

Molecular sabotage of plant defense by aphid saliva

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Aphids, which constitute one of the most important groups of agricultural pests, ingest nutrients from sieve tubes, the photoassimilate transport conduits in plants. Aphids are able to successfully puncture sieve tubes with their piercing mouthparts (stylets) and ingest phloem sap without eliciting the sieve tubes' normal occlusion response to injury. Occlusion mechanisms are calcium-triggered and may be prevented by chemical constituents in aphid saliva injected into sieve tubes before and during feeding. We recorded aphid feeding behavior with the electrical penetration graph (EPG) technique and then experimentally induced sieve tube plugging. Initiation of sieve tube occlusion caused a change in aphid behavior from phloem sap ingestion to secretion of watery saliva. Direct proof of "unplugging" properties of aphid saliva was provided by the effect of aphid saliva on forisomes. Forisomes are proteinaceous inclusions in sieve tubes of legumes that show calcium-regulated changes in conformation between a contracted state (below calcium threshold) that does not occlude the sieve tubes and a dispersed state (above calcium threshold) that occludes the sieve tubes. We demonstrated *in vitro* that aphid saliva induces dispersed forisomes to revert back to the nonplugging contracted state. Labeling Western-blotted saliva proteins with ⁴⁵Ca²⁺ or ruthenium red inferred the presence of calcium-binding domains. These results demonstrate that aphid saliva has the ability to prevent sieve tube plugging by molecular interactions between salivary proteins and calcium. This provides aphids with access to a continuous flow of phloem sap and is a critical adaptation instrumental in the evolutionary success of aphids.

calcium-binding | plant-aphid interaction | plugging | saliva proteins | sieve element

Aphids (Homoptera: Aphididae: Aphidinae) withdraw nutrients from sieve tubes, the photoassimilate-conducting channels of plants, using their stylet mouthparts and, by doing so, weaken the plant. An intriguing aspect of aphid feeding is that they apparently inactivate occlusion of the sieve tubes. This defense reaction in response to injury (1) prevents the loss of sieve-tube sap, which is pressed as a mass flow through the sieve tubes. The functional units of sieve tubes are sieve elements (SEs) that are elongated cells arranged end-to-end to form a continuous conduit; their abutting ends are modified as porous sieve plates to allow the flow of sap from one SE to the next. Because SEs are part of a continuous sap transport conduit, damage to a single SE would result in far more sap loss than simply the contents of a single cell; thus SE occlusion is a vital function for plants (2, 3).

Occlusion reactions have been documented extensively by confocal laser scanning microscopy [CLSM (4, 5)] and transmission electron microscopy [TEM (6)] for broad bean, *Vicia faba*. In response to injury in SEs of intact broad bean plants, spindle-like protein bodies (forisomes) disperse, and parietal proteins detach from the plasma membrane and SE reticulum (3), plugging the sieve plates (4), whereas callose deposition constricts the pores in the sieve plates from the apoplast side (7, 8). Protein meshworks inside SEs that collapse as a consequence of wounding are described for some dicotyledonous plants (6, 9) and water-soluble proteins, which become insoluble after wounding, thereby plugging the sieve plate, are postulated for Poaceae (10). Different combinations of these mechanisms can be observed in various plant

families (11) with exception of forisomes that are restricted to the Fabaceae (12, 13). Regardless of plant species and family, usually sieve plates are immediately occluded in response to the slightest mechanical damage that is initiated by an increase in free calcium (4, 5, 10) in the SEs or by a change in the redox state (14, 15).

Impaling SEs with a fluid-filled microcapillary with a tip diameter of 1 μm , fixed in a microelectrode holder, induces the deposition of "plugs" on the sieve plates of *V. faba* (4). In contrast, insertion of aphid stylets with a similar tip diameter does not appear to trigger such a plugging reaction that would stop the mass flow in the affected SE.

The contrasting SE response between the microcapillary and aphid stylet raises the question of how aphids prevent the natural blocking of the sieve plates (5), which includes plugging by different types of proteins and constriction by callose (2, 3). Before the plasma membrane seals in response to impalement by a microcapillary tip, extracellular calcium presumably gets into the sieve tube lumen through the wound site, inducing sieve plate occlusion (4).

During stylet penetration, aphids secrete two types of saliva, one of which gels almost immediately after exiting the stylet tip and forms a continuous sheath around the stylet. This type of saliva is called sheath saliva, and it also seals the puncture site in the SE cell wall before and during the stylet tip piercing the SE plasma membrane (16). Thus, sheath saliva may reduce the influx of extracellular calcium into the SE lumen through the punctured site (10). Calcium influx into sieve tubes may also be brought about by drastic changes in SE turgor (5, 17). The sheath saliva sealing the puncture site presumably also reduces loss of phloem sap through the wound and in this way decreases a loss of turgor pressure as well (10).

Furthermore, the microcapillary has a large compressible volume that can contribute to a loss of turgor pressure when the microcapillary pierces the SE, whereas the two canals in aphid stylets (salivary and food canals) have a much smaller width and length (10). Moreover, the stylet canals are initially closed by the precibarial valve in the head region (18) during a SE puncture, further restricting the loss of turgor through the lumens of the stylet canals. All in all, there are good reasons to believe that the structure of aphid stylets and the secretion of sheath saliva mitigate physical factors that are normally responsible for a sudden rise in calcium concentration in the SE (18).

The second type of saliva secreted by aphids is called watery saliva because, unlike sheath saliva, it does not gel (19). When aphids penetrate a SE with their stylet tips, they start injecting watery saliva into the SE (20), and, if the penetration is successful, after a period of secretion of watery saliva, the aphid then begins

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Abbreviations: EPG, electrical penetration graph; SE, sieve element.

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other systems to determine how universal the role of calcium binding is in aphid–SE-protein interactions.

Materials and Methods

Aphid and Plant Cultivation. *M. viciae* was reared on 14- to 20-day-old plants of *V. faba* L. cv. Witkiem major (Nunhem Zaden, Haelen, The Netherlands) in a controlled-environment room at 25°C and a 17/7-h light/darkness regime in Perspex cages with large gauze-covered windows.

V. faba plants used in leaf-tip burning experiments were grown in a greenhouse under 20°C with natural lighting plus additional lamp light (SONT Agro 400W; Phillips, Eindhoven, The Netherlands) with a 14/10-h light/dark period and used at the age of 20–25 days, having three to four true leaves. *V. faba* plants used for forisome isolation were cultured in a controlled-environment room at 20°C and a 14/10 h light/dark period and were used from 5 until 7 weeks after germination.

EPG Recording and Long-Distance Signaling in Plants. Randomly picked apterous adult aphids (*M. viciae*) were placed on the petiole base of a mature leaf of *V. faba*, ≈6 cm from the leaf tip. After aphids showed ingestion from a sieve tube, recognized by the E2 waveform (20), careful burning of the leaf tip (an area of maximum 1 cm²) for 3 seconds was used to trigger phloem-mediated electrical long-distance signals that induce sieve-plate occlusion (45).

Plant penetration behavior of the aphid was monitored by application of the dc EPG technique, and waveforms were displayed and recorded by using *Giga* hardware and *PROBE* software (both from Wageningen Agricultural University, Wageningen, The Netherlands) (30, 32, 39, 40). A gold wire electrode (2 cm long and 20 μm in diameter) was attached to the dorsum of each aphid by using electrically conductive silver glue (Electrolube, Swadlincote, U.K.), and the electrode was connected to the input of an EPG amplifier. The plant electrode was inserted in the soil of the potted plant. EPG waveforms were retrieved by using EPG signal analysis software (*PROBE* analysis module). In leaf-tip burning, as well as in the control experiments, 12 replicates were executed, each with fresh aphids and plants.

Statistical Analysis of EPG Data. Data from each aphid were scored as “1” if it changed from E2 to E1 and scored as “0” if it remained in E2 during 10 minutes after leaf-tip burning. Statistical analysis was done with the χ^2 test of Fisher’s exact test and the software SigmaStat 3.0 (SPSS, Chicago, IL).

Aphid Saliva Collection and Saliva Concentration. Aphids of *M. viciae* were collected by gently beating the infested plants, resulting in many aphids, mostly adults, falling from the plant. Aphids were weighed to calculate their number (the weight of an adult aphid was ≈1 mg) and ≈1,500–2,000 aphids were poured onto the Parafilm cover of each saliva-collection chamber. This custom-made device is made up of a Perspex block in which a shallow bath of 9-cm radius and a depth of 1 mm had been milled. The bath contained an artificial aphid diet composed of 100 mM serine, 100 mM methionine, 100 mM aspartic acid, and 15% sucrose [modified from Cherqui and Tjallingii (31)], pH 7.2 (KOH). The diet was filtered through a PVDF syringe membrane with a pore diameter of 0.45 μm (Rotilabo; Carl-Roth, Karlsruhe, Germany) under sterile conditions. Sterilized collection chambers were filled with 3 ml of diet under sterile conditions.

Aphids were placed on the Parafilm-covered diet for 24 h, during which time they secreted saliva into the diet solution. The diet/saliva solution was then collected under sterile conditions, and saliva collection chambers were washed with an additional 2 ml of diet that was pooled with the saliva-containing diet. In general, eight saliva collection chambers (with a total of 16,000 aphids) were used simultaneously, and diet/saliva solutions were pooled and stored at –80°C. Only ≈90% of these latter solutions could be recovered,

because diet solution was ingested by the aphids, and solute remnants stayed on the covering Parafilm and the surface of the collection chamber.

The diet/saliva mix was concentrated for protein separation from a starting volume of ≈35 ml (16,000 aphids) to an end volume of 160 μl (the saliva of 100 aphids per microliter) for 1D SDS/PAGE. For 2D SDS/PAGE, proteins were further concentrated to the saliva of 250 aphids per microliter. For protein concentration, centrifugal concentrators (Vivaspin 20; 2 and 500 with a 3,000 molecular weight cut-off and polyethersulfon membrane; Sartorius, Goettingen, Germany) were used. The final sample was parted in aliquots of 10 μl and kept frozen at –80°C.

The 1D SDS/PAGE and second-dimension control lane in 2D separation used the saliva of 1,000 aphids (10 μl), whereas for 2D (first-dimension), saliva of 3,000 aphids (12 μl) was used per lane. Protein concentration could not be determined owing to the low sample concentration and the high number of different proteins.

Forisome/saliva interaction experiments as well as labeling experiments needed a higher protein concentration. Therefore, samples of different saliva collection runs were pooled before concentrating and concentrated as mentioned in the respective sections.

Isolation of Forisomes. Forisomes were isolated from *V. faba* phloem tissue according to Knoblauch *et al.* (25). Forisome isolation media with EDTA concentration of 10 and 2 mM were outgassed and covered with argon gas, and 1 mM sodium sulfite was added to quench oxygen (M. Knoblauch and W. S. Peters, personal communication). The cortex of one 5- to 7-week-old *V. faba* stem was carefully pulled off, and phloem was scratched off with a scalpel. Plant material was transferred to forisome isolation medium containing 10 mM EDTA. After incubation time of 30 min, scratched phloem was homogenized with liquid nitrogen. For solubilization of homogenized plant material containing forisomes, 4 ml of 2 mM EDTA forisome-isolation medium was used to lower the EDTA concentration for subsequent experiments. After filtration of solubilized plant material through a 60 μm mesh filter freshly isolated forisomes were used for *in vitro* studies on saliva function.

In Vitro Interactions Between Forisomes and Aphid Saliva. Dispersion and contraction of forisomes in response to various treatments was observed with a bright-field microscope using a ×40 objective (Leica DMLB; Leica, Wetzlar, Germany) and documented with a digital camera (Digital Eyepiece Camera DEC-18; WPI, Sarasota, FL). Solutions were outgassed and covered with argon gas, and 1 mM sodium sulfite was added to quench oxygen (M. Knoblauch and W. S. Peters, personal communication). Four microliters of 1 mM CaCl₂ [with 50 mM KCl and 10 mM Tris buffer (pH 7.3)] was pipetted under the coverslip, followed by successive additions of 2 μl of 2 mM EDTA [with 50 mM KCl and 10 mM Tris buffer (pH 7.3)], 2 μl of 1 mM CaCl₂, 2 μl of 2 mM EDTA, again 2 μl of CaCl₂ and, finally, an aliquot of 4 μl of saliva concentrate (equivalent to watery saliva of 6,750 aphids), all dissolved in the same buffer. All steps were done in a 5-cm-deep custom-made Perspex bath chamber, perfused continuously with argon gas to protect proteins from oxygen attack (M. Knoblauch and W. S. Peters, personal communication).

To test the presence of nonprotein chelators in aphid watery saliva, salivary proteins were digested with proteinase K bound to acrylic beads (Sigma–Aldrich, St. Louis, MO). Proteinase K acrylic beads were used so that the proteinase could be removed easily after digestion. Two and a half milligrams of Proteinase K acrylic beads were activated by incubation in 200 μl of H₂O containing 1 mM CaCl₂ and 1 mM EDTA (pH 7.5) (KOH) for 30 min at 37°C and 750 rpm in a thermomixer (Thermomixer Comfort; Eppendorf, Hamburg, Germany). Activation solution was removed and replaced with concentrated saliva of ≈30,000 aphids (1,000 aphids per microliter). Digestion was done for 2 h at 37°C and 1,500 rpm. Acrylic beads were removed by centrifugation for 5 min at

≈11,000 × g; the supernatant contained the digested salivary proteins. Digestion was controlled by 1D SDS/PAGE versus a nondigested saliva protein sample. Supernatant with digested salivary proteins was tested against forisomes as described previously for the undigested salivary proteins.

One-Dimensional and 2D SDS/PAGE. SDS/PAGE of the saliva concentrate was carried out according to Laemmli (41) by using a 4% stacking gel and a 10% separation gel in a MiniProtean 3 Electrophoresis System (Bio-Rad, Hercules, CA). As protein size markers, Precision Plus Protein Standards; All Blue or Kaleidoscope (Bio-Rad Laboratories, Hercules, CA) were used for 1D SDS/PAGE, and Sigma Marker-Wide molecular weight range (Sigma-Aldrich) was used for 2D SDS/PAGE. For 1D SDS/PAGE and the first dimension of 2D SDS/PAGE, fourfold concentrated reducing sample buffer, (Roti-Load 1; Carl-Roth, Karlsruhe, Germany) was added to each saliva sample in the proportion of 1:3. Two-dimensional SDS/PAGE according to ref. 35 was used for detection of calcium-binding saliva proteins. Saliva samples for 2D SDS/PAGE were mixed with a diagonal marker (random polymers of Glu, Lys, and Tyr_(6:3:1); Sigma-Aldrich) before separation in the first dimension. All gels contained 2 mM EDTA in the first dimension, whereas the second-dimension separations were carried out with 2 mM CaCl₂, MgCl₂, or ZnCl₂ in the separation gel. Control gels contained 2 mM EDTA in both dimensions. First-dimension lanes excised for the secondary dimension were prepared with equilibration buffer (35) containing the appropriate ions as mentioned above. The SDS/PAGE gels were silver stained (42, 43).

Gels were documented with the Gel Doc XR documentation system (Bio-Rad) and analyzed by using the Quantity One 1-D Analysis Software (Bio-Rad).

Labeling with ⁴⁵Ca²⁺. Concentrated watery saliva proteins of 41,000 aphids (21 μl) separated by 10% SDS/PAGE mini gels, were blotted onto nitrocellulose membrane (Protran BA 85, pore size 0.45 μm;

Whatman/Schleicher & Schuell, Dassel, Germany) by using Towbin buffer (44) at 35 V overnight. For protein detection, the blot membrane was stained with Ponceau S (Roche Diagnostic, Mannheim, Germany) and destained with PBS [6.1 mM Na₂HPO₄/2 mM NaH₂PO₄/154 mM NaCl (pH 7.2)]. As a protein marker, 7 μl of Precision Plus Protein Kaleidoscope (Bio-Rad) was preferred to control the transfer quality during Western blotting. It showed an affinity to calcium, which was helpful in PhosphorImager (FUJIX BAS 1000; Fuji Photofilm, Tokyo, Japan) analysis.

The blot membrane was washed three times for 20 min each in 40 ml of incubation buffer [5 mM MgCl₂/60 mM KCl/10 mM imidazol-HCl (pH 6.8)]. Subsequently, the membrane was incubated for 10 min in 40 ml of buffer containing 1.5 MBq [⁴⁵Ca]Cl₂ (GE Healthcare U.K., Little Chalfont, U.K.). The membrane was washed two times in 20 ml of Millipore H₂O for 5 min each (33). Incubation time of the blot membrane onto the PhosphorImager screen was set to 72 h. The ⁴⁵Ca²⁺-labeled proteins were detected by making use of a PhosphorImager (FUJIX BAS 1000; Fuji Photofilm) and the acquisition and analysis software TINA.

Ruthenium Red Staining. Ruthenium red staining was preceded by Western blotting and Ponceau S staining in a way identical to that for ⁴⁵Ca²⁺ labeling. Final staining of calcium-binding proteins with ruthenium red [25 mg/liter of ruthenium red (Sigma-Aldrich) in 60 mM KCl, 5 mM MgCl₂, 10 mM Tris-HCl (pH 7.5)] lasted for 48 h (34). Ponceau S (Roche Diagnostic) as well as ruthenium red staining were documented with an Epson Perfection 3490 scanner (Epson, Meerbusch, Germany).

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