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The structural sheath protein of aphids is required for phloem feeding

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ABSTRACT

Aphids produce two types of saliva that mediate their interactions with plants. Watery saliva is secreted during cell penetration and ingestion, whereas gel saliva is secreted during stylet movement through the apoplast where it forms a sheath around the stylet to facilitate penetration and seal puncture sites on cell membranes. In order to study the function of the sheath when aphids interact with plants, we used RNA interference (RNAi) to silence the aphid structural sheath protein (SHP) in the pea aphid *Acyrthosiphon pisum*. The injection of 50 ng of double stranded RNA completely disrupted sheath formation, as confirmed by scanning electron microscopy. Aphid behavior was monitored using the electrical penetration graph technique, revealing that disrupted sheath formation prevented efficient long-term feeding from sieve tubes, with a silencing effect on reproduction but not survival. We propose that sealing the stylet penetration site in the sieve tube plasma membrane is part of a two-step mechanism to suppress sieve-tube occlusion by preventing calcium influx into the sieve tube lumen. The SHP is present in several aphid species and silencing has a similar impact to aphid-resistant plants, suggesting that SHP is an excellent target for RNAi-mediated pest control.

1. Introduction

Aphids (Hemiptera, Sternorrhyncha, Aphidoidea) are severe agricultural pests that deprive plants of nutrition and act as vectors for phytopathogenic viruses. Aphids feed on phloem sap from the sieve tubes of higher plants through specially adapted mouthparts known as stylets. Prior to feeding, the aphid stylet must penetrate the plant epidermis and move through the cortical layer. To facilitate this process, aphids secrete gel saliva which hardens to form a surface flange and a continuous tubular sheath encasing the full length of the stylet within the apoplast. When aphids are fed on an artificial diet, traces of gel saliva form structures reminiscent of pearls in a necklace, indicating that the salivary sheath is formed progressively from drops of saliva that hardens rapidly (Miles, 1965; Miles et al., 1964).

The stylet follows an intercellular pathway towards the sieve tube, but periodically probes adjacent plant cells and injects them with a small amount of watery saliva (Powell, 2005; Martin et al., 1997). The same watery saliva is also injected into the sieve tube immediately after penetration (Prado and Tjallingii, 1994) and this is thought to counteract plant defense mechanisms (Louis and Shah, 2013; Will et al., 2013). After the initial salivation phase, aphids begin to ingest phloem sap while intermittently secreting more watery saliva (Prado and Tjallingii, 1994).

Although the functions of watery saliva are understood in detail, little is known about the functions of gel saliva (Miles, 1999). The salivary flange on the epidermal surface is presumed to facilitate stylet penetration by serving as an anchor point (Pollard, 1973; Tjallingii, 2006). During stylet movement through the apoplast, the continuous sheath around the stylet may provide mechanical stability, lubrication and protect against chemical defenses (Kimmins, 1986; Tjallingii and Hogen Esch, 1993), which would explain why gel saliva contains anti-defense molecules such as enzymes that detoxify free radicals (Miles, 1999). Other plant-sucking pests such as whiteflies and planthoppers also form a salivary sheath by the secretion of gel saliva (Brentassi and Remes Lenicov, 2007; Freeman et al., 2001) and show feeding-associated secretion of watery saliva (Walling, 2008).

The salivary proteome of the aphid *Acyrthosiphon pisum* has been described in detail (Carolan et al., 2009, 2011) and we have published a comparative proteomic analysis of watery and gel saliva in the aphid *Megoura viciae* (Will et al., 2012). The latter study demonstrated that oxidation is required to polymerize the

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structural components of the gel saliva to achieve hardening, and that salivary sheaths adopt an amorphous form when the reducing agent dithiothreitol is included in the artificial diet (Will et al., 2012). One of the most abundant proteins in the gel saliva of *A. pisum* is the sheath protein (SHP), which is rich in cysteine residues and is thought to form a polymer matrix during sheath hardening via intermolecular disulfide bonds (Carolan et al., 2011).

To test this hypothesis, we used RNA interference (RNAi) to silence the expression of SHP and studied its impact on sheath structure (by scanning electron microscopy) and function (by observing aphid feeding behavior, survival and reproduction). We found that sheath formation was disrupted and that aphid feeding and reproduction (but not survival) were inhibited. We discuss our results in the context of aphid–plant interactions and agricultural pest management strategies.

2. Materials and methods

2.1. Aphid and plant breeding

We reared *Acyrtosiphon pisum* clone LL01 on 2–3-week-old bean plants (*Vicia faba* var. minor) in a climate cabinet (KBWF 720, Binder GmbH, Tuttlingen, Germany) with a 16-h photoperiod and a day/night temperature of 24/18 °C. Plants for experiments and aphid rearing were cultivated in a greenhouse with an average temperature of 20 °C and natural light plus additional illumination (SONT Agro 400 W, Phillips, Eindhoven, Netherlands) to maintain a 14-h photoperiod.

2.2. dsRNA production and injection

A 491-bp template for the production of dsRNA representing the *A. pisum* SHP sequence (ACYPI009881) was generated by PCR from plasmid DNA using gene-specific primers containing a 5′ T7 polymerase promoter sequence (AP-SHP-for 5′-TAATACGACTCACTATAGGGAGACGTTATTATTGCTGCTGCTGTG-3′ and AP-SHP-rev 5′-TAATACGACTCACTATAGGGAGAACAGCTACCCTGGCCGATCTT-3′).
We ensured this sequence did not have overlaps exceeding 19 bp with any other gene, to avoid off-target effects. The template was purified using the QIAquick PCR Purification Kit (Quiagen, Hilden, Germany) and dsRNA was prepared using the Ambion MEGAscript RNAi kit (Applied Biosystems, Austin, TX). The primers were designed with Primer3 (Rozen and Skaletsky, 2000) and were purchased from Sigma–Aldrich (Taufkirchen, Germany). We used dsRNA representing the *Galleria mellonella* insect metalloproteinase inhibitor gene (AY330624) as a control which is absent in aphids (Clermont et al., 2004).

We injected 15 nl of dsRNA solution under a stereomicroscope by using a Nanoliter 2000 injector together with a Sys-Micro4 controller (World Precision Instruments, Berlin, Germany). Glass microcapillaries for injection were pulled with a PN-30 puller (Narishige International Limited, London, UK). Prior to injection, aphids were immobilized with their dorsal thorax on a vacuum holder (van Helden and Tjallingii, 2000). The dsRNA was injected at a rate of 2 nl/s between the mesothorax and methathorax, as previously described (Mutti et al., 2006).

2.3. Rearing aphids during experimental treatments

Aphids were reared on detached, mature *V. faba* leaves cut from intact plants with a razor blade. A petiole section of 1–5 mm in length was cut again under water and the leaf was transferred to a Petri dish, filled to a height of 7 mm with 1.5% tap water agar (Carl-Roth GmbH, Karlsruhe, Germany) containing 0.03% methyl-4-hydroxybenzoate (Sigma–Aldrich). Leafs were inserted into the cooled agar upside down and the Petri dishes were maintained in a climate cabinet as described above. Senescent leaves were replaced.

2.4. Determining SHP expression level by real time PCR (qPCR)

RNA was isolated from aphids 5 days after injection of *impi* dsRNA and *shp* dsRNA respectively. 3 × 15 aphids of each treatment were collected and directly frozen in liquid nitrogen. RNA was extracted using TriReagent (Sigma–Aldrich) and a TissueLyser II with 3 mm steal beads (Quiagen, Hilden, Germany). Samples were centrifuged to remove aphid body remnants and were subsequently mixed with 95% ethanol. RNA was collected with Direct-zol™ RNA MiniPrep columns (Zymo Research, Freiburg, Germany). mRNA was converted to cDNA (First Strand cDNA Synthesis Kit; Fermentas, St. Leon-Rot, Germany) and subsequent qPCR was performed with the StepOnePlus™ Real-Time PCR system (Applied Biosystems, Darmstadt, Germany) using *Power* SYBR® Green Master Mix (Applied Biosystems). Appropriate primers were designed using Primer3 (Rozen and Skaletsky, 2000) (AP-SHP-qPCR-for 5′-AAA TGT TGC GTT GTG GAC TT-3′ and AP-SHP-qPCR-rev 5′-GGT AAT CCT TGA AGG GGA GA-3′) and were purchased from Sigma–Aldrich. The amplified sequence was different to the one used for production of *shp* dsRNA. As a reference gene we used 18srRNA (AP-18srRNA-qPCR-for 5′-CCT GCG GCT TAA TTT GAC TC-3′ and AP-18srRNA-qPCR-rev 5′-CCG CCT AGT TAG CAG GAC AG-3′). Calculation of ΔΔCt values was done with StepOne™ software v2.3 (Applied Biosystems).

2.5. Preparation and observation of aphid salivary sheaths

Aphids were reared on an artificial diet that mimics the cell-wall milieu (20 mM KCl, 1 mM $CaCl_2$, 10 mM MES, adjusted to pH 5.5 (Cosgrove and Cleland, 1983; Will et al., 2012)) to encourage secretion of gel saliva. The diet was sterile-filtered before use (pore size 0.45 μm) and 150 μl was placed in a Parafilm sachet. Parafilm sheets were previously sterilized with 30% H_2O_2 for at least 30 min. Five days after dsRNA injection, the time point were dsRNA mediated silencing reaches its maximum (Jaubert-Possamai et al., 2007), 15 aphids of each treatment were placed in groups of five per sheet. The sachet was located on one side of a plastic ring. Opposite to the diet sachet, the ring was closed with a single Parafilm sheet after the ring volume was filled with water. The diet sachet was then placed downwards on a small aphid cage and aphids were allowed to feed for 24 h. Sheets containing aphids were then placed downwards in a Petri dish and were searched for salivary sheaths with an inverse microscope (Olympus IMT-2). Regions of interest were labeled, SEM sample holders were placed on these regions and Parafilm was cut around the sample holders with a scalpel. The samples were dried for a minimum of 3 days in a desiccator with silica gel under vacuum, then gold-sputtered and observed with a Zeiss DSM982 Gemini SEM. Two replicas were prepared for each treatment and 20 randomly-chosen salivary sheaths were observed for each replica.

2.6. EPG analysis of aphid feeding behavior

Aphid feeding behavior was monitored using the electrical penetration graph (EPG) technique (Tjallingii, 1988). A gold wire electrode (1 cm × 20 μm) was attached to the dorsal abdomen of randomly selected apterous aphids 5 days after injection, using electrically conductive silver glue (Electrolube, Swadlincote, Derbyshire, UK) and a vacuum device for immobilization (van Helden and Tjallingii, 2000). The aphid electrode was connected to a DC EPG Giga-8 (Tjallingii, 1978, 1988) and the EPG output was recorded with Stylet+ (hardware and software from EPGSystems,