Intracellular salivation is the aphid activity associated with inoculation of non-persistently transmitted viruses

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Approximately 75% of aphid-vectored viruses are transmitted in a non-persistent (non-circulative) manner. Localization studies indicate that such viruses are acquired via ingestion and retained in the food canal of the maxillary stylets, but the inoculation mechanism has remained unresolved. Electrical recording of stylet penetration activities reveals that inoculation is associated with the first intracellular activity (subphase II-1) following maxillary puncture of an epidermal cell. Subphase II-1 may represent virus inoculation via egestion (regurgitation of virions with food-canal contents) or salivation (saliva-mediated release of virions from the common food-salivary duct at the tips of the maxillary stylets). Here, inoculation of the circulatively transmitted Pea enation mosaic virus was used as a marker for intracellular salivation during epidermal cell punctures. The results confirmed that inoculation of non-persistently transmitted viruses (subphase II-1) is associated with active injection of saliva directly into the cytoplasm.

Aphids are the most important group of plant virus vectors, transmitting at least 275 viruses (Nault, 1997). The majority (approx. 75%) of these viruses are transmitted in a non-persistent manner, a category of non-circulative transmission also known as stylet-borne (Kennedy et al., 1962; Nault, 1997). Although non-persistently transmitted viruses are a polyphyletic group, belonging to at least five genera (Nault, 1997; Pirone & Perry, 2002), the aphid transmission process is remarkably uniform. Acquisition and inoculation occur optimally during the brief (<1 min) epidermal stylet penetrations ('probes') that aphids make to assess host-plant suitability (Nault & Styer, 1972; Powell & Hardie, 2000). Considerable research effort has therefore focused on aphid probing activities and their consequences for the non-persistent transmission process. The mechanism of virus acquisition is well established as ingestion; virions are imbibed from virus-infected plants to their retention sites within the food canal of the maxillary stylets (Pirone & Perry, 2002). Much of our understanding of the acquisition and retention processes comes from studies on potyviruses, where a virally encoded helper component (HC) protein is required for transmission. When aphids acquire transmissible combinations of HC and potyvirus, virions are retained within the maxillary stylets (Berger & Pirone, 1986) where they show HC-mediated adherence to the cuticular lining of the food canal (Ammar et al., 1994; Wang et al., 1996).

Uptake of virions occurs when the maxillary stylet tips puncture the plasma membrane of an epidermal cell (Lopez-Abella et al., 1988; Powell, 1991). These brief (3–15 s) intracellular punctures can be monitored using the DC electrical penetration graph (EPG) technique (Tjallingii, 1988) and appear as distinct potential drops (pds) in the recorded signal (Tjallingii, 1985). Three successive intracellular activities occur during each short pd, characterized as subphases II-1, II-2 and II-3, respectively (Powell et al., 1995; Martin et al., 1997; Fig. 1a). The third intracellular activity (II-3) is associated with efficient uptake of non-persistently transmitted viruses and therefore represents active ingestion of cytosolic fluid by the aphid (Powell et al., 1995; Martin et al., 1997; Powell & Hardie, 2000).

How are ingested virions released from within the maxillary stylets into healthy plant cells during inoculation? EPG studies have shown that, like acquisition, virus inoculation requires maxillary puncture of the plasma membrane (Powell, 1991). Interrupting viruliferous aphids during the three subphases showed that only the first intracellular activity (II-1) was necessary for efficient release of virions (Martin et al., 1997). However, by contrast with the widely accepted ingestion scenario for acquisition, the mechanism of inoculation (i.e. the activity represented by II-1) remains unclear. The ‘ingestion–egestion’ hypothesis posits that virions are regurgitated (egested) from the food canal into the inoculated plant (Watson & Plumb, 1972; Harris & Bath, 1973; Harris, 1977). Microscopic examination of aphid stylets penetrating membranes of stretched Parafilm has suggested that ink particles moved both into and out of...
II-1 during subphase II-1, II-2 or II-3 (sample sizes: II-1, \( n = 62 \); II-2, \( n = 41 \); II-1 + II-2, \( n = 72 \)).

Fig. 1. (a) Representative EPG signal recorded during styllet penetration of an epidermal cell by a pea aphid. The intracellular phase (II) occurs as three successive subphases: II-1, II-2 and II-3. The figure reads from left to right. Bar, 0.5 s. (b) PEMV inoculation efficiency during intracellular subphases. Cell punctures by viruliferous aphids were interrupted artificially during subphase II-1, II-2 or II-3 (sample sizes: II-1, \( n = 41 \); II-1 + II-2, \( n = 62 \); II-1 + II-2 + II-3, \( n = 72 \)).

the food canal, leading to speculation that egestion occurs during penetration of this artificial system. If egestion also occurs during styllet penetration of plants, then this may be the mechanism of inoculation. An alternative hypothesis is that virions are released by salivation. This is possible because, although the salivary canal remains distinctly separate from the food canal for almost the entire length of the aphid styllet bundle, the two canals converge 2–4 \( \mu \)m from the tips (Forbes, 1969). At the point of convergence, the maxillary styllets form an enclosed common duct (Kimmins, 1986) where mixing of food and salivary canal contents may occur. Ingested virions adhering to the cuticular lining of the common duct may therefore be flushed out during saliva secretion into the plant, providing an alternative ‘ingestion–salivation’ hypothesis for transmission (Martin et al., 1997).

While the ‘egestion’ and ‘salivation’ scenarios provide intriguing alternatives for the inoculation mechanism, direct experimental evidence for either case is lacking. None of the three intracellular subphases has been experimentally associated with either egestion or salivation. Observations of aphids penetrating Parafilm and ejecting ink particles have been extrapolated to plants in support of the ingestion–egestion hypothesis (Harris & Bath, 1973; Harris, 1977), but there is no direct evidence that aphids egest during plant penetration. The ingestion–salivation hypothesis is also unproven: it has been proposed that subphase II-1 represents a distinctive phase of watery salivation by aphids (Cherqui & Tjallingii, 2000), but there is no definitive evidence that aphids inject saliva into cells during brief punctures. The aim of the present study was to exploit the unique properties of Pea enation mosaic virus (PEMV) to investigate the occurrence of intracellular salivation and its importance for the inoculation of non-persistently transmitted viruses.

PEMV represents an obligate symbiosis between two distinct viruses (de Zoeten & Skaf, 2001): an enamovirus (PEMV-1, a member of the family Luteoviridae) and an umbravirus (PEMV-2). PEMV-1 confers transmission by aphids in a circulative (non-propagative) manner and so, in common with luteoviruses, acquired PEMV accumulates in the accessory salivary glands and is inoculated via salivation (de Zoeten & Skaf, 2001; Reavy & Mayo, 2002; Gray & Gildow, 2003). PEMV-2 plays no known role in determining interactions with aphid vectors, but influences the site of successful virus inoculation within plants. By conferring cell-to-cell movement, PEMV-2 enables escape from the phloem limitation that is typical of luteoviruses, so that PEMV can be inoculated during the superficial, brief epidermal probes that also characterize optimal inoculation of non-persistently transmitted viruses (Nault & Gyrisco, 1966). This unique combination of features makes inoculation of PEMV an ideal marker for aphid salivation during styllet penetration of the epidermis. The occurrence of PEMV inoculation during intracellular (EPG-recorded) activities was therefore investigated in order to determine whether and when intracellular salivation occurs.

In order to maximize virus acquisition and subsequent experimental transmission, PEMV vectors (clone JF01/29 of the pea aphid, Acyrthosiphon pisum) were born and reared on virus-infected plants (tick bean, Vicia faba var. minor). Apterous aphids approaching the final moult to the adult stage (i.e. late IV instars) were collected from these plants and allowed access to healthy 'test' plants (newly germinated tick bean seedlings at the 'hook' stage growing in perlite/compost mix in individual 7.5 cm pots). Although PEMV is a circulatively transmitted (and therefore saliva-inoculated) virus, it was important to eliminate the possibility that additional inoculation occurred via carry-over of virus in or on the styles. This was achieved by confining aphids (under an inverted glass tube) to a first test plant overnight (20–26 h), allowing extended access to a virus-free food source. The following day, only aphids that had moulted to the adult stage (and therefore shed and replaced their styles) were selected as an additional precaution against inoculation of stylet-retained virus. These aphids were then starved in plastic Petri dishes for 2–4 h before being used in experiments, in order to encourage the probing behaviour that characterizes efficient inoculation of non-persistently transmitted viruses (Powell, 1993) and PEMV (Nault & Gyrisco, 1966). After the starvation period, each
aphid was attached to a fine (20 μm diameter) gold wire for EPG recording and allowed access to seven consecutive ‘EPG test’ plants for a single probe on each. EPG signals were recorded directly on to a PC hard disk (STYLET 3.7 software) and simultaneously displayed on a second PC running the Picoscope for Windows package (Pico Technology). The majority of probes terminated naturally within 20 s, but those that lasted up to 30 s were terminated artificially by lifting the gold wire. In addition, a subset of randomly selected probes \((n = 175)\) was terminated earlier than this, by lifting the wire during intracellular subphase II-1, II-2 or II-3. All test plants (eight plants per aphid: one ‘overnight’ test plus seven EPG tests) were transferred to a nicotine-fumigated glasshouse and symptoms of PEMV infection were scored visually 14–17 days after inoculation. A total of 80 aphids was used in the experiment, accounting for 640 test plants during the course of the study. Over the same period, a further 400 plants were not subjected to experimental aphid exposure but were grown in the same glasshouse environment; none of these test plants developed symptoms of infection. The efficiency of PEMV transmission to the first (overnight) test plant was very high (98.8%), but 10 insects did not inoculate any of the EPG test plants in the subsequent recordings and were excluded from the statistical analysis. The remaining aphids \((n = 70)\) inoculated PEMV to 32.0% of 490 EPG test plants overall and showed no significant decline in inoculation efficiency from the first (31.4%) to the last (28.6%) EPG test plant. Data for all seven EPG tests were therefore pooled for subsequent analysis \((n = 490)\).

The occurrence of virus inoculation was absolutely dependent on cell puncture by the maxillary stylets. All 157 occurrences of PEMV inoculation were associated with a clearly recorded pd (Table 1), confirming that the virus was delivered into cells during intracellular salivation. Analysis of those cell punctures that were interrupted artificially showed that only subphase II-1 was necessary for inoculation. Aphids allowed further intracellular activities, and interrupted during subphases II-2 or II-3, did not inoculate virus at a significantly higher efficiency than those allowed subphase II-1 only \((\chi^2 = 2.14; \text{d.f.} = 1; P > 0.05)\), may be related to the duration of II-1 activity; II-1 was inevitably cut short for insects that were interrupted during this subphase. A further analysis was therefore carried out (across all recorded pds) to assess whether the duration of each subphase was related to the occurrence of inoculation. Cell punctures effecting successful inoculation showed significantly longer duration of II-1 than punctures that did not inoculate \((t = 4.63; \text{d.f.} = 355; P < 0.001; \text{Fig. 2})\), a result that lends further support to the link between this first intracellular activity and virus release. By contrast, the durations of subphases II-2 and II-3 showed no relationship with virus inoculation \((\text{Fig. 2})\).

In supporting a link between PEMV inoculation and subphase II-1, these data indicated that the first intracellular activity represented injection of saliva directly into the punctured cell. Comparison with the data of Martin et al. \((1997)\) provides a link between this distinct salivation event and inoculation of non-persistently transmitted viruses. The results presented here therefore support the ingestion–salivation hypothesis; non-persistently transmitted viruses are most likely flushed from the common duct during intracellular secretion of watery saliva. Although aphids also secrete gelling saliva, forming a sheath around the stylets during penetration of the apoplast, sheath material is apparently excluded from the cell contents when the maxillary stylets perforate the plasma membrane \((\text{Tjallingii} \& \text{Hogen Esch}, 1993)\). Puncture of the membrane may therefore be followed by a very rapid switch to watery salivation \((\text{Cherqui} \& \text{Tjallingii}, 2000)\). The two types of saliva may have very different abilities to release retained virions from the common duct surface and an abrupt change to watery saliva secretion may be accompanied by a sudden increase in virion release \((\text{Martin et al.}, 1997)\).

A great deal of speculation has been based on reported egestation by aphids \((\text{Harris} \& \text{Bath}, 1973)\) but, following the ingestion–salivation hypothesis \((\text{Martin et al.}, 1997)\) and

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<th>Table 1. Contingency analysis of the association between the occurrence of EPG-recorded cell puncture and inoculation of PEMV</th>
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<tr>
<td><strong>PEMV inoculation</strong></td>
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<td>Cell puncture</td>
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\(\chi^2 = 86.1; P < 0.001\).

![Fig. 2. Mean duration (± SE) of intracellular subphases, categorized according to whether PEMV inoculation occurred (***, \(P < 0.001\); ns, no significant difference; results from \(t\)-tests).](http://vir.sgmjournals.org)
the present supportive findings, it now seems appropriate to question whether egestion occurs during epidermal penetration. While the first and third intracellular sub-phases appear to represent salivation and ingestion, respectively, the behaviour represented by the intermediate period (II-2) remains unclear. Harris & Harris (2001) have speculated that II-2 represents egestion and have presented a scheme whereby this subphase is responsible for inoculation of non-persistently transmitted viruses, but there is no evidence that II-2 is linked with ejection of styel contents. The data reported by Martin et al. (1997) showed clearly that the activity associated with virus inoculation was subphase II-1, not II-2.

The common duct at the maxillary styel tips represents less than 1% of the total aphid styel length (Forbes, 1969) and the vast majority of acquired virions are retained at more proximal sites within the food canal (Wang et al., 1996). However, very few (probably less than 100) virus particles are involved in the non-persistent transmission process (Pirone & Thornbury, 1988) and the presence of virions at more proximal locations does not provide evidence that they function in transmission (Pirone & Perry, 2002). The common duct represents the likely functional retention site and, although many virions flow past this position during ingestion, there may be no credible mechanism by which they can be inoculated.

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References


