Journal of General Virology (2002), **83**, 3163–3171. Printed in Great Britain

Cauliflower mosaic virus is preferentially acquired from the phloem by its aphid vectors

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Cauliflower mosaic virus* (CaMV) is transmitted in a non-circulative manner by aphids following the helper strategy. Helper proteins P2 and P3 act as a bridge between virions and the aphid cuticle. Electronic monitoring of aphid stylet activities (EPG technique), transmission tests and electron microscopy showed that CaMV is preferentially acquired from the phloem by its most common aphid vectors, *Brevycorine brassicae* and *Myzus persicae*. We also found that CaMV is semi-persistently transmitted and that the rate of acquisition does not follow a typical bimodal curve. Instead, the virus could be acquired from non-phloem tissues at a low and fairly constant rate after one or more intracellular punctures within a few minutes, but the probability of acquisition rose significantly when aphids reached the phase of committed ingestion from the phloem. The acquisition rate of CaMV did not increase with increasing number of intracellular punctures, but the total duration of intracellular puncture was one of the variables selected by the stepwise logistic regression model used to fit the data that best explained acquisition of CaMV. Furthermore, aphids reaching the phloem faster had a higher probability of acquiring the virus. Our results support the hypothesis that multiple intracellular punctures of epidermal and mesophyll cells result in loading aphids with the CaMV-encoded aphid transmission factor (P2), and that aphids, in most cases, subsequently acquire CaMV particles during phloem sap ingestion. Consistently, immunoelectron microscopy showed that P3–virions are frequently found in the sieve element lumen, whereas P2 could not be detected.

Introduction

Cauliflower mosaic virus (CaMV), the type member of the genus *Caulimovirus*, is transmitted by aphids in a non-circulative manner, meaning 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 (Gray & Banerjee, 1999).

CaMV uses the 'helper strategy' for the transmission process (Pirone & Blanc, 1996). The helper strategy implies that a non-structural viral protein (helper component, HC) mediates the interaction for attachment, and thus retention, of viral particles in the cuticle of the aphid stylet. For CaMV, the HC is the viral protein P2, which binds with one domain to a non-identified attachment site in the stylet and with another domain to the viral protein P3 associated with the virus capsid (Blanc *et al.*, 2001; Leh *et al.*, 1999, 2001; Woolston *et al.*, 1987). In order to be transmitted, a P2–P3–virion complex must form. However, in infected plant cells P2 and P3–virions are mainly sequestered in two different viral inclusion bodies. While electron-dense inclusion bodies (edIBs) contain 96% of the total number of virions associated with P3 and no P2, the electron-lucent inclusion bodies (elIBs) contain all of P2, some P3, and the remaining 4% of virus particles (Drucker *et al.*, 2002; Espinoza *et al.*, 1991). Consequently, it has been suggested that transmissible complexes do not predominantly form in plant cells but in the aphid mouthparts, where the P2 and P3-virion pools are bound during the aphids' feeding activities.

There are at least 27 aphid species listed as known vectors of CaMV (Kennedy *et al.*, 1962), but the main vectors in the

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field are *Brevicoryne brassicae* L. and *Myzus persicae* Sulzer (Broadbent, 1957). Markham *et al*. (1987) demonstrated that CaMV is transmitted in a semipersistent manner, although conflicting results were obtained in earlier work: Some authors have considered CaMV to be an atypical non-persistently (Hamlyn, 1955; van Hoof, 1958) or bimodally transmitted virus (Bouchery *et al*., 1990; Chalfant & Chapman, 1962). The term bimodal was proposed to describe the transmission rate of CaMV by *B. brassicae* as a curve with two peaks associated with optimum acquisition-access times of around 5 min and 8 h. This bimodal type of transmission has been described for only one other plant virus, the potyvirus *Pea seed-borne mosaic virus* (Lim & Hagedorn, 1977). However, the term bimodal is somehow misleading, because the optimum acquisition peaks of CaMV may vary and exhibit a bi- or multiphasic pattern depending on the species of vector used for the transmission experiments (Markham *et al*., 1987).

Electrical systems for monitoring insect probing and feeding behaviour provide detailed analysis of aphid stylet penetration and the vector–plant interactions involved in the transmission of plant viruses (see reviews by Fereres & Collar, 2001; Tjallingii & Prado, 2001). Using DC-amplifier monitoring systems, researchers can correlate recorded waveform patterns (so-called electrical penetration graphs, EPG) with specific stylet positioning and feeding activities of the insect associated with the transmission plant viruses. Acquisition of typical non-persistent viruses by aphids occurs during the last subphase (II-3) of superficial intracellular stylet puncture (Martin *et al*., 1997; Powell *et al*., 1995), while persistently transmitted viruses are acquired during the phloem ingestion phase (Prado & Tjallingii, 1994). However, information on specific probing or feeding behavioural events (or EPG waveforms) associated with the acquisition of semipersistent viruses by aphids is lacking.

Semipersistently transmitted viruses include members of seven genera (*Badnavirus*, *Caulimovirus*, *Closterovirus*, *Sequivirus*, *Trichovirus*, *Waikavirus* and *Crinivirus*) that are transmitted by aphids or other homopterans (Hull, 2001; Powell *et al*., 1995). The best-known semipersistent viruses are caulimoviruses and closteroviruses. The closterovirus *Beet yellows virus* (BYV) is found in the phloem (Esau *et al*., 1967), while caulimoviruses are present in most plant tissues (Francki *et al*., 1985; Kitajima *et al*., 1969). Limburg *et al*. (1997) found that the time threshold needed for acquisition of BYV was consistent with the mean time for aphids to reach the phloem. However, there is no experimental evidence showing whether caulimoviruses are acquired from epidermis, mesophyll or from vascular tissues.

A prerequisite for elucidation of the transmission mechanism of CaMV is a better characterization of the acquisition phase. In the present study, we investigated the probing and feeding behaviour of *B. brassicae* and *M. persicae* during the acquisition of CaMV using the EPG technique. The goal was to find out from which specific plant tissue(s) aphids usually acquire CaMV as well as to investigate aphid probing and feeding behavioural patterns associated with acquisition of the virus. We also compared the probing behaviour of the two main aphid vectors after short and long acquisition access periods to clarify the discrepancies seen in previous reports on the transmission mechanism of CaMV. To complement this study, we used immunoelectron microscopy to detect CaMV virions, P2 and P3 in the vascular tissue of infected plants.

Methods

Virus isolates, aphid clones and test plants. The aphid-transmissible isolate Cabb-S (Franck *et al*., 1980) was used in our study. The virus isolate was maintained in turnip plants (*Brassica rapa* L. cv. 'Just Right') and propagated by aphid transmission (*B. brassicae*). The virus was aphid-transmitted to healthy test plants (two-leaf stage) which were used as CaMV-source plants for transmission experiments 3–4 weeks later. Infected turnip plants were selected for consistency between batches and uniformity of symptom appearance. The last expanded leaf showing vein clearing symptoms was used to place aphids for virus acquisition experiments. All infected plants were kept inside an aphid-free growth chamber at 26/20 °C (day/night), and a photoperiod of 16/8 h (light/dark).

Non-viruliferous aphid clones of *M. persicae* and *B. brassicae* were reared under controlled conditions [(22/18 °C and 14/10 h (light/dark)] on *Brassica rapa* cv. 'Just Right'. The clones of *M. persicae* and *B. brassicae* were started from single virginiparous females collected at Alcala de Henares and Villa del Prado (Madrid, Spain), respectively.

Fifteen-day-old seedlings of *Brassica rapa* cv. 'Just Right' were used as test plants for all experiments because they show very clear symptoms when infected with CaMV. The seedlings were sprayed with Confidor (Bayer Hispania Industria) after the inoculation access period (18–24 h), and transferred to the aphid-free growth chamber where they were checked regularly for symptoms during a 3–5 week period.

Transmission efficiency of CaMV by its main vectors. The transmission efficiency of CaMV by *M. persicae* and *B. brassicae* was first tested under laboratory conditions. For this set of experiments, aphids were not connected to the EPG device. The transmission procedure was similar to the one described by Fereres *et al*. (1993). Groups of 25–30 aphids (young apterae adults) were placed inside plastic cages for a 1 h pre-acquisition period. Then, the last expanded leaf of a young infected turnip plant was detached from the plant and used for virus acquisition. Twenty aphids at a time were allowed to acquire the virus from the infected leaf. After a 5 min or 8 h acquisition access period (two different treatments), groups of five aphids were transferred to each turnip test plant for an 18–24 h inoculation access period.

EPG recording set-up. A gold wire (3 cm long × 20 mm 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 Inc., Reeding, CA, USA). The other end of the gold wire was attached to a copper wire (3 cm long × 1 mm diameter) which was connected to one of the electrodes of the EPG system. A second electrode was connected to a copper post (0·2 cm diameter × 10 cm long) which was inserted into the plant pot (Tjallingii, 1990).

EPG recordings were acquired at 100 Hz through a 4-channel Giga-99 DC-amplifier. This 1 giga-ohm input resistance DC-amplifier system has its own AD converter, which allows direct recording of the EPG

Table 1. EPG variables analysed for each of the treatments used to study behavioural events associated with acquisition of CaMV by *M. persicae* and *B. brassicae*

Treatment/EPG variables studied	Abbreviation
(a) Acquisition until the first intracellular puncture (= potential drop, pd) was recorded	
Time elapsed from the beginning of the 1st probe until the beginning of the 1st pd (s)	1^{st}C–1^{st}pd
Duration of pd (s)	pd duration
Duration of subphase II-1 of the pd (s)	II1 duration
Duration of subphase II-2 of the pd (s)	II2 duration
Duration of subphase II-3 of the pd (s)	II3 duration
Number of archlets	No. of archlets
(b) Acquisition during a 5 min access period	
Time from the beginning of the 1st probe until the beginning of the 1st pd (s)	1^{st}C–1^{st}pd
Total probing time (s)	T C duration
Number of pds	No. of pds
Total time on pd (s)	T pd duration
Time from the last pd to the end of recording	T Lpd–Z
Total number of archlets	T no. of archlets
(c) Acquisition during a 30 min access period	
Number of pds	No. of pds
(d) Acquisition until committed phloem ingestion (E2 > 15 min) was recorded	
Total probing time (min)	T C duration
Total non-probing time (min)	T np duration
Number of pds	No. of pds
Total time in pd (min)	T pd duration
Mean time in pd (min)	T pd/no. of pds
Time from last pd until E1 followed by E2 > 15 min	T Lpd–E1(E2 > 15 min)
Number of E1	No. of E1
Number of E2	No. of E2
Time from the beginning of first probe until the beginning of first E1	1^{st}C–1^{st}E1
Time from the beginning of first probe until the last E1	1^{st}C–LE1
Time from the beginning of first probe until E2 > 15 min	1^{st}C–E2 > 15 min
Duration of E1 followed by E2 > 15 min	TLE1
Duration of last E2	TLE2
Time from the beginning of first probe until first E2 < 15 min	1^{st}C–1^{st}E2 > 15 min
Total time in E1	TE1 duration
Total time in E2	TE2 duration
Time from last C until last E2 > 15 min	TLC–LE2 > 15 min

signals 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 and data analysis was performed with MacStylet v2.0 β10 (Febvay *et al.*, 1996) software after data conversion.

EPG recording during acquisition of CaMV. To investigate the behavioural events associated with acquisition of CaMV, aphids were first connected to the EPG device and then placed on a CaMV-infected source plant. Aphid probing was artificially interrupted by removing the aphid from the infected plant with a paintbrush just after the following events: (a) aphids were allowed to make a single intracellular puncture (= potential drop, pd) during the first probe. Aphids that were unable to make an intracellular puncture within 3 min of the beginning of the first probe were discarded; (b) aphids were allowed a 5 min acquisition access period starting after the beginning of the first probe; (c) aphids were allowed a 30 min acquisition access period starting after the beginning of the first probe; (d) aphids were allowed to carry out a committed phloem ingestion phase (E2 15 min). Aphids that were unable to reach the phloem within 3 h were discarded. To reduce the time to reach the phloem, aphids were placed on the abaxial surface of the last expanded leaf.

Groups of starved (for 1 h before virus acquisition) and non-starved aphids were used for the studies described under treatments (a) and (b). Table 1 shows the specific EPG variables that were calculated and analysed for each of the treatments described above.

Detection of CaMV particles, P2, and P3 in plant tissue by immunoelectron microscopy. Preparation of samples and immunogold labelling was essentially as previously described (Drucker *et al.*, 2002). Briefly, small pieces of Cabb-S-infected turnip leaves containing class I and II veins were fixed for 4 h at room temperature after vacuum infiltration with 50 mM sodium cacodylate buffer (pH 7·4) containing 0·5% glutaraldehyde and 2·0% paraformaldehyde. Samples were embedded in Unicryl Resin (TEBU) and polymerized at 4 °C under UV light

for 2 days. Ultrathin sections on grids were quenched with 50 mM NH_4Cl in PBS and blocked for 30 min in TBS complemented with 0·1% Tween 20 and 5% skim milk powder. Incubation with primary antiserum (1:25 for mouse P2 and 1:50 for rabbit P2 and P3 antisera) was for 1 h in the same buffer. After washing with TBS complemented with 0·1% Tween 20 and 0·5% skim milk powder, the sections were incubated with gold-conjugated secondary antibodies (30 nm particle rabbit anti-mouse and 10 nm particle goat anti-rabbit) for 1 h, rinsed extensively with TBS complemented with 0·1% Tween 20 and then with distilled water before contrasting with 2% uranyl acetate and 1% lead citrate. Grids were observed in a Zeiss EM 10C RC electron microscope operated at 60–80 kV.

Statistical analysis. Transmission rate, calculated as a percentage, was compared among the different treatment groups using a χ^2 analysis (Abacus Concepts, 1989) or by Fisher's Exact test (SAS Institute, 1996) when the expected values were lower than 5. The formula of Gibbs & Gower (1960) was used to calculate the probability of transmission by a single aphid when groups of aphids were used to determine transmission efficiency.

The behavioural variables obtained by EPG recording from aphids that transmitted CaMV were compared with those from aphids that did not transmit by means of a Mann–Whitney U test, because these variables followed a non-Gaussian distribution. These comparisons allowed us to correlate specific aphid behavioural events with their ability to acquire CaMV.

The 17 behavioural variables calculated for treatment d (Table 1) were introduced into a stepwise-forward logistic regression model (Afifi & Clark, 1990), using SPSS computer software (SPSS, 2001), to determine which were really critical for acquisition of CaMV. All EPG data obtained for transmitters and non-transmitters of both aphid species under treatment d (51 recordings) were pooled. The probability function used was of the type $P(C) = e^u/1+e^u$, where P(C) represents the probability of acquisition of CaMV. The function $u = b_o + b_1 f_1(X_1) + b_2 f_2(X_2) + \ldots 7 + b_n f_n(X_n)$ includes the 16 behavioural variables that determine virus acquisition according to the model. The model also calculates the constant values (b_i) and includes the transformations (f_i) necessary to achieve normality of the variables. The usefulness of the stepwise logistic regression method is to determine which of the variables considered in the experiment are most appropriate to estimate the probability for virus acquisition.

Results

Transmission efficiency of CaMV after short and long acquisition access times

B. brassicae transmitted CaMV at a significantly ($P < 0·05$) higher rate after long (8 h) compare with short (5 min) acquisition access periods (Table 2), whereas for *M. persicae* no such difference was found. However, comparison of transmission efficiencies between the two aphid species after short and long acquisition periods revealed no significant differences. When the transmission efficiency data were expressed as one aphid per test plant, the calculated values using the Gibbs and Gower formula were 13% and 41% for *B. brassicae* and 18% and 29% for *M. persicae*, after short and long acquisition access periods, respectively. These results showed that the connection of aphids to an EPG device did not reduce the ability of aphids to acquire CaMV as indicated by the similar transmission rates obtained when single aphids acquired the virus during EPG recording (see below).

Table 2. Comparison of rate of CaMV transmission (%) by *B. brassicae* and *M. persicae* after short and long acquisition access periods

Experiments were done with five aphids per plant. Numbers in parentheses indicate the no. of infected plants/total no. of test plants.

Aphid species	Acquisition time		
	5 min	8 h	χ^2/P
B. brassicae	50·0 (14/28)	92·8 (26/28)	**12·6/0·001***
M. persicae	64·3 (18/28)	82·1 (23/28)	**2·28/0·13**
χ^2/P...	**1·17/0·28**	**1·47/0·22**	

* Significant differences at the $P = 0·05$ level according to a χ^2 test or by Fisher's Exact test (when the expected values were lower than 5).

Acquisition of CaMV by aphids after a single intracellular puncture and after a short acquisition access period

The transmission rate after a single intracellular puncture was always very low (< 13%) (Table 3). There were no significant differences ($P < 0·05$) in the transmission rate of CaMV between fasted and non-fasted *B. brassicae* or *M. persicae* after single intracellular punctures, although the duration of intracellular punctures was significantly longer for fasted aphids. The probing behaviour of both aphid species was significantly different during the recording of single intracellular punctures: *B. brassicae* produced longer potential drops than *M. persicae* due to an increase in the duration of the II-3 subphase. Also, *B. brassicae* needed more time than *M. persicae* to produce the first pd after the beginning of the probe. However, we found no correlation between the EPG variables analysed and the acquisition of CaMV during single intracellular punctures (data not shown).

No significant differences were found in the transmission rate of CaMV by aphids that were allowed to produce a single intracellular puncture and those that were permitted a 5 min acquisition access period on CaMV-infected plants, except for the group of non-starved *B. brassicae* (2·3% vs 22·0%; $P = 0·029$). In all cases, the transmission rate was low, and always below 22%. (Table 3). The transmission rate obtained for aphids subjected to a 30 min acquisition access period was also low: 20·4% (11/54) for *B. brassicae* and 12·5% (5/40) for *M. persicae*.

There were significant differences ($P < 0·05$) in the probing behaviour between the two aphid species during the 5 min acquisition access period. We found that the total probing time (251·6 ± 10·37 s vs 110·1 ± 11·18 s, $P < 0·05$), the probing

Table 3. Transmission efficiency (%) of CaMV by *B. brassicae* and *M. persicae* after a single intracellular puncture (pd) and after a 5 min acquisition access period

Experiments were done with one aphid per test plant. Numbers in parentheses indicate the no. of infected plants/total no. of test plants. *P* values were calculated using a χ^2 test or Fisher's Exact test (when the expected values were lower than 5).

	Single pd			5 min		
	Starved		Not starved	Starved		Not starved
B. brassicae	12·8 (6/47)		2·3 (2/44)	19·1 (9/47)		22·0 (13/59)
P value		0·27			0·71	
M. persicae	4·8 (2/42)		7·3 (3/41)	13·3 (8/60)		15·1 (8/53)
P value		0·67			0·85	

Table 4. Relationship between EPG variables and CaMV transmission by *B. brassicae* and *M. persicae* allowed a 5 min acquisition access period on infected plants

Experiments were done with one aphid per plant. Data for groups of aphids that were starved and not starved were pooled.

	B. brassicae Transmission:			*M. persicae* Transmission:		
EPG variable	Yes ($n = 22$)	No ($n = 85$)	P^1	Yes ($n = 16$)	No ($n = 97$)	P^2
1^{st}C–1^{st}pd (s)	23·4 ± 4·6	55·5 ± 6·4	*	18·9 ± 6·6	15·0 ± 2·4	NS
T C duration (s)	222·8 ± 17·8	242·4 ± 8·6	NS	74·6 ± 9·9	131·7 ± 9·4	*
No. of pds	2·9 ± 0·30	2·5 ± 0·17	NS	2·4 ± 0·27	2·5 ± 0·14	NS
T pd duration (s)	17·2 ± 1·7	16·1 ± 1·1	NS	13·7 ± 1·4	12·6 ± 0·70	NS
TLpd–Z (s)†	68·8 ± 14·5	62·7 ± 6·7	NS	12·2 ± 4·8	34·7 ± 4·6	NS
T no. of archlets†	6·2 ± 0·9	6·3 ± 0·58	NS	7·9 ± 1·3	6·0 ± 0·45	NS

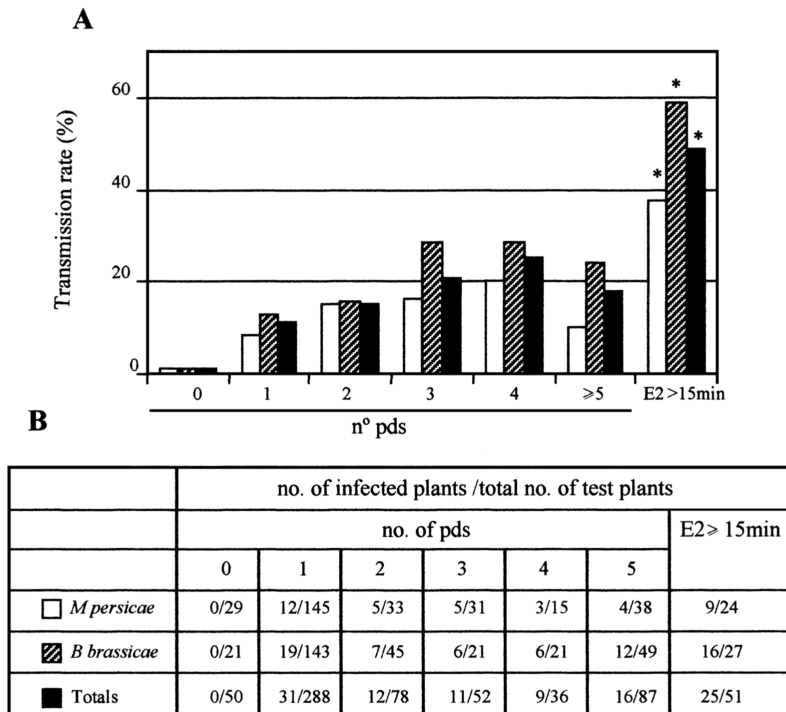
* Indicates significant difference ($P < 0·05$) according to a Mann–Whitney U test. P^1, pair-wise comparison between transmitters vs non-transmitters when using *B. brassicae*; P^2, pair-wise comparison between transmitters vs non-transmitters when using *M. persicae*. NS, Not significant.
† EPG variables that best explained acquisition of non-persistent viruses according to Collar *et al.* (1997).

time until the first pd was produced (52·1 ± 6·98 s vs 14·2 ± 3·05 s, $P < 0·05$), the duration of intracellular punctures (17·3 ± 1·49 s vs 11·4 ± 0·81 s, $P < 0·05$) and the time from the last pd to the end of the probe (63·7 ± 8·83 s vs 23·9 ± 4·86 s, $P < 0·05$) was significantly longer for *B brassicae* than for *M. persicae*. Also, the number of intracellular punctures was larger for *B. brassicae* than for *M. persicae* (2·8 ± 0·24 vs 2·2 ± 0·17, $P < 0·05$). In spite of the apparent behavioural differences observed between the two aphid species, there were no significant differences in their ability to transmit CaMV after a 5 min acquisition access time.

EPG variables that best explained the acquisition of non-persistent viruses during a 5 min acquisition access period were irrelevant for the acquisition of CaMV (Table 4). The time from the beginning of the probe until the first pd was produced (1^{st}C–1^{st}pd) was the only EPG variable that showed significant differences between the group of *B. brassicae* that was able to transmit and the one that failed to transmit CaMV. Aphids that transmitted CaMV produced the first pd faster than aphids that failed to transmit the virus. In the case of *M. persicae*, aphids that were able to acquire CaMV spent less time probing than the ones that did not transmit the virus (Table 4).

Acquisition of CaMV during committed phloem ingestion

The phloem access period to CaMV-infected plants under treatment d was standardized to 3 h because that was the time previously reported for successful penetration of phloem sieve elements by *B. brassicae* on susceptible brassicas (Cole, 1994). When aphids reached the phloem ingestion phase (pattern E2),



	no. of infected plants /total no. of test plants						
	no. of pds						E2≥ 15min
	0	1	2	3	4	5	
□ M persicae	0/29	12/145	5/33	5/31	3/15	4/38	9/24
▨ B brassicae	0/21	19/143	7/45	6/21	6/21	12/49	16/27
■ Totals	0/50	31/288	12/78	11/52	9/36	16/87	25/51

Fig. 1. (A) Relationship between the number of intracellular punctures and committed phloem ingestion produced by *B. brassicae* and *M. persicae* and the transmission rate of CaMV. *Indicates significant differences ($P < 0{\cdot}05$) for pair-wise comparisons of the transmission rate among different classes according to a χ^2 analysis, or Fisher's Exact test when the expected values were lower than 5. (B) Ratio between the number of infected plants and the total number of test plants under the different treatments used (see Methods). The number of potential drops recorded for all aphids under treatments a, b and c were pooled.

the EPG signal was recorded for at least a further 15 min (= committed phloem ingestion) and then aphids were transferred to test plants. The transmission rate for aphids acquiring CaMV during committed phloem ingestion was 59·2% (16/27) in the case of *B. brassicae* and 37·5% (9/24) for *M. persicae*. These results showed that committed ingestion from the phloem was the key factor that significantly increased the acquisition rate of CaMV. The EPG data obtained for aphids under treatments a, b and c were analysed to find out the relationship between the number of potential drops and the transmission rate of CaMV by *B. brassicae* and *M. persicae*. Aphids that reached the phloem and ingested for at least 15 min were able to acquire the virus more efficiently than the groups of aphids that produced one, two, three, four or five or more intracellular punctures without reaching the phloem phase (Fig. 1). Pair-wise comparisons between the behaviour of transmitters and non-transmitters subjected to treatment d also revealed significant differences. Aphids that transmitted CaMV spent significantly ($P < 0{\cdot}05$) less time to reach the phloem ($1^{st}C–1^{st}E1 = 59{\cdot}5 \pm 8{\cdot}2$ min; $1^{st}C–1^{st}E2 < 15$ min $= 68{\cdot}2 \pm 9{\cdot}6$ min) than aphids that failed to transmit the virus ($1^{st}C-1^{st}E1 = 79{\cdot}0 \pm 8{\cdot}0$ min; $1^{st}C–1^{st}E2 < 15$ min $= 85{\cdot}1 \pm 7{\cdot}2$ min). No significant differences between the behaviour of transmitters and non-transmitters were obtained for the rest of the EPG variables analysed. Furthermore, the stepwise logistic regression analysis indicated that aphids reaching the phloem faster had a higher probability of acquiring CaMV. The regression model identified three behavioural variables that best explained the acquisition of CaMV. These EPG variables were the total duration of intracellular punctures within the 3 h of recording (Tpd duration $= X_1$), the time until the first phloem contact was detected ($1^{st}C-1^{st}E1 = X_2$) and the time elapsed until committed phloem ingestion ($1^{st}C–E2 >$ 15 min $= X_3$). The following function explained 80% of the cases analysed: $Z = 0{\cdot}634 + 0{\cdot}503\ X_1 - 0{\cdot}012\ X_2 - 0{\cdot}031\ X_3$. Addition of any of the 14 remaining EPG variables analysed did not significantly improve the performance of the model.

Detection of P2, P3 and virions in infected turnip leaves

As the transmission rate rose significantly when aphids were allowed access to the phloem in addition to making superficial probes, we were interested to know which components of the transmissible complex were found in the phloem. We performed immunoelectron microscopy to detect P2, P3 and virus particles in the vascular tissues. Fig. 2 shows that, as previously reported (Kitajima *et al*., 1969), virus particles were easily observed in the phloem sieve cell lumen, either free or as clusters seemingly associated with membranous or fibrillar material (Fig. 2A), or in tubule-like elements (Fig. 2B). The virions were repeatedly gold-labelled using P3 antiserum, although the labelling was weaker than in electron-dense inclusions of mesophyll cells (not shown). In contrast, despite the use of different P2 antisera and extensive observation, we were unable to detect any P2-label in the lumen of sieve cells, though P2 was readily detected in electron-lucent inclusion bodies of epidermal, mesophyll and companion cells (not shown). We conclude that P2, unlike P3–virions, is probably absent from phloem sieve cells.

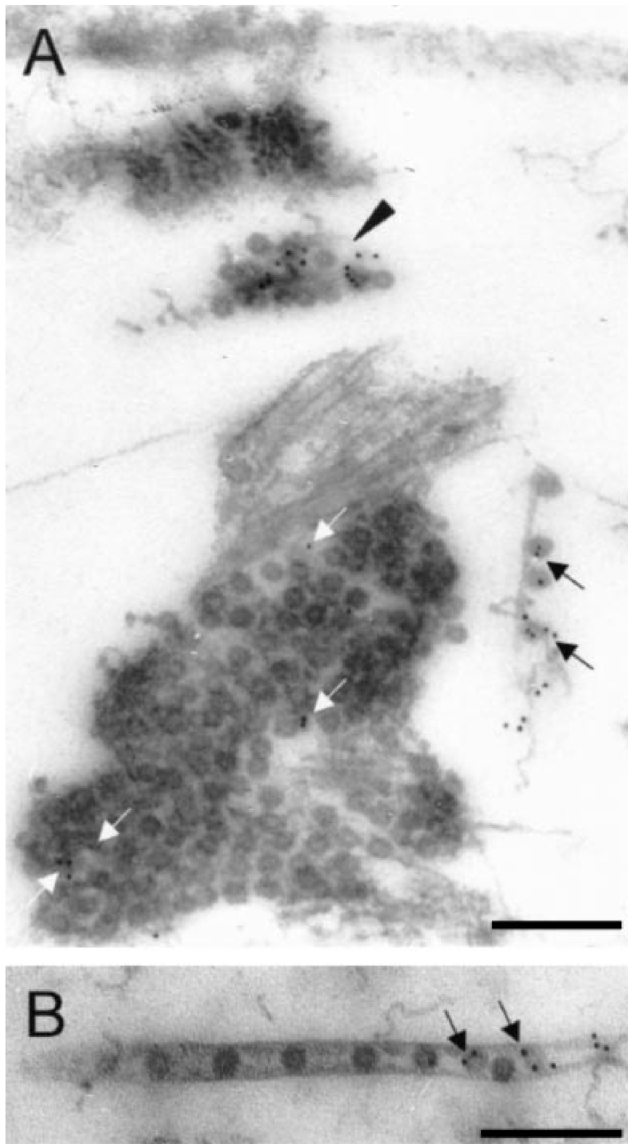


Fig. 2. Detection and localization of P2, P3 and virus particles in sieve cell lumen of infected plants. The micrographs show sections double-labelled with P2 (30 nm gold) and P3 (10 nm gold) antisera. Only the P3 antiserum (arrows) reacted with CaMV particles that are found (A) in clusters, in association with fibrillar material or seemingly free (arrowhead), or (B) in tubule-like structures, probably deriving from companion/sieve cell plasmodesmata through which the virus moves from cell to cell in similar tubules. Bar, 250 nm.

Discussion

Our results do not confirm a typical bimodal type of transmission for CaMV, as observed by Chalfant & Chapman (1962) when using *B. brassicae*, but rather demonstrated that there was only one peak for virus acquisition that occurred when aphids reached the committed phloem ingestion phase. The time needed to reach the committed phloem ingestion phase is very variable (Max/Min values ranged from 197·8 down to 12·2 min), and therefore, the acquisition rate will vary tremendously depending on the particular behaviour of each individual aphid. Our work shows that aphids that reach the committed phloem ingestion phase faster have a higher probability of acquisition of CaMV but we have no clear explanation for this. However, our results show that although the virus could also be acquired from non-phloem tissues at a low and fairly constant rate after one or more intracellular punctures within a few minutes, the probability of acquisition was significantly higher when aphids reached the committed phloem ingestion phase.

Chalfant & Chapman (1962) suggested that *M. persicae* transmitted CaMV in a non-persistent manner, but our results agree with those reported by Markham *et al.* (1987) that show a semipersistent type of virus–vector relationship. We found out that CaMV was preferentially acquired from the phloem by *M. persicae* and that pre-acquisition starvation and long acquisition access periods (8 h) did not change the rate of virus transmission. Furthermore, analysis of the data recorded during aphid probing and feeding activities revealed that EPG variables that best explained acquisition of non-persistently transmitted viruses (Collar *et al.*, 1997) are irrelevant for CaMV acquisition. However, in the case of *B. brassicae*, the individuals that transmitted CaMV during a 5 min acquisition access period produced the first pd faster than those that failed to transmit the virus. This result suggests that acquisition of P2 and P3–virion complexes during short probes is more efficient after a superficial (epidermal) than after deeper (mesophyll) intracellular stylet punctures.

Our work shows that aphids were able to acquire CaMV after just a single intracellular puncture, which was produced within the first minute after the beginning of the probe. Van Hoof (1958) showed that *M. persicae* needs at least 2 min to cross the epidermis of PVY-infected potato plants. Therefore, as opposed to other semipersistent viruses, we confirmed that CaMV is acquired from superficial plant tissues, including the epidermis. The rate of CaMV acquisition did not increase with increasing number of intracellular punctures (Fig. 1). However, the total duration of intracellular punctures was one of the variables selected by the stepwise logistic regression model to explain the acquisition of CaMV. This analysis shows that the virus is acquired more frequently when the total duration of intracellular stylet activities is long. The length of intracellular stylet punctures has been related to the volume of sap ingested by an aphid (Collar *et al.*, 1997; Powell *et al.*, 1995). It seems logical that aphids ingesting larger volumes of superficial cell contents are more likely to acquire CaMV electron-lucent inclusion bodies and therefore, are more competent for subsequent acquisition of CaMV virions.

The model of Drucker *et al.* (2002) for sequential acquisition of CaMV by aphids from infected cells has two steps that are consistent with the findings reported in the present work. The model proposes that an aphid stylet pierces the plasmalemma of an infected mesophyll cell and ingests part of the cell contents, possibly including an electron-lucent inclusion body.

Immediately after acquisition, P3 is liberated and ingested while P2, perhaps together with a few P3–virion complexes, attaches to the aphid stylet (or foregut) cuticle. Our results demonstrating that acquisition of CaMV after a single intracellular stylet puncture in superficial tissues is a rare but possible event supports the proposed model. The model further suggests that after the initial stylet punctures in infected cells, the aphid is P2-loaded and thus transmission competent, ready to acquire more P3–virion complexes during subsequent feeding. The fact that we here describe a significant increase of CaMV acquisition during the phase of committed phloem ingestion is also consistent with the proposed model. Indeed, while P2 could not be detected in the phloem sieve lumen, P3–virion complexes were very frequently observed. We propose that aphids mainly loaded with free P2 acquired during multiple intracellular stylet punctures of epidermal and mesophyll cells can act as a sieve for trapping CaMV virions during committed phloem ingestion. Beside further suggesting that the sequential acquisition of P2 and P3–virion complexes is predominant during CaMV transmission, our electron microscopic data represent the first indication that the CaMV operates long-distance movement in the vascular tissues of infected host plants in the form of viral particles complexed with P3.

Work by Limburg *et al*. (1997) suggests that the closterovirus *Beet yellows virus* (BYV), another semipersistent virus, is acquired from the sieve elements by *Aphis fabae* Scopoli during phloem ingestion. However, this work could not exclude the possibility that the virus is also acquired from non-phloem tissues because the electronic device they used (an AC-amplifed monitor) could not detect intracellular stylet punctures during stylet pathway activities (Reese *et al*., 2000). They were only able to correlate the average time needed to reach the sieve elements (20 min) with the minimum time needed for BYV acquisition. Therefore, it is not possible to conclude that closteroviruses are acquired exclusively from the phloem. However, closteroviruses seem to be phloem-restricted and no helper proteins have been formally proposed (Hull, 2001), suggesting that their transmission strategy is different from the one described for CaMV. This fact opens the debate that semipersistent viruses do not share uniform transmission properties and another classification such as the one proposed by Pirone & Blanc (1996) should be used when referring to viruses transmitted in a non-circulative manner.

The exact location of cuticle receptors in the mouthparts of vectors of semipersistently transmitted viruses remains unclear. Some authors have shown that semipersistently transmitted viruses are found mainly in the foregut of aphid vectors (Murant *et al*., 1976) or leafhoppers (Ammar & Nault, 1991; Childress & Harris, 1989). López-Abella *et al*. (1988) suggested that semipersistent viruses may differ from non-persistently transmitted viruses in the tenacity with which virions are carried in a transmissible state in the foreguts of aphids, or that perhaps there are two binding sites, one in the aphid stylet and another in the foregut. More studies are necessary to identify the specific retention sites of CaMV virions in a transmissible form within the aphid feeding apparatus.

We are indebted to the Spanish Ministry of Science and Technology (Research Grant, no. AGL:2000-2006) for funding this work. Also, we acknowledge the Comunidad Autónoma de Madrid (Spain) and CAPES (Brazil) for funding the fellowships awarded to I.P. and S.L., respectively. M.D. was awarded a fellowship from the ACI-JC of the French Ministry for Research. We thank Takii Ltd seed company for kindly providing turnip 'Just Right' seeds. We gratefully acknowledge the precious help of L. Barrios (CTI-CSIC) for assistance in statistical analysis and M. Ravallec in electron microscopy Finally, thanks to T. Hohn (FMI, Basel, Switzerland) for providing P3 antiserum.

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Received 13 May 2002; Accepted 26 July 2002