus with two ambisense RNA segments. In M RNA a large open reading frame (ORF) was localized on the viral complementary (vc) strand, encoding the precursor to both envelope glycoproteins, G1 (78 kDa) and G2 (58 kDa). The second, smaller ORF, located on the viral strand of M RNA, encoded a putative nonstructural protein, denoted NS_M, of 33.6 kDa. The amino acid sequence of the glycoprotein precursor revealed the presence of an RGD-motif in the N-terminal region of G2. It was therefore anticipated that the glycoproteins are involved in

acquisition and transmission by thrips via receptor binding.

Comparison with the genomes of the animal-infecting members of the *Bunyaviridae*, reveals that the second ORF within the M RNA of TSWV, i.e. the NS_M gene, represents an addition to the standard bunyaviral genome, suggesting that this gene might be involved in the adaptation of this bunyavirus to plant hosts. To gain more insight into the function of the nonstructural proteins of TSWV, both the NS_S and NS_M genes were cloned and expressed in heterologous expression systems. The proteins thus produced were purified and used for the production of polyclonal antisera. In Chapter 5, immunogold labelling analyses demonstrated the presence of the NS_S protein in fibrous structures within the cytoplasm of infected cells. Depending on the isolate, these structures were arranged in flexible or paracrystalline arrays. Similar studies were performed for the NS_M protein (Chapter 6), and revealed a transient character of this protein in a time course analysis of systemically infected plants. Immunogold analyses showed the association of NS_M with both non-enveloped nucleocapsid aggregates and plasmodesmata, though only during a short period early after infection. These results provided evidence that NS_M represents the viral movement protein, involved in cell-to-cell transport of non-enveloped ribonucleocapsids.

As a first step towards unravelling the maturation pathways of the glycoproteins, the gene for the glycoprotein precursor was cloned and expressed in the eukaryotic baculovirus/insect cell expression system. The results obtained, and presented in Chapter 7, demonstrated the actual glycosylation of the viral glycoproteins. Moreover, the precursor to the glycoprotein was found to be glycosylated, indicating that glycosylation takes place at the stage of the non-processed G1/G2 precursor protein. Finally, in Chapter 8 a general discussion is presented within the family *Bunyaviridae*. The evolutionary relationship to the animal-infecting members of the *Bunyaviridae* is emphasized.

Samenvatting

Dit proefschrift beschrijft onderzoek naar de opheldering van de genetische organisatie en expressiestrategie van het RNA-genoom van het tomatebronsvlekkenvirus (Engels: tomato spotted wilt virus, afgekort TSWV). Dit virus is de type-soort van het genus *Tospovirus*, een groep van plantevirussen behorend tot de grote familie *Bunyaviridae*, waarvan de meeste leden vertebraten infecteren.

Met behulp van specifieke cDNA-klonen, elk corresponderend met één van de drie genomische RNA-segmenten, is de synthese van virus-specifieke RNA-moleculen in systemisch geïnfecteerde *Nicotiana rustica* planten nader onderzocht (Hoofdstuk 2). Op basis van de kinetiek van de virale RNA-synthese kon worden bevestigd dat TSWV zich vermenigvuldigt als een negatief-strengig RNA-virus. Tevens kon worden aangetoond dat twee genen die gecodeerd liggen op het S RNA, getranscribeerd worden van complementaire strengen, waarmee het "ambisense" karakter van dit RNA segment definitief werd aangetoond. Detectie van M RNA-specifieke subgenomische RNA moleculen vormde een eerste aanwijzing dat ook het M RNA ambisense van karakter is.

Om meer informatie te verzamelen over de eerste stappen in het virale transcriptieproces werden de beide subgenomische boodschapper-RNA's (mRNA's), die van het S RNA worden overgeschreven, gedeeltelijk opgezuiverd en hun 5'-uiteinden geanalyseerd. Aldus kon de aanwezigheid van niet-virale nucleotidenvolgorden, met een lengte van 12 tot 20 residuen, worden aangetoond aan de 5'-uiteinden van deze mRNA's (Hoofdstuk 3). Hiermee werd aannemelijk gemaakt dat de transcriptie van het TSWV-genoom geïnitiëerd wordt met behulp van leaders van cellulaire mRNA's, een proces dat bekend staat onder de naam "cap-snatching", en voor het eerst beschreven is voor influenzavirussen.

De bepaling van de nucleotidenvolgorde van het M RNA, beschreven in Hoofdstuk 4, toonde aan dat dit RNA, evenals het S RNA, inderdaad ambisense is. In de streng die complementair is aan het virale M RNA bevindt zich een open leesraam coderend voor de precursor van de beide glycoproteinen, G1 (78 kDa) en G2 (58 kDa). De aminozuur-

volgorde van deze precursor bleek een RGD-motief in de amino-terminus van het G2 eiwit te bevatten, hetgeen suggereert dat dit virale eiwit een rol speelt in opname en overdracht door trips door middel van binding aan een receptor. Het tweede gen in het M RNA is gelegen op de virale streng, en codeert voor een niet-structureel eiwit, genaamd NS_M, met een M_r van 33.6 kDa.

Op basis van vergelijkingen met de genomen van de dier-infecterende leden van de *Bunyaviridae* kon geconcludeerd worden dat het NS_M-gen van TSWV een extra toevoeging is aan het standaard bunyavirale genoom. De aanwezigheid van dit extra gen in TSWV leidt tot de veronderstelling dat NS_M een rol zou spelen bij de aanpassing van dit bunyavirus aan planten als gastheer. Om meer inzicht te krijgen in de mogelijke functie van het NS_M-eiwit, en tevens van het tweede niet-structurele eiwit NS_S, werden de desbetreffende genen gekloneerd en in heterologe expressiesystemen tot expressie gebracht (*E.coli*, baculovirus/insektecel). De aldus geproduceerde eiwitten werden opgezuiverd en gebruikt voor de produktie van polyklonale antisera. Door middel van immuno-elektronenmicroscopische studies kon vervolgens de intracellulaire locatie van het NS_S-eiwit worden vastgesteld in fibrillaire structuren in het cytoplasma van de geïnfecteerde plantecel (Hoofdstuk 5). De functie van dit eiwit bleef echter onbekend. Analyse van de NS_M-eiwit-synthese toonde aan dat dit eiwit alleen vroeg in het infectieproces detecteerbaar is. Het NS_M-eiwit bleek specifiek geassocieerd te zijn met nucleocapside-aggregaten en met plasmodesmata waarin het lijkt te aggregeren tot buisvormige structuren (Hoofdstuk 6). Deze waarnemingen vormen een sterke aanwijzing dat NS_M funktioneert als een viraal transporteiwit, betrokken bij het transport van cel naar cel van de ribonucleocapside-structuren.

Als eerste stap tot opheldering van de maturatie van de glycoproteïnen is het gen coderend voor de precursor voor deze eiwitten, gekloneerd en tot expressie gebracht in het baculovirus/insektecel-systeem. Zowel de rijpe glycoproteïnen als het precursoreiwit blijken geglycosyleerd te worden, op grond waarvan wordt aangenomen dat glycosylering zeer vroeg, op het niveau van de ongemodificeerde G1/G2 precursor, plaats vindt (Hoofdstuk 7). Hoofdstuk 8 bevat, ter afsluiting, een algemene discussie, waarin de evolutionaire verwantschap van TSWV met de dier-infecterende leden van de *Bunyaviridae* wordt belicht.

Curriculum vitae

Richard J.M. Kormelink werd geboren op 5 januari 1964 te Stad-Delden geboren. Hij behaalde het diploma VWO aan het Twickel College te Hengelo (Ov.) in 1982 en begon in datzelfde jaar met een studie Planteziektenkunde aan de toenmalige Landbouwhogeschool Wageningen. In 1983 behaalde hij de propadeuse en vervolgde zijn studie met een specialisatie in de "Fysiologische en Moleculair Biologische Aspecten van de Gewasbescherming". De doctoraal fase werd afgerond met afstudeeropdrachten in de Moleculaire Biologie, Moleculaire Virologie en Biochemie. Tussentijds werd in de periode maart-oktober 1987 in het kader van een praktijktijd Virologie onderzoek verricht op de Plant-Pathology Department aan de University of Kentucky, Lexington (USA), onder leiding van Robert J. Shepherd. In september 1988 behaalde hij het ingenieursdiploma in de Planteziektenkunde.

Vanaf november 1988 tot november 1992 was hij als assistent in opleiding verbonden aan de vakgroep Virologie van de Landbouwuniversiteit. Onder leiding van Prof. R.W. Goldbach verrichtte hij onderzoek aan TSWV, waarvan de resultaten beschreven zijn in dit proefschrift. Vanaf mei 1993 tot mei 1995 is hij als post-doc verbonden aan de vakgroep Virologie waar hij in het kader van een STW project onderzoek uitvoert naar het TSWV NS_M gen t.b.v de ontwikkeling van een universele detectietoets en nieuwe resistentiestrategiën.

Account

De Haan, P., Kormelink, R., Peters, D. and Goldbach, R. (1991). Genetic organization and expression of the tomato spotted wilt virus genome. In: Virus-thrips-plant interactions of tomato spotted wilt virus. USDA ARS 87, 60-66.

De Haan, P., Kormelink, R., Resende, R. de O., 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.

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., De Haan, P., Peters, D. and Goldbach, R. (1991). Tomato spotted wilt virus: A Bunyavirus invading the plant kingdom. Proceedings of a NATO Advanced Study Institute on Plant Molecular Biology. Edited by R.G. Herrmann & B.A. Larkins, Plenum Press, New York, 49-56.

Resende, R. de O., De Haan, P., De Avial, 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.

De Avila, A.C., De Haan, P., Kitajima, E.W., Kormelink, R., Resende, R. de O., Goldbach, R.W. and Peters, D. (1992). Characterization of a distinct isolate of tomato spotted wilt virus (TSWV) from Impatiens sp. in the Netherlands. J. Phytopathol. 134, 133-151.

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

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

Kormelink, R., Van Poelwijk, F., Peters, D. and Goldbach, R. (1992). Non-viral heterogeneous sequences at the 5' ends of tomato spotted wilt virus (TSWV) mRNAs. J. Gen. Virol. 73, 2125-2128.

Kormelink, R., De Haan, P., Meurs, C., Peters, D. and Goldbach, R. (1992). The nucleotide sequence of the M RNA segment of tomato spotted wilt virus: A bunyavirus with two ambisense RNA segments. J. Gen. Virol. 73, 2795-2804.

Goldbach, R., Kormelink, R., De Haan, P., Resende, R. de O., De Avila, A.C., Van Poelwijk, F., Van Lent, J., Wijkamp, I., Prins, M. and Peters, D.. (1992). Tomato spotted wilt virus: genome organization, transmission, and symptom induction. Proceedings Fifth International Symposium on Biotechnology and Plant Protection, College Park, Maryland, U.S.A., October 91-21.

De Avila, A.C., De Haan, P., Smeets, M.L.L., Resende, R. de O., Kormelink, R., Kitajima, E.W., Goldbach, R.W. and Peters, D. (1993). Distinct levels of relationships between tospovirus isolates. Archives of Virology 128, 211-227.

Wijkamp, I., Van Lent, J.W.M., Kormelink, R., Goldbach, R. and Peters, D. (1993). Virus-vector interactions during the transmission of tospoviruses by thrips. Proc. Exper. and Appl. Entomol. 4, 193-198.

De Avila, A.C., De Haan, P., Kormelink, R., Resende, R. O. de, Goldbach, R.W. and Peters, D. (1993). Classification of tospoviruses based on phylogeny of nucleoprotein gene sequences. J. Gen. Virol. 74, 153-159.

Wijkamp, I., Van Lent, J.W.M., 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.

Kormelink, R., Storms, M., Van Lent, J., Peters, D. and Goldbach, R. (1994). Expression and subcellular location of the NS_M protein of tomato spotted wilt virus (TSWV), a putative viral movement protein. Virology 199, in press.