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Aphid stylet activities during potyvirus acquisition from plants and an *in vitro* system that correlate with subsequent transmission

Glen Powell[1], Thomas Pirone[2]* and Jim Hardie[1]
[1]*Department of Biology, Imperial College at Silwood Park, Ascot, Berks., SL5 7PY, UK*; [2]*Institute of Arable Crops Research, Rothamsted Experimental Station, Harpenden, Herts., AL5 2JQ, UK*; **Permanent address: Department of Plant Pathology, University of Kentucky, Lexington, KY 40546, USA*

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Abstract

The behavioural events associated with acquisition of tobacco etch potyvirus by starved *Myzus persicae* during single, electrically-recorded penetrations of plants or a Parafilm membrane were compared. Twenty nine percent of aphids acquired virus from plants and subsequently transmitted to test plants. Stylet puncture of the plasmalemma, indicated by a potential drop (pd) to the intracellular signal voltage level, occurred during 84% of penetrations, and virus transmission was always associated with this behavioural event during acquisition. Periods of intracellular stylet tip location, known as pd phase II, ranged from 3.6–12.2s, and always comprised three consecutive sub-phases, designated II1, II2 and II3. Ninety six percent of pds included distinct pulses during phase II3. A waveform which closely resembled these pulses was produced by 59% of aphids that probed a virus suspension through a Parafilm membrane; nineteen percent of the aphids subsequently transmitted membrane-acquired virus and transmission was significantly associated with the occurrence of the phase II3-like pulses during acquisition. The duration of occurrence of recorded phase II3 pulses, either on plants or the *in vitro* system, did not influence the virus transmission efficiency of aphids. The association of virus uptake from aqueous suspension with a particular behavioural activity is discussed as evidence for the 'ingestion-egestion' hypothesis for non-persistent transmission. Starved aphids acquiring virus from infected leaf tissue or the *in vitro* system had significantly higher transmission efficiencies than non-starved aphids. Starved and non-starved insects were electrically-recorded penetrating the artificial membrane, and again there was a clear difference in transmission efficiency (starved aphids, 26%; non-starved aphids, 2%). The higher transmission efficiency of starved insects could not be explained by behavioural differences, and the results lend support to the hypothesis that non-behavioural factors determine the enhancement of potyvirus transmission by pre-acquisition starvation.

Abbreviations: BMV = Beet mosaic virus; EMF = Electromotive force; HAT = Highly aphid-transmissible; HC = Helper component; pd = Potential drop; PVY = Potato virus Y; TEV = Tobacco etch virus.

Introduction

Non-persistent transmission of plant viruses by aphids involves acquisition and inoculation during brief stylet penetrations of the epidermal cell layer. Viruses transmitted in this manner belong to at least six taxonomic groups [Sylvester, 1989]. For the potyviruses, two viral gene products determine aphid transmissibility [Pirone, 1991]: the coat protein and 'helper component' (HC) protein [Govier and Kassanis, 1974]. Aphids can therefore acquire purified potyviruses *in vitro* from virus-

HC mixtures through artificial membranes of stretched Parafilm [Govier and Kassanis, 1974; Govier *et al.*, 1977; Pirone, 1981; Pirone and Thornbury, 1984], and the available evidence suggests that HC may help to bind virions to their retention site, within the aphid foregut [Berger and Pirone, 1986]. The acquisition characteristics from the '*in vitro*' system appear similar to natural acquisition from potyvirus-infected plants, with virus uptake occurring during brief (< 30s) stylet penetrations and rapid loss of vector efficiency during post-acquisition fasting [Raccah and Pirone, 1984].

The connection of aphid and feeding substrate in a simple circuit allows electrical recording of stylet penetration of plants [Tjallingii, 1978; 1988] or Parafilm-covered artificial diets [Tjallingii, 1978; 1985a]. This technique gives detailed information concerning stylet activities, and enables a distinction between intra- and extra-cellular stylet tip position in plants, according to the recorded signal potential level [Tjallingii, 1985a]. Stylet puncture of the plasmalemma is recorded as a potential drop (pd), and a correlation has been found between the occurrence of this activity and the acquisition and inoculation of potyviruses [Powell, 1991a; 1993; Powell *et al.*, 1992]. Potential drops occur in three phases [Tjallingii, 1985a]. The first phase (I) occurs when the membrane is initially punctured, and includes the drop to the intracellular signal level. The second phase (II) consists of a maintenance of this voltage level, and is accompanied by characteristic waveforms. The third phase (III) consists of a return to the original (extracellular) voltage level, and is presumably caused by stylet withdrawal from the plasmalemma. Phases I and III are dependent upon the presence of a transmembrane potential, and therefore occur exclusively on plants, but phase II waveforms have also been recorded on artificial diets, and therefore reflect intrinsic aphid activities [Tjallingii, 1985a].

In the present study, aphids were electrically-recorded penetrating potyvirus-infected plants, to investigate stylet activities during acquisition access and their consequences for virus transmission. The results from this system were compared with behaviour during acquisition of the same virus from an *in vitro* system, where aphids penetrated a potyvirus-HC suspension through a Parafilm membrane. The effect of pre-acquisition starvation, which has long been known to influence transmission of non-persistent viruses from plants [Watson, 1938], was also compared using the two acquisition systems.

Materials and methods

Virus and aphid culture. Tobacco plants (*Nicotiana tabacum* L. cv. White Burley) were used as sources of tobacco etch virus (TEV) 2–3 weeks after aphid inoculation, and as test plants at the 2–3 leaf stage. *Myzus persicae* (Sulz.) were reared on Chinese cabbage (*Brassica pekinensis* L. cv. Tip Top) at 15 °C and L16:D8 photoperiod. Adult apterae were usually starved before use, held in plastic Petri dishes for 2 ± 1 h. In some experiments non-starved aphids were also tested, immediately following careful disturbance [Powell, 1993] from a feeding position using a fine camel hair brush. After the acquisition behaviour experiments described below, the aphids were placed on test plants for at least 1h inoculation access. Aphids were then removed, and test plants transferred to a glasshouse. Virus symptoms were scored after 4–7 days.

Virus and helper component preparation in vitro. The highly aphid-transmissible (HAT) strain of TEV was purified as described by Pirone and Thornbury [1983]. Sucrose gradient purified potato virus Y (PVY) HC was prepared as described by Thornbury *et al.* [1985]. PVY HC was used with TEV because we are unable to obtain active preparations of TEV HC, and purified TEV is readily transmitted in the presence of PVY HC [Pirone, 1981]. The solution offered to aphids contained 340 μg ml^{-1} TEV and 50–80 μg ml^{-1} HC in TSM buffer (0.1M Tris, 0.02M $MgCl_2$, 20% sucrose, adjusted to pH 7.2 with H_2SO_4).

Observation and recording of aphid behaviour during acquisition access. Three techniques were employed to investigate virus acquisition by aphids:

(1) Electrical recording. Stylet activities were investigated using the electrical recording system described by Tjallingii [1988]. Con-

ductive silver paint was used to attach a 20 μm diameter gold wire to the aphid dorsum, enabling electrical contact with an amplifier input (10^9 Ohm resistance). An electrode was placed into the potting soil to connect the supplied voltage (± 100 mV). Virus acquisition from plants during electrically-recorded penetrations was investigated as described previously [Powell, 1991a].

The technique was modified to record stylet penetration of the *in vitro* system (Fig. 1). Each insect was wired as above, and immediately transferred to a Parafilm membrane. The Parafilm was stretched over one end of a perspex tube (9 mm internal diameter), and placed onto 15–20 μl of virus-HC mixture. The solution was in contact with a gold wire, providing electrical contact with the supplied voltage. A broad-band green filter (530 nm λ max; 65 nm half band width) was fitted over a cold light source in order to promote stylet penetration [Pelletier, 1990].

When a single stylet penetration had been recorded, either on a plant or the *in vitro* system, the gold wire was cut near the aphid body, and the insect transferred directly to an individual test plant for inoculation access. All electrically-recorded signals were stored on tape (Racal Store 4 recorder) and later replayed to a chart recorder (Graphtec WR7200, paper speed > 5 mm s^{-1}) or computer hard disk for analysis.

(2) Direct observation. In some experiments aphids were not tethered during acquisition access through Parafilm membranes, and penetration was then assessed by observing proboscis contact under a binocular microscope.

(3) Video recording. Acquisition of virus from infected leaf tissue by untethered aphids was

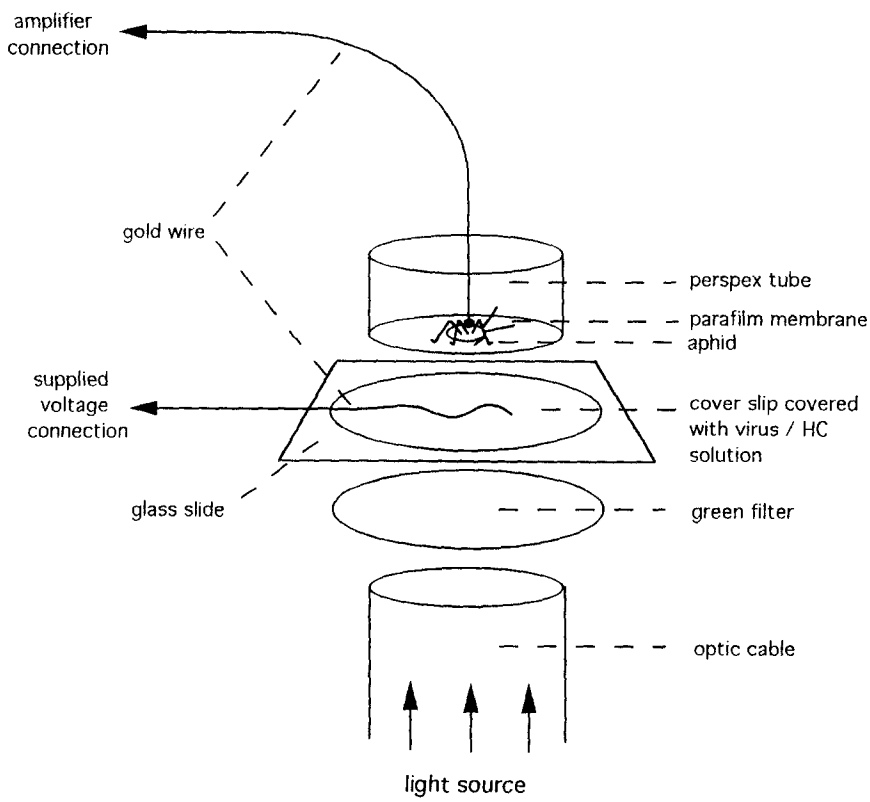

Fig. 1. 'Exploded' view of principal components used to electrically record stylet penetration of *in vitro* virus/helper component suspension. A gap of approximately 30 mm was maintained between the green filter and glass slide, but all other components were brought into contact for recording.

also investigated, using a close-up video technique [Hardie and Powell, 1995]. A 16mm diameter disc was cut from a TEV-infected leaf and floated on water in a transparent dish, positioned above a light source [Powell *et al.*, 1995]. Individual aphids were placed near the centre of the disc and movements continuously recorded using an overhead camera. Using antennal movements as behavioural markers for stylet insertion and withdrawal [Hardie *et al.*, 1992; Powell *et al.*, 1993], each aphid was allowed one penetration of the disc before being transferred directly to a test plant.

Analysis. The proportions of aphids that produced particular behavioural activities during acquisition access and transmitted virus were compared by χ^2 with Yates' [1934] continuity correction. The durations of behavioural parameters were compared using t tests when log-transformed distributions and sample sizes were suitable, and Mann-Whitney U tests [Siegel, 1956] when such criteria were not met.

Results

Comparative transmission of in vitro- *and plant-acquired virus.* Most penetrations on TEV-infected plants were between 10 and 20s duration (Fig. 2a), but penetrations of the Parafilm membrane were more variable (Fig. 2b). Virus acquisition thresholds associated with successful transmission were similar for both systems: 10.8s on plants and 8.4s on Parafilm. Virus transmission efficiency was 28.8% from plants (n = 80) and 19.0% from the *in vitro* system (n = 137) (χ^2 = 2.23; $P > 0.05$). With the *in vitro* system, acquisition penetrations that resulted in successful transmission were longer than those that did not (U = 735.5; $P < 0.001$), but this was not the case with plants (U = 618.5; $P > 0.05$).

Stylet activities associated with transmission. On virus-infected source plants, 83.8% of stylet penetrations included one or more electrically-recorded pds, and all transmissions of TEV were associated with pd occurrence on the source plant, although not all pds resulted in transmission

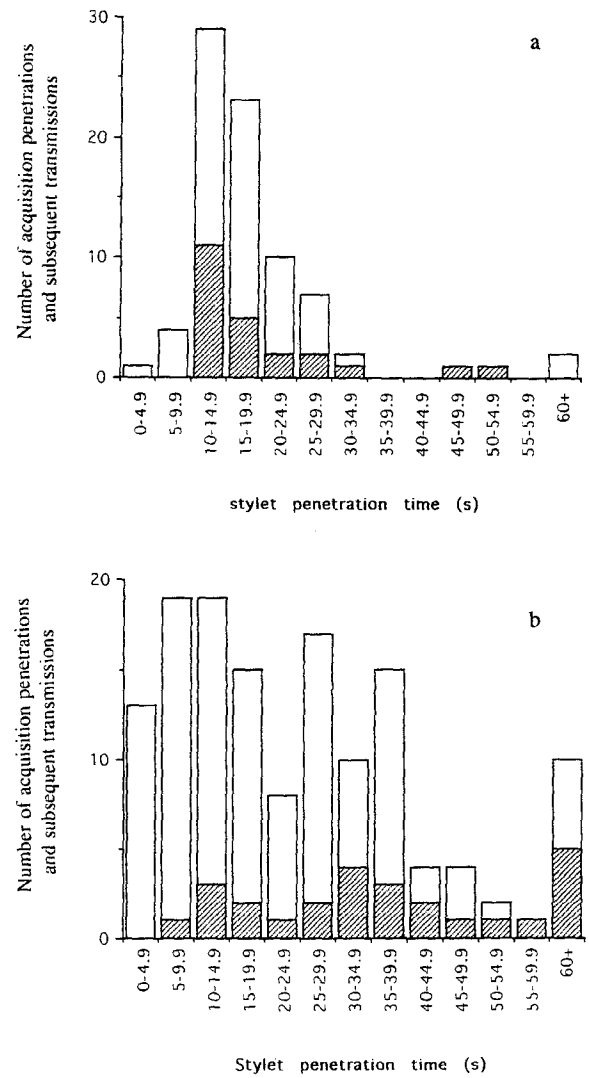

Fig. 2. Tobacco etch virus transmission following single acquisition penetrations by *M. persicae* on infected plants (a) and the *in vitro* system (b). Histograms show the distributions of stylet penetration times; hatched areas represent those penetrations which resulted in subsequent virus transmission.

(Table 1). Most aphids either produced 1 or 0 pds, but one penetration of the source plant included 3 pds. The duration of the intracellular phase (II) of each singly-occurring pd (Fig. 3ab) ranged from 3.6–12.2s, and did not differ for those aphids which did/did not subsequently transmit the virus (U = 440; $P > 0.05$).

Analysis of the recorded pds suggests that phase II can be sub-divided into three parts (Fig. 3b). The earliest part of the intracellular phase (II1)

Table 1. The association between tobacco etch virus transmission to test plants and the occurrence of potential drops recorded during acquisition penetration of the source plant

		Transmission to test plants:	
		no	yes
pd(s) recorded during acquisition:	no	13	0
	yes	42	23

$\chi^2 = 4.70$; $P < 0.05$

consists of a relatively low amplitude (5–8 mV) waveform with a frequency of 8–13 Hz. A second waveform (II2) then follows, with a lower frequency (5–7 Hz) but often higher amplitude (5–20 mV). There then occurs a change to a third part (II3), consisting of a lower amplitude (< 5 mV) waveform. This phase II3 'base' waveform is less regular than phases II1 and II2, and the signal often disappears below the noise level, making determination of the frequency difficult in our recordings from *M. persicae*. However, the II3 base waveform may appear more clearly, with a frequency of 8–14 Hz, when recorded from other aphid species [W.F. Tjallingii, pers. comm.]. Phase II3 usually includes a series of pulses, superimposed on the base waveform, having an amplitude of 5–30 mV and a frequency of 1–2 Hz. Each pulse has a duration of 100–300 ms, and occurs as a positively-directed increase in signal level. Phase II1, II2 and II3 waveforms are all predominantly electromotive force (EMF) component signals, since their polarity and relative amplitude are independent of the direction and magnitude of the supplied voltage [Tjallingii, 1985b]. The shape of the phase II3 waveform resembles pattern E1 [Tjallingii, 1990], but E1 occurs at a higher frequency (2–4 Hz) and seems to be linked with salivation inside phloem sieve elements [Prado and Tjallingii, 1993]. Phases II1, II2 and II3 occurred in all recorded pds, although the exact points of transition between the different subphases were sometimes ambiguous. Four percent of the pds showed phase II3 base with no pulses but, when the pulses occurred, they always continued until the end of phase II. All but one aphid that subsequently transmitted showed clear phase II3 pulses during the pd. When phase II3 pulses were recorded, their duration did not significantly differ according to whether transmission occurred ($U = 389.5$; $P > 0.05$). Phase II waveforms never occurred at the extracellular voltage level on plants.

Phase II3-like pulses were also observed in 59.1% of penetrations of the *in vitro* system; they occurred with similar frequency (1–2 Hz, occasionally increasing to 3 or 4 Hz after several seconds), amplitude (10–30 mV) and pulse duration (100–300 ms) as on plants, although the ascending part of each *in vitro* pulse was typically slower than the descending one (Fig. 3ab *vs.* cd). No preceding phase II1 or II2 waveforms were recorded *in vitro*, and neither were patterns that are associated with periods of sustained ingestion by aphids (E2/G; Tjallingii, 1988; 1990). The occurrence of one or more periods of phase II3-like pulses during penetration of the purified virus-HC mixture was associated with all except one TEV transmission (Table 2). However, the duration of occurrence of phase II3-like pulse activity did not appear to influence virus transmission ($U = 563$; $P > 0.05$). Thirty two aphids penetrated the *in vitro* system for less than 10s; only 4 of these insects produced phase II3-like pulses, and only 1 transmitted virus, so their inclusion in the contingency table falsely enhances the χ^2 value. However, excluding these insects from the analysis also resulted in a significant association ($\chi^2 = 7.2$; $P < 0.01$) between phase II3-like pulse occurrence and transmission.

Effect of prior starvation on aphid behaviour and virus transmission. Single video-recorded acquisition penetrations of TEV-infected leaf discs resulted in transmission by 32.4% of starved aphids, and 2.5% of non-starved aphids. Non-starved aphids took longer to initiate a penetration and penetrated for longer periods than starved aphids (Table 3).

In a preliminary test on the *in vitro* acquisition system, 67 non-starved aphids were allowed a single observed penetration, but none subsequently transmitted virus. However, during the same experiment, a group of 62 starved aphids transmitted virus with an efficiency of 32.3%. This apparent effect of pre-acquisition starvation on transmission of virus from the artificial system was investigated further using electrical recording.

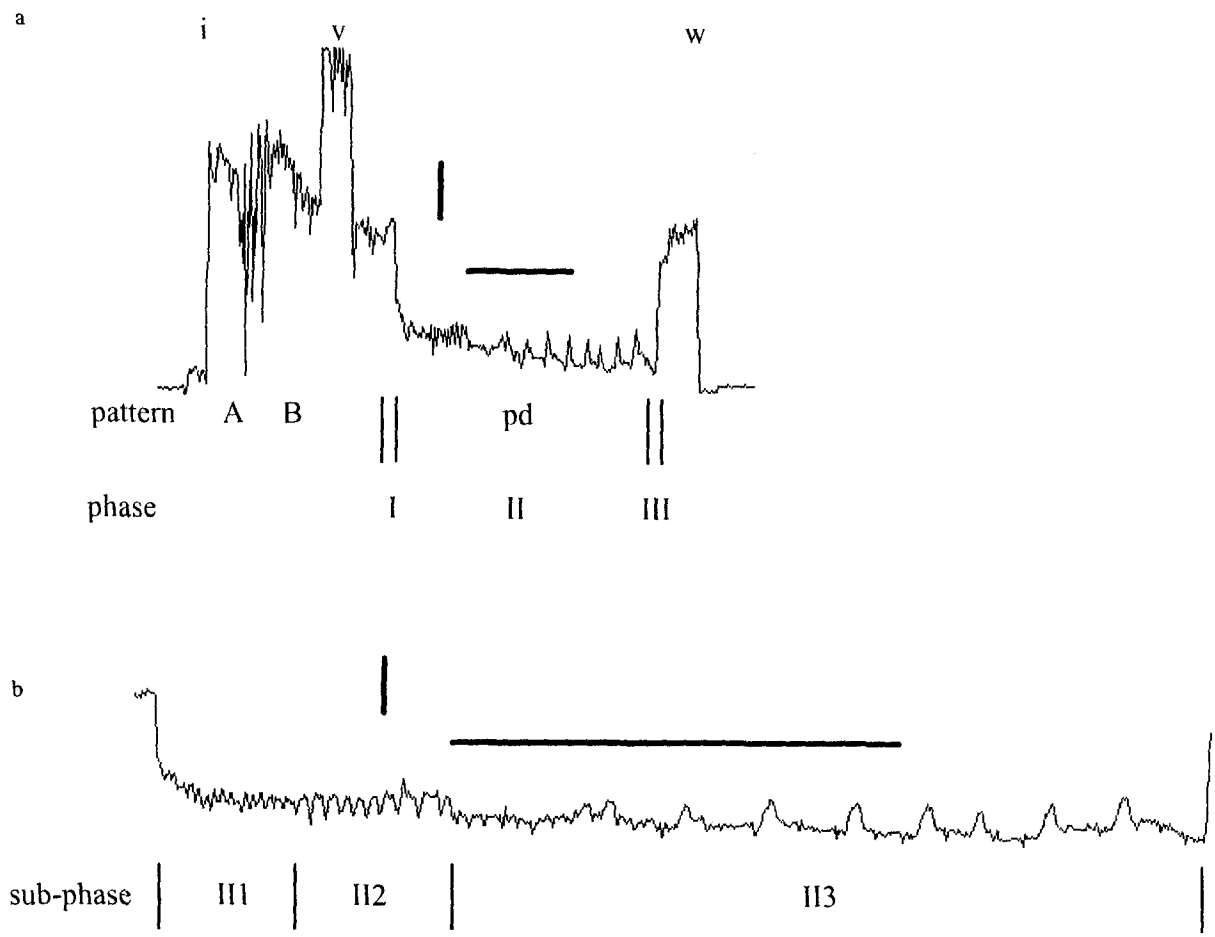

Fig. 3. Typical waveforms recorded on plants (a, b) and the *in vitro* system (c, d). Figure reads from left to right; horizontal bars = 5s; vertical bars = 10 mV; i = insertion of stylets; w = withdrawal of stylets; v = a brief 50 mV increase to the supplied voltage; patterns A, B and potential drop (pd) have been described previously [Tjallingii, 1988]. (a) Stylet penetration of a virus-infected plant showing a single potential drop (pd), with phases I, II and III; (b) Detail of phase II, divided into sub-phases II1, II2 and II3; (c) Stylet penetration of the *in vitro* system showing a period of the phase II3-like pulses; (d) Detail of the *in vitro* phase II3-like pulses.

Table 2. The association between tobacco etch virus transmission to test plants and the occurrence of one or more periods of phase II3-like pulses during recorded acquisition penetration of the *in vitro* system

		Transmission to test plants:	
		no	yes
Phase II3-like pulses recorded *in vitro*:	no	55	1
	yes	56	25

$\chi^2 = 16.4$; $P < 0.001$

Table 3. Transmission of tobacco etch virus by starved and non-starved *M. persicae* following single video-recorded acquisition penetrations on infected leaf discs (Number of aphids tested per treatment = 40; ** $P < 0.01$; *** $P < 0.001$; stylet penetration parameters were log transformed for analysis, but back-transformed means are given)

Prior treatment of aphids	Mean pre-penetration duration (s)	Mean penetration duration (s)	% Virus transmission
starved	13.2 ***	15.8 ***	32.5 **
non-starved	52.5	36.3	2.5

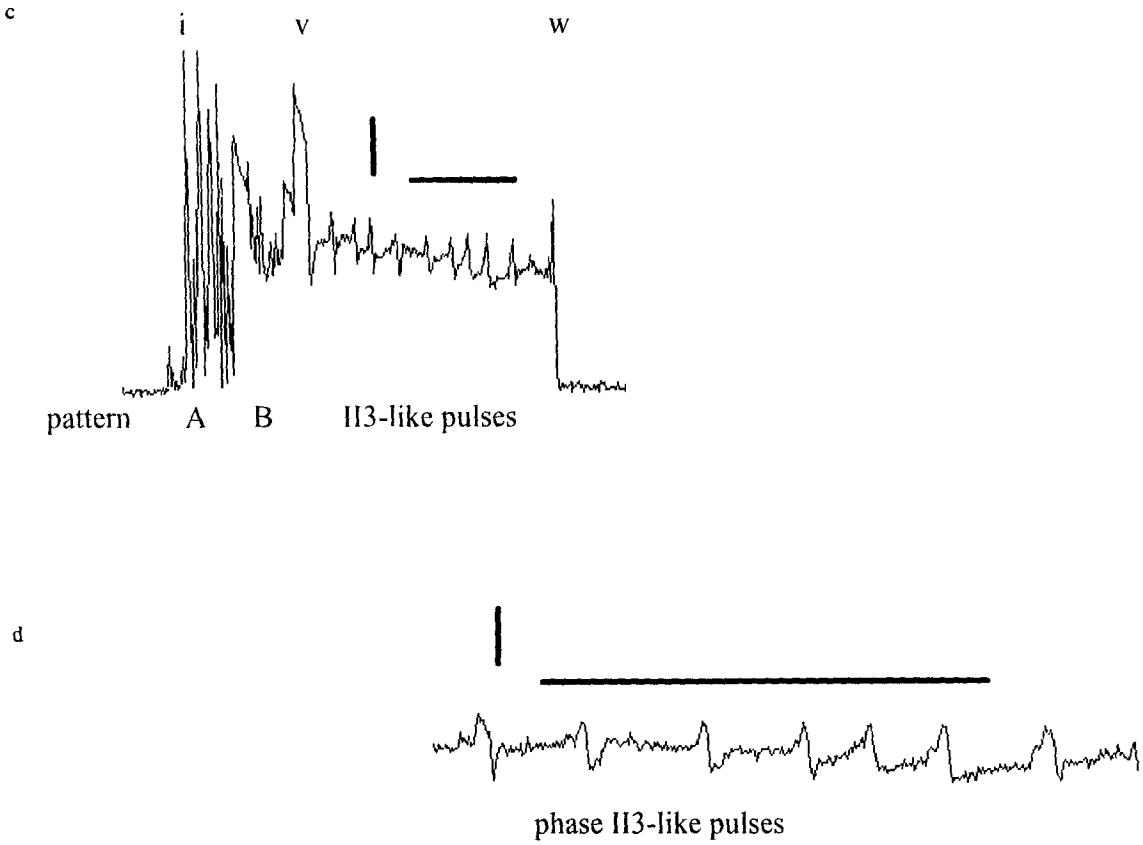

Fig. 3. Continued.

Twenty six percent of starved aphids given electrically-recorded acquisition penetrations through Parafilm membranes transmitted the virus (n = 50). This transmission efficiency was significantly higher than that (2%; n = 100) for non-starved aphids (χ^2 = 18.8; $P < 0.001$). Durations of stylet penetration and periods of phase II3-like pulses by starved aphids were longer than by non-starved aphids, but the occurrence of the phase II3 waveform was not affected by starvation (Table 4) (χ^2 = 0.00; $P > 0.05$).

Discussion

Electrical recordings indicated that acquisition of potyvirus from the natural (plant) and artificial (*in vitro*) systems occurred by very similar processes during brief stylet penetrations. Acquisition threshold times and transmission efficiencies were similar for both system, although durations of stylet penetrations were more varied on Parafilm than on plants. Acquisition of TEV by *M. persicae* occurred when stylets punctured plant cell membranes, as was found for two other potyviruses, beet mosaic virus (BMV) and PVY [Powell, 1991a]. The duration of recorded membrane punctures (pd phase II) was not related to TEV transmission efficiency, although punctures which resulted in PVY and BMV transmission were significantly longer than those that resulted in no transmission [Powell, 1991b]. Similarly, although a period of phase II3 pulses was recorded during 96% of pds in the present study, the duration of this activity did not affect virus transmission efficiency.

Similarities between activities associated with successful virus acquisition from suspension under

Table 4. Transmission of tobacco etch virus by starved and non-starved *M. persicae* following single electrically-recorded acquisition penetrations on the *in vitro* system (ns = no significant difference; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$)

Prior treatment of aphids	Median penetration duration (s)	% Phase II3 occurrence	Median phase II3 duration (s)	% Virus transmission	Number of aphids
starved	36.4	66.0	16.0	26.0	50
	*	ns	**	***	
non-starved	28.9	65.0	10.0	2.0	100

Parafilm and from plants are further strengthened by the association of phase II3-like waveforms with virus uptake from the *in vitro* system. The occurrence of these waveforms with the plant excluded from the electrical circuit indicates that they are generated by intrinsic aphid activities [Tjallingii, 1985a], and their association with *in vitro* virus acquisition suggests that they reflect a behaviour which brings virus/HC into contact with their aphid retention site. Carriage of non-persistent viruses on external stylet surfaces has been proposed as a transmission mechanism [Day and Irzykiewicz, 1954; Kennedy *et al.*, 1962; Bradley, 1964; Pirone, 1969], but acquisition of virus particles would then seem unlikely to be related to any specific behaviour during stylet penetration of a virus suspension. The aphid activity which causes the phase II3 pulses seems likely to involve a brief ingestion or 'sampling' behaviour, and the results are more supportive of the 'ingestion-egestion' hypothesis for non-persistent transmission [Watson and Roberts, 1940; Gamez and Watson, 1964; Harris, 1977] which implicates the aphid foregut as a probable retention site. This hypothesis is supported by autoradiographic and immunolabeling studies which localized potyvirus particles within the food canal and foregut [Berger and Pirone, 1986; Ammar *et al.*, 1994], and implies that inoculation occurs by regurgitation of virus.

Sampling the contents of plant cells may be an important feature of aphid host plant selection behaviour, serving to transfer intracellular metabolites to contact chemoreceptors in the epipharynx [Wensler and Filshie, 1969]. Aphids certainly seem able to discriminate hosts from non-hosts on the basis of brief probes [Wensler, 1962; Klingauf, 1987]. The phase II3 activity occurred as similar waveforms on plants and the TEV-HC suspension, perhaps indicating that plant cells are actively sampled, and that aphids do not rely entirely on cell turgor pressure to bring sap to the foregut. The recorded pulses may reflect the action of the cibarial pump. Aphids which showed a longer period of phase II3 pulses might have taken up larger volumes of the TEV-HC mixture but the period of occurrence of these pulses during acquisition, either on plants or *in vitro*, did not differ for aphids which subsequently transmitted virus vs. those that did not. In a previous *in vitro* study, the quantity of potyvirus particles acquired by aphids did not influence their ability to transmit [Pirone and Thornbury, 1988].

The electrically-recorded waveforms produced during stylet puncture of the plasmalemma by *M.persicae* may differ in frequency, amplitude and electrical origin from those produced by other aphid species [W. F. Tjallingii, pers. comm.]. Although the signals recorded during the intracellular phase were predominantly of EMF origin in the present study, equivalent waveforms from the cabbage aphid, *Brevicoryne brassicae* L., were reported to have a major resistance fluctuation component [Tjallingii, 1985a]. Such differences between species probably reflect differences in stylet activities during cell puncture, which may influence interspecific differences in potyvirus vector efficiency. Further comparisons between recorded stylet activities during membrane punctures by *M. persicae* and *B. brassicae* could be made on the same plant species.

Transmission of TEV from infected leaves was clearly increased by prior starvation of the aphids, as has been found for other non-persistently-transmitted viruses [e.g. Watson, 1938; Watson and Roberts, 1939; Day and Irzykiewicz, 1954; Sylvester, 1954; 1955; Lopez-Abella *et al.*, 1988]. On plants, starvation affected aphid behaviour as previously reported, causing shorter penetrations to be initiated earlier than by non-starved

aphids [Nault and Gyrisco, 1966; Hardie *et al.*, 1992; Powell, 1993; Powell *et al.*, 1993]. The effect of starvation on TEV transmission was at least as clear when using the *in vitro* acquisition system, and could not be accounted for by the differences in recorded stylet activities. Similarly, prior starvation did not affect the occurrence of electrically-recorded membrane punctures during acquisition access to PVY and BMV from infected plants by *M. persicae*, but transmission of these viruses was increased [Powell, 1993]. These findings support the suggestion that non-behavioural factors determine the enhancement of virus transmission by pre-acquisition starvation. Plant sap remaining in the foregut immediately following feeding may contain inhibitors or inactivators of virus and/or HC. Inhibitors or inactivators may also be present in aphid salivary secretions, and such compounds may accumulate during feeding activity, as originally proposed by Watson [1938].

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