# Intracellular ingestion and salivation by aphids may cause the acquisition and inoculation of non-persistently transmitted plant viruses

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**Transmission of non-persistent plant viruses is related to aphid behaviour during superficial brief probes. A widely accepted hypothesis postulates that virus acquisition occurs during ingestion of plant cell contents, and inoculation during egestion or regurgitation of previously ingested sap. Although conceptually attractive, this ingestion–egestion hypothesis has not been clearly demonstrated. Furthermore, it overlooks the anatomy of the tips of the stylets (mouthparts) and, consequently, the potential role of salivation in the inoculation process. Here, we used the electrical penetration graph (EPG) technique to investigate aphid-stylet activities associated with uptake (acquisition) and release (inoculation) of two non-persistently transmitted viruses. Our results show that acquisition occurs primarily during the last sub-phase (II-3) of intracellular stylet punctures, whereas inoculation is achieved during the first sub-phase (II-1). An alternative mechanism to the ingestion–egestion hypothesis is proposed on the basis of our findings.**

The transmission of non-persistent plant viruses is unique to aphids (Homoptera: Aphididae) because they exhibit specific and characteristic activities during brief (a few seconds or minutes) and superficial probes, involved in host plant recognition (Pollard, 1973). Two different hypotheses have been proposed to explain the mechanism of transmission. The first, so-called stylet-borne hypothesis (Kennedy *et al.*, 1962), suggests that virus particles are attached to the tip of the stylets (mouthparts), i.e. to their outside or to the inner surface of the distal part of the 20 μm maxillary food canal (Taylor & Robertson, 1974). Aphids would then act as 'flying needles', i.e. virions would mechanically attach to (acquisition) or detach from (inoculation) the aphid's stylets. The second, so called ingestion–egestion hypothesis (Gamez & Watson, 1964; Harris, 1977) suggests that aphids, behaving as 'flying syringes' rather than 'flying needles', contribute more actively to the acquisition and release of the virus. It presumes that virions are acquired when aphids ingest cell contents in the process of food selection and later inoculated during intracellular egestion (regurgitation) on a healthy plant. The ingestion–egestion hypothesis, widely accepted at present, was initially based on microscopical observations of stylet penetration through artificial membranes (Parafilm) into fluids containing ink particles (Harris & Bath, 1973). The particles that moved into the stylet tips and food canal also moved out of the food duct by egestion. Extrapolated to plants, the hypothesis suggests that virions are ingested from virus-infected cells into the food canal and distal foregut. Virions attached to the walls in these places have been detected (Berger & Pirone, 1986). Aphids moving to healthy plants after acquisition would release virions during regurgitation, which may be functional in removing blocking cell organelles (chloroplasts, etc.) from the food canal entrance, or in ejecting noxious plant components present in sampled sap.

Although the ingestion part of the ingestion–egestion hypothesis is rather well supported by experimental evidence, support for the egestion part is much less convincing. A model in which egestion through the food canal injects virus into the plant does not take account of the anatomical fact that the food and salivary canals in the maxillary stylets fuse at 2–8 μm from the tips (Forbes, 1969). Therefore, why could attached virus particles not be released equally well by saliva excretion? Recently, radiolabelled potyvirus particles were found to be retained especially in the distal third of the maxillary stylets (Wang *et al.*, 1996) (i.e. stylet tips).

Aphid transmission of non-persistent viruses is almost completely restricted to very brief (5–10 s) intracellular

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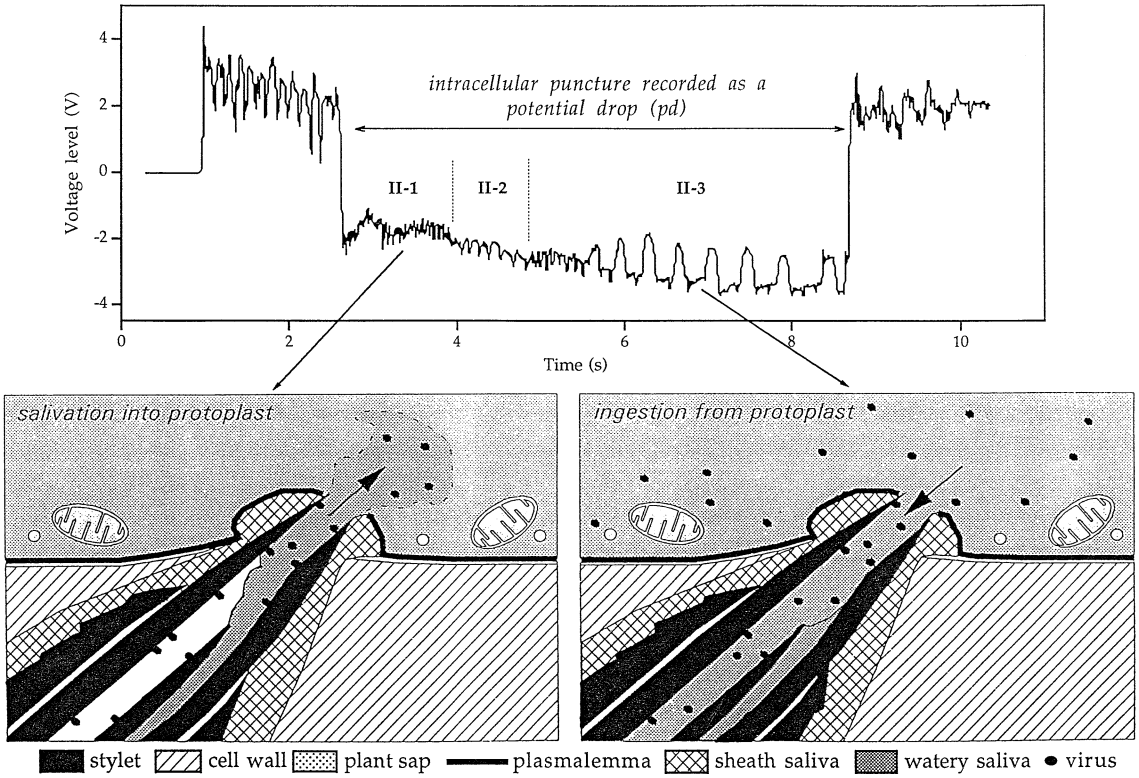


Fig. 1. Typical potential drop (pd) produced by *M. persicae* soon after the beginning of a probe and its intracellular sub-phases (II-1, II-2 and II-3) correlated with the new hypothesized mechanism of non-persistent virus inoculation (bottom left) and acquisition (bottom right). 'Cross-sections' through stylet tips (four parts) with fusing maxillary canals during an intracellular puncture of an epidermal or mesophyll plant cell.

punctures of epidermal or mesophyll cells (López Abella & Bradley, 1969; Powell, 1991). These intracellular punctures can be recorded and visualized as potential drops (pd) (Fig. 1) using the electrical penetration graph (EPG) technique (DC method; Tjallingii, 1978). Potential drops show three different phases (Tjallingii, 1985) and the second, truly intracellular phase has been sub-divided into three distinct sub-phases (II-1, II-2 and II-3; Fig. 1). Only phase II-3 has been associated with the acquisition of a non-persistent virus (tobacco etch virus, TEV, *Potyviridae*) (Powell *et al*., 1995). Inoculation, either by egestion or otherwise, must also occur during the pd (Powell, 1991). Salivation is abundant during probing and some saliva is excreted into the cells or into the adjacent plasmalemma during these intracellular punctures (Tjallingii & Hogen Esch, 1993) and can make contact with the protoplast contents, at least initially.

To investigate the behavioural events associated with inoculation or acquisition of non-persistently aphid-transmitted viruses we used an EPG device (DC, 1 GOhm input; Tjallingii, 1988) connected to a strip-chart recorder and a microcomputer. Aphid probing was recorded separately during acquisition and inoculation probes using two different vector–virus systems: *Aphis gossypii* (Glover) transmitting cucumber mosaic virus (CMV, *Bromoviridae*) to melon (*Cucumis melo* L.); and *Myzus persicae* (Sulzer) transmitting potato virus Y (PVY, *Potyviridae*) to pepper (*Capsicum annuum* L.). All aphids used were apterae adult virginoparae obtained from a clonal culture. Aphid cultures, virus sources and test plants were generated and maintained according to Fereres *et al*. (1993). The virus strains used were: a pepper-PVY isolate (pathotype 0) inoculated on 'Yolo Wonder' pepper plants and the Val-CMV-24 isolate described by Garcia-Luque *et al*. (1984). All aphids were subjected to a 1 h pre-acquisition starvation period before the beginning of the experiments. Aphids were allowed to carry out a single probe that was artificially interrupted by removing the aphid from the plant at the end of sub-phases II-1, II-2 or II-3 of the first potential drop observed. For virus acquisition, after the EPG-recorded single probe on an infected source plant, the aphid was transferred to a test plant for a 3 h inoculation access period. For virus inoculation, aphids were subjected first to an acquisition access period of 5 min on an infected source. Then, their (interrupted) inoculation probe on a healthy test plant was recorded and subsequently every aphid was transferred to a second test plant to assess its initial virus acquisition. Aphids that were unable to infect any of the test plants were discarded from the analysis of the inoculation data. Virus infection was checked for all test plants serologically (ELISA) and by symptoms after 3–4 weeks. The EPGs were

**Table 1.** Transmission efficiency of CMV and PVY by *A. gossypii* and *M. persicae*

**(*a*) Acquisition experiment**

| | CMV transmitted by *Aphis gossypii* on melon | | | PVY transmitted by *Myzus persicae* on pepper | | |
|---|---|---|---|---|---|---|
| Sub-phase | *n* | Infected plants (no.) | Transmission (%) | *n* | Infected plants (no.) | Transmission (%) |
| II-1 | 65 | 4 | 6.1 | 37 | 3 | 8.1 |
| II-1+II-2 | 64 | 6 | 9.4 | 16 | 3 | 18.7 |
| II-1+II-2+II-3 | 65 | 23 | 35.4 | 56 | 35 | 62.5 |
| | | $\chi^2$ | *P* | | $\chi^2$ | *P* |
| Overall | | 23.61 | < 0.0001 | | 30.93 | 0.0001 |
| II-1 vs II-1+ II-2 | | 0. 47 | 0.494 | | 1.26 | 0.2616 |
| II-1 vs II-1+II-2+II-3 | | 16.87 | < 0.0001 | | 27.28 | 0.0001 |
| II-1+ II-2 vs II-1+II-2+II-3 | | 12.52 | < 0.0001 | | 9.56 | 0.002 |

**(*b*) Inoculation experiment**

| | CMV transmitted by *Aphis gossypii* on melon | | | PVY transmitted by *Myzus persicae* on pepper | | |
|---|---|---|---|---|---|---|
| Sub-phase | *n* | Infected plants (no.) | Transmission (%) | *n* | Infected plants (no.) | Transmission (%) |
| II-1 | 53 | 24 | 45.3 | 12 | 6 | 50 |
| II-1+II-2 | 48 | 28 | 58.3 | 13 | 5 | 38.5 |
| II-1+II-2+II-3 | 60 | 27 | 45.0 | 27 | 9 | 33.3 |
| | | $\chi^2$ | *P* | | $\chi^2$ | *P* |
| Overall | | 2.35 | 0.3088 | | 0.97 | 0.6142 |
| II-1 vs II-1+ II-2 | | 1.72 | 0.1900 | | 0.34 | 0.5615 |
| II-1 vs II-1+II-2+II-3 | | 0.001 | 0.9759 | | 0.97 | 0.3234 |
| II-1+ II-2 vs II-1+II-2+II-3 | | 1.90 | 0.1684 | | 0.10 | 0.7501 |

analysed by the computer program MacStylet to confirm that each individual record (aphid) matched an appropriate category of the interrupted potential drops: records with only phase II-1, with phases II-1 + II-2 or with phases II-1 + II-2 + II-3 ( = complete pd).

Our results indicate that the last sub-phase of the potential drop (II-3) was mostly responsible for virus acquisition (Table 1*a*), as shown by the significant increase of transmission efficiency when the intracellular puncture was completed. The $\chi^2$ test indicates that phase II-3 contributes significantly more to virus acquisition than the two previous phases together ($\chi^2 = 12{\cdot}52$, $P < 0{\cdot}0001$ for CMV; $\chi^2 = 9{\cdot}56$, $P = 0{\cdot}002$ for PVY). Nevertheless, there were a few cases of positive acquisition during sub-phases II-1 and II-2. Conversely, inoculation efficiency was already high when the pd was interrupted after the first sub-phase (II-1) (Table 1*b*) and stayed on that level when further pd sub-phases were allowed. Hence, the first sub-phase of the intracellular puncture lasting only 1–2 s was responsible for virus inoculation.

The crucial importance of sub-phase II-3 for acquisition has been shown earlier (Powell *et al.*, 1995) in artificial-membrane probing studies. The sudden increase in transmission efficiency in phase II-3 supports the active uptake–acquisition linkage but our data suggest that some virus acquisition is also possible by passive binding (low acquisition efficiency related to II-1 and II-1 + II-2 sub-phases, Table 1*a*). Aphids apparently do ingest plant sap during cell punctures, presumably for gustatory-based food plant selection. As a result they also ingest virus particles.

The evidence presented here showing that the inoculation of non-persistent viruses (CMV and PVY) occurs during the first phase (II-1) of the intracellular stylet puncture suggests active ejection. The sequence of events shown makes salivation a better candidate for inoculation than egestion because

regurgitation seems to occur after any ingestion takes place when aphids feed on artificial systems (Harris & Bath, 1973). The very few virions (only about 100) that are needed for the transmission of potyviruses (TEV and tobacco vein mottling virus, TVMV) (Pirone & Thornbury, 1988) may be carried in the single canal structure at the end of the stylet tips of an aphid. Thus, we propose 'ingestion–salivation' as an alternative to the 'ingestion–egestion' hypothesis (Fig. 1): non-persistently transmitted viruses are acquired by sap ingestion during the last sub-phase (II-3) of intracellular punctures, whereas inoculation may be caused by ejection of saliva during the first sub-phase (II-1), especially while aphids are performing their first intracellular punctures in superficial plant tissues. However, further investigation is needed to elucidate if salivation, egestion or both occur during virus release from the aphid's stylets.

Evidence of regurgitation by aphids comes from experiments with radioisotopes on artificial membranes (Garrett, 1973) over considerably longer periods than the 15–60 s reported for optimum acquisition and inoculation of non-persistent viruses. Conversely, this brief period of time fits well with the idea that the crucial events are associated with the first intracellular puncture after probe initiation. Furthermore, inoculation during salivation would account for the fact that, after release of the particles from the common stylet tip canal, the ability to transmit is lost or significantly reduced and displays no correlation (Pirone & Thornbury, 1988) with the number of potyvirus particles acquired and retained in the proximal food canal and foregut. Conversely, a lack of, or a decrease in, virion retention in the stylets was highly correlated with lack or reduction in TEV transmission (Wang *et al.*, 1996).

Abundant salivation during aphid probing (visualized using EPGs as a specific waveform B; Tjallingii, 1978) continues until puncturing of the plasmalemma of an epidermal or mesophyll cell. At that point, we believe that saliva excretion flushes the virus particles into the cytoplasm. It remains unclear whether the saliva composition changes at this time from gelling sheath material (which is found inside the cell wall but not inside the plasmalemma after the withdrawal of the stylets; Tjallingii & Hogen Esch, 1993) to watery saliva (Miles, 1965) (Fig. 1). Such a change might explain the sudden inoculation observed if watery saliva specifically mediates the release of virions from the stylet tips (vs gelling saliva, initiated before cell puncture). It has been shown that watery saliva excretion into sieve elements causes the inoculation of persistently aphid-transmitted viruses (Prado & Tjallingii, 1994). On the other hand, Bradley (1959) found that artificial induction of salivation without aphid probing after PVY acquisition did not reduce transmission of the virus. However, this same work proves that aphids can eject saliva while probing on glass surfaces, which presumably leads to a reduction in the transmission efficiency. Also, Hashiba & Misawa (1969) found potyvirus-like particles on the saliva ejected by *M. persicae* while probing on a glass surface.

Finally, another piece of evidence that salivary secretions may be involved in non-persistent virus inoculation is the inhibition of virus transmission induced by the *Vat*-gene (Lecoq *et al.*, 1979). This gene acts exclusively against *A. gossypii* during the inoculation phase but not during the acquisition of several unrelated non-persistent viruses (cucumber mosaic virus, papaya ringspot virus and zucchini yellow mosaic virus). Probing behaviour differences between *A. gossypii* and *M. persicae* could not account for the complete inhibition of transmission that was found only to occur for *A. gossypii* on plants carrying the *Vat*-gene (Chen *et al.*, 1997). Therefore, this work suggests that the *Vat*-gene product interacts with specific components of aphid origin (most likely, the saliva) causing inhibition of virus release from the stylets.

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