Bulletin of Entomological Research (2004) **94**, 501–507 DOI: 10.1079/BER2004326

Specificity of accumulation and transmission of tomato spotted wilt virus (TSWV) in two genera, *Frankliniella* and *Thrips* (Thysanoptera: Thripidae)

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Abstract

The accumulation and transmission of tomato spotted wilt virus (TSWV) was examined in second instar larvae and adults of two thrips genera, *Frankliniella* and *Thrips*. The species tested were *F. occidentalis* (Pergande), *F. intonsa* (Trybom), *T. tabaci* Lindeman, *T. setosus* Moulton, *T. palmi* Karny and *T. hawaiiensis* (Morgan). In a standard petunia leaf disc assay, the efficiencies of TSWV transmission by two species of *Frankliniella* were higher than those of any *Thrips* species in the adult stage. A triple antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA) showed that large amounts of the TSWV-nucleocapsid (N) protein were present in the ELISA-positive larvae of each species, with the exception of *T. palmi*. The ELISA titre of and the proportion of virus-infected individuals of the two *Frankliniella* species increased or did not significantly change from the larval to the adult stages, whereas those of the four *Thrips* species decreased significantly. These results show that the specificity of virus transmission by adult thrips is probably affected by the amount of viral N protein accumulation in the adults and that the accumulation pattern from the larval to the adult stages is in between the two genera tested in the present study.

Introduction

Many plant viruses are transmitted by arthropod vectors (Nault, 1997). Tomato spotted wilt virus (TSWV), the type species of the genus *Tospovirus*, family Bunyaviridae (Murphy *et al.*, 1995), is exclusively transmitted by several thrips species in a propagative manner (German *et al.*, 1992; Ammar, 1994; Goldbach & Peters, 1994). During the last two decades, TSWV has emerged worldwide as a serious disease of vegetable and ornamental crops (German *et al.*, 1992; Goldbach & Peters, 1994; Mumford *et al.*, 1996; Ullman *et al.*, 1997), concurrent with the geographical expansion of its major vector species, the western flower thrips, *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae) (Brødsgaard, 1989; German *et al.*, 1992; Anon., 1993; Goldbach & Peters, 1994).

Specific associations exist between TSWV and the thrips vectors (Sakimura, 1962). Only thrips that acquire the virus in the larval stage can become transmitters, either as second instar larvae or as adults (Smith, 1931; Sakimura, 1963; Ullman *et al.*, 1992; Wijkamp & Peters, 1993; van de Wetering *et al.*, 1996), after the virus has replicated and circulated inside the host (Ullman *et al.*, 1993; Wijkamp *et al.*, 1993). Successful transmission of the virus requires

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heavy infection of the salivary glands during thrips development (Nagata *et al.*, 1999, 2002). Presently, at least seven species of thrips, belonging two genera, have been reported as vectors of TSWV: *Frankliniella schultzei* (Trybom), *F. occidentalis*, *F. intonsa* (Trybom), *F. fusca* (Hinds), *F. bispinosa* (Morgan), *Thrips tabaci* Lindeman and *T. setosus* Moulton (Thysanoptera: Thripidae) (Mound, 1996; Mumford *et al.*, 1996; Ullman *et al.*, 1997, 2002; Webb *et al.*, 1997). The variation in efficiency of TSWV transmission among several thrips species has been reported in previous studies (Paliwal, 1976; Fujisawa *et al.*, 1988; Wijkamp *et al.*, 1995). Wijkamp *et al.* (1995) indicated that the most and least efficient known vectors were *F. occidentalis* and *T. tabaci*, respectively. A recent study showed that the ELISA titre for TSWV-N protein remained high in three-day-old larvae through the process of pupation to adult emergence in *F. occidentalis*; however, it decreased rapidly during the pupal stage in *T. tabaci* (Nagata *et al.*, 2002). These findings indicate that the pattern of TSWV-N protein accumulation during thrips development could be an important determinant of vector competence, although little is known about the relationship.

In the present study, the accumulation and transmission of TSWV by second instar larvae and adults were investigated to reveal whether an interspecific variation in TSWV vector competence is caused by distinct patterns of virus accumulation during thrips development. Six thrips species, which are widespread in Japan, were tested with this objective: *F. occidentalis*, *F. intonsa*, *T. tabaci*, *T. setosus*, *T. palmi* Karny, and *T. hawaiiensis* (Morgan). *Thrips palmi* is recognized as a vector of other tospoviruses, such as watermelon silver mottle virus (WSMV) and groundnut bud necrosis virus (GBNV) (Yeh *et al.*, 1992; Lakshmi *et al.*, 1995; Ullman *et al.*, 1997); however, *T. hawaiiensis* has never been investigated for vector–tospovirus relationships.

Materials and methods

Thrips

The mass rearing of virus-free thrips was based on an *in vitro* technique using germinated broad beans, *Vicia faba* L. (Fabaceae), and tea pollen from *Camellia sinensis* (L.) Kuntze (Theaceae) (for details see Murai & Loomans, 2001). However, the oviposition of *T. setosus*, the species feeding on leaf tissue, was accomplished by a leaf cage method (Ohnishi *et al.*, 1999) using the leaves of kidney bean, *Phaseolus vulgaris* L. (Fabaceae). Adult thrips were collected for rearing from the following host plants and locations in the western part of Japan. Populations of *F. occidentalis* and *F. intonsa* were collected from eggplants, *Solanum melongena* L., and sweet peppers, *Capsicum annuum* L. (Solanaceae), respectively in Kochi Prefecture. A thelytokous population of *Thrips tabaci* from onion, *Allium cepa* L. (Liliaceae), and *T. setosus* from soybean, *Glycine max* (L.) Merr. (Fabaceae), were collected in Shimane Prefecture. *Thrips palmi* and *T. hawaiiensis* were collected from eggplants and roses, *Rosa* spp. (Rosaceae), respectively in Okayama Prefecture. All thrips species used in this study had been reared for one to four years in the laboratory. The laboratory-reared cultures were maintained at 25 ± 0.5°C and L16:D8 photoperiod.

Virus isolate and plant materials

A TSWV isolate from sweet pepper, *C. annuum*, originating from Ibaraki Prefecture, Japan in 1993, was used for transmission tests (Tsuda *et al.*, 1993). This isolate was maintained on *Datura stramonium* L. (Solanaceae) by regular inoculation with viruliferous thrips. The inoculated plants were maintained in an incubator at 22 ± 1°C and L16:D8. To determine virus transmission, leaves of *Petunia* × *hybrida* cv. Polo Blauw (Solanaceae) were used for assay 40–45 days after sowing. These plants were kept in a growth chamber at 22 ± 1°C and L10:D14.

Virus acquisition by thrips

Newly hatched larvae, 0–4 h old, were placed on a systemically TSWV-infected detached leaf of *D. stramonium* sandwiched between two plastic plates for an acquisition access period (AAP) of 4 h (fig. 1). For details of this modified leaf cage method see Ohnishi *et al.* (1999). Larvae were reared on healthy germinated broad bean plants, *V. faba*, until adult emergence. Larvae caged on a virus-free *D. stramonium* leaf were used as controls. All experiments were carried out at 25 ± 0.5°C and L16:D8.

Assay for TSWV transmission efficiency

The vector competence of six thrips species was compared. The petunia leaf disc assay (Wijkamp & Peters,

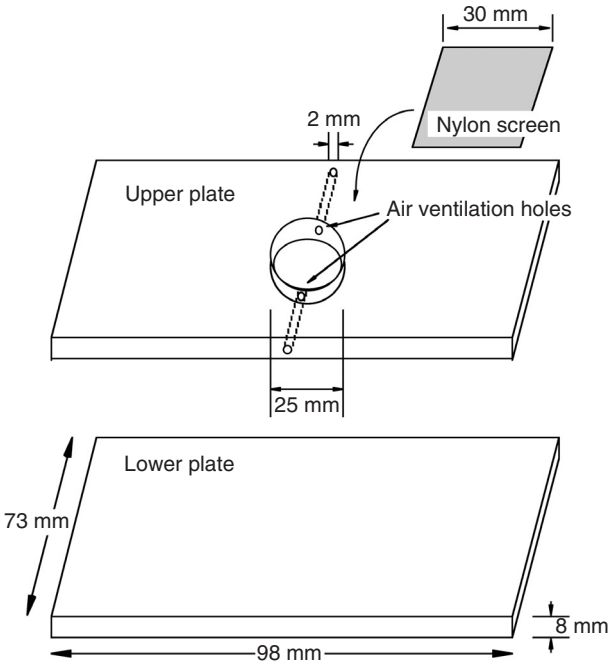

Fig. 1. The experimental cage used for exposing thrips to tomato spotted wilt virus (TSWV). Newly hatched larvae were given an acquisition access period of 4 h to a TSWV-infected detached leaf of *Datura stramonium* sandwiched between two plastic plates. Larvae fed a virus-free *D. stramonium* leaf were used as control. Each of the opening holes was sealed with a piece of nylon screen to prevent the thrips from escaping.

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1993) was used for the thrips that were subjected to an acquisition access period of 4 h. Individual thrips on the third day after hatching into larvae and the fourth to fifth day after emergence as adults were allowed an inoculation access period (IAP) of 24 h, using 9 mm diameter leaf discs of *Petunia* × *hybrida* cv. Polo Blauw. After the inoculation access period, larvae and adults were removed and stored directly at –30°C for testing using a triple antibody sandwich, enzyme-linked immunosorbent assay (TAS-ELISA) (Clark & Adams, 1977). The leaf discs were floated on water for 48 h in 24-well plates (Corning Glass Works, Corning, New York, USA) to observe symptom development. Infections of petunia leaf discs sub-samples showing local lesions were confirmed by ELISA. Leaf discs from healthy plants were used as controls. The virus transmission efficiency was calculated as the percentage of leaf discs that developed local lesions (Wijkamp & Peters, 1993). All experiments were carried out at 25 ± 0.5°C and L16:D8.

Virus detection in entire thrips body by ELISA

Thrips larvae and adults were analysed individually to detect internal virus accumulation using TAS-ELISA. It was performed with antibodies against the nucleocapsid (N) protein, which is one of the indicators of TSWV multiplication in vectors and plays a central role in the viral life cycle (Wijkamp *et al.*, 1993; Uhrig *et al.*, 1999; see review by Adkins, 2000). The TAS-ELISA procedure was performed and the enzyme reaction was amplified as described by Sakurai *et al.* (1998). The absorbance values were measured on an ELISA-micro plate reader (MPR-A4, TOSOH, Tokyo, Japan) at 405 nm. Blank values were read for wells without thrips in the sample incubation step. Six healthy thrips were added to each plate as negative controls. The ELISA-positive samples were taken as those that gave reading values higher than the mean of the healthy control value plus three times the standard deviation. The ELISA values were corrected by subtracting the mean of the blank absorbance values from the sample values.

Statistical analysis

The Mann-Whitney *U* test (Sokal & Rohlf, 1995) was applied to detect differences in virus accumulation between the larval and adult stages. Fisher's exact probability test (Sokal & Rohlf, 1995) was performed for the comparison of frequencies of virus transmission in the leaf disc assay and of the virus positive individuals in the TAS-ELISA. These analyses were conducted by using StatView, version 5 (SAS Institute Inc., 1998) and the open source software, R, version 1.7.1 (R Development Core Team, 2003; available from http://www.r-project.org/).

Results

Transmission of TSWV by thrips

No transmission of TSWV by second instar thrips larvae was observed with any of the species tested using the petunia leaf disc assay. In the adult stage, the virus was transmitted by four species, *F. occidentalis*, *F. intonsa*, *T. tabaci* and *T. setosus*, but was not transmitted by *T. palmi* and *T. hawaiiensis* (fig. 2). The most efficient vectors were the two species of *Frankliniella*: *F. occidentalis* and *F. intonsa* with 31.1% and 32.8% transmission, respectively. By contrast, the least efficient vectors were the two species of *Thrips*: *T. tabaci* and *T. setosus* with 8.6% and 5.6% transmission, respectively. A comparison using the Mann-Whitney *U* test showed that the frequencies of transmission differed significantly between the four species (fig. 2).

Virus accumulation in larval and adult stages

No significant change in virus accumulation was observed between the larval and adult stages of the two species of *Frankliniella* (fig. 3a). A significant increase in virus accumulation was found in the ELISA-positive samples of *F. occidentalis*, whereas a significant difference was not observed in those of *F. intonsa* (fig. 3b). On the other hand, significant reductions in the values were observed for both the total and the ELISA-positive samples of the four species of *Thrips*, except for the ELISA-positive samples of *T. palmi* (fig. 3). The proportion of individuals that were ELISA-positive was also compared between the larval and adult stages for each species. The difference between the stages was not significant in the case of the two species of *Frankliniella*; however, it was significant for all four species of *Thrips* (fig. 4).

Discussion

The present study confirmed that four of the six thrips species tested, *F. occidentalis*, *F. intonsa*, *T. tabaci* and *T. setosus*, vectored TSWV in the adult stage (fig. 2), although the efficiencies of virus transmission differed substantially between them. Specificity in the transmission of TSWV by thrips vectors has been reported by several workers (Paliwal, 1976; Fujisawa *et al.*, 1988; Wijkamp *et al.*, 1995). A comparison of the virus transmission efficiencies in the petunia leaf disc assay showed that *F. occidentalis* was a more efficient vector than *F. intonsa*, *F. schultzei* and *T. tabaci* (Wijkamp *et al.*, 1995). The present study showed that TSWV

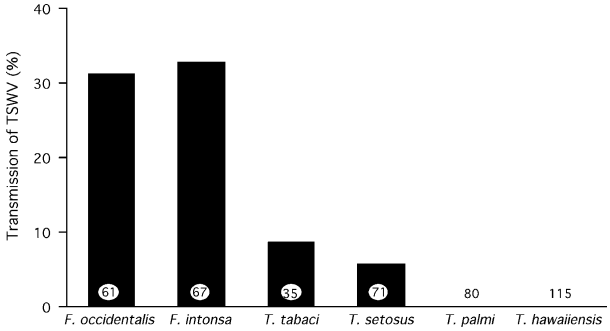

Fig. 2. Efficiency of tomato spotted wilt virus (TSWV) transmission by six adult thrips species determined by petunia leaf disc assay. The adults were given an inoculation access period of 24 h to petunia leaf discs. There was a significant difference between four species that transmitted the virus: *Frankliniella occidentalis*, *F. intonsa*, *Thrips tabaci* and *T. setosus* (Fisher's exact probability test, $P < 0.0001$). The number in or above each bar represents the number of thrips tested.

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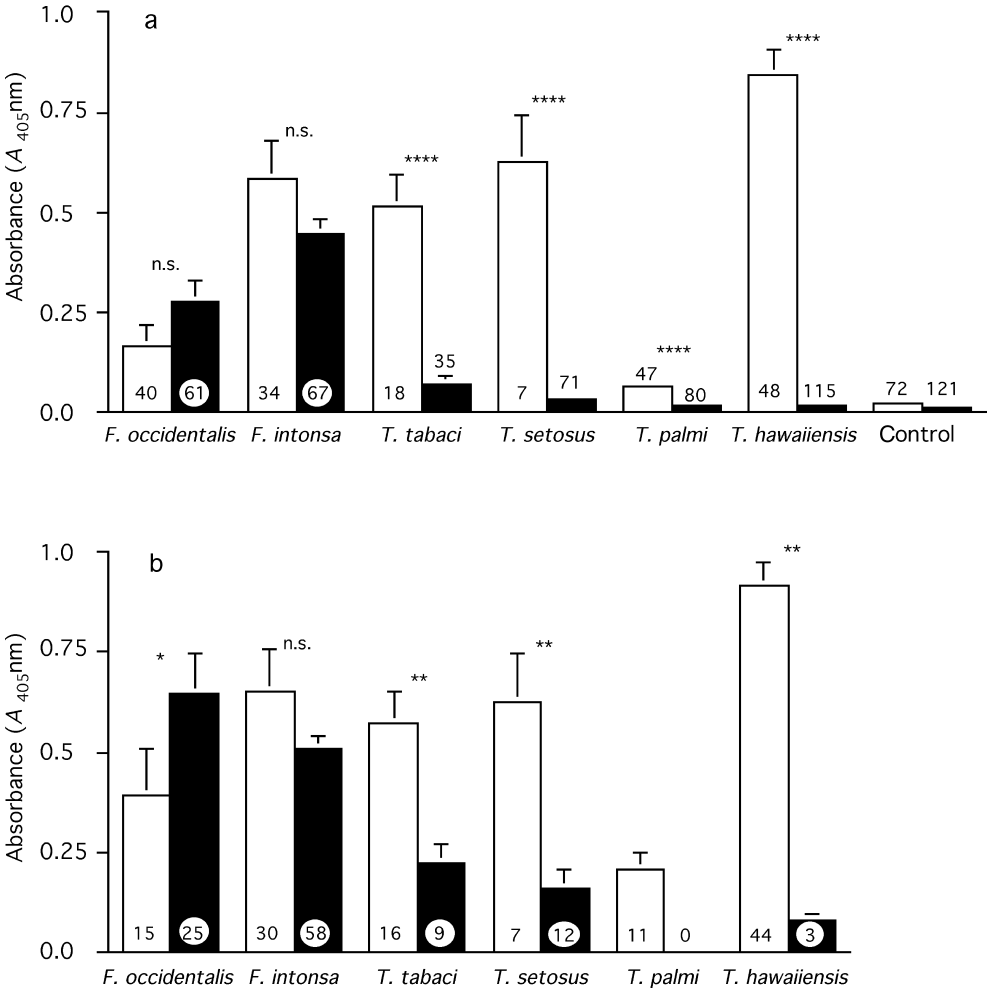

Fig. 3. ELISA values for the larvae (□) and adults (■) of six thrips species using antiserum against the N protein of tomato spotted wilt virus (TSWV) isolate. The thrips were individually assayed. The data give the mean values and standard errors of (a) total and (b) ELISA-positive thrips. n.s. designates not significant at $P > 0.05$. *, **, **** indicate significant differences between stages in each species at $P < 0.05$, 0.01, and 0.0001, respectively (Mann-Whitney *U* test). In panel (b), no data for adult *Thrips palmi* are given, owing to negative samples. The number in or above each bar represents the number of thrips tested.

transmission efficiencies of *F. occidentalis* and *F. intonsa* were higher than those of *T. tabaci* and *T. setosus*.

None of the six tested species, however, managed to transmit TSWV during the larval stage. Some studies have shown that the transmission efficiency of TSVW by larvae decreases with increasing temperatures, as in the case of *F. occidentalis* (Wijkamp & Peters, 1993) and *T. tabaci* (Chatzivassiliou *et al.*, 2002). Although the larval transmission efficiencies examined in the present study need to be investigated at different temperatures, the large amounts of viral N protein and high proportions of ELISA-positive individuals in the non-transmitting larvae observed in the present test may be explained on the basis of insufficient accumulation or an inability of the virus to establish in the salivary glands. This is because heavy infection of the salivary glands by the virus is necessary in order for virus transmission to be successful (Nagata *et al.*, 1999, 2002). Immunohistochemical investigation might provide additional information on this aspect.

The N protein plays a central role in the viral life cycle of TSWV as well as other members of the Bunyaviridae and other negative-stranded RNA viruses (see review by Tordo *et al.*, 1992; Adkins, 2000; Portela & Digard, 2002). Its primary function is to encapsidate the virus genome for packaging and protection. This protein is also crucial in the early stage of RNA synthesis (Steinecke *et al.*, 1998), particularly for switching from transcription to replication (Tordo *et al.*, 1992; Portela & Digard, 2002). Furthermore, the N protein appears to be necessary to initiate the budding of virions through host membranes in association with virus glycoproteins during virus maturation (Matsuoka *et al.*, 1991). Thus, the N protein must interact with viral RNA in RNA synthesis and other proteins during the maturation process. Recent reports have suggested that these activities are probably responsible for multiple RNA binding capacity, homotypic interaction and multimerization of the N protein (Richmond *et al.*, 1998; Uhrig *et al.*, 1999). Since the N protein performs these multiple functions during a viral life cycle, its increase during

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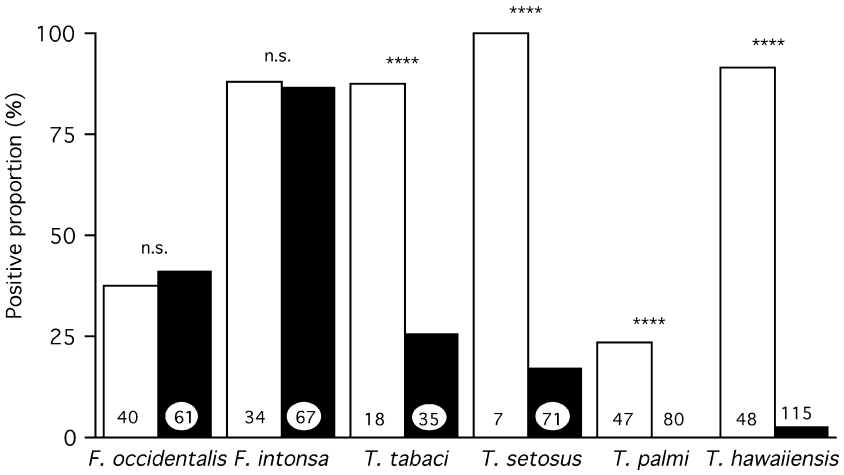

Fig. 4. Frequencies of ELISA positive larvae (□) and adults (■) for tomato spotted wilt virus (TSWV) in six thrips species. The difference between stages in each species was tested by Fisher's exact probability test (n.s. $P > 0.05$, and **** $P < 0.0001$). The number in or above each bar represents the number of thrips tested.

thrips development may result in completion of the viral life cycle. Consequently, if there is sufficient accumulation of viral N protein during the adult stage, the possibility of successful transmission is improved. In the present study, higher concentrations of TSWV-N protein in the adults of *F. occidentalis* and *F. intonsa* appeared to contribute to a higher frequency of virus transmission by these species. In contrast, lower concentrations of N protein in the adults of *T. tabaci*, *T. setosus*, *T. palmi* and *T. hawaiiensis* appeared to lead to a lower frequency of virus transmission (figs 2 and 3).

A specific pattern of N protein accumulation was observed in the present study (figs 3 and 4). The ELISA values and proportions of ELISA-positive thrips increased or were not significantly different between the larval and adult stages of the *Frankliniella* species. In contrast, they declined significantly in the adult stage of four species of *Thrips*, including the non-vector species, *T. palmi* and *T. hawaiiensis*, although an increase of the N protein accumulation was observed during the second instar larval stage of these species. These results suggest that the patterns of TSWV-N protein accumulation from the larval to the adult stages differ between *Frankliniella* and *Thrips*; the ELISA values of the former increased or were stable during development, whereas those of the latter declined sharply. So far, only a few observations suggesting a species-specific pattern of TSWV-N protein accumulation have been reported. Nagata *et al*. (2002) reported that the amount of N protein increased during the larval stages, except for the first 4 h after virus ingestion in *F. occidentalis* and *T. tabaci*, and then remained at a high level in *F. occidentalis* adults; however, the amount decreased rapidly in the case of *T. tabaci*. In the case of *T. setosus*, a rise in the ELISA value for TSWV-N protein was observed during the larval stages, whereas a marked decline was observed in the adults (Ohnishi *et al*., 1996; Tsuda *et al*., 1996). Mau *et al*. (1991) showed that the proportions of ELISA-positive larvae that acquired TSWV ranged from 61% in *F. occidentalis*, 72% in *F. schultzei*, 46% in *T. tabaci*, to 25% in *T. palmi*; however, those of ELISA-positive adults were 44%, 2%, 0% and 0%, respectively. This implies that the N protein continued to accumulate during the development of *F. occidentalis*, whereas it almost disappeared in the adults of the other species. Thus, the distinct patterns of N protein accumulation observed between *Frankliniella* and *Thrips* spp. in the present study appear to agree with the earlier reports, except for those of *F. schultzei*, as observed by Mau *et al*. (1991). Although the pale-coloured forms of *F. schultzei*, which were possibly used by Mau *et al*. (1991), have been shown to be extremely inefficient vectors (Sakimura, 1969; Wijkamp *et al*., 1995), Sakurai (2004) reported that the dark-coloured adults of *F. schultzei* were able to accumulate and transmit TSWV with high efficiencies. Thus, the pattern of TSWV-N protein accumulation of the latter appears to agree with the pattern of *Frankliniella* observed in the present study.

In conclusion, this research shows that the pattern of TSWV-N protein accumulation from larva to adult differs between species of *Frankliniella* and *Thrips*, and this in turn affects virus transmission by adults of these species. Further studies are required to elucidate how distinct patterns of N protein accumulation occur during the developmental stages of other TSWV-vector and non-vector species of *Frankliniella*.

Acknowledgements

The authors would like to thank Dr S. Tsuda, National Agricultural Research Center, for supplying the virus isolate and the antiserum products. We also express our thanks to Dr T. Nagata, Universidade Católica de Brasília, for his critical revision of an earlier manuscript and to two anonymous referees for their suggestions. We are grateful to Dr S. Okajima, Tokyo University of Agriculture, for identifying the thrips and to Dr H. Tsumuki, Okayama University, for hospitality and advice. The present study was supported in part by a grant from the Ohara Foundation for Agricultural Research.

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(Accepted 12 July 2004)