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ORIGINAL ARTICLE

Identification of two clip domain serine proteases involved in the pea aphid's defense against bacterial and fungal infection

Li Ma[1,*], Feng Chen[1,*], Wen Wang[1], Lu Xu[1] and Zhi-Qiang Lu[1,2]

[1]*Department of Entomology, College of Plant Protection, Northwest A&F University, Yangling, Shaanxi, China* and [2]*Key Laboratory of Plant Protection Resources and Pest Management, Ministry of Education, Northwest A&F University, Yangling, Shaanxi, China*

Abstract Phenoloxidases (POs) are required for the pea aphid's defense against bacterial and fungal infection. Prophenoloxidases (PPOs) are proteolytically converted to its active form PO through a clip domain serine protease cascade. In this study, we identified five clip domain serine proteases in the pea aphids. The messenger RNA levels of two of them, Ap_SPLP and Ap_VP, were upregulated by Gram-positive bacterium *Staphylococcus aureus* and fungus *Beauveria bassiana* infections. Double-stranded RNA-based expression knockdown of these two genes resulted in reduced PO activity of the aphid hemolymph, higher loads of *S. aureus* and *B. bassiana* in the aphids, and lower survival rates of the aphids after infections. Our data suggest that Ap_SPLP and Ap_VP are involved in PPO activation pathway in the pea aphid.

Key words defense; pea aphid; prophenoloxidase; RNA interference; serine proteases

Introduction

Extracellular serine proteinases (SPs) and non-catalytic serine proteinase homologs (SPHs) are extensively present in arthropods. Some of these SPs and SPHs comprise modules, such as LDLa (low-density lipoprotein receptor class A repeat), Sushi, Wonton, SR (scavenger receptor) domains and so on, usually located at the amino-termini (Cao *et al.*, 2015). These domains probably mediate protein–protein interaction between SPs and other proteins in complex physiological processes. The largest group of modules in arthropod SPs is the clip domain family, so called clip-SPs (Kanost & Jiang, 2015; Veillard *et al.*, 2016). The clip domain was first identified in the horseshoe crab hemolymph clotting system (Muta *et al.*, 1990). They usually contain 35–55 residues with three conserved disulfide bonds. Clip-SPs play roles in dorsal-ventral pattern determination (Jiang & Kanost, 2000), hemolymph coagulation (Jiang & Kanost, 2000), phenoloxidase (PO)-mediated melanization (Jiang *et al.*, 1998; Volz *et al.*, 2005; Kan *et al.*, 2008; An *et al.*, 2011; An *et al.*, 2013; Zhang *et al.*, 2016), Toll pathway-mediated antimicrobial peptide induction (Buchon *et al.*, 2009; Roh *et al.*, 2009; Issa *et al.*, 2018), and wound healing (Patterson *et al.*, 2013).

Compared to other insects, the pea aphid (*Acyrthosiphon pisum*) has a reduced immune system. It lacks key genes in the immune deficiency (IMD) signaling pathway and antimicrobial peptides, which are conserved in most insect species (Gerardo *et al.*, 2010; Laughton *et al.*, 2011). PO-mediated melanization is a core component in the insect immune system (Cerenius *et al.*, 2008). We have already shown that POs are required for the pea aphid's defense against bacterial and fungal infections (Xu *et al.*, 2019). However, little is known about prophenoloxidase (PPO) activation in the aphid. Several clip-SPs have been annotated in the pea aphid genomic database (http://bipaa.genouest.org/is/aphidbase). We are interested in the roles of these clip-SPs in the immune responses and pathways of the pea aphid,

Correspondence: Zhi-Qiang Lu, Department of Entomology, College of Plant Protection, Northwest A&F University, Yangling, Shaanxi 712100, China. Email: zhiqiang.lu@nwsuaf.edu.cn

*These authors contributed equally to the work.

particularly in the PPO activation pathway. Herein, we report our study of two clip-SPs, Ap_SPLP and Ap_VP, which are involved in the pea aphid's defense against bacterial and fungal infections, and suggest that these two serine proteases participate in the PPO activation pathway.

Materials and methods

Aphid rearing

The *A. pisum* strain used in the present study was originally collected from Yunnan, China and derived from a single parthenogenetic female. The aphid colonies were maintained on broad bean (*Vicia faba*) seedlings in a growth chamber at 21 ± 1 °C, 70 ± 5% relative humidity under a 16 h light (L) : 8 h dark (D) photoperiod. Ten adult females were placed on each bean plant and allowed to produce offspring for 2 days. These adults were then removed from the plant. The nymphs were reared on the plant until they reached wingless adults. These adults were used in the following experiments.

Bacterial and fungal infections

For bacterial infection, *Pseudomonas aeruginosa* (PAO1, from Dr. Xihui Shen at Northwest A&F University) and *Staphylococcus aureus* (ATCC43300, from Dr. Xihui Shen at Northwest A&F University) were cultured in Luria-Bertani liquid medium at 37 °C and their growth was monitored by taking absorbance reading at 600 nm until the optical density reached around 1. Then the cells were harvested by centrifugation. The pellets were subsequently resuspended in sterilized 0.85% NaCl solution to bring the final *P. aeruginosa* cell suspension to 1×10^{10} colony formation units (CFU)/mL and the *S. aureus* cell suspension to 2×10^{11} CFU/mL, respectively. The adult aphids were anesthetized with CO_2 and pricked by a sterile capillary dipped into bacteria suspensions or sterilized 0.85% NaCl solution according to the method previously described (Altincicek *et al.*, 2011).

For fungal infection, *Beauveria bassinana* (strain 242, from Dr. Michael Bidochka at Brock University) was cultured on a potato dextrose agar (PDA) plate at 28 °C for 7 days until the plate was full of white hyphae and conidia. The mycelium was then picked up with a pipette tip and placed in an Eppendorf tube with sterile H_2O. Hyphae and spores were isolated by centrifugation with a 0.5 mL Eppendorf tube with a filter of sterile degreasing cotton that had been inserted into a 1.5 mL Eppendorf tube. The harvested spore pellets were suspended in 0.05% Tween-20. The fungal spores were counted using a hemocytometer under an inverted microscope and diluted to 2×10^7 spores/mL with 0.05% Tween-20. One microliter of conidia preparation or 0.05% Tween-20 buffer were pipetted onto the dorsal side of the abdomen of CO_2-anesthetized newly emerged aphid adults.

The inoculated aphids were placed on sterile plates until recovery and then placed on fresh broad bean seedlings.

RNA extraction and real-time quantitative polymerase chain reaction (qPCR)

Total RNA from five aphids in each group was extracted using Trizol reagent (Roche, Basel, Switzerland). To remove genomic DNA, the RNA samples were further purified using the Direct-zol RNA Miniprep Kit (Zymo Research, Irvine, CA, USA). The first-strand complementary DNA (cDNA) was synthesized using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland).

qPCRs were performed on a Rotor Q thermocycler (Qiagen, Hilden, Germany) with the synthesized cDNA using the Faststart Essential DNA Green Master (Roche, Basel, Switzerland). The *A. pisum* ribosomal protein L7 (*Rpl7*) gene was used as an endogenous reference for normalization (Nakabachi *et al.*, 2005). The results were calculated using a relative quantitative method ($2^{-\Delta\Delta Ct}$). All analyses were performed with three biological replicates. The primers used in qPCR are listed in Table S1.

Synthesis of double-stranded RNA (dsRNA) and RNA interference

The *Ap_SPLP* and *Ap_VP* target fragments for RNA interference (RNAi) were amplified by PCR using the primers listed in Table S1. The PCR products purified using the Gel Extraction Kit (Promega, Madison, WI, USA) were used to synthesize dsRNA with the T7 RiboMAX™ Express RNAi System (Promega, Madison, WI, USA) according to the manufacturer's instructions. *dsGFP* was also synthesized and served as RNAi control. The purified dsRNA were quantified by spectrophotometry analysis and purity and integrity was verified by agarose gel electrophoresis, and then stored at −80 °C.

Newly emerged adult aphids were CO_2-anesthetized and injected with 50 nL of dsRNA at the dorsal site of

the abdomen using a Nanoject III micro-injector (Drummond Scientific, Broomall, PA, USA) equipped with glass capillaries prepared using a P-97 Micropipette Puller (Sutter Instrument Co., Novato, CA, USA). After injection, the aphids were transferred to fresh broad bean seedlings and collected at each time point for RNA preparation. RNAi efficiency was verified by measurement of the transcriptional level of the targeted genes using qPCR.

Aphid survival recording, bacterial CFUs and fungal spores counting

Two days after dsRNA injection, the aphids were infected with bacteria as previously described. Twenty aphids in each group were used in the survival assays in the following week at 1-day intervals.

The bacterial CFUs were determined as previously described (Xu *et al.*, 2019). Briefly, at least eight aphids were surface-sterilized with 75% ethanol and washed twice with 0.85% NaCl solution to clear residual ethanol. Then, each aphid was ruptured in sterilized 0.85% NaCl. After diluting to a suitable concentration, 100 μL of the mixture was evenly spread on a Luria-Bertani plate and the bacterial colonies were counted after culturing at 37 °C overnight.

For fungal spores counting, the homogenate of aphids were obtained as above and distributed on PDA plates. The fungal colonies were counted after incubation at 28 °C for 5 days.

Phenoloxidase activity assay

To determine phenoloxidase activity, adult aphids were injected with dsRNA 2 days prior to infection. The hemolymph of aphids was collected at different time points. For each group, 20 head-cut aphids were contained in a 0.5 mL Eppendorf tube with a filter of sterile degreasing cotton that had been inserted into a 1.5 mL Eppendorf tube. The equipment was then placed into a 1.5 mL Eppendorf tube and centrifuged at 500 × *g* for 10 min at 4 °C to collect the hemolymph. Two microliters of hemolymph and 100 μL L-dopamine (2 mmol/L in 50 mmol/L Tris-HCl, pH8.0) were promptly added to a pre-chilled 96-well plate and the absorption at 490 nm was recorded every 30 s for 30 min on a microplate reader (Tecan Pro200, Tecan, Männedorf, Switzerland). PO activity was shown as the maximum slope, which was defined as the increase of absorbance at 490 nm/min (Jiang *et al.*, 2003). Three independent biological replicates were performed for each treatment.

Statistical analysis

All the data in this experiment were plotted using GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, CA, USA). Student's *t*-test was used to determine the significant differences between the controls and treated samples in mRNA levels, bacterial CFUs counting, fungal spores counting and phenoloxidase assays. The log-rank (Mantel-Cox) test was used to analyze the aphids' survival curves.

Results

Sequence features of Ap_SPLP and Ap_VP

We searched the AphidBase by BLASTP using typical clip domain serine proteases, *Manduca sexta* hemolymph protein 21 (Ms_HP21, AAV91019.1) and 17-short (Ms_HP17s, AAV91015.1) and identified five clip domain serine proteases. Sequence analysis showed that they contain a clip domain at the amino-termini and protease domain at the carboxyl-termini (Fig. S1). We focused on two clip-SPs, ACYPI000297 (designated as Ap_SPLP) and ACYPI009131 (designated as Ap_VP). Using BLASTP search results, we retrieved protein sequences of clip-SPs that have been studied in other insect species. Sequence comparison revealed that Ap_SPLP is similar to *M. sexta* HP21 (percent identity: 38.46), *T. molitor* SAE (Spätzle-processing enzyme-activating enzyme) (percent identity: 37.40), *A. mellifera* snake (percent identity: 39.84), *A. aegypti* AAEL011991-PA (percent identity: 37.14) and *A. gambiae* AGAP008835-PA (percent identity: 39.12) (Fig. 1A). Ap-VP is similar to *M. sexta* HP17s (percent identity: 41.19), *A. mellifera* VSP (percent identity: 43.47) and *B. mori* Clip-SP4 (percent identity: 37.70) (Fig. 1B).

Staphylococcus aureus and Beauveria bassiana infection but not Pseudomonas aeruginosa infection induced the expression of Ap_SPLP and Ap_VP

To determine whether Ap_SPLP and Ap_VP respond to microbial challenge in the pea aphid, we first measured the mRNA levels of *Ap_SPLP* and *Ap_VP* after bacterial and fungal infections. Our results showed that Gram-positive bacterium *S. aureus* and fungus *B. bassiana* infections induced up-regulation of expression of *Ap_SPLP* and *Ap_VP*, whereas Gram-negative bacterium *P. aeruginosa* did not (Fig. 2). This result suggests that *Ap_SPLP* and *Ap_VP* respond to immune challenge in the pea aphid.

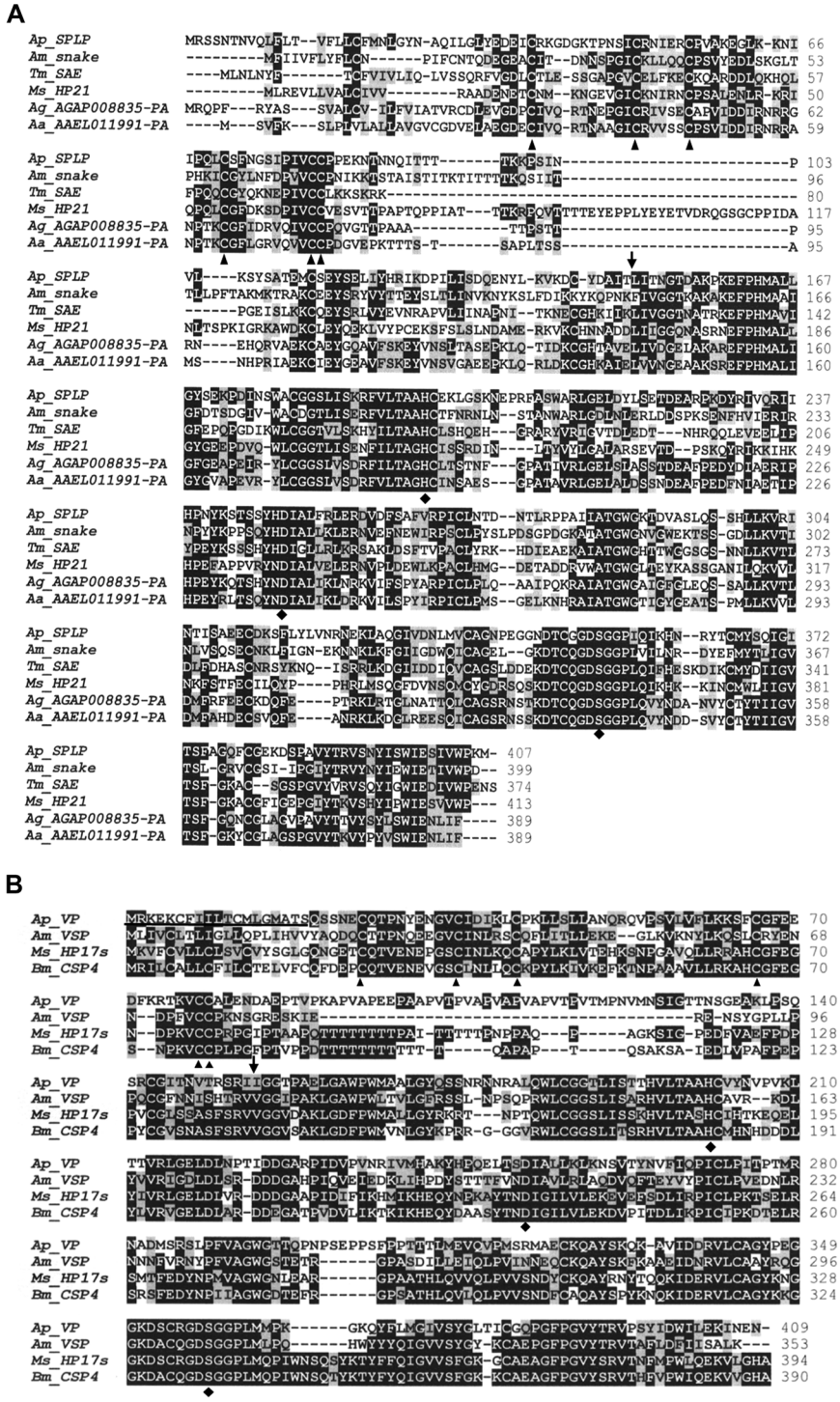

Fig. 1 Multiple amino acid sequence alignment of Ap_SPLP (A) and Ap_VP (B) with clip-SPs from other insect species: Aa_AAEL011991-PA (*Aedes aegypti*), Ag_AGAP008835-PA (*Anopheles gambiae* str. PEST), Am_snake (*Apis mellifera*, XP_001120043.2), Am_VP (*Apis mellifera*, XP_006560620.1), Bm_CSP4 (*Bombyx mori*, NP_001036891.1), Ms_HP17s (*Manduca sexta*, AAV91015.1), Ms_HP21 (*Manduca sexta*, AAV91019.1), Tm_SAE (*Tenebrio molitor*, AB363979.1). These sequences were aligned by the Clustal Omega program with default settings. The six conserved cysteine residues in the clip domain are marked by "▲", and the residues of the catalytic triad are marked by "♦" under the sequences. Arrows indicate the activation site.

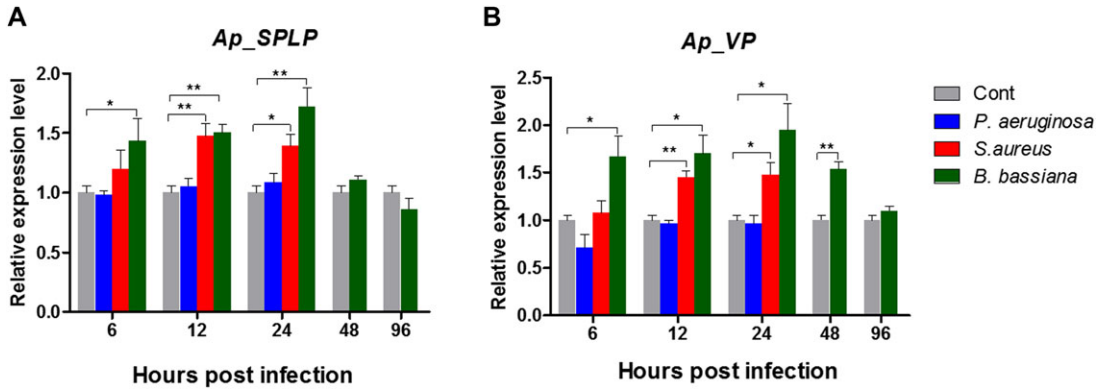

Fig. 2 Relative expression levels of *Ap_SPLP* (A) and *Ap_VP* (B) in the pea aphids after *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Beauveria bassiana* infections. Cont, control aphids injected with sterile 0.85% NaCl. The messenger RNA abundances of *Ap_SPLP* and *Ap_VP* were determined by quantitative real-time polymerase chain reaction. The expressions of *Ap_SPLP* and *Ap_VP* were normalized with *A. pisum* ribosomal protein L7 gene (*Rpl7*), and their expression in the infection groups were compared to the expression in the control group at each time point. Values shown are the mean (± SEM) of three independent experiments. The statistical differences between the control group and infection groups were denoted with asterisks. $*P < 0.05$; $**P < 0.01$.

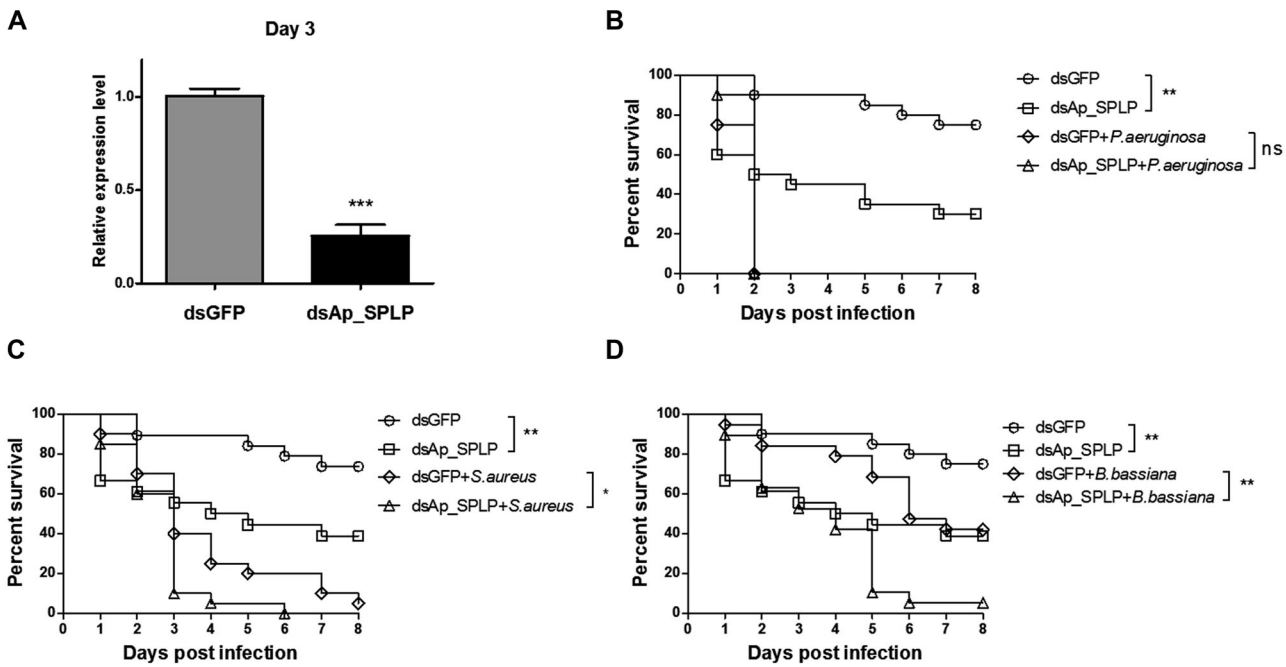

Fig. 3 Knockdown of expression of *Ap_SPLP* by RNA interference (A) and its effect on survival of pea aphids after *Pseudomonas aeruginosa* (B), *Staphylococcus aureus* (C) and *Beauveria bassiana* (D) infections. Relative expression level of *Ap_SPLP* was examined by quantitative real-time polymerase chain reaction after double-stranded RNA injections. Asterisks denote statistical differences between two experimental groups.$*P < 0.05$; $**P < 0.01$; ns, not significantly different.

Knockdown of Ap_SPLP and Ap_VP caused higher susceptibility to S. aureus and B. bassiana infections

To confirm whether Ap_SPLP and Ap_VP contribute to the pea aphid's defense against infection, we knocked down the expression of *Ap_SPLP* and *Ap_VP* by injection of their dsRNA into the hemocoel, and monitored the survival of the aphids after infection. Expression analysis showed that the relative mRNA levels of *Ap_SPLP* and *Ap_VP* significantly decreased 3 days after

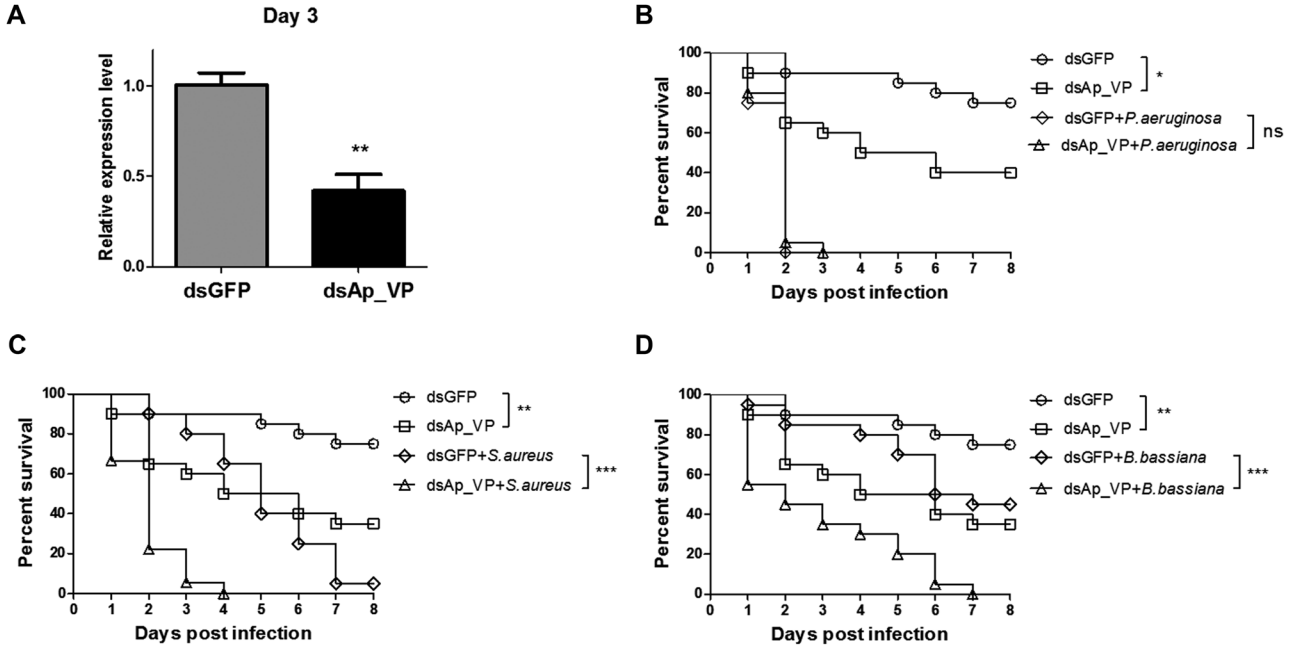

Fig. 4 Knockdown of expression of *Ap_VP* by RNA interference (A) and its effect on survival of pea aphids after *Pseudomonas aeruginosa* (B), *Staphylococcus aureus* (C) and *Beauveria bassiana* (D) infections. Relative expression level of *Ap_VP* was examined by quantitative real-time polymerase chain reaction after double-stranded RNA injections. Asterisks denote statistical differences between two experimental groups. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; ns, not significantly different.

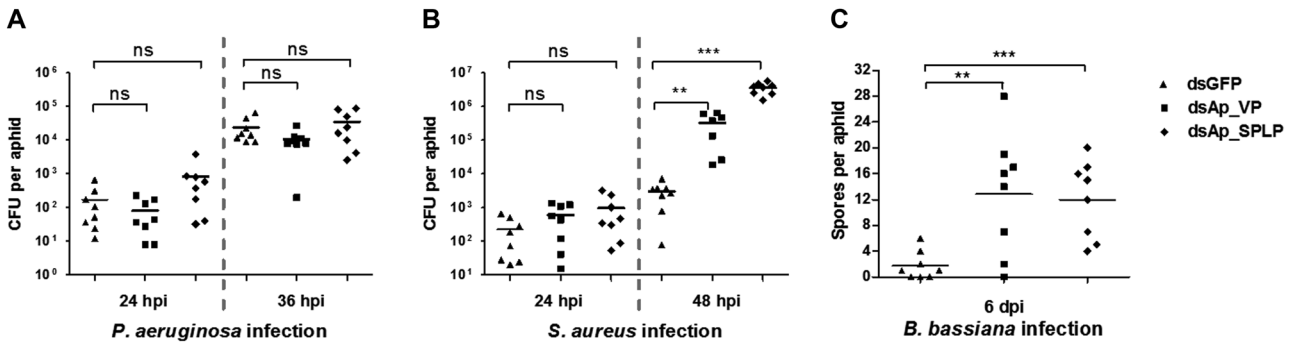

Fig. 5 Microbial loads of pea aphids after *Pseudomonas aeruginosa* (A), *Staphylococcus aureus* (B) and *Beauveria bassiana* (C) infections. After infections at indicated time points, the aphids were homogenized and bacterial colony-forming units and fungal spores were counted after culturing. The *dsGFP*-injected aphids were used as controls. The statistical differences were denoted with asterisks. $**P < 0.01$; $***P < 0.001$; ns, not significantly different; hpi, hours post-infection; dpi, days post-infection.

injection of dsRNA, indicating successful knockdown of expression of these two genes (Fig. 3A and Fig. 4A, respectively). The knockdown of *Ap_SPLP* and *Ap-VP* resulted in a higher mortality of the aphids after *S. aureus* (Fig. 3C and Fig. 4C) and *B. bassiana* infections (Fig. 3D and Fig. 4D), whereas no apparent effect was observed on aphid survival after *P. aeruginosa* infection (Fig. 3B and Fig. 4B). Therefore, Ap_SPLP and Ap-VP play roles in the aphid's defense against Gram-positive bacterium *S. aureus* infection and fungus *B. bassiana* infection.

Knockdown of Ap_SPLP and Ap_VP resulted in higher loads of S. aureus and B. bassiana in the pea aphids

We challenged aphids with live bacteria and fungi, then investigated propagation of the bacterial and fungal cells

inside the aphids. CFU counting indicated that knockdown of *Ap_SPLP* and *Ap_VP* did not affect the growth of *P. aeruginosa* in the aphids (Fig. 5A). On the contrary, knockdown of *Ap_SPLP* and *Ap_VP* resulted in higher loads of *S. aureus* and *B. bassiana* inside the aphids (Fig. 5B and 5C). These results suggest that knockdown of *Ap_SPLP* and *Ap_VP* impaired the aphids' immune responses to *S. aureus* and *B. bassiana* infections, but not to *P. aeruginosa* infection. This is consistent with our observation on aphids' survival (Fig. 3 and Fig. 4).

Knockdown of Ap_SPLP and Ap_VP reduced PPO activation of the pea aphids

It has been shown that no antimicrobial peptides were produced in the pea aphid after bacterial infection (Gerardo *et al.*, 2010; Laughton *et al.*, 2011). Our unpublished results also confirmed there was no antibacterial activity detected in the pea aphid hemolymph after bacterial infection. We then investigated the change of PO activities in the *Ap_SPLP* and *Ap_VP* knockdown aphids. We collected hemolymph from the knockdowns and placed the samples on a bench. Two hours later, the hemolymph from *dsGFP*-injected aphids melanized, the sample from *dsAp_SPLP*-injected aphids showed partial melanization, and the sample from *dsAp_VP*-injected aphids did not melanize (Fig. S2). Knockdown of *Ap_SPLP* resulted in decrease in the basal PO activity 24 and 48 h after injection of sterilized 0.85% NaCl solution. Knockdown of *Ap_SPLP* resulted in lower PO activity 24 h after *S. aureus* and *B. bassiana* infections (Fig. 6B and 6C), whereas no effect on PO activity was detected in the *P. aeruginosa* infections (Fig. 6A). We obtained similar results in the *Ap-VP* knockdown aphids (Fig. 7). Collectively, PO activity data revealed that in the pea aphid *Ap_SPLP* and *Ap_VP* are involved in the PPO activation pathway in response to infections by Gram-positive bacterium *S. aureus* and fungus *B. bassiana*.

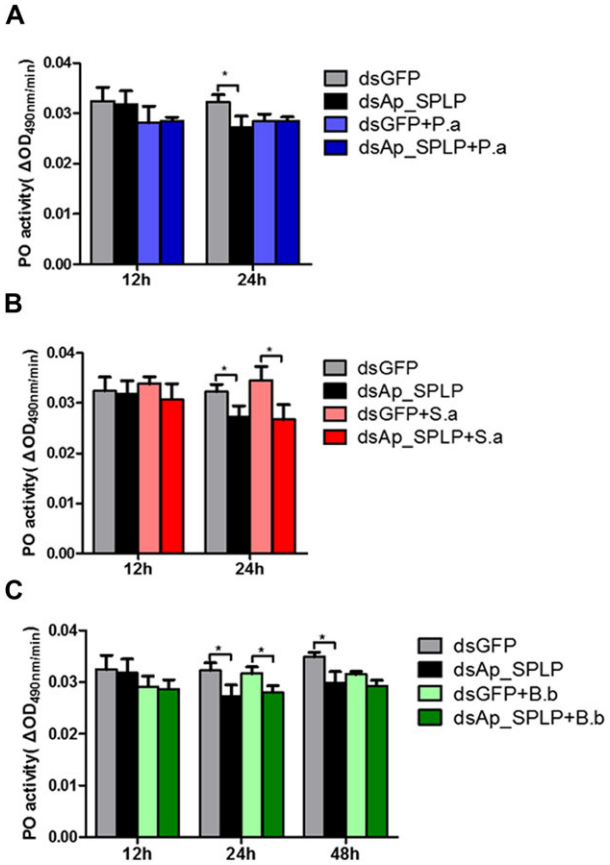

Fig. 6 Phenoloxidase activity of the *Ap_SPLP* knockdown pea aphids after *Pseudomonas aeruginosa* (A), *Staphylococcus aureus* (B) and *Beauveria bassiana* (C) infections. Adult aphids were injected with double-stranded RNA and 2days later were challenged with microbes. The hemolymph samples were collected from 20 aphids in each group at different time points and subjected immediately to phenoloxidase activity assays on a microplate reader. The statistical differences between the control group (*dsGFP* injection) and dsAp_SPLP groups were denoted with asterisks. $*P < 0.05$. Three independent biological replicates were performed for each treatment.

Discussion

PO-mediated melanization plays a critical role in the insect immune system (Cerenius *et al.*, 2008; Nappi *et al.*, 2009; Yassine *et al.*, 2012; Binggeli *et al.*, 2014). We recently showed that PO is required in the pea aphid for survival against microbial infections (Xu *et al.*, 2019). The activation of PPO relies on a serine protease cascade (Jiang *et al.*, 1998; Volz *et al.*, 2005; Kan *et al.*, 2008; An *et al.*, 2013; Zhang *et al.*, 2016). In this study, we showed that knockdown of expression of two serine proteases, *Ap_SPLP* and *Ap_VP*, resulted in decrease of PO activity in the pea aphid, and higher mortality of the aphids after bacterial and fungal infections as a consequence. This study suggests that these two serine proteases are involved in the activation of the pea aphid PPO and reflects the critical role of PO in the pea aphid immune system, given the fact that the aphid lacks the IMD pathway (Gerardo *et al.*, 2010).

Based on the length and secondary structures between the third cysteine and the fourth cysteine, the clip domains can be separated into three groups. The group 1 clip domains typically have 15–17 residues containing one helix between Cys3 and Cys4, whereas group 2 have 22–24

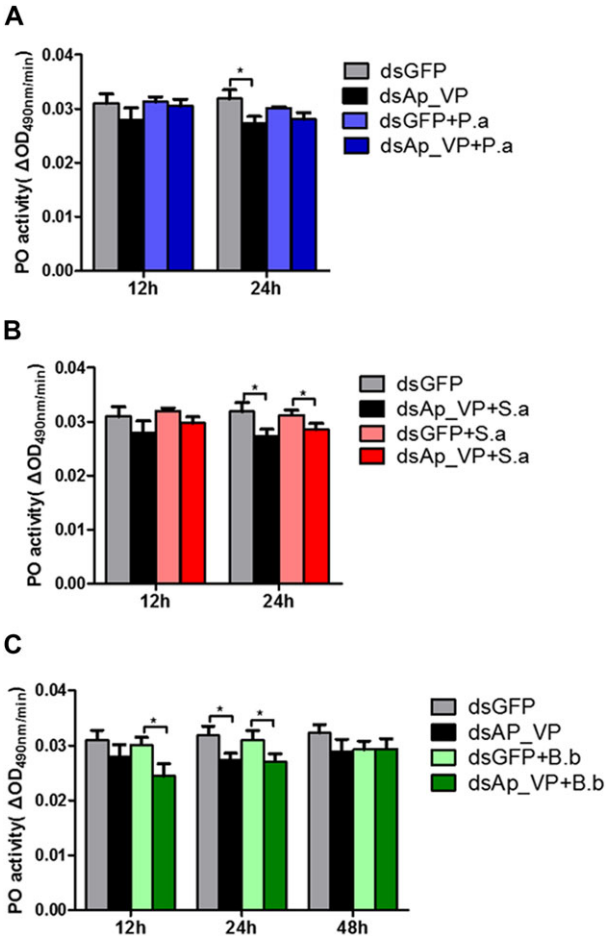

Fig. 7 Phenoloxidase activity of the *Ap_VP* knockdown pea aphids after *Pseudomonas aeruginosa* (A), *Staphylococcus aureus* (B) and *Beauveria bassiana* (C) infections. Adult aphids were injected with double-stranded RNA and 2 days later were challenged with microbes. The hemolymph samples were collected from 20 aphids in each group at different time points and subjected immediately to phenoloxidase activity assays on a microplate reader. The statistical differences between the control group (*dsGFP* injection) and dsAp_VP groups were denoted with asterisks. $*P < 0.05$. Three independent biological replicates were performed for each treatment.

residues containing two helixes between these two cysteines, and the third group has no helix at the same region (Kellenberger *et al.*, 2011). In general, type 1 clip domain-containing SPs activate type 2 clip domain-containing SPs and the latter further activate effector proteins PPO and pro-Spätzle. Spätzle is the ligand for Toll pathway (Kanost & Jiang, 2015). We compared sequences of the five selected serine proteases from pea aphid (Fig. S1) and found that there are 24 residues between Cys 3 and Cys 4 in the clip domains of Ap_VP and ACYPI004531, and 15–16 residues between Cys 3 and Cys 4 in the clip domains of the other three SPs. Secondary structure prediction revealed that there are two alpha-helixes between Cys 3 and Cys 4 in the clip domains of Ap_VP and ACYPI00531, and only one helix between Cys 3 and Cys 4 in the clip domains of the other three SPs. Secondly, Ap_VP and ACYPI00531 have an arginine residue at their activation site, whereas the other three are leucine or threonine. According to the evolutionarily conserved clip domain serine protease cascade (Kanost & Jiang, 2015), we predict that Ap_VP and ACRPI004531 might be the terminal proteases in the PPO activation cascade, whereas the other three might be at upstream positions. In fact, our sequence analysis showed that Ap_SPLP is quite similar to *M. sexta* HP21 and *T. molitor* SAE (Fig. 1A), both of which are the penultimate proteases in the cascade. The observation of a weak melanization of hemolymph from the *Ap_VP* knockdown aphids than from the *Ap_SPLP* knockdown aphids (Fig. S2) partially support our prediction. However, further biochemical study is definitely needed to examine this prediction.

Acknowledgments

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Disclosure

The authors declare no conflicts of interest.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Primers used in this study

Fig. S1. Protein sequences alignment of five clip-SPs identified from the AphidBase. The amino acid residue numbers of the clip-SPs are labeled on the right side of the sequences. The six conserved cysteines in the clip domain are indicated by the numbers and the helix structures between C3 and C4 are predicted by the SOPMA program and highlighted with yellow. The proteolytic activation site is indicated by an arrow in the SP domain.

Fig. S2. Melanization of hemolymph from *dsGFP*, *dsAp_SPLP* and *dsAp_VP* injected aphids. On the 3rd day after the aphids were injected with dsRNA, 5 μL of hemolymph collected from the aphids (as described in the Methods) were mixed with 15 μL of phosphate-buffered saline in Eppendorf tubes. The samples were placed at room temperature for 2 h and photographed.