Intracellular Salivation Is the Mechanism Involved in the Inoculation of Cauliflower Mosaic Virus by Its Major Vectors *Brevicoryne brassicae* and *Myzus persicae*

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ABSTRACT Cauliflower mosaic virus (CaMV) is transmitted to crucifers in a noncirculative manner by several aphid species. CaMV is preferentially acquired from the phloem, although acquisition also occurs after brief intracellular stylet punctures of aphid vectors in nonvascular leaf tissues. In the present work, we used the electrical penetration graph technique to study the specific aphid stylet activities and behavioral events leading to the inoculation of CaMV to turnip plants by its two major vectors, *Brevicoryne brassicae* (L.) and *Myzus persicae* (Sulzer). Aphids subjected to an 8-h acquisition access time on infected plants were transferred to test plants and removed immediately after specific behavioral events were recorded. CaMV was readily inoculated after the first intracellular puncture in nonvascular tissues by both vector species. Inoculation rate of CaMV by *B. brassicae* was the highest after a 3-h inoculation access period, regardless of whether aphids had reached the phloem phase during that period. Consistent interspecific differences also were found in the ability of both aphid vectors to retain CaMV. *B. brassicae* could retain the virus after several intracellular punctures, whereas *M. persicae* readily lost the virus after performing the same number of intracellular stylet punctures. We concluded that salivation by aphids during successive intracellular stylet punctures in the epidermal and mesophyll cells before reaching the phloem phase are the key behavioral events associated to the inoculation of Cauliflower mosaic virus. The likely location of the viral retention site inside the aphid mouthparts is discussed.

KEY WORDS CaMV, *Myzus persicae*, *Brevicoryne brassicae*, vector transmission, inoculation

INSECT-TRANSMITTED VIRAL DISEASES cause severe economic losses in horticultural crops. Cauliflower mosaic virus (CaMV) is one of the most widely spread viruses in *Brassica* crops in Spain (Moreno et al. 2004) and worldwide (Jenkinson 1995, Raybould et al. 1999, Pallett et al. 2002). CaMV, the type member of the genus *Caulimovirus*, has an 8-kpb double-stranded circular DNA genome. The virion is an icosahedral particle with a diameter of 53.8 nm made of 420 subunits of the viral coat protein (CP) (Plisson et al. 2005). CaMV is transmitted by at least 27 aphid species (Kennedy et al. 1962) in a noncirculative manner, which means that the virus particles do not cross the vector cell membranes and are carried externally on the cuticle lining of the vector's mouthparts or foregut. The main vectors in the field are *Myzus persicae* (Sulzer) and *Brevycorine brassicae* (L.) (Broadbent 1957). Other types of transmission (seeds, pollen, and insect vectors other than aphids) have not been reported.

The relationship between the virus and its aphid vectors has long been unclear. Some studies concluded that *M. persicae* transmits CaMV in a nonpersistent manner (e.g., Kennedy et al. 1962), and others have considered CaMV to be a bimodally transmitted virus (e.g., Chalfant and Chapman 1962). The bimodal transmission was described for *B. brassicae* that happened to exhibit features of both nonpersistent and semipersistent transmission. As opposed to nonpersistent transmission, there is no preacquisition starvation effect for semipersistent viruses, which are typically phloem-restricted and require longer acquisition and inoculation periods (Hull 2002). Later, research conducted by Markham et al. (1987) showed that the term bimodal was misleading, because the optimum acquisition peaks of CaMV may vary and show a bi- or multiphasic pattern, depending on the vector species used for the transmission experiments. These authors concluded that CaMV is transmitted in a semipersistent manner.

Advances in molecular interactions between viruses and vectors have shown that CaMV uses a helper strategy for transmission (Pirone and Blanc 1996). The helper component (HC) acts as a reversible molecular bridge between the virion and the cuticular binding site in the vector mouthparts. In CaMV, the HC is encoded by the ORFII. This gene codes for an 18-kDa nonstructural protein, P2, which recognizes both the

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attachment sites in the vector's mouthparts and the viral protein P3, itself forming a complex with the virus particle (for review, see Blanc et al. 2001). Although it is known that the α-helical C terminus of P2 is involved in self-association (Hebrard et al. 2001) and interacts with P3–virion complexes (Leh et al. 1999), there is no information about the domain of P2 that interacts with the aphid's cuticle. Nevertheless, a recent report has confirmed that P2 is the only viral product that is retained when acquired alone by aphids and that its acquisition before that of P3–virion complex is mandatory for the success of transmission, thus further proving that P2 interacts indeed with aphids' mouthparts (Drucker et al. 2002). Within the insect's feeding apparatus, the retention sites for semipersistent viruses have been determined only in the leafhopper-transmitted viruses (Childress and Harris 1989). Similar data on semipersistent aphid-transmitted viruses have not been reported; thus, information on the precise location of the aphid's receptor(s) recognized by CaMV P2 is not known.

The use of electronic feeding monitoring systems (EMSs) allows study of the relationship between the feeding behavior of insects and their ability to transmit viruses. This technique, also called electrical penetration graph (EPG), has been used to identify specific waveform patterns associated with transmission of persistent (Prado and Tjallingii 1994), nonpersistent (Powell et al. 1995, Martin et al. 1997), and semipersistent (Palacios et al. 2002) viruses by aphids. Acquisition and inoculation of typical nonpersistent viruses, such as Cucumber mosaic virus or Potato virus Y, occur during specific subphases of brief intracellular stylet punctures (potential drop, pd) in nonvascular leaf tissues (Martin et al. 1997). In contrast, the phloem ingestion phase (E2) and the phloem salivation phase (E1) are associated with the acquisition and inoculation, respectively, of persistently transmitted luteoviruses (Prado and Tjallingii 1994). Recent studies have shown that CaMV does not share most of the properties of nonpersistent virus acquisition. Preacquisition fasting does not affect the transmission rate of CaMV, and the virus can be acquired from either nonvascular or phloem tissues. Furthermore, although the rate of CaMV acquisition does not depend on the number of intracellular punctures produced by the vector, it increases sharply after phloem ingestion (Palacios et al. 2002). These findings are consistent with the model of a "sequential acquisition" of the various components of the CaMV transmissible complex (Drucker et al. 2002), where P2 is acquired in specific inclusion bodies before P3–virion complexes acquisition from another type of inclusion or from the phloem.

In contrast to acquisition, information on the relationship between different behavioral events and inoculation of CaMV is still lacking. In the present work, we studied aphid probing and feeding behavior during the inoculation of CaMV to *Brassica rapa* L. plants by its two major vectors, *B. brassicae* and *M. persicae.* A better understanding of the behavioral events associated with retention and inoculation processes of the virus can help to elucidate the mechanism and the location of the specific cuticular binding sites involved in the transmission process of CaMV.

Materials and Methods

Aphid Clones, Virus Isolates, and Test Plants. Aphid clones of the two species used in the study, *M. persicae* and *B. brassicae*, were started from single virginiparous females collected in the central region of Spain at Alcalá de Henares and Villa del Prado, respectively. Nonviruliferous aphids were reared on turnip plants, *Brassica rapa* L. 'Just-Right', in an environmental growth chamber under controlled conditions (23:16°C [day:night] and a photoperiod of 16:8 (L:D) h.

An aphid-transmissible isolate of CaMV, Cabb-S (Franck et al. 1980), was propagated and maintained on turnip plants by aphid transmission. The virus was transmitted to two-leaf healthy seedlings 3–4 wk before the experiments began. CaMV-infected source plants were kept inside an aphid-free growth chamber at 26:20°C (day:night) and a photoperiod of 16:8 (L:D) h. Two-leaf, noninfected turnip seedlings grown under similar conditions were used as test plants for experiments. All test plants were sprayed after the inoculation access period with imidacloprid (Confidor, Bayer Hispania Industria, Barcelona, Spain) and placed in an aphid-free growth chamber for 3–6 wk to check for virus symptoms.

Aphid Probing and Feeding Behavior Associated with Inoculation of CaMV. Infected turnip plants used as virus sources were selected for consistency between batches and uniform appearance. A thin gold wire (20 μm in diameter) was attached to the dorsum of a young adult apterae aphid by immobilizing it with a vacuum-operated plate and touching the aphid with a small droplet of silver conducting paint (Pelco Colloidal Silver no. 16034, Ted Pella, Redding, CA). Aphids with the attached gold wire on their dorsum were placed on the youngest expanded leaf of a CaMV-infected plant for an acquisition access time of 8 h. Then, aphids were removed from the infected leaf and were connected to the EPG device after attaching the opposite end of the gold wire to a copper electrode (3 cm in length by 1 mm in diameter). A second electrode was connected to a copper post (0.2 cm in diameter by 10 cm in length), which was inserted into the plant pot.

After virus acquisition, aphids connected to the EPG device were placed on the youngest expanded leaf of a healthy test plant and removed after specific waveform patterns or inoculation periods were recorded. The following treatments were used: group I, first probe interrupted before the first intracellular puncture (pd) was produced; group II, probe interrupted after the first intracellular puncture ended; group III, probe interrupted after 5–10 intracellular punctures were produced; group IV, aphids removed after 5 min of inoculation access time on test plants; and group V, aphids removed after continuous phloem ingestion (EPG waveform E2 $>$ 15 min) during a 3-h inoculation access period. All aphids were then trans-

Table 1. EPG variables calculated for each treatment group in the study of behavioral events associated with inoculation of CaMV by *M. persicae* and *B. brassicae*

EPG variable	Abbreviation	Group II	Group III	Group IV	Group V
Total probing time (s)	T C duration	X*	X	X	X
Total pd time (s)	T pd duration	X	X	X	X
Total time of subphase II-1 of pd (s)	T II-1 duration	X	X	X	
Total time of subphase II-2 of pd (s)	T II-2 duration	X	X	X	
Total time of subphase II-3 of pd (s)	T II-3 duration	X	X	X	
Total no. of archlets	T no. of archlets	X	X	X	
Time from the beginning of the register until the beginning of first pd (s)	First np-first pd	X	X	X	
Time from the beginning of first probe until the beginning of first pd (s)	First C-first pd	X	X	X	
Time from the beginning of the register until the beginning of first probe (s)	First np-first C	X	X	X	
No. of pds	No. of pd	X	X	X	X
No. of probes	No. of C	X	X	X	X
Mean probing time (s)	Mean C duration	X	X	X	X
Mean of pd duration (s)	Mean pd duration	X	X	X	
Mean of subphase II-1 duration (s)	Mean II-1 duration	X	X	X	
Mean of subphase II-2 duration (s)	Mean II-2 duration	X	X	X	
Mean of subphase II-3 duration (s)	Mean II-3 duration	X	X	X	
Mean no. of archlets	Mean no. of archlets		X	X	
Time from the beginning of the register until the beginning of last pd (s)	First np-Lpd		X	X	
Time from the beginning of first probe until the beginning of last pd (s)	First C-Lpd		X	X	
Time from the beginning of last pd to the end of recording (s)	T Lpd-Z		X	X	
Total nonprobing time	T np duration				X
No. of E1	No. E1				X
No. of E2	No. E2				X
Total time in E1	TE1 duration				X
Total time in E2	TE2 duration				X
Duration of the last E1	TLE1 duration				X
Duration of the last E2	TLE2 duration				X
Time from the beginning of first probe until the beginning of first E1	First C-first E1				X
Time from the beginning of first probe until the beginning of E1 > 15 min	First C-E2 > 15 min				X
Time from the beginning of last probe until the beginning of last E1	TLC-LE1				X
Time from the beginning of last probe until the beginning of last E2 > 15 min	TLC-LE2 > 15 min				X
Time from the beginning of last pd until the beginning of last E1 followed by E2 > 15 min	TLpd-E1(E2 > 15 min)				X

* Indicates the variables that were calculated for each particular treatment group.

ferred to a second test plant for a 24-h inoculation access period after each specific treatment was completed. The second test plants were used to assess the initial virus acquisition rate as well as the retention capacity of the virus by the vector. Aphids that were unable to infect any of the two test plants were discarded from the analysis. A complete randomized design was used for the five treatment groups by using a minimum of 14 replicates per treatment.

EPG recordings were acquired at 100 Hz through a four-channel Giga-99 DC-amplifier. This 1 giga-ohm input resistance DC-amplifier system has its own AD converter, which allows direct real-time recording of the EPG signal onto the PC hard disk at the time that the EPG waveforms are displayed on the computer monitor. Data acquisition and screen display were controlled by Stylet 3.0 software (Tjallingii 1999), and data analysis was performed with MacStylet version 2.0 β10 (Febvay et al. 1996) software after data conversion.

Statistical Analysis. To correlate specific aphid behavioral events with their ability to inoculate CaMV, all the behavioral variables obtained by EPG recording under each treatment group (Table 1) from aphids that transmitted CaMV were compared with those from aphids that were unable to transmit the virus by means of a Mann–Whitney *U* test (when the variables followed a non-Gaussian distribution) or by an analysis of variance (ANOVA) test (for Gaussian variables). Pairwise comparisons between the transmission rate under the different treatments were analyzed using a χ^2 test and by Fisher's exact test when expected values were lower than 5. All analyses were conducted using StatView 4.0 software for Macintosh (Abacus Concepts 1992).

Table 2. Relationship between probing behavior of *M. persicae* and *B. brassicae* and inoculation of CaMV

Aphid species	*M. persicae*					*B. brassicae*				
Treatment group	C without pd	1 pd	5–10 pds	5-min inoculation access period	E2 > 15 min	C without pd	1 pd	5–10 pds	5-min inoculation access period	E2 > 15 min
Transmission rate (no. of infected plants/no. of test plants)	0% a (0/17)	40.7% b (11/27)	65.3% bc (17/26)	66.6% bc (16/24)	93.3% c (14/15)	0% a (0/19)	18.5% b (5/27)	48% c (12/25)	45.8% c (11/24)	92.8 d (13/14)
		χ^2	*P*				χ^2		*P*	
1 pd vs. 5–10 pd		3.228	0.072				5.127		0.02*	
1 pd vs. 5 min		3.428	0.064				4.403		0.035*	
1 pd vs. E2 > 15 min		11.07	0.0009*				20.687		<0.0001*	
1 pd vs. C without pd		9.235	0.0024*				3.948		0.04*	
5–10 pd vs. 5 min		0.009	0.923				0.23		0.879	
5–10 pd vs. E2 > 15 min		4.029	0.069				7.847		0.005*	
5–10 pd vs. C without pd		18.38	<0.0001*				12.54		0.0004*	
5 min vs. E2 > 15 min		3.69	0.11				8.403		0.0037*	
5 min vs. C without pd		18.587	<0.0001*				11.7		0.0008*	
E2 > 15 min vs. C without pd		28.207	<0.0001*				29.111		<0.0001*	

* Significant differences ($P < 0.05$) according to a χ^2 test and to Fisher's exact test when expected values were lower than 5.

Results

Transmission of CaMV by *M. persicae* and *B. brassicae* after Brief Inoculation Access Periods. For *B. brassicae*, the transmission rate after the first single intracellular puncture (pd) (18.5%) was significantly lower than after 5–10 punctures (48%) or when aphids were subjected to a 5-min access to test plants (45.8%) (Table 2). For *M. persicae*, the inoculation rate was rather high after the first intracellular puncture (40.7%) and also increased to 65.3% after 5–10 punctures and to 66.6% after a 5-min inoculation access period. For both vector species, infection was never detected on test plants where aphids were removed during the first probe before starting the first intracellular puncture (pd). Comparison of the transmission rate revealed no significant differences between the two aphid species, whatever the treatments studied (Table 3).

When pooling all the comparable data from all the brief inoculation access periods (a single pd, 5–10 pds, and a 5-min-inoculation access period), there were no significant differences between transmitters and nontransmitters for any of the EPG variables analyzed in the case of the experiments conducted with *M. persicae* (Table 4). However, for *B. brassicae*, the total number of pds was higher for aphids that inoculated CaMV compared with those that failed to inoculate the virus. Moreover, the variable time from the beginning of first probe until the beginning of first pd showed significant differences. Aphids that transmitted the virus produced the first pd faster than aphids that failed to transmit CaMV. The rest of the EPG variables analyzed showed no significant differences between the behavior of transmitters and nontransmitters (Table 4).

The capacity of retention of CaMV by aphids was estimated by counting the proportion of aphids transmitting the virus to the first test plant but not to the second. *B. brassicae* always retained CaMV after a single or after 5–10 intracellular punctures and after 5-min inoculation access period, whereas *M. persicae* often lost the virus after performing the same number of stylet punctures (Table 5). These data, together with the fact that *M. persicae* could inoculate the virus with high efficiency after a single pd, indicate that CaMV is more persistent in *B. brassicae* than in *M. persicae.* Thus, during short inoculation access pe-

Table 3. Statistics for the comparison between the transmission rate of CaMV by *M. persicae* and *B. brassicae* subjected to the different treatments

Treatment	χ^2	*P*
M. persicae 1 pd vs. *B. brassicae* 1 pd	3.197	0.073
M. persicae 5–10 pds vs. *B. brassicae* 5–10 pds	1.57	0.21
M. persicae 5 min vs. *B. brassicae* 5 min	2.116	0.1457
M. persicae E2 > 15 min vs. *B. brassicae* E2 > 15 min	0.002	>0.99

Significant differences ($P < 0.05$) according to a χ^2 test and to Fisher's exact test when expected values were lower than 5.

Table 4. Relationship between EPG parameters and the transmission of CaMV by *B. brassicae* and *M. persicae*

EPG variable	*B. brassicae*			*M. persicae*		
	Transmitters (mean ± SE)	Nontransmitters (mean ± SE)	*P*	Transmitters (mean ± SE)	Nontransmitters (mean ± SE)	*P*
No. pd	4.296 ± 0.514	2.486 ± 0.337	0.0027*	3.651 ± 0.411	2.588 ± 0.41	0.08
Mean pd duration (s)	6.802 ± 0.226	7.013 ± 0.394	0.827	5.025 ± 0.23	5.247 ± 0.277	0.6
Mean II-1 duration (s)	2 ± 0.97	2.118 ± 0.141	0.59	1.994 ± 0.15	1.807 ± 0.123	0.4
Mean II-2 duration (s)	1.25 ± 0.054	1.718 ± 0.221	0.08	0.982 ± 0.05	0.979 ± 0.069	0.65
Mean II-3 duration (s)	3.459 ± 0.19	3.364 ± 0.295	0.38	2.315 ± 0.187	2.66 ± 0.24	0.34
First np-first pd	91.62 ± 16.778	124.334 ± 18.331	0.269	128.403 ± 20.172	157.63 ± 35.025	0.62
First C-first pd	25.681 ± 5.296	58.252 ± 12.171	0.034*	52.043 ± 13.051	39.704 ± 14.91	0.11

Significant differences ($P < 0.05$) according to Mann–Whitney *U* test.

riods (5 min) and similar number of intracellular stylet punctures *M. persicae* releases CaMV from the stylet much faster than *B. brassicae.*

CaMV Transmission Rate during Long Inoculation Access Periods. The mean time for a successful penetration of phloem sieve elements by *B. brassicae* on susceptible brassicas is known to be 3 h (Cole 1994). For this reason, we allowed aphids 3 h to reach the phloem phase in our experimental setup. The phloem ingestion phase (E2) was recorded for at least 15 min before removing the aphids from the test plants. Aphids that were unable to reach the phloem phase on the test plant after a 3-h interval were transferred to a second test plant to assess their rate of transmission.

The proportion of aphids reaching the phloem phase (first E1 waveform) in the 3-h period was 70.4% (31/44) for *M. persicae* and 58.2% (32/55) for *B. brassicae.* The transmission rate for aphids inoculating CaMV after a sustained phloem ingestion phase was 93.3% (14/15) for *M. persicae* and 92.8% (13/14) for *B. brassicae* (Table 2). For *M. persicae*, no significant differences were found between aphids that were able to perform a sustained phloem ingestion phase and those that were exposed to shorter inoculation access periods (Table 2). The transmission rate obtained by individuals of *M. persicae* that were unable to reach the phloem phase after a 3-h interval was 60% (three-fifths). However, for *B. brassicae*, a prolonged inoculation access period of 3 h significantly increased the rate of transmission. This increase was similar whether the aphids failed to reach the phloem and remained under stylet pathway activities (100%, 12/12) or were able to reach a sustained phloem ingestion phase (92.8%, 13/14). The average number of potential drops for aphids that reached continuous phloem ingestion and for those that failed to reach the phloem phase during the 3-h inoculation access period was rather similar, 48.8 ± 9.2 and 40.6 ± 6.4 (mean ± SE), respectively. Pairwise comparisons between the behavior of transmitters and nontransmitters subjected to a continuous phloem ingestion phase did not reveal any significant differences. There were no significant differences ($P > 0.05$) in the transmission rate between aphid species after a continuous phloem access period (Table 3).

Table 5. Proportion of aphids transmitting the virus to the first but not to the second test plant (no. of cases/total no. of aphids)

Treatment	Transmission			
	M. persicae	*B. brassicae*	χ^2	*P*
1 pd	4/27	0/27	4.32	0.05*
5–10 pds	17/26	0/25	15.09	<0.0001*
5-min inoculation access period	16/24	0/24	9.6	0.0019*

Significant differences ($P < 0.05$) according to a χ^2 test and to Fisher's exact test when the expected values were lower than 5.

Discussion

The relationship between CaMV and its various vector species has always been difficult to classify under one of the established classical types of noncirculative transmission (nonpersistent or semipersistent) (Sylvester 1962, Harris 1983). In fact, there have been contradicting results, depending on the methodology and the aphid species used in the transmission studies. All earlier studies were conducted by calculating vector efficiency after fixed acquisition or inoculation access time periods. However, aphid behavior is very unpredictable and probing or feeding activities leading to virus transmission does not always occur at the same precise time interval. Electrical recording of aphid stylet activities allows real-time monitoring of the specific behavioral events leading to virus transmission. In the last decade, it has become the most powerful and reliable tool for understanding and elucidating the mechanisms of transmission of plant viruses (Prado and Tjallingii 1994, Martin et al. 1997, Palacios et al. 2002).

In the present work, we used an EPG device and revealed that differences exist in the way CaMV is transmitted by its two major vectors, *M. persicae* and *B. brassicae.* First, the inoculation rate after a single intracellular puncture was higher for *M. persicae* (40.7%) than for *B. brassicae* (18.5%) ($P = 0.073$) and then increased by 25–30% as the number of pds increased to 5–10 for *M. persicae* and *B. brassicae*, respectively (Table 2). Second, we observed that the persistence and retention capacity of CaMV varied depending on the vector species. *B. brassicae* always retained CaMV after producing one or more intracellular probes in the test plants (whether the virus was transmitted or not), whereas *M. persicae* often lost the virus after performing the same number of cell

membrane punctures (Table 5), suggesting that CaMV is more persistent in *B. brassicae* than in *M. persicae.* These results are consistent with those reported by Chalfant and Chapman (1962), who showed a longer retention time of CaMV by *B. brassicae* than by *M. persicae* after a series of postacquisition feeding or starvation periods.

CaMV can be acquired from nonphloem tissues, but the probability of acquisition was significantly higher when aphids reached the continuous phloem ingestion (Palacios et al. 2002) However, transmission rate did not increase when viruliferous aphids reached the phloem phase during inoculation probes. Data in Table 2 show that individuals of *B. brassicae* reaching the continuous phloem ingestion phase actually transmitted the virus more efficiently than those producing a limited number of potential drops (5–10). However, the increase in transmission rate cannot be attributed to stylet activities within the phloem tissues, because aphids that never reached the phloem phase during the 3-h inoculation access period transmitted CaMV equally well (13/14 versus 12/12).

During CaMV inoculation, we found that the total duration of the potential drops was not related to the success in transmission, although for *B. brassicae*, individuals that transmitted CaMV produced a higher number of cell punctures than those that failed to transmit the virus (Table 4). In contrast, previous work on CaMV acquisition indicated that the total duration of intracellular punctures was one of the variables that best explained the probability of subsequent transmission of the virus. Collar et al. (1997) and Powell et al. (1995) suggested that the duration of intracellular stylet punctures was related to the volume of sap ingested by an aphid, increasing the chances of acquisition of a given virus from an infected cell. It is also known that the duration of the subphase of the potential drop leading to virus acquisition (II-3) is variable and is often longer during the first intracellular punctures. However, the duration of subphase II-1 of the potential drop leading to virus inoculation is very short and does not vary over successive intracellular punctures (Collar and Fereres 1998). Therefore, it is not surprising that the success in acquisition of CaMV is associated with a longer duration of intracellular punctures, whereas inoculation of the virus is not. The occurrence of a series of consecutive intracellular punctures, but not their individual duration, is the key factor leading to the inoculation of CaMV. In *M. persicae* the first potential drop was enough to obtain a high transmission rate, which further increased after additional potential drops within the stylet pathway phase. Moreover, the transmission rate of CaMV obtained by *M. persicae* after completion of the first inoculation stylet puncture (40%) is similar to the one obtained for nonpersistent viruses such as Potato virus Y and Cucumber mosaic virus (Martin et al. 1997).

The differences in the retention times of nonpersistent and semipersistent viruses such as CaMV are rather more quantitative than qualitative, and possibly the stability of the transmissible complex within the aphid's cuticle at the tip of the stylets is what really determines the degree of persistence of the virus. So, both nonpersistent and semipersistently transmitted viruses could share similar retention sites in the tip of the aphid's maxillary stylets. The hypothesis that the retention sites of nonpersistent viruses are located on the stylet tips is not new and was supported by a series of early experiments conducted long ago that showed that viruliferous aphids lost their transmission ability after treating their stylets with formaldehyde and UV (Bradley and Ganong 1955a, b). However, treatment of stylets with formaldehyde prevented transmission of CaMV by *B. brassicae* after short acquisition access periods but did not abolish transmission after long acquisition periods, although there was a decline in transmission rate as concentration of formaldehyde was increased (Chalfant and Chapman 1962). Therefore, the existence of a second receptor site located behind the stylet tips cannot be excluded.

Aphids produce two types of saliva during the interaction with their host plants: the gelling saliva, produced extracellularly during stylet pathway; and the watery saliva, secreted intracellularly during cell punctures. The composition and properties of the saliva may vary between different aphid species and seems to be associated with processes such as inoculation of nonpersistent virus by aphids and inactivation of plant resistance to aphid feeding and virus transmission (Miles 1999). The reducing properties of watery saliva may facilitate the release of nonpersistent viruses into the cytoplasm soon after stylet penetration of the plasmalemma. It has been suggested that differences in the composition of watery saliva may explain why Cucumber mosaic virus can be transmitted by *M. persicae* but not by *Aphis gossypii* Glover to resistant melons carrying the *Vat* gene (Chen et al. 1997). Also, qualitative or quantitative differences in the composition of watery saliva of *M. persicae* and *B. brassicae* could explain why the virus is inoculated more efficiently by the former than the latter after identical behavioral events.

At present, and considering the success in transmission after the first intracellular stylet punctures, we conclude that CaMV is inoculated in a similar manner as nonpersistent viruses, and therefore intracellular salivation is very likely involved in the inoculation process. Studies conducted so far on the behavioral events associated with transmission of noncirculative and circulative viruses by aphids suggest that the ingestion–salivation mechanism is the general rule. Furthermore, recent findings reported by Powell (2005) by using inoculation of circulatively transmitted Pea enation mosaic virus as a marker confirms that injection of saliva directly into the cytoplasm occurs during subphase II-1 of the first intracellular stylet puncture on epidermal cells.

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