Plant Biotechnology Journal

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Plant Biotechnology Journal (2015), pp. 1–9 doi: 10.1111/pbi.12322

Silencing the expression of the salivary sheath protein causes transgenerational feeding suppression in the aphid *Sitobion avenae*

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Received 29 August 2014;
revised 7 November 2014;
accepted 4 December 2014.
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Keywords: host-induced gene silencing, RNAi, aphid, salivary sheath protein, *Sitobion avenae*, transgenerational silencing.

Summary

Aphids produce gel saliva during feeding which forms a sheath around the stylet as it penetrates through the apoplast. The sheath is required for the sustained ingestion of phloem sap from sieve elements and is thought to form when the structural sheath protein (SHP) is cross-linked by intermolecular disulphide bridges. We investigated the possibility of controlling aphid infestation by host-induced gene silencing (HIGS) targeting *shp* expression in the grain aphid *Sitobion avenae*. When aphids were fed on transgenic barley expressing *shp* double-stranded RNA (*shp*-dsRNA), they produced significantly lower levels of *shp* mRNA compared to aphids feeding on wild-type plants, suggesting that the transfer of inhibitory RNA from the plant to the insect was successful. *shp* expression remained low when aphids were transferred from transgenic plants and fed for 1 or 2 weeks, respectively, on wild-type plants, confirming that silencing had a prolonged impact. Reduced *shp* expression correlated with a decline in growth, reproduction and survival rates. Remarkably, morphological and physiological aberrations such as winged adults and delayed maturation were maintained over seven aphid generations feeding on wild-type plants. Targeting *shp* expression therefore appears to cause strong transgenerational effects on feeding, development and survival in *S. avenae*, suggesting that the HIGS technology has a realistic potential for the control of aphid pests in agriculture.

Introduction

The family Aphididae encompasses more than 4300 aphid species, all of which are specialized in phloem sap feeding (Blackman and Eastop, 1994). Aphid populations proliferate rapidly reflecting their parthenogenetic and viviparous mode of reproduction during the asexual life cycle (International Aphid Genomics Consortium, 2010). The evolution of telescoping generations, wherein embryonic development begins before the mother is born, allows nymphs to reach maturity a few days after birth (Dixon, 1987). Aphids have a severe impact on their host because they deprive plants of nutrition and act as vectors for phytopathogenic viruses; annual crop losses due to aphids therefore cost hundreds of millions of dollars (Blackman and Eastop, 2000). The control of aphid pests is becoming more challenging due to the spread of insecticide-resistant populations, so more effective control measures are urgently required (Elzen and Hardee, 2003).

RNA interference (RNAi) has been developed as a novel method for pathogen and pest control (Price and Gatehouse, 2008; Huvenne and Smagghe, 2010; El-Shesheny *et al.*, 2013; Wang *et al.*, 2013; Zhang *et al.*, 2013; Koch *et al.*, 2013; for review see Koch and Kogel, 2014). RNAi is an ancient and highly conserved form of gene regulation present in almost all eukaryotes (Fire, 2007; Fire *et al.*, 1998), which involves two pathways: post-transcriptional gene silencing (PTGS) and transcriptional gene silencing (TGS) (Vaucheret, 2006; Vaucheret and Fagard, 2001). PTGS occurs in the cytoplasm acting at the mRNA level and begins with the cleavage of a precursor double-stranded RNA (dsRNA) by RNase III-like enzymes of the Dicer family, producing 21–25 nt short interfering RNA (siRNA) duplexes (Baulcombe, 2004). These siRNAs assemble with proteins to form an RNA-induced silencing complex (RISC) that subsequently binds to a complementary mRNA, degrading it and thereby inhibiting translation (Brodersen and Voinnet, 2006; Vaucheret *et al.*, 2004).

More recently, systemic RNAi methods have been developed by studying dsRNA uptake in model organisms such as the beetle *Tribolium castaneum* (Tomoyasu *et al.*, 2008). Several protocols have been developed for the delivery of dsRNA to insects, including dietary supplements with purified dsRNA (Baum *et al.*, 2007), microinjection (Arakane *et al.*, 2004; Suzuki *et al.*, 2008) and delivery by feeding on plants expressing dsRNA transgenes (Will and Vilcinskas, 2013). The latter strategy is known as host-induced gene silencing (HIGS) (Nowara *et al.*, 2010) and has been proposed as a method to engineer crops for resistance to

Please cite this article as: Abdellatef, E., Will, T., Koch, A., Imani, J., Vilcinskas, A. and Kogel, K.-H. (2015) Silencing the expression of the salivary sheath protein causes transgenerational feeding suppression in the aphid *Sitobion avenae*. *Plant Biotechnol. J.*, doi: 10.1111/pbi.12322

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pathogens and pests such as aphids, including the prevalent pest species *Sitobion avenae* and *Myzus persicae* (Bhatia *et al.*, 2012; Pitino *et al.*, 2011; Xu *et al.*, 2014).

Aphids feed on phloem sap from sieve tubes, the nutrient transport channels of the vascular bundles in higher plants (van Bel, 2003). The aphids penetrate the cuticle with specialized mouthparts known as stylets and push the stylet through the apoplast, probing and penetrating cells along its pathway, possibly in response to plant signals (Hewer *et al.*, 2010; Tjallingii, 2006). Stylet movement is accompanied by the secretion of gel saliva, which forms a salivary flange on the epidermis and an enveloping salivary sheath in the apoplast, both of which may provide stability, lubrication and protection during feeding, while the latter also seals the plasma membrane at stylet penetration sites (Miles, 1999; Will and van Bel, 2006; Will *et al.*, 2012). Arnaud (1918) compared the salivary sheath with the padding of an oil borehole and suggested that the sheath prevents sap leaking from the stylet as well as the ingress of undesirable plant material into the feeding channel. Most phytophagous hemiptera form a salivary sheath during feeding, emphasizing its biological relevance (Morgan *et al.*, 2013; Will *et al.*, 2013).

The hardening of the salivary sheath is thought to be caused by the oxidation of cysteine sulfhydryl groups to form intermolecular disulphide bonds (Miles, 1965; Tjallingii, 2006). This view is supported by the observation that salivary sheath formation is inhibited under anoxic and reducing conditions (Will *et al.*, 2012). The *A. pisum* sheath protein (SHP) contains the highest number of cysteine residues in the aphid saliva proteome and is a pivotal component of the sheath hardening process (Carolan *et al.*, 2009), making it a promising candidate for the disruption of sheath hardening by RNAi. Here, we show that silencing *shp* expression in the grain aphid *S. avenae* by HIGS strongly inhibits feeding and reproductive behaviour of the aphid and negatively impacts its survival. Remarkably, we found that *shp* silencing was transmitted to offspring across several generations of aphids even after switching to wild-type plants.

Results

Barley plants expressing *shp*-dsRNA induce *shp* silencing in feeding aphids

Barley plants (cultivar Golden Promise) were transformed with either the silencing vector p7i-Ubi-*shp*-dsRNA or the empty-vector p7i-Ubi-RNAi2. The former contains two inverted ubiquitin promoters (*ubi*) driving the constitutive expression of sense and antisense copies of a 491-bp fragment of *shp* to generate the corresponding dsRNA, while, in the latter, this fragment is replaced by a fragment of the *ß-glucuronidase* (*GUS*) gene (Figure S1). Six transgenic lines with independent transformation events were assessed by quantitative real-time PCR (qRT-PCR) analysis for expression of *shp*-dsRNA. Strongest expression of the inhibitory RNA was found in lines L26, L28 and L33 (Figure S2).

Next, we assessed whether the expression of the inhibitory RNA affects target gene expression in aphids fed on transgenic barley lines. To this end, grain aphids (*Sitobion avenae*) were fed for 2 weeks on L26 which showed the highest level *shp*-dsRNA expression. The relative expression level of the aphid's *shp* gene was reduced 10-fold compared to aphids that fed on wild-type and empty-vector controls (Figure 1a). To determine whether the aphids recovered from silencing, those fed on L26 and empty-vector control plants for 2 weeks were subsequently transferred to wild-type plants, and *shp* mRNA levels were measured after 1 and 2 further weeks of feeding. In aphids that had previously fed on L26 plants, the *shp* gene remained strongly silenced. After 1 week of recovery, *shp* mRNA levels had risen from 10% to only 34% of the control level, and after 2 weeks, they had risen further but only to 38% of the control level (Figure 1b).

HIGS-mediated silencing of *shp* impairs the fitness of adult aphids

We investigated the impact of *shp* silencing on sheath formation and various fitness parameters. The aphids were fed for 2 weeks on either L26 or empty-vector/wild-type control lines and were subsequently transferred to an artificial diet on parafilm sachets. After 2 further days, 30 salivary sheaths formed by gel saliva secreted onto the underside of the parafilm were inspected using an inverse microscope. The salivary sheaths deposited by the control aphids showed a typical necklace-like structure (Figure 2a–b), whereas sheath formation was largely inhibited in aphids that had initially fed on L26 (Figure 2c). The salivary sheath of *shp* silenced aphids showed a significant size reduction of approx. 81% (0.21 ± 0.2 mm^2; $P < 0.001$) while there was no impact on sheath size on empty-vector plants (1.104 ± 0.3 mm^2) compared with wild type (1.143 ± 0.4 mm^2; $P < 0.74$).

The impact of *shp* silencing on reproduction was determined by placing synchronous 8-day-old adults onto *shp*-dsRNA or control plants and scoring the number of offspring. The number of nymphs was significantly ($P < 0.001$) lower when aphids were fed on the *shp*-dsRNA lines (28, 37 and 39 nymphs per adult on L26, L28 and L33, respectively) compared to 63 and 58 nymphs per adult on the wild-type and empty-vector controls, respectively (Figures 3a; S3a). Reproduction ceased after 41 days on the empty-vector controls and after 42 days on the wild-type controls, but after 31 days on L26. The average daily reproduction rate rose to a maximum of ~4 nymphs per day on control lines and ~2.8 nymphs per day on L26 (Figure 3b). Comparable results were obtained with transgenic lines L28 and L33 (Figure S3b, c). The overall survival of aphids feeding on L26 was significantly ($P < 0.05$) lower than aphids fed on the control lines (Figure 3c). Comparable results were obtained with transgenic lines L28 and L33 (Figure S3d).

Aphids feeding on *shp*-dsRNA lines experience a developmental delay

Ten synchronous one-day-old nymphs were taken from wild-type control lines and placed individually inside clip cages, which were placed on the surface of L26 or control leaves. Development was monitored until maturity. The nymphs feeding on control plants remained at developmental stages 2 and 3 for 4 days and developed normally, reaching maturation within 8 days. By contrast, the nymphs feeding on L26 remained at developmental stages 2 and 3 for 9 days and reached maturation only after 12–14 days (Figure 4a). We also measured the offspring body plan area (BPA) at maturity and found that those feeding on L26 was approximately 50% smaller than those feeding on control lines (Figure 4b).

Feeding on *shp*-dsRNA lines induces transgenerational silencing in aphids

Silencing in insects can be vertically transmitted, that is parents subjected to RNAi and can transmit the effect to their progeny (Bucher *et al.*, 2002). We investigated the potential transgenerational effects of *shp* silencing by feeding aphids for 2 weeks on L26 and allowing them to reproduce on wild-type plants for 24 h. We then monitored *shp* expression in successive generations of

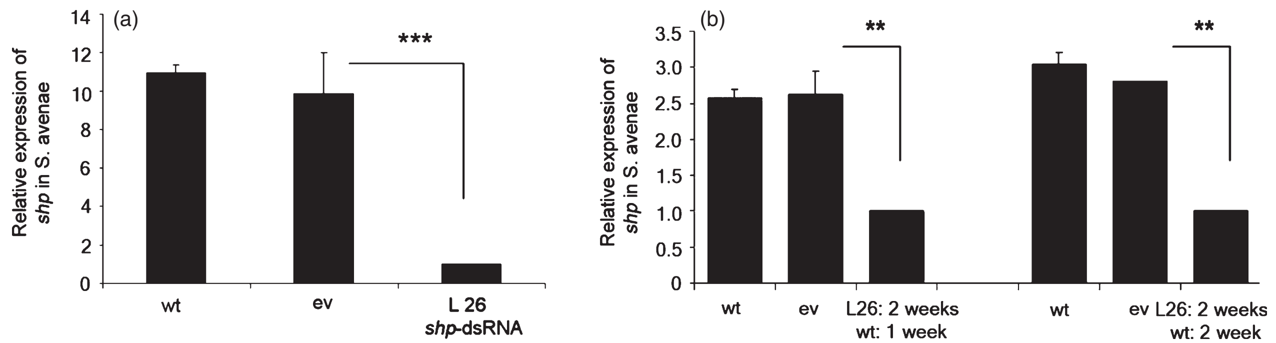

Figure 1 Quantification of *shp* transcripts in *Sitobion avenae* by qRT-PCR. The cDNA was generated from total RNA extracted from aphids after feeding on the *shp*-dsRNA line L26 as well as empty-vector (ev) and wild-type (wt) control plants. (a) The abundance of *shp* mRNA after 2 weeks feeding on L26, empty-vector and wild-type control lines. The lower *shp* mRNA levels in aphids fed on L26 was statistically significant (***$P < 0.001$, *t*-test). (b) Abundance of *shp* mRNA after 2 weeks feeding on L26 before transfer to wild-type plants for 1 or 2 weeks. The lower *shp* mRNA levels in aphids fed on L26 was statistically significant (**$P < 0.01$, *t*-test). Bars represent mean values ± SDs for three independent samples with 10 aphids each.

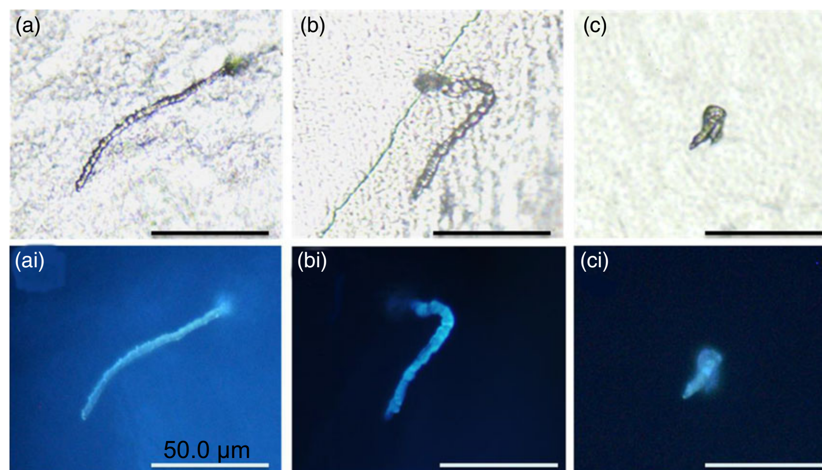

Figure 2 Influence of *shp* silencing on salivary sheath formation. (a) and (b) salivary sheath formation by aphids maintained on wild-type (wt) control (a) or empty-vector (ev) control (b) plants after transfer to artificial diet and incubation for 48 h, showing typical necklace structure. (c) Salivary sheath formation by aphids maintained on L26 before transfer to artificial diet for 48 h, showing that sheath formation on the artificial diet is disrupted. (a), (b) and (c) were observed by bright-field microscopy, (ai), (bi) and (ci) by fluorescence microscopy. Thirty randomly selected salivary sheaths from a total of 10 aphids were observed for each treatment, and images show typical examples.

aphids fed solely on wild-type plants. Remarkably, we found that *shp* expression was not only inhibited in the parental generation that had fed on L26, but remained significantly reduced in the six subsequent generations that fed only on wild-type plants (Figure 5). Aphids recovered only slowly from *shp* silencing over successive generations, with relative expression levels peaking at 2.5%, 4.2%, 21%, 19%, 44%, 77% and 94% of control levels in generations 1–7, respectively). The offspring were also characterized by prolonged development (15.5, 15, 13.7, 14.2, 12.75, 11 and 9.2 days in generations 1–7, respectively) compared to 8 days in controls (Table 1) and a higher percentage of winged adults (80%, 70%, 68%, 69%, 70%, 50% and 30% more than controls in generations 1–7, respectively; Table 2). These data show that aphid feeding can induce long-lasting *shp* silencing via HIGS in successive aphid generations.

Discussion

RNAi is a prospective tool for pest control that has been experimentally validated in a large number of studies (e.g. Baum *et al.*, 2007; Huvenne and Smagghe, 2010; Mao *et al.*, 2007; Zhang *et al.*, 2013). Although RNAi has been used to silence targets in several aphid organs (Jaubert-Possamai *et al.*, 2007; Sapountzis *et al.*, 2014; Zhang *et al.*, 2013), the salivary glands appear to be a promising target because aphid saliva (and particularly the proteins in it) are indispensable for the infestation of plants (Will *et al.*, 2013). Previous work has suggested that the efficacy of gene silencing against salivary effectors is species/method dependent. For example, the silencing of salivary protein C002 in the green peach aphid *Myzus persicae* by HIGS reduced the reproduction rate but did not affect survival (Pitino *et al.*, 2011). By contrast, Mutti *et al.* (2006, 2008) achieved a reduction in feeding, reproduction and survival by targeting the same protein by microinjection in the pea aphid *Acyrthosiphon pisum*, suggesting that targets must be explored on a case-by-case basis.

The composition of salivary proteins differs among aphid species even if they feed on similar host plants (Cooper *et al.*, 2011; Rao *et al.*, 2013; Vandermoten *et al.*, 2014). Furthermore, even similar aphid salivary effectors are species restricted and are adapted to particular hosts (Pitino and Hogenhout, 2013). By

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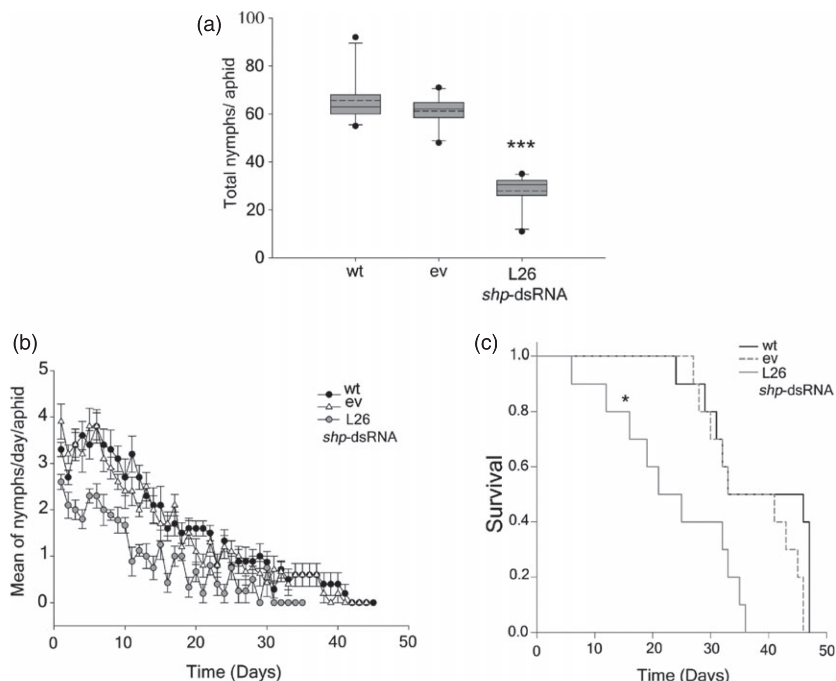

Figure 3 Fitness analysis of aphids fed on *shp*-dsRNA plants. (a) The reproduction of aphids fed on *shp*-dsRNA line L26 was significantly lower than that of aphids fed on empty-vector (ev) and wild-type (wt) control lines. Synchronous eight-day-old nymphs were placed either on *shp*-dsRNA or on control lines. (***$P < 0.001$, *t*-test). (b) Aphids fed on *shp*-dsRNA L26 show a significant decline in reproduction rate and a shorter overall duration of reproduction than aphids fed on ev and wt control plants. (c) Mortality was recorded for 48 days. Aphids fed on *shp*-dsRNA lines died earlier than those fed on ev and wt control lines, and the total survival rate was significantly lower in the *shp*-dsRNA line L26 compared to the controls (*$P < 0.05$, *t*-test). All experiments were repeated three times, with 15 aphids, and each replicate was similar to the results shown here. Bars represent mean values $\pm$ SDs of 15 single animals. Whiskers of box plots defining the 10th and 90th percentiles.

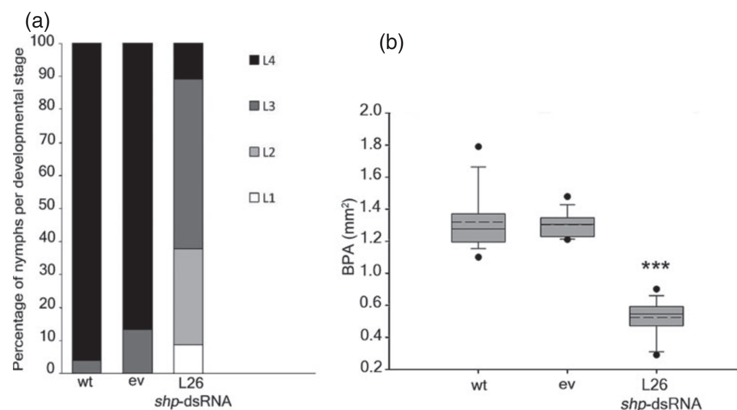

Figure 4 Aphid feeding on *shp*-dsRNA line disrupts offspring nymph development and reduces the adult size. (a) Aphid feeding on *shp*-dsRNA line L26 delays nymph maturation compared to nymphs fed on empty-vector (ev) and wild-type (wt) controls after 8 days. The experiment was carried out three times with similar results. (b) Adults were smaller when the nymphs were fed on *shp*-dsRNA L26 compared to nymphs fed on ev and wt controls. The body plan area of aphids fed on the *shp*-dsRNA was significantly smaller than aphids fed on ev and wt control plants (***$P < 0.001$, *t*-test). All experiments were repeated three times, with 10 aphids, and each replicate was similar to the results shown here. Bars represent mean values $\pm$ SDs of 10 single animals.

contrast, the highly conserved sheath protein SHP (Rao *et al.*, 2013) is present in diverse aphid species such as *A. pisum* and *S. avenae* (Carolan *et al.*, 2009, 2011) and therefore represents an ideal target to control different aphid pests infesting various crops. SHP is necessary for sheath formation and is found not only in aphids but also in other hemipteran pests such as white flies, making it a candidate target with broad applicability (Freeman *et al.*, 2001). SHP is not found in nontarget and beneficial insects, which reduces the risk of off-target effects (Carolan *et al.*, 2009).

We have demonstrated that aphids feeding on transgenic barley plants expressing *shp*-dsRNA experienced potent *shp* silencing, which prevented the formation of normal sheathes and thus confirmed the pivotal role of SHP in the structural integrity of the salivary sheath (Carolan *et al.*, 2009). The

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Table 1 Transgenerational effects of *shp* silencing on seven successive aphid generations.

Line	Maturation time (mean)/days						
	1st generation	2nd generation	3rd generation	4th generation	5th generation	6th generation	7th generation
Wild type	8.5 ± 0.0	8.5 ± 0.0	8.5 ± 0.0	8.5 ± 0.0	8.5 ± 0.0	8.5 ± 0.0	8.5 ± 0.0
Empty vector	8.5 ± 0.0	8.5 ± 0.0	8.5 ± 0.0	8.5 ± 0.0	8.5 ± 0.0	8.5 ± 0.0	8.5 ± 0.0
shp-dsRNA L26	15.5 ± 0.7**	15 ± 0.7**	13.75 ± 1.7**	14.25 ± 2.4*	12.75 ± 2.0	11 ± 1.4	9.25 ± 0.3

Maturation time: delayed maturation (mean values ± SDs) was observed in 50 nymphs fed on *shp*-dsRNA L26 through seven generations. Until the sixth-generation nymphs matured after 11–15 day compared to 8–9 days for nymphs fed on wt plants. Maturation time in the aphid with background feeding on *shp*-dsRNA compared to feeding on empty-vector and wild-type controls was statistically significant (**$P < 0.01$, *$P < 0.05$ Student's *t*-test).

Table 2 Transgenerational effects of *shp* silencing on seven successive aphid generations.

Line	Winged adult (proportion and percentage)						
	1st generation (%)	2nd generation (%)	3rd generation (%)	4th generation (%)	5th generation (%)	6th generation (%)	7th generation (%)
Wild type	3.5 ± 1/50 (7)	4.5 ± 2/50 (9)	4.0 ± 2/50 (8)	2.0 ± 2/50 (4)	2.0 ± 1/50 (4)	7.5 ± 2/50 (15)	4.5 ± 2/50 (9)
Empty vector	5.0 ± 1/50 (10)	3.5 ± 0.7/50 (7)	4.5 ± 3/50 (9)	7.5 ± 3/50 (15)	9.0 ± 1/50 (18)	5.5 ± 2/50 (11)	8.0 ± 1/50 (16)
shp-dsRNA L26	40 ± 1/50** (80)	35 ± 2/50** (70)	34 ± 4/50* (68)	34.5 ± 4/50* (69)	35 ± 4/50* (70)	25 ± 4/50* (50)	16 ± 4/50 (32)

Winged adults: wing development (mean values ± SDs) was observed in 50 nymphs until maturation through seven generations. Until the sixth generation, 80–50% of nymphs fed on *shp*-dsRNA L26 developed wings compared to 7–15% for nymphs fed on wild-type control plants. There was a statistically significant difference in the development of wings when the first generation of aphids were initially fed on the *shp*-dsRNA line compared to empty-vector and wild-type controls (**$P < 0.01$, *$P < 0.05$, *t*-test).

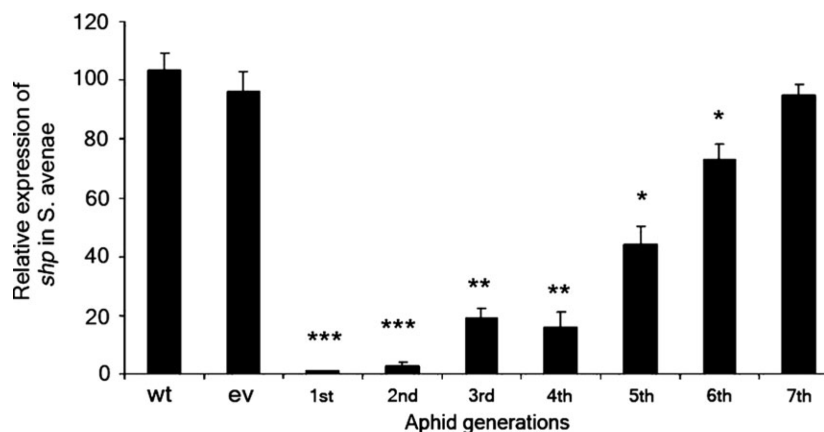

Figure 5 Transgenerational effect of *shp* silencing. Aphids were fed for two weeks on *shp*-dsRNA L26 and empty-vector (ev) and wild-type (wt) controls and subsequently allowed to reproduce on wt. The *shp* transcript level was determined in seven successive aphid generations. The *shp* transcript levels in aphids initially fed on the *shp*-dsRNA lines were significantly lower than those fed on ev and wt controls (***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, *t*-test). Each generation included 50 aphids. Bars represent mean values ± SDs of 10 single animals, and three replicates were conducted.

disruption of sheath formation prevented normal food uptake, and thus prolonged development, produced aphids with a smaller body plan area and reduced fecundity and survival. In addition to these direct effects of *shp* silencing, we observed indirect yet potent transgenerational effects lasting at least seven generations. These effects included prolonged development and a higher percentage of winged aphids (alatae), a morph type that correlates with reduced parental nutrition (Braendle *et al.*, 2006).

The induction of RNAi by the microinjection of siRNA or dsRNA was shown to cause silencing that lasted up to 7 days in *A. pisum* (Jaubert-Possamai *et al.*, 2007), 25 days in the honeybee *Apis mellifera* (Amdam *et al.*, 2003) and more than 6 months in the beetle *Tribolium castaneum* (Miller *et al.*, 2012). In the latter case, there was a positive correlation between the amount of dsRNA and the duration of the silencing effect. Silencing could be achieved by continuous feeding with either single-stranded or double-stranded siRNAs (Bhatia *et al.*, 2012). We found that *shp* silencing by HIGS lasts for more than 2 weeks when aphids were transferred onto wild-type plants after feeding on *shp*-dsRNA lines. This suggests that long-term feeding could potentially cause the accumulation of new inhibitory RNA species, as observed for *T. castaneum* (Miller *et al.*, 2012).

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In insects, parental RNAi was first reported as the delivery of dsRNA to the mother and the induction of RNAi in the offspring in *T. castaneum* (Bucher *et al.*, 2002) and *Locusta migratoria* (He *et al.*, 2006). We found that the parental transfer of RNAi to offspring also occurs in *S. avenae* but lasts for seven generations, at least when the target is *shp*. We observed a slow but continuous weakening of *shp* silencing over successive generations, accompanied by a decreasing percentage of alataes and prolonged development in the offspring, which appears to be directly induced by reduced nutritional uptake in the nymphs.

Bucher *et al.* (2002) speculated that parental RNAi could be caused either by a specific cellular uptake mechanism for dsRNA or secondary amplification of small amounts of incidentally incorporated dsRNA. The transfer of siRNA/dsRNA to following generations could in aphids be facilitated by the phenomenon of telescoping generations, meaning that a parthenogenetically adult already carries its developing grandchildren in it. The long-lasting silencing of *shp* in *S. avenae* cannot adequately be explained by the uptake of dsRNA into the ovaries resulting in the delivery of dsRNA or siRNA to the developing embryos. More likely, the observed transgenerational effects reflect an attenuated amplification process, in which the total amount of siRNA must decline in successive generations. However, we cannot exclude the possibility that long-term transgenerational silencing of *shp* reflects a direct interaction between the delivered inhibitory RNA and the aphid's transcriptional machinery, resulting in an epigenetic modification directed by small RNAs (Castel and Martienssen, 2013).

We have demonstrated that HIGS targeting the structural sheath protein in aphids is a powerful strategy for aphid control. Direct effects on adults and indirect effects on the offspring are induced by the inhibition of sheath formation as well as a long-term parental RNAi effect, and this should lead to a significant reduction of plant infestation. The high percentage of alataes among the offspring could result in the emigration of a large proportion of the offspring in subsequent generations, but the parental RNAi effect will limit the effectiveness of infestations caused by those migrating aphids, that is population growth will be delayed compared to aphids fed on wild-type plants. The presence of SHP in all aphid species studied thus far (Rao *et al.*, 2013) makes it unlikely that population decline in one aphid species will lead to the settlement of a new aphid species. Our results demonstrate the substantial potential of HIGS as an alternative or complementary strategy for insect pest control in agriculture.

Experimental procedures

Maintenance of plants and aphids

Barley (*Hordeum vulgare*) cv. Golden Promise (GP) and all transgenic GP plants were grown in a climate chamber with a 16-h photoperiod (260 μmol/m^2/s) at 18 °C/14 °C (light/dark) with 65% relative humidity. Grain aphids (*S. avenae*) were reared on three-week-old barley plants in a climate chamber. To obtain synchronized insects, reproductive mature aphids were placed in clip cages (one aphid per cage) on GP plants for 24 h. The adults were then removed, and the offspring were used for experiments as previously described (Gaupels *et al.*, 2008; Schmitz *et al.*, 2012). All experiments were conducted in a climate cabinet under the conditions stated above.

Construction of *shp* templates and generation of transgenic barley plants

For the constitutive overexpression of *shp*-dsRNA in barley, a 491-bp cDNA template fragment (Figure S1) from the 3621-bp *Acyrthosiphon pisum* (Ap) *shp* cDNA (XM_001943863, ACYPI009881) was amplified using primers Ap-HindII_F and Ap-AflII_R (Table S1) and inserted into the binary RNAi vector p7i-Ubi-RNAi2 (DNA Cloning Service, Hamburg, Germany) to replace the *GUS* gene. The resulting vector (p7i-Ubi-*shp*-RNAi), containing the *shp* fragment under the control of inverted plant ubiquitin (*ubi*) promoters, was transferred by electroporation into *Agrobacterium tumefaciens* strain AGL1 (Lazo *et al.*, 1991), which was used for the transformation of immature barley embryos as described (Imani *et al.*, 2011; Kogel *et al.*, 2010). PCR analysis was used to confirm integration of the transferred DNA using primers *shp*-RNA_F and *shp*-RNA_R. Primers 35sP_F and Hyg_R were used to identify the empty-vector (ev) lines that contained p7i-Ubi-RNAi2 (Table S1).

RNA extraction and qRT-PCR analysis

The expression of *shp*-dsRNA was analysed by qRT-PCR following the isolation of RNA from barley leaves using TRIzol® reagent (Ambion, Carlsbad, CA, USA) according to the manufacturer's instructions. Freshly extracted mRNA from 10 aphids was converted into cDNA using the QuantiTect® Reverse Transcription Kit (Qiagen, Hilden, Germany) and 40 ng of cDNA was used as the template for qRT-PCR in an Applied Biosystems 7500 FAST real-time PCR system. Each reaction comprised 7.5 μL SYBER Green JumpStart Taq ReadyMix (Sigma-Aldrich, Steinheim, Germany) and 0.5 pmol of the gene-specific primers *shp*-RNA-qpcr-F1 and *shp*-RNA-qpcr-R1 (Table S1). After initial heating to 95 °C for 5 min, the target was amplified by 40 cycles at 95 °C for 30 s, 52 °C for 30 s and 72 °C for 30 s. Ct values were determined using 7500 Fast software (Applied Biosystems, Darmstadt, Germany). The *shp*-dsRNA transcript levels were determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) by normalizing against barley ubiquitin mRNA (GenBank M60175). The *shp* transcript levels in aphids were determined using the same primers but normalizing against 18*S* ribosomal RNA (GenBank APU27819).

Electrical penetration graph (EPG) technique

Aphid feeding behaviour was monitored using the electrical penetration graph (EPG) technique (Tjallingii, 1988). A 1 cm × 20 μm gold wire was attached to the dorsal abdomen of apterous aphids, 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 amplifier (Tjallingii, 1978, 1988), and the EPG output was recorded with stylet hardware and software (EPG Systems, Wageningen, the Netherlands). A second electrode was inserted into the soil of potted plants. The experimental set-up was placed in a Faraday cage to shield it from electromagnetic interference. Aphids starved for 3–4 h were placed onto the abaxial side of a mature leaf of a 3-week-old barley plant. EPG recordings commenced immediately and continued for 1 h while behaviour was observed continuously. Only aphids that secreted gel saliva during the observation period were used for *shp* expression analysis by qRT-PCR. If the aphid behaviour differed from gel saliva secretion during the observation period, the

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aphids were forced to withdraw their stylets penetrate the plant again. EPG waveforms were interpreted as described by Prado and Tjallingii (1994).

Aphid salivary sheath formation on the artificial diet

An artificial diet that mimics the plant cell wall milieu (20 mM KCl, 1 mM $CaCl_2$, 10 mM MES, pH 5.5) as described by Cosgrove and Cleland (1983) was used to stimulate the secretion of gel saliva. The food was filter sterilized through a 0.45-μm PVDF membrane, and 150 μL was placed in a parafilm sachet (Verheggen *et al.*, 2009) sterilized with 30% H_2O_2 for approximately 30 min prior to use. After feeding for 3 weeks on the *shp*-dsRNA and control lines, a single aphid was placed on a diet sachet and 10 single aphids were observed per plant line. The diet sachet was placed downwards on a small aphid cage, and aphids were allowed to feed for 48 h. Parafilm foils pierced by the aphids were placed facing upwards in a Petri dish, and bright-field/fluorescence microscopy using an inverse microscope (Leica DMLB; Leica Microsystems, Mannheim, Germany) was used to locate the salivary sheaths. Three replicates (10 aphids each) were prepared for each treatment, and 10 randomly chosen salivary sheaths were observed in each replicate. ImageJ v1.42q (Wayne Rosband; National Institute of Health, Bethesda, Maryland) was used to measure the area of the randomly selected salivary sheaths on parafilm, and data of aphids from *shp*-dsRNA plants were compared with controls using *t*-test.

Evaluation of aphid fitness parameters after feeding on *shp*-dsRNA lines

Aphid survival ($n = 3$) and fecundity assays ($n = 3$) used for each replicate and a total of 10 synchronized mature aphids in individual clip cages placed on the abaxial side of leaves (one cage per leaf) from intact barley plants. Parameters were checked daily throughout the aphid lifespan. Survival was analysed using the Kaplan–Meier log-rank test (Kaplan and Meier, 1985) in Sigma Plot v11. For the analysis of nymph development and growth ($n = 3$), 10 synchronous one-day-old nymphs born on wild-type barley plants were segregated into individual clip cages on *shp*-dsRNA and control lines. Nymph development was recorded after 8 days and scored from 1 to 5 according to the developmental stages listed by Chau and Mackauer (2000). Growth was measured by determining the body plan area (Daniels *et al.*, 2009) after nymphs reached maturity. For this purpose, images of individual aphids were captured using a Leica MZ16FA (Leica Microsystems) and analysed using ImageJ v1.42q. Obtained data for reproduction and body plan area were compared with *t*-test.

Permanency and transgenerational analysis of *shp* silencing

Recovery from *shp* silencing was observed in 10 aphids that fed for 2 weeks on *shp*-dsRNA plants before switching to wild-type plants for a further 1–2 weeks. The *t*-test was applied for the comparison of normalized $2^{-\Delta\Delta Ct}$ values obtained from aphids that fed on *shp*-dsRNA plants with aphids that were feeding on control plant lines. Transgenerational *shp* silencing was investigated by feeding 50 synchronized aphids on *shp*-dsRNA plants for 2 weeks and again transferring them to wild-type plants. Offspring produced over the next 24 h were maintained on wild-type plants until maturity and the birth of the next-generation nymphs. The 50 adults were then used for transcript analysis. Each time, aphids were allocated to 3 individual plants. Controls were treated in a similar way without incubation on *shp*-dsRNA plants. We assessed the maturation time, frequency of winged aphids and *shp* expression levels until the seventh generation whereas each single aphid was considered as an experimental unit. Data from *shp*-dsRNA were compared with control lines for each individual generation by *t*-test. Both experiments were repeated three times.

Acknowledgements

We thank Dr. Edgar Schliephake (Julius Kuehn Institute—Federal Research Centre for Cultivated Plants, Institute for Resistance Research and Stress Tolerance) for the starter colony of *S. avenae*. We thank Marlène Sophie Birk for her assistance with experiments and data acquisition. This work was supported by the Landes-Offensive zur Entwicklung Wissenschaftlich-ökonomischer Exzellenz (LOEWE) programme (project Insect Biotechnology) of the Hessian State, Germany, to T.W., A.V. and K.-H.K., the German Research Council (Deutsche Forschungsgemeinschaft, DFG) (to K.-H.K.) and Deutscher Akademischer Austauschdienst (DAAD) to E.A. The authors thank Richard M. Twyman for valuable comments concerning manuscript editing.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 Partial sequences of the *shp* gene.
Figure S2 Quantification of *shp*-dsRNA expression in leaves of transgenic barley.
Figure S3 Fitness analysis of aphids fed on *shp*-dsRNA barley lines L28, L33 and empty-vector (ev) and wild-type (wt) control lines.
Table S1 List of primers used for PCR and qRT-PCR analysis.

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