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RESEARCH ARTICLE

An aphid's Odyssey – the cortical quest for the vascular bundle

Angela Hewer, Alexander Becker and Aart J. E. van Bel*

Plant Cell Biology Research Group, Institute of General Botany, Justus–Liebig–University, Senckenbergstraße 17–21, D-35390 Gießen, Germany

*Author for correspondence (Aart.v.Bel@bot1.bio.uni-giessen.de)

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SUMMARY

Sensing pH and sucrose concentration (with a preference for pH values of 7.0–7.5 and sucrose concentrations of approximately 400 mmol l^{-1}) enables aphids to recognise sieve tubes inside vascular bundles. However, it is still unclear how aphids find their way to the vascular bundles. Membrane potentials in the cortex of *Vicia faba* stems were measured along a radial transect from the epidermis to the sieve elements and there was no gradient detected that could be used by aphids to guide their stylets to the sieve elements. Additionally, aphids did not demonstrate a preference between artificial diets with low or high levels of dissolved oxygen, making it unlikely that oxygen gradients in the cortex assist orientation towards the phloem. Tracks of salivary sheaths indicate that aphids search for vascular bundles in a radial direction (perpendicular from the stem surface to the vascular bundle) with regular side punctures in a pre-programmed fashion. Optical examination and electrical penetration graph (EPG) recordings suggest that aphids (*Megoura viciae*) probe the vacuolar sap of cortex cells. Acidic pH (5.0–5.5) and low sucrose concentrations in vacuoles, therefore, may provoke aphids to retract their stylets and probe the next cell until a favourable cell sap composition is encountered. The importance of sucrose as a cue was demonstrated by the experimental manipulation of *Ricinus communis* plants that cause them to transport hexoses instead of sucrose. Aphids (*Aphis fabae*) ingested less phloem sap of plants transporting hexoses compared with plants transporting the normal sucrose. The proposed rejection–acceptance behaviour provides a universal plant-directed mode of how aphids orientate their stylets towards the phloem.

Key words: aphids, *Aphis fabae*, *Megoura viciae*, *Ricinus communis*, *Vicia faba*, cortical stylet pathway, orientation cues.

INTRODUCTION

Numerous studies on aphid growth, reproduction and survival on diets (e.g. Auclair, 1963; Auclair, 1969; Mittler and Dadd, 1963; Simpson et al., 1995) have demonstrated that aphids perform best on diets that mimic the sucrose and pH composition of sieve-tube sap (pH values of approximately 7.0 and a high concentration of sucrose). A preference for similar diets in choice chambers (Mittler and Dadd, 1964; Hewer et al., 2010) indicates that aphids have the sensory means to identify sieve tubes in plant tissues (Hewer et al., 2010). The sensors must be located in the stylets, i.e. the highly specialised mouthparts that penetrate the plant tissues. The stylets consist of four components: two outer, mandibular mouthparts, which contain neuronal canals with mechano-sensitive dendrites, and two inner, maxillary mouthparts, which are interlocked to form the food canal and the salivary canal (Kimmins, 1986; Tjallingii and Hogen Esch, 1993), which fuse to a single canal at the stylet tip (Kimmins, 1986; Tjallingii and Hogen Esch, 1993; Prado and Tjallingii, 1994). Sieve tubes are the ultimate destination of the stylets; however, the cues by which aphids orientate their stylets towards sieve tubes are unknown.

Stylet penetration is accompanied by the secretion of gel saliva, which solidifies into a salivary sheath that surrounds the entire length of the stylets in the plant. The salivary sheath remains in the plant after stylet withdrawal and thus enables us to microscopically follow the pathway of the stylet tip towards vascular bundles (Stafford and Walker, 2009). These tracks disclose a relatively straightforward stylet path mostly through secondary cell walls (Kimmins, 1988; Tjallingii and Hogen Esch, 1993), although the stylets are sometimes retracted over a distance of several cells for unknown reasons and then stylet penetration is resumed along another path (e.g. Hennig, 1966; Botha et al., 1975; Tjallingii and Hogen Esch, 1993). Transmission electron microscopic reconstructions reveal a stylet progression pathway that is identical to that observed by light microscopy (Tjallingii and Hogen Esch, 1993).

Aphid behaviour can be monitored and recorded with the electrical penetration graph (EPG) technique [introduced by McLean and Kinsey (McLean and Kinsey, 1964); modified for direct current (DC) acquisition by Tjallingii (Tjallingii, 1988)]. Aphid and plant are incorporated into an electrical circuit, which is closed as soon as the aphid's stylets are inserted into the plant (Tjallingii, 2006). As a consequence, aphid and plant act as a variable resistor (e.g. Walker, 2000; Will et al., 2009). The resulting signal waveforms have been correlated with distinct foraging activities (Tjallingii, 1985; Tjallingii, 1988; Tjallingii and Hogen Esch, 1993; Prado and Tjallingii, 1994; Tjallingii, 1994; Tjallingii, 1995a; Tjallingii, 1995b), such as saliva secretion or sap ingestion. In this way, three main behavioural phases of functionally related activities (pathway, xylem and phloem phase) were identified (Tjallingii, 2006). During the pathway phase, the extracellular path of stylet penetration is interrupted regularly by punctures of cells along the stylet pathway as demonstrated by potential drops in the EPG (Tjallingii, 1985). The specific drops in potential are associated with the membrane potentials of the plant cells, which act as additional voltage sources (Walker, 2000). During potential drops, minute amounts of cell sap are ingested, presumably to be 'analysed' by the aphid (Miles, 1987; Tjallingii and Cherqui, 1999).

The question, therefore, is do gradients from the plant surface to the vascular bundle act as orientation cues for stylet progression? Membrane potentials of successive cells along a radial transect from the epidermis to the vascular bundle (van der Schoot and van Bel, 1990), for instance, may provide information on the direction to be followed. Similarly, decreasing oxygen levels from the epidermis to the vascular bundle (van Dongen et al., 2003) may also present a gradient for orientation.

The mode of orientation, however, may be of an entirely different nature. Sucrose is the principal sugar in the cytosol of parenchyma cells whereas hexoses such as glucose and fructose are the main carbohydrates in vacuoles of, for example, *Hippeastrum* sp., *Tulipa* sp. (Wagner, 1979), *Nicotiana tabaccum* (Heineke et al., 1994), and *Alonsoa meridionalis* (Voitsekhovskaja et al., 2006). Moreover, cytoplasmic pH is between 7.0 and 7.5 [e.g. pH 7.2–7.4 in *Riccia fluitans* (Bertl et al., 1984); pH 7.35 in *Eremosphaera viridis* (Bethmann et al., 1998); pH 7.5 in *Nitella flexilis* (Plieth et al., 1997)], while the vacuolar pH is about pH 5 (Roberts et al., 1980; Bertl et al., 1984; Strack et al., 1987; Taiz, 1992). The composition of vacuolar sap thus strongly contrasts with that of parenchyma cytosol and sieve-tube sap with potential consequences for aphid feeding (Hewer et al., 2010).

The possibility that the stylets penetrate parenchyma cells, pierce the tonoplast and enter the vacuole is not excluded (Will and van Bel, 2006; Pettersson et al., 2007). As the composition of vacuolar sap deviates from the ideal pre-set conditions for ingestion, aphids might withdraw their stylets and continue penetrating and probing until the desired sap composition is sensed in the sieve tubes.

In this study, we used a diversity of methods to investigate: (i) the existence or impact of chemical gradients from the plant surface to the vascular bundle; (ii) the configuration of stylet sheath tracks; (iii) the possibility that when stylets penetrate a parenchyma cell they reach the vacuolar sap; and (iv) the effects of manipulating sieve-tube sugars on aphid feeding behaviour.

MATERIALS AND METHODS

Cultivation of host plants and rearing of aphids

Vicia faba (L. cv. Witkiem major; Nunhem Zaden, Haelen, The Netherlands) was cultivated in a greenhouse under daylight plus artificial light sources (IP65 and SON-T Agro 400, Philips, Eindhoven, The Netherlands) at 20–22°C, with a relative humidity of 65%, and a 17 h:7 h light:dark photoperiod. *Ricinus communis* (L. cv. Sanguineus; Benary Samenzucht GmbH, Hannoversch Münden, Germany) seedlings were cultivated under the above-mentioned conditions except that water-soaked seeds were planted in wet vermiculite in a humid atmosphere at 27°C (Kallarackal et al., 1989).

Under the same greenhouse conditions, the aphids *Megoura viciae* (Buckton) and *Aphis fabae* (Scopoli) were reared on *V. faba* in Perspex® boxes (*ca*. 50 cm×50 cm×60 cm), covered with a gauze cloth for better air circulation. Under these conditions, parthenogenetically generated, apterous aphid colonies developed.

Measurement of membrane potentials in the cortex of *V. faba* stems

In very dense colonies, as in our rearing conditions, individuals of *M. viciae* also feed on the youngest mature internodes of *V. faba*. Therefore, cell membrane potentials along a radial transect of cortex cells extending from the epidermis to the phloem of the youngest mature internode of stems of 4-week-old *V. faba* plants were recorded. Stem pieces of *V. faba* of 3 cm in length were excised with a razor blade under a physiological buffer medium (modified from Hafke et al., 2005): 2 mmol l^{-1} KCl, 1 mmol l^{-1} $MgCl_2 \cdot 6\,H_2O$, 1 mmol l^{-1} $CaCl_2$, 50 mmol l^{-1} mannitol, 2.5 mmol l^{-1} MES (NaOH, pH 5.7). While the tissue remained moist, radial transects of approximately 500 µm in thickness were cut, which included all cell types from the epidermis to the stem cavity. Sections were placed on a modified microscope slice into a groove of hardened, waterproofed two-component adhesive (UHU plus, UHU GmbH, Bühl, Germany), soldered with small drops of dental wax (Plano W. Plannet GmbH, Marburg, Germany) at the dry radial edges of the section as described previously (Hofmann et al., 2009), and immediately immersed in buffer medium. After approximately 1 h of recovery time, electrophysiological measurements started. Borosilicate microcapillaries and reference electrodes were prepared as described previously (van der Schoot and van Bel, 1989; Hafke et al., 2005). Microcapillaries were back-filled with 500 mmol l^{-1} KCl solution and fixed in an Ag/AgCl pellet electrode holder (World Precision Instruments, Sarasota, FL, USA), which was connected to the pre-amplifier of a DUO 773 intracellular amplifier (DUO 773 high-input impedance differential electrometer, World Precision Instruments). The Ag/AgCl reference electrodes were also filled with 500 mmol l^{-1} KCl solution placed in the buffer medium. Membrane potentials of a radial transect of cells, one layer beneath the cut surface, were recorded until they stabilised (approximately 15 min).

Impact of oxygen levels on feeding behaviour

In order to determine the preference of aphids for different levels of dissolved oxygen, a modified choice chamber was constructed based on the flow system described previously (Will et al., 2008) (Fig. 1). Two separate flow systems each with two serially connected flow chambers (4 cm×2 cm×0.3 cm) were combined to create one choice chamber. Each flow system was supplied with basic diet solutions (Hewer et al., 2010) from separate reservoirs. One diet was aerated with synthetic air (20.5% O_2 in N_2; AIR LIQUIDE Deutschland GmbH, Düsseldorf, Germany; high oxygen diet); the other diet was flushed with argon (AIR LIQUIDE Deutschland GmbH; low oxygen diet). The diets were pumped through the systems by a peristaltic pump (Reglo Digital ISM596; Ismatec® Index Corporation, Wertheim-Mondfeld, Germany) at a rate of 2 ml min^{-1}. Behind either outlet, an oxygen micro-sensor (PreSens Flow-Through Cell Housed Oxygen Microsensor: FTCH-PSt1-L5-TS-YOP, PreSens Precision Sensing GmbH, Regensburg, Germany) was installed and connected to a PreSens 4-Channel Oxygen Meter OXY-4 micro (PreSens Precision Sensing GmbH). The oxygen levels were recorded every 15 min. Adult *M. viciae* (10 replications with 200 adults per replicate) were confined in the choice chambers for 24 h (17 h:7 h light:dark) under diffused light. At the end of the 24 h period, a digital image was taken and the number of aphids in the respective chambers was counted (cf. Hewer et al., 2010). Statistical analysis was performed using the software SigmaStat® 3.0 (SPSS Inc., Chicago, IL, USA) executing the *t*-test with a significance limit of P=0.05.

Optical examination of stylet penetration of *M. viciae* in the cortical tissues of *V. faba*

To observe aphid stylets of *M. viciae* in *V. faba* stems, stylets of feeding adults were microcauterised (HF-microcautery unit CA-50, Syntech, Hilversum, The Netherlands) (Fisher and Frame, 1984). The stem tissue was cut down to the site of stylet insertion under a stereomicroscope and thin tissue slices, including the stylet stump, were cut with a razor blade. The slices were contrasted with L-dihydroxyphenylalanine [L-DOPA, 1% (wt/vol.)] and examined under a light microscope (Leica TCS 4D, Leica Microsystems, Heidelberg, Germany). Additionally, gel saliva tracks in stem tissue

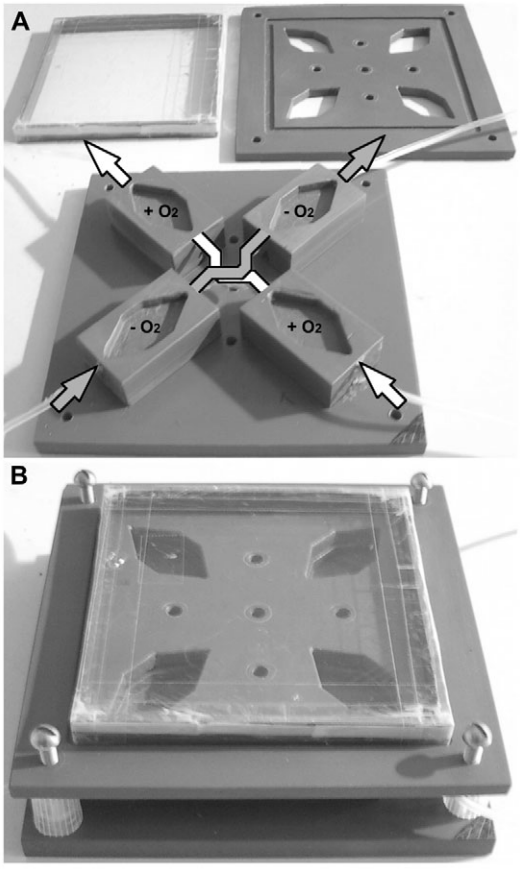

Fig. 1. Oxygen choice chamber system. The dissolved oxygen choice chamber system was manufactured from grey Perspex®, and was based on the design described previously (Will et al., 2008). (A) Two separate flow systems (one designated with white arrows, the other with grey arrows), each with two flow chambers with an in- and outflow, connected in series, were mounted on a base plate (15 cm×15 cm×0.5 cm) and combined to create a choice chamber (A, lower part). Each flow chamber was covered with a transparent plastic film and sealed with a ring of BlueTac®. To keep the flow chambers in place and to fix the plastic films, a second plate having the same dimensions as the base plate with relevant cut-outs (A, upper part right) was screwed (4 outer and 5 inner screws) onto the base plate. Diets, with or without oxygen, were pumped through the systems by a peristaltic pump (direction of flow indicated by arrows). Behind both terminal outlets (outward arrows), a micro-sensor recorded the oxygen level of the perfused solutions every 15 min. (B) During experiments, the aphids (*Megoura viciae*) were confined inside a tight-fitting plastic frame covered with transparent plastic film (A, upper part left).

were observed by removing aphids from the stems, making free-hand cross sections of the stems, fixing/staining the cross sections in 0.2% acid fuchsine in 1:1 95% ethanol:glacial acetic acid for approximately 1 min and then destaining them several times in 1:1:1 lactic acid:glycerol:water until only the gel saliva tracks remained stained (Stafford and Walker, 2009). Finally, entire gel saliva tracks were extracted from the stem tissue by incubating pieces of infested stem internodes in an enzyme mixture (Hafke et al., 2007) with 0.55% cellulase 'Onozuka' RS (Yakult Honsha, Tokyo, Japan) and 0.35% pectolyase Y-23 (Seishin, Tokyo, Japan) in a shaker at approximately 30°C until most of the plant tissue had been degraded. The tissue conglomerate was stained with acid fuchsine as described above, and stained saliva tracks were liberated using thin glass needles under a stereomicroscope.

Analysis of intracellular punctures by *M. viciae* stylets in cortical tissues of *V. faba*

A DC EPG Giga-8 system and PROBE software (W. F. Tjallingii, Wageningen Agricultural University, Wageningen, The Netherlands) was used as described previously (Will et al., 2008; Will et al., 2009) to monitor *M. viciae* feeding on the youngest mature internode of a *V. faba* stem. EPG recordings were made from three classes of aphids: (i) aphids directly transferred from the breeding plant to the EPG-recorded plant (i.e. no starvation) (10 replicates); (ii) aphids transferred to the EPG-recorded plant after a mean starvation period of approximately 2.5 h and produced only short phloem-ingestion phases (<45 min) or no phloem ingestion on the EPG-recorded plant (13 replicates); and (iii) aphids transferred to the EPG-recorded plant after a mean starvation period of approximately 2.5 h and produced at least one long ingestion phase (of at least 90 min to 5 h) on the EPG-recorded plant (8 replicates). Recordings were 6 h in duration starting immediately after placing an aphid onto the plant. Most of the pathway of aphid stylets from the epidermis to the phloem is in extracellular space but aphids frequently make brief (*ca*. 10 s) penetrations of cells that they pass along the way. EPGs record a distinctive waveform called potential drop, when the stylets penetrate into, reside in and retract from the punctured cell (Tjallingii, 1985). The structure of the potential drops and their chronological order were compared among the three classes of aphids.

Stylectomy during potential drop EPG waveforms in the cortex of *V. faba*

EPGs were recorded from adult *M. viciae* feeding on the two youngest mature internodes of a *V. faba* plant. After registration of nine successive potential drops, the stylets were microcauterised at the start of the tenth potential drop (when the stylet tip was assumed to be inside a cortex parenchyma cell) after disconnection of the EPG amplifier (within 1 s). Tissue segments of 2 mm×2 mm×3–4 mm (downwards to the inner cavity of the stem) around the stylet stump were immediately fixed in a solution of 3% glutaraldehyde in 50 mmol l^{-1} sodium cacodylate fixation buffer containing 2 mmol l^{-1} $CaC1_2$, pH 7.2 (Ehlers et al., 2000) and stored overnight in a refrigerator. The samples then were transferred into fresh fixative for 2 h on ice. Following fixation, samples were washed for 1 h on ice in 50 mmol l^{-1} sodium cacodylate buffer containing 2 mmol l^{-1} $CaCl_2$ (pH 7.2; buffer medium was replaced every 15 min). Dehydration was performed on ice in a graded ethanol series [twice for 45 min at 30%, 50%, 70% (at this point samples were switched from cool to room temperature), 90%, 96%, 100% and 100% dry ethanol] and propylene oxide [once for 45 min in dry propylene oxide, then propylene oxide with Spurr epoxy resin (Spurr, 1969)]. Subsequently, the samples were transferred into pure Spurr epoxy resin (once for 45 min of every mixture step, and twice of pure Spurr epoxy resin). Samples were embedded in flat embedding molds and polymerised for 24 h at 70°C. Serial semi-thin cross sections were cut with a glass knife on a Reichert Om U2 ultramicrotome (Leica, Bensheim, Germany), and were collected on microscope slides and stained with a dilute solution of Gentian Violet. Sections were examined with a microscope (Leica DM-LB, Leica, Wetzlar, Germany), photographed with a Canon PowerShot S40 (Canon Inc., Tokyo, Japan) and processed using Corel® PhotoPaint (Corel Corporation, Ottawa, Canada).

Manipulation of phloem loading in *R. communis* seedlings and analysis of phloem sap

Roots of 6–7-day-old *R. communis* seedlings were freed from vermiculite, and then rinsed and incubated in the above-mentioned physiological buffer medium. After removal of the endosperm, the

bare-lying cotyledons, which were still attached to the rest of the seedling, were incubated in other buffer solutions (cf. Kallarackal et al., 1989) containing 300 mmol l^{-1} sucrose, 500 mmol l^{-1} glucose or 1000 mmol l^{-1} glucose. After incubation times of various length (2 h, 4 h, 8 h, 20 h and 24 h), the seedlings were cut at the hypocotyl hook and the spontaneously exuding sap was collected by disposable 1 µl and 5 µl capillaries (Hirschmann Laborgeräte, Eberstadt, Germany). The samples were prepared for HPLC [HPAE-PAD installed in a DX 500 system (Dionex, Sunnyvale, CA, USA) with an analytical CarboPacTM PA20 column (Dionex, Sunnyvale, CA, USA)], with a length of 250 mm and a diameter of 3 mm (Dionex) with a 50 mm guard column for isocratic separation (35 mmol l^{-1} NaOH) as described previously (Hewer et al., 2010).

EPGs of *A. fabae* foraging on *R. communis* seedlings with manipulated phloem sugar composition

To investigate the effects of a modified composition of sieve-tube sap, adult individuals of *A. fabae* were prepared for EPG as described previously (Will et al., 2008; Will et al., 2009). EPG recordings (8 h duration) were started immediately after placing an aphid onto the hypocotyl hook of a *R. communis* seedling that had had its cotyledons pre-incubated in sugar solutions for 2 h (Fig. 2), as described above, with 6 replicates for each treatment. Statistical analysis was performed using the software SigmaStat® 3.0 (SPSS Inc.) executing the analysis of variance (significance limit P=0.05) for the aggregated duration of each waveform.

RESULTS

Measurement of membrane potentials in the cortex of *V. faba*

Radial series of cortical and vascular cells were measured electrophysiologically in succession to determine their membrane potentials. In three measurements, which each lasted 12 h, we succeeded in recording all membrane potentials along an entire stretch of cells from the epidermis to the sieve elements (Fig. 3). Additional data (Fig. 3) were collected from shorter cell arrays (maximum of three cells) in other broad bean plants. The radial potential profile (Fig. 3) does not reveal a distinct electrical gradient through the cortex or a steep jump in membrane potential between cortex cells and sieve elements.

Lack of impact of oxygen levels on the feeding behaviour

In view of the radial oxygen gradient across stem tissues (van Dongen et al., 2003), oxygen level was tested as a potential orientation parameter for aphids in the plant cortex. In choice chambers, identical diets were offered to aphids under a normal oxygen (20%) and a low-oxygen (3%) atmosphere (Fig. 1). No preference for a normal or a low-oxygen level in the diets was observed in 10 replicates (51.2% *vs* 48.8% of the aphids, respectively; P=0.717). It may, therefore, be unlikely that the oxygen gradient presents a guideline for stylet penetration.

Optical examination of stylet progression of *M. viciae* in cortical tissues of *V. faba*

Tracks of stained salivary sheaths (e.g. Hennig, 1966; Botha et al., 1975) or electron microscopic images (e.g. Spiller et al., 1985; Tjallingii and Hogen Esch, 1993) reveal a stylet pathway through the cell wall continuum. Our free-hand sections show saliva tracks localised in cell walls and oriented mainly in a radial direction (Fig. 4A,B). This can only be observed clearly if the stylets and saliva tracks lay exactly in same focal plane of the microscope (Fig. 4A,B). Free-hand cross sectioning was surprisingly successful and often yielded cross sections containing the whole length of the salivary track

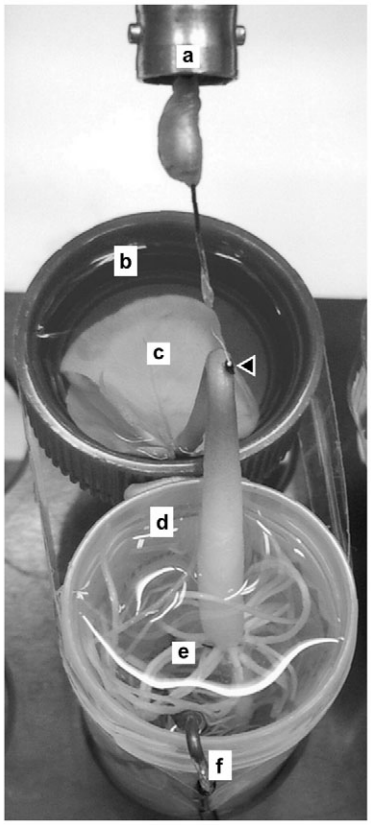

Fig. 2. Electrical penetration graph (EPG) arrangement of *Aphis fabae* foraging on *Ricinus communis* seedlings. (a) An EPG pre-amplifier connected with a gold-wire electrode dorsally fixed onto an adult *A. fabae* individual (arrow head) sitting on the hypocotyl hook; (b) container for buffer solutions containing various sugars; (c) incubated cotyledons; (d) container for buffer medium; (e) submersed roots; and (f) EPG substrate electrode. To prevent evaporation both containers were covered with Parafilm® during the experiment.

because the stylets largely move within a plane perpendicular to the plant surface. These observations are confirmed by serial sectioning of pre-infested plants (Fig. 4B). Moreover, isolated stylet sheath systems extracted from degraded tissues contain multiple branches that occur mostly in the same plane (Fig. 4C). Quite often, the stylet is withdrawn over a distance of several cell layers for unknown reasons, but the renewed penetration also runs in the same plane (Fig. 4A). Unusually frequent stylet retraction was observed when stylets encountered sclerenchyma cells, implying that fortified cell walls present an insurmountable barrier for stylet penetration (Fig. 4B). All in all, it appears that aphids orientate their stylet penetration perpendicular to the plant surface in a radial direction.

Potential drop analysis during the progression of *M. viciae* stylets in cortical tissues of *V. faba*

As in previous reports (Tjallingii, 1985; Powell et al., 1995; Martin et al., 1997), the potential drops that we recorded were composed of three sub-phases. Potential drops begin with a sharp voltage drop followed by a slight voltage increase (Fig. 5). After voltage stabilisation, potential waves with a high frequency are followed by potential waves with a lower frequency and a stage with irregular electrical oscillations until the original extracellular voltage level is reached. EPGs showing the three distinct sub-phases during potential drops were amplified at values between 0 V and +1 V (Fig. 5A).

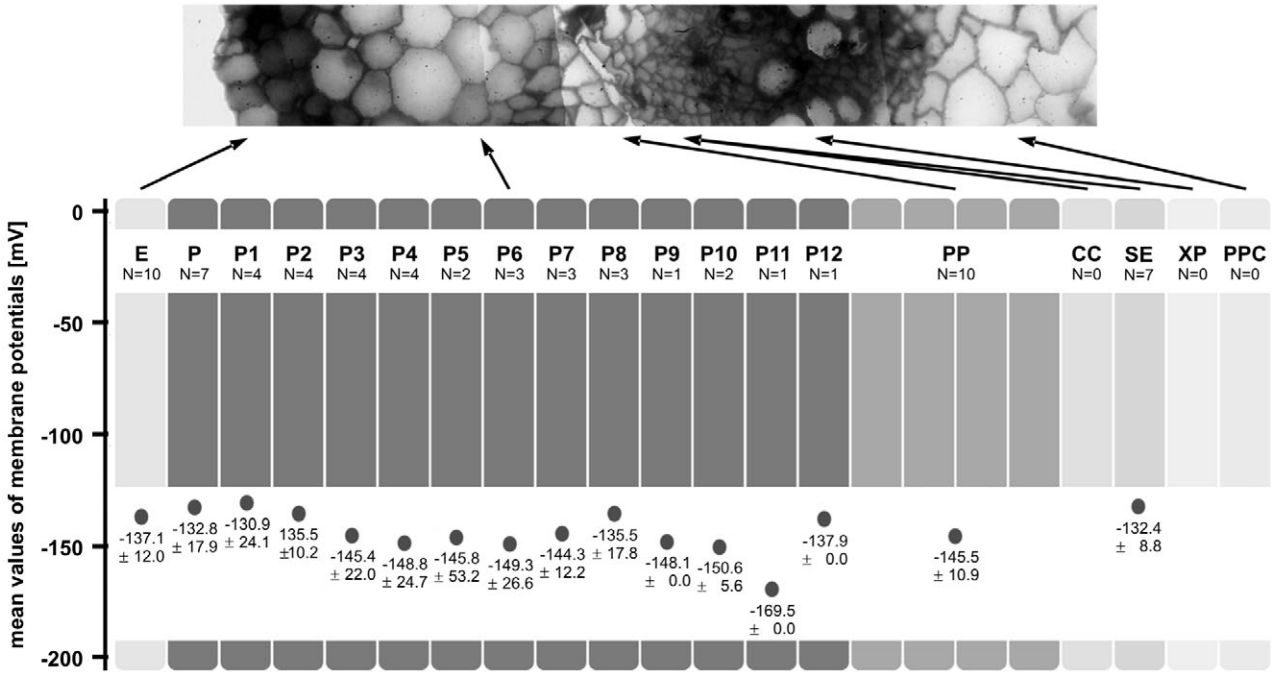

Fig. 3. Membrane potential profile through radial transects of *Vicia faba* stems. Membrane potentials of cells from the epidermis to the vascular bundles were measured by electrophysiology. The mean values of successive cells, the number of measured cells (*N*) and the respective standard deviations are presented. Grey scaling represents diverse cell types. E, epidermis cell; P, cortex parenchyma cell (the mean for single measurements of scattered cells); P1–P12, successive cortex parenchyma cells (successive cell measurements); PP, phloem parenchyma cells (phloem parenchyma cells are mostly positioned between the cortex parenchyma and sieve tubes, but the radial orientation is variable); CC, companion cell; SE, sieve element; XP, xylem parenchyma cell; and PPC, pith parenchyma cell. (For CC, XP and PPC, no data are available, but they are included for completeness.)

By contrast, EPGs focusing on the phase of return to the extracellular voltage level were recorded at amplifications between +4 V and +5 V to reach more distinctive patterns (Fig. 5B). The return to the extracellular voltage level is intermitted by one or several sharp voltage drops (Fig. 5B). Three categories of voltage level restoration were distinguished (Fig. 5B): one sharp voltage drop followed by a sudden and mostly permanent return to extracellular voltage levels (category 1); a gradual return to extracellular voltage levels intermitted by a few sharp voltage drops (category 2); and an abrupt return to extracellular voltage level with occasional rapid voltage drops (category 3). Categories 1 and 2 were further subdivided in two sub-categories each (see legend Fig. 5B).

Characteristics of voltage level restoration (Fig. 5B) turned out to be correlated with precedent feeding behaviour of the respective aphids. With increasing duration of foraging, category 2 predominated among aphids that had been feeding on plants irrespective of a precedent diet deprivation whereas category 1 was more frequent among aphids that had not or had hardly been feeding on plants (Table 1).

Stylectomy during potential drop EPG waveforms in the cortex of *V. faba*

To localise the stylet tip inside punctured cortical cells, stylets were microcauterised during the initial stage of cell penetration and the tissue was fixed, embedded and sectioned. An EPG recording was used to determine the appropriate time point for cauterisation. Serial sections were intended to provide information on the location of the stylet tip. In only 2 out of 5 series, the stylet had remained in place during sectioning (Fig. 6), probably due to the heterogeneity of the stylet and plant material. Given the thinness of the cytoplasmic layer, the stylet seems to reside inside the vacuole of cortical cells (Fig. 6).

Manipulation of phloem loading in *R. communis* seedlings and analysis of phloem sap

Ricinus communis seedlings demonstrated the ability to translocate glucose instead of sucrose (Kallarackal et al., 1989). When glucose was applied to bare-lying cotyledons, the sugar composition of the phloem sap shifted. HPLC analysis of carbohydrates in exudate samples collected from seedlings of *R. communis* detected different proportions of translocated sugars depending on the sugar supplied to the cotyledons. Untreated seedlings and seedlings with bare-lying cotyledons, treated with 300 mmol l^{-1} sucrose, translocated mainly sucrose and minor amounts of glucose (Fig. 7A,B). In contrast, seedlings with bare-lying cotyledons, treated with either 500 mmol l^{-1} or 1000 mmol l^{-1} glucose solutions, translocated predominantly glucose with low amounts of sucrose (Fig. 7C,D).

EPGs of *A. fabae* foraging on *R. communis* seedlings with manipulated phloem sugar composition

The feeding behaviour of *A. fabae* on *R. communis* seedlings manipulated to translocate different proportions of carbohydrates was monitored by EPG (Fig. 2). *Aphis fabae* was used because *M. viciae* does not settle on *R. communis* seedlings. The percentages of time that the aphids spent in different waveforms (behavioural activities) are presented in Table 2. Transitional phases precluded the recognition of a distinct E1 (secretion of watery saliva into sieve elements) waveform in a number of measurements (transitional phase X) (Table 2), because it is not possible to distinguish different waveform patterns due to the waveform overlay. As the E2

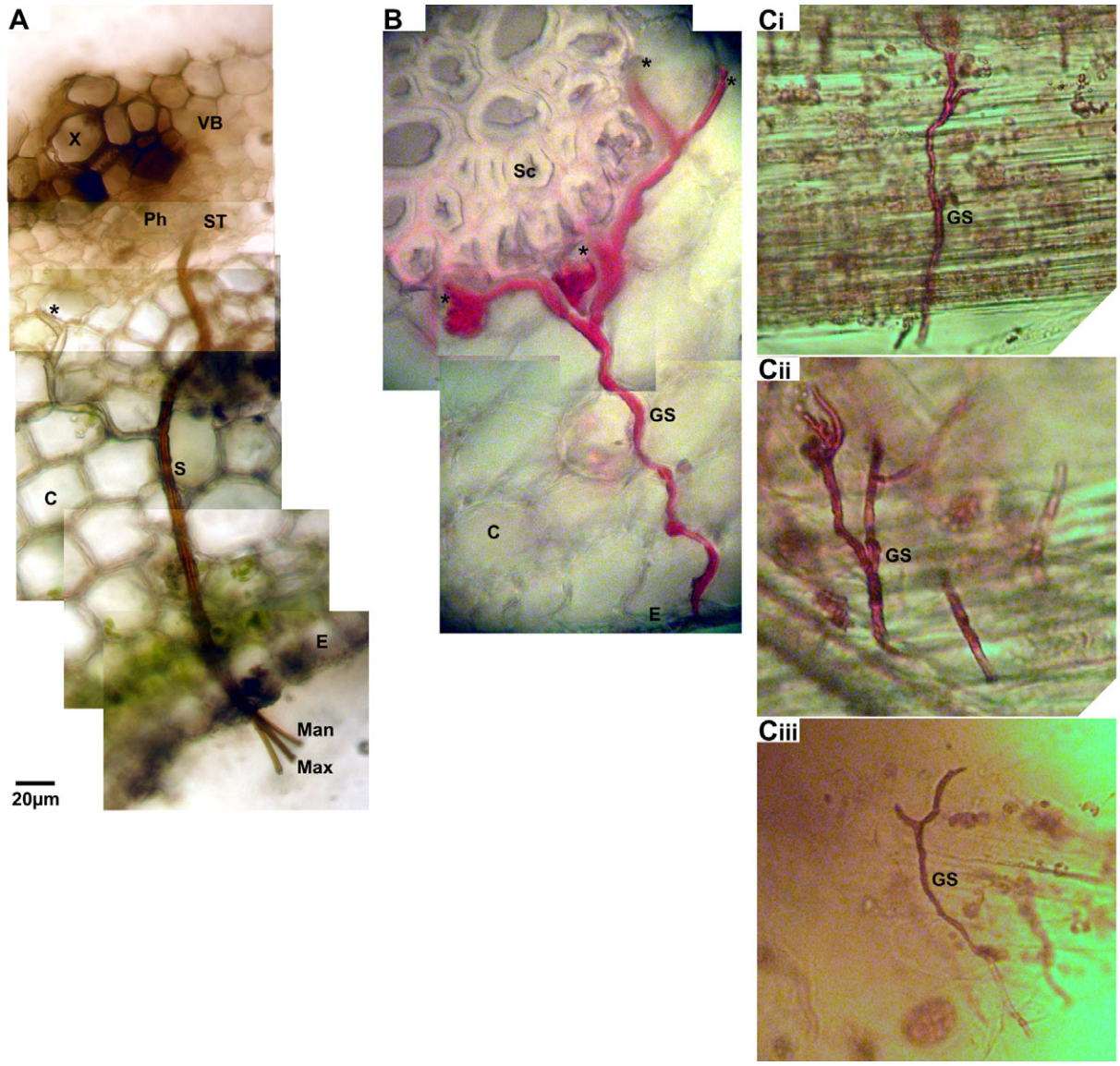

Fig. 4. Optical examination of stylet pathway of *Megoura viciae* in cortical tissues of *Vicia faba*. (A) Composite picture of a cut stylet of *M. viciae* inside a stem cross section of *V. faba*. The severed ends of the mandibular and maxillary stylets protrude from the plant surface in the lower right of the micrograph. An asterisk (*) marks the terminal end of a dead-end branch of gel saliva from which the stylets were retracted. Image scanning reveals that the stylet progresses through the cell walls. (B) Repetitive retraction of a stylet encountering fortified sclerenchyma cell walls. Composite picture of gel saliva sheath of *M. viciae* in a stem cross section of *V. faba*. (Ci–Ciii) Diverse isolated stylet sheath systems with increasing dissolution of *V. faba* stem tissue. C, cortex tissue; E, epidermis; GS, gel saliva sheath; Man, mandibular mouthparts; Max, maxillar mouthparts; Ph, phloem; S, stylet bundle; ST, stylet tip; Sc, sclerenchyma; VB, vascular bundle; X, xylem; and *, dead-end stylet track.

waveform is always preceded by an E1 phase, there are a number of E1 waveforms included in the transitional phases. Time spent in waveform E2 [sieve element sap ingestion with concurrent salivation (Prado and Tjallingii, 1994)] was the longest for seedlings with intact endosperm and those without endosperm and treated with 300 mmol l^{-1} sucrose (Table 2), but was lower for seedlings without endosperm treated with 500 mmol l^{-1} glucose and only about one quarter for those treated with 1000 mmol l^{-1} glucose in comparison with the sucrose treatment (Table 2) Due to the heavy statistical spread of the results, significance (P=0.05) between the treatments has not obtained. The trend, however, is obvious: it becomes more difficult to identify the sieve tubes with increasing glucose concentrations in the sieve-tube sap (Table 2).

DISCUSSION

Lack of impact of apparent radial gradients on feeding behaviour

The data lead us to conclude that the location of vascular bundles by aphids relies heavily on pre-programmed mechanisms of radial orientation, as we were unable to detect any dependence of searching behaviour on radial gradients. As in tomato stems (van der Schoot and van Bel, 1990), membrane potentials do not provide a distinct radial gradient in the stem cortex (Fig. 3). Furthermore, it is highly unlikely that aphids employ cortical oxygen gradients (cf. van Dongen et al., 2003) for their orientation given the identical preference for artificial diets offered under aerobic or anaerobic conditions (Fig. 1). Differences in the sugar concentrations in the

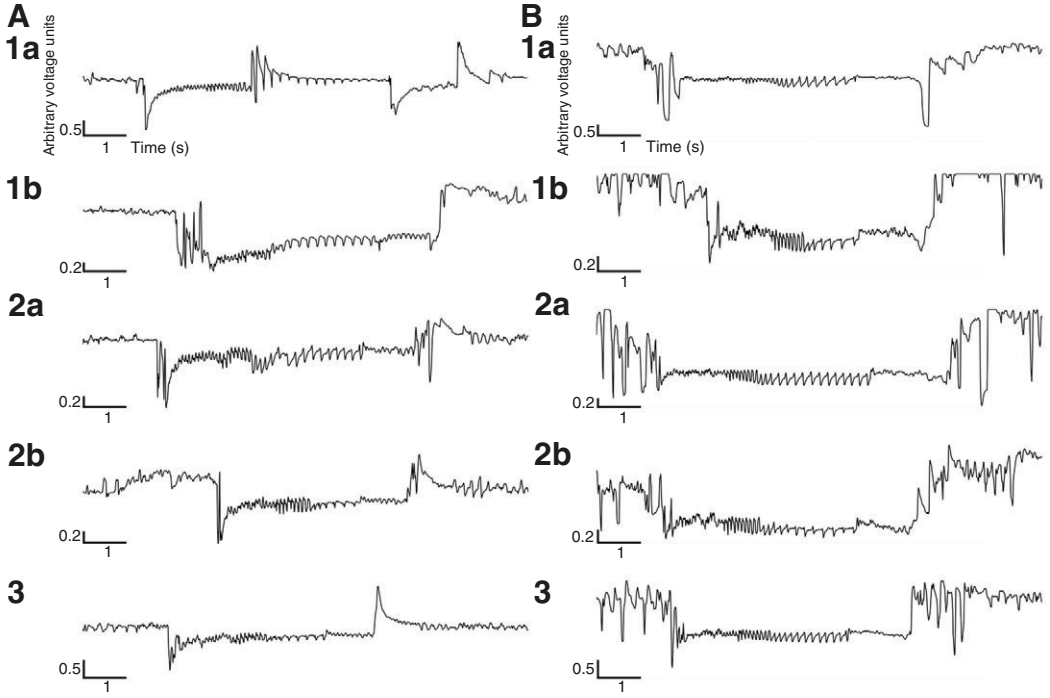

Fig. 5. Exemplary electrical penetration graphs (EPGs) of potential drop categories during punctures of cortical cells. Prior to a potential drop, an extracellular, positive voltage level is recorded. Then, when the stylets pierce through a plasma membrane, a sharp voltage drop is recorded as the stylet tips enter the intracellular, negatively charged sap. Recorded voltage remains low until the aphid retracts its stylet tip back into extracellular space and a sharp rise in voltage level is recorded. (A) Five typical potential drop patterns recorded between 0 V and +1 V set by manual adjustment. (B) Typical potential drop patterns recorded between +4 V and +5 V set by manual adjustment. As for the return to the extracellular voltage level, the waveforms were divided into three categories. In two of the three categories, two sub-categories were distinguished: (1) abrupt strong voltage drops (a) or more gradual (b) voltage drops before a final return to extracellular voltage level; (2) a gradual return to extracellular voltage level with one (a) or several (b) voltage drops; and (3) an abrupt return to extracellular voltage level occasionally followed by several voltage drops.

cortex [if such sugar gradients exist (Verscht et al., 2006)] would probably be too small in magnitude given the aphid's limited discriminative ability for different sugar concentrations (Hewer et al., 2010). In addition, sugar gradients might not provide reliable set points for orientation as abrupt concentration jumps rather than absolute concentrations are used for cell identification (Hewer et al., 2010). To this end, pH values may be more suitable orientation cues as previously argued (Hewer et al., 2010).

Optical examination of stylet progression of *M. viciae* in cortical tissues of *V. faba*

Our micrographs (Fig. 4) and those of others (e.g. Hennig, 1966; Botha et al., 1975; Spiller et al., 1985; Tjallingii and Hogen Esch, 1993) appear to demonstrate stylets progressing through cell walls. Biomechanically it is most plausible that stylets preferably progress through the weak primary lamellae sandwiched between the two layers of harder secondary cell wall material at either side as postulated by McAllan and Adams (McAllan and Adams, 1961). Mouthparts may be driven forward in incremental fashion, while the secondary cell walls are pushed apart and shots of gel saliva are being secreted. Progress is probably facilitated by cell wall degradation by digestive enzymes present in gel saliva such as pectinase and cellulase (T. Will, K. Steckbauer, M. Hardt and A.J.E.v.B., unpublished results). The pearl necklace appearance of gel saliva deposits onto Parafilm® appears to result from pulsed secretions of sheath saliva (T. Will, K. Steckbauer, M. Hardt and A.J.E.v.B., unpublished results). These salivary sheaths possess side branches indicating regular sideward gel secretion probably associated with cell probing (T. Will, K. Steckbauer, M. Hardt and A.J.E.v.B., unpublished results).

The direction of stylet penetration tends to be radial and perpendicular to the plant surface with occasional retraction of the mouthparts (Fig. 4A,B). Retraction may be due to cell wall barriers, which have been postulated long ago (Hennig, 1966), but have not been investigated in more detail since. This hypothesis is corroborated by the fact that aphids make a stylet detour when encountering arches of tough sclerenchyma cells along the phloem in *V. faba* (Fig. 4B). All in all, it seems that aphids use a pre-programmed motor pattern resulting in radial orientation and regular sideward puncturing. This claim calls for future studies on an 'automatic pilot' in the aphid brain.

Assessment of potential drop analysis during stylet penetration in cortical tissues

EPGs obtained during puncturing of parenchyma cells show a high degree of similarity (Fig. 5). This seems to reflect a fixed behavioural programme. As postulated previously (Martin et al., 1997; Powell, 2005; Pettersson et al., 2007; Marchetti et al., 2009; Tjallingii et al., 2010), aqueous saliva is secreted during a brief period of high-frequency voltage fluctuations immediately after cell puncturing, followed by an as yet undefined period (lower frequency fluctuations) and terminated with a brief period of sap ingestion. Yet, the EPG profiles of sub-phases II-1 and II-2 are reminiscent of E1 and E2 waveforms, respectively, after sieve element puncture. The profile of sub-phase II-3 resembles a mixed E1/E2 behaviour (e.g. Will et al., 2009). We have, therefore, an

Table 1. Potential drop analysis of *Megoura viciae* on *Vicia faba*

Potential drop category	A (%)	B (%)	C (%)
1	22.9	**52.6**	25.4
2	**61.3**	37.5	**57.3**
3	15.8	9.9	17.3

Potential drop analysis of (A) aphids directly transferred from the breeding plants to the electrical penetration graph (EPG)-recorded plant (i.e. no starvation) (*N*=10), (B) aphids transferred to the EPG-recorded plant after a mean starvation period of approximately 2.5 h and produced only short phloem-ingestion phases (<45 min) or no phloem ingestion on the EPG-recorded plant (*N*=13), and (C) aphids transferred to the EPG-recorded plant after a mean starvation period of approximately 2.5 h and produced at least one long ingestion phase (of at least 90 min to 5 h) on the EPG-recorded plant (*N*=8). For starvation, aphids were transferred from the breeding plants into a Petri dish for a mean time of approximately 2.5 h and then transferred onto the feeding plants of EPG. Potential drops classified according to the categories (1–3) defined in Fig. 5 were given as their respective proportional occurrence in percentages. *N*, number of individuals measured. The values in bold indicate the category with the highest percentages with respect to the aphid groups.

alternative interpretation of the EPGs: secretion of aqueous saliva during the initial high-frequency period, ingestion for probing during the subsequent lower frequency period, and sap analysis, data processing and retraction during the final period. This interpretation has the charm of an essentially universal probing behaviour for all cell types.

Location of stylets in cortical parenchyma cells

The phases in EPGs during potential drops have been identified on the basis of elegant experiments with viruses. Apparent virus inoculation by salivation during sub-phase II-1 (Martin et al., 1997; Powell, 2005; Marchetti et al., 2009; Tjallingii et al., 2010) and virus ingestion during sub-phase II-3 (Martin et al., 1997; Marchetti et al., 2009) seem to provide compelling evidence for salivation and ingestion during the sub-phases in question. As virus multiplication occurs in the cytoplasmic space (Martelli and Castellano, 1971; Shalla et al., 1980), the stylet tip is supposed to be inserted into the cytoplasmic compartment during the entire potential drop (Powell et al., 2006). In the following, we explore if conclusions on stylet location and aphid behaviour leave room for doubts, especially because aphid behaviour during sub-phase II-2 remains unclear (Marchetti et al., 2009).

The crucial point here, of course, is the optical examination of the stylet location during potential drops. In order to determine their location, stylets were microcauterised just after the EPG monitored that a cell had been punctured. Our micrographs (Fig. 6) suggest that the stylets penetrated the tonoplast, because they were located deeper into the cell than the thin cytoplasmic layer, which can be observed more clearly in the neighbouring cell. However, there is a caveat: microcautery might cause a shock reaction in the aphid that could cause stylet retraction or advancement in a split second, resulting in the histological sections showing the stylet tips being slightly shifted from their natural position. Older research indicates that aphids retract their stylets in response to shock reactions (Kimmins and Tjallingii, 1985; Tjallingii and Hogen Esch, 1993; Caillaud and Niemeyer, 1996; Calatayud et al., 1996) and, hence, the stylet tip may have been penetrated more deeply than reflected in the pictures (Fig. 6).

Pettersson et al. advanced the hypothesis that during intracellular punctures of parenchyma cells, the maxillary stylets pierce into the vacuole (Pettersson et al., 2007). On mechanistic grounds, it seems plausible that the thrust needed to breach the cell walls would drive the stylet tips through the thin parietal cytoplasm and into the vacuole. Pieces of circumstantial evidence for this idea include irreversible damage to intracellular membranes presumably caused by aphid stylets (Spiller et al., 1985) (see also Fig. 6), the location of stylet tips in *V. faba* sieve tubes deep into the sieve element lumen (Tjallingii and Hogen Esch, 1993), and, in lettuce, sieve tubes opposite the site of penetration (W. F. Tjallingii, unpublished results).

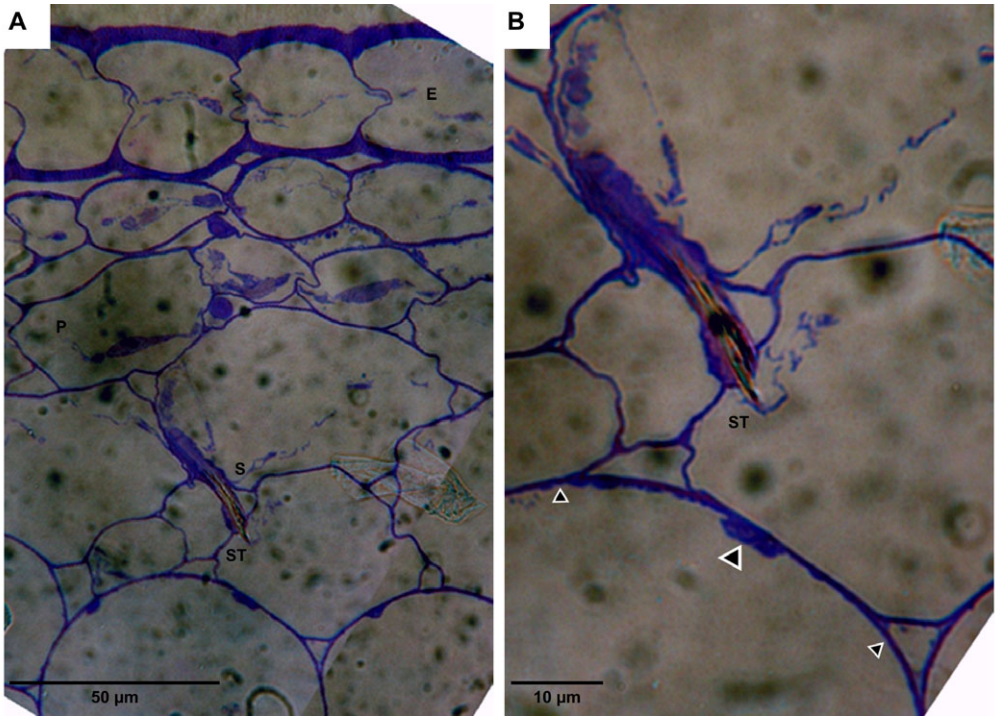

Fig. 6. Stylet tip location in the cortex cells of *Vicia faba* during potential drop electrical penetration graph (EPG) waveforms. Fixed and embedded stem cross sections with a stylet tip of *Megoura viciae* in *V. faba* cortex tissue microcauterised after the onset of a potential drop. (A) Stylet tip having punctured a cortex cell. (B) Detail of the stylet tip in A. A thin cytoplasmic layer of cortex cells is indicated with arrowheads; a chloroplast is indicated with a large arrowhead. E, epidermis cell; P, cortex parenchyma cell; S, stylet; and ST, stylet tip.

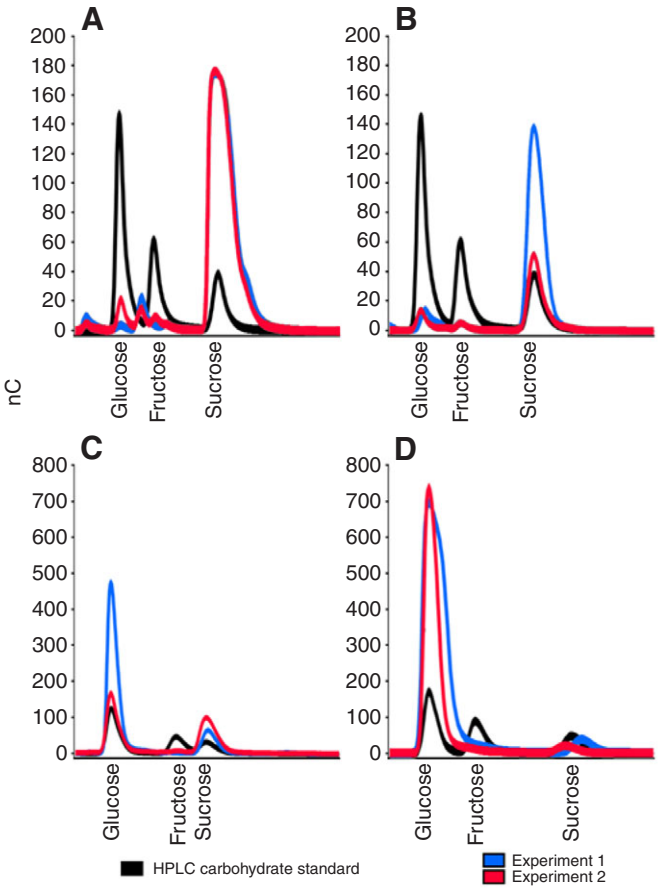

Fig. 7. High-performance liquid chromatography (HPLC) profiles of sugars in phloem exudates of *Ricinus communis* seedlings after differential treatment. Seedlings were incubated in either sucrose or glucose solutions for various times (2 h, 4 h, 8 h, 20 h, 24 h) (see Materials and methods), after which the seedlings were cut at the hypocotyl hook and the spontaneously exuding sap was sampled and analysed with HPLC (two independent experiments are indicated in blue and red). The composition of phloem exudates after 2–24 h incubation periods was essentially similar; data shown are from the 2 h incubation periods. Untreated seedlings with intact endosperm (control) (A) and seedlings with the endosperm removed and incubated in 300 mmol l^{-1} sucrose solution (B) both show high amounts of sucrose as the translocate. Seedlings with the endosperm removed and incubated in 500 mmol l^{-1} (C) or 1000 mmol l^{-1} glucose solutions (D) both show high amounts of glucose as the translocate. nC, nanoCoulomb.

As stylet penetration of the tonoplast is the key issue of this concept, arguments against vacuolar penetration need to be addressed. One argument states that traces of gel saliva have never been observed inside punctured cells (Tjallingii and Hogen Esch, 1993); this is in contrast to the deposits on the plasma membrane that are hypothesised to seal the puncture site. This argument is based on the assumption that stylet advancement is always accompanied by sheath saliva secretion regardless of whether the stylets are penetrating through the apoplast or into a living cell. We interpret deposition of gel saliva at the puncture site to function as a holdfast for the penetrating stylet and as a means to repair the cell wall to prevent leakage of cell components that would interfere with the stylet orientation or that would react with other cell contents to form poisonous compounds. Furthermore, gel saliva may not be needed to seal a pierced membrane because the plasma membrane and tonoplast are perfectly able to repair themselves from punctures,

Table 2. Percentage of total recording time occupied by different electrical penetration graph (EPG) waveforms of *Aphis fabae* feeding on *Ricinus communis* seedlings with manipulated phloem sugar composition

EPG waveform	Untreated seedlings with intact endosperm		Seedlings without endosperm treated with 300 mmol l^{-1} sucrose		Seedlings without endosperm treated with 500 mmol l^{-1} glucose		Seedlings without endosperm treated with 1000 mmol l^{-1} glucose	
	%	*N*	%	*N*	%	*N*	%	*N*
np	34.3	114	55.8	65	37.6	171	45.2	167
A, B and C	49.0	1324	31.1	783	49.7	1567	41.0	1512
E1	**0.3**	**4**	**0.0**	**1**	**2.0**	**6**	**0.0**	**0**
E2	**4.2**	**5**	**5.6**	**4**	**3.7**	**13**	**1.4**	**1**
F	6.4	11	3.4	6	0.6	4	8.9	19
Potential drop	3.9	1230	2.2	725	3.4	1403	3.0	1329
Transitional phase X	**0.2**	**7**	**0.4**	**7**	**1.3**	**14**	**0.1**	**1**
Transitional phase Y	1.7	24	1.4	9	1.8	7	0.3	4

EPGs lasted 8 h, with six replicates for each treatment, with apterous adult individuals of *A. fabae*. The waveforms were defined according to Prado and Tjallingii (Prado and Tjallingii, 1994): np, non-probing; A, cuticle penetration; B, sheath salivation; C, pathway activities; E1, secretion of watery saliva into sieve elements; E2, ingestion of sieve element sap and concurrent salivation of watery saliva into the food canal; and F, penetration problems (Tjallingii, 1995b). The transitional phase X (C→X→E1 / E1↔X↔E2) describes the short intermediate phases between two clear waveforms concerning the E waveforms. The transitional phase Y (wf→Y→wf / Y→np) describes the short intermediate phases between two clear waveforms (wf) excluding the E waveforms. The total number of events of the respective waveform in six EPG experiments is expressed as N. Values in bold indicate the phloem waveforms, which are essential for the interpretation of the results.

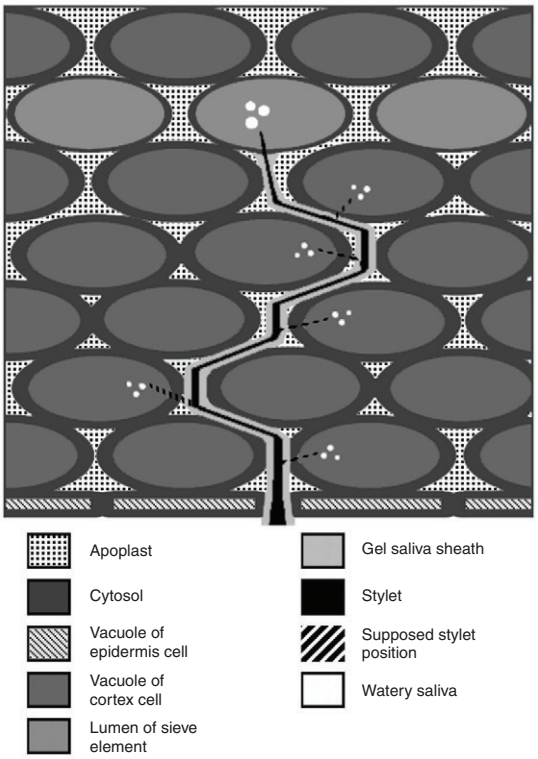

Fig. 8. Hypothetical mode of aphid orientation towards the sieve tubes. Stylets of aphids penetrate their host plants at anticlinal cell walls of the epidermis and progress mainly through the cell wall continuum in a pre-programmed fashion. The aphids use programmed coordinates that enable steering of the stylet tips in a plane perpendicular to the plant surface in a predominantly radial direction while gel saliva is being secreted. Gel saliva is a lubricating substance that, after hardening, encases the entire length of the stylets in the plant tissue except for the stylet tip. The progress is intermitted by automated sideward punctures into cells during which watery saliva is secreted and cell sap is imbibed and assayed. If the sampled sap does not contain the required carbohydrate contents and the appropriate pH (which is the case if the vacuolar sap of parenchymatous cells is probed), the stylets are withdrawn. This procedure is repeated until a cell (a sieve element) is punctured with the desired sucrose concentration (in the range of several hundred mmol l^{-1} sucrose) and a pH value of 7.0–7.5. After intense secretion of watery saliva into the sieve element, the aphid starts ingestion while pulses of watery saliva are continuously secreted to keep the food canal free of clogging plant proteins.

as shown during impalement and withdrawal of microelectrodes. Further, it is unlikely that chemically incompatible materials such as proteinaceous gel saliva and lipids in the plasma membranes amalgamate in a satisfactory way. A second argument claims that the tonoplast is too flexible to allow stylet penetration (Powell et al., 2006), but is this true when the stylets advance at an angle of approximately 90 deg into a turgescent tonoplast? A third argument is that viral multiplication associated with parenchyma cell penetration could not take place in vacuoles (Powell et al., 2006). However, virus particles may be able to contaminate the cytoplasm during stylet retraction.

Positional assessment of EPG waveforms during potential drops

One may speculate on how EPG waveforms (Fig. 5) and virus multiplication (Martin et al., 1997; Powell, 2005; Marchetti et al., 2009; Tjallingii et al., 2010) are compatible with tonoplast penetration. During residence of stylet tips in vacuoles, aqueous saliva is secreted (sub-phase II-1, Fig. 5) and vacuolar sap probed (sub-phase II-2; Fig. 5). The stylet is slowly retracted and the tip traverses the cytoplasm while secreting and probing in alternation (mixed waveform, sub-phase II-3; Fig. 5). After tonoplast penetration, viruses may leak into the cytoplasm through the puncture site (cf. Spiller et al., 1985) during sub-phase II-1 (Fig. 5) or are able to move across the tonoplast to the cytoplasm, a potential phenomenon that has not been investigated seriously to the best of our knowledge. Occasional probing of cytoplasmic sap during sub-phase II-3 (Fig. 5) provides a good explanation for virus ingestion (Martin et al., 1997; Powell, 2005).

Another assessment of EPG waveforms, but more unlikely at this stage, would be that sub-phases II-1–II-3 (Fig. 5) occur with the stylet inserted into the vacuole, while sub-phase II-3 represents sample assay and data processing. Steep spikes below the potential drop level during the return to the extracellular voltage level might then show lower voltage levels in the cytoplasm and represent a shock-wise stylet retraction passing the plasma membrane once (Fig. 5B, category 1) or several times (Fig. 5B, category 2).

The impact of vacuolar stylet insertion on aphid orientation

Provided that stylets probe vacuolar contents in parenchyma cells, the hexose content and the low pH strongly differ from those of sieve-tube sap. Choice chamber experiments provided compelling evidence that aphids are capable of pH and sucrose sensing (Hewer et al., 2010) and would reject cells with much lower pH values than the preferential pH (7.0–7.5) and with sucrose concentrations lower than in the desired 400 mmol l^{-1} range. Acidic vacuolar pH values (Roberts et al., 1980; Bertl et al., 1984; Strack et al., 1987; Taiz, 1992) and low concentrations of sucrose (Wagner, 1979; Heineke et al., 1994; Voitsekhovskaja et al., 2006), which would be encountered in a parenchyma cell vacuole, would urge aphids to reject the cells. When sieve element conditions are not met during probing, aphids withdraw their stylets. Stylet retraction coincides with a sharp increase in EPG voltage level. Perhaps, the cell wall is finally sealed by gel saliva which might explain the one to a few abrupt voltage drops (Fig. 5B, category 1 and 2). Similarly, steep voltage drops precede the potential drops (Fig. 5B) indicative of gel saliva deposition as part of the preparation to puncturing (Tjallingii et al., 2010).

Consequences of phloem sap manipulation on aphid feeding behaviour

As reported previously (Kallarackal et al., 1989), treatment of cotyledons with sugars causes drastic changes in carbohydrate composition of phloem sap (Fig. 7). In plants translocating high hexose concentrations in phloem sap, aphids have considerable problems finding sieve tubes as inferred from the much lower ingestion times (Table 2). Similarly, *Macrosiphum euphorbiae* individuals feeding on antisense *StSUT1* lines of potato plants (Pescod et al., 2007), which have a reduced sucrose content of the sieve-tube sap, experience increasing difficulties in sieve-tube identification and feeding. Recognition of sieve tubes is facilitated by a sharp borderline between cortex cells and sieve tubes (low *vs* high sucrose or/and glucose *vs* sucrose in cortex cells *vs* sieve tubes), which is lacking in glucose-manipulated plants (Fig. 7). Nevertheless, aphids retain a reduced ability to identify sieve tubes despite the lack of indicator carbohydrate; this ability may be ascribed to pH sensing.

Concluding remarks

Based on our results, we propose the following model of sieve-tube searching and recognition by aphids (Fig. 8), with the caveat that it

is not verified in all aspects, and should be explored further. The feeding behaviour of aphids is composed of two components. A pre-programmed radial orientation of stylet progression *via* cell walls which is intermitted by regular punctures into cells along the pathway. During intracellular punctures, aqueous saliva is secreted and the cell content is sampled. The start and end of the puncture are marked by the secretion of gel saliva. Low sucrose concentrations and low pH in the probed cell sap are stimuli for stylet retraction. Aphids continue probing until appreciable sucrose concentrations and a neutral pH are sensed and then they start ingesting sap. The attractiveness of the concept is that it provides a universal model for aphid orientation towards sieve tubes and that EPG waveforms are interpreted in a universal fashion for parenchyma cells and sieve tubes.

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