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Stylet penetration behavior resulting in inoculation of a semipersistently transmitted closterovirus by the whitefly *Bemisia argentifolii*

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Abstract

The electrical penetration graph (EPG) technique was used to determine what part of stylet penetration behavior by the whitefly vector, *Bemisia argentifolii* Bellows & Perring (Homoptera: Aleyrodidae), is lettuce chlorosis virus (LCV) inoculated in the host plant *Malva parviflora* L. LCV is a semipersistently-transmitted closterovirus. Since closteroviruses generally are found in the phloem of their plant hosts, this study tested the hypothesis that virus inoculation occurs during the phloem phase of stylet penetration behavior. Virus-exposed whiteflies were allowed to feed on uninfected host plants, and the whiteflies were divided into two experimental groups: group 1 attained phloem phase on the uninfected plants, and group 2 did not attain phloem phase. Two series of tests were conducted, one where whiteflies were manipulated so that the amount of time spent in non-phloem phase stylet penetration behaviors was similar between group 1 and group 2, and a second series of tests where whiteflies were manipulated so that the number of intracellular punctures made during stylet penetration was similar between group 1 and group 2. Both series of tests indicated that virus inoculation took place primarily during phloem phase. Considering only individual whiteflies shown to be capable of transmitting virus, 11 of 23 whiteflies (48%) in the phloem phase treatment successfully inoculated the virus whereas only one of 19 whiteflies (5%) in the non-phloem phase treatment successfully inoculated the virus ($P = 0.00008$).

Introduction

Semipersistently transmitted plant viruses are responsible for some of the most economically important diseases of cultivated crops, yet this group has received less attention in the scientific literature than nonpersistently and persistently transmitted plant viruses (Raccah et al.,1989). For a detailed description of transmission characteristics for nonpersistently, persistently, and semipersistently transmitted viruses, see Nault (1997). The largest group of semipersistently transmitted plant viruses are the closteroviruses (Candresse & Martelli, 1995; Nault, 1997) which are transmitted by three families within the homopteran suborder Sternorrhyncha: Aphididae, Aleyrodidae, and Pseudococcidae (Nault, 1997). Each specific closterovirus is transmitted by only one or a limited number of vector species within the same insect family (Raccah et al., 1989).

The vector behaviors responsible for transmission of plant viruses have been studied for numerous homopteran vectors and the viruses they transmit. The earliest studies of stylet penetration behaviors that result in nonpersistent transmission of potyviruses by aphids used transmission electron microscopy to examine the stylet pathway in detail, and found that acquisition and inoculation depended on the aphid stylet tips puncturing plant epidermal cells (Lopez-Abella & Bradley, 1969; Lopez-Abella et al., 1988). More recent studies using the Electrical Penetration Graph (EPG) technique confirmed that transmission of nonpersistently transmitted potyviruses and cucu-

moviruses occurs during brief intracellular punctures by the stylet tips early in a probe (Powell, 1991, 1993; Powell et al., 1992, 1995; Perez et al., 1996; Collar et al., 1997; Martín et al., 1997). Powell et al. (1995) and Martín et al. (1997) examined the sequence of behaviors that occurs during brief intracellular punctures by aphid stylets, and the role of the sequential behaviors in inoculation and acquisition of nonpersistently transmitted potyviruses. They demonstrated that potyvirus acquisition takes place primarily during the last sequential behavior (presumed ingestion) during an intracellular puncture while inoculation of these viruses takes place primarily during the first sequential behavior (presumably salivation) in the intracellular puncture.

The EPG technique also has been used to study the transmission of persistently transmitted viruses by aphid, leafhopper, and whitefly vectors. Inoculation of persistently transmitted viruses primarily takes place when the insect salivates into a phloem sieve element during the first period of sieve element penetration by the vector's stylets (Prado & Tjallingii, 1994; Kimmins & Bosque-Perez, 1996; Jiang et al., 2000). Acquisition of persistently transmitted viruses also is dependent on sieve element penetration, but takes place later during sieve element penetration when the phloem sap ingestion phase occurs (Prado & Tjallingii, 1994).

There is only one study of which we are aware that examined stylet penetration behavior responsible for transmission of a semipersistently transmitted virus. Wayadande & Nault (1993), utilized the EPG technique to determine that maize chlorotic dwarf waikavirus is inoculated by the leafhopper *Graminella nigrifrons* (Forbes) (Homoptera: Cicadellidae) when the vector's stylets initially penetrate a phloem sieve element, prior to phloem ingestion.

At present, there has been only one study determining stylet penetration behavior that results in inoculation of a whitefly-transmitted virus; the virus in this case being a persistently transmitted geminivirus (Jiang et al., 2000). Additionally, there have been no studies on stylet penetration behavior that result in transmission of any closterovirus by whiteflies or by any other vector.

The objective of the current study is to identify the whitefly stylet penetration behavior that results in inoculation of lettuce chlorosis closterovirus (LCV). Since closteroviruses are generally, but not exclusively, found in the phloem of their plant hosts (Esau, 1968), this study focuses on the sieve element phase of stylet penetration.

Materials and methods

Virus source and maintenance

The original isolate of LCV used in these experiments was obtained from lettuce, *Lactuca sativa* L., grown in the Imperial Valley of California (McLain et al., 1998). The virus was maintained in *Malva parviflora* L. (cheeseweed) plants by periodic transmission from virus infected cheeseweed to healthy plants via the vector *Bemisia argentifolii* Bellows & Perring (Homoptera: Aleyrodidae). LCV-infected cheeseweed was grown in a vector-free greenhouse at approximately 26 °C. The greenhouse was sprayed weekly with Malathion and Pentac in order to keep it vector-free. Confirmation of LCV infection in the virus culture was based on enzyme-linked immunosorbent assay (ELISA) (protocol described later).

Source and maintenance of insects

The original colony of *B. argentifolii* was initiated from adults collected in 1991 from *Brassica oleracae* L. in Imperial Valley, California. Voucher specimens are deposited in the Entomological Research Museum at the University of California, Riverside. The original colony was reared in cages in a greenhouse on potted *Phaseolus lunatus* L. (lima bean) and *Brassica juncea* (L.) Czernov (Florida broadleaf mustard). Whiteflies used in earlier replicates were taken from this colony. Subsequently, 150–200 adults from this colony were used to establish a second colony reared on LCV-infected cheeseweed maintained either in a growth chamber (L16:D8, $\approx$ 32 °C) or in a greenhouse. Later replicates in this study used whiteflies from the LCV infected colony in order to improve virus transmission efficiency.

Viruliferous whiteflies used in inoculation experiments were obtained as follows. In each of the earlier replicates, approximately 20–30 *B. argentifolii* adults were taken from the original colony, described above, and were placed in a moistened acquisition dish with 2–3 excised LCV-infected cheeseweed leaves for a 48 h acquisition access period (AAP). Following the 48 h AAP, the insects were removed from the acquisition dish and used in the experiments. In later replicates, viruliferous whiteflies were obtained di-

rectly from the colony maintained on LCV-infected cheeseweed.

To standardize the vectors, only females were used because in previous transmission studies, the females of the whitefly *Bemisia tabaci* Gennadius showed a greater ability than males to transmit several viruses (Cohen & Nitzany, 1966; Costa & Bennett, 1950). The age of the females was not standardized.

Plants

All plants used in the experiments were virus-free, 2–3 week old cheeseweed grown from seed (purchased from Valley Seed Service, Fresno, California) in pots (10 cm) in a vector-free greenhouse.

Enzyme linked immunosorbent assay (ELISA) procedures

Plant extracts, LCV IgG, goat anti-rabbit IgG alkaline phosphatase conjugate (GARG-conjugate [Sigma, St. Louis, MO]), and p-nitrophenylphosphate substrate were each incubated in the sequential order given, for approximately 2 h at 37 °C on Immulon 2 polystyrene plates (Dynatech, Chantilly, VA). LCV IgG was utilized at a 1/8,000 dilution, and GARG-conjugate was used at a 1/2,000 dilution. One sample per plant (leaf disk, 2.5 cm diameter from a middle leaf), was collected with a #15 cork borer, inserted between rollers of a sap expressor (Ravenel Specialties Co., Seneca, SC) and rinsed with coating buffer (0.05 M sodium carbonate, pH 9.6) until 2 ml of sap/buffer were collected. There were two 200 μl wells per sample. Each ELISA plate assayed included a buffer control, and a negative and positive control from a healthy and a known LCV-infected cheeseweed plant, respectively. Absorbence measurements (405 nm) were taken when positive controls (known LCV-infected plants) had an absorbence of at least 1.0. Samples were considered positive when absorbence were greater than 2.5 times the mean absorbence of the two wells for the healthy cheeseweed control.

Recording equipment

Both AC and DC electronic feeding monitors were used to record electrical penetration graphs (EPGs) of whitefly stylet penetration. For both monitors, the output probe from the monitor was placed in the soil of the potted plant, and the EPGs were recorded from insects and plants that were enclosed in a Faraday cage. Output from both monitors was recorded using CODAS software and analogue-to-digital hardware (Dataq Instruments, Akron, OH) on a computer (IBM Personal System/2 Model 70 386). Analogue to digital conversion rate was 100 samples per second per channel as recommended by Tjallingii (2000).

The AC recordings were made using a 4-channel Missouri Electronic Feeding Monitor type 2.1, a modification of the original Missouri monitor described by Backus & Bennett (1992). The modifications were a 10^6 input impedance, an increase in gain capabilities, and an increase in the cutoff frequency of the final low pass filter to 17 Hz (Backus, personal communication). AC substrate voltage applied to the soil of the plant was approximately 300 mV at a frequency of 250 Hz.

The DC recordings were made using a Giga-4 EPG with a 1 Giga ohm input resistance (Tjallingii, 1988). The DC substrate voltage initially was set at 30 mV DC and then adjusted to fit into the +5 V to −5 V window provided by the CODAS software that was used to record the EPGs. The gain was set at 100×.

General experimental procedures

Attaching wires to whiteflies

Adult female whiteflies were attached to Wollaston process platinum wire, 2.5 μm diameter (Sigmund Cohn, Mount Vernon, NY) using the method described by Walker & Janssen (2000). During attachment of the wires, the whiteflies were immobilized with cold rather than CO_2 because CO_2 may have adverse effects on virus transmission (Caciagli, 1991). Following wire attachment, whiteflies were allowed an acclimation period on LCV-infected cheeseweed for at least 1 h before EPG recordings were made.

Test plants, indicator plants, and sentinel plants

EPGs were recorded from whiteflies feeding on the abaxial surface (normal feeding surface) of one of the first true leaves of a virus-free 'test plant'. During recording, the leaf was held abaxial side up on a plexiglass stand using long narrow strips of Parafilm® (American Can Co., Greenwich, Connecticut, USA) to secure the leaf in position.

EPGs were recorded from the whitefly's first contact with the test plant until the desired stylet penetration behavior (described later) was completed. After completion of the desired behavior, the whitefly was removed from the test plant and placed on a second virus-free plant, referred to as an indicator plant (EPGs were not recorded on the indicator plant). Then the

wire attached to the whitefly was cut and a plastic cage was placed over the entire indicator plant to confine the whitefly. The whitefly was allowed to remain on the indicator plant for 48 h.

After 48 h, the whitefly was removed from the indicator plant and all whitefly eggs were removed from both the test and indicator plants. All plants then were sprayed with Resmethrin® and placed in a vector-free greenhouse for approximately 6–8 weeks to await the development of LCV symptoms and ELISA testing.

The test plants, on which the whiteflies' stylet penetration behavior was recorded electronically, were used to determine which stylet penetration behavior results in inoculation of LCV, as described below. The indicator plants were used to help determine whether or not a given whitefly was viruliferous since prefeeding on, or being reared on a virus infected plant does not guarantee that the whitefly is viruliferous. It was assumed that if the whitefly did not inoculate either the test plant or the indicator plant, after 48 h exposure to the latter, that the whitefly was not viruliferous. Only approximately 10–20% of whiteflies used in the experiments were shown to be viruliferous (i.e., inoculated the test plant and/or the indicator plant).

During the time that the test and indicator plants were kept in the vector-free greenhouse awaiting development of LCV symptoms and ELISA testing, virus-free cheeseweed plants, which had not been exposed to the vector, were placed in the greenhouse along with the test and indicator plants. These plants were referred to as 'sentinel' plants because if they tested positive for LCV, they signal unwanted invasion by the virus in the greenhouse which would confound the experimental results. There was one sentinel plant per row of indicator and test plants (four plants per row total). None of the sentinel plants tested positive for LCV, indicating any test or indicator plants that tested positive for LCV, obtained LCV from the insects in the experiment rather than from insects in the greenhouse.

Experimental treatments

The hypothesis tested in this study is that inoculation of LCV takes place during phloem phase behavior (phloem phase is when the stylet tips are positioned in a phloem sieve element and is associated with salivation and ingestion in the sieve element). Phloem phase is preceded by various non-phloem phase behaviors, including intercellular advancement of the stylets, and usually one or more of the following: partial intercellular stylet withdrawal, brief intracellular punctures, xylem ingestion, and 'penetration difficulties' (Janssen et al., 1989; Walker & Perring, 1994; Walker & Janssen, 2000; Jiang & Walker, 2001). In this study, there were two series of tests, each series with essentially the same two treatments: (1) phloem phase treatment (+PP treatment) in which whiteflies engaged in non-phloem phase behavior and eventually achieved and naturally terminated one bout of phloem phase behavior; and (2) no phloem phase treatment (−PP treatment) in which whiteflies engaged in non-phloem phase behavior only .

Equalizing time spent in non-phloem phase behavior in the +PP and −PP treatments

The first series of tests used an AC EPG, and whiteflies were manipulated as follows so that the total time in non-phloem phase behavior was similar for whiteflies in both treatments. After termination of a single phloem phase by each whitefly in the +PP treatment, the whitefly was manually removed from the test plant, and the EPG recording was examined to determine the total time that the whitefly engaged in non-phloem phase behavior before it initiated phloem phase. These data were used to construct a frequency distribution of the time spent in non-phloem phase behavior for whiteflies in the +PP treatment. In obtaining whiteflies for the −PP treatment, the objective was to manipulate the whiteflies so that their range and average time spent in non-phloem phase behavior was similar to the range and average time in non-phloem phase behavior for whiteflies in the +PP treatment.

Whiteflies in the −PP treatment were allowed to penetrate the plant until the amount of time in non-phloem phase behavior reached the desired value (based on the frequency distribution of time in non-phloem phase behavior for whiteflies in the +PP treatment), and then their exposure to the test plant was manually terminated. Whiteflies from both treatments (−PP, $n = 123$; +PP, $n = 125$) were tested interspersed with one another over time.

Phloem phase, in AC EPGs of whiteflies, is represented by the high-flat waveform (Walker & Perring, 1994). Another waveform in AC EPGs of whiteflies, the pseudotransition waveform, represents general intracellular punctures, and is homologous to the well known potential drops (pds) in DC EPGs (Johnson & Walker, 1999). In AC EPGs, the high-flat waveform and pseudotransition waveform are very similar in shape, and the distinction between the two is based primarily on the duration of their intracellular phases (phases 2 + 3, Johnson & Walker, 1999). In this

study, a waveform was considered to be a pseudotransition waveform (general intracellular puncture) if the intracellular phase was ≤ 91 s; and a waveform was considered to be a high-flat waveform (phloem phase) if the intracellular phase was ≥ 268 s. Most pseudotransition waveforms (90%) had intracellular phases < 20 s, and most high-flat waveforms (89%) had intracellular phases > 989 s.

Equalizing number of intracellular punctures in the +PP and −PP treatments

Examination of EPGs of both treatments in the first series of tests indicated that whiteflies in the +PP treatment tended to make more brief intracellular punctures by the stylet tips (pseudotransition waveforms) during non-phloem phase behavior than whiteflies in the −PP treatment. This was concerning because it did not allow us to decisively rule out intracellular punctures as an important behavior in inoculation of LCV. In order to rule out this possibility, the data were re-analyzed after all whiteflies with < 2 intracellular punctures were discarded (one from the +PP treatment, and eight from the −PP treatment), which made the numbers of intracellular punctures similar between the two treatments. However, discarding those replicates reduced the sample size to the point where differences between the treatments were no longer statistically significant. Therefore, a second series of tests was initiated to augment the sample size of comparisons between the −PP and +PP treatments when numbers of brief intracellular punctures were similar between the two treatments.

The second series of tests used the DC EPG. On this monitor, brief intracellular punctures are represented as pds while phloem phase is represented by waveform E (Janssen et al., 1989). The subpatterns of pds and waveform E are distinctly different, allowing them to be clearly distinguished from each other (Jiang et al., 1999). In our DC recordings, the longest pd was 44 s and the shortest phloem phase was 920 s. This helps justify our decision in the AC EPGs to distinguish an intracellular puncture (pseudotransition waveform) from phloem phase (high flat waveform) when the intracellular phase was ≤ 91 s and ≥ 268 s, respectively, as noted previously.

In the second test series, whiteflies in the +PP treatment were manipulated the same as in the first series of tests. After recording the EPG of each whitefly in the +PP treatment, the total number of intracellular punctures was noted. These data were used to construct a frequency distribution of the total number of intracellular punctures per whitefly in the +PP treatment. Whiteflies in the −PP treatment were manipulated so that the range and average number of intracellular punctures per whitefly was similar to that in the +PP treatment. Whiteflies in the −PP treatment were allowed to penetrate the plant until the number of intracellular punctures reached the desired value (based on the frequency distribution of intracellular punctures per whitefly in the +PP treatment), and then their exposure to the test plant was manually terminated.

When it was necessary to obtain whiteflies with a large number of intracellular punctures (> 4) in the −PP treatment (in order to match whiteflies with a large number of intracellular punctures in the +PP treatment), the −PP whiteflies were allowed a maximum of four intracellular punctures within the same probe, and then the probe was interrupted by gently lifting the whitefly off the leaf and then placing the whitefly back on the leaf where it could initiate new probes. This was repeated until the desired number of intracellular punctures was achieved, and then the whitefly was removed permanently from the test plant. Interruption of probes after four intracellular punctures was necessary to facilitate obtaining whiteflies in the −PP treatment with large numbers of intracellular punctures because after approximately four intracellular punctures, whiteflies had a strong tendency to initiate phloem phase; and if phloem phase was initiated, then the whitefly was included in the +PP treatment rather than the −PP treatment. Whiteflies from both treatments (−PP, $n = 43$; +PP, $n = 41$) were tested interspersed with one another over time.

Statistical analysis

Only viruliferous whiteflies (i.e., those that inoculated the test plant and/or indicator plant) were included in the statistical analyses. This resulted in a small number of usable replicates, consequently, the proportion of test plants inoculated with LCV by viruliferous whiteflies was compared between the two treatments using Fisher's exact test (Daniel, 1990) because it is a more appropriate test than the χ^2 test when sample size is small.

The first analysis compared inoculation rate between the −PP and +PP treatments when the time in non-phloem phase behavior was manipulated so that it was similar in the two treatments. The first series of tests yielded 15 viruliferous whiteflies in the −PP treatment and 18 viruliferous whiteflies in the

+PP treatment. To verify that the total time in non-phloem phase behavior by the viruliferous whiteflies did not differ between the two treatments, total time in non-phloem phase behavior was compared between treatments using a 2-sample *t*-test for samples with different variances (Minitab, 1996).

The second analysis compared inoculation rate between the −PP and +PP treatments when the number of intracellular punctures was similar in the two treatments. This analysis included 11 viruliferous whiteflies in the −PP treatment and 22 viruliferous whiteflies in the +PP treatment. Four of the viruliferous whiteflies in the −PP treatment and five of the viruliferous whiteflies in the +PP treatment came from the second series of tests, and seven and 17 viruliferous whiteflies in the −PP and +PP treatments, respectively, came from the first series of tests. Whiteflies that were used from the first series of tests included all viruliferous whiteflies that made ≥ 2 brief intracellular punctures. To verify that the numbers of brief intracellular punctures per whitefly did not differ between the two treatments, total number of intracellular punctures was compared between treatments using a Mann Whitney test (due to a non-normal data distribution in the −PP treatment) (Minitab, 1996).

Results

+PP versus −PP when time in non-phloem phase behavior is similar in both treatments

The mean time that the viruliferous whiteflies engaged in non-phloem phase behavior did not differ significantly between treatments (mean ± S.D.: +PP treatment, 58.6 min ± 41.7 ($n = 18$); −PP treatment, 83.2 min ± 42.8 ($n = 15$); P = 0.11, 2-sample *t*-test). Thus, the objective of the experimental protocol (to achieve similar time in non-phloem phase behavior in both treatments) was successful.

The proportion of viruliferous whiteflies that inoculated the test plant with LCV differed significantly between the two treatments (Table 1). Inoculation rate was 44% for whiteflies in the +PP treatment and 7% for whiteflies in the −PP treatment (P = 0.018, Fisher's exact test). These results implicate phloem phase behavior as the primary behavior responsible for inoculation of LCV. However, whiteflies in the +PP treatment on average made more intracellular punctures that those in the −PP treatment. Eight of the 15 viruliferous whiteflies in the −PP treatment and 1 of the 18 viruliferous whiteflies in the +PP treatment made less than two intracellular punctures (range: 0–43 intracellular punctures per whitefly). Thus, an alternate explanation for the significantly lower inoculation rate in the −PP treatment compared to the +PP treatment could be that more whiteflies in the −PP treatment made only one or fewer intracellular punctures.

+PP versus −PP when numbers of intracellular punctures are similar in both treatments

The mean number of intracellular punctures did not differ significantly between the two treatments (mean ± S.D.: +PP treatment, 6.5 ± 3.66, median = 5.5 ($n = 22$); −PP treatment, 10.5 ± 11.4, median = 9 ($n = 11$); $P = 0.34$, Mann–Whitney test). Thus, the objective of the experimental protocol (to achieve a similar number of intracellular punctures in both treatments) was successful.

The proportion of viruliferous whiteflies that inoculated the test plant differed significantly between the two treatments (Table 1). Inoculation rate was 50% for whiteflies in the +PP treatment and 9% for whiteflies in the −PP treatment series ($P = 0.024$, Fisher's exact test). These data strongly suggest that phloem phase is the primary behavior responsible for inoculation of LCV, and that brief intracellular punctures do not play a major role in its inoculation.

Combined analysis

When whiteflies from both series of tests are pooled, 11/23 (48%) of whiteflies in the +PP treatment inoculated the test plant in contrast to only 1/19 (5%) in the −PP treatment ($P = 0.00008$, Fisher's exact test).

Discussion

Results from the present study implicate phloem phase as the main *B. argentifolii* stylet penetration behavior responsible for LCV inoculation. Non-phloem phase, including intracellular punctures plays only a minor role in inoculation of LCV. However, a viruliferous whitefly's engagement in phloem phase behavior does not guarantee that successful virus inoculation will take place (only 48% of proven viruliferous whiteflies engaging in phloem phase inoculated the test plant). Nonetheless, unless phloem phase is achieved, the probability of successful LCV inoculation is low (5%). These results are congruent with the distribution of the

Table 1. Proportion of viruliferous whiteflies that inoculated test plants with LCV during electronically recorded stylet penetration behavior

Treatment[a]	Proportion of viruliferous whiteflies that inoculated the test plant[b]	Percentage[c]	Fisher's exact test *P*-value
Analysis when total time in non-phloem phase behavior was similar between −PP and +PP treatments[d]			
−PP	1/15	6.7	
			0.018
+PP	8/18	44.4	
Analysis when number of intracellular punctures was similar between −PP and +PP treatments[e]			
−PP	1/11	9.1	
			0.024
+PP	11/22	50.0	

[a] −PP: whiteflies engaged in non-phloem phase only; +PP: whiteflies engaged in non-phloem phase followed by phloem phase (see *Materials and methods* for details).
[b] Number of whiteflies inoculating LCV in test plant divided by total number of viruliferous whiteflies tested.
[c] Calculated from proportions in column to the left in order to facilitate comparisons when denominators of proportions differ between treatments.
[d] All replicates were from first test series (see *Materials and methods* for details).
[e] Selected replicates in test series 1 were pooled with all replicates in test series 2 (see Materials and methods for explanation).

virus in the plant, which is located primarily in the phloem (Dolja et al., 1994).

Only a single whitefly in the −PP treatment inoculated the test plant (it was included in both analyses in Table 1). It is possible that one or more of the nine intracellular punctures made by this whitefly were intrusions into phloem cells, and that this may have been the cause of LCV inoculation. In support of this, Tjallingii & Hogen Esch (1993) demonstrated that the stylet tips of the aphid *Aphis fabae* (Scopoli) (Homoptera: Aphididae) punctured several sieve elements before one was eventually accepted and phloem phase was initiated, but the EPG potential drops associated with intracellular punctures of sieve elements prior to phloem phase were indistinguishable from pds associated with intracellular punctures of other types of cells. Jiang et al. (2000) made a similar conclusion on inoculation of a persistently transmitted geminivirus, tomato yellow leaf curl virus, by *Bemisia argentifolii*. Inoculation occurred primarily during phloem phase, but a residual low level of inoculation occurred prior to phloem phase, which the authors hypothesized as being caused by intracellular punctures into phloem companion cells.

The results from this study were similar to those found in a previous study on another semipersistently transmitted phloem-limited virus. Wayadande & Nault (1993), using an AC EPG, demonstrated that the leafhopper *Graminella nigrifrons* (Forbes) inoculated maize chlorotic dwarf waikavirus early in phloem phase (during the X-waveform which precedes phloem ingestion).

This study and Wayadande & Nault (1993) both indicate that inoculation of semipersistently transmitted viruses is more similar to the persistently transmitted viruses than to the nonpersistently transmitted viruses in regard to the plant tissues where inoculation takes place. The persistently transmitted plant viruses are inoculated in phloem tissue (Leonard & Holbrook, 1978; Prado & Tjallingii, 1994; Kimmins & Bosque-Perez, 1996; Scheller & Shukle, 1986) while the nonpersistently transmitted viruses are inoculated in epidermis and outer mesophyll tissue (Lopez Abella & Bradley, 1969; Lopez-Abella et al., 1988; Powell, 1991, 1993; Powell et al., 1992, 1995; Perez et al., 1996; Collar et al., 1997; Martín et al., 1997).

Although the same plant tissue (phloem sieve element) appears to be involved in inoculation of persistently- and semipersistently transmitted viruses, the insect behaviors within the sieve element may differ between inoculation of these two types of viruses. The persistently transmitted viruses are ingested (acquired) by the vector while feeding on virus infected host plants, and the viral particles then move across the gut wall, circulate through the vector, and migrate to the salivary glands. Inoculation occurs during subsequent feeding when the insect injects virus laden saliva into the phloem. In contrast, the semipersis-

tently transmitted viruses do not circulate within the vector. Following ingestion by the vector, viral particles adhere to sites in the anterior alimentary canal, primarily in the region of the pharynx, cibarium, and precibarium, and in one recorded case, the maxillary food canal (Murant et al., 1976; Childress & Harris, 1989; Ammar & Nault, 1991). According to the ingestion-egestion hypothesis (Harris, 1977), after a vector acquires a semipersistently transmitted virus, the virus is inoculated during subsequent bouts of feeding, when egestion dislodges viral particles from their binding site in the vector's alimentary canal, and they are then injected into the plant. An alternative to the ingestion-egestion hypothesis recently has been proposed for inoculation of nonpersistently transmitted viruses (Martín et al., 1997), and could be equally applicable for semipersistently transmitted viruses. This hypothesis notes that the food and salivary canals of aphids are confluent near the apex of the maxillary stylets, and consequently, viral particles adhering to the area of confluence could be dislodged during salivation into plant cells, resulting in inoculation. This is referred to as the ingestion-salivation hypothesis.

Whiteflies also have an area of confluence of the food and salivary canals near the apex of the maxillary stylets (Rosell et al., 1995), and thus the ingestion-salivation hypothesis could apply to whiteflies as well. However, we presently do not know the binding site of LCV in *B. argentifolii*. Nor do we know what behavior during phloem phase, salivation or egestion, is responsible for inoculation of LCV. Future studies are planned to identify the binding site of LCV in the whitefly, and to identify the specific behavior during phloem phase that is responsible for virus inoculation. As a starting point, we will focus on the DC system waveforms E1 and E2 described during whitefly phloem phase by Lei et al. (1997). E1 has been correlated with salivation into sieve elements (Prado & Tjallingii, 1994; Jiang et al., 2000), whereas E2 includes ingestion, salivation, and possibly egestion (Prado & Tjallingii, 1994). If LCV is inoculated according to the ingestion-salivation hypothesis, we expect inoculation to occur primarily during E1. In contrast, if LCV is inoculated according to the ingestion-egestion hypothesis, we expect inoculation to occur primarily during E2.

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