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Salivary secretions by aphids interacting with proteins of phloem wound responses

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Abstract

Successful phloem feeding requires overcoming a number of phloem-related plant properties and reactions. The most important hurdle is formed by the phloem wound responses, such as coagulating proteins in the phloem sieve elements of the plant and in the capillary food canal in the insect’s mouth parts, i.e. the stylets. It seems that in order to prevent protein clogging inside a sieve element, ejection of watery saliva plays an important role. This ejection is detected in the electrical penetration graph (EPG) as E1 salivation and always precedes phloem sap ingestion. During this feeding from sieve elements, another regular and concurrent salivation also occurs, the watery E2 salivation. This E2 saliva is added to the ingested sap and, it probably prevents phloem proteins from clogging inside the capillary food canal. Whatever the biochemical mode of action of the inhibition of protein coagulation might be, in some plants aphids do not seem to be able to prevent clogging, which may explain the resistance to aphids in these plants. The relevance of this hypothesis is demonstrated by new experimental results and is related to new EPG results from plants with phloem-located resistance.

Key words: Aphids, clogging, phloem protein, saliva, sieve elements, wound response.

Introduction

Aphids and some other Homopterans feed on phloem sieve elements while delicately keeping these cells alive and their sieve plate pores open by preventing coagulation of phloem proteins (p-proteins) and, later, callose formation (Tjallingii and Hogen Esch, 1993; Prado and Tjallingii, 1994). Thus aphids can ingest phloem sap continuously for many hours or even days from a single sieve element (Tjallingii, 1995). Plant penetration by aphids and other herbivores with piercing mouthparts can be monitored electrically by the electrical penetration graph (EPG) technique (Tjallingii, 1988). This technique allows the recording of signal waveforms reflecting different insect activities, such as mechanical stylet work, saliva secretion, and sap ingestion. The position of the stylet tips in the plant has also been established for some of the waveforms. The waveforms are grouped in distinguishable patterns, representing three main behavioural phases of functionally related activities, i.e. pathway, xylem, and phloem phase. During the pathway and phloem phases, four periods of saliva secretion with at least two types of saliva have been shown by EPGs, one period of gelling salivation and three periods of watery, non-gelling salivation. Gelling salivation occurs during the pathway phase and forms a sheath of saliva enveloping the stylets in the plant tissue intercellularly. Watery salivation occurs: (i) during brief intracellular punctures that occur regularly throughout pathway activity, (ii) at the start of phloem phase behaviour, and (iii) during phloem feeding. The two latter salivation activities are supposed to be used by the insects to cope with responses evoked in sieve elements when wounded. In particular, the fast wound responses dominated by mobilization and clogging of several p-proteins (Knoblauch and Van Bel, 1998; Eckardt, 2001) are thought to be suppressed (Will and Van Bel, 2006).

An overview of EPG and other experimental evidence of aphid salivation phases is provided here. Furthermore, an hypothesis of the sequential aspects of the wound reactions and how salivation might avoid or suppress the wound responses is discussed.

EPG technique

In the electrical penetration graph (EPG) technique an insect and plant are made part of an electrical circuit that
also includes a low DC voltage source and an input resistor of $1 \Omega$ (Fig. 1). An amplifier connects at the ‘measuring point’ between insect and resistor. The amplifier does not influence the circuit as such, due to its very high input impedance ($10^{15} \Omega$) compared with the input resistor. Only the DC EPG system is presented here, which is the most common and sensitive of the two existing EPG systems (Tjallingii, 2000). Voltage fluctuations at the measuring point are transmitted to the recording system after moderate 50–100-times gain by the amplifier. When mouthparts (stylets) are inserted the circuit is completed and the voltage source is adjusted to obtain a signal between ±5 V at the amplifier output. By convention, the plant voltage is adjusted so that when the stylet tips are inserted intercellularly the signal voltage is positive and when the tips are intracellularly the signal voltage is (mostly) negative (Fig. 2). To minimize behavioural disturbance, the insect electrode is a thin, flexible gold wire (mostly 20 μm in diameter and 2 cm long) attached on the aphid’s dorsum by conductive silver glue (water-based). The plant electrode is inserted into the potting soil.

The signal (Fig. 2) shows a clear distinction between probing (stylet penetration) and non-probing. Within probing, the pathway (path), xylem, and phloem phase each contain one or more patterns of voltage fluctuation, called waveforms. After characterizing the waveforms in terms of amplitude, frequency, voltage level (intra- or extracellular), and electrical origin (resistance or electromotive force), the waveforms have been related experimentally to probing activities of the insects and to locations in the plant tissue of the stylet tips (Tjallingii, 1978, 1985; Tjallingii and Hogen Esch, 1993).

### Salivation periods during plant penetration by aphids

On the plant surface, aphids secrete a small amount of gelling saliva (called salivary flange) before stylet insertion. Then stylets enter the plant epidermis starting at the border between two cells, following a pathway between the fibres of the secondary cell wall of one of these cells. This intercellular pathway may cross air spaces in the mesophyll and it eventually leads to the vascular bundle. Gelling saliva is continuously excreted during the pathway phase. This saliva will envelop the stylets and is referred to as the salivary sheath; it remains in the plant after stylet withdrawal. The saliva gels within a second as a reaction with oxygen in the air (flange) or in the tissues (sheath). The EPG signals during the pathway phase show a cyclic activity of mechanical stylet penetration and secretion of gelling saliva (waveforms A, B, and C).

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**Fig. 1.** The EPG circuit. The amplifier (amp) does not influence the circuit, it just amplifies (50×) the signal and the output is connected to any recording device, mostly a computer hard disk. The input resistor has a value of $1 \Omega$, about the mean value of the aphid. The plant voltage is adjustable by the voltage source electrode in order to record the EPG in the conventional way, extracellular signals positive and intracellular signals negative (see Fig. 2). A thin gold wire attached to the insect’s dorsum by silver glue allows the aphid to move.

**Fig. 2.** The EPG signal represented by an example of a 1 h recording. Three probes, the periods of stylet penetration are shown, separated by non-probing periods (np). The waveforms can be grouped into three behavioural phases, each comprises one or more waveforms. The major part of the signals have a positive voltage, as adjusted by the voltage source (Fig. 1). These reflect extracellular probing activities. The spikes during the path, as well as during the complete phloem phases, are on a negative, intracellular voltage level due to the membrane potential of the punctured plant cell. The first phloem phase only shows waveform E1, referred to as a single E1 (sgE1) in text, whereas in the second phloem phase waveform E1 and E2 do occur. Such E1 and E2 periods are referred to as E1 and E2 fractions, respectively.
Most cells along the stylet pathway are briefly (typically for 5–10 s) punctured intracellularly, but the stylets are always withdrawn from the cells and then continue along the intercellular pathway (Tjallingii and Hogen Esch, 1993). The intracellular punctures appear as the potential drop (pd) waveform in the EPG. At the beginning of a potential drop, the signal shows a sharp negative edge when the stylet tips pass through the plasmalemma, reflecting the membrane potential of the punctured plant cell. At the end of a potential drop, a sharp positive edge marks the withdrawal of the stylets from the cell. Most cells survive these punctures with little or no effect. During the puncture, some watery saliva is injected into the cell (Martía et al., 1997).

Two more cases of watery salivation have been found during phloem phase. First, every phloem phase starts with a salivation period, reflected as waveform E1. When E1 saliva is injected for about 1 min into the sieve element, in general, a subsequent period of passive phloem sap ingestion will occur with continuous salivation (waveform E2). However, during E2 the saliva will not reach the plant; rather it is immediately mixed with the phloem sap that is forced into the food canal by the high hydrostatic pressure in the sieve elements. The reason E1 saliva is injected into the sieve element and E2 saliva is not, is that in the head of the insect, the food canal has a valve, which is closed during E1 and open during E2. Since the outlet of the saliva is at some distance (a few μm) from the stylet tip (Fig. 3) an open valve during E2 allows phloem sap to flow up through the food canal and to catch up the saliva in this stream, preventing the saliva from entering the sieve element. Conversely, when the valve is closed during E1 there is no flow of sap up to the food canal to prevent the saliva from entering the sieve element (Prado and Tjallingii, 1994). A phloem phase can consist of only E1 (1st phloem phase, Fig. 2), referred to as a single E1 (sgE1), or in combination with E2 (2nd phloem phase, Fig. 2), which will be referred to as an E1 fraction (E1fr). Sometimes, depending on the sieve element ‘suitability’, after E2 the aphid may return to E1 or may alternate between E1 and E2 now and then, but E1 is always shown first.

Thus four salivation periods are distinguished in the EPG (Fig. 4). Considering salivation from the point of view of the plant tissue, it can be stated that the sheath saliva predominantly occurs intercellularly, pd salivation is watery and is injected into cells in all tissues, including sieve element. Pd-punctures also occur in most sieve elements as shown by electron micrographs (Tjallingii and Hogen Esch, 1993). Both E1 and E2 salivation are watery and occur when the stylet tips are in the sieve element.

### Salivary glands and saliva composition

The salivary glands of aphids are paired and the right and left glands have two glandular units, a large principal gland and a smaller accessory gland. The salivary ducts of both glandular units on one side join together and then their common duct joins the one coming from the contralateral side. The principal gland is innervated and contains eight secretory cells, possibly secreting different components (Ponsen, 1972). This gland seems to play a major role in the sheath saliva production. The accessory gland does not appear to be enervated, and its cells do not show much differentiation. Transmission studies of persistent/circulative

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**Fig. 3.** EPG of the transient of waveform E1 to waveform E2 and a diagram with the two activities reflected by each of them. Stylet tips, mostly the maxillary tips only, are inserted into the sieve element. During E1 the cibarial valve in the insect’s head closes the food canal so that excreted saliva is allowed to move into the sieve tube. During E2 the cibarial valve is open and the sap is forced into the food canal by the high hydrostatic pressure in the sieve element. During E2 there is a continuous salivation, but this saliva does not reach the plant. It will be mixed with the sap and directly moved into the food canal.
plant viruses have shown that the accessory glands transfer the virus from the haemolymph to the salivary canal in the stylets and into plants (Gray and Gildow, 2003). From this, it has been inferred that the watery E1 saliva must come from the accessory glands since E1 salivation is responsible for inoculation of these viruses (Prado and Tjallingii, 1994). It remains unclear whether the principal glands exclusively produce the sheath saliva and the accessory glands the watery saliva. It cannot be excluded that saliva composition comes from both glands. Although there is no experimental evidence, the innervation of the principal gland suggests that aphids may adjust at least the saliva contribution from this gland on the basis of gustatory information.

Recently, Peter Miles (1999) reviewed the current aphid saliva knowledge. The published protein components show a lot of contradictions, not only between but also within aphid species (Miles and Harrewijn, 1991; Baumann and Baumann, 1995; Urbanska et al., 1994; Madhusudhan and Miles, 1998; Cherqui and Tjallingii, 2000; Kornemann, 2005). Parafilm® covered diets have mostly been used to collect saliva. Possibly, on the basis of the sensory aspects mentioned above, the saliva composition might vary due to the different diet compositions. Moreover, using diet collection, sheath saliva has inevitably been mixed with watery saliva while E1 and E2 salivation periods are mostly short in fluid diets. Sampling saliva from separate behavioural phases is the most difficult aspect of salivary research. It cannot be excluded that within watery saliva, the composition differs between pd-salivation, E1 salivation, and E2 salivation.

**Phloem wound responses and aphid salivation**

As demonstrated by Knoblauch and Van Bel (1998) some proteins appear to play a key role in fast wound responses in sieve elements. When sieve elements are severed by a glass needle or by UV radiation these proteins – stored at different locations in sieve tube cells – are released and subsequently coagulate, causing a blockage of the downstream sieve plate. Injury by aphid stylets apparently do not lead to such wound responses since aphids start ingesting from a sieve element within a few minutes after a puncture and may continue to do so for hours at least. If the p-proteins had blocked the sieve plates, ingestion would be interrupted as soon as the pressure in the punctured sieve element was no longer supported by their unrestricted connection to adjacent sieve elements. Also, if aphids did not inhibit sieve element wound responses, punctured sieve elements would show a similar coagulation when wounded by a glass needle of a similar diameter. Neither feeding difficulties nor coagulated protein masses have been observed so far in sieve elements punctured by aphid styles. It seems likely, therefore, that E1 salivation may suppress the wound responses. How the suppression works and in what stage the E1 saliva interferes with the wound responses, is not clear.

Stylectomy (fast stylet amputation) during E2 waveforms can be used to collect phloem sap exuding from severed stylets (Van Helden and Tjallingii, 1994). However, exudation of the phloem sap often stops soon after stylectomy. Electron micrographs of the stylet stump in the plant showed the presence of coagulated lumps of protein inside the food

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**Fig. 4.** All salivation periods detected by the EPG so far. (i) Sheath salivation (grey area with red arrowheads), gelling saliva that will envelop the stylets along the track. (ii) Intracellular watery salivation (open blue arrowheads) during the potential drop (pd) waveform; a pd reflects a brief intracellular puncture into any cell along the stylet track but the track itself remains intercellular. (iii) E1 salivation into a sieve element (purple arrows), always the first phloem phase activity. (iv) E2 salivation, secreted in pulses and directly ingested (dotted long black arrow in stylet), mixed with the phloem sap. Phloem: CC, companion cell; SE, sieve element.
canal (Tjallingii and Hogen Esch, 1993). This suggests (i) that there is free (unbound and not coagulated) protein in the sieve elements; and (ii) when E2 salivation stops due to stylectomy, this protein will clog in the food canal. Thus it seems likely, that E1 salivation does not prevent the release of bound p-protein but it may only prevent its coagulation, at least in the sieve tube. It is probable that continuously adding E2 saliva to the imbibed phloem sap at the beginning of the food canal prevents clogging of p-proteins in the food canal. The reason why the protein solution does not clog in the sieve tube but may do so in the food canal is not clear, it might be that at the chitin wall of the thin and long capillary of the food canal, some ion exchange occurs. A small increase in calcium concentration has been shown to play a role in many cases of protein coagulation in animals and will trigger p-protein coagulation as well (Eckardt, 2001; Knoblauch et al., 2001).

**Phloem-based plant resistance to aphids**

In a number of cases, EPG studies on plant resistance have indicated that a phloem-located mechanism is involved. One example shown here concerns lettuce resistance (Lactuca sativa × Lactuca virosa) to the aphid Nasonovia ribisnigri. The lettuce studies used a resistant line containing the NR gene (single dominant resistance gene) indicated by NRR (Van Helden and Tjallingii, 1993) and a near iso-genic susceptible control, indicated by NRS. Two more examples shown here are a melon (Cucumis melo) cultivar, TGR-1551, resistant to Aphis gossypii (Garzo et al., 2002), and a potato (Solanum tuberosum) cultivar, Kardal, resistant to Myzus persicae. Data from the susceptible controls of the latter two are not shown, as they are similar to the NRS data from the lettuce study. All data are from new 4H EPG recordings, 12–17 replicates per cultivar, obtained under the same conditions as described by Van Helden and Tjallingii (1993), Garzo et al. (2002), and Alvarez et al. (2006), respectively.

On NRR plants, fewer aphids showed phloem phase (E1 initiation) than on susceptible NRS plants, whereas on TGR and Kardal, the percentage of aphids showing phloem phase was similar to the susceptible controls (about 90%; Fig. 5). This suggests that the first step in sieve element recognition was more difficult to accomplish on NRR, transient difficulties from sieve element puncture [pd] activity to E1 salivation, but not on NRS, TGR and Kardal. Since aphids on NRR and NRS plants showed an equal time lapse between stylet insertion into the epidermis and E1 initiation there is no indication that the lower percentage on NRR would be due to sieve element location problems.

Initial E1 salivation may or may not be followed by phloem ingestion (E2). If not, the phloem phase remains a single E1 period (sgE1). On susceptible NRS, 23% of the aphids showed a single E1 period (three aphids, and only one period each, average number = 0.2; Fig. 5), on NRR it was 35% (average number = 0.4), but on TGR and Kardal 75% (average = 1.8) and 87% (average number = 4.5), respectively, indicating transient difficulties from E1 salivation aspects and one E2 ingestion aspect on phloem-resistant plants. NRR is a lettuce accession originating from a lettuce hybrid (Lactuca sativa × L. virosa) which is resistant to Nasonovia ribisnigri, TGR (−1551) is a melon accession with resistance to Aphis gossypii, and Kardal is a potato accession with resistance to Myzus persicae. NRS is a near isogenic susceptible lettuce used as a control. On susceptible melon and potato accessions, the respective aphids behaved similarly but results are not shown here for simplicity. % of aphids with gives the fraction of aphids showing any E1, any single E1 periods, and sustained (>10 min) E2 (sE2), respectively. The total number (avg) of single E1 periods per aphid is shown in graph 4 and the total duration (avg) of single E1 periods and E1 fractions is given last. On TGR and Kardal, aphids show more and longer E1 periods, whereas E2 is very much reduced. On NRR, E2 is also reduced but the number and the duration of E1 periods are similar to the susceptible NRS, although fewer aphids showed any E1 at all. It is concluded that TGR and Kardal seem to support the hypothesis that the resistance might be due to a reduced ability to suppress phloem wound responses.
salivation to E2 ingestion. Apart from the number of sgE1, the duration of the E1 periods (both sgE1 and E1fr) was considerably increased on resistant TGR and Kardal plants (Fig. 5; note the log scale), but not on resistant NRR. Thus, in TGR and Kardal in particular, E1 salivation occurs substantially more often and for longer durations than on susceptible plants, suggesting initial difficulties to initiate phloem sap ingestion.

It is unclear on what basis aphids decide to start E2 ingestion, but fewer aphids did so on all three resistant plants. On resistant plants fewer aphids showed sustained E2 (sgE2) activity longer than 10 min (Fig. 5), which is a threshold time often used as a ‘phloem acceptance’ indicator (Tjallingii, 1990). Garzo et al. (2002) suggested that prolonged E1 salivation and reduced E2 would indicate a reduced ability to suppress the phloem wound responses in TGR. Also in these new experiments, aphids on TGR and Kardal showed a sustained E1 and reduced E2, thus supporting this hypothesis. However, phloem phase behaviour of aphids on NRR plants does not show the same changes. So presumably, the resistance in NRR is due to a completely different phloem-located mechanism.

Resistance to A. gossypii and M. persicae in TGR and Kardal, respectively, does, however, not imply resistance to other aphid species on these plants. It should be noted here that resistance to aphids is mostly very species-specific. Kardals resistance to M. persicae, for example, does not affect phloem phase behaviour of Macrosiphon euphorbiae, another common aphid on potato. Sieve element wound responses seem Ca++ triggered (Knoblauch and Van Bel, 1998; Knoblauch et al., 2001). Thus calcium binding might be the key factor of E1 salivation, disabling the wound responses. But when E1-disabling by one aphid species no longer works in resistant plants, why would it still work with another aphid species? In other words how to explain that the resistant plant discharges Ca binding of E1 saliva by one aphid species and not by another? Many more questions arise that can only be answered when more knowledge about wound response and salivary proteins are gained. In addition to the resistance measurements in young leaves of TGR and Kardal plants, it appeared that old leaves became susceptible. Although is not clear whether this is in conflict with the hypothesis, it will be the challenge of further research to show whether this specificity and age-dependent expression agrees with the hypothesis or not. Evidence of long-distance signals in sieve tubes and phloem-phase behavioural changes will be presented elsewhere in this symposium (Will and Van Bel, 2006).

There is now worldwide interest in the molecular aspects of constitutive as well as induced resistance to phloem feeders (Klingler et al., 2005; Kaloshian and Walling, 2005), but there still is wide gap between the molecular backgrounds and the actual mechanisms. With respect to phloem–insect interactions the composition and timing of salivary secretions is apparently a key factor.

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References


