Short Communication

Differences in the mechanism of inoculation between a semi-persistent and a non-persistent aphid-transmitted plant virus

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Inoculation of the semi-persistent cauliflower mosaic virus (CaMV, genus *Caulimovirus*) is associated with successive brief (5–10 s) intracellular stylet punctures (pd) when aphids probe in epidermal and mesophyll cells. In contrast to non-persistent viruses, there is no evidence for which of the pd subphases (II-1, II-2 and II-3) is involved in the inoculation of CaMV. Experiments were conducted using the electrical penetration graph (EPG) technique to investigate which particular subphases of the pd are associated with the inoculation of CaMV to turnip by its aphid vector *Brevicoryne brassicae*. In addition, the same aphid species/test plant combination was used to compare the role of the pd subphases in the inoculation of the non-persistent turnip mosaic virus (TuMV, genus *Potyvirus*). Inoculation of TuMV was found to be related to subphase II-1, confirming earlier results, but CaMV inoculation appeared to be related exclusively to subphase II-2 instead. The mechanism of CaMV inoculation and the possible nature of subphase II-2 are discussed in the scope of our findings.

Most of the plant viruses transmitted by aphids follow a non-circulative strategy (Fereres & Moreno, 2009). The basic characteristics of the non-circulative viruses include the ability to be acquired in a short time period (seconds to minutes) and to be transmitted in a similar time interval with no latent period (Ng & Falk, 2006). Based on the inoculation and acquisition periods and the retention site in the vector, it is possible to distinguish two categories of non-circulative virus: non-persistent and semi-persistent (Sylvester, 1962).

Different viral proteins binding to the specific receptor in the vector mediate relationships between the vector and non-persistent viruses. For potyviruses, it is known that the interaction between the capsid protein (CP) and the receptor in the cuticle of the aphid stylet tip is mediated by the helper component protein (HC-pro) (Syller, 2005).

Knowledge of the retention and transmission features of semi-persistent viruses in their vectors is not as broad as in the case of non-persistent viruses. Cauliflower mosaic virus (CaMV, genus *Caulimovirus*), which needs two non-structural viral proteins (P2 and P3) as helper to be transmitted, is retained in the tip of the aphid's maxillary stylets (Uzest *et al.*, 2007, 2010), the same as was proposed long ago for non-persistent viruses (Bradley & Ganong,

1955a, b). However, it is known that treatment of stylets with formaldehyde prevents transmission of CaMV by *Brevicoryne brassicae* L. after short acquisition access periods (AAPs), but does not abolish transmission after long AAPs (Chalfant & Chapman, 1962).

Stylet penetration behaviour is not directly observable, but can be monitored using the electrical penetration graph (EPG) technique (Tjallingii, 1985; Tjallingii *et al.*, 2010). Using EPG, the successive intracellular stylet punctures in the epidermal and mesophyll cells done by aphids before reaching the phloem phase can be visualized as potential drops (pd) (Fig. 1). This technique has allowed association of two of the pd subphases, II-1 and II-3, with the salivation and ingestion events by experimental evidence from inoculation and acquisition of non-persistent viruses, respectively (Powell *et al.*, 1995; Martín *et al.*, 1997; Powell, 2005).

CaMV can be acquired from epidermal and mesophyll cells during brief intracellular punctures, but acquisition efficiency increases sharply after phloem ingestion (Drucker *et al.*, 2002; Palacios *et al.*, 2002). Conversely, salivation by aphids during successive pds in the epidermal and mesophyll cells is the key behavioural event associated with the inoculation of CaMV (Moreno *et al.*, 2005). As opposed
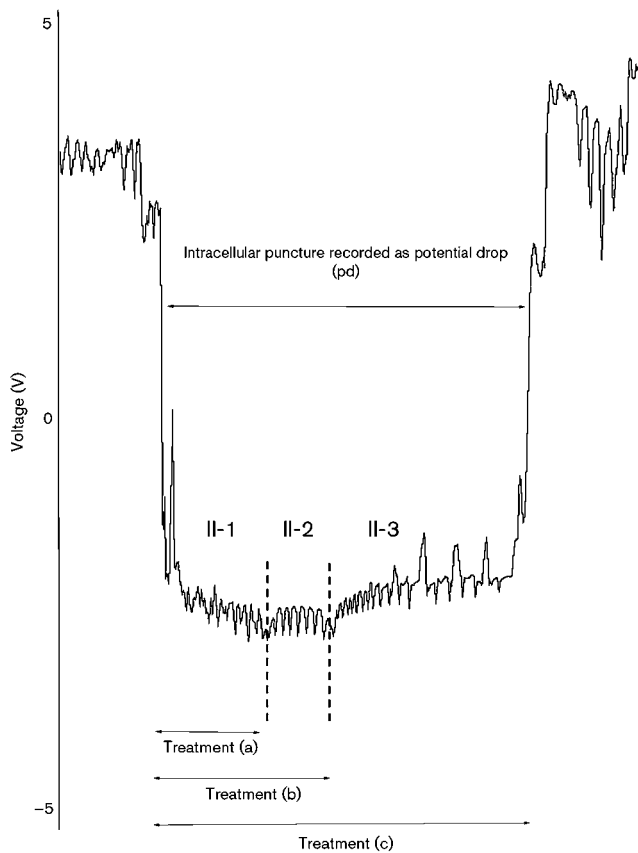
Fig. 1. Representative EPG signal recorded during intracellular stylet penetration, showing one potential drop (pd) and its intracellular subphases: II-1, related to intracellular salivation; II-2, of unknown biological meaning; and II-3, related to intracellular ingestion. Three distinct experimental treatments linked to specific aphid stylet activities during the inoculation of non-circulative viruses were studied: (a) aphid probe was interrupted after the II-1 subphase; (b) aphid probe was interrupted after the II-2 subphase; and (c) aphid was allowed to carry out one complete pd on the receptor plant.

to non-persistent virus, there is no evidence for which pd subphase is involved in CaMV inoculation and for the specific mechanism involved in the inoculation process.

To compare the behavioural events associated with the inoculation of CaMV with those of the non-persistent turnip mosaic virus (TuMV, *Potyvirus*) and to determine what particular pd subphase is associated with CaMV inoculation, aphid stylet penetrations were monitored and recorded. We used an EPG device (Giga-4; EPG-Systems) connected to a USB AD card (DI-158U; DATAQ Instruments) and a laptop PC. Signals were acquired and analysed using WindaqLite software for Windows (DATAQ Instruments).

Experiments were conducted on turnip (*Brassica rapa* cv. 'Just Right') using *B. brassicae* as a vector of CaMV (isolate Cabb-S, kindly provided by Dr S. Blanc, INRA-CIRAD-SupAgro, Montpellier, France) (Franck *et al.*, 1980) and of TuMV (isolate UK-1, kindly provided by Dr F. Ponz,

CBGP-UPM-INIA, Madrid, Spain). The experiments with TuMV allowed us to compare the results obtained under our experimental conditions with those obtained by Martín *et al.* (1997) with other non-persistent viruses.

Non-viruliferous aphids and virus-infected source and test plants were generated and maintained according to Moreno *et al.* (2005).

For transmission experiments conducted using TuMV- and mixed TuMV/CaMV-infected sources, aphids were individually allowed to acquire the virus for an AAP of 5 min after a 1 h pre-acquisition starvation period, as described previously (Fereres *et al.*, 1993). For transmission experiments conducted with CaMV-infected plants as viral sources, the AAP was 8 h.

Thereafter, aphids were removed from the infected plants and connected to the EPG device to monitor the inoculation access period (IAP) on receptor plants. The following treatments were recorded during the inoculation process on the receptor plants (Fig. 1): (a) aphid probe was interrupted after the first subphase (II-1) of the first pd; (b) aphid probe was interrupted after the second subphase (II-2) of the first pd; and (c) aphid was allowed to carry out one complete pd on the receptor plant.

Finally, the aphid was left overnight on a second clean test plant without monitoring its behaviour to check its ability to acquire and transmit the virus under optimal conditions. Aphids that were unable to infect any of the two test plants were discarded from the analysis. Virus infection was checked serologically (ELISA) and by symptom expression 4–5 weeks after inoculation. A completely randomized design was used.

Statistical analyses were conducted using Statview 4.0 software for Macintosh (Abacus Concepts) and SPSS 17.0 statistical package for Windows (SPSS Inc.).

The results show that *B. brassicae* was able to transmit TuMV at the same inoculation frequency after the three different subphases of the pd (38.8, 48.0 and 50.0 %, respectively; $P_{\text{II-1 vs II-2}}=0.485$, $P_{\text{II-1 vs complete pd}}=0.360$, $P_{\text{II-2 vs complete pd}}=0.879$), showing that the inoculation process occurs mainly during the first of these subphases (II-1) (Table 1). These results are in agreement with those of Martín *et al.* (1997), who proposed the ingestion–salivation model for non-persistent virus transmission. Furthermore, no significant differences were observed in TuMV transmission efficiency between treatments ($P_{\text{II-1 vs II-2}}=0.214$, $P_{\text{II-1 vs complete pd}}=0.731$, $P_{\text{II-2 vs complete pd}}=0.390$). The virus transmission efficiency obtained under each treatment was calculated by dividing the number of receptor plants that became infected (either first, second or both receptor plants) by the total number of test plants in each treatment to determine whether the aphid had actually acquired the virus from the source plant and inoculated it to the receptor plants.

For experiments using single CaMV-infected source plants, significant differences were observed between the inoculation

Table 1. Transmission efficiency of TuMV and CaMV by *B. brassicae* to turnip plants from single-infected virus source plants

Significant differences ($P<0.05$) were tested by a χ^2 test and by the Fisher exact test when expected values were <5, and are indicated by different superscript letters.

Subphase	TuMV inoculation frequency*			CaMV inoculation frequency*		
	n	Infected plants (no.)	Transmission (%)	*n*	Infected plants (no.)	Transmission (%)
II-1	31	12	38.8[a]	19	0	0[a]
II-2	25	12	48.0[a]	24	8	33.3[b]
Complete pd	34	17	50.0[a]	53	17	32.1[b]
Subphase	**TuMV transmission efficiency†**			**CaMV transmission efficiency†**		
	n	Infected plants (no.)	Transmission (%)	*n*	Infected plants (no.)	Transmission (%)
II-1	62	31	50.0[a]	134	19	14.2[a]
II-2	65	25	38.5[a]	106	24	22.6[a]
Complete pd	73	34	46.6[a]	251	53	21.1[a]

*Calculated by dividing the number of first receptor plants that became infected (where the inoculation treatment was monitored) by the total number of test plants in each treatment.

†Calculated by dividing the number of receptor plants that became infected (either first, second or both receptor plants) by the total number of test plants in each treatment to determine whether the aphid had actually acquired the virus from the source plant and inoculated it to the receptor plants.

frequencies obtained when aphids were removed after the different inoculation access, EPG-controlled treatments were applied (Table 1). No plants at all were infected by CaMV after subphase II-1 was produced on the first receptor plant. However, the inoculation efficiencies obtained when aphids were allowed to carry out subphases II-2 and II-3 of the pd on the receptor plants were 33.33 and 32.07 %, respectively, without significant differences between these two treatments ($P_{\text{II-1 vs II-2}}=0.006$, $P_{\text{II-1 vs complete pd}}=0.004$, $P_{\text{II-2 vs complete pd}}=0.913$). These results show that subphase II-1 of the pd is not involved in the inoculation of CaMV.

No significant differences were observed between the CaMV transmission efficiencies obtained under each treatment ($P_{\text{II-1 vs II-2}}=0.094$, $P_{\text{II-1 vs complete pd}}=0.102$, $P_{\text{II-2 vs complete pd}}=0.779$). This fact indicates that the observed differences in the CaMV inoculation efficiency after each subphase of the pd are only due to the inoculation process and not to differences in the number of viruliferous aphids used in each treatment.

Similarly, there were no significant differences in the transmission efficiency for each inoculation treatment for experiments where the AAP was conducted on double-infected donor plants ($P_{\text{II-1 vs II-2}}>0.999$, $P_{\text{II-1 vs complete pd}}>0.999$ and $P_{\text{II-2 vs complete pd}}>0.999$) (Table 2). Some receptor plants (considering first and second receptor plants) were double-infected by both viruses, indicating that a single aphid is able to inoculate the same test plant with both viruses simultaneously. As for experiments using single TuMV-infected source plants, the inoculation of TuMV from mixed-infected donor plants showed no significant differences between treatments: 33.3 % after

subphase II-1, 75 % after subphase II-2 and 33.3 % after a complete pd ($P_{\text{II-1 vs II-2}}=0.134$, $P_{\text{II-1 vs complete pd}}>0.999$ and $P_{\text{II-2 vs complete pd}}=0.486$). Once again, no receptor plants were infected by CaMV during the first subphase of the pd (II-1), confirming that subphase II-2 of the pd is required for virus inoculation. No significant differences were observed on CaMV inoculation efficiency after subphase II-2 (50 %) or after a complete pd (60 %) ($P_{\text{II-1 vs II-2}}=0.021$, $P_{\text{II-1 vs complete pd}}=0.027$, $P_{\text{II-2 vs complete pd}}>0.999$).

The duration of the different subphases of the pd obtained under the different treatments from aphids that transmit the virus were compared with those from aphids that did not transmit by means of an ANOVA test. Such comparison was made to determine whether the time that aphids spend on the different probes is involved in their ability to inoculate the virus. No significant differences were observed in subphase II-1 duration between aphids able to transmit and those unable to transmit TuMV after they were removed during the first subphase of the pd on the receptor plant (mean $\pm$ SEM for aphids that transmitted TuMV, 1.71 ± 0.71 s; for aphids that did not transmit TuMV, 1.68 ± 0.059 s; $P=0.74$).

Moreover, we analysed whether the probing duration determines the differences in the inoculation efficiency observed between the viruses. Comparisons between subphase II-1 duration from aphids that were able to inoculate TuMV after subphase II-1 and those that were not able to inoculate CaMV after subphase II-2 did not show significant differences (mean $\pm$ SEM for aphids that transmitted TuMV, 1.71 ± 0.71 s; for aphids that did not transmit CaMV, 1.87 ± 0.14 s; $P=0.46$). The analysed variables for each virus

Table 2. Transmission efficiency of TuMV and CaMV by *B. brassicae* from TuMV/CaMV mixed-infected turnip plants to receptor turnip plants

Significant differences ($P<0.05$) were tested by a χ^2 test and by the Fisher exact test when expected values were <5, and are indicated by different superscript letters.

Subphase	TuMV inoculation frequency*			Mixed infections‡	CaMV inoculation frequency*		
	n	Infected plants (no.)	Transmission (%)		*n*	Infected plants (no.)	Transmission (%)
II-1	15	5	33.3[a]	0	9	0	0.0[a]
II-2	4	3	75.0[a]	2	4	2	50.0[b]
Complete pd	3	1	33.3[a]	1	5	3	60.0[b]

Subphase	*n*	Infected plants (no.)	Transmission (%)	Mixed infections‡	No. of plants infected by TuMV	No. of plants infected by CaMV	
II-1	43	16	37.2[a]	8	7	1	
II-2	15	5	33.3[a]	3	1	1	
Complete pd	17	6	35.5[a]	2	1	3	

*Calculated by dividing the number of first receptor plants that became infected (where the inoculation treatment was monitored) by the total number of test plants in each treatment.
†Calculated by dividing the number of receptor plants that became infected (either first, second or both receptor plants) by the total number of test plants in each treatment in order to determine whether the aphid had actually acquired the virus from the source plant and inoculated to the receptor plants.
‡Number of receptor plants where single aphids were able to co-inoculate both viruses during each of the specific pd subphases.

were selected because processes in these phases have been considered determinant for inoculation of non-persistent and semi-persistent viruses, respectively.

Finally, when subphase II-1 duration was compared between aphids that inoculated CaMV after subphase II-2 and those that did not, no significant differences were found either (mean $\pm$ SEM for aphids that transmitted CaMV, 1.89 ± 0.26 s; for aphids that did not transmit CaMV, 1.85 ± 0.17 s; $P=0.9$). Comparison of subphase II-2 duration from the same batch of aphids showed that there were no differences between aphids that transmitted CaMV and aphids that did not (mean $\pm$ SEM for aphids that transmitted CaMV, 1.62 ± 0.22 s; for aphids that did not transmit CaMV, 1.44 ± 0.10 s; $P=0.4$).

Our study confirms that inoculation of TuMV occurs during subphase II-1 of the first intracellular puncture, as has been demonstrated earlier with other viruses transmitted in a non-persistent manner (Martín et al., 1997; Powell, 2005; Tjallingii et al., 2010). The saliva injected into the plant cell during this first subphase is supposed to release the virus particles retained at the cuticular tips of the stylets. Uzest et al. (2007) showed that, similar to non-persistent viruses, CaMV particles are also attached to a special area in the common duct of the stylet tips that was named the 'acrostyle' (Uzest et al., 2010).

CaMV inoculation results show that, unlike non-persistent TuMV, inoculation occurs during pd subphase II-2. The same result was observed for experiments when both viruses were acquired from single- or double-infected donor plants. We also observed that both viruses were co-inoculated from a double-infected source to test plants at the same time by the same single aphids during each of the specific pd subphases (II-1 for TuMV and II-2 for CaMV) (Table 2).

The idea that the release of CaMV from the cuticular receptor would just need more time to be released by the same saliva does not seem likely. Comparison of subphase durations showed no relationship between the time spent in each of them and the ratio of successful inoculation. Also, the duration of subphase II-1 is very short and does not vary over successive intracellular pds (Collar & Fereres, 1998), supporting our results that show no influence of subphase II-1 duration on the ability of the aphid to inoculate either TuMV or CaMV.

Therefore, we suggest two alternative explanations for our results. The first hypothesis postulates an alternative mechanism of CaMV inoculation during subphase II-2. The candidate might be the old ingestion–egestion hypothesis proposed by Harris (1977) for viruses transmitted in a non-persistent manner. When aphids are salivating, the cibarial valve is presumably closed, thus avoiding passive egestion of foregut contents and saliva ingestion into the food canal (Forbes, 1969). The pharyngeal valve is then opened when the suction pump starts, allowing the plant sap to move in through the food canal during virus-acquisition subphase II-3

of the pd. One could argue that, during CaMV-inoculation subphase II-2, the pharyngeal valve might open and facilitate egestion of components retained in the foregut, enabling virus release and inoculation. The reverse movement of previously ingested sap back to the plant through the food canal (egestion or regurgitation) was proposed by McLean & Kinsey (1984). Whether such fluids egested from the foregut might dislodge CaMV virions from the stylet binding site or might contain any CaMV virions retained in the pharyngeal cavity should be investigated.

Alternatively, our second hypothesis postulates that subphase II-2 activity might represent another salivation phase, presumably injecting a different type of saliva into the punctured cell (e.g. gelling saliva). Both watery and gelling saliva components are secreted from the very beginning of intercellular stylet penetration into the intercellular spaces from the accessory and principal salivary glands, respectively (Moreno et al., 2011). These secretions could be regulated depending on the type of environment, and two end products may be obtained. Thus, salivary components are able to release TuMV particles from the stylet tips during subphase II-1 and different salivary components might release CaMV particles during subphase II-2.

We do not understand why the II-2 waveform is so different from those of II-1 and II-3. Also, we do not understand the function of II-1 salivation. Anyhow, there is no reason to suppose that the three pd subphase activities are for the benefit of virus transmission. Their primary function in aphid–plant interactions needs further investigation.

Acknowledgements

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