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Stylet penetration activities of the whitefly *Bemisia tabaci* associated with inoculation of the crinivirus *Tomato chlorosis virus*

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Abstract

Bemisiatabaci is an important vector of numerous plant viruses, including the emergent semi-persistently transmitted crinivirus *Tomato chlorosis virus* (ToCV). Its vector feeding behaviour is complex, with important implications for virus transmission, epidemiology and control. Thus, the objective of this study was to investigate the role of the stylet penetration activities of *B. tabaci* in the inoculation of ToCV in tomatoes by using the electrical penetration graph (EPG) technique. EPG recordings were classified into six categories depending on the waveforms observed. The results showed that ToCV inoculation is mainly associated with stylet activities in phloem sieve elements (E_1 waveform), as there was a significant increase in the rate of transmission when whiteflies performed waveform E_1. The precise stylet activities – either salivation or egestion – associated with virion release, presumably from retention sites in the foregut, need further investigation.

The relationships between whiteflies and the plant viruses they transmit are complex, and some biological and behavioural aspects of these interactions vary considerably depending on the species of virus, vector and host plant, as well as on the environmental conditions and mode of transmission [1–3]. Changes in host selection and vector feeding behaviour can significantly influence transmission rate [4].

A clear assessment of the impact of behavioural changes on transmission probabilities requires an understanding of the specific vector stylet activities involved in virus acquisition and inoculation, and where in the plant tissues these activities take place. Whitefly feeding behaviour associated with virus transmission has been studied for the begomovirus *Tomato yellow leaf curl virus* (TYLCV) [5] and the crinivirus *Lettuce chlorosis virus* (LCV) [6]. In those studies, the electrical penetration graph (EPG) technique was used to correlate virus inoculation with the occurrence of specific EPG waveforms that represent different insect activities associated with the feeding process in various plant tissues. EPG is an important tool for studying feeding behaviour of Hemiptera, in which the insect and plant are connected through electrodes in an electrical circuit with an input voltage. Oscillations in the system voltage due to specific insect probing activities are measured by the EPG device, and then digitalized and displayed in real time as typical waveforms on a computer screen.

Among the numerous plant viruses transmitted by *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae), the crinivirus *Tomato chlorosis virus* (ToCV) is of particular importance because it is an example of an emerging virus worldwide [7]. All known criniviruses, ToCV included, are phloem-limited and are semi-persistently transmitted by whiteflies from the genera *Bemisia* and *Trialeurodes*. ToCV is the only crinivirus transmitted by species from both genera. Three species of the *B. tabaci* complex have been reported as vectors of ToCV: New World (NW1, formerly biotype A), Middle East-Asia Minor 1 (MEAM1, formerly biotype B) [8] and Mediterranean (MED, formerly biotype Q) [9, 10]. ToCV is also transmitted by *T. vaporariorum* and *T. abutilonea* [8].

Significant yield losses occur as a result of ToCV infection in tomato, but they are usually not as devastating as with

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Abbreviations: AAP, acquisition access period; CaMV, *Cauliflower mosaic virus*; EPG, electrical penetration graph; IAP, inoculation access period; LCV, *Lettuce chlorosis virus*; MEAM1, Middle East-Asia Minor 1; MED, Mediterranean; NW1, New World 1; pd, potential drop; ToCV, *Tomato chlorosis virus*; TYLCV, *Tomato yellow leaf curl virus*.

some begomoviruses such as TYLCV [7]. Although ToCV primarily affects tomato, it has been found infecting pepper [11, 12], potato [13, 14], tobacco [15] and other vegetable and ornamental crops [10]. Given the importance of *B. tabaci* as a vector of many plant viruses and the increasing importance of ToCV worldwide, knowledge of whitefly feeding behaviour associated with the transmission of ToCV may be strategic to defining methods of control.

In the present work, we conducted a study using the EPG technique to determine the *B. tabaci* stylet penetration activities and plant tissues involved in the inoculation of ToCV in tomato plants.

ToCV (isolate P1-1-2) used for the inoculation experiments was obtained from a naturally infected tomato plant sampled in Malaga, Spain, and maintained on tomato plants (*Solanum lycopersicum* cv. Moneymaker). Plant sources of the virus were obtained by placing groups of 25 adult whiteflies of *B. tabaci* MED in clip cages for a 72 h acquisition access period (AAP) on symptomatic tomato leaves of whole plants, followed by groups of 25 adults in clip cages being transferred to healthy two- to four-true-leaf-stage tomato plants (cv. Moneymaker) for a 7-day inoculation access period (IAP). After the IAP, leaves infested with insects were removed from the plants together with the clip cage to eliminate any remaining eggs and nymphs. The plants were placed in insect-proof cages in a greenhouse (25 : 21 °C day:night temperature, 80 % relative humidity and 14 : 10 h light:dark photoperiod).

A virus-free colony of *B. tabaci* MED originally obtained from Malaga was maintained in whitefly-proof cages on eggplant (*S. melongena*) under greenhouse conditions at the ICA-CSIC. Genetic identity was confirmed periodically by amplifying and sequencing the cytochrome oxidase I mitochondrial gene according to the protocol of Frolich *et al.* [16]. For the inoculation experiments, healthy two- to three-leaf-stage tomato plants were used as test plants. To run the EPG-assisted inoculation experiments, non-viruliferous adult females (1–10 days old) were acclimated for 72 h on healthy tomato plants and then given a 24 h AAP on symptomatic leaves of ToCV-infected tomato plants.

Viruliferous whiteflies collected in a glass tube were chilled in an ice bath for 5–10 min for immobilization. Insects were then placed on a Petri dish cover that was set atop crushed ice under a dissecting microscope. A thin gold wire (2 cm in length, 12.5 µm in diameter; EPG Systems, Wageningen, The Netherlands) was attached to the pronotum of each whitefly by using a small droplet of silver conductive paint glue (Colloidal Silver Liquid, Ted Pella). The opposite end of the gold wire was glued with a droplet of silver paint to a thin copper wire (2 cm in length), which was connected to the EPG probe. Another copper electrode (10 cm in length, 2 mm in diameter) was inserted into the soil of the plant container.

After a 1 h starvation period, each wired whitefly was placed on the abaxial surface of one of the first true leaves of a virus-free tomato test plant (two- to three-leaf stage) and the EPG waveforms were recorded and visualized in real time on a computer screen, by using a direct current eight-channel EPG device, model Giga-8d, with Stylet+ for Windows software (EPG Systems). The recordings were carried out in a room at 25±1 °C inside a Faraday cage, an enclosure used to block electric fields.

Stylet penetration activities on the test plants were classified into six groups (Table 1, Fig. 1): (I) short stylet pathway (waveform C<5 min); (II) stylet pathway (C)+1 or more potential drops (pds); (III) stylet pathway + a single E_1 waveform; (IV) stylet pathway + a transition E_1/E_2 phase; (V) stylet pathway phase + a single E_1+E_2 episode; and (VI) stylet pathway + more than one E_1+E_2 episodes.

Whiteflies were lifted and removed from the test plant (plant A) immediately after each specific group treatment. Next, the gold wire was cut and each whitefly was individually transferred to a second tomato test plant (plant B) for a 72 h IAP, in order to assess its initial virus acquisition. Finally, both test plants (plants A and B) were placed in an insect-free greenhouse for 4–5 weeks. Insects that were

Table 1. Relationship between treatments (groups) and stylet activities of *Bemisia tabaci* MED

Group	Waveforms shown	Associated stylet activities
I	Stylet pathway (waveform C) <5 min	Short stylet pathway phase (without potential drops, pds), which represents movement of the stylets in the intercellular apoplastic space.
II	Stylet pathway + one or more pds	Stylet pathways followed by one or more pds lasting between 3–10 s each. Pds are correlated with intracellular punctures [27].
III	Stylet pathway + waveform E_1*	Stylet pathway phase followed by a single E_1 (>10 s). Waveform E_1 is associated with salivation into phloem sieve elements at the beginning of the phloem phase [5].
IV	Stylet pathway + (E_1/E_2) transition phase*	Stylet pathway phase followed by E1 and a short E_1/E_2 transition phase (15–20 s), in which waveforms E_1 and E_2 overlap. Waveform E2 is correlated with passive phloem sap uptake from sieve elements [25, 34].
V	Stylet pathway + a single E_1+E_2 episode*	Stylet pathway phase followed by a single E_1+E_2 phase and return to stylet pathway phase (C).
VI	Stylet pathway + more than one E_1+E_2 episode*	Stylet pathway phase followed by two or more E_1+E_2 phases (each E1+E2 episode was followed by a pathway phase).

*Insects in groups III, IV, V and VI performed one or more pd before reaching the phloem phase.

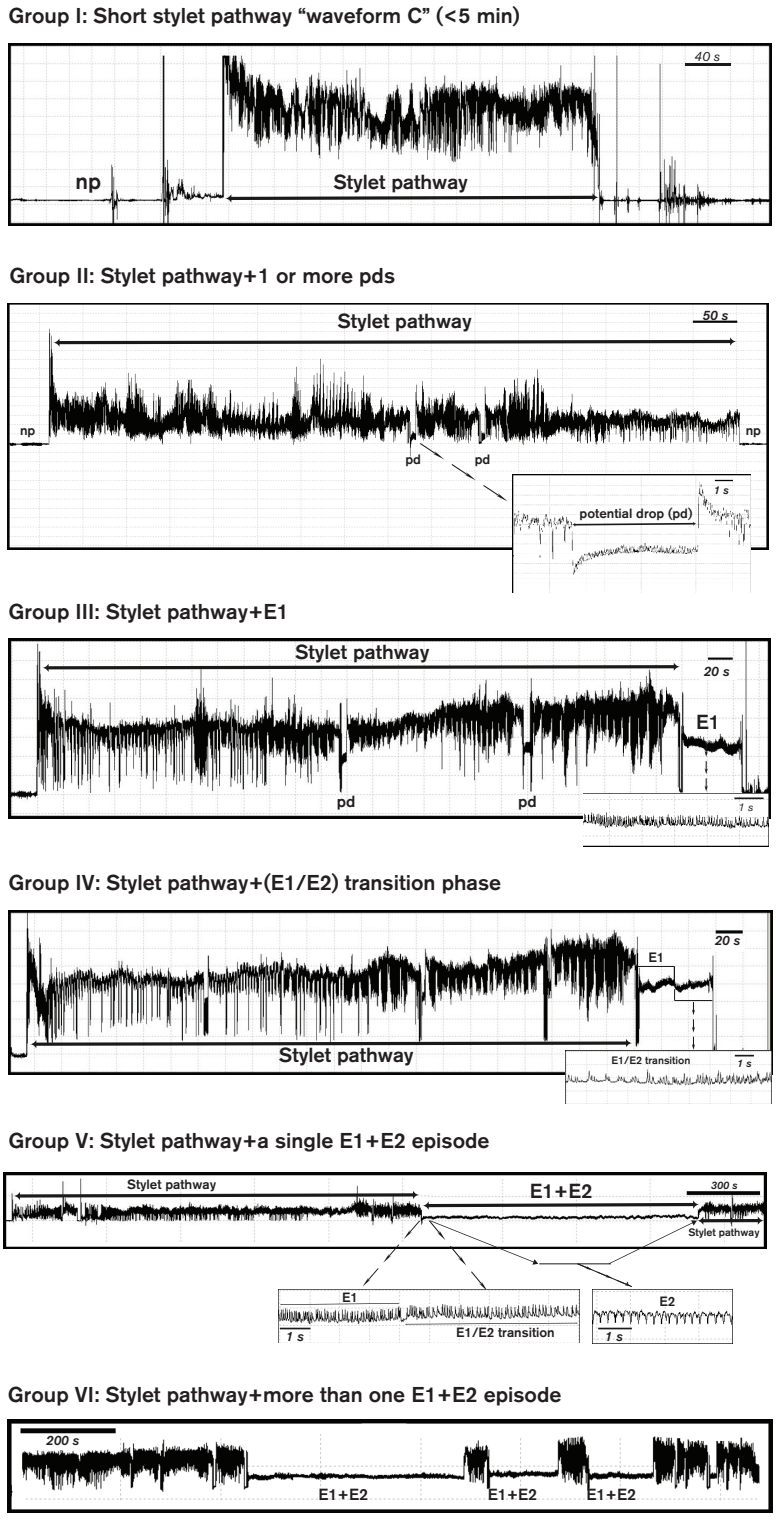

Fig. 1. Typical EPG signals obtained in the six different groups used to correlate specific patterns of the probing behaviour of *B. tabaci* and the transmission of ToCV.

unable to infect either of the two test plants were considered as non-viruliferous and discarded from the analysis. The virus transmission rate obtained for each treatment was calculated by dividing the number of recordings where plant A became infected by the total number of recordings where either A, B or both test plants became infected (Table 2).

Table 2. Relationship between stylet activities of *B. tabaci* MED and the transmission efficiency of ToCV to tomato plants

Group	Waveforms shown	No. of test plants		Discarded data (A- B-)	Total	Transmission efficiency (%)*
		Plant A+ (A+B+ or A+B-)	Only B+ (A- B+)			
I	Stylet pathway (waveform C)<5 min	0	32	29	0/32	0 a
II	Stylet pathway + one or more pds	1	50	62	1/51	1.96 a
III	Stylet pathway+E_1	12	11	15	12/23	52.2 b
IV	Stylet pathway + (E_1/E_2) transition phase†	11	4	8	11/15	73.3 bc
V	Stylet pathway + a single E_1+E_2 episode	19	6	8	19/25	76.0 bc
VI	Stylet pathway + more than one E_1+E_2 episodes	11	0	12	11/11	100.0 c

*Different letters indicate significant differencesbetween groups according to a chi-square test and Fisher's exact test, when the expected values were lower than five [18]. Insects that were unable to infect either of the two test plants (A or B) were considered as non-viruliferous and were discarded from the analysis. See main text for a complete explanation of the calculation procedure.

†Whiteflies were removed from the test plant at least 15 s after onset of the transition phase E_1/E_2.

Infection of plants was confirmed by RNA hybridization of stem prints. For tissue printing, leaf petiole cross-sections (fourth leaf counted from the bottom up) were blotted on positively charged nylon membranes (Roche Diagnostics) four weeks after EPG recording and hybridized with a digoxigenin-labelled negative-sense RNA probe specific for the coat protein gene, as described previously [17]. Hybridization signals were detected on X-ray film (X-Omat AR, Kodak) after treatment with CDP-Star (Roche) and developed following a conventional photographic process. The tissue-printing molecular hybridization technique has been proven to be a sensitive and reliable method for detection of ToCV [17].

Transmission rates calculated as percentages were compared among different treatment groups, using the chi-square test and Fisher's exact test, when the expected values were lower than five [18].

The results (Table 2) show that ToCV inoculation is mainly associated with waveform E_1 (preceded by various stylet pathway episodes), as there was a substantial increase in transmission rate when whiteflies performed the first E_1 (52.2 %; group III). There was no statistically significant increase in transmission rate when whiteflies were interrupted during the E_1/E_2 transition phase (73.3 %; group IV), or when the insects performed one episode of E_1+E_2 (76 %; group V). However, there was a significant increase (P<0.05) in transmission rate after two or more E_1+E_2 episodes (group VI; 100 %).

It is important to note that we also observed a single ToCV inoculation event before whiteflies performed E_1 (group II; Table 2). This result suggests that virus infection could also occur at a very low rate (below 2 %) during stylet puncture either in mesophyll, companion or parenchyma cells or even during brief stylet punctures in phloem sieve elements, culminating in plant infection by the virus. However, our results show that stylet activity in the phloem sieve elements is the primary feeding behaviour involved in the inoculation of this crinivirus.

Similar results were observed by Johnson *et al.* [6], who found the highest percentage of infected plants with the crinivirus LCV when *B. argentifolli* (*B. tabaci* MEAM1) fed in phloem sieve elements (44–50 % of inoculation rate). Those authors also observed a low rate of transmission of LCV before whiteflies exhibited any phloem phases (7–9 % of inoculation rate). They suggested that whiteflies made intracellular punctures in the phloem cells. In our study we considered intracellular punctures as those in the 3–10 s range, unlike Johnson *et al.* [6] who considered pd durations as long as 44 s. Such long intracellular punctures could actually reflect short E_1 signals and not pds, a fact that would justify the relatively high inoculation rate they found.

In general, semi-persistently transmitted viruses in the genera *Waikavirus*, *Crinivirus* and *Closterovirus* are acquired from and inoculated into phloem tissues [6, 19, 20]. In contrast, the semi-persistent *Cauliflower mosaic virus* (CaMV; *Caulimoviridae: Caulimovirus*) can be acquired from and inoculated into any plant tissue. Unlike ToCV, which is believed to be foregut-borne based on similarity to the related crinivirus *Lettuce infectious yellows virus* [21], the retention of CaMV particles occurs in the acrostyle, which is located in the common ducts at the tips of the maxillary stylets [22]. In another study involving CaMV, Moreno *et al.* [23] concluded that aphid salivation during successive pds in epidermal and mesophyll cells, before reaching the phloem phase, is critical for CaMV inoculation.

The main difference between aphid and whitefly intracellular stylet puncture is that of whiteflies occurs much less frequently than that of aphids and does not occur during brief probes [24–26]. Reported frequencies of brief intracellular puncture before the phloem phase (E waveforms) range from 0.3 to 1.5 per hour for whiteflies [27–29] compared to approximately 0.5 pd per minute for aphids [30]. However, pds in mesophyll, phloem cells or in any other type of plant cells are indistinguishable when using the EPG technique [31].

In our study, 84 % of the ToCV-viruliferous whiteflies included in group II performed 1 to 4 pds, but the only case

where ToCV was transmitted was when *B. tabaci* performed 6 pds. Intracellular punctures have been associated with non-persistent virus transmission by aphids [32], but the role of intracellular punctures in the transmission of whitefly-borne viruses is unknown.

The transmission of the begomovirus TYLCV, a phloem-restricted circulative virus, occurred when *B. tabaci* reached phloem sieve elements, but whiteflies were able to transmit the virus during the stylet pathway phase [5]. In that work, a low TYLCV transmission rate before whiteflies reached the phloem salivation phase (2.4 %) was found, which is consistent with the low transmission rate that we found for ToCV. Similarly, they found that TYLCV transmission efficiency increased significantly when the insects performed several E_1+E_2 episodes (37.5 %) compared to whiteflies that performed a stylet pathway phase (C) followed by a single E_1+E_2 episode (23.4 %).

In conclusion, our work shows that whiteflies transmit ToCV to tomato plants when reaching the phloem phase E_1, but the specific activities and stylet tip positions during the inoculation events are still unknown. In a previous study, Johnson *et al.* [6] associated inoculation of another crinivirus, LCV, with the phloem phase, but could not associate waveform E_1 with inoculation. They compared the transmission rate of LCV for two group treatments only: one containing a phloem phase and another with a non-phloem phase. Conversely, in our work we were able to compare insects that performed only E_1 (group III) to those removed during the E_1/E_2 transition phase (group IV) and those that engaged in single or multiple phloem E_1+E_2 episodes (groups V and VI), showing that inoculation occurs mostly during E_1, which is thought to represent salivation into the phloem sieve elements. Our work does not support, but also cannot rule out, the ingestion–egestion hypothesis as suggested by Chen *et al.* [21], because the specific EPG signals associated with egestion by whiteflies remain to be determined. We cannot rule out that egestion (or extravasation) of liquids present in the foregut may occur in addition to salivation during either E_1 or the E_1/E_2 transition phase of whiteflies. This would explain how virions retained in the foregut are released into the phloem cells, as suggested by Chen *et al.* [21].

Therefore, histological analysis should be conducted to determine which specific plant cells are punctured by whiteflies, eventually resulting in virus inoculation and infection of tomato plants. Studies on the histological correlations between specific EPG waveforms and stylet tip positions of aphids have been conducted in the past using light and transmission electron microscopy together with stylet amputation [31, 33]. Similar studies using histology coupled with microscopy will be necessary to determine whether crinivirus transmission can also occur during brief intracellular punctures either in the mesophyll, companion or sieve element cells.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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