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High expression of a unique aphid protein in the salivary glands of *Acyrthosiphon pisum*☆

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ABSTRACT

Salivary proteins play key roles in the co-evolution of aphids and their host plants. This study characterizes the aphid-specific and small secretory protein ACYPI006346 in the pea aphid *Acyrthosiphon pisum*. The *ACYPI006346* transcript is enriched in the salivary glands, particularly in cell types 5 and 7 of the principal salivary glands. Transcript knockdown by dsRNA injection, which lasts for 24 h does not influence the survival of aphids on plants. The transcript levels of *ACYPI006346* were different in three pea aphid colonies adapting to *Vicia faba*, *Vicia villosa*, or *Medicago truncatula*, respectively.

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Introduction

Aphids are a unique group of insects because of their special piercing–sucking feeding behavior. Aphids use modified mouth parts called stylets to collect phloem sap from their host plants. During this process, substance exchange occurs between aphids and their hosts [1]. Aphids obtain carbohydrates, proteins, and nucleic acids from the phloem sap while secreting saliva into the host tissues [2]. Saliva contains bioactive compounds, particularly proteins, which can modulate, suppress, or circumvent plant defenses [3,4]. The composition of saliva proteins differs in different aphids and when aphids are fed on different plants [5,6], indicating that saliva proteins play key roles in the co-evolution of aphids and their host plants. Most salivary proteins of aphids lack homologs in other organisms and co-evolve in aphids at a fast rate [7]. Understanding the biochemical nature and physiological function of salivary proteins is crucial to produce plants that are resistant or tolerant to aphids.

Many saliva proteins have already been identified in aphids. Some of these saliva proteins are enzymes, such as glucose oxidase, glucose dehydrogenase, NADH dehydrogenase, α-glucosidase, α-amylase, angiotensin-converting enzyme, M1 zinc-dependent metalloprotease, and glucose-methanol-choline-oxidoreductase [8–10]. Salivary proteins with unknown functions are also important for aphid feeding; Protein C002 is an example of such proteins. Interference of *C002* expression suppresses phloem passive ingestion and thus increases the death ratio of the pea aphid *Acyrthosiphon pisum* [11,12]. Salivary proteins influence aphid reproduction. The Mp55 protein of the green peach aphid *Myzus persicae* increases aphid reproduction by decreasing the amounts of 4-methoxyindol-3-ylmethylglucosinolate, callose, and hydrogen peroxide in *Arabidopsis thaliana* [13]. MpC002 promotes *M. persicae* colonization on *Nicotiana benthamiana* and *A. thaliana* [14]. Me10 and Me23 of the potato aphid *Macrosiphum euphorbiae* also enhance aphid fecundity on *N. benthamiana* [15]. However, some salivary proteins are deleterious to aphid reproduction. Mp10 and Mp42 of *M. persicae* trigger a defense response in *N. benthamiana* and decrease aphid reproduction [16]. Mp10 also suppresses flg22-induced oxidative burst [17].

Despite recent progress in identifying saliva proteins, only a few studies have focused on the function or characteristics of the salivary proteins within insects. In addition to Protein C002, the salivary protein ACYPI39568 has been analyzed for its physical and chemical characteristics in *A. pisum*. ACYPI39568 is a zinc-binding protein of the aphid-specific cysteine-rich protein family [18]. A calcium-binding protein, Armet, is detected in *A. pisum* watery saliva and in the phloem sap of fava beans fed on by aphids. Knockdown of the *Armet* transcript disturbs aphid feeding behavior and leads to a shortened life-span on fava beans [19]. Angiotensin-converting enzyme 1 (ACE1) has been detected in *A. pisum* saliva [10], but knockdown of *ACE1* transcript fails to affect aphid survival [20]. When *ACE1* and *ACE2* (another ACE gene of *A. pisum*) are simultaneously knocked down, aphid feeding is enhanced but

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aphid mortality rate on plants increases [20]. Protein C002, ACYPI39568, Armet, and ACE1 belong to the 42 salivary gland enriched proteins in *A. pisum*, 32 of which are non-annotatable [7]. In the present study, we characterized the non-annotatable salivary protein ACYPI006346 for its expression pattern in development and in different tissues of *A. pisum*. ACYPI006346 is the sixth protein on the top of the 42 salivary gland-enriched proteins in *A. pisum*. That "ranking" is of extent of enrichment of the transcript in salivary glands compared to whole body [7]. The potential function of ACYPI006346 in aphid–plant interaction is explored in this paper.

Materials and methods

Insect rearing

The laboratory colony of *A. pisum* originated from the offspring of several parthenogenetic female aphids from a field population collected from the pea *Pisum sativum* at Yuxi (102° 55′ E, 24° 35′ N), Yunnan, China, in 2010. Since then, this colony has been reared on the fava bean *Vicia faba* at the laboratory and named YYC [19]. Some *V. faba* colony aphids were transferred to and reared on *Vicia villosa* and *Medicago truncatula* cv. A17 to form two stable colonies. All plants and aphids were grown in environmental chambers at 21 ± 1 °C with a photoperiod of 16 h: 8 h (light: dark) and a relative humidity of 60% ± 5%.

RNA extraction and gene cloning

Total RNA was extracted from the whole bodies and different tissues of aphid using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. After removing DNA contamination by using the TURBO DNA-free kit (Ambion, Austin, TX, USA), 1 μg of RNA was reverse transcribed into cDNA using M-MLV reverse transcriptase (Promega, Madison, Wisconsin, USA) in accordance with the manufacturers' protocol. A pair of specific primers, 5′-ATGACGGTTAAAATTTCTGT-3′ and 5′-AATAATTCAC-GATCGATG-3′, was designed to amplify the open reading frame (ORF) of *A. pisum ACYPI006346* on the basis of its sequence registered in GenBank (AK341542) with reverse transcription PCR (RT-PCR). RT-PCR was performed using the Mastercycler thermal cycler (Eppendorf, Hamburg, Germany) with cycling conditions of 95 °C for 5 min, 35 cycles of 95 °C for 35 s, 57 °C for 30 s, and 72 °C for 45 s, followed by 72 °C for 10 min. The PCR product was confirmed by sequencing.

Protein sequence analysis of ACYPI006346

The ORF of *ACYPI006346* was translated to its amino acid sequence in http://web.expasy.org/translate. Signal peptide and transmembrane domain were predicted by using SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/) and TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM/), respectively. The molecular weight and theoretical pI were computed through the ExPAsy ProtParam tool (http://web.expasy.org/protparam/). Possible glycosylation sites were searched in http://www.cbs.dtu.dk/services/NetOGlyc. Secondary structure was predicted in https://www.predictprotein.org/. Homologous proteins of ACYPI006346 from other species were identified with BLASTP software at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and aligned using ClustalW at EBI (http://www.ebi.ac.uk/Tools/msa/clustalw2/). A phylogenetic tree was constructed with the maximum likelihood method under the Jones–Taylor–Thornton model using the MEGA 6.0 software. Bootstrap analysis (1000 replicates) was carried out to evaluate the internal support of the tree topology.

Temporal–spatial expression analysis of ACYPI006346

First to the fourth instar larvae and adults were collected for RNA extraction. The expression of *ACYPI006346* was measured in each developmental stage using real-time quantitative PCR (qPCR). Six biological replicates and five individuals per replicate were prepared for each sample. Differences among the developmental stages were analyzed by using one-way ANOVA, followed by a Tukey's test for multiple comparisons with SPSS 17.0 software.

Brain, salivary glands, whole gut, and ovaries from the fourth instar larvae were dissected in 0.9% normal saline and used for RNA extraction. Expression of *ACYPI006346* was measured in each tissue by qPCR. Six biological replicates and 20 tissues per replicate were prepared. Differences in *ACYPI006346* expression among the four tissues were evaluated by Tamhane's T2 test in one-way ANOVA by using SPSS 17.0 after log transformation of the relative transcript levels.

Expression analysis of ACYPI006346 in three aphid colonies

Adult aphids from *V. faba*, *V. villosa*, and *M. truncatula* colonies were collected for RNA extraction. The expression of *ACYPI006346* was measured by using qPCR. Six biological replicates and five adults per replicate were prepared for each sample. The relative transcript levels of *ACYPI006346* in the *V. villosa* and *M. truncatula* colonies were compared with those in the *V. faba* colony, and the differences were evaluated by *t*-test by using SPSS 17.0.

qPCR

The expression of *ACYPI006346* was quantified by qPCR using the SuperReal PreMix plus SYBR Green Kit (TIANGEN, Beijing, China). The primers 5′-GAAGTGTCTACAACTACCATAC-3′ and 5′-GAATGTCGTCGTCTACTGT-3′ were designed to amplify a 200 bp fragment of *ACYPI006346*. A 108 bp fragment of the *A. pisum* ribosomal protein *L27* gene (CN584974) was amplified as an internal control with primers 5′-TCGTTACCCTCGGAAAGTC-3′ and 5′-GTTGGCATAAGGTGGTTGT-3′. The qPCR was carried out on a Roche Light Cycler 480 (Roche, Mannheim, Germany). The thermal cycling steps were 95 °C for 2 min, followed by 40 cycles of 95 °C for 20 s, 58 °C for 20 s, and 68 °C for 40 s. The melting curve was collected in the end of PCR with one cycle of 95 °C for 30 s, 58 °C for 30 s, and 95 °C for 10 s. The PCR products were sequenced to confirm the identity of the amplified gene. The relative transcript level of *ACYPI006346* compared with that of the *L27* gene was reported as the mean ± SEM.

Immunohistochemistry

Localization of the ACYPI006346 protein in *A. pisum* salivary glands was visualized by immunohistochemistry as described by Mutti et al. [12]. The primary antibody was the purified rabbit polyclonal anti-ACYPI006346 antibody, which was made from Escherichia coli-expressed full-length ACYPI006346 (Beijing Protein Innovation, Beijing, China), and was used at 1:100 dilution. The secondary antibody was Alexa fluor 488 (Life Technology, Carlsbad, CA, USA) at 1:1000 dilution. Cell nuclei were stained by using Hoechest 33258 (Sigma–Aldrich, Saint Louis, MO, USA) at 1:500 dilution. Images were captured by a Zeiss LSM 710 Confocal Microscope (Zeiss, Oberkochen, Germany).

Knockdown of transcript ACYPI006346

A 197 bp fragment in the ORF of *ACYPI006346* was cloned as the template for the synthesis of double-stranded RNA (dsRNA) using

the primers 5′-TCGTTCTGATTGTGACTTGT-3′ and 5′-CGTATGGTAGTTGTAGACACT-3′. The 420 bp fragment of the green fluorescent protein (GFP) gene was used as a negative control after amplification with the primers 5′-CACAAGTTCAGCGTGTCCG-3′ and 5′-GTTCACCTTGATGCCGTTC-3′. DsRNA was synthesized by using the T7 RiboMAX Express RNAi system (Promega) and then purified with Wizard SV Gel and PCR Clean-Up System (Promega) in accordance with the manufacturer's protocol. Third-instar aphids were injected into the abdomen through a glass needle by using Nanoliter 2000 (World Precision Instruments, Sarasota, FL, USA). For each aphid, 23 nL of dsRNA at 9 μg/μL was injected, and then the aphids were raised on *V. faba* leaves. After 1 d and 3 d of injection, the heads, containing salivary glands, were used to detect gene interference efficiency by using qPCR through comparison between the dsACYPI006346-RNA and dsGFP-RNA injection groups. Six biological replicates and five heads per replicate were prepared for each group. Differences were statistically estimated by *t*-test using SPSS 17.0. Survivals of aphids were recorded every 24 h after the dsACYPI006346-RNA or dsGFP-RNA injection. Survival curves of knockdown and control groups were statistically compared with the Kaplan–Meier method by using the Log Rank (Mantel–Cox) test in SPSS 17.0 within 7 d after injection. Six replicates and 15 individuals per replicate were used in each group. Values are presented as mean ± SEM.

Results

Sequence characteristics of ACYPI006346

ACYPI006346 is a single-copy gene on the genome of *A. pisum* [21]. The ORF of *ACYPI006346* is 426 bp, encoding a 16.2 kD protein. One intron of 428 bp partitions the gene into two exons of 36 and 390 bp, respectively. A secretory signal peptide with the cleavage site between amino acid residues 19 and 20 was predicted at the N-terminal of the protein (Fig. 1A) using SignalP 4.1 and TMHMM 2.0, and no transmembrane region exists, indicating that ACYPI006346 is secreted. ProtParam predicted the molecular weight of the mature protein as 14.1 kD with a theoretical pI of 5.8. An O-glycosylation site possibly exists at Thr28 (Fig. 1A) based on NetOGlyc analysis. The secondary structure of ACYPI006346 was predicted to contain 50% helix, 37% loop, and 13% strand by PredictProtein server. ACYPI006346 has homologs within aphids, such as *Acyrthosiphum kondoi* (FO005338), *M. persicae* (EE571823), *Myzus ascalonicus* (FO037272), *Rhopalosiphum padi* (FO070159), *Rhopalosiphum maidis* (FQ990319), *Toxoptera citricida* (CB833235), and *Aphis gossypii* (GW571758), indicating that ACYPI006346 is an aphid-specific protein. The homologs within the same genus of aphids are closest to each other in phylogeny (Fig. 1B).

Temporal–spatial expression patterns of ACYPI006346

The expression patterns of *ACYPI006346* along development and in four organs of the fourth instar larvae are shown in Fig. 2. The expression of *ACYPI006346* reduced from the first instar to the third instar larvae and then increased until it reached a peak at the adult stage, when the expression level was 29.4% higher than the first instar and 79.2% higher than the third instar stage (Fig. 2A). *ACYPI006346* was predominantly expressed in the salivary glands, in which the transcript level was approximately 9-fold higher than in the brain, 240-fold higher than in the gut, and 1260-fold higher than in the ovaries (Fig. 2B). The principal salivary glands of aphids contain eight types of secretory cells [22]. Immunohistochemistry using the anti-ACYPI006346 polyclonal antibody showed that *ACYPI006346* was principally expressed in cell types 5 and 7 of the principal salivary glands (Fig. 2C).

Interference efficiency and effect on aphid survival

ACYPI006346 is a protein of unknown function. We used RNA interference (RNAi) to investigate the possible effect of ACYPI006346 on aphid survival on *V. faba*. The dsRNA of *ACYPI006346* was synthesized in vitro and micro-injected into the aphid abdomen. At 1 d after injection, the transcript level of *ACYPI006346* decreased by 47% ($P = 0.039$) in the heads containing the salivary glands of aphids (Fig. 3A). However, no significant decrease in *ACYPI006346* transcript was observed in the interference group at 3 d after the injection, indicating recovery from dsRNA-based interference (Fig. 3A). No significant difference was observed between the survival curves of the dsACYPI006346-injected and the control dsGFP-injected aphids on *V. faba* within 7 d after injection ($P = 0.22$, Fig. 3B). This result indicates that the 24 h of gene expression interference had no significant effect on aphid survival on plants.

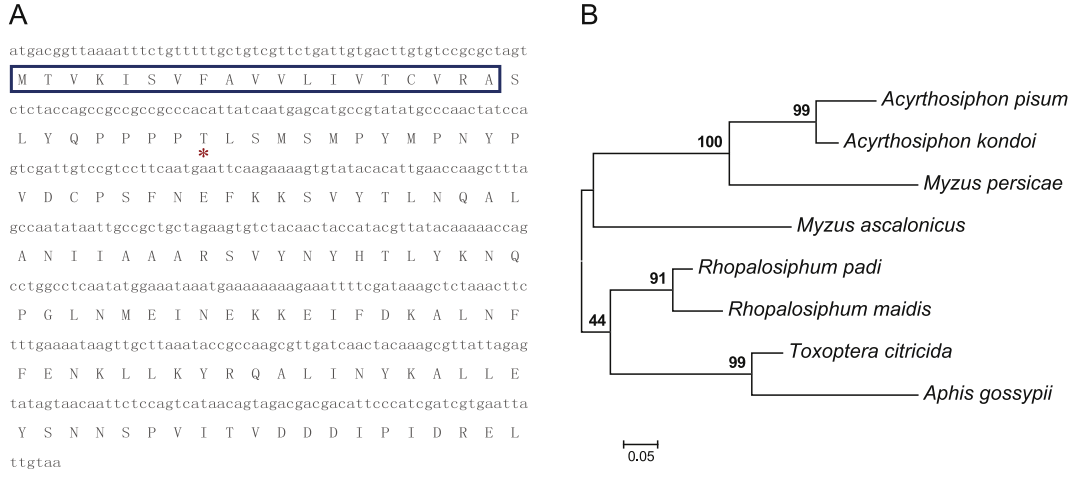

Fig. 1. Sequence characteristics of ACYPI006346. (A) The open reading frame and protein sequence of *ACYPI006346*. The secretory signal peptide is boxed. The predicted O-glycosylation site is marked with an asterisk. (B) The phylogenetic tree of ACYPI006346 and its homologs from other aphids constructed with the maximum likelihood method under the Jones–Taylor–Thornton model. Bootstrap supporting values (1000 replicates) are shown at the branch nodes.

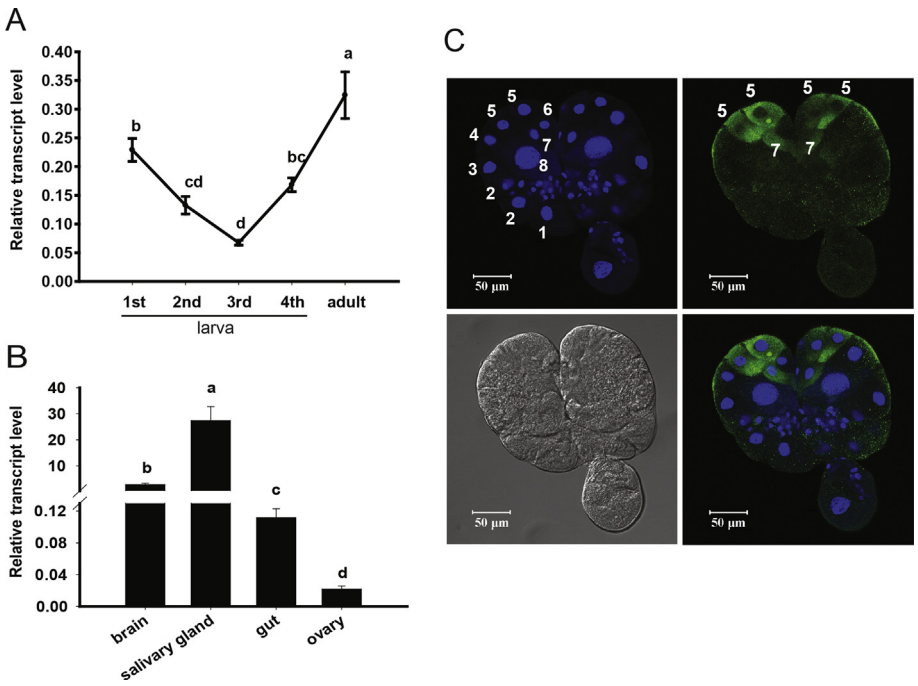

Fig. 2. Temporal–spatial expression pattern of ACYPI006346. Relative transcript level of *ACYPI006346* along developmental stages (A) and in four tissues (B) measured with real-time quantitative PCR. The transcript level of *Acyrthosiphon pisum* ribosomal protein *L27* was used as an internal control. The relative ratio of the two genes was reported as mean ± SEM. Differences among developmental stages were analyzed using one-way ANOVA, followed by a Tukey's test. Differences among the four tissues were evaluated by Tamhane's T2 test in one-way ANOVA after log transformation of the relative transcript levels. (C) Immunohistochemical location of ACYPI006346 in the salivary glands of aphids. Top left, cell nuclei stained in blue with Hoechest 33258. Numbers near the cells delegate the cell type. Top right, green positive signal of anti-ACYPI006346 polyclonal antibody staining. Down left, differential interference contrast image to show the configuration of salivary gland. Down right, the merged image of cell nuclei staining and positive signal of anti-ACYPI006346 polyclonal antibody staining.

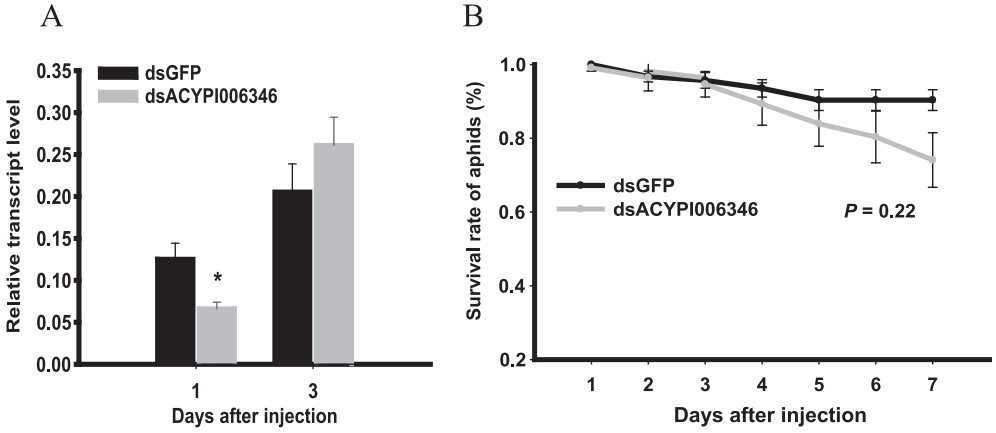

Fig. 3. Gene expression interference of ACYPI006346 by dsRNA injection and effect on aphid survival. (A) Knockdown efficiency measured by real-time quantitative PCR within 3 d after dsRNA injection. The transcript level of *Acyrthosiphon pisum* ribosomal protein *L27* was used as an internal control. The relative ratio of *ACYPI006346* and *L27* was reported as mean ± SEM. Differences were statistically estimated by *t*-test. *, $P < 0.05$. (B) The survival curves of *A. pisum* after dsACYPI006346-RNA or dsGFP-RNA injection. Values were presented as mean ± SEM. The survival curves were statistically compared with the Kaplan–Meier method using the Log Rank (Mantel–Cox) test.

Different response of ACYPI006346 to host plants

The transcript levels of *ACYPI006346* in the three *A. pisum* colonies adapted to *V. faba*, *M. truncatula*, and *V. villosa* were measured by qPCR. The *V. villosa* colony had the highest transcript level, approximately 13-fold greater than that of the *V. faba* colony, followed by the *M. truncatula* colony, about 5-fold greater than that of the *V. faba* colony (Fig. 4). The result indicates that *A. pisum* requires different quantities of ACYPI006346 during adaptation to different host plants.

Discussion

Salivary proteins play key roles in aphid–plant interaction. Aside from identifying salivary proteins, analyzing the gene expression and proteins within the insect is indispensable in understanding their function as effector proteins to manipulate aphid–plant interaction. In this study, we characterized a protein of unknown function ACYPI006346 whose transcript was previously shown to be enriched in the salivary glands of *A. pisum* [7]. We found that ACYPI006346 is an aphid-specific protein, whose

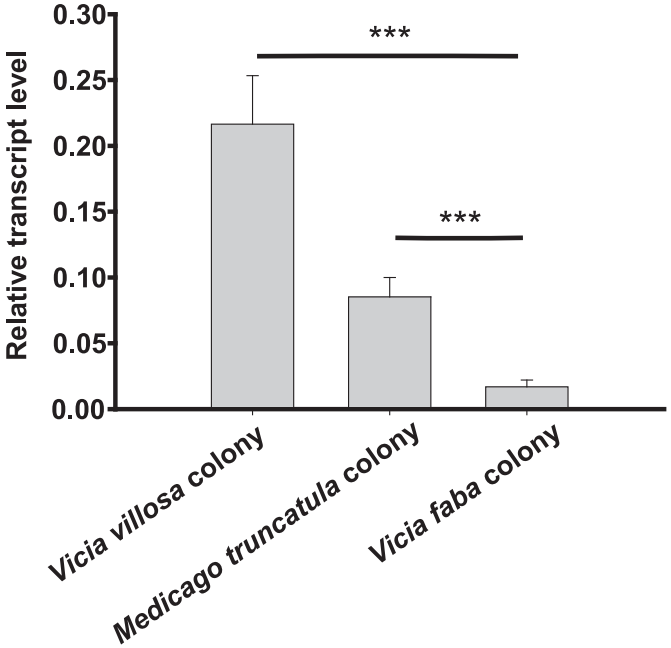

Fig. 4. Relative transcript levels of *ACYPI006346* in three *Acyrthosiphon pisum* colonies adapted to *Vicia faba*, *Medicago truncatula*, or *Vicia villosa* measured with real-time quantitative PCR. The transcript level of *A. pisum* ribosomal protein *L27* was used as an internal control. The relative ratio of *ACYPI006346* and *L27* was reported as mean ± SEM. The relative transcript levels of *ACYPI006346* in the *V. villosa* and *M. truncatula* colonies were compared with that in the *V. faba* colony, respectively, and the differences were evaluated by *t*-test. ***, $P < 0.001$.

transcript is more abundant in the salivary glands than in other tissues. Furthermore, *ACYPI006346* is only expressed in specific secretory cells of the salivary glands. When adapted to different host plants, aphids require different amounts of ACYPI006346, suggesting that this protein plays a role in aphid–plant interaction.

The salivary glands of aphids consist of two principal glands and two accessory glands. The principal gland is a symmetrical and bi-lobed organ. The principal gland in *M. persicae* contains eight cell types that are morphologically different and may serve various functions [22]. Individual salivary proteins are probably secreted by specific secretory cells of salivary glands. Several prominent salivary proteins, namely, 66/69 and 154 kD proteins, are specifically expressed in the posterior part of the principal salivary glands in *Schizaphis graminum* [9]. Based on the cell types of *M. persicae* salivary glands, we summarized the reported expression location of several salivary proteins in *A. pisum* salivary glands. The ACYPI39568 protein is unevenly expressed in both primary and accessory glands but absent in the cell type 8 of the primary glands [18]. Protein C002 is located in cell types 3 and 4 [12], whereas ACYPI006346 is expressed in cell types 5 and 7. Another salivary protein, Armet, is predominantly expressed in cell type 8 [19]. Therefore, every secretory cell possibly produces different types of salivary proteins to undertake different functions within aphids or in the host plants.

The salivary proteins of aphids usually function as effectors to induce or overcome the defense reaction of plants. De Vos and Jander found that 3 kD to 10 kD protein components of *M. persicae* induce defense responses in *A. thaliana* [23]. A specific salivary protein of *M. persicae*, namely, Mp10, was later shown to specifically induce chlorosis and local cell death in *N. benthamiana* [17]. Salivary proteins can also suppress the production of abhorrent chemical compounds in plants upon aphid feeding. For example, Mp10 suppresses flg22-induced oxidative burst [17]. Whether or not ACYPI006346 induces or overcomes the defense reaction of plants as an effector protein remains unknown. However, the different transcriptional levels of *ACYPI006346* in the aphids adapted to different plants imply that this protein serves as an effector that mediates aphid–plant interaction. The three *A. pisum* colonies have different fitness levels on their respective host plants. The *V. faba* colony has the best fitness and the largest body size, whereas the *V. villosa* colony has the worst fitness and the smallest body size (unpublished data). Interestingly, the *V. villosa* colony has the highest *ACYPI006346* expression, whereas the *V. faba* colony has the lowest *ACYPI006346* expression. The alternative hypothesis would be that the colonies expressed different quantities of ACYPI006346 because of random genetic drift. The present study is rather inconclusive and further research on the effector function of ACYPI006346 is underway in our laboratory.

RNAi sabotages the gene expression in vivo by using an RNA sequence that is homologous to the targeted gene [24]. As the first hemipteran insect with an available genome, *A. pisum* shows an unexpected expansion of the microRNA pathway, containing two copies of *dicr-1* and *ago 1* genes and four copies of *pasha* a cofactor of *drosha* involved in microRNA biosynthesis [25]. The RNAi-mediated knockdown of *A. pisum* genes can be achieved by microinjecting dsRNAs or small-interfering RNAs, or by feeding the aphids on artificial diets that contain small RNAs [26,27]. Similar levels of gene silencing can be achieved in plant-mediated RNAi [28,29]. However, the knockdown effect is dependent on the administration method. The injection of dsRNA provides an efficient spread to all the tissues in aphids, whereas administration by feeding results in a clear gene knockdown in aphid guts [27]. The duration of gene expression knockdown is an important factor that influences the success of RNAi experiments. The best inhibition is observed from 72 h after the treatment in aphids [11,18,26,27,30]. However, the transcript level of *ACYPI006346* decreased to 47% at 24 h after the injection of dsRNA and then recovered to the normal level after 72 h in the present study. Shakesby et al. [31] reported a similar phenomenon, in which the *aquaporin* transcript was depressed by more than 2-fold within 24 h after dsRNA oral administration and then progressively increased over the subsequent 4 d [31]. The impaired potency of RNAi may come from the high abundance of the transcript *ACYPI006346*, one of the top 10 secretory-protein-encoding genes that are expressed in the salivary glands of *A. pisum* (unpublished data).

Acknowledgments

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References

[1] Elzinga DA, Jander D. The role of protein effectors in plant-aphid interactions. Plant Biol 2013;16:451–6.
[2] Douglas AE. Phloem-sap feeding by animals: problems and solutions. J Exp Bot 2006;57:747–54.
[3] Howe GA, Jander G. Plant immunity to insect herbivores. Annu Rev Plant Biol 2008;59:41–66.
[4] Miles PW. Aphid saliva. Biol Rev 1999;77:71–85.
[5] Cooper WR, Dillwith JW, Puterka GJ. Comparisons of salivary proteins from five aphid (Hemiptera: Aphididae) species. Environ Entomol 2011;40:151–6.
[6] Vandermoten S, Harmel N, Mazzucchelli G, De Pauw E, Haubruge E, Francis F. Comparative analyses of salivary proteins from three aphid species. Insect Mol Biol 2014;23:67–77.

[7] Carolan JC, Caragea D, Reardon KT, Mutti NS, Dittmer N, Pappan K, et al. Predicted effector molecules in the salivary secretome of the pea aphid (*Acyrthosiphon pisum*): a dual transcriptomic/proteomic approach. J Proteome Res 2011;10:1505–18.
[8] Harmel N, Létocart E, Cherqul A, Giordanengo P, Mazzucchelli G, Guillonneau F, et al. Identification of aphid salivary proteins: a proteomic investigation of *Myzus persicae*. Insect Mol Biol 2008;17:165–74.
[9] Cherqui A, Tjallingii WF. Salivary proteins of aphids, a pilot study on identification separation and immunolocalisation. J Insect Physiol 2000;46:1177–86.
[10] Carolan JC, Fitzroy CI, Ashton PD, Douglas AE, Wilkinson TL. The secreted salivary proteome of the pea aphid *Acyrthosiphon pisum* characterised by mass spectrometry. Proteomics 2009;9:2457–67.
[11] Mutti NS, Park Y, Reese JC, Reeck GR. RNAi knockdown of a salivary transcript leading to lethality in the pea aphid, *Acyrthosiphon pisum*. J Insect Sci 2006;6:1–7.
[12] Mutti NS, Louis J, Pappan L, Pappan K, Begum K, Chen MS, et al. A protein from the salivary glands of the pea aphid, *Acyrthosiphon pisum*, is essential in feeding on a host plant. Proc Natl Acad Sci U S A 2008;105:9965–9.
[13] Elzinga DA, De Vos M, Jander G. Suppression of plant defenses by a *Myzus persicae* (Green Peach Aphid) salivary effector protein. Mol Plant Microbe Interact 2014;27:747–56.
[14] Pitino M, Hogenhout SA. Aphid protein effectors promote aphid colonization in a plant species-specific manner. Mol Plant Microbe Interact 2012;26:130–9.
[15] Atamian HS, Chaudhary R, Cin VD, Bao E, Girke T, Kaloshian I. In planto expression or delivery of potato aphid *Macrosiphum euphorbiae* effectors Me10 and Me23 enhances aphid fecundity. Mol Plant Microbe Interact 2013;26:67–74.
[16] Rodriguez PA, Stam R, Warbroek T, Bos JI. Mp10 and Mp42 from the aphid species *Myzus persicae* trigger plant defenses in *Nicotiana benthamiana* through different activities. Mol Plant Microbe Interact 2014;27:30–9.
[17] Bos JI, Prince D, Pitino M, Maffei ME, Win J, Hogenhout SA. A functional genomics approach identifies candidate effectors from the aphid species *Myzus persicae* (Green Peach Aphid). PLoS Genet 2010;6:1–13.
[18] Guo K, Wang W, Luo L, Chen J, Guo Y, Cui F. Characterization of an aphid-specific, cysteine- rich protein enriched in salivary glands. Biophys Chem 2014;189:25–32.
[19] Wang W, Dai H, Zhang Y, Chandrasekar R, Luo L, Hiromasa Y, et al. Armet is an effector protein mediating aphid-plant interactions. Faseb J 2015;29. http://dx.doi.org/10.1096/fj.14–266023.
[20] Wang W, Luo L, Lu H, Chen S, Kang L, Cui F. Angiotensin-converting enzymes modulate aphid–plant interactions. Sci Rep 2015;5:8885.
[21] The International Aphid Genetics Consortium. Genome sequence of the pea aphid *Acyrthosiphon pisum*. PLoS Biol 2010;8:e1000313.
[22] Ponsen MB. The site of potato leafroll virus multiplication in its vector, *Myzus persicae*: an anatomical study. Wageningen, Netherlands: H. Veenman; 1972.
[23] De Vos M, Jander G. *Myzus persicae* (green peach aphid) salivary components induce defence responses in *Arabidopsis thaliana*. Plant Cell Environ 2009;32:1548–60.
[24] Meister G, Tuschl T. Mechanisms of gene silencing by double-stranded RNA. Nature 2004;431:343–9.
[25] Jaubert-Possamai S, Rispe C, Tanguy S, Gordon K, Walsh T, Edwards O, et al. Expansion of the miRNA pathway in the Hemipteran insect *Acyrthosiphon pisum*. Mol Biol Evol 2010;27:979–87.
[26] Jaubert-possamai S, Le Trionnaire G, Bonhomme J, Christophides GK, Rispe C, Taqu D. Gene knockdown by RNAi in the pea aphid *Acyrthosiphon pisum*. BMC Biotechnol 2007;7:1–8.
[27] Sapountzis P, Duport G, Balmand S, Gaqet K, Jaubert-Possamai S, Febvay G, et al. New insight into the RNA interference response against cathepsin-L gene in the pea aphid, *Acyrthosiphon pisum*: molting or gut phenotypes specifically induced by injection or feeding treatments. Insect Biochem Mol Biol 2014;51:20–32.
[28] Pitino M, Coleman AD, Maffel ME, Ridout CJ, Hogenhout SA. Silencing of aphid genes by dsRNA feeding from plants. PLoS One 2011;6:1–8.
[29] Bhatia V, Bhattacharya R, Uniyal PL, Singh R, Niranjan RS. Host generated siRNAs attenuate expression of serine protease gene in *Myzus persicae*. PLoS One 2012;7:e46343.
[30] Whyard S, Singh AD, Wong S. Ingested double-stranded RNAs can act as species-specific insecticides. Insect Biochem Mol Biol 2009;39:824–32.
[31] Shakesby AJ, Wallace IS, Isaacs HV, Pritchard J, Roberts DM, Douglas AE. A water-specific aquaporin involved in aphid osmoregulation. Insect Biochem Mol Biol 2009;39:1–10.