Competence and Specificity of Thrips in the Transmission of Tomato Spotted Wilt Virus

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Cover: Laser scanning microscopic image of TSWV-infected midgut and salivary glands of *Frankliniella occidentalis* using FITC labeled nucleocapsid protein antiserum.

Propositions

1. To be transmitted tospoviruses have to reach the salivary glands before the thrips pre-pupates.

   This thesis

2. Tomato spotted wilt virus isolates containing defective interfering RNAs are less efficiently transmitted due to their reduced replication rate in the midgut of the vector.

   This thesis

4. Currently, the whole mount immunofluorescent staining (WMIS) is the most appropriate method to analyse the infection of tospoviruses within thrips.

   This thesis

5. The absence of squash leaf curl virus in the haemocoel of *Trialeurodes vaporarorium* can not be explained by an incompetence of this virus to cross the gut system of this whitefly.

   Rosell, R.C., Torres-Jerez, I. and Brown, J.K. (1999) Tracing the Geminivirus-whitefly transmission pathway by polymerase chain reaction in whitefly extract, saliva, hemolymph and honeydew. Phytopathology 89: 239-246.

6. The implication of a GroEL homologue of endosymbiotic bacteria in the white fly *Bemisia tabaci* in the transmission of tomato yellow leaf curl virus further rules out the possibility that this virus multiplies in its vector.

   Morin, S., Ghanim, M., Zeidan, M., Czosnek, H., Verbeek, M and van den Heuvel, J.F. (1999) A GroEL homologue from endosymbiotic bacteria of he whitefly Bemisia tabaci is implicated in the circulative transmission of tomato yellow leaf curl virus. Virology 256: 75-84

7. The environmental conditions in Europe are not favourable for a permanent establishment of *Thrips palmi* in this continent.

   Bayart, J.-D., Reynaud, P., Lemonnier, R., Cabazan, P. (1999) Éviter l'importance en Ile-de France de *Thrips palmi*. Bilan de trois années de contrôle. Phytoma 514: 53-55.

8. Thelotoky of *Thrips tabaci* may be regulated by a *Wolbachia*-like system.

9. Winged aphids seem to preferentially alight on the open eyes of the bicycle-rider.

Stellingen behorende bij het proefschrift
Competence and specificity of thrips in the transmission of
tomato spotted wilt virus

Wageningen, 22 juni, 1999

Tatsuya Nagata

To my parents and brother

Kaiko, Yoshihiro and Tsuyoshi

my wife

Alice Kazuko

and to my son Hiro

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Chapter 1

Introduction

Background

During the last two decades, a marked increase of outbreaks of tomato spotted wilt virus (TSWV) disease occurred in a great variety of agricultural and ornamental crops. This disease was originally described in tomato in Australia (Brittlebank, 1919). Its etiology was recognised a few years later (Samuel *et al.*, 1930). The discovery was followed by numerous reports on many other plant species being infected by this virus, the identification of various thrips species as its vector, and protocols to purify this virus for preparation of antisera (Best, 1968; Francki and Hatta, 1981; Peters *et al.*, 1991). The increase of disease incidence in the eighties attracted a great interest for this virus which resulted in the recognition that this virus had several common properties with the *Bunyaviridae*, as was proposed several years before by Milne and Francki (1984). Based on the 5' and 3' terminal sequences of its three genomic RNA segments, the virus was placed within the *Bunyaviridae*, as the type species of a new genus, denoted *Tospovirus*, embracing at this moment at least twelve distinct virus species.

Except for the members of the genus *Hantavirus*, bunyaviruses are transmitted by arthropods. The animal-infecting bunyaviruses utilise bloodsucking arthropods like mosquitoes, phlebotomine flies and ticks as vectors, while tospoviruses use phytophagous thrips (Mound, 1997). As far as determined, transmission is proceeded by active replication of the virus in the vector. The biology of the various vectors implies that certain developmental stages are not involved in acquisition and/or transmission of viruses. Except when transovarial transmission occurs, acquisition and transmission of mosquito-borne viruses are restricted to the adults, since the vector's larvae live in water and do not feed on vertebrates. In case of tick vectors, larvae and nymphs acquire virus, whereas the nymphs and the adults are the stages which will transmit the virus. An even more complex situation exists in thrips for their tospovirus transmission. The larvae and the adults are the only stages involved in acquisition and transmission as only they feed on plants. Both the first and the second instar larval stages acquire these viruses, while

the larvae at the end of their second stage and adults transmit them after a temperature-dependent latent period. Two developmental stages, the prepupae and pupae, *i.e.*, the stages in between the second larval and adult stages, do not feed and, hence, can neither acquire nor transmit the virus. The separation of stages which acquire and which transmit implies that the virus has to survive in moulting and pupation during which part of the tissues are replaced. The tospoviruses have to be transtadially maintained during the pupal stages in which dramatic transformations occur. The objective of the present study was to understand, in the thrips-tospovirus system, the processes determining and regulating vector competence and specificity, the virus tissue tropism during these stages, and the fate of the virus in transmitting and non-transmitting populations. Before presenting the results an introduction is given on the transmission of plant viruses with special reference to tospoviruses.

The tospoviruses

TSWV is the type species of the genus *Tospovirus* within the family *Bunyaviridae* (Murphy *et al.*, 1995). This virus has been considered for many years as a single member of a monotypic group of plant viruses. Over the past ten years, eleven additional tospovirus species, differing in serology, genome sequence, vector specificity and natural host range, have been described (Bezzera *et al.*, 1999). The symptoms caused by the tospoviruses generally consist of necrotic and chlorotic (ring)spots on the inoculated leaves, and mosaic, mottling, necrosis or chlorosis on the systemically infected leaves, stems and fruits. In addition, top distortion and stunting can be found on plants of many vegetable, fibre and ornamental plant species. The most frequently affected plants are groundnut, lettuce, pea, potato, sweet pepper, tobacco, tomato and many plant species used in the horticultural industry.

The number of susceptible plant species differs considerable for each of the various tospovirus species. Notably, TSWV can infect over 900 plant species within 80 botanical families. *Impatiens* necrotic spot virus (INSV) has been found to infect approx. 180 species occurring in 70 families (D. Peters, personal communication). A considerable lower number of plant species is susceptible to the other tospovirus species. This may reflect the number of studies made on these viruses, the more local significance of these viruses or the lower number of families in which susceptible species occur.

The tospovirus particle has a quasi-spherical shape, ranging from 70-110 nm in diameter. The virus particle consists of a lipid envelope on which two types of glycoproteins (G1 and G2) are anchored and which enclose three genomic RNA segments. These segments are denoted L (large), M (medium) and S (small) RNA

according to their sizes, and are encapsidated by multiple copies of the nucleocapsid (N) protein to form pseudo-circular structure. The L RNA is completely negatively stranded while both M and S RNA are ambisense (Goldbach and Peters, 1994). The L RNA consists of 8897 bases and encodes the RNA-dependent RNA polymerase protein (331.5 kDa) in an ORF in the viral complementary sense (De Haan *et al.*, 1991; Van Poelwijk *et al.*, 1993). The M RNA is 4821 bases long and encodes the glycoprotein precursor (127.4 kDa) in the viral complementary sense and a nonstructural (NS_M) protein (33.6 kDa) in its viral sense (Kormelink *et al.*, 1992). The latter protein has been shown to represent the viral movement protein (Kormelink *et al.*, 1994, Storms *et al.*, 1995 and 1998). The glycoprotein precursor matures into G1 (78 kDa) and G2 (58 kDa) glycoproteins forming the protruding spikes on the envelop. The S RNA is 2916 bases long and encodes the N protein (28.8 kDa) in the complementary sense and a nonstructural (NS_S) protein (52.4 kDa) in the viral sense orientation (De Haan *et al.*, 1990). The function of the latter protein is still not known. For some tospovirus isolates, this protein occurs in large paracrystalline aggregates or flexible filaments (Kormelink *et al.*, 1991) in the infected plants and accumulates in well-detectable amounts in the salivary glands of thrips (Wijkamp *et al.*, 1993).

Thrips as vector of tospoviruses

Thrips is the common name for the insect species belonging to the order Thysanoptera. This order includes more than 5000 species (Mound and Teulon, 1995). Because of their minute body size (0.5 to 10 mm), they often remain unnoticed and when found, difficult to identifiy. Estimates suggest that only two thirds of the total number of thrips species is currently known (Mound, 1997). The order is divided into two suborders, *Tubulifera* and *Terebrantia*. The *Tubulifera* consists of a single, but large family, the *Phlaeothripidae*, including of more than 3000 recorded species. The *Terebrantia* embraces seven families, of which the *Thripidae* is the largest one. This family counts more than 1700 species (Mound, 1997). The thrips species vectoring tospoviruses are all classified in the *Thripinae*, a most diverse subfamily within the *Thripidae*. Most species in the *Thysanoptera* are saprophytic. Only a few hundreds are reported as pests of cultivated plants. All tospovirus vector species reported are known as important crop pests. Until now, eight members of only two genera, the genus *Frankliniella* (5 species) and the genus *Thrips* (3 species), are reported to transmit tospoviruses (Mound, 1996, Webb *et al.*, 1998). The status of two species, *Scirthothrips dorsalis* (D.V.R. Reddy, ICRISAT, India; personal communication) and *Thrips flavus*, (Mound, 1996; Singh and Krishnareddy, 1996) as vector has been disputed. For many years *T. tabaci* was

considered to be the main vector species for TSWV. This position has recently been challenged by *F. occidentalis*. This species, which was found to be an efficient vector (Wijkamp *et al.*, 1995), expanded in the last thirty years from the western states in the USA to reach almost a global occurrence. This dispersal had a great impact on the incidence and emergence of TSWV and some other tospoviruses. The impact of *T. palmi* as tospovirus vector, which seems presently to be in a phase of world-wide dispersal (Mound, 1997), has not yet be estimated. Although this species has been reported as a vector of TSWV (Fujisawa *et al.*, 1988), its vector potency needs to be confirmed.

Thrips feed themselves by a piercing-sucking type of mechanism. Their unique feeding apparatus consists of one mandible and two maxillae forming an elongated stylet. Larvae and adults use a similar 'punch and suck' feeding technique. The mandible punches a hole through which the maxillary stylets penetrates the cell. The content is then subsequently ingested. Feeding on plants results in a range of symptoms. Silvering, a result of air entering the emptied cell, is often observed. Scarring is observed on fruits and the development of corky tissue has been noticed on some fruits. Heavy infestation of thrips can cause total deformation of the plants occasionally resulting in a total loss of a crop.

Thrips oviposit on younger leaves, stem, flowers and fruits. The life cycle of the *Thripinae* encompasses the egg stage, two larval stages, two pupal stages and the adult stage. For instance, in *F. occidentalis* and *T. tabaci*, it takes 12-15 days to complete a life cycle from egg to adult at 25 °C (Jarosik and Lapchin, 1998). Adult thrips can survive more than one month old and produce 100 - 200 eggs in this period.

Anatomy of thrips

Most of the studies on the anatomy of a tospovirus transmitting thrips has been made on *F. occidentalis* (Ullman *et al.*, 1989; Moritz, 1997). The information obtained on the anatomy of this species can also be applied to a large extent to other species of the subfamily *Thripinae* due to the high similarities of their internal structures. As no significant differences between the anatomy of *F. occidentalis* and *T. tabaci* were found in the present study, only the internal anatomy and digestive system of *F. occidentalis* will be described here and is illustrated for *Hercinothrips femoralis* in Fig. 1. The digestive tract consists of a single tube formed by the foregut, midgut and hindgut. The foregut and hindgut are embryonically of ectodermal origin. The thick cuticle surface lining these organs are highly impermeable. The midgut is of endodermal origin with a soft inner epithelial cell layer. The midgut is formed by two loops resulting in three distinct parts, denoted anterior (Mg1), middle (Mg2) and posterior midgut (Mg3) (Ullman *et al.*, 1989).

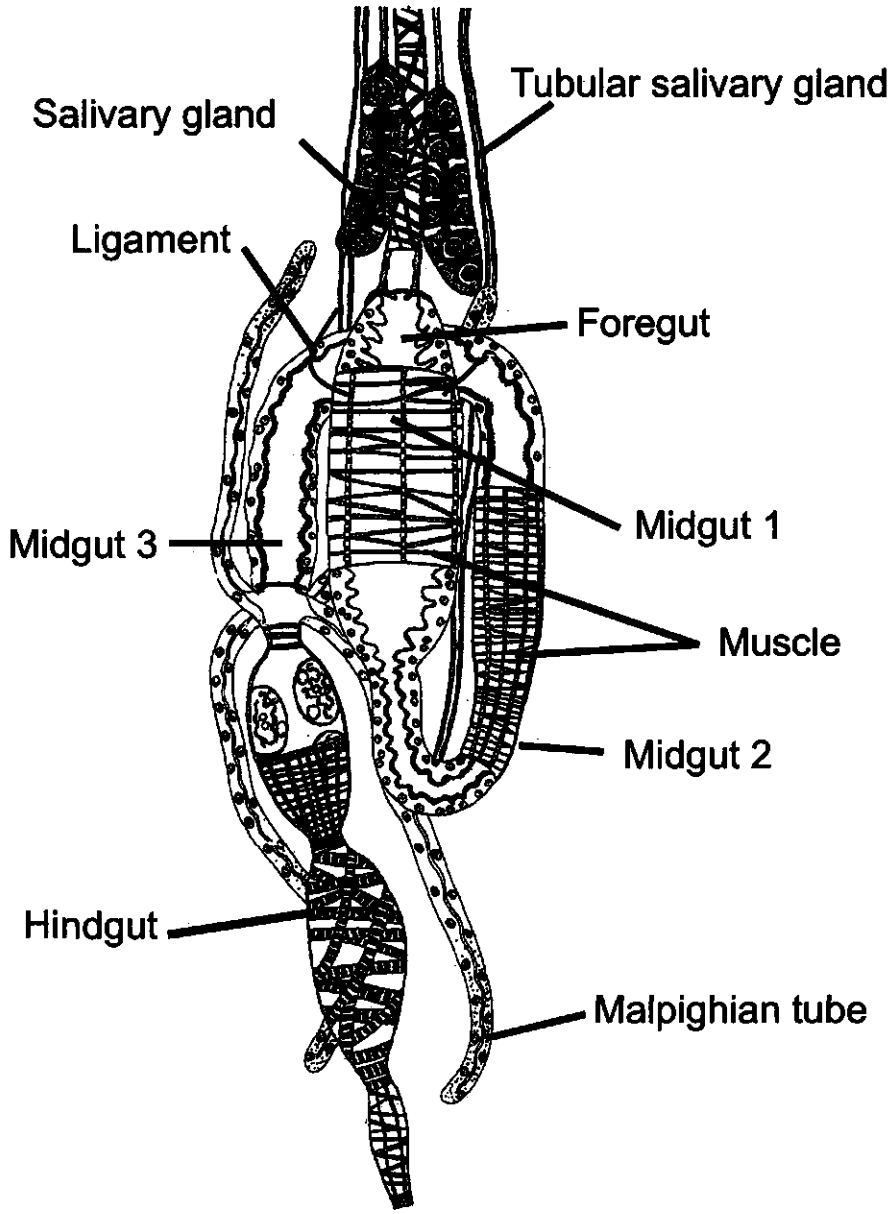

Fig. 1. Composite drawing of the alimentary tract and associated organs of the thrips *Hercinothrips femoralis*. Courtesy of Dr. H. Moritz and modified by T. Nagata

Each part may have a specific function in the digestion process as reported for other insects (Baldwin *et al.*, 1997; Billingsley and Lehane, 1997). The midgut lumen is composed by a well developed microville and glycocalix complex. Until now, the occurrence of a distinct peritrophic membrane or matrix (PM), which is often considered

to form a potentially physical barrier against virus infections (Kaslow and Welburn, 1997), has not been reported in thirps (Moritz, 1997; Moritz and Schreiter, 1998).

The salivary gland complex is formed by two lobular and two tubular glands. The tubular glands may correspond to the accessory salivary glands found in other insects. The function of these glands has remained unknown. Evidence has been provided that they form a bridge between the Mg1 and the salivary glands. It has been suggested that this organ act as a connecting tube through which virus may be transported from the midgut to the salivary glands (Ullman *et al.*, 1989). However, the existence of a direct connection between the tubular glands and midgut could not be confirmed in other studies (Del Bene *et al.*, 1991). Inaddition, thin thread-like structures, called ligaments, connected the anterior midgut with the lobular salivary glands (Ullman *et al.*, 1989). This ligament is connective tissue-like structure, but the real function of this tissue is not known.

Virus-vector relationship

Most plant viruses depend on insects for their dissemination. The common insects which are involved in plant virus spread are aphids, leafhoppers, planthoppers, beetles, whiteflies and thrips. According to the type of transmission, three mechanisms can roughly be distinguished in the virus-vector relationship. They are referred to as non-persistent, semi-persistent and persistent transmission, reflecting the period that the vector is viruliferous after virus acquisition. Viruses, transmitted in the first way, are acquired while the vector probes a host, and remain restricted to the food channel of the stylets and/or the anterior part of the intestinal tract. These viruses are released together with some saliva to the plant during the next probe or feeding activity (Martin *et al.*, 1997). This type of transmission is completely or almost completely restricted to aphids. The non-persistently transmitted viruses are either isometric shaped (caulimo- and cucumoviruses) or elongated (poty-, potex-, and carlaviruses). Due to the close association of these viruses with the stylets of the vector, this transmission type is referred to as "stylet-borne".

The semi-persistent way is often considered as intermediate between the non-persistent and persistent manner of transmission. The viruses, transmitted in this way, are acquired in a period of several hours. Although being directly infectious after whatever acquisition period, the vector remains able to transmit the virus for several days. The virus particles are believed to move beyond the stylets and to attach to the foregut. The mechanism by which these viruses are transmitted does not basically differ from the stylet-borne viruses except for the retention time and location in the foregut. As in the

case of the non-persistently transmitted viruses, the infectivity is lost when nymphs moult. These viruses can be referred to as "foregut-borne" viruses (Nault and Ammar, 1989). Examples of such viruses are the closteroviruses.

The persistently transmitted viruses are divided into two groups, the circulative and the propagative viruses. The circulative viruses are acquired from the phloem in an acquisition access period of, at least, several hours. After acquisition, the virus passes through the gut wall and the basal lamina and circulates via the haemocoel to the (accessory) salivary glands. The passage through the aphid may take some days, a period defined as the latent or incubation period. Luteoviruses are examples of persistently transmitted circulative viruses. One of which, potato leafroll virus (PLRV), seems to pass the posterior midgut (Garret *et al.*, 1996), while, another luteovirus, barley yellow dwarf virus passes the hindgut via a process of receptor-mediated endocytosis (Gildow, 1993). Upon reaching the salivary glands, the virus is transmitted after its release in the salivary duct and addition to the saliva. During the movement through the haemocoel, PLRV is protected against the proteolytic activities by a protein excreted by the endosymbiotic bacteria *Buchnera sp* (Van den Heuvel *et al.*, 1994; Hogenhout *et al.*, 1998). The viruses transmitted in this way by aphids belong to the *Luteoviridae* and those by leafhoppers or whiteflies to the *Geminiviridae*.

The viruses of the second group, the propagative viruses, not only circulate through the vector like the previous group, but also multiply in the vector before they can be transmitted. Due to this feature, these viruses could also be considered as insect viruses. Plant viruses belonging to the *Reo-* and *Rhabdoviridae* and the genus *Tospovirus* propagate in their vectors (Fukushi, 1933 and 1940; Sylvester, 1969; Sylvester and Richardson, 1992; Peters and Black, 1970; Wijkamp *et al.*, 1993). There is also some published evidence that tenui- and marafiviruses propagate in their vectors (Shinkai, 1962; Rivera and Gamez, 1986). Leafhopper- and aphid-borne rhabdoviruses, and leafhopper-borne reoviruses are well known viruses in this group. The location and maturation of these viruses in their insect vectors have been studied in the 70's. Due to their size and the formation of large aggregates in infected tissues, these viruses are readily recognised in light and electron microscopic immunohistological studies. After being ingested, rhabdoviruses infect the alimentary canal lining, escape into the haemocoel, infect the muscles, fatty body, tracheal and nervous systems, heart, haemocytes, reproductive and epidermal tissues, and the salivary glands (Sylvester and Richardson, 1992). Infection of the reproductive organs suggests that they may be transmitted transovarially, as has been demonstrated for some of these viruses (Sylvester, 1969). The wide spectrum of tissues and organs that can be infected by these viruses without any pathogenic effect implies a long co-evolutional history with their vectors.

The tospoviruses also propagate in their thrips vector and thus are transmitted in a persistent way. However, the way they are transmitted differs from the mechanisms established for the other propagatively transmitted viruses. In contrast to the other propagative (and circulative) viruses, they are acquired from and transmitted to the mesophyll cells instead of the phloem, while the acquisition and infection access periods are considerably shorter than for the other persistently transmitted viruses (Wijkamp *et al.*, 1993).

Interactions between tospoviruses and thrips

T. tabaci was the first identified vector of TSWV (Pittman, 1927), followed a few years later by species of the genus *Frankliniella* (Gardner *et al.*, 1935; Samuel *et al.*, 1930). At this moment eight thrips species are definitively identified as vector for tospoviruses. The transmission of tospoviruses by thrips is characterised by some unique features. Only the larvae, but not adult, can acquire the virus, a process which leads to multiplication (Wijkamp *et al.*, 1993; Ullman *et al.*, 1993a) and usually also to transmission of the virus (Chapter 4). The competence to acquire tospoviruses decrease with the age of the larvae (Van de Wetering *et al.*, 1996). Virus can be transmitted by thrips, late in their second larval stage when the virus is acquired by first instars early in their development and by adults after a temperature-dependent latent period (Wijkamp *et al.*, 1993). The median latent period (defined as the period at which 50 % of thrips have made their first transmission) was 84 and 171 h when they were kept at 27 or 20 °C, respectively (Wijkamp *et al.*, 1993). The median acquisition and inoculation access period were 67 min on *Impatiens* and 59 min on petunia (Wijkamp *et al.*, 1996). These parameters show that TSWV, hence probably all tospoviruses, are transmitted in relatively short periods when the thrips is viruliferous. This type of transmission may be referred to as persistent or circulative propagative. However, it may also be designated "biological transmission" as done for the bunyaviruses infecting animals and transmitted after replication in arthropods.

A recent study (Wijkamp *et al.*, 1995) showed that vector specificities exist between several tospoviruses and their vectors. INSV could only be transmitted by *F. occidentalis* at a high efficiency (Wijkamp *et al.*, 1995), whereas TSWV was found to be transmitted by four different thrips species at variable efficiencies. The factors determining virus-vector specificities, the processes leading to the ability to transmit the virus and the high variability of the virus content in thrips are not yet fully understood. The multiplication of TSWV in thrips has been demonstrated by an increase of N protein titre in the larvae and adults after their emergence from pupae (Wijkamp *et al.*, 1993; Ullman *et al.*, 1993a). The

accumulation of the NS_S protein, which can only be synthesised after replication of the S RNA is another proof of TSWV multiplicaiton in thrips (Wijkamp *et al.*, 1993; Ullman *et al.*, 1993a). Histological studies revealed that the infection was restricted to the midgut epithelium, midgut muscle tissue and salivary glands (Ullman *et al.*, 1992b, 1993a and 1995; Wijkamp *et al.*, 1993). The viral proteins have been detected in various cell compartments of the midgut (Ullman *et al.*, 1995). In larvae, the N, G1 and G2, and NS_S proteins were found in dense masses, amorphous inclusions and paracrystalline arrays in cells of the midgut epithelium, the first tissue expected to become infected (Ullman *et al.*, 1995). However, any process describing the temporal order in the infection process, which eventually converse thrips into a transmitter, is lacking.

Thrips cell cultures

Continuous cell lines of vector insects are of great importance in studies on the molecular processes involved in the virus infections in vector cells (Black, 1984). Since the first successful establishment of insect cell lines (Grace, 1962) and their infection with a propagatively transmitted plant virus (Chiu *et al.*, 1966; Chiu and Black, 1967), several insect cell lines were established and used in studies on the kinetics by which these cells were infected, and the characterisation of the genome of wound tumor virus. These studies evoked a series of studies in which continuous leafhopper cell lines have been used to study several plant infecting reo- and rhabdoviruses (Hsu and Black, 1973; Chiu *et al.*, 1970, Kimura, 1984; Kimura and Omura, 1988). The establishment of the insect cell lines often requires a long period of incubation in completely sealed vessels before continuous cell growth is initiated (Grace, 1962). Initiation of growth will mainly depend on the composition of the medium, but also on other factors such as the osmolarity, the pH and incubation temperature. Some attempts have been made to establish continuous cell lines of aphids, the most important group of plant virus vectors. However, these trials have not yet resulted in continuous cell lines, possibly due to the lack of the suitable medium or suboptimal use of any other factors involved in the establishment of the cell cultures (D. Peters, personal communication). Some efforts to establish thrips cell lines were recently made (Hunter and Hsu, 1995 and 1996). Although cell cultures with some outgrowth of the explants were obtained, real secondary cultures were not obtained. Such cultures are required to answer questions regarding virus entry into cells, the existence of receptors, virus replication and maturation and virus escape from the insect cells. The establishment of continuous cell lines of the natural vector host is an essential tool, thus, efforts to establish such lines from the proper vector are still highly justified.

Scope of this study

The study described in this thesis aims to elucidate the fate and pathway of ingested TSWV in thrips during their development from larvae to adult. Insight in this process will contribute to a better understanding of the factors regulating and determining vector competence and specificities.

Analysis of the differences in virus susceptibility among thrips species or populations was approached by infection of cell cultures. The methodology developed and the media used to prepare primary cell cultures of the species *F. occidentalis* and *T. tabaci* are described and discussed in Chapter 2. The cultures obtained were derived from an efficiently transmitting *F. occidentalis* population and from a non-transmitting *T. tabaci* population which was not able to transmit the virus. The results obtained by inoculation of these cultures with preparations of purified TSWV particles are described in Chapter 3. To analyse the tissue tropism of TSWV in thrips in relation to its vector competence, a novel histological technique, called whole mount immunofluorescent staining (WMIS) was developed (Chapter 4). Using this technique and other immuno-histochemical techniques, infection of the midguts and salivary glands during the development of *F. occidentalis* thrips was described (Chapter 4). By the combination of all techniques, the temporal development of the virus infection in larvae and adults could be elucidated. To define the various barriers which may regulate the development of virus infection, specific TSWV mutants were used which failed either to infect the thrips or to convert the thrips in a transmitter after infection. Definite barriers were observed at the level of virus entry in the midgut epithelium or virus escape from the midgut to the salivary glands (Chapter 5). The pathway of the virus within the thrips and the mechanism determining the vector specificities were further unravelled by analysing the infection in thrips of a transmitting F. occidentalis population and a non-transmitting T. tabaci population (Chapter 6). Concluding remarks of this study is presented in Chapter 7.

Chapter 2

The establishment of thrips cell lines to study tomato spotted wilt virus replication

SUMMARY

Two world wide occurring thrips species *Frankliniella occidentalis* and *Thrips tabaci*, reported to be the main vectors of tomato spotted wilt virus (TSWV), were used to establish cell cultures from developing embryos. Among the five different insect cell culture media and their modifications tested, Kimura's medium gave the best results for both thrips species. Migration of fibroblast-like cells from the tissue fragments started within 24 h after explanting. The first multicellular vesicles, consisting of a monolayer of cells already developed one week after incubation of the explants. These vesicles, showing also a tendency to form clusters, evidently increased in number and size in *F. occidentalis* derived cultures. They were less conspicuously present in *T. tabaci* cultures. One line consisting of these vesicles from *F. occidentalis* was maintained for more than two years and subcultured fifty times. The formation of vesicles consisting of a monocellular layers was confirmed by electron microscopy.

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INTRODUCTION

In spite of the fact that many plant viruses are transmitted by insects, only a few are propagated in these vectors (Matthews, 1991). This constitutes one of the reasons why only a few plant viruses have so far been studied in cell cultures of their vectors (Chiu and Black, 1967; Black, 1979). A second reason can be found in the difficulties encountered in the establishment of cell lines from vectoring insect species. Studies on plant viruses in insect cell cultures are not only of interest for analysing the intimate interactions between the propagatively transmitted viruses and their vectors on the cellular level, but also for the fact that they are often not mechanically transmissible to their plant hosts (Peters and Black, 1970; Adam and Sander, 1976). The investigations made so far have mainly been restricted to leafhoppers (Black, 1979; Kimura, 1984) because continuous cell lines were readily obtained from these vectors. Attempts to establish continuous cell lines failed thus far for aphids (Peters and Black, 1971; Adam and Sander, 1976), and thrips (Hunter and Hsu, 1995 and 1996) which vector rhabdo- and tospoviruses, respectively, in a propagative way.

The tospoviruses, forming a genus within the *Bunyaviridae*, cause serious diseases in a large number of plant species (Goldbach and Peters, 1994). The type species of this genus, tomato spotted wilt virus (TSWV), has been known to be transmitted by several thrips species (Sakimura, 1962a, b; Wijkamp *et al.*, 1995). It has been shown that TSWV replicates in the western flower thrips, *Frankliniella occidentalis* (Wijkamp *et al.*, 1993; Ullman *et al.*, 1993a). Establishment of continuous cell lines of this and other thrips species would provide a powerful tool in the study of tospoviruses and their replication strategies. At present, continuous cell lines of thrips are not available (Hunter and Hsu, 1995 and 1996). Here, we report our studies to establish continuous cell lines of thrips and the further characterisation of a selected cell line of *F. occidentalis* which has been maintained for more than two years.

MATERIALS AND METHODS

Setting up and maintaining cell cultures of thrips

A population of *F. occidentalis* Pergande and of *T. tabaci* Lindeman were reared in glass jars at 26 °C and 16 h photoperiod on fresh bean pods or leek, respectively. In nature, both thrips species lay eggs (250-300 µm in length) inside the plant tissue, making collection of large numbers of eggs exceptionally difficult. Recently, Loomans and Murai (1997) developed a simple and efficient technique (Murai cage technique) by which the caged thrips could oviposit in water between a layer of parafilm and a plastic Petri dish, hence

enabling us to collect large numbers of eggs within a few minutes.

To set up a primary cell culture, approximately 200 to 500 of two to three days old eggs were collected from these Murai cages. At this stage, the red eye spots could clearly be distinguished in the head region. The eggs were then surface-sterilised with 70% ethanol, gently crushed with a sterile pestle in a balanced salt solution (Solution F, Peters and Black, 1970), and subsequently incubated with trypsin (0.25%) or collagenase (200U/ml) for 1 h at room temperature or overnight at 4 °C. After washing with medium to remove the added proteases, the treated fragments and dissociated cells were explanted with 0.5 ml medium in a 3.5 cm in diameter plastic Petri dish. Half of the medium was weekly replaced by fresh medium.

Five different media, Kimura's (Kimura 1984), Mitsuhashi and Maramorosch (M&M, Mitsuhashi and Maramorosch, 1964), Hink's (Hink, 1970), Grace's (Grace, 1962), TC-100 (Gardner and Stockdale, 1975), with some modifications (Table 1) as well as mixtures of these media, were evaluated for their suitability to support cell growth for a period of one month or longer by visual observation with an inverted field microscope. Attempts to subculture the primary cells were made in the wells of 24 multiwell plastic plate (Nunc) or in flat sided tubes (Nunc).

Electron microscopy

The cultured cells of *F. occidentalis* were collected by microcentrifugation in an Eppendorf tube, washed 3 times with PBS, and fixed in a solution of 3 % paraformaldehyde and 2 % glutaraldehyde for 30 min. The pellets were embedded in 5% gelatine and cut in small pieces. After staining with 2% uranyl acetate and 1% osmium tetroxide, the samples were dehydrated in a series of 50 to 100 % ethanol solutions, and embedded in LR-white (London Resin). After solidification at 60 °C for 16 h, they were sectioned at 60 to 70 nm. The sections were stained with uranyl acetate and Reynold's lead citrate, and studied with an electron microscope (Philips CM12).

RESULTS

Evaluation of the primary cultures and their evolution

The number of tissue fragments which were able to attach within a couple of days to the bottom of a Petri dish varied with the medium used. In addition, single cells varying in form and size adhered to the bottom, but they never showed any sign of growth. Non-attached cells, tissue fragments and embryo sacs which remained floating in the medium were removed at the first change of medium one week after seeding. Most attached tissue fragments were observed to originate from the head region as could be judged from the

presence of eye spots. Cell fragments from abdomen or other parts were supposedly washed off at the first medium change. Similar observations have also been made for the first stages of primary cell cultures of leafhoppers (I. Kimura, personal communication). Fibroblast-like cells (Fig. 1A) started to migrate from the tissue fragments as early as 24 h post-seeding, followed by spread over the surface of the plastic dish. This cell migration continued during the first two weeks. After this period, migration gradually reduced in the following weeks and became ultimately undetectable.

Five different media, as well as some modifications and mixtures of these media were evaluated for their efficiency to support cell growth in the primary cell cultures of both *F. occidentalis* and *T. tabaci.* This was assessed by direct observation of cell migration and the rate at which the attached cells deteriorated (Table 1).

Modifications were based on varying the Na/K ratio or the concentrations of yeastolate and lactalbumin hydrolysate. However, none of these modified media resulted in better growth rates as compared to the original media (Table 1). Among the media tested, only Kimura's and M&M medium showed strong cell migration and high survival rates of the cells up to one month after seeding. With the other media tested only weak cell migration of fibroblast-like cells could be seen, and cells started to degrade before one month post-explanting.

In Kimura's medium many cells could survive longer and expanded in number, although a mild degradation of cells or detachment was observed over a period of 3 months. In this medium monolayer cell sheets migrating on the bottom of Petri dishes from tissue fragments were occasionally seen (Fig. 1C). These sheets increased in size for approximately one month. Some of the monolayer sheets transformed into vesicular structures floating in the medium (Fig. 1D), whereas the remaining tissue of the sheet showed deterioration.

In Kimura's and M&M media, hollow sphere- or balloon-like structures (Fig. 1B), formed by a monolayer of cells, started to appear one week post-seeding. These sphere-like multicellular structures, in literature also referred to as vesicles or vesicular structures, have also been reported in primary or continuous cell lines of *Nephotettix cincticeps* (Mitsuhashi, 1965), *Blattella germanica* (Kurtii and Brooks, 1977), *Aedes aegypti* (Peleg and Shahar,

Fig. 1. Light microscopy graphs of a nine months (A,B) and a two years old (C,D) cell lines and electron micrography of two years old *F. occidentalis* cells (E,F). A) Fibroblast-like cells migrating from a tissue fragment. B) Vesicular structures floating in the medium. C) Cell sheet adhering on the bottom surface. D) Generation of vesicles from a cell sheet on the bottom. E) Vesicular structure consisting of monolayer. F) Higher magnification of the cells forming vesicles. Bar = 2 µm (F), 20 µm (E), 40 µm (A,C), 100 µm (B,D)

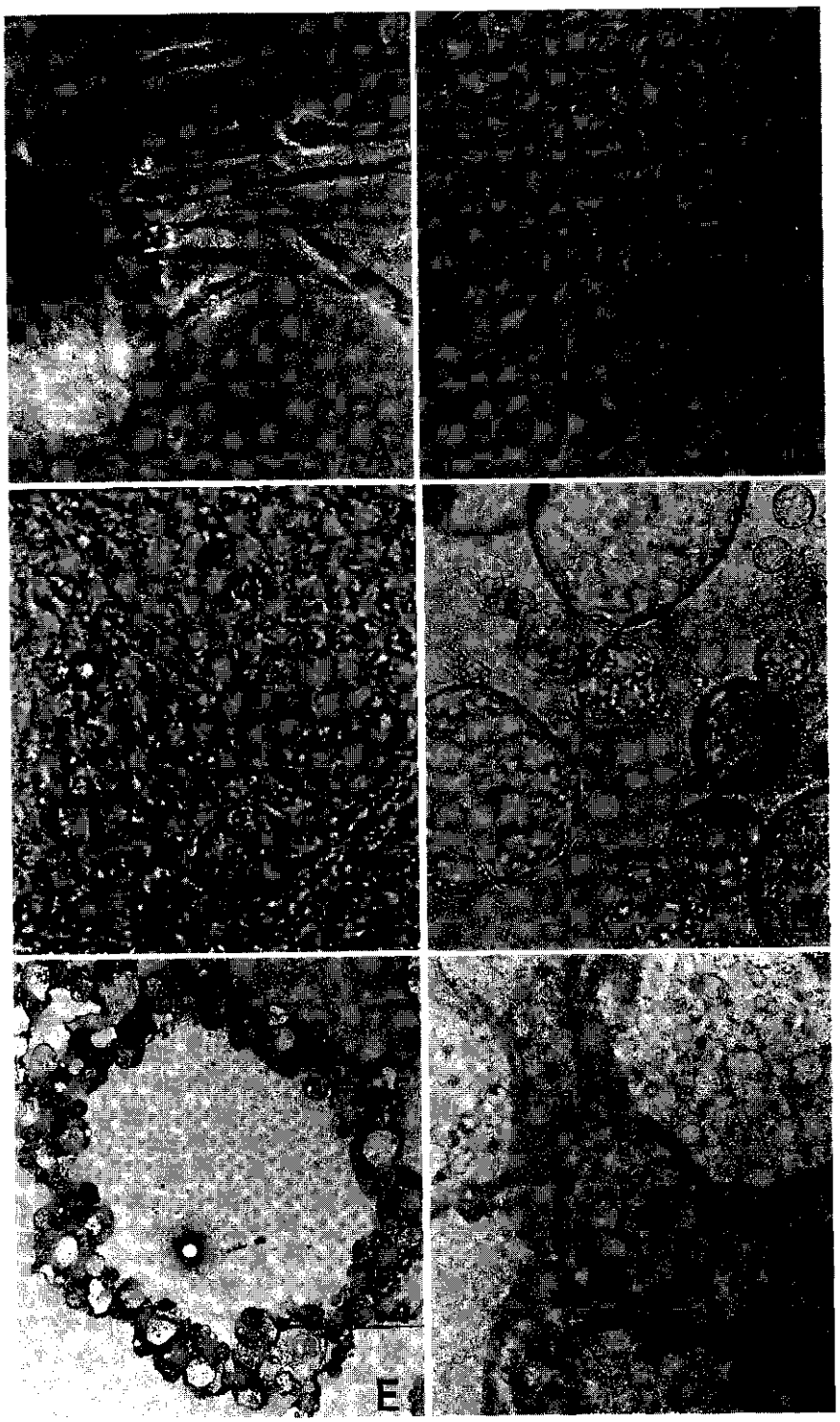
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Table. 1. Evaluation of cell growth in primary cell cultures of the thrips *F. occidentalis* in the media used.

Medium	FBS (%)	Osmolarity	Cell growth [5]
Kimura	12	282	+++
Kimura + M&M (4:1)	14	nt	+++
Kimura + M&M (1:1)	16	nt	++
Kimura + M&M (1:4)	18.4	nt	++
M&M	10	320	++
M&M	20	308	+
M&M	30	nt	+
M&M without NaCl	20	nt	-
M&M $-^1/_2$x NaCl [1]	20	257	+/-
M&M - NaCl, + KCl [2]	20	319	+/-
M&M - $^1/_2$x NaCl, + KCl [3]	20	320	+/-
M&M -0.2x NaCl, + KCl [4]	20	320	+
Grace	10	369	+
Hink + M&M (1:1)	15	351	+
Hink	10	370	+
Hink + 2x Yeastolate	10	408	+/-
Hink+ 2x Lactalbumin hydrolysate	10	399	+
Hink + 2x Yeast. & L. hydrolysate	10	443	+/-
TC-100	10	223	+

1) This medium contained half of the amount of NaCl originally present in M&M medium. 2) NaCl was replaced by the same molarity of KCl. 3) Half amount of NaCl was replaced by KCl on molar base. 4) 20% of NaCl was replaced by the same amount of KCl on molar base. 5) Levels at which cell growth is supported. nt= not tested.

1972), and *Leptinotarsa decemlineata* (Dübendorfer and Liebig, 1992). The growth of these vesicles in size and number was invariably better in Kimura's medium than in M&M medium. In M&M medium, growth and proliferation of vesicular structures ceased after one to two months and deteriorated thereafter very fast.

The use of Kimura's medium with non-heat inactivated fetal bovine serum (Dübendorfer and Liebig, 1992) 4 months after seeding resulted in a slow, but prolonged development of vesicles of *F. occidentalis*. Some of them reached a diameter of 1 mm but then stopped growing. These vesicles always showed increased levels of melanisation and/or degradation.

Attempts to establish and maintain lines of these vesicular structures of *F. occidentalis* using Kimura's medium were made. The first subculturing was done 6 months after seeding. Treatment of these structures with proteases as trypsin, collagenase or dispase did not affect the structures and failed to release single cells to form monolayers on the bottom.

Occasionally, some vesicles adhered to the surface of the dish by the attachment of a couple of cells, which migrated from the vesicles. However, this normally did not result in a continuous cell migration from the vesicles. The vesicles increased in number and size either by budding or pinching off. These generation of new vesicles occurred often clustered around a centre, resulting in a complex of vesicles. In attempts to subculture them, the vesicles collapsed when they were centrifuged or forcefully pipetted, but it was often noticed that they recovered.

Changing the pH of the medium from 6.5 to 6.0 at the second subculturing resulted in an intense proliferation of these structures. Small and numerous vesicular structures (30-300 µm in diameter) were then present, mostly in groups of 5 to 10 vesicles. At this pH, vesicles never became as large as at pH 6.5 (up to 1 mm), although cell deterioration occurred less. One of the continuous cultures, being subcultured fifty times, is presently more than two years old.

Embryos of the onion thrips *T. tabaci* were also used for starting a cell culture similarly as has been done with embryos of *F. occidentalis*. A comparison of the performance of cells of the two species showed a less active growth of *T. tabaci* cells. The migration of fibroblast-like cells in *T. tabaci* culture was moderately stronger than those of *F. occidentalis*, however, this type of cells did not show any sign of multiplication and stopped migration and deteriorated after three months. Growth of vesicular structures was less pronounced, therefore it was not possible to subculture the cells.

Electron microscopy

The characteristics of the two-year maintained cell line of *F. occidentalis* was further studied by electron microscopy. Monolayer of cells formed the vesicle-like structures (Fig. 1E), confirming the observations made by light microscopy. These cells were always accompanied with well-developed lysosome-like organelles (Fig. 1F). Their presence suggested that these cells originated from an organ with high digestive activities, e.g. midgut epithelium. Since their morphology and character may change during the long time of incubation, the cells may have been adapted to the new environment and therefore difficult to be identified.

DISCUSSION

The establishment of stable continuously growing insect cell lines is often a time consuming process. Periods as long as four years have been reported (Dübendorfer and Liebig, 1992). A continuous thrips cell line producing vesicular structures has now been obtained and maintained for more than two years. This line is rather difficult to manipulate in experimental

studies.

The formation of vesicular structures is not a specific feature of thrips cells. They have been reported in several primary and continuous insect cell lines (Mitsuhashi, 1965; Kurtti and Brooks, 1977; Peleg and Shahar, 1972; Dübendorfer and Liebig, 1992). Kurtti and Brooks (1977) and Dübendorfer and Liebig (1992) established cell lines from these vesicles. The thrips cells which formed the vesicular structures did, thus far, not adopt to monodispersable cells while they grew either in suspension cultures or in monolayers adhering to the bottom.

Hunter and Hsu (1995) reported that Kimura's medium supplemented with small amounts of L15 (20%) and M&M (5%) medium showed a comparatively higher cell growth than without these additions. The fibroblast-like and epithelial-like cells they found in *F. occidentalis* primary cell cultures could survive for 7 months in this medium (Hunter and Hsu, 1996). Since cell growth was as large in Kimura's original medium as in Kimura's medium supplemented with M&M medium (Table 1), Kimura's original medium was preferred in our studies for establishing continuous cell lines. Although no cell growth was observed solely in M&M medium by Hunter and Hsu (1995), cell growth was immediately obvious when this medium was used in our condition.

A great progress on the maintenance of cell cultures could be obtained by switching from heat-inactivated to non-inactivated FBS. This may be one of the answers to get continuous cell lines of thrips. Osmolarity and pH are also factors that could affect insect cell growth (Sohi, 1980). Although established cell lines often show a wide tolerance for changes in the pH or osmotic pressure, primary cell cultures are rather sensitive. A better development of the vesicular structures was noticed when the medium had a pH of 6.0.

The primary cell cultures of *T. tabaci* did not perform as good as those of *F. occidentalis*. This difference might be explained by species dependent factors or by differences in ploidy. Our population of *F. occidentalis* consisted of males and females, whereas the *T. tabaci* population was parthenogenic and produced only females. Genetic variability of *T. tabaci* is considered monotypic and very narrow, by which their cells may adapt poorly to any unnatural environment.

Chapter 3

Multiplication of tomato spotted wilt virus in primary and continuous cell cultures derived from two thrips species

SUMMARY

Primary cell cultures prepared from embryos of the thrips species *Frankliniella occidentalis* and *Thrips tabaci* were tested for their potential to support replication of tomato spotted wilt virus (TSWV). Using polyclonal antibodies against the viral nucleocapsid protein (N) and indirect immunofluorescent staining, discrete spots with strong signals were observed in the cytoplasm at 48 h post-inoculation in the cell cultures of a transmission competent *F. occidentalis* population and a *T. tabaci* population which failed to transmit the virus. The infection was found in approximately 40 % of the monolayer cells of both thrips species. Using antibodies against a nonstructural protein (NS_S) of TSWV, uniform and more diffused staining was observed throughout the cytoplasm of these cells, underlying active genome replication. The NS_S protein accumulated slower than the N protein in the cells of both thrips species. Infection of the continuous cell line of *F. occidentalis* was further confirmed by electron microscopy, which also revealed that the N protein accumulated in dense-masses characteristic for nucleocapsid aggregates described previously. No multiplication of TSWV was observed in a heterologous insect cell line, *i.e.*, from *Spodoptera frugiperda*, suggesting the existence of specific host factors in the thrips-derived cells.

A condensed version of this chapter has been published as: Tatsuya Nagata, Marc M. H. Storms, Rob Goldbach and Dick Peters (1997) Multiplication of tomato spotted wilt virus in primary cell cultures derived from two thrips species. *Virus Research* **49**, 59-66.

INTRODUCTION

Cell cultures of insect vectors represent strong tools to study the replication cycle both of true insect viruses and of insect-vectored plant and animal viruses (Black, 1979; Nuss and Dall, 1990; Mitsuhashi, 1994; Uyeda *et al.*, 1995). The first continuous cell cultures of a plant virus vector which could successfully be infected were established for *Agallia constricta,* the vector of wound tumor reovirus (WTV) (Chiu and Black, 1967). Vector cell cultures were also used in studies on other plant viruses, such as potato yellow dwarf virus, a rhabdovirus (Chiu *et al.,* 1970; Hsu and Black, 1973), and rice dwarf virus, a reovirus (Kimura, 1984). The application of insect cell lines for studying other plant virus-vector interactions has been hampered by the difficulties encountered during establishment of continuous cell lines for other vector species. As an alternative, primary cell cultures can be exploited to study virus-vector interactions on the cellular level. Primary cell cultures of the aphid, *Hyperomyzus lactucae,* have been used to demonstrate the replication of sowthistle yellow vein virus (a rhabdovirus) in the cells of its vector (Peters and Black, 1970). Recently, it has been shown that tomato spotted wilt virus, type species of the genus *Tospovirus* within the arthropod-borne family *Bunyaviridae* (Goldbach and Peters, 1994), replicates in its thrips vector *Frankliniella occidentalis* (Wijkamp *et al.,* 1993; Ullman *et al.,* 1993a). As extensive transmission experiments have revealed major differences in tospovirus transmission efficiency, not only among various thrips species but also between different populations of single thrips species (Wijkamp *et al.,* 1995), it would be very worthwhile to study the specificity of tospovirus-thrips interactions on the cellular level. However, all attempts to establish continuous cell lines from any thrips species have failed so far (Hunter and Hsu, 1995 and 1996).

Here, we report the successful use of primary cell cultures (see Chapter 2) derived from two reportedly major vector species, *F. occidentalis* and *Thrips tabaci* (Sakimura, 1963; Fujisawa *et al.,* 1988; Wijkamp *et al.,* 1995), and of a continuous cell line of *F. occidentalis* for study of tospovirus-thrips interactions at the cellular level. The infection of thrips cells by TSWV is discussed in relation to the different transmission specificities of the thrips populations from which the cells have been derived.

MATERIALS AND METHODS

Setting up primary cell cultures

Three to five hundred healthy adult thrips from the Dutch population of *F. occidentalis* (NL-03) or a Dutch thelotokous population of *T. tabaci* were transferred to Murai cages

(Murai and Ishii, 1982). Sterilised demineralized water was placed on the top of a parafilm layer which sealed the Murai cages and covered with plastic lid. The adults were allowed to lay eggs into the water placed on the parafilm sealing the Murai cage. The eggs of 0.2 to 0.3 mm in length were collected after 24 h and incubated in water for 3-4 days at 27 °C until the eye spots became red. After sterilising them with 70 % ethanol for 5 min, eggs were washed with a balanced salt solution, designated F (Peters and Black, 1971), and then, subsequently crushed with a sterilised pestle. The tissue fragments were washed with Kimura's (K) medium (Kimura, 1984) and seeded into several wells of a sterilised 96 microwell plate (Coster). The cells were incubated at 27 °C for 4 days in K medium to obtain firm cell attachment and cell growth. Established *S. frugiperda* (Sf 21) cell lines were used as a control.

Inoculation of the cell cultures with TSWV

Purified virus preparations were obtained according to the method described by Black *et al.*, (1963) from *Nicotiana rustica* plants inoculated two weeks before. The virus preparations were resuspended in double distilled water and kept at -80 °C after quick freezing in liquid nitrogen. The virus concentration was determined using a protein assay kit (Bio-Rad). The virus was diluted to 30 µg/ml in 0.1 M histidine buffer (Kimura and Omura, 1988), pH 6.8, and sterilised by filtration through a 0.22 µm pore size low protein binding filter (Millipore). The primary cell cultures obtained were inoculated for 2 h at room temperature by adding the virus suspension of 50 µl to cell cultures previously washed with solution F. After inoculation, the cells were washed, then incubated in K medium for 48 h at 27 °C.

Immunofluorescence staining

After incubation, the cells were washed with 1x PBS, pH 7.2, and fixed with 3% paraformaldehyde and 0.1% glutaraldehyde for 30 min. The fixed cells were washed with PBS-0.1% glycine to block unwanted binding by the glutaraldehyde residues, treated with PBS-0.5% Triton X-100 for 5 min, and washed with PBS. Specific polyclonal antibodies of rabbit against the nucleoprotein (N) and the non-structural protein (NS_S) encoded by S RNA (Kormelink *et al.*, 1991) were diluted to 0.5 and 2 µg per ml, respectively, in PBS-10% bovine serum albumin (BSA) and incubated for 45 min. All IgG preparations were cross-absorbed with extracts from healthy cell material of *F. occidentalis*, *T. tabaci* and *S. frugiperda* in PBS and subsequently purified through a DEAE-Sephacel column (Sigma). After washing with PBS, the cells were incubated for 30 min with the second antibody (pig anti-rabbit fluorescein isothiocianate (FITC) conjugate) diluted to 10 µg/ml with PBS-BSA. After washing, the cells were observed in an inverted field microscope (Leica) at 400 times magnification under UV irradiation.

Transmission tests

The populations of *F. occidentalis* and of *T. tabaci* were reared in glass jars. First instar larvae of up to 4 h old were collected and allowed to acquire virus from *Datura stramonium* leaves infected with the Brazilian TSWV isolate BR-01 (De Ávila *et al.*, 1993). One half of each leaf was used for feeding *F. occidentalis* and the other half for *T. tabaci.* After 24 h of acquisition, all thrips were transferred to healthy *D. stramonium* leaves in Tashiro cages (Tashiro, 1967) and kept until all thrips became adult, which normally took approximately 12 days at 27 °C. Each individual adult thrips was tested for its infectivity for 2 days on a petunia (*Petunia x hybrida*) leaf disc, which was subsequently incubated on water in a 24 well plate (Coster) for another 2 days for the development of local lesions. The transmission ability of the thrips was evaluated by the appearance of local lesions on the discs. The virus accumulation in these thrips individuals was confirmed by amplification ELISA as described by Wijkamp *et al.* (1995).

Electron microscopy

Inoculated cells of a continuous line of *F. occidentalis* were fixed in a 3 % paraformaldehyde-2 % glutaraldehyde solution for 30 min. After collecting and embedding in 5% gelatine, the fixed cell clumps were cut in pieces of 1 to 2 mm^2 cubes, dehydrated in a series of 50 to 100 % ethanol solutions, and embedded in LR-Gold (London Resin). The resin was polymerised by ultraviolet light irradiation at –20 °C. The 60-70 nm thick sections were incubated with 0.8 µg/ml pre-absorbed polyclonal antibodies to the N protein or glycoprotein (G1 and G2) for 2 h, washed with 30 droplets of PBS, and incubated with gold-conjugated protein-A for 1 h. After washing with PBS, the sections were post-fixed in 1 % glutaraldehyde, contrasted with uranyl acetate and lead citrate, and studied with an electron microscope (Philips CM 12).

RESULTS

Transmission of TSWV by *F. occidentalis* and *T. tabaci* populations

Wijkamp *et al.* (1995) reported that *F. occidentalis* transmitted TSWV efficiently, whereas the thelotokous population of *T. tabaci* used did not. Since the populations of *F. occidentalis* and *T. tabaci* used for the generation of cell lines differed from those originally studied by Wijkamp *et al.* (1995), we analysed the transmission efficiency of both populations used. Adult thrips of both populations which fed TSWV as new born larvae up to 4 h old, were tested for transmission and accumulation of virus by ELISA. No transmission was observed by any of the 109 *T. tabaci* adults tested, whereas 55 out of

113 *F. occidentalis* adult thrips transmitted TSWV. Moreover, in none of the *T. tabaci* thrips, accumulation of virus could be detected by ELISA, while on the other hand, 83 out of 113 *F. occidentalis* adults were ELISA positive. The results showed that thrips of the used *F. occidentalis* population were efficient vectors, while the thelotokous population of *T. tabaci* used for primary cell cultures failed to transmit and to support virus accumulation.

Inoculation of *F. occidentalis* cells with TSWV

F. occidentalis cell cultures were prepared and inoculated with a TSWV suspension adjusted to 30 µg/ml. Pulsation of some tissue fragments could still be observed 48 h post-inoculation (p.i.), indicating that the inoculation procedure was not harmful to the cell survival. No cytopathogenic effects could be seen in the cells during the incubation period as judged by light microscopy. Infected cells treated with antibody against N and stained with FITC showed strong signals inside the cells 48 h p.i. (Fig. 1A, panel 1). These signals were extensively observed in the cytoplasm, but not in the nuclei (Fig. 1A, panel 1). In repeated inoculation experiments, approximately 40 % of the monolayer cells scored consistently positive using antibodies against N protein, demonstrating that a significant number of cells was able to support TSWV replication. No fluorescence was observed in non- inoculated control cultures or in inoculated cultures at time zero. Virus replication in cells could be confirmed using antibodies against the nonstructural viral protein (NS_S) which is only produced after tospovirus genome replication (Kormelink *et al.*, 1991; Wijkamp *et al.*, 1993). Positive NS_S signals were observed in the cultures at 48 h p.i., as a uniform staining throughout the cytoplasm (Fig. 1A, panel 2). Again, no staining was observed in non-inoculated cultures and in inoculated cultures at time zero (Fig. 1A, panel 5). The reaction with anti-NS_S was always less pronounced than with anti-N, and could be detected in approximately a quarter of the cells. Some faint signals were observed both at 0 and 48 h p.i. using anti-N, when the inactivated virus by UV irradiation for 10 min was used as a negative control. These signals could easily be distinguished from the signals in infected cells as they occurred only at the periphery of the cells. Since no signals were observed in these cultures when tested with anti-NS_S at 0 or 48 h p.i., we concluded that the faint reaction with anti-N was considered to be the input virus attached to the surface of the cells during inoculation.

The infectivity of the virus suspension was investigated with a dilution series of 30, 3 or 0.3 µg virus per ml. Positive signals of both N and NS_S antibodies were obtained 48 h p.i. in the cell cultures inoculated with 30 and 3 µg/ml, but not with the 0.3 µg/ml inoculum. Hence, in the present conditions, the end dilution point of the virus lays between 3 and 0.3 µg/ml.

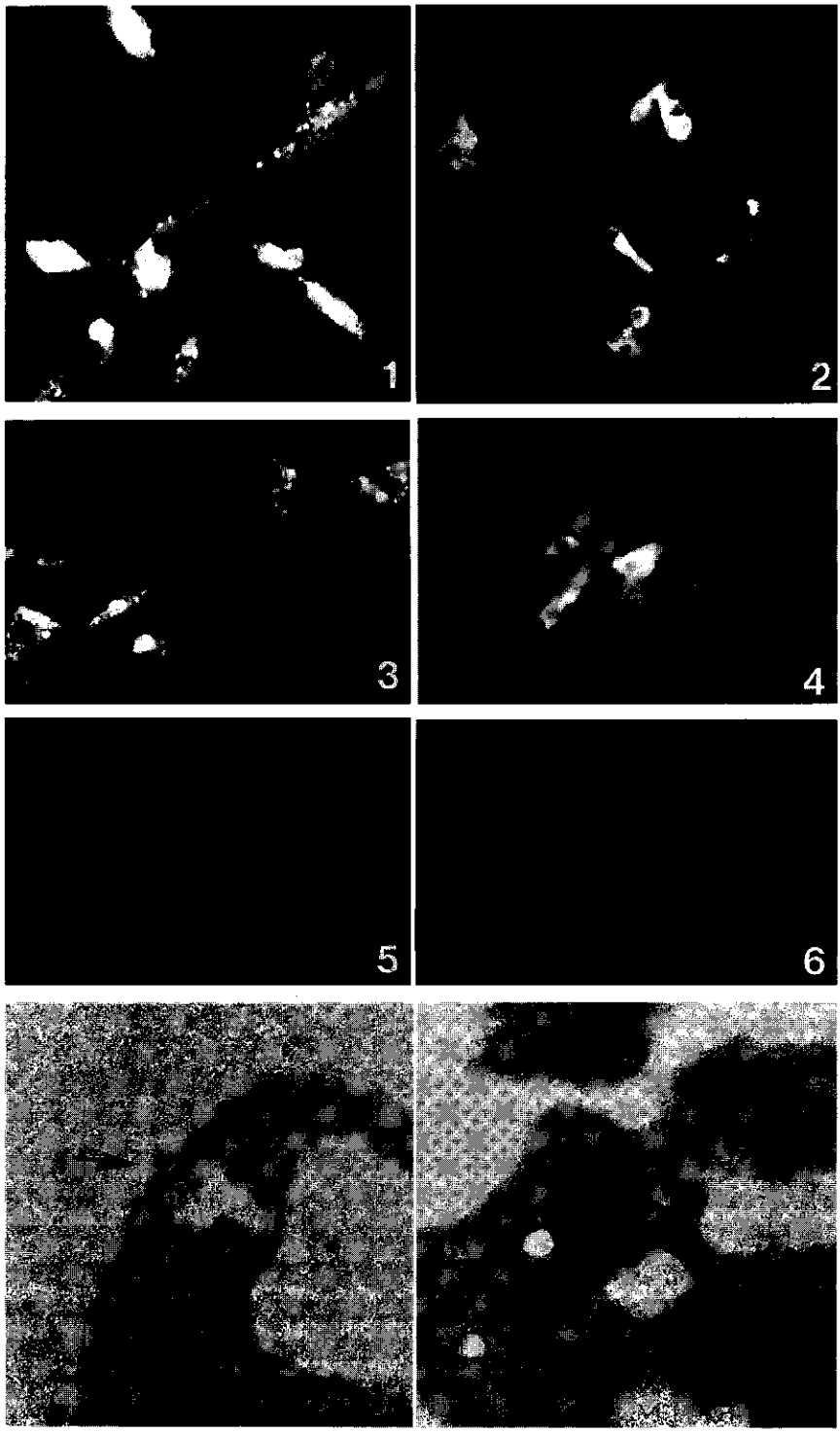
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Fig. 1A. Detection of viral proteins by immunofluorescence in TSWV infected monolayer cells of *F. occidentalis* (1, 2) and *T. tabaci* (3, 4). Specimens were examined by indirect fluorescence at 48 h p.i. using anti-N (1, 3) and anti-NS_S (2, 4) immunoglobulins. Inoculated *F. occidentalis* cells 0 h p.i. (5) and inoculated *S. frugiperda* (6) cells 48 h p.i., treated with anti-N, are shown as control. Magnification is the same 1-6, Bar = 30 µm. **Fig. 1B.** Inoculation and infection of TSWV in continuous cell culture of *F. occidentalis*. Putative virus entry by endocytosis at 0 p.i. labeled anti G1 and G2 protein (1), and nucleocapsid aggregates labelled anti-N protein (2). Bar = 500 nm.

Initially, enzymes such as trypsin or collagenase were used to dissociate the tissue fragments prior to seeding the cultures. However, the infection rate was always low in these cultures (data not shown), indicating that possible virus receptors on the cells were digested during this treatment. Since a sufficient number of cells attached, enzymatic dissociation of thrips tissues was not further used in the inoculation procedure.

Inoculation of *T. tabaci* cells with TSWV

A primary cell culture derived from embryos of *T. tabaci* was inoculated by the procedure described for *F. occidentalis* cells. Immunostaining showed that TSWV could also replicate with comparable efficiency in the cells of this *T. tabaci* population. Both anti-N and anti-NS_S gave signals as intense as in *F. occidentalis* 48 h p.i. Again, discrete spots in the cytoplasm were observed using anti-N serum (Fig. 1A, panel 3), while dispersed fluorescence could be seen using anti-NS_S (Fig. 1A, panel 4). Hence the localisation of viral antigens in *T. tabaci* cells was similar to *F. occidentalis* cell cultures. Approximately one third of monolayers scored as positive when anti-N was used, and a quarter of the cells, using anti-NS_S.

Time course of TSWV infection

Inoculated *F. occidentalis* and *T. tabaci* cell cultures were immunostained at different times post-inoculation (p.i.). The results are shown in Table 1. The first signals were observed at 9 h p.i. in *F. occidentalis* and 12 h p.i. in *T. tabaci* cell cultures using antibody against N protein (Table 1). The first positive reaction with NS_S antibodies could be seen only after 24 h in cells of both species. Hence, NS_S protein appeared to accumulate slower than N protein in these cells. Images obtained at 48 and 72 h p.i. were almost the same for both antibodies, although more intense than at 24 h p.i. These results showed that the latent incubation period was about 9-12 h p.i. in *F. occidentalis* and 12-24 h p.i. in *T. tabaci* cell cultures, and that virus accumulation reached a maximum level between 24 and 48 h p.i.

Table 1. Detection of viral proteins in TSWV-inoculated cells of *F. occidentalis* and *T. tabaci*

	IgG	0 *[a]	3	6	9	12	24	48	72
F. occidentalis	N	-	-	-	+-	+	+	++	++
	NS_S	-	-	-	-	-	+	++	++
T. tabaci	N	-	-	-	-	+-	+	++	++
	NS_S	-	-	-	-	-	+	++	++

*[a]: Numbers indicate incubation time in hours post-inoculation. Immunofluorescent staining was categorised into 4 classes, - indicating no staining, ++ the strongest immuno-staining.

pH effects on the inoculation

As the pH of the virus inoculation buffer has been shown to be critical for the inoculation to insect cells (Black, 1979; Kimura and Omura, 1988), we tested the effect of a 0.1 M histidine inoculation buffer at pH values ranging from 5.8 to 7.2 using *F. occidentalis* primary cell cultures. As control, the suspended virus preparations were simultaneously inoculated to detached petunia leaves to test their infectivity. Higher infectivities were obtained using buffers at neutral pH, while the numbers of infected cells dropped with decreasing pH values (approx. from 50% at pH 7.2 to 10 % at pH 6.2). Although positive reactions were observed at each pH value tested, stronger signals with antibody against N protein could be seen in cell cultures using buffers of pH 6.8 to 7.2. However, the pH of 7.0 and 7.2 resulted in a drastic cell detachment during the inoculation. On the other hand, applying a pH of 5.8 or 6.0 caused non-specific binding of the input virus to the plastic surface and to the cells. This lower pH might change the conformation of viral membrane or nucleoprotein and make them easy to attach non-specifically to any surface. Considering the results of mechanical inoculation of petunia and cell inoculation, a pH of 6.8 was selected as standard pH for inoculation.

Infectivity of the culture fluids of infected cell cultures

Production of progeny virus in the cultures was studied by inoculating the culture fluids of *F. occidentalis*, *T. tabaci* and *S. frugiperda* cell cultures at 6, 48 and 72 h p.i. to *F. occidentalis* cells. Infection of the inoculated cells was found only when the *F. occidentalis* culture fluids collected at 48 and 72 h p.i. were used as inoculum. The infection rate was very low, only 0.1 % to 0.8% of cells became infected. No positive results were obtained when the fluids of *T. tabaci* and *S. frugiperda* cell cultures were inoculated to *F. occidentalis* cell cultures.

Correlation between cell susceptibility and vector specificity

The results obtained demonstrated that primary cell cultures of both thrips species have the capacity to replicate TSWV. To test whether susceptibilty to TSWV is limited to thrips cells, a continuous cell line of *S. frugiperda* was inoculated. This insect cell line failed to support replication and accumulation of the virus. No positive immunofluorescent signals, not even a faint reaction, could be seen (Fig. 1A, panel 6) at the tested conditions. This suggests the existence of a high specificity of tospovirus-thrips interactions at the cellular level.

Infection of a continuous cell culture of *F. occidentalis*

TSWV was inoculated to the cells of a continuous *F. occidentalis* cell line (see Chapter 2) by the same method used for the inoculation of the primary cell cultures. Inoculated cells at 0, 12, 24 and 48 h p.i. were collected and observed by electron microscopy. Virus infection was detected by immunolabeling with antibodies against the viral N and glycoprotein (G) conjugated with protein A-gold. At 0 h p.i., no viral proteins were detected inside the cells. However, virus particles which was entering the cells, possibly by endocytosis, could occasionally be seen at locations on the plasma membrane (Fig. 1B, panel 1). Large N protein aggregates were seen from 24 h p.i., which became more intense at 48 h p.i. (Fig. 1B, panel 2). No accumulation of G protein or enveloped virus particles could be observed in the Golgi apparatus or in the plasma membrane of the infected cells. These results suggest that mature enveloped virus particles are not produced in these cells.

DISCUSSION

The use of continuous cell lines of insects offers the possibility to study the replication of plant viruses in their vector cells and to elucidate the nature of virus-vector relationship in more detail (Black, 1979; Nuss and Dall, 1990; Uyeda *et al.*, 1995). Since, so far, all attempts to establish cell cultures of thrips were not successful (Hunter and Hsu, 1995 and 1996), primary cell cultures were first used as the best alternative. Our results show that cells in primary cultures obtained from *F. occidentalis* and *T. tabaci* could support replication of TSWV. Approximately 20 to 50 % cells in the monolayers, which developed in 4 days after seeding, became infected. The infection rate differed for each aggregate of cells, which can be explained by differences in susceptibility of the cells.

The viral NS_S protein accumulated later in inoculated primary cell cultures than the N protein. A delay of accumulation of NS_S, compared with N, was also found in

Uukuniemi (UUK) virus-infected BHK cells (Simons *et al.*, 1992). NS_S of UUK virus, which is expressed by the same strategy as NS_S of TSWV, was detected 8 h p.i. whereas N was detected 4 h p.i., using immunoprecipitation of [^{35}S]-methionine labeled cell extracts. A dispersed distribution of NS_S was observed in cells of primary thrips cultures, similar as found in cells of TSWV-infected *N. rustica* plants (Kormelink *et al.*, 1991) and in UUK virus-infected BHK cells (Simons *et al.*, 1992). The function of NS_S protein is still unknown, but it may have a similar role in plant and animal cells. Based on the late expression of this protein, Simons *et al.*, (1992) suggested that this protein might be involved in virus assembly.

The fluids of the inoculated *F. occidentalis* cultures collected 48 and 72 h p.i. appeared to be infectious. No infectivity was found in the supernatant of cultures collected 6 h p.i. These results show evidently that the virus could replicate in *F. occidentalis* primary cell cultures and that replication resulted in an infectious progeny. The supernatant fluid of *T. tabaci* cell cultures was not infectious. This result may be explained by a lower replication rate of TSWV in the *T. tabaci* cells and by lower number of *T. tabaci* cells attached to the bottom of Petri dishes to *F. occidentalis* primary cell cultures.

Although *T. tabaci* has been reported as a widely spread and efficient vector of TSWV (Sakimura, 1962b; Fujisawa *et al.*, 1988; Lemmetty and Lindqvist, 1993), other authors have demonstrated that *T. tabaci* failed to transmit it (Paliwal, 1974; Zawirska, 1976). Recently, it has been shown that an arrhenotokous population of *T. tabaci* could transmit TSWV at a low efficiency, whereas three thelotokous populations of *T. tabaci* were not able to transmit this virus (Wijkamp *et al.*, 1995). The primary cell cultures used in the current study were derived from a thelotokous population of *T. tabaci*, which, though incapable to transmit TSWV, supported TSWV multiplication with a comparable efficiency of a cell culture from an efficient vector, *F. occidentalis*. This lack of specificity may be indicative for the occurrence of a possible barrier for virus acquisition in the midgut cells. Evidence for the existence of such barriers, causing differences in vector competence, has been reported for vectors of some animal bunyaviruses (reviewed by Beaty and Bishop, 1988; Beaty and Calisher, 1991; Ammar, 1994). Intrathoracial injection of La Crosse (LAC) virus revealed that the midgut and salivary gland barriers of mosquitoes were the factors which determined the vector competence (Paulson *et al.*, 1989). Furthermore, studies using reassortants of LAC virus and snowshoe hare virus showed that the vector competence was genetically linked to the M RNA (Beaty and Bishop, 1988). Our results, showing that virus replication takes place in both *F. occidentalis* and *T. tabaci* cells, but not in *S. frugiperda* cells, is suggestive for the existence of specific host factors essentially involved in TSWV infection process. Whether such factor represents a virus-recognising receptor protein or a host factor supporting the

intracellular replication process remains to be elucidated.

A continuous *F. occidentalis* cell line was obtained, which could be maintained for more than two years, and thus may be regarded as an established cell line. The possible scene of TSWV entry into the thrips cells by endocytosis was observed, but no evidence for membrane fusion at virus entry. Alphaviruses also enter their mosquito vector mesenteron epithelium cells via endocytosis (Houk *et al.,* 1985), whereas entry by membrane fusion appears to be case in cultured cells (Irurzun *et al.,* 1997). These observations suggest that the mechanism used by enveloped viruses to enter cells may depend on the cell type. The results of our study do not exclude the possibility that TSWV enters the cell by membrane fusion, and, hence TSWV may use both strategies to enter thrips cells in nature.

The continuous cell line generated from *F. occidentalis* has been shown to produce the 94 kDa protein (data not shown) which was shown to bind enveloped TSWV particles *in vitro* (Kikkert *et al.,* 1998). This protein has been proposed to play a role in the infection process as cellular receptor for TSWV. Obviously, the use of our *in vitro* vector cell culture may contribute to unravel the precise function of this virus binding protein in the TSWV infection cycle, as well as the tool to establish the molecular interactions between TSWV and its vector more in general.

Chapter 4

Tissue tropism related with vector competence of *Frankliniella occidentalis* for tomato spotted wilt virus

SUMMARY

The development of tomato spotted wilt tospovirus (TSWV) infection in the midgut and salivary glands of transmitting and non-transmitting thrips, *Frankliniella occidentalis*, was studied to elucidate tissue tropism and the viral pathway within the body of this vector. Immunohistological techniques used inthis study showed that the midgut, foregut and salivary glands were the only organs in which virus accumulated. The first signals of infection, observed as randomly distributed fluorescent granular spots, were found in the epithelial cells of the midgut, mainly restricted to the anterior region. The virus subsequently spread to the circular and longitudinal midgut muscle tissues, a process which occurred late in the larval stage. In the adult stage, the infection occurred in the visceral muscle tissues, covering the whole midgut and foregut, and was abolished in the midgut epithelium. The infection of the salivary glands was first observed 72 h post-acquisition, and simultaneously in the ligaments connecting the midgut with the salivary glands. The salivary glands of transmitting individuals appeared heavily or completely infected, while no or only a low level of infection was found in the glands of non-transmitting individuals. Moreover, the development of an age-dependent midgut barrier against virus infection was observed in second instar larvae and adults. The results show that the establishment of TSWV infection in the various tissues and the potential of transmission seems to be regulated by different barriers and processes related to the metamorphosis of thrips.

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INTRODUCTION

Tomato spotted wilt virus (TSWV) is the type species of the genus *Tospovirus*, harbouring the plant-infecting viruses within the family *Bunyaviridae*. Other members of this family includes important viruses infecting humans and animals, which are mainly transmitted by mosquitoes, ticks or other bloodsucking arthropods. Tospoviruses are exclusively transmitted by phytophagous thrips in a propagative fashion (Wijkamp *et al.*, 1993; Ullman *et al.*, 1993a).TSWV became one of the most world-widely distributed plant viruses (Goldbach and Peters, 1994) due to the recent global expansion of one of the most efficient vectors, *Frankliniella occidentalis* Pergande (Mound and Teulon, 1995, Wijkamp *et al.*, 1995), and its impressive host range (Peters, 1998).

The virus replicates and circulates after acquisition in its thrips vector and is transmitted by larvae late in their second stage and by adults (Sakimura 1962a, b and1963, Wijkamp and Peters, 1993). An increase of viral antigens in thrips during their development, particularly the nucleocapsid (N) and the non-structural (NS_S) proteins, is considered to be an indication for the replication of TSWV in thrips (Wijkamp *et al.*, 1993, Ullman *et al.*, 1993a). Replication of this virus has also been shown in *in vitro* cultured thrips cells (Chapter 3).

Only larvae can acquire virus leading to replication and virus transmission (Sakimura, 1962a, b and 1963, Van de Wetering *et al.*, 1996). Ingestion of virus by adults does not lead to replication and, hence, to the ability to transmit (Ullman *et al.*, 1992a, Van de Wetering *et al.*, 1996).The existence of this restriction may be explained by a differential expression of barriers during the development of thrips.

Studies on the transmission efficiency and the virus load of individual viruliferous and non-viruliferous adults revealed the existence of three categories of thrips (Wijkamp and Peters, 1993, Wijkamp *et al.*, 1995, Van de Wetering *et al.*, 1996). The individuals of the first category could successfully transmit the virus and possessed high titres in ELISA. The second category was not able to transmit the virus, but was positive in ELISA. The third category was negative in both assays. The existence of these three categories within a single popultion reflects different physiological potentials to replicate TSWV among the individual thrips. These potentials are often explained by the existence of different barriers (Paulson *et al.*,1989, Grimstad and Walker, 1991). Such barriers, which determine vector competence, may exist at the level of virus infection or escape from the midgut, dissemination through the insect, infection of the salivary glands and release into the saliva. (Hardy *et al.*, 1983, Hardy 1988). In plant virus vectors, the existence of such barriers has not yet been elucidated.

Most of our knowledge of the tospovirus-thrips relationships has been obtained in

transmission studies. Although some histological studies have been made (Ullman *et al.*, 1993a, b and 1995., Wijkamp *et al.*, 1995, Tsuda *et al.*, 1996), little is known about the tissue tropism related to the vector competence of thrips to transmit tospoviruses. This report describes the results of a study on TSWV tissue tropism in the thrips *F. occidentalis*. For this purpose, an immunohistochemical technique has been developed to study virus infections in plant virus vectors.

MATERIALS AND METHODS

Thrips rearing and virus acquisition

A Dutch population of *F. occidentalis* was reared in glass jars on fresh bean pods. The cultures were incubated in a climate chamber at 25 °C with a photoperiod of 16 h. Newborn larvae, up to 4 h old, were given an acquisition access period (AAP) of 16 h on *Datura stramonium* leaves infected with TSWV strain BR-01 (De Ávila *et al.*, 1993) in Tashiro cages and then transferred to non-infected leaves in these cages (Tashiro, 1967, Peters *et al.*, 1997). Samples of these thrips were collected at several time intervals after ingestion of the virus for immunocytochemical studies and to test the virus transmission efficiency when they became adults.

Virus transmission assay and ELISA

Transmission by thrips was tested using the local lesion assay method on petunia leaf disks (Wijkamp and Peters, 1993). Each newly emerged adult was individually placed on a leaf disk in an Eppendorf tube for 2 days at 25 °C. The disks were then transferred to a 24-well plate and incubated for 3 days on water for the development of local lesions. The virus accumulation in these thrips was determined by ELISA using antibodies to the nucleocapsid (N) protein and involved an amplification step as described by Wijkamp *et al.* (1995). The minimum threshold for positive ELISA values was the average of the readings of ten non-infected thrips plus three times their standard deviation.

Midgut preparation and whole mount immunofluorescent staining (WMIS)

Thrips were dissected in distilled water and the midguts transferred to object glasses coated with poly-L-lysine (0.5 % in water) and subsequently air-dried. After 1 h of fixation with cold acetone, the preparations were stored at -20 °C until further processing. After storage, the midguts were incubated in PBS, pH 7.2, containing 10 % bovine serum albumin (BSA) for 1 h to block non-specific reactions. Polyclonal antibodies against the viral N protein (2 µg/ml) raised in a rabbit were used as a first overlay for 2 h in 10 % BSA-PBS. To remove non-specific antibodies, this antiserum was first absorbed with a

crude extract from uninfected thrips (10 % in PBS, v/v) that was heated at 100 °C for 3 min before adding to the antiserum. After removing the precipitate by microcentrifugation at 10,000 r.p.m. for 10 min, the antibodies were extracted from the supernatant by a two-step method of ammonium sulfate precipitation (33 and 50 % saturation). The antibody fraction was dialysed and fractionated on a DEAE-Sephacel column (SIGMA) with PBS. The midgut preparations were incubated with this pre-absorbed antibodies for 2 h at room temperature in 10% BSA-PBS, washed three times for 10 min with PBS, and then incubated with 10 µg/ml pig anti-rabbit FITC conjugate (Nordic, Tilburg) in 10% BSA-PBS for 1 h. After washing, the specimens were mounted in CitiFluor (Agar) and studied by fluorescence microscopy (Leica).

Preparing sections for immunocytochemical studies

The legs and antennas were removed from the body under a stereomicroscope in Bouin's Hollande sublime (Smid., 1998). After removing the legs, the bodies were incubated in this fixative at room temperature under vacuum for 1 h and then overnight at 4 °C, dehydrated in a series of 70-100 % ethanol solutions, and finally incubated in amyl acetate for 1 h. They were then embedded in Paraplast (Oxford Labware), in 5 µm thick sections which were mounted on an object glass precoated with poly-L-lysine (0.1 % in distilled water). The sections were deparaffinized with xylene, rehydrated in a series of 100 to 70 % ethanol solutions and incubated in PBS. After this treatment, they were incubated with 10 % normal pig serum in PBS for 30 min, then with pre-absorbed anti-N protein antibodies (1 µg/ml PBS) for 2 h, washed with PBS and incubated with horseradish peroxidase (HRP)-conjugated pig anti-rabbit antibodies (5 µg/ml PBS containing 10% normal serum) for 1 h (Dako). After washing, the sections were incubated for 5 min with a substrate solution consisting of 0.05 % (w/v) diaminobenzidin (DAB) and 0.01 % (v/v) hydrogen peroxide in 50 mM Tris-HCl, pH 7.6. This reaction was stopped by washing with distilled water. The sections were then stained with Mayer's haematoxylin (Sigma), dehydrated, mounted with DPX (Fluka) and studied by light microscopy (Leica).

Electron microscopy

Thrips midguts were dissected and fixed in a 3 % paraformaldehyde-2 % glutaraldehyde solution for 30 min, dehydrated in a series of 50-100 % ethanol solutions, and embedded in LR-Gold (London Resin). The 60-70 nm thick sections were incubated with 0.8 µg/ml pre-absorbed antibodies to the N protein for 2 h, washed with 30 droplets of PBS, and incubated with gold-conjugated protein-A for 1 h. After washing with PBS, the sections were post-fixed in 1 % glutaraldehyde, contrasted with uranyl acetate and lead citrate, and studied with an electron microscope (Zeiss EM 109).

RESULTS

Thrips midgut infection in different developmental stages

The midgut *of F. occidentalis* consists of two loops (Ullman *et al.*, 1989 and 1992b, Moritz, 1997), which divide the midgut into three distinct regions, designated Mg1, Mg2 and Mg3 (See Chapter 1, Fig. 1). The foregut ends in the anterior Mg1 region, which ends at a constricted bending where the next region (Mg2) starts. This part runs in the direction of the head and continues after a second loop into the third region (Mg3), which goes towards the hindgut.

The development of TSWV infection in the midgut was studied by the WMIS technique (see Materials and Methods) using antiserum to the viral N protein. This technique was applied to larvae at 0, 24 (first instar), 48, 72, or 96 h (second instar) post-acquisition (p.a.) and to adults that were 4-5 days old. Infection was discernible by greenish positive FITC signals (Fig. 1). These signals were not observed in the intestinal tract of larvae or adults kept on uninfected plants (Fig. 1C). Moreover, the midgut of thrips fed on cucumber mosaic virus (CMV)-infected plants did not give any positive signal when antibodies to either the coat protein of CMV or the N protein of TSWV were used.

The first infection signals, discernible as granular fluorescent spots, were mainly restricted to the Mg1 (Fig. 1A) and occasionally also to the anterior part of Mg2. They occurred in the epithelial cells directly after the end of the 16 h AAP (0 h p.a.), and became more intense and clear in the following hours when the midguts were studied 24 h p.a. (Fig. 1A). The fluorescence signals were weaker at 48 h p.a. than at 24 h p.a. and again more intense at 72 and 96 h p.a. Apparently, the partial renewal of the epithelium during the moulting process, which occurred between 24 and 36 h p.a., resulted in a temporal loss of infected cells and, hence, of the intensity of positive reaction. Interestingly, the infection was completely abolished in the midgut epithelial cells when the thrips became of adults. This observation suggests that the resorption of the midgut epithelium during pupation results in a complete loss of the virus from this tissue.

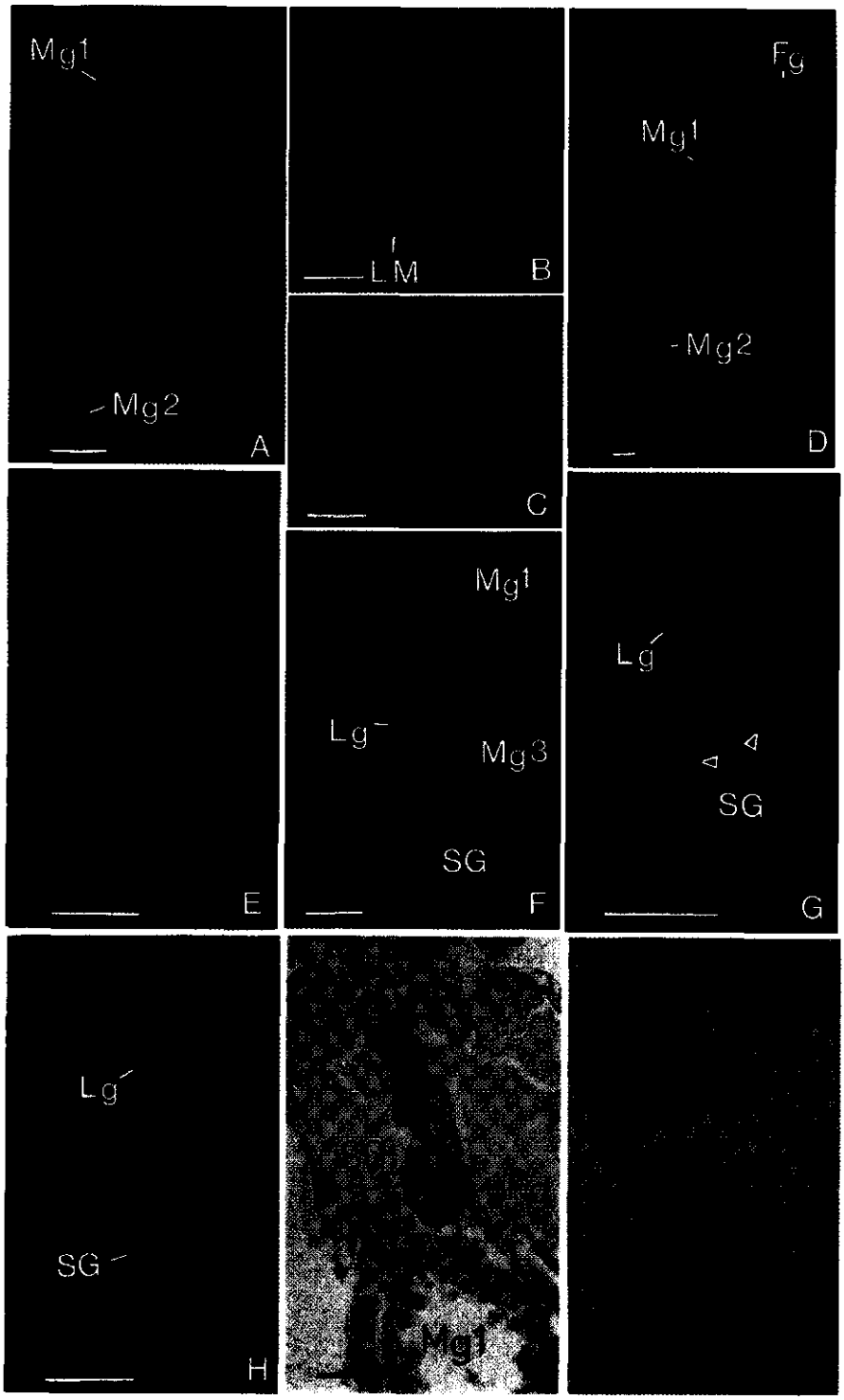
Mg1
Mg2
A
LM
B
C
Fg
Mg1
Mg2
D
E
Mg1
Lg
Mg3
SG
F
Lg
SG
G
Lg
SG
H
Mg1

Fig 1. Infection of the midgut and salivary glands of thrips at different developmental stages after ingesting the virus in a 16 h AAP given to 0-4 h old larvae. A) Mg1 of 24 h p.a.; B) Mg1 and Mg2 of 72 h p.a.; C) Healthy control of 72 h p.a.; D) Whole midgut of adult transmitter thrips; E) Mg1 of adult transmitter thrips: F and G) Midgut and salivary glands of 72 h p.a.; H) Midgut and salivary glands of adult transmitter thrips; I) Thin section of adult transmitter thrips; J) Thin section of a healthy control. A), D), E), H) were made using a 12/3 filter (Leica) and B), F), G) using a FITC filter (Leica). Mg: midgut, LM: longitudinal muscle, Fg: foregut, Lg:ligament, SG: salivary gland. Bar=50 µm

Infection of the epithelial cells was followed by virus invasion in the visceral circular and longitudinal muscle tissues lining the midgut, visible as a fluorescent lattice pattern. This infection was initially observed 72 h p.a. (Fig. 1B) and became more intense 96 h p.a. In the adult stage, the infection was often present in the visceral muscle tissues of the entire midgut (Fig. 1 D, E). This infection was usually discernible as a lattice pattern, but, in some cases, restricted to a few rows of longitudinal muscle cells in the posterior midgut (Mg3). The presence of the virus in the Mg2 and Mg3 muscle tissues in the adult stage and its almost absence in epithelium of these regions during the larval development suggests a lateral cell-to-cell migration of the virus in visceral muscle cells from the Mg1 to those of other midgut regions.

Besides infection of the muscle cells lining the midgut of the adult, the infection was also observed in the foregut (Fig. 1D). During larval development, this organ did not show any detectable infection, suggesting that the ingested virus can not infect foregut epithelium from the intestinal tract lumen. Infection of this organ, late in the infection process, represents a novel aspect of tissue tropism for TSWV infections in thrips.

In cells of the salivary glands, the first infection signals were seen at 72 or 92 h p.a. as spots in the border region of the salivary glands (Fig. 1F, G). The salivary glands in a large percentage of adults appeared to be completely infected (Fig. 1H). No or limited infection in one or a few cells could be detected in the salivary glands of a small group of adults. Furthermore, the infection in the ligament connecting the salivary glands with the anterior part of Mg1 was often observed at 72 h p.a. or later (Fig. 1F-H).

Signals of infection were never encountered in the tubular salivary glands or in the Malpighian tubes, which were co-dissected with the midgut.

The infection of the midgut of larvae 72 h p.a. could further be confirmed by electron microscopy. Large aggregates of N protein were observed in the cytoplasm of the epithelial cells of the midgut (Fig. 2A) and also in the circular and longitudinal muscle cells (Fig. 2B). Infection was observed only in Mg1 (two out of three of the larvae tested), confirming the results obtained by WMIS.

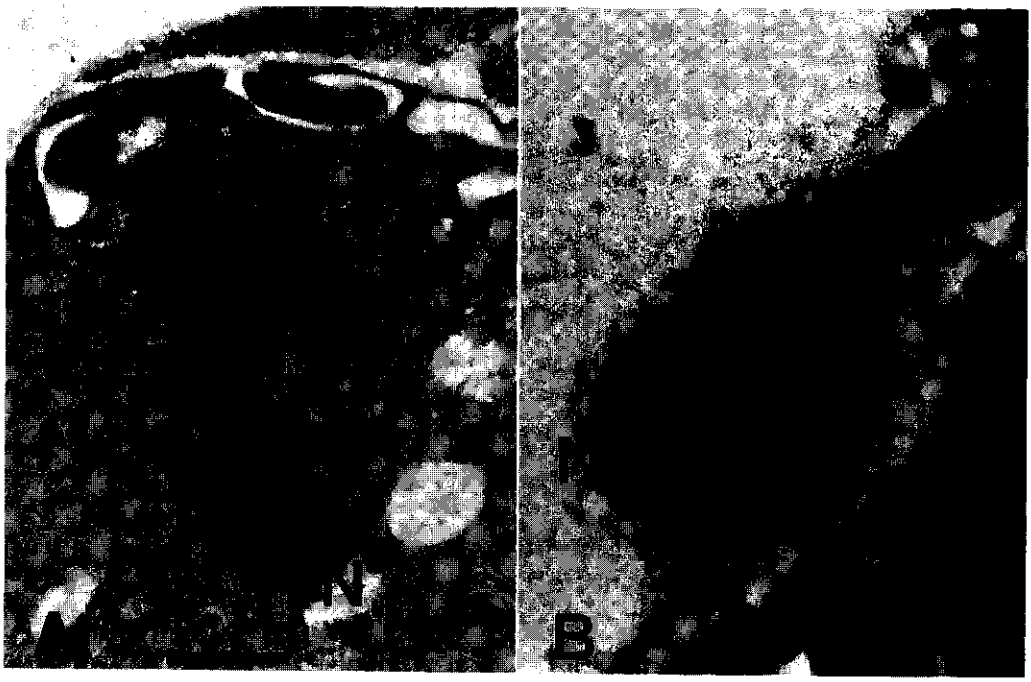

Fig 2. Electron micrograph of an infected epithelial (A) and a muscle cell (B) 72 h p.a. in the Mg1. N = gold particles labelled with antibodies to the nucleocapsid protein; M = muscle cell. Bar (A) = 500 nm and (B) = 200 nm.

Correlation of midgut infection and transmissibility

To understand the mechanism underlying the vector competence of *F. occidentalis*, the effect of the AAP on the infection in the tissues and the transmission rate was studied using the same cohorts of thrips. First instar larvae (up to 4 h old) were given an AAP of either 3 h or 16 h. Some of these thrips were analysed by WMIS for midgut infection 24, 96 h p.a. or after a few days becoming adult (Table 1). To test the transmission rate, larvae were grown to adults and individually tested for their ability to transmit. The virus content of these adults was analysed by amplification ELISA or immunohistologically for the infection of their tissues.

The WIMS results revealed that all thrips became infected when they were given AAPs of either 3 h or 16 h. Infections were mainly found in the epithelial cells of Mg1 and occasionally also in other midgut regions 24 h after the AAP of 3h. (Table 1). The percentage of larvae with infected midguts (epithelia and muscle cells) increased from 83 % (n=60) 24 h p.a. to 97 % (n=69) 96 h p.a and to 100 % when they became adults

Table 1. Infection of midgut regions as determined with anti-N-serum at several intervals after acquisition of TSWV by 0-4 h old *F. occidentalis* larvae. Infection development in midgut regions detected by anti-N-serum after acquisition of TSWV by 0-4 h old *F. occidentalis* larvae

AAP (h)	h p.a.	Midgut infected (%)		Infected midgut regions*(%)			Individuals
		No	Yes	Mg1	Mg2	Mg3	tested (N)
3	24	16.7	83.3†	63.3	15.0	5.0	60
	96	2.9	97.1‡	78.3	17.4	1.4	69
	adult	0.0	100.0§	38.9	22.2	38.9	18
16	24	14.1	85.9†	56.2	25.0	4.7	64
	96	0.0	100.0‡	71.6	28.0	0.0	67
	adult	0.0	100.0§	26.7	3.3	70.0	30

* Mg1: infection restricted to region Mg1; Mg2: infection in region Mg1 and Mg2; Mg3: infection in region Mg1, Mg2 and Mg3. † Infection restricted to the epithelium. ‡ Infection in the epithelium and muscle cells of midgut. § Infection only in midgut muscle cells.

(n=18). These percentages were slightly higher for larvae that acquired the virus in an AAP of 16h, indicating that almost no quantitative differences existed in the development of virus infection in the midguts of larvae when given AAPs of 3 h or 16 h. However, significant differences were observed when the intensity of infection in the midgut muscles of the adults was studied. The portion of infected vesicular muscle cells was smaller in thrips fed on infected plants for 3 h than those which fed for 16h (Table 1).

The effect of the AAP on the transmission rate was studied by testing individual adults using the local lesion leaf-disk assay. The virus was transmitted by 117 out of 180 (65 %) and by 92 out of 147 (62 %) adults of the cohorts given AAPs of 3 h or 16 h, respectively. These results revealed that the length of AAP did not affect the transmission rate. Analysis of the midguts by WMIS revealed that only a part of the population could transmit the virus although the midguts of all thrips were infected.

The amount of the N protein in adults was measured by amplification ELISA after determining the transmission rate. The results showed that 96 % (n=22) and 100 % (n=15) of the transmitters were positive in ELISA, whereas 70 % (n=29) and 60 % (n=20) of the non-transmitters were positive in the cohorts that had fed for 3 h or 16 h on infected plants.

Table 2. Number of thrips with salivary glands infected with TSWV in transmitting and non-transmitting adult thrips.

	No infection	Restricted to one gland*	Infections in both glands†
Transmitter	0	4	16
Non-transmitter	17	20	3

* Infection was restricted to one or a few cells in one of the salivary glands. † The glands were either completely or almost completely infected in both glands.

Histological study of virus infection in whole thrips sections

To correlate tissues tropism with the transmission capacity, the infection in adults was studied in paraffin sections of whole thrips bodies. After testing their capacity to transmit by the leaf-disk assay, transmitter and non-transmitter adults were embedded in paraffin and sectioned in 5 µm thick longitudinal sections. N protein was targeted with specific antibodies followed by second antibodies conjugated with HRP and then stained with DAB. Positive reactions (Fig. 1I), observed as a dark brown colour, were exclusively detected only in the midgut visceral muscle, foregut and salivary glands of the adult thrips, but not in any other organs, including midgut epithelial cells, hence confirming the absence of virus infection in the adult midgut epithelial cells. No positive signals could be seen in the sections of control thrips reared on uninfected *D. stramonium* leaves (Fig. 1J).

The infection in both salivary glands was often unevenly distributed (Table 2). Virus antigen was readily detected in the salivary glands of all transmitter thrips (n=20). On the other hand, infection of the salivary glands was completely absent 17 out of the 40 non-transmitter thrips and very restricted (one or few cells in one gland) in 20 out of the 40 non-transmitters (Table 2). These results show that a limited infection of the salivary glands is not sufficient for a thrips to become a transmitter.

Effect of vector age on infection of the midguts

Vector competence is drastically affected by the age at which TSWV is acquired (Sakimura, 1962b and 1963, Van de Wetering *et al.,* 1996). This effect has been explained by the existence or development of a midgut barrier in adult thrips (Ullman *et al.,* 1992a). To elucidate the existence of an age-dependent midgut barrier, WMIS was applied to larvae and adults which acquired the virus at different times in their development. First instar (up to 4 h old) and second instar (72-76 h old) larvae were given an AAP of 16 h, and studied by

Table 3. Midgut infection in thrips after ingesting of virus by first and second instar larvae and adults.

Age when fed on virus source	Transmission efficiency	Midgut analysed	Analysed (N)	Midgut infections*				
				No	Faint	Mg1	Mg2	Mg3
0-4 h old larvae	47.3 %	24 h p.a.	8	0	0	8	0	0
		Transmitters†	8	0	0	0	1	7
		Non-transmitters‡	27	0	0	0	9	18
72-76 h old larvae	12.4%	24 h p.a.	11	9	1	1	0	0
		Transmitters†	4	0	0	0	4	0
		Non-transmitters‡	26	16	3	6	0	1
1-2 days old adults	0 %	1 day p.a§	12	12	0	0	0	0
		3 days p.a.	8	8	0	0	0	0
		6 days p.a.	19	18	1	0	0	0
		9 days p.a.	37	36	1	0	0	0

* No: no infection discernible, Faint: reaction not discernible using a 100-time magnification but was with a 400-time magnification, Mg1: infection restricted to region Mg1, Mg2: infection was restricted to regions Mg1 and Mg2, Mg3: infection was seen in whole midgut. † ‡ Transmission was evaluated after adult emergence by leaf disk assay. § Post acquisition incubation time in days before midgut dissection. All adult thrips in this category did not show any transmission

WMIS 24 h p.a. Some of these thrips, when they were two-day old adults, were tested for their ability to transmit the virus and then histologically analysed. Adults, when fed for 16 h on an infected plant, were likewise studied 1, 3, 6 and 9 days p.a. (Table 3). The transmission efficiency was 47.3 % (n=201), 12.4 % (n=169) and zero (n=152) for the thrips which acquired virus as first, second larvae or as adults. These results confirmed the generally accepted idea that adults are unable to acquire virus, and secondly that second instar larvae can also acquire virus albeit to a lower extent than first instars.

This study showed that the midguts of all (n=8) of the first instar and 18 % (n=11) of the second instar larvae were infected 24 h after acquistion of the virus. After emergence of the adults, midgut muscle cell infection was found in all adults (n=35) which acquired virus as first instar larvae, and in 42 % (n=30) of the adults which acquired virus as second instars. No infection could be discerned in thrips which had acquired the virus as adults 3 days p.a. (n=8). A weak positive reaction was found in one out of 18 (5 %), and in one out of 36 (2.7 %) thrips analysed 6 and 9 days p.a., respectively. This infection was observed as

faint localised positive spots only in the epithelial cells of Mg1 but not in the muscle cells of these midguts.

DISCUSSION

The pathway of TSWV in the tissues of thrips and the intrinsic factors related to the transmission of the virus are of major interest for understanding the relationship between this virus and its vector. Evidence of infection of midgut epithelial cells, visceral muscle cells, and salivary glands of *F. occidentalis* by TSWV has already been obtained by electron microscopic studies (Ullman *et al.*, 1993a and 1995, Wijkamp *et al.*, 1995). However, these studies did not provide information on the development of the infection in the individual thrips at different periods after acquisition of the virus, or in thrips populations which have been given similar AAPs to larvae of the same age. In addition, the relation between the tissues infected and the capacity to transmit the virus has not been elucidated. These questions can be answered by histological immunostaining techniques using the isolated intestinal tract and salivary glands, or the whole thrips bodies.

The results obtained in this study showed that the first infection is initially restricted to the epithelial cells of the Mg1 region, followed by infection of the visceral and longitudinal muscle cells bordering this region, and subsequent invasion of the visceral and longitudinal cells of the Mg2 and Mg3 region by the virus. After emergence of the adults, infection could also be found in the foregut. The processes leading to virus spread from Mg1 to Mg2, Mg3 and foregut regions still remain to be studied. We presume that this may occur by a lateral spread of the virus by cell-to-cell movement through the muscle tissue, or by release of virus from the Mg1 region either into the haemocoel or into the intestinal lumen. The latter possibility, though, is less plausible as infection of the intestinal lumen did not occur upon ingestion of the virus and the susceptibility of the midgut to infection decreased during larval development. Our observation that the infection in the posterior midgut remained occasionally restricted to a few rows of longitudinal muscle cells, while the circular muscle cells did not become infected, strongly supports the idea that the virus spread laterally in a cell-to-cell manner.

Restricted foci of the first viral infections in midgut have also been reported in some vectors of arboviruses. For instance, dengue-2 virus infects a small region of the posterior portion of mesenteron (midgut) of *Aedes albopictus* (Kuberski, 1979), while the initial infection of Japanese encephalitis (JE) virus is restricted to small isolated foci of epithelial cells of *Culex triaeniorhynchus* and *C. pipiens* (Doi *et al.*, 1967; Doi, 1970). This infection can be explained by the existence of a limited number of specific virus entry sites or by an

extra or intracellular digestive activity resulting in the degradation of a large number of virus particles. Degradation of the TSWV particles may also explain their failure to infect the epithelium of the Mg2 and 3 regions.

Virus acquisition early in the larval development resulted in an infection of all thrips individuals, as demonstrated by the newly adapted histological technique (WMIS). Previous studies (Wijkamp *et al.*, 1993; Van de Wetering *et al.*, 1996) showed that the percentage of ELISA positive thrips was higher than that of the transmitters when the virus was acquired by first instars. We attribute this discrepancy between the transmission tests and ELISA to a lower virus load in the saliva and hence failing to transmit the virus. Comparison of the results by WMIS with those of the transmission experiments and ELISA showed that the midgut muscles and salivary glands of the transmitter thrips were heavily infected as shown by the almost complete infection of these tissues and the high virus titres. Two groups of non-transmitters could be distinguished. In one group, infections occurred in the midgut and in a very restricted area of the salivary glands. They were ELISA positive, but contained lower virus titers than the transmitters. The infection was restricted to the midgut muscle cells of the non-transmitters that were ELISA negative. These results suggest that the inability of thrips to transmit the virus after an early acquisition was not due to a failure to infect thrips, but to different quantitatively effects of some barriers operating in different tissues or to partial abortion of the infection in some cells.

A virus, circulative and propagative in its vector, has to pass several tissues, the barriers of which finally determine the vector competence (Hardy *et al.*, 1983; Hardy, 1988). These barriers for TSWV may include a midgut infection, midgut escape, dissemination, salivary gland infection and salivary gland escape barriers. In this study, two important barriers were recognized. The midgut escape barrier in adults of *F. occidentalis* was believed to be the first barrier (Ammar, 1994; Wang *et al.*, 1994). Our results showed that the first effective barrier is the midgut infection barrier. This barrier became increasingly effective during the development of larvae and almost complete in adult thrips. A similar barrier may be present in the leafhopper *Agaria constricta*, the vector of wound tumor reovirus and potato yellow dwarf rhabdovirus. The efficiency by which this vector transmits these viruses also decreased with its age (Sinha, 1963), again suggesting the development of an infection in the midgut. However, this type of barrier is not common in mosquitoes-arboviruses interactions, as acquisition and transmission of arboviruses is restricted to the adult stage except when the virus is transovarially passed from one generation to another.

To reach to and infect salivary glands, TSWV has to pass three more barriers, *i.e.*, the midgut escape, midgut to salivary gland dissemination, and the salivary gland infection. Since escape from the midgut and dissemination can not be determined and the pathway of TSWV to the salivary gland is not known, the factors which limit the infection of the salivary glands remain to be elucidated.

In our study, evidence was found for the existence of a salivary gland escape barrier since transmission seems to be dose-dependent. The observation that a low infection level of the salivary glands was associated with an inability to transmit suggests a failure of the virus to spread to other cells or to be released in sufficiently large amounts in the saliva. The immunohistological studies of transmitters showed that the salivary glands of such individuals become completely infected. A dose-dependent barrier is the most common model of the salivary gland escape barrier for the arbovirus-mosquito relationship (Hardy *et al.*, 1983). Similar observations have also been made for JE virus in the salivary glands of *C. triaeniorhynchus* (Takahashi and Suzuki, 1979, Takahashi, 1982). Their results suggest that heavily infected salivary glands are required to transmit the virus, indicating that a successful transmission is a dose-dependent phenomenon.

It has been suggested (Ullman *et al.*, 1992b) that the virus can be transported from the midgut through the tubular salivary glands to the salivary glands. The former organ was thought to form a channel-like structure between the midgut and the salivary glands. This possible role in virus transport is not confirmed in the present studies as no virus antigen was detected in this organ. Rather, this study supports the absence of any direct connection between the midgut and salivary glands as has been suggested by Del Bene *et al.* (1991). A possible new pathway by which the salivary glands became infected could be deduced by detecting infection in the ligaments which connect the Mg1 region with the salivary glands. These positive signals were always found in the late larval stage (72 h p.a. or later) and in adults, associated with infected salivary glands (Fig. 1 F-H).

In conclusion, using the immunohistological techniques, infection of TSWV can be followed in thrips during their development. The development of infection in all young larvae which acquired virus showed that the susceptibility to virus entry and midgut infection are stable characters of the thrips population used in this study. Thus, it can be concluded that the inability to transmit (often observed) does not originate from a lack of viral entry, but is largely dependent on existing barriers which must be passed through during infection. The ability to transmit TSWV by thrips is therefore the outcome of successful virus replication, spread and passage through several barriers resulting in a release of virus in the saliva.

Chapter 5

Impeded thrips transmission of defective tomato spotted wilt virus isolates by *Frankliniella occidentalis*

SUMMARY

Two defective RNA containing isolates (Pe-1 and 16-2) and an envelope deficient (env$^-$) isolate of tomato spotted wilt virus (TSWV) were tested for their transmissibility by *Frankliniella occidentalis*. The isolate Pe-1 contained a truncated L RNA segment which barely interfered with symptom expression and replication of the wild type (wt) L RNA segment. The isolate 16-2 contained a genuine defective interfering (DI) L RNA, as concluded from its ability to suppress wt L RNA synthesis and viral symptom expression. A transmission efficiency of 51% was obtained with Pe-1, which was comparable to the transmission (54%) of the wild type, while isolate 16-2 was not transmitted at all. Northern blot analysis revealed that the defective RNA segment of isolate Pe-1 was successfully acquired and transmitted. The midguts of all larvae that ingested Pe-1 became infected, whereas only 24 % of the larvae exposed to isolate 16-2 showed some slight infections in the midgut. This result was explained by the presence of low amounts of infectious units in the inoculum as verified by Northern blot analysis. The env$^-$ isolate completely failed to infect the midgut when ingested, and could not be transmitted by any thrips stage. As this isolate also failed to infect primary thrips cell cultures, it was concluded that it lacked the ability to enter thrips cells. This indicates that the envelope of TSWV contains the determinants required for binding and subsequent infection of the thrips cells by the virus.

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INTRODUCTION

Tomato spotted wilt virus (TSWV), the type species of the genus *Tospovirus* in the *Bunyaviridae* (Murphy *et al.*, 1995), is biologically transmitted by thrips, a group of minute insects. Thus far, eight thrips species have been reported as vectors (Mound, 1996; Webb *et al.*, 1998), of which *F. occidentalis* is the most efficient one (Wijkamp *et al.*, 1995). The competence of thrips to transmit tospoviruses shows some unique features. Tospoviruses are acquired by young larval stages (Van de Wetering *et al.*, 1996), and transmitted by larvae at the end of their second instar stage and by adults after a latent period during which the virus replicates (Wijkamp and Peters, 1993; Ullman *et al.*, 1993a; Wijkamp *et al.*, 1993). Replication of TSWV has also been demonstrated in primary thrips cell cultures (Chapter 3). The ability to acquire virus decreases with the development of the larvae and is completely lost when the thrips becomes adult (Ullman *et al.*, 1992a; Van de Wetering *et al.*, 1996). Hence ingestion of virus by adults does not lead to transmission. The decreasing ability to become viruliferous is apparently caused by an age-dependent midgut infection barrier (Ullman *et al.*, 1992a; Chapter 4).

Two distinct types of TSWV mutants, generated during multiple mechanical passages in plants, have been described (Resende *et al.*, 1991). One type contains defective RNAs, which are generated by a single deletion in the polymerase-encoding L RNA segment (Resende *et al.*, 1991; Inoue-Nagata *et al.*, 1997a and 1998). Most of these defective RNAs cause symptom attenuation in plants and represent true defective interfering (DI) RNAs. Some defective RNAs, however, hardly interfere with the synthesis of L RNA. (Inoue-Nagata *et al.*, 1997a). The location of the deleted region in the L RNA varies, but always occurs internally, preserving both termini of the molecule (Resende *et al.*, 1992; Inoue-Nagata *et al.*, 1998). The second type of mutants is characterised by the lack of the viral envelop which contains the viral encoded glycoproteins (Ie, 1982; Resende *et al.*, 1991).

In this paper, the acquisition and infection of thrips by both types of TSWV mutants and their transmission has been studied. For this purpose, three mutants were selected: two defective RNA-containing isolates (denoted Pe-1 and 16-2, Inoue-Nagata *et al.*, 1997a and 1998), of which only 16-2 contained a true DI RNA, and an envelop deficient (env$^-$) isolate (Resende *et al.*, 1991). The ability of these mutants to infect thrips larvae after acquisition was analysed using a whole mount immunofluorescent staining technique (WMIS, Chapter 4) and inoculation to thrips primary cell cultures (Chapter 3).

MATERIALS AND METHODS

Mutant virus isolates

The wild type (wt) of the Brazilian TSWV isolate BR-01 (De Ávila *et al.*, 1993), and two of its derived lines containing defective L RNAs (Pe-1 and 16-2) were used in this study. They were genérated by repeated mechanical passage of BR-01 wt on *Capsicum annuum* (Pe-1) or *Nicotiana rustica* (16-2) (Inoue-Nagata *et al.*, 1997a and 1998). An envelop deficient line of isolate NL-04 (env$^-$, Resende *et al.*, 1991) and its wt (NL-04 wt) were also included in the present study. This env$^-$ isolate was free of any contaminating defective L RNA species as shown by Northern blotting (Inoue-Nagata *et al.*, 1997b). The presence or absence of defective L RNA species in the isolates used was verified by Northern blot hybridisation prior to and after transmission tests.

ELISA

Before the infected *Datura stramonium* plants were used for virus acquisition by thrips, the amount of virus was determined by DAS-ELISA using anti-nucleocapsid (N) protein (De Ávila *et al.*, 1990). Leaf disks of 5 mm in diameter were triturated in 0.01 M potassium phosphate buffer, pH 7.2, containing 0.14 M NaCl and 0.05 % Tween-20 (PBS-T) in a ratio of 1 mg leaf material/ml PBS-T. This ratio was used to avoid saturation of the antigen-antibody reaction (Inoue-Nagata *et al.*, 1997a).

Thrips rearing and virus acquisition.

A Dutch population of *F. occidentalis* (NL-03, Van de Wetering *et al.*, 1996) was reared in glass jars on bean pods. Cultures were incubated in a climate chamber at 25 °C with a photoperiod of 16 h. New-born larvae, up to 4 h old, were given an acquisition access period of 16 h on TSWV infected *D. stramonium* leaves in Tashiro cages (Tashiro, 1967; Peters *et al.*, 1997) and then transferred to cages with healthy *D. stramonium* leaves. Samples of these larvae were collected for WMIS (Chapter 4) at 24, 72 and 96 h post-acquisition (p.a.) during their larval development or of 4-5 days old adults after determining their transmission ability.

Virus transmission assay.

The transmission efficiency of thrips was tested using a petunia leaf disk assay (Wijkamp and Peters, 1993). Newly emerged adults were individually placed on a petunia leaf disk in an Eppendorf tube for 2 days at 25°C. The disks were then transferred to wells in a 24-well

plate and incubated for three days while floating on 2 ml of tap water for the development of local lesions.

Whole mount immunofluorescent staining (WMIS) of thrips midguts.

To detect and locate virus infection in the midgut, the WMIS technique was performed essentially as described by Nagata *et al.* (1999). In brief, midguts of dissected thrips were mounted on an object glass coated with poly-L-lysine, fixed with cold acetone and incubated in phosphate buffered saline (PBS) with 10% bovine serum albumin (BSA) for 1 h to block aspecific binding of antibodies. Polyclonal antibodies against the viral nucleoprotein (N) (2 mg/ml) were used as a first overlay for 2 h in 10% BSA-PBS. Prior to its use, this antiserum had been cross-absorbed with an extract from uninfected thrips (10% in PBS, w/v) and fractionated on DEAE-Sephacel column (SIGMA). After washing the specimens with PBS, the midguts were incubated with pig anti-rabbit FITC conjugate (Nordic, 10 µg/ml) for 1 h at room temperature. Finally, the specimens were mounted with CitiFluor (Agar) and examined by UV microscopy (Leica) at 400 times magnification.

Total RNA extraction from plants and Northern blot analysis

Before and after transmission, the presence of defective RNA molecules in TSWV isolates was demonstrated by Northern blot analysis of total RNA extracts from 0.02 g systemically infected leaves (Inoue-Nagata *et al.,* 1997a). Extracts were subjected to electrophoresis in a 1% agarose gel containing methylmercuric hydroxide and transferred onto a nylon membrane (Hybond N, Amersham). The hybridisation procedure was performed with a mixture of two distinct ds-DNA probes, which were ^{32}P-labelled by random priming. One probe was specific to the 5' terminus (nucleotide position 1 to 1178) and the other to the 3' terminus (7749 to 8897) of the reported viral complementary strand (vc) of TSWV L RNA (De Haan *et al.,* 1991).

Virus inoculation of primary cell culture system of *F. occidentalis*

To analyse whether the NL-04 env$^-$ isolate was able to infect thrips cells, purified virus preparations were inoculated to primary cell cultures of *F. occidentalis.* Purified preparations of wt TSWV, BR-01 and NL-04, were also used as control. The primary cell cultures were prepared by crushing approximately three days old thrips embryos and transplanted into modified Kimura's insect medium (Kimura, 1986, Chapter 3). Four-day old cultures were overlayed with a purified virus suspension for 2 h. The infectivity of the virus preparation of fresh and after 2 h incubation, containing 10 µg virus/ml, was confirmed by mechanical inoculation to *C. quinoa* plants. Following this incubation, the cells were washed with a physiological salt solution, incubated in medium for 48 h, and fixed with 3% paraformaldehyde-0.1 % glutaraldehyde. Infection of the cells was evaluated by

immunofluorescent assay (Chapter 3) using antibodies to the N and nonstructural (NS_S) protein with UV microscope (Leica) at 400 times magnification. These proteins are abundantly expressed in infected thrips cells (Wijkamp *et al.*, 1993; Ullman *et al.*, 1993a).

RESULTS

Titre of defective isolates in acquisition hosts

The amount of virus ingested may affect the transmission efficiency of the vector. Therefore, the virus contents in *D. stramonium* plants infected with the isolates Pe-1, 16-2 or env⁻ were determined prior to their use as virus source in transmission studies. Leaf disks were sampled for measuring their virus contents at three different locations in systemically infected leaves. The mean ELISA values of these samples are shown in Table 1. The two wt (BR-01 and NL-04) and env⁻ isolates reached similar titres in the leaves, while lower amounts of virus were found in the plants infected with isolates Pe-1 or 16-2.

Table 1. Response of *D. stramonium* plants to infection by different TSWV isolates and mutants, and the rate at which these isolates were transmitted by the thrips *F. occidentalis*

TSWV	Symptom severity	Mean ELISA value	Transmission rate (%)
BR-01 wt	+++	1.26	53.1 (n=64)
Pe-1	+++	0.60	50.8 (65)
DI 16-2	+	0.89	0 (66)
NL-04 wt	+++	1.26	34.3 (67)
NL-04 env⁻	+++	1.26	0 (66)

Effect of defective viral RNAs on transmission

Isolates Pe-1 and 16-2, each containing a defective L RNA, were selected for this study in view of their different virulence on plants (Inoue-Nagata *et al.*, 1997a and 1998). Pe-1 causes a stunting of *N. rustica* and *D. stramonium* plants and a systemic mosaic, followed by a severe necrosis. These symptoms were indistinguishable in character and severity from those produced by wt virus (BR-01). The symptoms caused by 16-2, however, were very mild, showing only a few necrotic spots on the inoculated leaves and a systemic

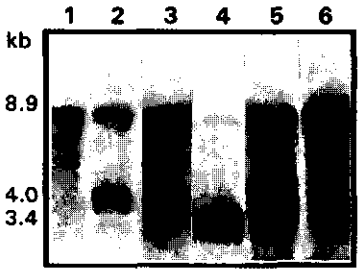

Fig. 1. Northern blot hybridisation of a total RNA extract from infected *D. stramonium* plants on which thrips acquired the TSWV isolates studied. 1) BR-01 wt, 2) Pe-1, 3) Pe-1 transmitted by thrips. 4) DI 16-2, 5) NL-04 wt, 6) NL-04 env⁻.

mottling, while no clear stunting was observed. Thus, 16-2 was characterised as a genuine defective interfering (DI) isolate, whereas Pe-1 was not as it did not cause clear symptom attenuation. Totally different results were obtained in the transmission experiments with these two defective isolates. Pe-1 was transmitted with an efficiency of 50.8%, a level similar to that of the wt TSWV isolate (53.1%) (Table 1). On the other hand, isolate 16-2 was not transmitted at all. Remarkably, plants infected with isolate Pe-1 contained lower virus titres than those infected with 16-2 (Table 1). Since BR-01 wt and Pe-1 were transmitted with comparable efficiency, it can be concluded that the acquisition period given in this study was long enough to exclude any effect of virus concentration in the leaves on which the thrips acquired the virus.

Northern blot hybridisation was applied to detect intact and defective L RNAs in *D. stramonium* leaves used for virus acquisition, and after transmission of Pe-1 by thrips. The amount of intact genomic L RNA of Pe-1 was considerably higher per gram of leaf material than that of 16-2 infected plants (Fig. 1, lane 2 vs. lane 4), suggesting that the truncated RNA of Pe-1 did not strongly interfere with the replication of intact L RNA. On the other hand, isolate 16-2 contained relatively high amounts of the defective L RNA, apparently suppressing the wt L RNA accumulation, and thus representing a DI RNA.

Furthermore, it was verified whether the defective L RNA species of Pe-1 was transmitted along with its helper virus by thrips. Leaf disks inoculated by thrips were ground and mechanically inoculated to two *D. stramonium* plants. After two weeks, the presence of L RNA specific fragments was analysed in these plants by Northern blotting (Fig. 1, lane 3), confirming thrips transmission of the defective RNA of Pe-1.

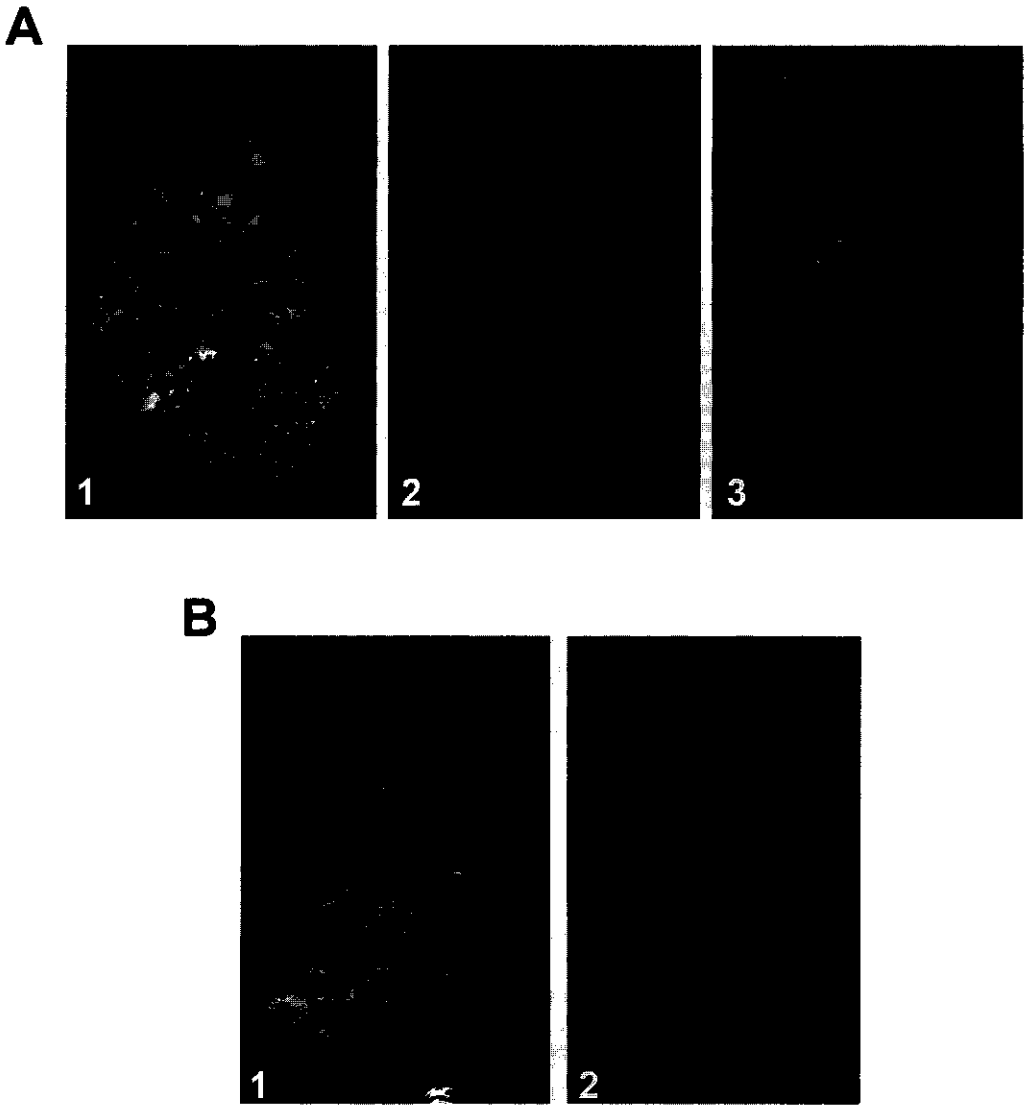

Fig. 2. TSWV infection of the midgut of thrips larvae 24 h p.a. as demonstrated by whole mount immunofluorescent staining (WMIS). A :1) BR-01 wt, 2) Pe-1, 3) DI 16-2, B: 1) NL-04 wt, 2) NL-04 env$^-$.

Midgut infection by the defective isolates Pe-1 and 16-2

Infection of the midgut epithelial cells is the first sign that refers to the vector competence of thrips to transmit tospoviruses. Whole mount immunofluorescent staining (WMIS)

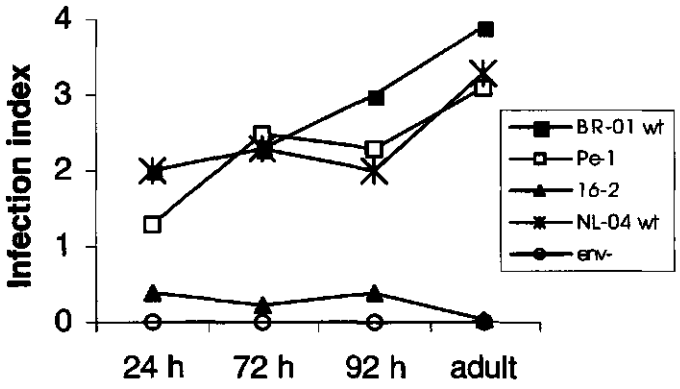

Fig. 3. Development of infection in the thrips midguts as rated by the infection index.

(Chapter 4) was applied to investigate whether the DI isolate 16-2, which could not be transmitted by thrips, would possibly infect the thrips midgut or not. Wt TSWV (BR-01) and the transmissible isolate Pe-1 were used as positive controls.

The fast positive signals of wt TSWV infections can be discerned by green coloured FITC spots in the epithelial cells in the anterior midgut region (Mg1) at 24 h post-acquisition (p.a.) (Fig 2A-1). Subsequently, the virus migrates to the visceral circular and longitudinal muscle cells of this region within 72 to 96 h p.a. and later on, to the muscle cells of the middle and posterior midgut regions (Mg2 and Mg3) (Chapter 4). Whole midgut visceral muscle tissue in adult stage becomes infected, while the infection in the midgut epithelial cells was clearly abolished after emergence of adults (Chapter 4).

To evaluate the infection of the midgut and muscle cells by the mutant and wt isolates, first instar larvae of 24 h p.a., second instar larvae of 72 and 96 h p.a., and four-day old adults were studied by WMIS. All larvae (100%) which ingested wt TSWV (both BR-01and NL-04) and Pe-1 showed clear signals of virus infection in the midgut, but only 23 % of the larvae which ingested 16-2 showed infection. Beside the low number of infected larvae, the severity of the 16-2 infections in the midgut was low (Fig. 2A-3). The level of infection was indexed by the proportion of infected midgut epithelium and/or muscle cells in thrips, viz., 0 (when no infection is found), 1 (when a faint infection is restricted to the Mg1 region), 2 (when heavy infections occur in Mg1), 3 (when infections occur in both Mg1 and Mg2), and 4 (when the whole midgut is infected). The average index values found for the midguts infected by the various isolates are given in Fig. 3. While strong reactions were observed for both wt virus isolates, the first signals of

Table 2. *Infectivity of enveloped and non-enveloped tomato spotted wilt virus preparations on primary cell cultures of F. occidentalis and Chenopodium quinoa leaves.*

	N[1]	NS_S[1]	Local lesions[2]
NL-04 wt	+	+	63
NL-04 env⁻	-	-	117
BR-01 wt	+	+	42
BR-01 RNP	-	-	121

[1] positive reaction of immunofluorescence in primary cells with the antibodies to N and NSs proteins
[2] Number of local lesions formed 5 days after inoculation of the virus preparations on *C. quinoa* leaves.

infection of both Pe-1 and 16-2 isolates were restricted to small areas in the Mg1 24 h p.a. (Fig. 2A-2,-3). The index value was slightly lower for Pe-1 than for wt virus (Fig. 3). The index reached maximum values in midguts of adults infected with the two wt (BR-01 and NL-04) and Pe-1 isolates. With the isolate 16-2, however, the infections were restricted to only a small proportion of the midgut of the larvae 72 and 96 h p.a. (Fig. 3). During all larval stages, the infection remained weak and was virtually absent in the midgut after emergence of the adult thrips. Hence, the WMIS data on isolate 16-2 support the interpretation from the transmission experiments that the lack of its transmissibility is associated with the low levels of virus accumulation in the thrips.

Lack of the viral membrane results in transmission deficiency

The envelope deficient (env⁻) isolate was used to test whether the viral envelope, containing the viral glycoproteins, was required for thrips transmission. Although the env⁻ isolate reached titres in the acquisition source plant comparable to those of wt virus (Table 1), the tested thrips were not able to transmit this isolate. As this isolate was shown to be free of any defective RNAs (see Materials and Methods, and Fig. 1 lane 5), the reason for this transmission deficiency must be due to the absence of an envelope containing the G1 ad G2 proteins.

The env⁻ mutant fails to infect the midgut and primary cell cultures of *F. occidentalis*

To investigate whether the env⁻ mutant was completely deficient in its capacity to infect the thrips, the midguts of thrips larvae, fed on env⁻ infected hosts, were analysed using WMIS. Signals of infection could neither be discerned in the midgut of any larvae in their

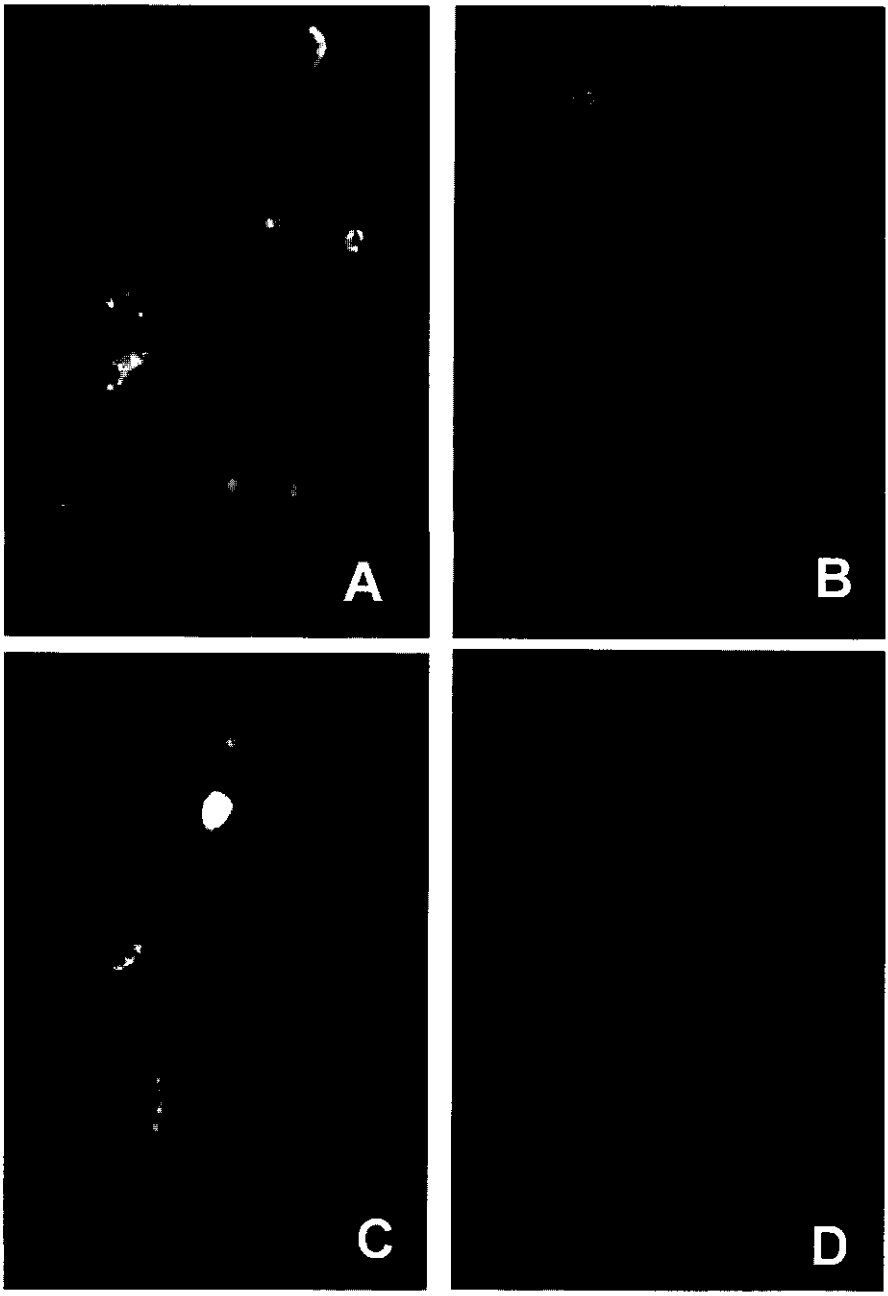

Fig. 4. Immunofluorescent staining of primary thrips cell cultures after infection with wt and env⁻ NL-04 isolates using antiserum to the N (A, B) and NSs protein (C, D).

L1 and L2 stages nor in their adult stage (Fig. 2B-2, Fig. 3). To verify whether the env⁻ mutant was completely unable to infect thrips cells, a primary cell culture derived from *F. occidentalis* (Chapter 3) was inoculated with this isolate. The susceptibility of these cells was shown in a previous study when intact TSWV particles were inoculated (Chapter 3). Preparations of complete wt virus particles (BR-01 and NL-04) and nucleoprotein of wt BR-01 were inoculated as controls. Infectivity of these virus preparations to plants was confirmed by mechanical inoculation (Table 2).

Infection of thrips cell cultures was monitored by detection of viral N and NS_S proteins using FITC labelled antibodies. Approximately, 30 to 40 % of cells became infected when intact particles of TSWV BR-01 or NL-04 were inoculated. However, no infection was found when the env⁻ isolate was inoculated (Table 2, Fig. 4). Nucleoprotein preparations of wt BR-01, devoid of the envelope, also failed to infect the cell cultures. Hence, it is concluded that the viral envelope contains the crucial determinants needed for virus entry into thrips cells. The absence of TSWV antigen in the midgut epithelial cells confirms that the transmission incompetence of the env⁻ isolate is based on a complete blockage of virus entry.

DISCUSSION

Neither infection of the midgut nor transmission to plants occurred when an env⁻ isolate of TSWV was ingested by thrips. This isolate, incapable of producing enveloped virus particles, as well as wt TSWV nucleocapsids failed to infect primary thrips cell cultures, although these preparations were infectious on plants. These features shared with purified nucleocapsids from wt TSWV indicate that the initial steps of the infection process differ in both hosts. The failure to infect thrips demonstrates that free nucleocapsids are not able to enter the midgut epithelial cells. This conclusion is confirmed by the results with primary cell cultures which are a mixture of cells originating from various types of tissues or organs. The failure to infect thrips and cell cultures has to be explained by the absence of any domain or motif on the nucleocapsids to be recognised by receptors on the midgut epithelium and on cultured cells. Since the membrane is absent in both the nucleocapsid and env⁻ inocula, it can be concluded that the receptor recognition sites occur on the virus envelope, *i.e.*, on one or on both viral glycoproteins. In analogy of the entry of animal viruses to their host cells, the G1 and/or G2 proteins of TSWV may possess one or even several receptor binding sites leading to endocytosis or membrane fusion of the virus with the cell and the release of nucleocapsids into the cytoplasm. Two thrips proteins of 94 and 50 kDa have been reported as possible candidates as receptor for binding of TSWV to thrips cells (Kikkert *et*

al., 1998; Bandla *et al.*, 1998).

Our results showed that the defective L RNA containing isolate which causes wt-like symptoms (isolate Pe-1) was efficiently transmitted, whereas a DI isolate (16-2) which produced attenuated symptoms on plants, was not transmitted at all. The inability of thrips to transmit this DI RNA containing isolate can be explained by the existence of a quantitative or dose-dependent barrier in the midgut and other tissues. Comparing the amount of RNA detected on Northern blots, it was concluded that the replication of the DI L RNA segment in isolate16-2 strongly interferes with the replication of the wt L RNA species. As a consequence, inocula of this DI isolate may have only low titres of infectious units compared to the wt and the defective L RNA containing isolate (Pe-1) and, hence, can establish infections only in a low number of midgut cells. This view is supported by the low levels of infection in the midgut after ingestion of this DI isolate. Northern blot analysis (Fig. 2, lane 2 and 3) showed that the defective RNA species of the isolate Pe-1 did not dramatically affect the replication of wt L RNA species. However, the slow start of Pe-1 accumulation in the midgut, compared to wt infection, implies that its multiplication is also slightly hampered or retarded.

The current study shows that thrips completely fail to transmit the DI isolate 16-2 despite limited initial replication in the midgut epithelium. On the other hand Pe-1, which accumulated somewhat slower than wt, was transmitted at a similar rate as wt. These observations suggested that dose-dependent processes regulate the virus accumulation in the midgut. One of these processes may be the rate at which sufficient infectious units become available by replication in the midgut for further spread to other organs. The moment at which the virus has to escape from the midgut, the virus has to be projected before pupation of the larvae. Pupation is characterised by a drastic renewal of the midgut epithelium (Müller, 1926; Hardy *et al.*, 1983; Hardy, 1988; Kaslow and Welburn, 1997; Moritz, 1997) which may cause an almost complete elimination of TSWV from this tissue. The virus which can be found in the muscular midgut tissues when the thrips become adult has apparently overcome this abortive process by escaping from the midgut epithelia before pupation.

One of the possible factors of the dose-dependent midgut barrier may consist of digestive activities at the intracellular level, autophagolysis, suggested by Ullman and co-workers (1992a and 1995). Autophagy seemed to attack viral inclusions in the infected cells to remove them. However, the real role of this system for the intracellular digestion of viral proteins should further be elucidated.

The results in this study suggest that thrips can play an important role in the elimination of TSWV mutants from natural virus reservoirs. Our studies showed that also defective interfering RNAs will be eliminated or suppressed in their occurrence, *in vivo* of their dose-dependent effect on transmissibility.

Chapter 6

The factors determining vector competence and specificity for tomato spotted wilt virus transmission

SUMMARY

Populations of the thrips species *Frankliniella occidentalis* are efficient vectors of tomato spotted wilt virus (TSWV), whereas thelotokous populations of *Thrips tabaci* do not transmit this virus. This difference in vector competence was further investigated. Larvae of thelotokous *T. tabaci* had the capacity to acquire the virus and to support its multiplication during their development, but virus accumulated in lower amounts than in larvae of *F. occidentalis.* After pupation and emergence of adult, the virus titre decreased to undetectable levels in *T. tabaci,* whereas high titers were readily detected in *F. occidentalis* adults. Immunohisto-cytochemical studies using whole mount immunofluorescent staining technique (WMIS) and sectioned specimens showed considerable differences in virus tropism between the two thrips species. In *F. occidentalis,* the first infections during the larval development were detected in the midgut epithelial cells and subsequently in midgut muscle cells and the ligaments, and finally in the salivary glands. In *T. tabaci* larvae, however, only weak infections were seen in their midgut epithelial cells, followed by a virtual absence of infection in the ligaments, and a complete lack of infection in the salivary glands during their further life span. Analysis by electron microscopy revealed accumulation of viral aggregates and virus particles budding from the midgut epithelial cells of *F. occidentalis* larvae. The enveloped virus particles were also seen in midgut muscle cells of *F. occidentalis* larvae. In *T. tabaci* larvae and adults, aggregates of viral proteins were observed, but never enveloped virus particles. The rate and extent of virus migration from the midgut to the visceral muscle cells and the salivary glands are probably the crucial factors in the determination of vector competence.

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INTRODUCTION

Tomato spotted wilt virus (TSWV) is a type species of the genus *Tospovirus* within the *Bunyaviridae*. This virus family contains many animal-infecting viruses, including very important human viruses (Murphy *et al.*, 1995). In nature, these viruses are mainly transmitted by mosquitoes and ticks (Calisher, 1996). Species of the genus *Tospovirus* infect plants and are transmitted by minute insects, thrips, which can form important pests in crops (Goldbach and Peters, 1994). The virus is transmitted by its vector in a propagative-circulative way by its vector (Wijkamp *et al.*, 1993; Ullman *et al.*, 1993a). TSWV has a unique vector relationship with thrips in that the virus is acquired by the larvae but not by adults, while the ability to acquire virus declines during larval development (Van de Wetering *et al.*, 1996; Chapter 4). After a temperature-dependent latent period, larvae at the end of their second stage and adults are able to transmit the virus (Wijkamp *et al.*, 1993). Until now, only eight thrips species within the genera *Thrips* and *Frankliniella* have been reported as vectors of tospoviruses (Mound, 1996; Webb *et al.*, 1998).

In a study combining four tospoviruses and four thrips species of six distinct populations, interspecific as well as intraspecific differences in vector competence was found (Wijkamp *et al.*, 1995). Out of the four viruses tested, TSWV was transmitted by all four thrips species, while *F. occidentalis* was the sole, but very efficient vector of *Impatiens* necrotic spot virus (INSV). Distinct differences were found among four *T. tabaci* populations. Three thelotokous populations of *T. tabaci* did not transmit TSWV, whereas an arrhenotokous population transmitted the virus with a low efficiency (Wijkamp *et al.*, 1995). These results suggested the existence of different factors that regulated the vector competence for tospovirus transmission on both thrips species and population level. The factors determining these differences in vector competence have not yet been elucidated.

The amount of TSWV present in the adult thrips body is probably one of the main factors that affect the vector competence and efficiency (Wijkamp *et al.*, 1995; Van de Wetering *et al.*, 1996). Virus accumulation, most likely, depends on the invasion and rate of replication in thrips tissues, presumably the midgut and salivary glands. Besides the accumulation of the virus in these tissues, the barriers, which may exist to prevent virus migration, must also play an important role (Chapter 4). The existence of such barriers may explain the failure of thelotokous *T. tabaci* populations to transmit TSWV, as the cells in primary cultures of such populations are susceptible (Chapter 3). This result suggests that the thelotokous *T. tabaci* are susceptible to TSWV, but lack the ability to become transmitters. Here, we report a comparative study performed to elucidate the stepwise development of the infection in the midgut and salivary glands of an efficiently transmitting *F. occidentalis* population and a non-transmitting thelotokous *T. tabaci* population.

MATERIALS AND METHODS

Transmission of TSWV and amplification ELISA

A *F. occidentalis* (NL-03, Van de Wetering, 1996) and a thelotokous *T. tabaci* (Wijkamp *et al.*, 1995, Chapter 3) population were reared in glass jars at 25 °C on bean pods and pieces of leek leaves, respectively. New-born larvae of less than 4 h old were collected and allowed to acquire the virus for 16 h on *Datura stramonium* leaves infected with the TSWV isolate BR-01 (De Ávila *et al.*, 1993). Following acquisition, the larvae were reared on healthy *D. stramonium* leaves until they were sampled for the transmission and immunohistochemical studies.

The infectivity of the thrips was tested using the local lesion assay method on petunia leaf discs (Wijkamp and Peters, 1993). Each newly emerged adult was individually placed on a leaf disc in an Eppendorf tube for 2 days at 25 °C. The discs were then transferred to a 24-well plate and incubated for three days on water for the development of local lesions.

The virus accumulation was determined by ELISA targeting the viral N protein. The reaction was amplified as previously described (Wijkamp *et al.* 1995). Five larvae were sampled at 0, 2, 4, 12, 24, 48, 72 h post acquisition (p.a.), and thrips in the prepupal and adult stages, and triturated together with 100 µl sample extraction buffer (Wijkamp *et al.*, 1995), half of which was used in the ELISA and another half was stored for repetition tests.

Midgut preparation and whole mount immunofluorescent staining (WMIS).

Thrips midgut and/or salivary glands were dissected and fixed with cold acetone on object glasses at -20 °C (Chapter 4). The specimens were incubated in PBS, containing 10 % bovine serum albumin (BSA) for 1 h to block non-specific reactions. Pre-absorbed polyclonal antibodies to the N protein (2 µg/ml) raised in a rabbit were used as a first overlay for 2 h in 10 % BSA-PBS. Then, the samples were incubated with 10 µg/ml pig anti-rabbit FITC conjugate (Nordic, Tilburg) in 10% BSA-PBS for 1 h. After washing, the specimens were mounted in CitiFluor (Agar) and studied by fluorescence microscopy (Leica).

Immunohistochemical studies on thrips.

Thrips bodies were fixed after amputating the legs and antenna in Bouin's Hollande sublime (Smid., 1998) and incubated under vacuum (Chapter 4). After fixation, the specimens were embedded in Paraplast (Oxford Labware) and sectioned in 5 µm thick sections, which were mounted on object glasses. The sections were deparaffinized with

xylene, rehydrated and incubated in phosphate buffered saline (PBS), pH 7.2. After this treatment, the specimens were incubated with N protein antibodies pre-absorbed with healthy thrips cell extracts (1 µg/ml PBS) (Chapter 4) for 2 h, washed with PBS and incubated with horseradish peroxidase conjugated pig anti-rabbit antibodies (5 µg/ml PBS containing 10% normal serum) for 1 h (Dako). After washing, the sections were incubated for 5 min with a substrate solution containing 0.05 % (w/v) diaminobenzidin (DAB) and 0.01 % (v/v) hydrogen peroxide in 50 mM Tris-HCl, pH 7.6. Some specimens were stained with Mayer's hematoxylin (Sigma) to visualise organs in detail and localise them correctly in the immunostained tissue. The samples were, then, mounted with DPX (Fluka) after dehydration and studied by light microscopy (Leica).

Electron microscopy.

Whole thrips specimens or dissected midguts were fixed in a 3 % paraformaldehyde-2 % glutaraldehyde solution for 30 min, dehydrated in a series of 50 to 100 % ethanol solutions, and embedded in LR-Gold (London Resin). Polymerisation of the resin was done by irradiation with UV light at –20 °C. The 60-70 nm thick sections were incubated with 0.8 µg/ml pre-absorbed antibodies to the N protein for 2 h, washed with 30 droplets of PBS, and incubated with gold-conjugated protein-A for 1 h. After washing with PBS, the sections were post-fixed in 1 % glutaraldehyde, contrasted with uranyl acetate and lead citrate, and studied with an electron microscope (Philips CM 12).

RESULTS

Accumulation and transmission of TSWV by *F. occidentalis* and *T. tabaci* populations.

After an acquisition access period of 16 h, first instar larvae of *F. occidentalis* and *T. tabaci* were transferred onto healthy *D. stramonium* leaves. The virus titre was determined by ELISA in five thrips immediately after acquisition and at various times later. The amount of virus ingested by the *F. occidentalis* larvae decreased within the first 2 to 4 h post-acquisition (p.a.) (Fig 1). This decrease in virus titre is, most likely, caused by the elimination of virus from the intestinal tract together with the faeces and by partial digestion of the virus. After this decrease, the ELISA values rapidly increased between 4 and 12 h p.a. Although a small decrease was observed in the larvae 24 h p.a. and in the prepupal stage, the amount of virus remained high in all stages (Fig. 1). The accumulation of viral proteins after acquisition supports previous conclusions that the virus multiplies in thrips (Wijkamp *et al.*, 1993; Ullman *et al.*, 1993a; Chapter 4). The decline in the titre found at 24 h p.a. and in the prepupal stages may be caused by histolysis of tissues

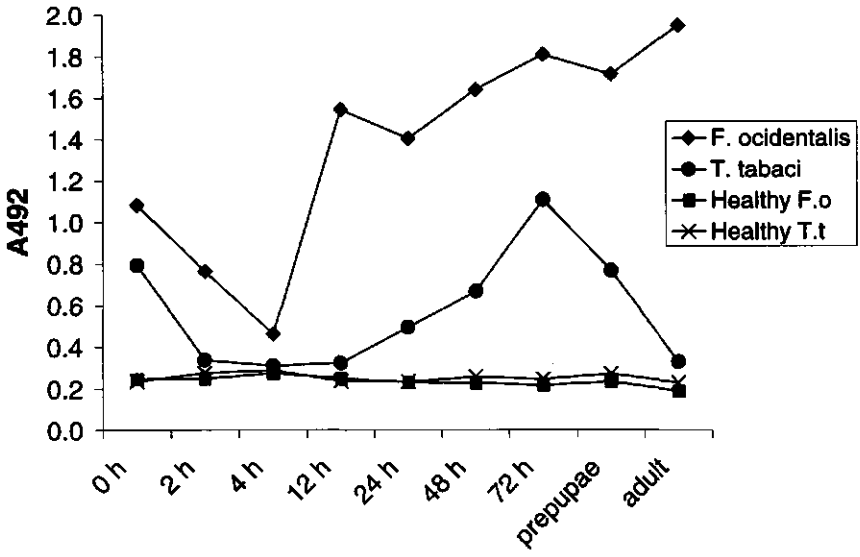


Fig. 1. Virus accumulation in *F. occidentalis* and *T. tabaci* as assayed by ELISA.

containing infected cells during the moulting processes.

A distinct pattern of virus accumulation was found in the thelokotous *T. tabaci* thrips studied (Fig. 1). Like in *F. occidentalis*, the amount of virus also decreased in the first 2 to 4 h p.a. after ingestion. The virus then started to accumulate, indicating virus multiplication, but at a slower rate than in *F. occidentalis*. The highest amount of virus was found before pupation at 72 h p.a. During the pupation, the virus decreased dramatically in amount and no resumption of virus accumulation could be detected in the adults of *T. tabaci*. These results show that this *T. tabaci* population could support the multiplication of TSWV in the larval stages, albeit with a lower efficiency than *F. occidentalis*, but that the infection was almost aborted during the subsequent pupation.

Adults of the *F. occidentalis* population transmitted TSWV at a rate of 67.3 % (206/306) and those of *T. tabaci* at 0 % (0/456) in three independent experiments, using the sensitive leaf disc test (Wijkamp and Peters, 1993).

Table 1. Incidence of TSWV infection in the midgut of two thrips species

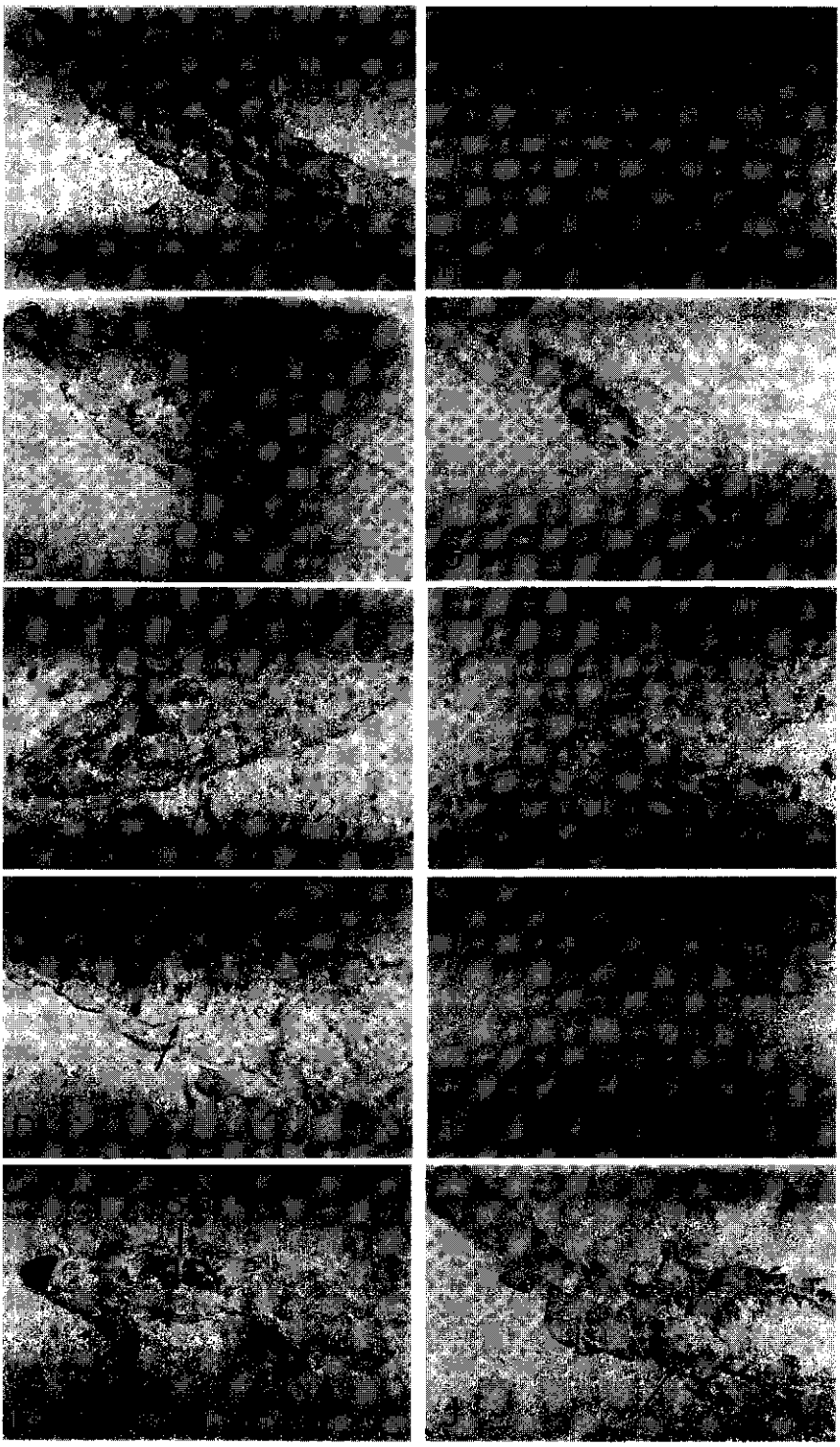
		Infection in Mg		Infected midgut regions (positive in WMIS)			
	Post-acquisition	-	+	Faint Mg 1[*a]	Mg1[*b]	Mg2[*c]	Mg3[*d]
F. occidentalis	72 h	0	20	1	12	7	0
	96 h	0	30	1	13	12	4
	Adult	0	37	0	1	13	23
T. tabaci	72 h	8	19	4	9	6	0
	96 h	4	26	4	14	8	0
	Adult	32	57	51	6	0	0

a) reaction not discernible using a 100-time magnification but with a 400-time magnification by fluorescent microscopy, b) infections restricted to region Mg1, c) infections restricted to regions Mg1 and Mg2, d) infections in the three regions in whole midgut.

Different levels of midgut infections in *F. occidentalis* and *T. tabaci* as determined by WMIS

Infection of the intestinal tract of the vector is the first discernible process, which can lead to transmission of the virus by the vector. Since a dramatic difference in the accumulation of virus was detected by ELISA in the late larval and following stages, the midguts of larvae of 72 h and 96 h p.a. (prepupae or pupae) and adults were analysed by WMIS (see Materials and Methods) for their infection by TSWV. In all stages, all midguts of *F. occidentalis* tested were found to be infected (Table 1). The infection was found in midgut epithelial cells, mostly restricted to the anterior midgut (Mg1), and midgut muscle cells in the Mg1 region at 72 and 96 h p.a. In the adult stage, no infection could be discerned in the midgut epithelium, but occurred in the muscle cells of, almost, the entire midgut (Mg1, 2 and 3). These observations confirmed the previous results that the virus was eliminated from the epithelial cells, but not from the muscle cells during pupation (Chapter 4).

Fig. 2. Immunohistochemical detection of TSWV in thrips tissues. Viral N protein was recognised by a dark colour of DAB precipitation. A-E, *F. occidentalis* and F-J, *T. tabaci*. A,F: 24 h p.a., B,G: 48 h p.a., C, H: 96 h p.a. D,I: 144 h p.a., and E, J: adult thrips. Arrows show the DAB precipitation. Mg1=anterior midgut, SG= salivary glands. Bar=100 µm.



A considerably lower susceptibility was found in the larvae of the *T. tabaci* population studied. Eight out of 27 larvae at 72 h p.a., 4 out of 30 prepupae (96 h p.a.) and 32 out of 89 adults did not show any signal of infection in the midgut (Table 1). The infection of *T. tabaci* differed not only in the proportion of infected individuals, but also in the intensity of infection. The largest differences were observed among the adult stages of both species. *F. occidentalis* showed strong signals in the muscle cells of the three midgut regions in 23 out of 37 specimens studied, while in most *T. tabaci* (51 out of 57) only faint infections, visible as few small spots, in their midgut muscle cells were observed (Table 1). This study shows also that WMIS is a more sensitive technique to detect infection of TSWV in thrips than ELISA. No response was found in any *T. tabaci* adult tested by ELISA, while weak, but positive reactions were found in 57 out of 89 adults using WMIS.

Development of TSWV infection in thrips of both populations

ELISA studies did not provide information on which tissues were infected and to which extent occurred. The use of WMIS targeting midgut, on the other hand, did not show the infection in the salivary glands, its infection is the most essential for virus transmission. Therefore, thrips of both populations were collected for thin sectioning analysis at 24 (1st instar), 48, 72 (2nd instar), 96 (2nd instar or prepupae), 144 h p.a. (pupae) and in the adult stage. The sections were analysed by targeting the N protein using the DAB substrate reaction, visualising positive reactions as dark brown precipitates. These reactions were not observed in thrips of both populations fed on healthy plants during the same intervals (data not shown).

Obvious infections were observed in the midgut epithelial cells of *F. occidentalis* 24 h p.a. (Fig. 2A). These infections were only noticed in the anterior midgut (Mg1), and mostly remained in this region during the whole larval development of this thrips species (Fig. 2A and B). With the pupation, large changes were noticed in the retention of the virus as a consequence of this metamorphological process. As the midgut epithelium is almost renewed during this process (Müller, 1926), most, if not all, of the virus present in these cells was eliminated (Fig. 2C). No infection could be discerned in the midgut epithelium of adults, showing that multiplication was not resumed in this renewed tissue. Infections in the midgut of adults were only noticed in the visceral muscle cells, mostly in the whole midgut region. In the adult stage of *F. occidentalis*, infection in the foregut was often observed.

The first infections in the salivary glands of *F. occidentalis* were seen at 96 h p.a in most specimens. As infections in these glands were not observed before 72 h p.a., migration of the virus to the salivary glands possibly took place between 48 and 72 h p.a. Analysis of adult transmitters revealed heavy infections in the salivary glands (Fig. 2E).

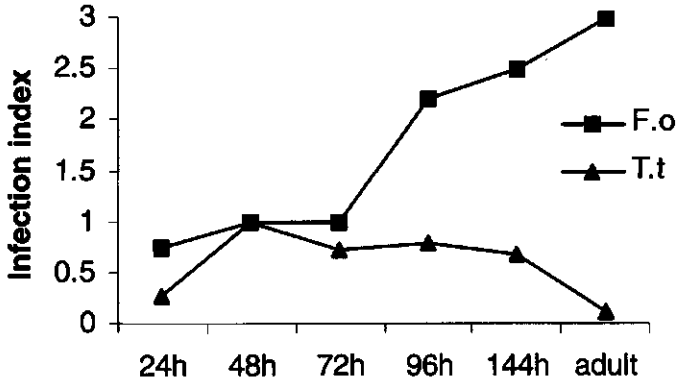


Fig. 3. Development of TSWV infection in thrips tissues evaluated by the infection index. 0: no infection, 1: infection restricted to the midgut, 2: infection of the midgut and foregut, 3: infection of the midgut, foregut and salivary glands.

Infection of the larvae and adults of the *T. tabaci* population was less pronounced (Fig. 2F-J) than that of *F. occidentalis*, especially in larvae of 24 h p.a. and in adults. The infection became more prominent in the midgut of larvae at 48 (Fig. 2B) and 72 h p.a. Since *T. tabaci* moulted to prepupae almost one day earlier (72-96 h p.a.) than *F. occidentalis* (96-144 h p.a.), elimination of epithelial cells by histolysis together with the virus consequently occurred one day earlier. No infection could be found in the salivary glands before pupation, during the prepupal stage and in the adults in any of the *T. tabaci* tested.

The development of the infection in the thrips of both populations was evaluated by an infectivity index, in which 0 stands for no infection, 1 for an infection restricted to the midgut, 2 for infection of the midgut and foregut, and 3 for infection of the midgut, foregut and salivary glands. For each species, an average of 8.25 individual thrips was examined at each moment after virus acquisition. The data obtained (Fig. 3) ran almost parallel with the graphs found for the accumulation of virus in thrips of both populations (Fig. 1). This indicates that the virus accumulation and the extent to which the tissues are infected, are positively correlated. The distinct vector specificity was therefore probably not due to a differential susceptibility of the virus at the cellular level, but by impeded migration to the salivary glands in *T. tabaci*.

Pathway by which the salivary glands become infected

Infection of the salivary glands is a prerequisite to transmit TSWV. However, the pathway by which the virus migrates from the midgut to the salivary glands in the thrips still remained to be elucidated. Two pathways of virus migration between these two organs can be proposed. The virus may migrate through the haemocoel from the midgut to the salivary glands. This is the pathway by which arboviruses in mosquito vectors are thought to reach the salivary glands (Hardy *et al.*, 1983). The second pathway may involve translocation of virus via a fine connective tissue-like structure, the ligament (Chapter 4), which connects the midgut and salivary glands (Ullman *et al.*, 1989). The presumed migration of TSWV through this structure has already been discussed in a previous study (Chapter 4). Evidence for this pathway can be obtained by making the time course study of the midgut, the ligament and the salivary glands during infection using WMIS. Since the first infection of the salivary glands was expected to occur late in the second larval stage, these tissues were studied in larvae 72 and 96 h p.a. and in adults.

Although many thrips were dissected, only a small number of midgut-ligament-salivary gland complexes were obtained. In *F. occidentalis*, the initial infected salivary glands were already observed at 72 h p.a. These infections often consisted of small patches, visible as a row of positive signals at the connection between the ligament and salivary glands. Infections in the ligaments always preceded those in the salivary glands. Infections only restricted to the ligament were occasionally seen, while infections in salivary glands were never observed without infected ligaments (Table 2).

These observations support the hypothesis that the salivary glands become infected by virus migration through the ligaments. Infected ligaments were found in three out of 48 *T. tabaci* adults and not in any of the 16 larval individuals. No infection was encountered in any of the salivary glands of *T. tabaci* analysed. These results show that the incompetence of the studied *T. tabaci* population to transmit the virus may be explained by a poor translocation of the virus through the ligament and hence a failure to reach and infect the salivary glands.

Table 2. TSWV infections in the midgut (Mg), the ligaments (Lg) and the salivary glands (SG) in each of the developmental stages of *F. occidentalis* and *T. tabaci*.

	Infected tissue					
	Mg	Lg	SG	72 h p.i.	96 h p.i.	Adult
F. occidentalis	+	+	+	2	5	13
	+	+	-	2	0	2
	+	-	-	0	1	4
	-	-	-	0	0	0
				72 h	96 h	Adult
T. tabaci	+	+	+	0	-	0
	+	+	-	0	-	3
	+	-	-	8	-	26
	-	-	-	8	-	19

Electron microscopy observations on midgut cells

Infection of the midgut epithelium and muscle cells was already immunohistologically demonstrated by light microscopy in both thrips species. To analyse the development of the infection, ultra-thin sections of the midgut of second instar larvae 72 h p.a. and adults were studied by electron microscopy. Gold labelled antibodies to the viral N and glycoproteins (G1/G2) were used to follow the viral infection process.

In *F. occidentalis* larvae 72 h p.a., many large nucleocapsid aggregates were observed in the cytoplasm of the epithelial cells of the Mg1 (Fig. 4A). In two out of four thrips examined, virus particle-like structures with a clear bilayer membrane were found only in the extracellular spaces of the basal labyrinth of the Mg1 epithelium (Fig. 4B, D) and in the cytoplasm of visceral muscle cells in Mg1 (Fig. 4C, E). These structures were specifically labelled with antibodies to both the N protein (Fig. 4B-D) and the G1 and G2 proteins (Fig. 4E), and hence could be identified as genuine TSWV particles. They were roughly spherical and their diameter was estimated to be approximately 100 nm (Fig. 4C-E), similar to the size of TSWV particles seen in plants.

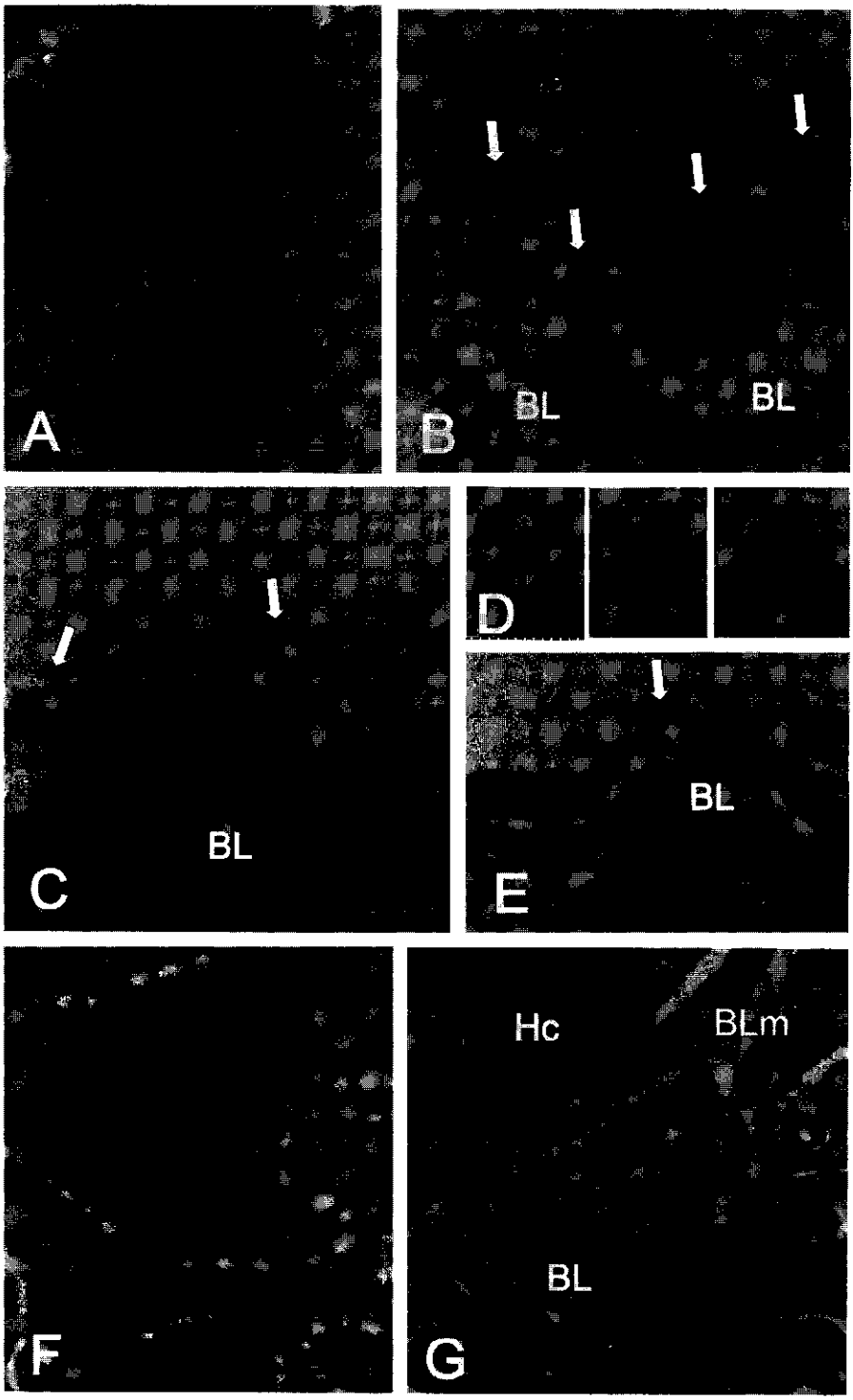

A
B
BL
BL
C
BL
D
E
BL
F
Hc
BLm
BL
G

Fig. 4. Immunolabeling of viral nucleocapsid or glycoproteins in thrips midgut. A-E, *F. occidentalis* at 72 h p.a. F,G, *T. tabaci* 72 h p.a. A) nucleocapsid aggregate (arrowhead) in the cytoplasm of epithelium tissue. B) Intermediate form of virus (open arrow) budding to the extracellular space of basal labyrinth. C) Enveloped virus particles (open arrow) in the cytoplasm of midgut visceral muscle. D) phases of virus budding observed at the basal plasma membrane of epithelium. E) Virus particles labelled with anti-G serum. F) Nucleocapsid aggregates found in the cytoplasm of epithelium tissue. G) N protein detected in the cytoplasm of midgut muscle tissue. Mc= muscle, BL= basal labyrinth, BLm= basal lamina, Hc= haemocoel, LMc= longitudinal muscle, CMc= circular muscle, Bar= 500nm (A,B,C,E,F,G), Bar= 100 nm (D).

Clear bilayer membrane appeared in the basal membrane of midgut epithelium where the virus budding was occuring (Fig. 4B), and maturing steps of budding was observed in this region (Fig. 4D). This is the first clear observation of bunyavirus maturation by budding through plasma membrane in the vector's body. Unlike for other bunyaviruses (Matsuoka *et al.*, 1991; Jäntti *et al.*, 1997) and TSWV in plant tissue (Kikkert *et al.*, 1999), virus maturation at the Golgi apparatus was not observed in the midgut. Enveloped virus particles, however, were not seen in the midgut muscle cells of adults. While nucleocapsid aggregates were found in the visceral muscle cells in two out of three *F. occidentalis* adults analysed. No infection could be detected in midgut epithelium of adults. The assembly of enveloped virus particles in larvae thereafter seems to be a transient event in the midgut basal labyrinth and midgut visceral muscle tissues.

In *T. tabaci*, enveloped virus particles could not be found in the midgut of larvae 72 h p.a. or of adults. However, nucleocapsid aggregates were the only viral inclusions found in the Mg1 epithelium (Fig. 4F) and visceral muscle cells (Fig. 4G) of larvae and adults. These aggregates were less abundant in *T. tabaci* than in *F. occidentalis*. From these observations, it may be concluded that the virus can less frequently escape to muscle cells or haemocoel in *T. tabaci* than in *F. occidentalis*.

DISCUSSION

A comparative study was made on the accumulation and translocation of TSWV within the body of an efficiently transmitting thrips, *F. occidentalis,* and a non-transmitting population of *T. tabaci* (Wijkamp *et al.*, 1995). It is shown that in the larvae of both vector competent and non-competent thrips the primary infections were established in the midgut epithelium. This means that differences in vector competence can not be explained at the level of receptor-mediated virus entry into the midgut epithelium. The current study unequivocally shows that this tissue can be infected in both transmitting *F. occidentalis* and non-transmitting *T. tabaci* thrips larvae. Following pupation and

emergence of adults, the virus was apparently eliminated from the midgut epithelium in both competent (*F. occidentalis*) and non-competent (*T. tabaci*) thrips. The virus could be found in the muscle cells of all three midgut regions in adults of the competent thrips, but barely in these tissues of non-competent thrips. The salivary glands of the competent thrips became infected before pupation, whereas no virus could be detected in these glands in larvae and adults of the non-competent thrips. The early infection in the larval salivary glands strongly indicates that the virus has to reach these glands before pupation to become transmitters. This view is supported by the observation that most larvae of *F. occidentalis* are able to transmit before pupation when they acquire the virus early in the first stage (Wijkamp and Peters, 1993).

One of the factors determining vector competence apparently is an efficient virus multiplication in the midgut epithelial cells followed by timely migration to the salivary glands. Lower amounts of virus accumulated not only in the midguts of the non-competent *T. tabaci* population compared in *F. occidentalis*, but also in the primary cell cultures derived from this population (Chapter 3).

The restricted virus spread in the visceral muscle tissue in *T. tabaci* (Fig. 3) implies that the less efficient virus escapes from the midgut epithelium than in *F. occidentalis*. Budding of enveloped particles from the midgut may be essential to enter midgut muscle cells, as the envelope plays a fundamental role in virus release and re-entry into neighbouring cells by endocytosis or membrane fusion. Since the midgut muscle cells of *T. tabaci* can become infected, it is tempting to speculate that virus has to be released from the midgut epithelium as enveloped particles by budding. To infect the muscle cells, the virus should pass a physical barrier, the basal lamina, which is a thick extracellular matrix lying on the basal membrane of the midgut (Lerdthusnee *et al.*, 1995; Kaslow and Welburn, 1997). The thickness of this layer may play a role in the transmission efficiency as shown for La Crosse virus. Small sized mosquitoes (*Aedes triseriatus*) reared on a nutritionally poor diet, could transmit this virus more efficiently than larger ones reared on a rich diet (Grimstad and Walker, 1991). The large sized mosquitoes developed a morphologically thicker midgut (mesenteron) basal lamina, a strong escape barrier, resulting in a low transmission rate in this population.

Viral infection was readily detected in the ligaments and salivary glands in the larvae and adults of competent *F. occidentalis*. The observation that infection in the ligament preceded the infection of the salivary glands, and that the salivary gland infection was always accompanied by ligament infection (Table 2) may suggest that virus migration to the salivary glands occurs through this tissue. TSWV had been thought to migrate, like other arboviruses, from the midgut to the salivary glands through the haemocoel, although TSWV particles have not yet been encountered in the haemolymph in any study (Ullman *et al.*, 1995). The failure of thrips to become viruliferous after injecting adults with

infectious virus particles (data not shown) supports the idea that the virus in the haemolymph does not serve as source for infection of the salivary glands.

Vector competence may also be determined by heavy infection in the salivary glands. Partial or weak infections in salivary glands do not often lead to virus transmission (Chapter 4). A heavy infection in the salivary glands and transmission (50% of male individuals) was observed for the males of an arrhenotokous *T. tabaci* population, while the salivary glands of its non-transmitting females were not infected (data not shown). This observation suggests that the salivary glands of *T. tabaci* are genetically susceptible and confirms that absence of infection of the salivary glands is due to poor virus delivery to this organ before pupation.

Chapter 7

General discussion and conclusion

Plant viruses belonging to the family *Reoviridae*, *Rhabdoviridae* and *Bunyaviridae* are all transmitted in a circulative propagative manner by phytophagous insects, colonising on plants. They circulate through the bodies of their vectors, while being replicated, from the intestine lumen via the midgut to the salivary glands where the virus particles are released with saliva as to infect a next susceptible plant. In analogy to the animal infecting arboviruses (Hardy *et al.*, 1983) the plant viruses have to cross several barriers during this circulation before they can be transmitted. These barriers may be encountered at the midgut level where the virus has to enter and to leave the midgut epithelium to infect the salivary glands, and at the salivary gland level where the virus has to enter and subsequently to be added to the saliva in the salivary ducts.

Tospovirus-vector relationships

Most of our knowledge on the relationship between tospoviruses and their vectors has been obtained in studies with Tomato spotted wilt virus (TSWV) and its major vector, the Western flower thrips, *Frankliniella occidentalis*. Following virus ingestion by larvae, successful transmission occurs after a temperature-dependent latent period. The virus has to be acquired by larvae while the adults fail to acquire although they ingest the virus. This ability to acquire the virus decreases with the age of the larvae (Van de Wetering *et al.*, 1996). It has been shown that only a part of each western flower thrips population, which have ingested virus, become transmitters whereas others fail to do so. The virus is readily detectable in all transmitting thrips, but only in some of the non-transmitters. Besides this variation in vector competence within a given population, a notable variability in vector competence also exists between the various *F. occidentalis* populations. A transmission efficiency of 80% can be obtained with some populations, while values for other populations are as low as 20 %. Even larger differences in TSWV vector competence may exist between populations of the onion thrips, *Thrips tabaci*. Some populations, which appeared to be thelotokous, completely fail to transmit, whereas an arrhenotokous population was able to transmit though at a low percentage (Wijkamp *et*

al., 1996).

Recognition of the existence of different levels of competence has stimulated a study on the fate and pathway of the tospoviruses in thrips. The primary aim of this chapter is to develop a coherent picture describing the events within a thrips leading either to transmission or to the failure to transmit tospoviruses. These events will determine whether the virus ingested will initiate an infection in the epithelium of the midgut and reach, after productive multiplication in midgut tissues, the salivary glands where another round of multiplication will be required to produce sufficient virus to be released in the salivary ducts. The requirements for the initial infection of the midgut are basically well documented, but our concepts of which subsequent organs the virus has to invade before it eventually can be transmitted through the saliva is less clear. Nonetheless, a general concept emerges from the study made as will be described.

Infection of thrips cell lines.

The first infection studies done during this PhD research concerned different (primary and one continuous) cell lines generated form to major vector thrips, *i.e. F. occidentalis* and *T tabaci.* As shown in Chapters 2 and 3 suitable cell lines were obtained, allowing inoculation with purified TSWV particles and resulting in successful infection. This was even the case for a primary cell line derived from a non-transmitting *T tabaci* population. As a heterologous insect (*Spodoptera exigua*) cell line, used as control in these experiments, was unable to support multiplication of TSWV, it may be concluded that thrips cells have a sort of basic compatibility (susceptibility) to TSWV. Whether this is based on the presence of specific receptors on the thrips cell's plasma membrane, or on the requirement of essential host factors, e.g. the viral transcriptase, remains to be elucidated. Nevertheless, the initial infection studies using the developed thrips cell lines (Chapters 2 and 3) indicate that they may represent important tools to unravel thrips-tospovirus interactions on the cellular and subcellular level.

Infection of the midgut

After ingesting virus and transferring the larvae to healthy plants, the virus titre decreases sharply within the first hours. This decrease can be explained by digestion of the virus in the intestinal lumen and its removal with the faeces. Transfer of larvae which fed on plants to sucrose sachets showed that the green colour resulting from the plant material ingested is flushed out within a few hours (unpublished results). This observation shows that the virus has to enter the midgut epithelium cells during or within the first hours after ingestion. Only a small amount of virus enters the midgut epithelium, as can be concluded from the decrease in the virus titre. After this drop, the virus titre starts to

increase reaching high levels within two to three days (Wijkamp *et al.*, 1993; Chapter 6). This is indicative for active replication in the midgut epithelium and implies that the virus has crossed the first barrier, midgut infection barrier. Feeding the thrips on plants infected with wild type virus and an envelop defective mutant (env$^-$) showed that thrips only become infected when they acquire intact virus particles. (Chapter 5). The env$^-$ mutant was also unable to infect primary cell cultures derived from a competent *F. occidentalis* population (Chapter 3) and the same holds true for nucleocapsids extracted from wt virus. These results strongly suggest that the viral envelope, *i.e.*, the viral glycoprotein contain the binding site for a receptor, which is supposed to be located at the midgut apical membrane, in casu, on the microvilli. Two different thrips-encoded proteins that may function as receptor, have been identified (Bandla *et al.*, 1998, Kikkert *et al.*, 1998) and await further characterisation.

All larvae of a competent *F. occidentalis* population appeared to be infected in midgut as shown by the whole mount immunofluorescent staining technique (WMIS), when fed for 8 h on infected leaf material (Chapter 4). The first infections were observed 24 h post acquisition (p.a.). These infections increased in intensity in the following hours, but remain restricted to the epithelium of the Mg1 region. Following pupation and having become adult a complete different picture emerges in the insect. The infection is no longer observed in the epithelium of the Mg1 region, but is evident in the muscle cells in all three (Mg1, Mg2 and Mg3) midgut regions as well as in the foregut. Apparently, the complete absence of infection in the epithelium cells was due to the entire renewal of this tissue during pupation. Earlier, serological studies indeed have shown that a considerable amount of virus is lost during the pupation process (Wijkamp *et al.*, 1993).

The way and the mechanisms by which the virus spreads from the Mg1 muscle cells to the Mg2 and 3 muscle cells and the foregut are not clear. Since the virus infection expanded in the direction from the Mg1 to Mg2, a longitudinal spread from cell to cell is likely. To which extend the Mg1 region is infected is difficult to appreciate by the histological techniques applied as the infection signals in the epithelium are too dominant.

The electron microscopic studies indicate that TSWV buds from the basal plasma membrane of midgut the epithelium into the extracellular space of the basal labyrinth (Chapter 6). This suggest that this virus matures and is released in this way from midgut epithelium, since maturation in Golgi complex and release by exocytosis as shown for other bunyaviruses in cultured cells has not been observed. Alternatively, TSWV maturation has reported in the Golgi apparatus of plant cells (Kikkert *et al.*, 1999) showing that this virus will probably mature, depending on the cell type, by two different strategies. The released virus particles should pass the basal lamina to infect the midgut muscle cells and most possibly also the ligament. Also, enveloped virus particles were observed in the cytoplasm of midgut muscle cells. The formation of enveloped virus

particles may be a prerequisite for migration of the virus to the muscle cells, ligaments and eventually to the salivary glands. Enveloped virus particles in the midgut were observed only in the larval stage of *F. occidentalis* but not in larvae or adults of *T. tabaci.* The lower intensity of virus infection in midgut muscles cells of *T. tabaci* implies that the virus can not timely escape in sufficient amounts form the midgut epithelium to infect the salivary glands.

The vector competence can be affected not only by the vector's characteristics, but also by the virus isolates of the same virus. The primary infection of the wt TSWV isolates of BR-01 and NL-04 in the midgut had similar titres when first and young second instar larvae were analysed (Chapter 5). However, the virus spread of NL-04 in adult midgut was less intense and a lower number of adults transmitted the virus compared to BR-01 (Chapter 5). These results suggest that a lower capacity of virus spread in the thrips midgut affects the vector competence. The existence of a dose-dependent infection, which affects the ability to transmit, could be confirmed using two mutant lines of the TSWV BR-01 isolate. One line contained a defective L RNA segment, which barely interfered with the replication of L RNA, accumulated at a lower rate than wild type BR-01 in the midgut epithelium and is transmitted at a slightly lower efficiency. The other line, of which the defective L segment strongly interfered with the replication of the wt L RNA genomic segment, multiplied very poorly in the thrips' midgut and could not be transmitted (Chapter 5). The gradual differences in the transmission efficiency found between these two lines compared to wild type virus, are reflected by the intensity by which the epithelium of Mg1 region becomes infected and the visceral muscle cells in the successive regions after pupation (Chapter 5).

Infection of the salivary glands

Analysis of the salivary glands revealed that these organs can become infected only after a thorough infection of the midgut epithelium. The first infections in the salivary glands were observed at 48 h p.a. (Chapter 5) showing that the virus can reach these glands within this time span. Intense infections could be observed in the salivary glands of several larvae between 72-96 h p.a. Early infection of the salivary glands of some larvae remained restricted to a small region where a ligament is connected with the salivary glands (See Fig. 1F,G in Chapter 4). Several pathways have been proposed by which the salivary glands may become infected. It is generally accepted, though not yet proven for arboviruses, that the virus particles are released from the midgut into the haemocoel to reach the salivary glands. Thus far no cytological evidence could be obtained for the release of TSWV particles from the midgut basal lamina into the haemocoel. Moreover, virus particles "floating" in the haemolymph have never been observed. Intrathoracical injection of infectious virus particles into more than 500 *F. occidentalis* adults resulted in

only three thrips were able to transmit several days post injection (unpublished data). These results and the failure to detect any virus in the haemolymph suggest that the main pathway of the virus to infect the salivary glands is not through the haemocoel. The suggestion that these glands become infected by virus transport from the midgut through the tubular glands (Ullman *et al.*, 1992b) was not supported in our studies as no infection signals were observed in these glands. Instead, infections were frequently observed in thin thread-like structures, ligaments, which connect the midgut with the salivary glands. Transport of the virus through these ligaments may be the main pathway by which the salivary glands become infected. This idea is supported by the observation that the salivary glands of the *F. occidentalis* larvae and adults were infected, when the ligaments were also infected (Chapter 6). In thrips of a non-competent *T. tabaci* population, only limited infections were observed in some ligaments close to the midgut, corroborating the idea that successful infection of the salivary glands must be preceded by full infection of the ligaments (Chapter 6)

Conversion of thrips into transmitters

Evidence is accumulating that the thrips will transmit TSWV only when the virus has reached the salivary glands before pupation. A large percentage of the larvae becomes transmitters before pupation when the virus is early acquired by first instars (Wijkamp and Peters, 1993). Only few thrips make their first transmissions after becoming adult. The failure of these thrips to transmit in their larval stage has to be explained by the short interval between the moment that they become viruliferous and pupation (Wijkamp and Peters, 1993). A rapid decline of the ability of thrips to become transmitters was reported when the virus was acquired later in their development (Van de Wetering 1996, Chapter 4). This decline may be attributed to a smaller interval between acquisition and moment that sufficient virus is produced to infect the salivary glands before pupation. Analysis of the thrips, which ingested virus a second instar larvae, showed that the midgut became less intensively infected than found for first larval instars (Chapter 4). Similar observations were also made for the salivary glands. The failure of non-competent thrips to transmit has to be explained along this line. A limited accumulation of the virus in the midgut epithelium before pupation, followed by complete loss of the virus by replacement of this tissue during pupation (Müller, 1926) will prevent infection of the salivary glands.

Conclusions

The results described in this thesis have shed more light on the complicated interactions between TSWV and its thrips vectors. Primary and continuous cell lines of two major vector species were obtained which not only gave first information with respect to the virus-vector interactions on the cellular level, but also represent important tools to unravel

these interactions more precisely in the near future. In further studies, focusing mostly on *F. occidentalis* and using both wild type and mutant lines of TSWV, a first model could be proposed as how the virus initiate infection in the midgut, and next is further virus translocation to the salivary glands during the development from larvae to adult.

Samenvatting

Tomatebronsvlekkenvirus (in het Engels tomato spotted wilt virus, afgekort TSWV) geldt momenteel als een van de meest schadelijke plantenvirussen. TSWV heeft een zeer breed waardplantbereik en kan veel schade veroorzaken in belangrijke gewassen zoals aardappel, aardnoot, erwten, paprika tabak, tomaat, en diverse siergewassen. Het virus wordt door een achttal tripssoorten overgebracht, waarvan de soorten *Frankliniella occidentalis* (de Californische trips) en *Thrips tabaci* (de tabaks- of uientrips) de meest belangrijke vectoren zijn. Taxonomisch is TSWV met een aantal andere virussen ondergebracht in het genus *Tospovirus* binnen de *Bunyaviridae*, een virusfamilie dat met nog vier genera telt en waarvan de meeste leden door muskieten, teken en andere arthropoden op warmbloedigen worden overgebracht.

De overdracht van TSWV en de andere tospovirussen vindt op een circulatief-propagatieve wijze plaats. Dat wil zeggen dat het virus na opname uit de plant zich tijdens de circulatie in de vector vermeerdert. Na afgifte aan het speeksel in de speekselklieren kan het virus dan weer op een gezonde plant worden overgebracht. De efficiëntie waarmee tripsvectoren het TSWV overbrengt verschilt, zelfs binnen een soort, sterk van populatie tot populatie, waarbij onder *T. tabaci* populaties voorkomen die het virus in het geheel niet kunnen overbrengen. Het doel van het in dit proefschrift beschreven onderzoek, was om deze verschillen in vectorcompetenties te analyseren. Enerzijds werd daartoe gebruik gemaakt van celcultures van tripsen, en anderzijds werd op immunohistologische wijze de ontwikkeling van virusinfecties in larven en adulten geanalyseerd.

Aangezien er bij de aanvang van deze studie geen celcultures van tripsen beschikbaar waren, was het noodzakelijk deze cultures eigenhandig op te zetten. Er kon een medium worden samengesteld waarin het geëxplanteerde embryonale weefsel tot celdeling kwam (Hoofdstuk 2). Het virus bleek in dergelijke primaire trips celcultures vermeerderd te worden, ongeacht of deze afkomstig waren van een overdrachtscompetente *F. occidentalis* populatie of van een niet-competente *T. tabaci* populatie (Hoofdstuk 3). Uit de verkregen resultaten kon geconcludeerd worden, dat *T. tabaci* cellen in principe vatbaar zijn voor het virus, ondanks dat deze afkomstig waren van een populatie die het virus niet kon overbrengen.

Om de ontwikkeling van de infectie in tripsen van zowel overdrachtscompetente als niet-overdrachtscompetente populaties verder te bestuderen werd een histologische techniek ("whole mount immunostaining technique", afgekort tot WMIS) ontwikkeld om de infectie in intacte middendarmen en speekselklieren te bestuderen (Hoofdstuk 4). In *F. occidentalis* larven werden de eerste infecties reeds 24 uur na virusinoculatie in het

epitheel van de middendarm waargenomen. Deze infecties bleven tot het eerste gedeelte van de middendarm beperkt. Aan het einde van het tweede larvale stadium werd het virus ook in de spiercellen van de middendarm en in de speekselklieren gevonden. Een structuur, ligament genoemd, dat de middendarm met de speekselklier lijkt te verbinden, bleek ook geinfecteerd te worden. In de adulte *F. occidentalis,* dus na de verpopping, bleek het middendarm epitheel geheel virus vrij te zijn. Uit deze waarneming werd de conclusie getrokken dat het virus tijdens de verpopping met het oude epitheel was verwijderd. De infectie had zich niettemin uitgebreid tot de spiercellen in het tweede en derde gedeelte van de middendarm en tot de voordarm. Een duidelijke correlatie kon gemaakt worden tussen de intensiteit waarmee in adulte tripsen de speekselklieren werden geïnfecteerd, en het vermogen van adulten om het virus over te brengen. In het geval dat de infectie tot een paar cellen in de speekselklier beperkt bleef, waren de adulten niet in staat tot virusoverdracht.

Met de in Hoofdstuk 4 beschreven immunohistologische techniek (WMIS) werd inzicht verkregen in het virus-opnameproces in de middendarm. Een mutant, die het vermogen had verloren om de nucleocapsiden met een envelop te omgeven, bleek niet meer door tripsen overgedragen te worden en was evenmin in staat de primaire tripscel cultures te infecteren. Na opname van deze mutant door *F. occidentalis* larven werd geen enkele infectiehaard in het middendarmepitheel aangetroffen, wat er op wijst dat de glycoproteinen op de virale envelop essentieel zijn voor binding aan (een receptor van) de middendarm. Tevens bleek dat infectie van de middendarm ook een dosis-afhankelijke component heeft. Een mutant met een defect L RNA segment ("DI RNA") dat de replicatie van het virale genoom sterk onderdrukt, bleek een zeer beperkte infectie in de middendarm teweeg te brengen. Een mutant met een defect L RNA dat nauwelijks interfereerde met de replicatie van het virale genoom, kwam nagenoeg, ofschoon wat langzamer, even efficient tot vermeerdering in het middendarmepitheel als het wild type virus. De tripsen die met de DI mutant waren geïnfecteerd brachten dit virus niet over, terwijl de tripsen die de laatstgenoemde mutant met het defecte L RNA hadden opgenomen, dat wel deden, zij het duidelijk minder efficiënt (Hoofdstuk 5). Virusopname door larven van de niet overdrachtscompetente *T. tabaci* resulteerde in een lage besmettingsgraad van het middendarmepitheel. In de speekselklieren werden geen infecties in de speekselklieren gevonden en slechts in enkele larven een beginnende infectie in de ligamenten. In de adulten van deze populatie werden sporadisch infecties in de middendarmspiercellen gevonden. Deze resultaten toonden aan dat het virus wel tot replicatie kan komen in deze niet-competente larven, maar dat er kennelijk een onvoldoende hoge titer bereikt wordt om de speekselklieren te infecteren, met als gevolg dat zij in het volwassen stadium geen virus kunnen overbrengen (Hoofdstuk 6).

Resumerend kan geconcludeerd worden dat de eerste infecties in het

middendarmepitheel plaatsvinden en dat dan van hieruit het virus, vermoedelijk via de ligamenten, richting speekselklieren wordt getransporteerd. Het virus dient deze klieren voor de verpopping bereikt te hebben. *T. tabaci* populaties die het virus niet kunnen overbrengen bleken alleszins vatbaar te zijn. Echter de virustiter die in het middendarmepitheel werd ontwikkeld was niet groot genoeg om de speekselklieren tijdig en effectief infecteren.

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Tatsuya Nagata was born in January 11, 1967 in Gifu-city, Province of Gifu, Japan. He obtained his Bachelor degree in Agriculture in 1989 and his Master degree in Agriculture at Hokkaido University, Sapporo-city, Hokkaido, Japan, under the supervision of Dr. Eishiro Shikata, Dr. Ikuo Kimura, Dr. Ichiro Uyeda and Dr. Teruo Sano. In 1991, he arrived in Brazil as a research fellow in the National Center of Vegetable Research (CNPH/EMBRAPA) by the program of Japan International Cooperation Agency (JICA). His work was focused on the characterisation of tomato and pea viruses, co-working with Dr. André N. Dusi and Dr. Antonio C. de Ávila.

From 1995 to 1998, he worked at the Department of Virology in Wageningen Agricultural University as a visiting researcher. His main theme was the study of virus-vector interaction under the supervision of Dr. Dick Peters and Dr. Rob Goldbach.

Currently, he works at CNPH as a research fellow on the relationship of Brazilian tospovirus-thrips vectors.

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