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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,

Wageningen, Netherlands). A second electrode (plant electrode) was inserted into the soil of potted plants. The experimental setup was placed in a Faraday cage to shield it from electromagnetic interference. Aphids were placed on the lower side of the petiole of a mature leaf on a 10-day-old plant, and EPG recordings were started immediately, running for 8 h. We carried out 14 biological replicates of each treatment. EPG waveforms were analyzed by pattern and autopower spectra as described (Prado and Tjallingii) using the Stylet + analysis module. Further analysis was performed with the workbook for automatic parameter calculation of EPG data version 4.4 (Sarria et al., 2009).

2.7. Survival and reproduction assay

Survival assays ($n = 3$) and reproduction assays ($n = 1$) were conducted separately using 15 aphids per group in each test. Aphids were maintained on a single leaf in an agar plate as described above. Parameters were checked once every day from the first day after injection until the final aphid died. Plates were placed in a climate cabinet using the conditions described above.

2.8. Statistical analysis

Real time PCR data were compared with *t*-test. Descriptive statistical analysis of aphid behavior was carried out and treatments were compared with ANOVA and Kruskal–Wallis ANOVA on ranks. The Wald–Wolfowitz test (SigmaPlot 11) was used to analyze non-parametric class-arranged behavior data. Because of the small sample size for non-parametric data analysis, Z and *p*-values were corrected (Siegel, 1956). Survival analysis was carried out with Kaplan–Meier Survival Analysis Log-Rank, and ANOVA was used to compare the median and maximum survival rates. Reproduction data were analyzed by ANOVA. The level for significance for the statistical tests was set to $p = 0.05$, whereas for behavior analysis *p*-values between 0.05 and 0.075 were seen to indicate a trend with marginal significance. For statistical analysis SigmaPlot 11 was used (Systat Software Inc., London, UK).

3. Results

3.1. Formation of the aphid salivary sheath is disrupted by shp silencing

We injected aphids with 25 ng dsRNA corresponding to the major salivary sheath protein (SHP) and compared them to non-treated controls and non-relevant dsRNA controls (injected with 25 ng dsRNA) corresponding to the insect metalloprotease inhibitor (IMPI), which is specific to the greater wax moth *G. mellonella* (Wedde et al., 2007). After 5 days feeding on an artificial diet on Parafilm, salivary sheaths were prepared for scanning electron microscopy. This revealed that salivary sheaths secreted by the control aphids adopted the typical necklace-like structure that forms on this substrate (Fig. 1a–d, white arrows), whereas those secreted by the *shp* RNAi aphids (injected with 25 ng dsRNA) showed the remnants of a bead-like structure but were predominantly amorphous (Fig. 1e,f). The injection of 50 ng of dsRNA SHP, lead to knockdown of approximately 33% (Fig. 2) and almost completely prevented the formation of bead-like structures, with minimal gel saliva deposits observed at the stylet penetration sites (Fig. 1g,h; white arrows).

3.2. The silencing of shp increases aphid probing activity and interrupts feeding

We selected aphids injected with 50 ng of dsRNA due to their more complete disruption of sheath formation (Fig. 1g,h) for further

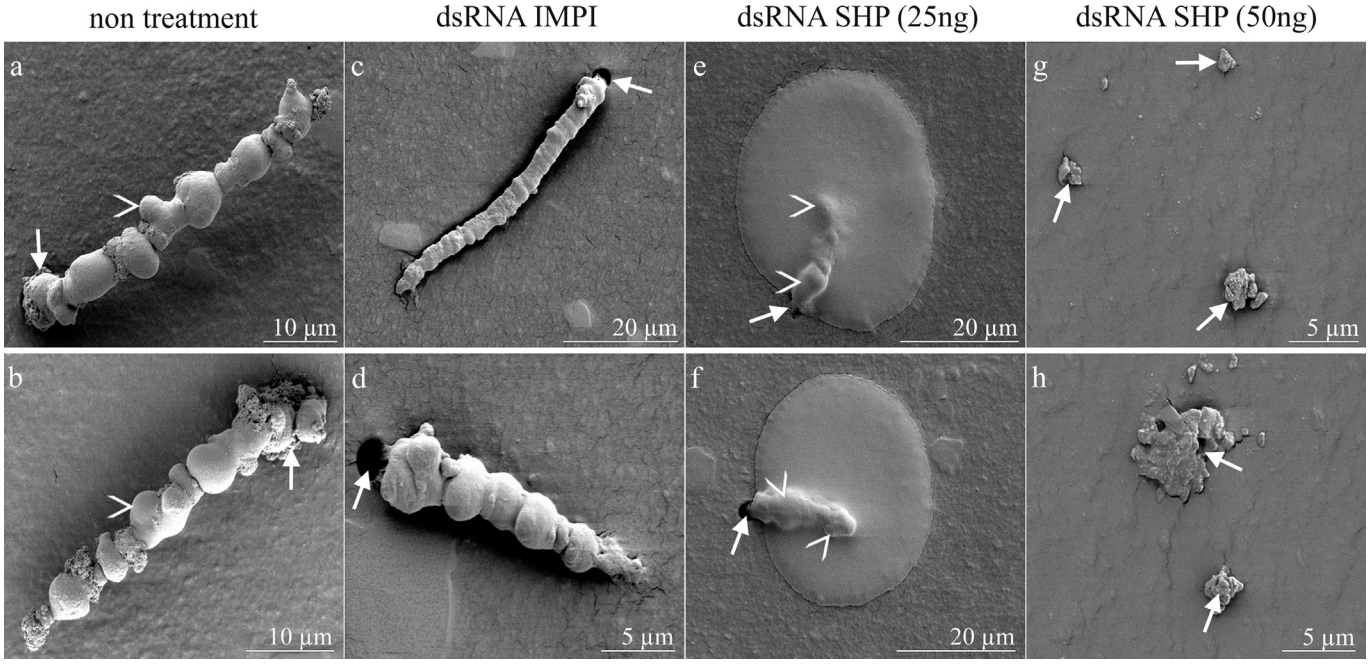

Fig. 1. Influence of *shp* silencing on sheath formation. Salivary sheaths from untreated aphids reared on an artificial diet (a, b) show a typical necklace structure and the sheaths are wider at the stylet penetration site (white arrow) than at the tip. Each bead represents one gel saliva secretion event (white arrowheads). Aphids injected with *impi* dsRNA form similar sheaths (c, d). The hole caused by stylet penetration through the Parafilm sheet is visible (white arrows). The silencing of *shp* disrupts sheath formation (e–h). In aphids injected with 25 ng *shp* dsRNA (e, f) the first two beads are clear and the next four or five appear less distinct. Additional gel saliva material appears to be distributed over the surrounding Parafilm sheet surface. In aphids injected with 50 ng *shp* dsRNA there are no visible beads (g, h) and only a small amount of gel saliva material covering the hole in the sheet (white arrow).

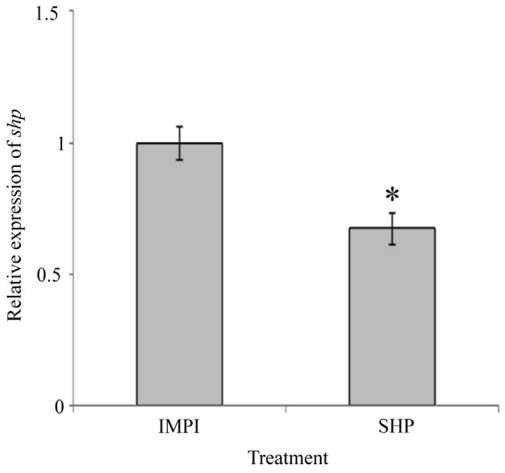

Fig. 2. Silencing of shp expression. *shp* mRNA level was measured by qPCR 5 days after injection in aphids injected with *impi* dsRNA (control) and *shp* dsRNA. For each sample the *shp* transcript level was normalized against *18S-rRNA*.

structural and behavioral analysis. We considered the possibility that *shp* silencing could affect interactions with the epidermis, mesophyll and phloem and therefore analyzed 29 of the 132 calculated parameters (Table 1) listed in the workbook v 4.4 for automatic parameter calculation of electrical penetration graph (EPG) data (Sarria et al., 2009) (Table 1). The parameters were numbered and numbers are given in square brackets in the following text, which points out the most relevant observations.

A marginal difference between the *shp* and *impi* control groups ($p = 0.069$; $n = 14$ for each treatment) was detected at the initiation of stylet penetration, as indicated by the time interval between the start of EPG recording to the first probe [P1]. Other parameters reflecting the accessibility of the epidermis and mesophyll showed no significant differences [P2–8]. However, there were significant differences between the *shp* group and controls in terms of stylet propagation and the secretion of gel saliva as shown by the increased duration of EPG waveform C [P19]. With regard to phloem-related parameters [P9–18], an increase in the number of individual E1 salivation events [P10] was observed in the *shp* group, i.e. the secretion of watery saliva into sieve tubes ($p = 0.012$), but the total duration of E2 (ingestion from sieve tubes [P16]) was lower ($p = 0.03$). The percentage of sustained E2 from total E2 (ingestion phases that last longer than 10 min [P29]) was significantly decreased for the *shp* group ($p = 0.032$). Aphids of the *shp* group ingested less sap, indicated by the mean duration of E2 [P18] and the duration of the longest E2 [P28]. The total probing time [P21] did not differ between the groups ($p = 0.646$).

Table 2
Non-parametric analysis of phloem localizing-parameters in s from the *shp* treatment and *impi* control group. Data from selected parameters (Table 1) were sorted as events in classes representing 2-h intervals and analyzed using the non-parametric Wald–Wolfowitz test.

Time	Start of EPG to 1st E2		1st Probe to 1st E2		Start of EPG to 1st sustained E2		1st Probe to 1st sustained E2	
	IMPI	SHP	IMPI	SHP	IMPI	SHP	IMPI	SHP
0–2 h	4	6	4	6	4	5	4	5
2–4 h	8	2	8	2	8	1	8	1
4–6 h	0	2	1	2	0	4	0	4
6–8 h	2	1	1	2	2	1	2	1
no detection	0	3	0	1	0	3	0	3
Z (corr.)	1.3481		1.3481		2.1184		2.1184	
P	0.123		0.178		0.021		0.034	

Table 1
Behavior analysis using 8-h EPG recordings in aphids from the *shp* treatment and *impi* control group. Statistical analysis was carried out by ANOVA (*) and ANOVA on ranks (**) with parameter definitions as previously described (Sarria et al., 2009).

Tissue specificity		No.	Parameters	IMPI			SHP			P value
				N	Mean [s]	SE [s]	N	Mean [s]	SE [s]	
Plant acceptability	Epidermis	1	Time to 1st probe from start of EPG	8	65.94	33.35	11	126.44	30.95	0.069**
	Epidermis and Mesophyll	2	Number of probes to the 1st E1	13	23.69	3.90	11	23.36	6.58	0.984*
		3	Number of F	14	1.36	0.27	14	0.71	0.19	0.064*
		4	Total duration of F	11	5082.76	939.58	8	2688.34	1073.73	0.113*
		5	Mean duration of F	11	3452.07	780.43	8	1772.87	537.15	0.137**
		6	Average number of pd per probe	14	14.5	6.15	13	10.39	1.85	0.544**
		7	Time from start of EPG to 1st E	14	11,020.36	1853.27	14	13,327.35	2756.92	0.783**
		8	Time from 1st probe to 1st E	14	10,982.69	1854.87	14	13,228.01	2765	0.854**
	Phloem	9	Number of E1	14	2.64	0.52	14	3.14	0.66	0.558*
		10	Number of single E1	14	0.07	0.07	14	0.64	0.27	0.012*
		11	Number of E2	14	2.5	0.48	14	2.29	0.55	0.64**
		12	Number of sustained E2 (longer than 10 min)	14	2.14	0.33	14	1.43	0.34	0.145*
		13	Contribution of E1 to phloem phase (%)	13	2.98	0.48	11	13.81	8.61	0.339**
		14	Total duration of E	13	9320.18	1167.81	11	5882.98	919.45	0.035*
		15	Total duration of E1	13	249.02	43.04	11	426.13	173.98	0.885**
		16	Total duration of E2	13	9071.16	1164.11	11	5456.85	990.77	0.03*
		17	Mean duration of E1	13	108.68	26.39	11	88.1	29.53	0.06**
		18	Mean duration of E2	13	4905.67	1221.21	11	2281.98	583.28	0.06**
	All tissues	19	Total duration of C	14	10,585.98	962.67	14	14,854.8	1516.52	0.025*
		20	Number of probes	14	34.71	4.74	14	37.86	4.24	0.625*
		21	Total probing time	14	24,893.48	1952.43	14	24,611.54	4025.88	0.646**
		22	Mean duration of np	14	152.68	15.38	14	1674.9	1440.95	0.818**
		23	Total duration of np	14	5564.7	1137.94	14	8734.3	2014.78	0.408*
		24	Time from start of EPG to 1st sustained E2 (10 min)	14	11,269.14	1896.99	14	16,681.34	2873.88	0.408**
		25	Time from 1st probe to 1st sustained E2 (10 min)	14	11,231.46	1898.74	14	16,282.83	2815.42	0.491**
		26	Time from start of EPG to 1st E2	14	11,254.16	1899.55	14	13,542.19	2866.63	0.748**
		27	Time from 1st probe to 1st E2	14	11,216.48	1901.29	14	14,897.81	2821.31	0.818**
Phloem acceptability	Phloem	28	Duration of the longest E2	13	6264.13	1233.51	11	3550.96	888.01	0.068**
		29	% E2 >10 min	13	92.86	4.85	11	66.84	10.75	0.032**

Several parameters associated with phloem localization, such as time to first E2 event [P26] and time to first sustained E2 event [P24, 25], were delayed in the *shp* group albeit not to a statistically significant extent, possibly reflecting the small sample size (Table 1). To refine the analysis, we sorted the data into groups representing intervals of 2 h, and each parameter [P24–27] was analyzed using non-parametric statistics (Table 2). In the *shp* group, there was a significant increase in the time from start to first sustained E2 event ($p = 0.021$) and the time from first probe to the first sustained E2 event ($p = 0.034$) but no difference in the time from the start of EPG recording ($p = 0.123$) or from first probe to the first E2 event ($p = 0.178$).

The late occurrence of sustained E2 in the shp group is also shown by displaying the percentage change of EPG waveforms (non-probing (np), stylet pathway activities (C), cell penetrations (pd), and phloem-related activities (E1, E2)) over the EPG recording time of 8 h in 30-min intervals (Fig. 3). During the first 1.5 h, there was a maximum of 21% np activity and 36% waveform C activity in the control group, and phloem activities (E1 and E2) increased from 14% after 1 h to 43% after 2.5 h. Approximately 50% of the aphids ingested sap after 7 h. In contrast, the *shp* group showed a higher frequency of np (~30%) and waveform C (29–57%) behavior but reduced phloem activities (7% after 1 h, increasing to 7–14% after 2.5 h and stabilizing at 21%).

3.3. The silencing of shp inhibits aphid reproduction

We monitored the reproduction of aphids in the *shp* and control groups throughout their lifespan. In all groups, the reproduction rate increased rapidly at the beginning of the observation period and reached a maximum after 4 days (Fig. 4a). The maximum reproduction rate in the control groups was approximately eight nymphs per day, whereas in the *shp* group it was six nymphs per day. Furthermore, reproduction in the control groups was maintained for 27 days (untreated control) or 22 days (*impi* control) whereas the reproduction rate dropped off after 4 days in the *shp* group and ceased after 17 days. There was a highly significant difference ($p < 0.001$) in the total mean reproduction rate (Fig. 4b) between the *shp* group (45.6 nymphs per adult) and untreated controls (88.2 nymphs per adult), and a significant difference ($p = 0.052$) between the *shp* group and *impi* control group (68.9 nymphs per adult in the latter). There was no significant difference between the two control groups ($p = 0.083$).

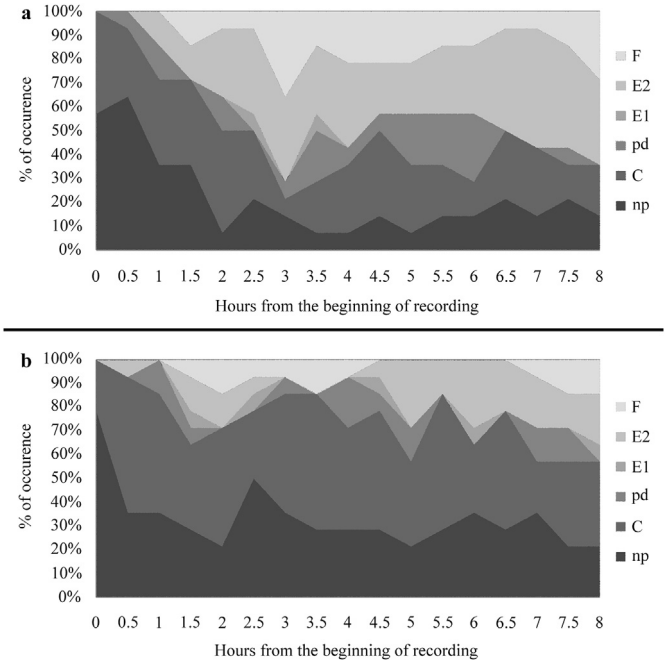

Fig. 3. Temporal evolution of the behavior of aphids in the *shp* treatment and *impi* control group. The data shown the percentage of individuals in the control group (a) and *shp* treatment group (b) that show a specific behavior in 30-min intervals over a total recording time of 8 h. Behavior is shown as EPG waveform codes: np – non-penetration, C – stylet movement and secretion of gel saliva, pd – potential drop, E1 – secretion of watery saliva in a penetrated sieve tubes, E2 – ingestion of phloem sap, F – penetration problems.

3.4. The silencing of shp does not affect aphid survival

We compared the *shp* group and controls using Kaplan–Meier survival analysis and found that the log-rank showed no difference in survival between the groups, where n = 3 (Fig. 4c, Table 3). There were also no differences in mean median survival (50% of animals alive) or mean maximum survival between the groups (Table 3).

4. Discussion

Aphids feed on phloem sap from sieve tubes, located deep inside the tissues of higher plants. While penetrating the epidermis and moving the stylet through the apoplast of the cortical layer towards the sieve tubes, aphids secrete gel saliva forming a sheath that envelops the stylet. This salivary sheath remains in the plant after stylet retraction (Tjallingii and Hogen Esch, 1993). The stylet sheath contains a number of proteins but the structural protein SHP is the most abundant and is therefore likely to be the most important component of sheath integrity (Carolan et al., 2009). Sheath hardening is probably caused by SHP polymerization, induced by the oxidation of sulfhydryl groups on multiple cysteine residues to form intermolecular disulfide bonds (Miles, 1965; Carolan et al., 2009; Will et al., 2012). We used RNAi to specifically target *shp* mRNA for degradation, thus reducing the amount of SHP in the saliva. We then observed the impact of thin intervention on sheath formation and aphid feeding behavior, survival and reproduction.

When aphids are fed on an artificial diet presented in Parafilm sachets, the salivary sheath forms a necklace-like structure wherein each bead represents an individual secretion event (Miles, 1965). Similar structures were produced by untreated aphids and those injected with 25 or 50 ng *impi* dsRNA, a *G. mellonella* sequence which does not have a natural homolog in *A. pisum* (Fig. 1a–d). In contrast, these bead-like structures progressively broke down following the injection of 25 ng (Fig. 1e,f) or 50 ng (Fig. 1g,h) *shp* dsRNA. The small deposits of gel saliva observed at the Parafilm stylet penetration site produced by aphids treated with 50 ng *shp* dsRNA probably form because there is sufficient oxygen on the Parafilm surface to polymerize gel saliva even with a low concentration of SHP (expression reduced to 67%). The impact of *shp* silencing on sheath formation confirms that SHP is an essential component of the sheath structure (Carolan et al., 2009).

We assumed that the inability to produce a hardened sheath would influence aphid feeding behavior such as probing, stylet movement through the apoplast and ingestion from sieve tubes, because these functions are probably facilitated by the stylet sheath (Miles, 1999; Will and van Bel, 2006). EPG analysis revealed significant differences in probing and feeding parameters between the *shp* treatment group and the *impi* control group, including delayed penetration, prolonged stylet movement, more watery saliva secretion events without subsequent ingestion, a lower mean duration of watery saliva secretion into the sieve tubes, a lower mean and total duration of ingestion, a lower (and delayed) total

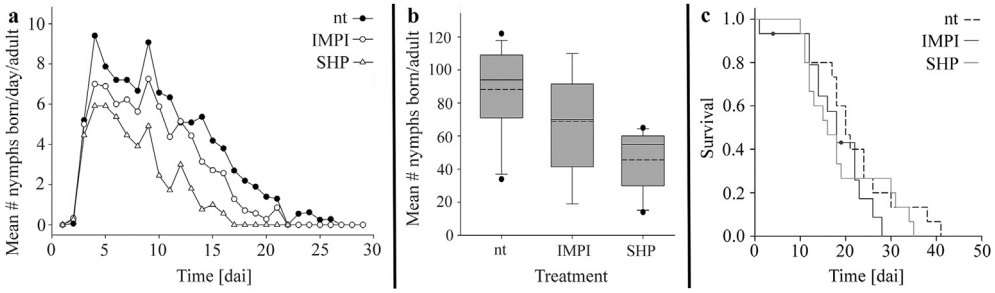

Fig. 4. Influence of *shp* silencing on aphid reproduction and survival. Each group contained 15 aphids. (a) Aphids in the *shp* treatment group show a lower reproduction rate and a shorter overall duration of reproduction than untreated and *impi* controls. (b) Total reproduction in the *shp* treatment group is significantly lower than the control groups. (c) Survival analysis by Kaplan–Meier log-rank analysis shows no difference in survival between the *shp* treatment group and control groups. Aphids that died for unrelated reasons are censored (black circles).

duration of sustained ingestion, and fewer instances of derailed stylet mechanics.

The formation of the salivary flange during the first period of gel saliva secretion is probably delayed in the *shp* treatment group but eventually succeeds as shown by the small deposits formed at the stylet penetration site (Fig. 1g,h), probably explaining the delayed penetration behavior we observed. The inability to form a completely hardened sheath, together with the potential diffusion of gel saliva components into the surrounding apoplast milieu, results in a loss of sheath functions potentially including lubrication, cell wall digestion and detoxification (Cherqui and Tjallingii, 2000). These losses are likely to explain the prolonged stylet movement, but it is notable that the time from first probe to first ingestion is similar in the *shp* treatment group and controls. This suggests that the loss of SHP does not delay or prevent the stylet reaching the phloem and calls into question the proposed lubrication function of the sheath (Miles, 1999). Instead, the bottleneck appears to be stable access to the sieve tubes after first contact (reduced phloem-associated behavior), suggesting that the absence of a functional sheath triggers sieve-tube occlusion mechanisms (Knoblauch and van Bel, 1998; Furch et al., 2007, 2010). The shorter overall ingestion period and the lower percentage of sustained ingestion events indicate that after successful stylet penetration, aphids with impaired sheath formation find it difficult to maintain access to their feeding site and cannot use it as a long-term food source.

The presence of calcium-binding proteins in the saliva of the aphid *Megoura viciae* suggests that plant defenses can be overcome by calcium sequestration (Will et al., 2007). Calcium-binding proteins are also found in the watery saliva of *A. pisum* (Carolan et al., 2009, 2011). Nevertheless, recent studies involving the cryofixation of aphids at different feeding stages demonstrate that sieve tube occlusion is not triggered at the beginning of sieve tube penetration before the secretion of watery saliva, suggesting there must be accessory upstream mechanisms that prevent calcium influx (Medina-Ortega and Walker, 2013). Our findings indicate that the salivary sheath prevents such an influx of calcium from the apoplast as previously suggested (Will and van Bel, 2006) by forming a seal at the stylet penetration site in the sieve-tube plasma membrane. The increase in watery salivation events without subsequent ingestion in the *shp* treatment group is most likely induced by the loss of turgor pressure inside penetrated sieve tubes as a consequence of an occlusion event (Will and van Bel, 2006; Gould et al., 2004). As observed in artificial feeding systems, aphids can detect a pressure drop and react by secreting watery saliva and pausing ingestion (Will et al., 2008). Although, *shp* silencing impedes the ingestion of phloem sap, we observed no impact on aphid survival but a significant impact on reproduction. This suggests that the reduced availability of nutrients forces a trade-off, in which the aphids sacrifice their reproductive ability in order to ensure survival.

Our findings confirm that SHP is a major structural protein of the salivary sheath that is required for sheath hardening. Based upon the behavior of aphids in the *shp* treatment group, we propose that the salivary sheath seals the stylet penetration site in the sieve-tube plasma membrane to prevent the influx of calcium from the apoplast. In this manner, it acts together with calcium-binding proteins in the watery saliva part of a two-step mechanism to suppress sieve-tube occlusion by preventing calcium influx into the sieve tube lumen. SHP is as a component of the gel saliva in several aphid species (Rao et al., 2013) so the development of plant-protection strategies that target this protein would be a universally applicable for aphid pest control. The modified behavior we observed was similar to the behavior of aphids feeding on aphid-resistant plants (Klingler et al., 1998). The RNAi-mediated protection of plants against aphids has already been demonstrated (Mao and Zeng, 2013; Bhatia et al., 2012; Pitino et al., 2011; Sapountzis et al., 2014) so transgenic plants expressing dsRNA targeting the *shp* mRNA of specific aphid pests should provide an efficient and environmentally sustainable approach to reduce the impact of aphid pests in agriculture (Will and Vilcinskas, 2013).

Table 3
Survival analysis. Kaplan–Meier survival log-rank analysis and survival analysis by median and maximum survival time in aphids from the *shp* treatment and control groups.

Experiment	Treatment	Median Survival time	P-value	Maximum survival time	P-value	Kaplan–Meier log-rank P-value	
						vs. nt	vs. IMPI
1	nt	13	0.586	50	0.772		
	IMPI	15		37		0.788	0.113
	SHP	19		40		0.824	
2	nt	20		41			
	IMPI	16		28		0.218	0.648
	SHP	15		35		0.274	
3	nt	18		38			
	IMPI	15		50		0.781	0.87
	SHP	18		45		0.567	

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