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Did the evolutionary transition of aphids from angiosperm to non-spermatophyte vascular plants have any effect on probing behaviour?

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Abstract

The probing behaviour of *Macrosiphum ptericolens* Patch on *Pteridium aquilinum* (L.) Kuhn was monitored using the electrical penetration graph (EPG) technique (DC system). This first EPG study on fern-associated aphids showed all characteristic features of probing behaviour typical of aphids on angiosperm plants. No special adaptation of stylet penetration and phloem feeding appeared necessary in the evolutionary switch from angiosperm to vascular non-spermatophyte plants, in spite of the special structure of their sieve elements. The waveforms related to pathway, phloem, and xylem activities by aphids and the sequence of events during probing are very similar to those known from angiosperm-associated aphid species. Stylet penetration in plant tissues of both plant groups appear extracellular with short probes into cells adjacent to stylet route and phloem sap ingestion from *P. aquilinum* seems passive as well and may last for several hours.

Key words: *Macrosiphum ptericolens*, electrical penetration graph, EPG, stylet penetration.

Introduction

The world fauna of aphids (Homoptera Aphididae) comprises approximately 4700 species (Blackman and Eastop, 2007). At present, the specifically fern-feeding Aphididae are represented by 44 species of 15 genera (Jensen and Holman, 2000; Blackman and Eastop, 2006), which makes approximately 23% of the sucking insect species known from ferns (Hendrix, 1980). The evolution of fern-feeding aphids is very interesting. At the time of origin in the late Jurassic, aphids were associated with gymnosperm woody plants, although ferns were an important component of vegetation at that time (Heie, 1987). It is generally believed that at the beginning, the radiation of aphids followed the evolutionary changes in the plant kingdom, i.e., firstly the transition from gymnosperms to dicotyledonous angiosperm trees and shrubs occurred, then to herbaceous and woody monocotyledons. Mosses, horsetails and ferns were acquired as host plants only recently (Shaposhnikov, 1987; von Dohlen and Moran, 2000). The cladistic analysis by Jensen and Holman (2000) showed that the largest group of the fern-feeding aphids, i.e., the 16 species of the genus *Macrosiphum* originated as a result of the recent adaptive evolution from spermatophyte plant feeders.

Almost all aphids on angiosperm plants feed solely on the phloem sap, using highly specialized stylet-like sucking-piercing mouthparts, called stylets (Pettersson *et al.*, 2007). The stylet route in the plant tissues is intercellular and passes through the secondary wall material (Tjallingii and Hogen Esch, 1993), frequently interrupted by brief intracellular punctures. Presumably these punctures aim gustatory purposes and leave the punctured cells intact (Tjallingii, 1995; Martin *et al.*, 1997). Occasionally, aphids may 'drink' from xylem vessels to relieve water stress. During the phloem phase of the periods of stylet penetration (probes), sieve element feeding is always preceded by sieve element salivation to suppress phloem wound responses (Will *et al.*, 2007). If suitable, the sieve element is fed upon for hours up to days continuously (Pettersson *et al.*, 2007). The structure of vascular tissues in angiosperm and non-spermatophyte plants differs substantially. In angiosperms, the assimilate conducting vessels consist of sieve tubes, which are elongated ranks of individual sieve-tube member cells connected by sieve plates with large pores (0.2 - 1 μm in diameter) (Ehlers *et al.*, 2000). The sieve pores are essentially open in the functional state, which enables the mass transfer of solutes (Knoblauch and van Bel, 1998). Due to the high hydrostatic pressure in the sieve tubes, the ingestion of phloem sap by aphids seems mainly passive (Tjallingii, 1995). In vascular non-spermatophyte plants, such as leptosporangiate ferns, elongate sieve elements (i.e., sieve cells) are 30 to 40 mm long, with horizontal or slightly oblique end walls and with sieve areas on both side and end walls. The sieve areas are numerous, elongate, and equally differentiated on the lateral and end walls (Smoot, 1985). The sieve-area pores are small (0.12-0.25 μm) and contain variable amount of ER membranes, but the membranes do not occlude the pores entirely (Warmbrodt and Evert, 1979; Warmbrodt, 1980; Evert *et al.*, 1989; van Bel, 1999). Even in bracken *Pteridium aquilinum* (L.) Kuhn (Dennstaedtiaceae), which is one of the evolutionarily recent leptosporangiate ferns (Smith *et al.*, 2006), the pores of various sizes are not aggregated into sieve plates; the larger pores occur individually in side walls and not in the sieve areas of end walls (Smoot, 1985).

In the present study, the probing behaviour of *Macrosiphum ptericolens* Patch that is monoecious and holocyclic on *P. aquilinum* (Holman, 1974; Jensen and Holman, 2000; Halarewicz, 2005) was monitored using

the electrical penetration graph (EPG) technique. This study was carried out to investigate whether the angiosperm-fern host-plant switch might include adaptations in aphid probing due to the different structures of the phloem systems of these plants. Aphid probing and feeding on ferns has not been documented before.

Materials and methods

Aphid probing behaviour, i.e. plant penetration by aphid stylets was monitored using the electrical penetration graph (EPG) technique EPG (DC system, Tjallingii, 1988), which is commonly applied in hemipteran-plant studies. In this experimental set-up, aphid and plant are made parts of an electrical circuit, which is completed when the aphid inserts its stylets into the plant. A weak voltage is supplied to the plant, and all voltage changes are recorded as EPG waveforms that have been correlated with aphid activities and stylet tip positions in plant tissues (Tjallingii, 1994). The values of parameters derived from EPG recordings, e.g., the duration of probing, duration of phloem sap ingestion, number of probes, etc., reflect the level of suitability of plants to aphids (Mayoral *et al.*, 1996). *P. aquilinum* plants and *M. ptericolens* apterous females were randomly collected from a natural site in a conifer forest in the Ślęża Landscape Reserve (Ślęża Massif, Lower Silesia, Poland). In the laboratory, clean, mature, fully developed leaves were used for EPG recording. Leaves used for EPG recording were detached from the plants immediately before the experiments and their petioles were inserted in a plastic vial with water, in which the plant electrode was inserted. After silver glue attachment of the gold wire electrode, aphids were starved for 1 hour prior to the experiment. Probing behaviour of 10 apterous females was monitored for 8 hours continuously. Signals from a 4-channel EPG device were stored on the computer hard disc (100HZ sample rate) and analysed subsequently by using PROBE 2.1 software (EPG-Systems, Wageningen, The Netherlands; www.epgsystems.eu). The following EPG patterns were distinguished: np (non probing - aphid stylets outside the plant), A, B, C (stylet pathway phase - penetration of tissues), E1 (sieve element salivation), E2 (sieve element ingestion), and G (xylem ingestion). A number of sequential and non-sequential parameters were calculated, i.e. referring to the sequence of events during recordings or waveform occurrence frequencies, total and average durations, etc., respectively (van Helden and Tjallingii, 1993).

Results and discussion

The EPG study of *M. ptericolens* probing on *P. aquilinum* revealed all waveforms that represent three major behavioural phases that have previously been described from various aphid species on angiosperm plants, which are: pathway (C and F), phloem (E1 and E2), and xylem (G) phases. The pathway waveform C had a typical appearance, i.e., it was composed of periods of waves at extracellular level and potential drops to intracellular level, which reflect stylet progress in the secondary cell walls and brief cell punctures along the stylet track, respectively. During the phloem phase, E1 and E2 waveforms had typical appearance known from most aphids on angiosperm plants and the sap ingestion periods E2 (if present) were always preceded by watery salivation E1. Waveforms F and G had also the typical shape and they occurred sporadically (figure 1).

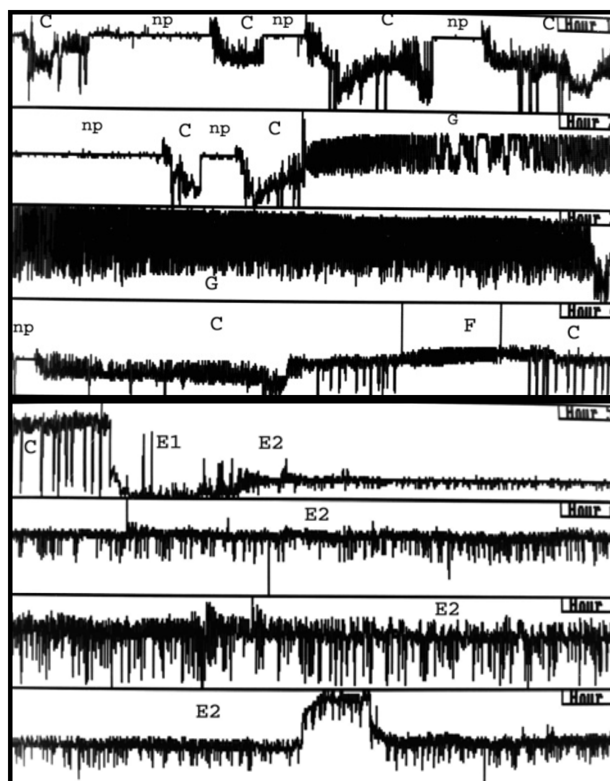

Figure 1. Representative 8-hour EPG recording of *M. ptericolens* on *P. aquilinum*. np = non probing; C = pathway (probing in peripheral plant tissues); E1 = phloem watery salivation; E2 = phloem sap ingestion; F = derailed stylet activities; G = xylem sap ingestion.

The average EPG recording from *M. ptericolens* on *P. aquilinum* started with a short (a few minutes long) period of non-probing, then aphids inserted stylets into the plant (figure 1). At the beginning, the probes were relatively short and included only pathway phase. There were 20 probes during the 8-hour recordings on average, but the variation in probe number among the recorded aphids was relatively high (table 1). On average, 19.3 (± 12.1) exclusively pathway probes preceded a probe that ended in the phloem. The period of pre-phloem activities lasted for 3.6 hours from the onset of the experiment, and 2.5 hours within an E-probe, on average. Almost all aphids on *P. aquilinum* showed phloem sap ingestion periods (E2) during the 8-hour recording. The success ratio of phloem acceptance, i.e. the proportion of aphids showing a sustained phloem ingestion (E2 waveform) period, was 85%, which means that almost all contacts with phloem elements ended in ingestion. The proportion of E2 in the phloem phase was ca. 93%. The average duration of the first phloem phase

Table 1. EPG-derived parameters related to probing activities of *M. ptericolens* on *P. aquilinum*.

EPG parameter		*Macrosiphum ptericolens*
Total duration of probing	h	6.9 (± 0.6)
Total duration of pathway phase*	h	5.5 (± 1.5)
Total duration of phloem phase E1 + E2	h	1.3 (± 1.5)
Total duration of sap ingestion E2	h	1.3 (± 1.4)
Number of probes	#	20.4 (± 12.3)
Number of probes before phloem phase	#	19.3 (± 12.1)
Number of phloem phases E1 and E1 + E2	#	1.3 (± 0.5)
Number of E2 phases	#	1.1 (± 0.4)
Time from 1st probe to 1st phloem phase	h	3.6 (± 2.3)
Time to phloem phase within a probe	h	2.5 (± 2.7)
Duration of 1st phloem phase	h	1.3 (± 1.4)
Duration of 1st sap ingestion phase E2	h	1.7 (± 1.5)
Proportion of aphids with phloem phase	%	70
Proportion of aphids with sustained sap ingestion phase	%	70

Values represent means (n = 10) (± SD). * F and G waveforms occurred sporadically and were included in pathway phase.

was 1.3 hours. The maximum recorded duration of uninterrupted E2 phase was at least 3.8 hours (i.e., the EPG experiment ended before the E2 phase ended) (figure 1). The proportion of non-probing decreased with time, whereas the proportion of pathway and phloem activities increased in the course of time (figure 2).

Not only the waveforms related to pathway, phloem, and xylem aphid activities have a similar shape but also the sequence of events during probing resembles that known from various aphid species on angiosperms. Probing by *M. ptericolens* as well as by angiosperm aphids is composed of a series of probes separated by periods of non-probing. The probes increase in duration with time and the aphids mostly need several hours to reach the first sieve element waveforms and sap feeding. Also, sustained phloem ingestion is often maintained in *M. ptericolens* for more than one hour as in other aphids (Tjallingii, 1988; 1995; Gabryś and Tjallingii, 2001; Halarewicz-Pacan *et al.*, 2003; Pettersson *et al.*, 2007; Kordan *et al.*, 2011).

In part, this EPG study on *M. ptericolens* supports the findings of Evert *et al.* (1989) considering *Myzus persicae* Sulzer on *Pellaea viridis* (Forsskal) Prantl (Polypodiales Pteridaceae). Based on the electron microscopy studies, Evert *et al.* (1989) reported that the penetration of the epidermis, hypodermis and mesophyll by the aphid stylets was entirely intercellular but at the endodermis and within the veins the stylets followed an intracellular pathway. Our EPG data also show that the stylet penetration in peripheral plant tissues, epidermis and mesophyll is extracellular with short probes into cells adjacent to stylet route. The image of intracellular position of stylets in endodermis and veins that was seen by Evert *et al.* (1989) might have concerned necrotic or disintegrating cells, like in their previous study (Evert *et al.*, 1973), which was explained by Tjallingii and Hogen Esch (1993) basing on the EPG/transmission electron microscopy correlation studies. Our EPG results clearly demonstrate that the penetration in the deeper tissue layers is also extracellular, which can be seen especially during the C/E1 (i.e., pathway/phloem salivation) extracellular/intracellular transition phase

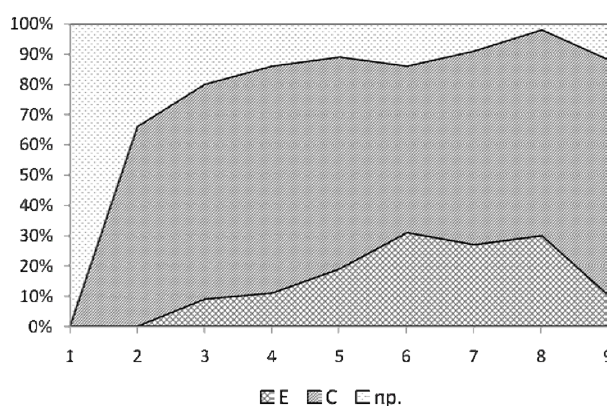

Figure 2. Sequence of probing activities of *M. ptericolens* on *P. aquilinum* during 8-hour EPG recording (mean of 10 aphids). np = non probing; C = pathway phase; E = phloem phase (E1 salivation and E2 sap ingestion). F and G waveforms occurred sporadically and were included in pathway phase.

that always indicates the beginning of aphid stylet activities in the sieve elements (Tjallingii, 1995). Evert *et al.* (1989) showed that aphid stylet tips were found in the fern sieve elements and were devoid of salivary sheath material, which suggested that the aphids were feeding on the conducting sieve elements. At the same time, they found no apparent damage in sieve elements containing aphid mouthparts. Indeed, once the aphids start with specific phloem activities (show phloem phase waveforms), they stop producing the salivary sheath material and the sieve elements that the aphids feed upon remain intact (Tjallingii and Hogen Esch, 1993). Moreover, in our study we found that the phloem sap from *P. aquilinum* is ingested in a passive way by *M. ptericolens* (E2 waveform; Tjallingii, 1995) and the aphids may ingest sap from one phloem element for several hours. This finding may be indirect evidence that the membranous material in the sieve pores of the fern phloem cells does not stop or hamper a continuous flow of the phloem sap.

Generally, the results of this first, preliminary, EPG study suggest that the fern-associated aphid *M. ptericolens* retained all characteristic features of probing behaviour typical of aphids on angiosperm plants. It seems that the evolutionarily recent switch to vascular non-spermatophyte plants and the special structure of their sieve elements had little effect on aphid probing behaviour and stylet penetration. However, EPGs from other aphid species on ferns but also on horsetails and mosses will certainly be interesting for comparison; ideally, in combination with microscopy.

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