Rate of *Tomato yellow leaf curl virus* Translocation in the Circulative Transmission Pathway of its Vector, the Whitefly *Bemisia tabaci*

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ABSTRACT

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Whiteflies (*Bemisia tabaci*, biotype B) were able to transmit *Tomato yellow leaf curl virus* (TYLCV) 8 h after they were caged with infected tomato plants. The spread of TYLCV during this latent period was followed in organs thought to be involved in the translocation of the virus in *B. tabaci*. After increasing acquisition access periods (AAPs) on infected tomato plants, the stylets, the head, the midgut, a hemolymph sample, and the salivary glands dissected from individual insects were

subjected to polymerase chain reaction (PCR) without any treatment; the presence of TYLCV was assessed with virus-specific primers. TYLCV DNA was first detected in the head of *B. tabaci* after a 10-min AAP. The virus was present in the midgut after 40 min and was first detected in the hemolymph after 90 min. TYLCV was found in the salivary glands 5.5 h after it was first detected in the hemolymph. Subjecting the insect organs to immunocapture-PCR showed that the virus capsid protein was in the insect organs at the same time as the virus genome, suggesting that at least some TYLCV translocates as virions. Although females are more efficient as vectors than males, TYLCV was detected in the salivary glands of males and of females after approximately the same AAP.

Tomato yellow leaf curl virus (TYLCV) is the name given to a complex of geminiviruses (family *Geminiviridae*, genus *Begomovirus*) affecting tomato cultures worldwide (11,12). TYLCV has a monopartite genome (30) and is transmitted by the whitefly *Bemisia tabaci* in a persistent circulative manner (10,34).

Parameters of acquisition and transmission of TYLCV by adult *B. tabaci* have been studied in depth (1,10,24,25,36). Single insects are able to acquire TYLCV and transmit it to tomato plants. In a one insect/one plant inoculation test, female *B. tabaci* are more efficient than male insects (10). Minimum acquisition access periods (AAP) and inoculation access periods (IAP) are approximately 10 to 20 min. The rate of transmission increases with longer AAPs and IAPs. The minimal latent period reported was 21 h (10) but was 24 h for the closely related TYLCV strain from Egypt (25) and 17 h for the more distant virus from Sardinia (6).

The geminiviral coat protein (CP) possesses determinants needed for virus acquisition and transmission by the insect vector. Exchanging the CP gene of the whitefly-transmitted *African cassava mosaic virus* (ACMV) with that of the leafhopper-transmitted *Beet curly top virus* (genus *Curtovirus*) produced a leafhopper-transmitted ACMV chimera (4). Replacing the CP of the non-transmissible *Abutilon mosaic virus* (AbMV) with that of the transmissible *Sida golden mosaic virus* produced a whitefly-transmissible chimeric AbMV (21). Mutating the CP gene of *Bean golden mosaic virus* resulted in the loss of acquisition by *B. tabaci* (2). Two amino acid replacements in the CP of TYLCV abolished virus transmission by *B. tabaci* but not its acquisition (32). Insect proteins also play a role in virus transmission. A GroEL homologue produced by the coxoid endosymbionts of *B. tabaci* is involved in the circulative transmission of TYLCV (26).

Geminiviruses translocation in their insect vector is still not fully understood. A parallel has been drawn between transmission of luteovirus and geminivirus, suggesting that these viruses move out of the gut into the haemocoel on their way to the salivary glands (20). Anatomical and immunolocalization studies performed during the last few years have allowed us to better understand the circulative pathway of geminiviruses in their whitefly vector (18,19,22). As observed for luteoviruses in aphids (15–17), geminivirus particles are thought to be ingested along with phloem sap of infected plants through the stylets and enter the esophagus and the filter chamber. Virions are subsequently transported through the gut wall into the hemocoel and from there they reach the salivary glands. The virus is translocated into the salivary duct and is finally excreted with the saliva during feeding. The time it takes for a geminivirus to complete this path is reflected in the minimal period of time that elapses from beginning of feeding on infected plants to transmission to test plants (latent period). The development of polymerase chain reaction (PCR) has been instrumental in analyzing the velocity of geminivirus translocation in *B. tabaci*. Using print capture-PCR, TYLCV DNA was detected in the head of whiteflies as early as after a 5-min AAP on infected tomato plants, in the thorax after 10 min, and in the abdomen after 25 min. TYLCV DNA was detectable in test plants as early as after a 5-min IAP (1). In another investigation, the transmission pathway of the *Squash leaf curl virus* (SLCV) was traced by DNA from *B. tabaci* extracts, saliva, hemolymph, and honeydew as substrate for PCR (33). SLCV was detected in insect extracts after a 30 min AAP on infected pumpkin. The virus was found in the hemolymph after 2 h and in the saliva and honeydew after 8 h. In the same study, the whitefly *Trialeurodes vaporariorum* was able to acquire but not transmit SLCV. In this insect, SLCV was not detected in the hemolymph and in the saliva.

In the present study, we asked the question of whether the Old World monopartite TYLCV transits through the same organs and with the same velocity as the New World bipartite SLCV. Using

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whitefly stylets, head, midgut, hemolymph, and salivary glands dissected from a single insect as substrate for PCR and immunocapture-PCR, we have determined the timing of TYLCV translocation and passage from one organ through another, from acquisition to transmission.

MATERIALS AND METHODS

Maintenance of virus cultures, whiteflies, and plants. Cultures of an Israeli isolate of TYLCV (30) were maintained in tomato plants (*Lycopersicon esculentum* cv. Daniella). Whiteflies, *B. tabaci*, B biotype (7), were reared on cotton plants (*Gossypium hirsutum* cv. Akala) grown in insect-proof wooden cages at 24 to 27°C (36). Unless specified otherwise, all insects used were females.

Acquisition of TYLCV by adult insects. All experiments were conducted in insect-proof wooden cages kept at 24 to 27°C in an insect-proof growth chamber. TYLCV was acquired by whiteflies 5 to 8 days after emergence. The insects were caged with the youngest true leaf from the infected tomato plant (at the five- to seven-leaf stage, 6 weeks after whitefly-mediated inoculation) for the AAP indicated in the experiments.

Determination of the latent period. The latent period was determined in two different ways. In the first experiment, approximately 1,000 whiteflies were caged with infected tomato plants for a 2-h AAP. The insects were then collected with a mouth aspirator and caged with 10 uninfected tomato test plants (at the five-leaf stage). After an IAP of 1 h, the insects were collected and caged with a second group of 10 tomato test plants for an additional 1-h IAP. The first 10 plants were treated with the insecticide Talstar 35 (active ingredient: bifenthrin; FMC Corporation, Rockland, ME) to ensure that no insect was left alive. The insects were collected from the second group of plants after the 1-h IAP and caged with a third group of 10 plants. The procedure was repeated until the insects were serially transferred to 13 groups of 10 plants. Plants from groups 1 to 8 were caged with the whiteflies for a 1-h IAP, plants from group 9 were caged with insects for a 2-h IAP, plants from groups 10 to 12 for a 4-h IAP, and plants from group 13 were caged with insects for a 24-h IAP. After each transfer, we made sure that each plant bore at least 10 whiteflies.

In the second experiment, approximately 2,000 whiteflies caged with infected tomato plants for a 2-h AAP were distributed among 13 groups of 10 plants each, ensuring that each plant had at least 10 insects. The insects were removed from each group of plants after 1, 2, 3, 4, 5, 6, 7, 8, 10, 14, 18, 22, or 48 h, and the plants were treated with insecticide. The appearance of symptoms was monitored during 5 weeks, and the presence of viral DNA was assessed by squash blot hybridization 14 days postinoculation (29).

Dissection of organs from single whiteflies. After a given access period, the insects were collected by aspiration and exposed to acetone vapors for 2 to 3 min to arrest movement. The insects were dissected on a glass slide under a binocular microscope ($\times 40$). Dissections were performed in insect physiological saline (IPS: 4.5 g of NaCl, 0.1 g of KCl, 0.1 g of CaCl₂, 0.1 g of MgCl₂, 0.1 g of NaHCO₃, and 2.0 g of glucose, per liter) with or without 1% toluidine blue, on a glass slide as described (3). The isolated tissues were placed in a PCR test tube containing 3 μ l of sterile double distilled water (ddw). To dissect the midgut, the abdomen was separated from the thorax at the connection between them. Its content was expelled in a drop of IPS (without toluidine blue) by pushing gently on the abdomen. The midgut was isolated, cleaned from other tissues, flushed several times with ddw, and collected with a fine metallic thread fixed on a wooden handle. To isolate the salivary glands, the prothorax was separated from the mesothorax and abdomen. The glands were teased away from the body and kept for 2 to 5 min in IPS-toluidine blue until they absorbed the dye, allowing identification and dissection. The pair of primary glands and the much smaller adjacent secondary

salivary glands were flushed several times with ddw and collected. The stylets were pulled out from the whole head. Hemolymph was extruded and collected with a glass micropipette as described (26).

Observation of *B. tabaci* organs with the scanning electron microscope. Insects were dissected as described above with the exception that they were first placed on a microscope cylindrical mount using double-sided adhesive tape. The organ of interest (midgut, salivary glands, or stylets) was washed several times with distilled water by a Pasteur pipette with a narrow tip and fixed for 3 to 5 min with 2.5% glutaraldehyde and 4% paraformaldehyde in phosphate-buffered saline, as described (28). The sample was incubated with hexamethyldisilazane for 45 min two times, allowed to air dry for 18 h, and observed in a low vacuum scanning electron microscope (JOEL 5410, Tokyo).

Amplification of TYLCV DNA by PCR with organs dissected from single whiteflies as substrate. The insect organs, without any further treatment, were subjected to PCR (20- μ l reaction). A $\approx$ 410-bp TYLCV DNA fragment was amplified by two primers designed from the nucleotide (nt) sequence of the TYLCV genome (30): V61 (nt 61-80, viral strand, 5'-ATACTTG-GACACCTAATGGC3') and C473 (nt 473-457, complementary strand, 5'-AGTCACGGGCCCTTACA3'). Oligonucleotides were purchased from Biotechnology General, Rehovot, Israel. The cycling protocol (using a Techne PHC-2 thermocycler) was as follows: initial denaturation for 3 min at 95°C, annealing of primers for 1 min at 55°C, extension for 2 min at 72°C, and denaturation for 1 min at 94°C; subsequent cycles were: 1 min at 55°C, 2 min at 72°C, and 1 min at 94°C; after 30 cycles, the reaction was terminated by a 10-min incubation at 72°C (31). The PCR products were analyzed by electrophoresis in a 1% agarose gel and photographed. The amplified virus DNA was identified after blotting, and hybridization with plasmid pTYH19 containing a full-length copy of the TYLCV genome labeled with ³²P-dCTP by a random-primed DNA labeling kit (Boehringer GmbH, Mannheim, Germany) (30). Autoradiography was for 1 to 8 h with X-ray films (Fuji) or 1 to 2 h with a Phosphor imager (FujiX-Bio Imaging Analyzer, IPR-1000; Fuji, Tokyo).

Detection of TYLCV CP. The virus CP was detected by immunocapture-PCR (23) by an antibody raised against the CP of a TYLCV isolate from the Dominican Republic overexpressed in *Escherichia coli* (provided by R. Gilbertson). The buffers used for immunocapture-PCR are described and were used as indicated by the manufacturer (Bioreba, Ebringen, Germany). PCR tubes were filled with 200 μ l of antiserum (1:1,000 diluted in coating buffer), incubated for 3 h at 37°C and washed five times for 5 min each with 200 μ l of washing buffer. Whitefly or plant homogenates in 200 μ l of extraction buffer were incubated in the coated PCR tubes for 18 h at 4°C. The tubes were washed five times for 5 min each with 200 μ l of washing buffer and dried. PCR-amplification of the viral DNA from the virions bound to the antibody-coated tubes was performed with the TYLCV specific primers V61 and C473 (13).

RESULTS

Whiteflies efficiently transmit TYLCV to tomato test plants 8 h after they started to acquire virus. We have determined the latent period using two different ways. In the first experiment, whiteflies provided access to infected tomato plants for 2 h and were caged with uninfected tomato test plants. They were transferred serially to new test plants at 1-h intervals, until 10 h after the beginning of the initial AAP (later the IAPs lasted longer). In the second experiment, whiteflies that accessed the infected tomato plants for 2 h were caged with the test plants for IAPs of increasing length, from 1 to 46 h. The results summarized in Table 1 show that in both experiments, whiteflies were able to transmit the virus 8 h after the start of the AAP, as determined by subse-

quent appearance of symptoms and of viral DNA. The rate of plant infection raised as the time span between the beginning of the AAP and the end of the IAP increased. Uninfected test plants remained symptomless and devoid of TYLCV DNA. These results showed that in our hands the latent period was 8 h.

TYLCV DNA detection by PCR in organs dissected from a single viruliferous whitefly. Organs of the whitefly vector believed to participate in the acquisition/transmission pathway of TYLCV were dissected. Because of the rather tight timing of translocation and the fragility of the tissues, we needed to ensure that virus movement was arrested at the time of sampling the insects. We found that using acetone vapors was adequate. Whereas the midgut of insects processed this way is immobile, that of whiteflies treated with CO₂ is still contracting. Freezing insects for a short time did not prevent the pumping movements that resumed under the heat of the binocular light. Freezing the insects for long periods of time also produced damage to the tissues.

Figure 1 (upper panel) shows the isolated stylets, digestive tract, and salivary glands, as observed with the scanning electron microscope. The major elements of the stylets can be seen, including the labium (outside the head) and the stylet bundle (inside the head), together with the cibarial pump protractor muscles. The digestive tract includes the midgut (descending and ascending), the hindgut, the caeca, and the filter chamber. For PCR studies, the hindgut was severed from the midgut and discarded. One of the pair of primary salivary glands is shown; in the PCR studies, both glands were used, together with the pair of secondary salivary glands.

Organs isolated from a single viruliferous whitefly served as a sufficient source of template DNA for PCR detection of TYLCV DNA. Except for washing thoroughly the midgut and salivary glands, there was no need to treat the organs in any way before subjecting them to the PCR. Attempts to extract DNA from these organs and using this DNA as substrate in the PCR provided poor results; the viral DNA was amplified with a frequency much lower than that obtained with untreated organs. Female whiteflies were caged with infected tomato plants. After a 12-h AAP, the stylets, the midgut, a hemolymph drop, and the salivary glands were isolated from a single insect and subjected to PCR by the TYLCV-specific primer pair V61 and C473. One nonviruliferous and two viruliferous whiteflies were analyzed. Figure 1 (lower panel) shows that the expected ~410-bp viral DNA fragment was amplified from each of the organs isolated from the two viruliferous insects assayed, but not from a nonviruliferous insect. The identity of the amplified viral DNA fragment was confirmed by hybridization with a TYLCV DNA probe.

Temporal detection of TYLCV DNA in the stylet, head, midgut, hemolymph, and salivary glands of single whiteflies, as a function of the AAP. Female whiteflies were caged with infected tomato plants for various AAPs, from 5 min to 24 h. Ten insects were analyzed individually for each time point. After a given AAP, whiteflies were collected, exposed to acetone vapors and immediately dissected. The stylet, the head (without the stylet), the midgut, a drop of hemolymph, and the salivary glands from each one of the 10 insect were subjected to PCR by the TYLCV-specific primer pair V61 and C473. Figure 2 shows the PCR products obtained with the selected organs of one representative whitefly for each of the AAPs. Figure 3 summarizes the results obtained after analyzing the organs of 10 insects for each AAP.

TYLCV DNA was undetectable in the insects after an AAP of 5 min (Fig. 3) as well as in nonviruliferous insects (Fig. 2), confirming that whiteflies from the insect colony were virus-free. As shown in Figure 3, we were unable to detect viral DNA in the stylets for up to 2 h of acquisition feeding (1 of 10 samples tested contained detectable viral DNA at this time). The efficiency of detection of viral DNA in the stylet did increase with time, but was very erratic. TYLCV DNA was never detected in the stylet of all 10 insects sampled, even after a 9-h AAP when the virus DNA was found in the head and midgut of all insects tested.

Viral DNA was detected in the head of the insect (minus the stylets) after a 10-min AAP (in 3 of 10 insects) but was not detected in any of the samples tested after a 5-min AAP. Because the stylet and the salivary glands were removed, we assume that virus was present only in the small segment of esophagus remaining in the head. The frequency of detection of TYLCV DNA in the whitefly head increased with the AAP. It certainly reflected the time when the individual insects started to feed on the infected plant. At 3 h, all 10 insects had acquired the virus. It should be noted that even after relatively long AAP, based on visual observation, some insects still were not feeding on infected tomato tissues (insect six at 7 h; insect eight at 8 h; and insects five and eight at 12 h).

As the length of the AAP increased, viral DNA was found in the midgut, then in the hemolymph, and finally in the salivary glands. Once acquired, TYLCV enters the digestive system via the esophagus, reaches the filter chamber and the midgut. Whether the virus spreads beyond the anterior part of the midgut is not known. TYLCV DNA was first found in the insect midgut after an AAP of 40 min. At this time the midgut of 2 of the 10 whiteflies analyzed contained detectable viral DNA. The virus was still not detectable in the midgut after an AAP of 20 min. We used five

TABLE 1. Determination of the latent period of *Tomato yellow leaf curl virus* in the whitefly *Bemisia tabaci*

Experiment 1			Experiment 2		
Time after AAP started (h) ^a	Successive IAP (h) ^b	Infection rate ^c	Time after AAP started (h) ^a	Length of IAP (h) ^d	Infection rate ^c
0	0	0/10	0	0	0/10
3	1	0/10	3	1	0/10
4	1	0/10	4	2	0/10
5	1	0/10	5	3	0/10
6	1	0/10	6	4	0/10
7	1	0/10	7	5	0/10
8	1	5/10	8	6	3/10
9	1	7/10	9	7	4/10
10	1	6/10	10	8	4/10
12	2	8/10	12	10	6/10
16	4	8/10	16	14	5/10
20	4	9/10	20	18	8/10
24	4	9/10	24	22	8/10
48	24	10/10	48	46	10/10

^a AAP = acquisition access period. Whiteflies were caged with infected tomato plants for a 2-h AAP; 0 indicates tomato plants tested before they were caged with viruliferous whiteflies.

^b IAP = inoculation access period. After the IAP indicated, the whiteflies were collected and transferred to uninfected tomato plants for a new IAP. The process was repeated until 12 groups of 10 test plants were treated.

^c Number of infected plants per total plants.

^d Following the 2-h AAP, the whiteflies were distributed among groups of 10 plants for the IAPs indicated.

insects to determine that viral DNA was undetectable after an AAP of 30 min (data not shown). After a 40-min AAP, the frequency of TYLCV DNA amplification increased with the length of the AAP. Viral DNA was found in the midgut of 8 of the 9 insects containing viral DNA after an AAP of 2 h and in all 10 insects analyzed after 3 h. These results showed that TYLCV reached the midgut approximately 30 min after it was first detected in the head of the insect.

The suggested pathway of a begomovirus in its whitefly vector involves a step where the virus crosses the gut walls and enters the hemolymph (20). Figure 3 shows that TYLCV DNA was first detected in the whitefly midgut after a 30-min AAP. At this time, TYLCV was not detected in the hemolymph. TYLCV DNA was amplified from the hemolymph of viruliferous whiteflies after an AAP of 2 h. Three hemolymph samples of the 10 analyzed contained detectable TYLCV DNA at this time. The viral DNA was never detected in the hemolymph after a 1-h AAP. To determine more precisely the time when TYLCV crosses the gut/hemolymph barrier, these tissues were analyzed every 15 min, between 1 and 2 h of AAP. Figure 4 shows that TYLCV was detected in the hemolymph 30 min after it was first detected in the midgut, i.e., 90 min after the beginning of acquisition feeding.

Once in the hemolymph, begomoviruses reach the salivary glands where they are inoculated into plants with saliva. Whereas TYLCV DNA was detected in the hemolymph after a 90 min AAP, the virus was not found in any of the salivary gland samples for up to 7 h (Fig. 3). At this time, the salivary gland of 2 of the 10 insects tested contained detectable viral DNA. Therefore, the

transit of the virus into the salivary gland constitutes the longest phase in the circulative transmission process. The frequency of virus detection in salivary glands increased with time; nevertheless, the virus was not detected in all the insects tested in any of the groups, even after AAPs of 10 h and more. The time the viral DNA was first detected in the salivary gland was very close to the 8-h latent period. Hence, it seems that enough virus reaches the salivary gland during an 8-h AAP to exceed the threshold of infectivity.

Detection of TYLCV DNA associated with the virus CP in the stylet, head, midgut, hemolymph, and salivary glands. We do not know how and in which form (naked DNA, virion, nucleoprotein) TYLCV or any other begomovirus translocates in its vector. We have followed the translocation of TYLCV DNA associated with the virus CP using the immunocapture-PCR technique. The CP-DNA complex (possibly virions) contained in homogenates of whitefly tissues binds to a CP antibody coating a test tube. The bound complex is then heat-disrupted, and the viral genome is amplified by PCR.

Because this immunocapture-PCR is less sensitive than PCR, samples extracted from 25 insects were pooled and assayed. Figure 5 shows that a positive signal was found in the stylets, head, and midgut of insects after a 1-h AAP, but not in the hemolymph or the salivary glands. After 2 h, the virus was also detected in the hemolymph, but still not in the salivary glands. After 12 h, the virus was detected in the salivary glands. In all cases, detection of TYLCV was dependent on the presence of the antibody. TYLCV DNA was not amplified when the antibody was omitted (Fig. 5).

Fig. 1. Top panel: Scanning electron micrographs of organs involved in the circulative transmission pathway of *Tomato yellow leaf curl virus* (TYLCV) isolated from whiteflies. **A**, Stylet; L = labium (outside the head), S = stylet bundle (inside the head), and M = cibarial pump protractor muscles. **B**, Digestive tract; DM and AM = descending and ascending midgut, H = hindgut, C = ceca, and FC = filter chamber. **C**, Salivary gland; PSG = one of the pair of primary salivary glands. Bottom panel: Detection of TYLCV by subjecting the stylets (ST), the head (HD), the midgut (MG), a hemolymph drop (HL), and the salivary glands (SG) to the polymerase chain reaction with the TYLCV-specific primer pair V61 and C473. All samples were dissected from a single viruliferous insect after a 12-h acquisition access period on an infected tomato plant. Two viruliferous whiteflies (1 and 2) and one nonviruliferous insect (0) were processed. The products of the reaction were subjected to agarose gel electrophoresis, blotted and hybridized with a virus-specific DNA probe. The arrow points to the ~400-bp amplified viral DNA fragment.

Therefore the viral DNA associated with the CP was detected by immunocapture-PCR at the same time the virus genome was detected by PCR. These results suggest that at least part of the TYLCV DNA that translocates in the insect is encapsidated in a virion.

TYLCV translocates with the same velocity in males and females. Females transmit TYLCV with higher efficiency than males (10). In our study, one female (1 week after emergence) was

enough to infect a tomato test plant, whereas five males of the same age are needed to achieve the same results (M. Ghanim and H. Czosnek, *unpublished data*). The reason for this difference is not known. We investigated the possibility that TYLCV reaches the salivary glands of females earlier than that of males. The midgut and salivary glands of females and males were subjected to PCR after a 7-h AAP, at a time when the virus appeared in the salivary glands of the female insects (Fig. 3). Figure 6 shows that

Fig. 2. Presence of *Tomato yellow leaf curl virus* (TYLCV) DNA in the stylets (ST), head (HD), midgut (MG), hemolymph (HL), and salivary glands (SG) of single whiteflies, as a function of the length of the acquisition access period (AAP) on infected tomato plant. All samples were dissected from a single insect after the indicated AAPs. The organs were subjected to polymerase chain reaction by the TYLCV-specific primer pair V61 and C473. The products of the reaction were examined by agarose gel electrophoresis and stained with ethidium bromide. P = plasmid pTYH20.7 containing the TYLCV DNA clone; 0 = nonviruliferous insects. The thick arrow points to the amplified ≈ 410 -bp viral DNA fragment; thin arrow points to the position of the primers. The number of the insect refers to one of the 10 whiteflies tested for each AAP (Fig. 3).

the virus was detected in the midgut of the two insects tested; the rate of detection of TYLCV DNA in the salivary gland of the females was equal to that of the males (three of four). Therefore TYLCV reaches the salivary gland at approximately the same time in male and female whiteflies.

DISCUSSION

Begomoviruses are transmitted by their whitefly vector in a circulative manner. Similar to the model proposed for luteovirus translocation in aphids (15–17), it is not likely that geminiviral

particles ingested through the stylets enter the esophagus and the digestive tract, then penetrate the gut membranes into the hemolymph, reach the salivary glands, and finally enter the salivary duct from where they are ejected with the saliva (18,19,22,33). Values given for the time necessary to complete the acquisition/inoculation pathway, the latent period, vary from 7 (27) to 24 h (8,9). This wide range of values may reflect the efficiency with which a given virus establishes a systemic infection in a plant rather than differences in the velocity of translocation in the insect vector. In this study we found a much shorter latent period for TYLCV from Israel than that reported 25 years ago, 8 h versus

Fig. 3. Time course of Tomato yellow leaf curl virus (TYLCV) translocation as analyzed by subjecting stylets (ST), head (HD), midgut (MG), hemolymph (HL), and salivary glands (SG) of single whiteflies to polymerase chain reaction (PCR). Whiteflies were caged with infected tomato plants for the acquisition access period indicated. At each time, 10 whiteflies (numbered 1 to 10) were dissected, and the relevant organs were subjected to PCR. The results obtained as in Figure 2 were scored as follows; white box indicates viral DNA not detectable and black box indicates TYLCV DNA detected.

Fig. 4. Passage of *Tomato yellow leaf curl virus* (TYLCV) from the digestive tract to the hemolymph. Midgut and hemolymph of whiteflies were subjected to polymerase chain reaction (PCR) at 60, 75, 90, 105, and 120 min after the insects were caged with infected tomato plants. Two replicates representing one insect each were analyzed. The products of the reaction by primers were examined by agarose gel electrophoresis, blotted and hybridized with a virus-specific DNA probe. P = plasmid pTYH20.7 containing the TYLCV DNA clone. 0 = no DNA in the PCR reaction.

Fig. 5. Detection of *Tomato yellow leaf curl virus* (TYLCV) DNA associated with the virus coat protein (CP) in whitefly tissues by immunocapture-polymerase chain reaction (PCR), as a function of the length of the acquisition access period (AAP). After the AAPs indicated, the stylets (ST), head (HD), midgut (MG), hemolymph (HL), and salivary glands (SL) were dissected from three groups of 25 insects each, and extracts of each of the pooled 25 organs were prepared. Two extracts were incubated in tubes coated with the CP antibody (+Ab), one extract was incubated with nontreated test tubes (-Ab). The virus bound to the antibody was subjected to PCR using the TYLCV primer pair V61 and C473. The reaction products were examined by agarose gel electrophoresis and stained with ethidium bromide. P = plasmid pTYH20.7 containing the TYLCV DNA clone; W* = extract of 20 viruliferous whiteflies; 0 = no DNA in the PCR reaction. The thick arrow points to the ~400-bp reaction product; thin arrow points to the position of the primers.

21 h (10). This change may be due to modifications that occurred in the virus or in the vector population.

Information related to the temporal passage of the virus in the various tissues participating in the transmission process is scarce. TYLCV was detected by hybridization in DNA extracted from the head (including the first segment of the thorax containing the salivary glands) and the abdomen of *B. tabaci* after a 24-h AAP (5), a time period greater than the latent period (6). The first attempt to measure the velocity of circulation of a geminivirus used PCR to track the New World bipartite begomovirus SLCV from the beginning of access feeding until it was excreted with the saliva (33). In this study, we have followed TYLCV DNA during its transit in the circulative pathway. We have investigated TYLCV translocation by subjecting organs of 10 insects to PCR to obtain a representative image of the presence of the virus in organs of an insect population at a given time after the beginning of the AAP.

The stylets were pooled from the head, and the salivary glands (primary and secondary) were dissected from the base of the head. Consequently, the only site in the dissected head the virus transited through was a small portion of the esophagus. The midgut was used after the hindgut was discarded. Hemolymph was collected from the thorax where the esophagus is very small and the midgut is far away. We are confident that the hemolymph sampled was devoid of contamination from virus leaking from the gut as a result of the dissection. We never found hemolymph with TYLCV DNA in the early AAPs (up to 1 h; Fig. 3) when virus was already detected in the midgut. Even later (from 2 to 6 h), the number of hemolymph samples with viral DNA was always lower than the number of midgut samples.

Detection of TYLCV in the insect stylets was erratic. We do not think that this was because the viral DNA was not amenable to PCR. Rather, we believe that the plant sap-containing virus is quickly ingested due to differences in pressure between plant and

Fig. 6. Presence of *Tomato yellow leaf curl virus* (TYLCV) in the digestive tract and in the hemolymph of female and male whiteflies, after a 7-h acquisition access period. The midgut and salivary glands of females (F) and males (M) was subjected to polymerase chain reaction. Four (salivary glands) or two (midgut) replicates representing one insect each were analyzed. The products of the reaction were subjected to agarose gel electrophoresis, blotted and hybridized with a virus-specific DNA probe. P = plasmid pTYH20.7 containing the TYLCV DNA clone.

insect and to a reflex mechanism occurring when the insect is removed from the plant. Therefore, the head represents a better object to detect virus acquisition. TYLCV was detected in the head of whiteflies as early as after a 10-min AAP. SLCV was detected by PCR of *B. tabaci* homogenates after a 30-min AAP; however, shorter AAPs were not tested (33). The passage of TYLCV from esophagus to midgut is rather rapid, probably because it does not have to cross membranes. TYLCV-DNA was detected in the midgut approximately 30 min after it was first found in the head of the insect. At this point, some of the virus might be excreted; for example SLCV was found in honeydew after an 8-h AAP (33). We do not know whether TYLCV is present in all parts of the gut or whether it is confined to a particular compartment (filter chamber, ascending or descending midgut, caeca). Immunolocalization of two New World bipartite begomoviruses (*Tomato mottle virus* [ToMoV] and *Cabbage leaf curl virus* [CaLCV]) in whiteflies has indicated that these viruses were present mainly in the filter chamber and in the anterior part of the midgut (22).

The crossing of TYLCV from the midgut to the hemolymph is surprisingly fast. The virus reached the hemolymph 30 min after it was first detected in the midgut, 90 min after the beginning of acquisition feeding. SLCV was also found in the hemolymph of *B. tabaci* after a 2-h AAP (33). We do not know whether the crossing of the gut is passive or whether it is an active process involving specialized receptors as in aphids (15,16). The passage of geminiviruses in the hemolymph of their whitefly vector seems a critical step in the virus circulative pathway. During this relatively long period, viruses are in the midst of a hostile environment where they may be subject to degradation by cells and enzymes. To avoid degradation, it seems that geminiviruses, like luteoviruses in aphids (35), interact with chaperonins produced by the insect vector endosymbiotic bacteria and excreted in the hemolymph (26).

TYLCV was detected in the salivary glands approximately 5.5 h after it was first detected in the hemolymph. Seven hours after the beginning of the AAP, approximately 1 h before the insects were able to infect tomato plants. Therefore, it seems that once the virus reaches the salivary gland, it is almost immediately excreted into the salivary pump and from there into the plant with the saliva. As a comparison, SLCV was detected in the excreted saliva after an 8-h AAP (33). Reaching the salivary duct implies crossing several cell walls that may constitute selective barriers as in aphids (15).

The temporal virus detection sequence was always the same, reflecting the polarity of virus translocation in the insect. In all 150 insects examined in the experiment summarized in Figure 3 (and in any all instances), we never detected the virus in a temporal order other than head-midgut-hemolymph-salivary glands. The timing of translocation of TYLCV was basically similar to that of SLCV (33), indicating that the circulation of begomoviruses may be independent of the identity of the virus (as long as it is transmissible) and of the geographical origin of *B. tabaci*.

Females transmit TYLCV with higher efficiency than males (10). One of the possible hypotheses we tested was that TYLCV reaches the salivary glands of females earlier than that of the males. PCR analysis indicated that TYLCV DNA was present in the salivary gland of both females and males 7 h after the beginning of acquisition feeding. It is possible that differences in virus amounts in the salivary gland are the cause for the differential inoculation abilities of males and females.

We do not know whether begomoviruses circulate in *B. tabaci* as encapsidated virions. The CP of two Old World bipartite begomoviruses ToMoV and CaLCV has been detected in the midgut and the salivary glands of *B. tabaci* anatomical sections by immunofluorescent labeling, suggesting that encapsidated virions were present in these tissues (22). Whereas PCR can account only for viral DNA (single-stranded and double-stranded), immunocapture-PCR allows detection of viral DNA associated with the

CP in insect extracts (13,23). Our results show that the TYLCV translocation timing obtained by PCR and by immunocapture-PCR overlap, although the later analysis was less extensive than the former. We never detected only viral DNA or CP unattached at any time during the circulative pathway. These results suggest that at least part of the virus is moving as a virion.

TYLCV has several features of an insect pathogen. Acquired by whiteflies after emergence, the virus (DNA and infectivity) remains associated with the insect for its entire adult life. This long-term relationship is associated with a decrease in the host life expectation and fecundity (34). TYLCV invades the *B. tabaci* reproductive system (14) and may be transmitted to other insects in a sexual-related manner (13). Therefore, it seems that most of the virus acquired by the insect leaves the circulative pathway at some unknown point and is stored in unknown tissues for periods of time much longer than the latent period. Similarly, SLCV (8) and other begomoviruses (5,27) are present in the insect as infective entities much longer than the latent period. Hence, the circulative pathway is only one of the tracks begomoviruses may follow in their acquired insect vector.

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