

## ORIGINAL ARTICLE

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

Li Ma<sup>1,\*</sup>, Feng Chen<sup>1,\*</sup>, Wen Wang<sup>1</sup>, Lu Xu<sup>1</sup> and Zhi-Qiang Lu<sup>1,2</sup> <sup>1</sup>Department of Entomology, College of Plant Protection, Northwest A&F University, Yangling, Shaanxi, China and <sup>2</sup>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 (*Acyrtosiphon 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  $21 \pm 1$  °C,  $70 \pm 5\%$  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  $1 \times 10^{10}$  colony formation units (CFU)/mL and the *S. aureus* cell suspension to  $2 \times 10^{11}$  CFU/mL, respectively. The adult aphids were anesthetized with CO<sub>2</sub> 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 bassiana* (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<sub>2</sub>O. 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  $2 \times 10^7$  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<sub>2</sub>-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 ( $2^{-\Delta\Delta C_t}$ ). 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<sub>2</sub>-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  $\mu$ L 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  $500 \times g$  for 10 min at 4 °C to collect the hemolymph. Two microliters of hemolymph and 100  $\mu$ L 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.

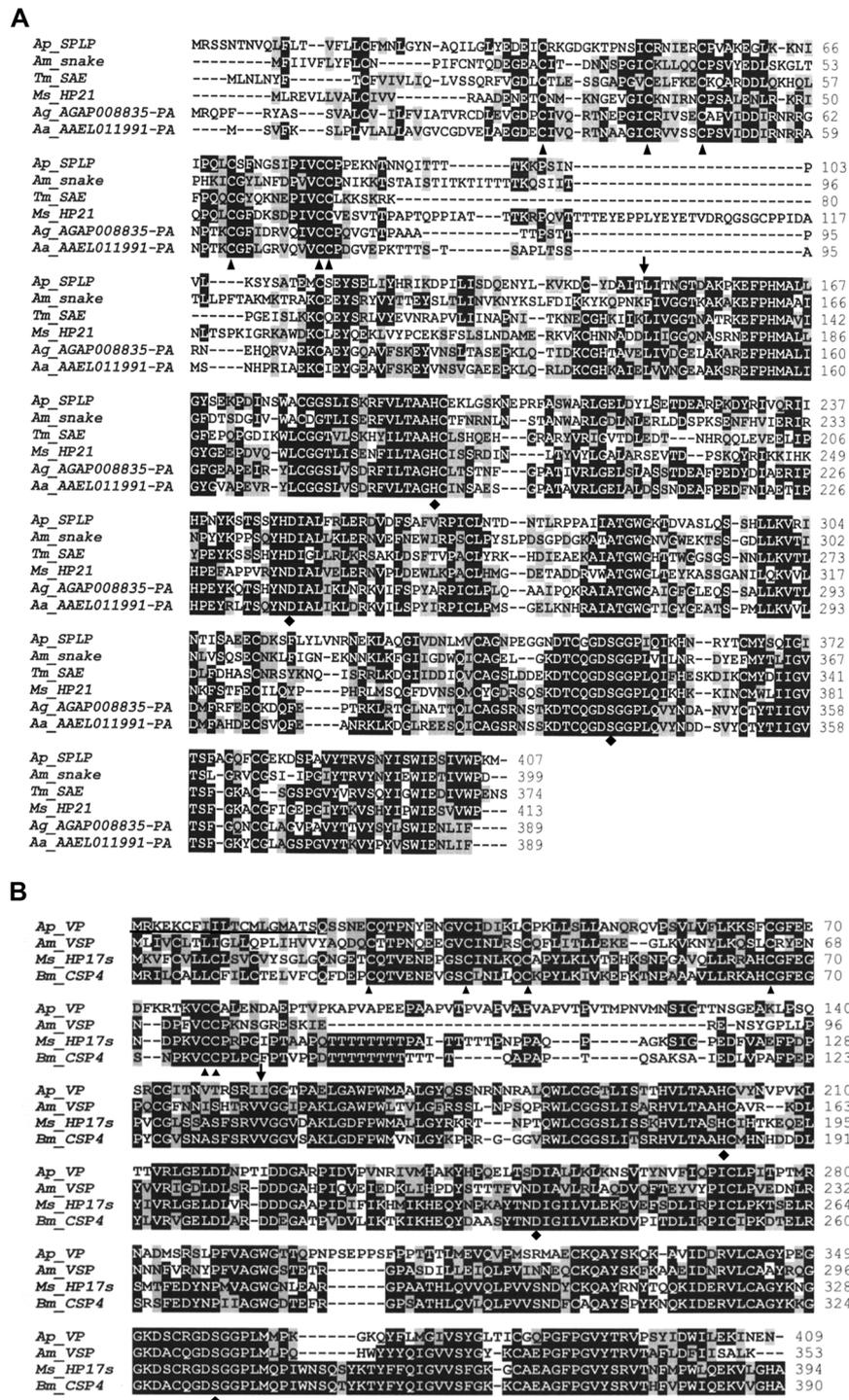
## Results

#### *Sequence features of Ap\_SPLP and Ap\_VP*

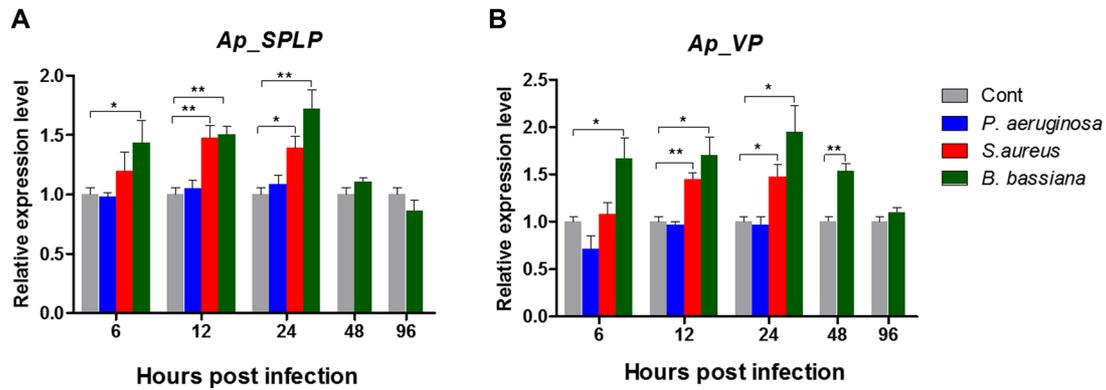
We searched the AphidBase by BLASTP using typical clip domain serine proteases, *Manduca sexta* hemolymph protein 21 (Ms\_HP21, AAV91019.1) and 17-short (Ms\_HP17s, AAV91015.1) and identified five clip domain serine proteases. Sequence analysis showed that they contain a clip domain at the amino-termini 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*

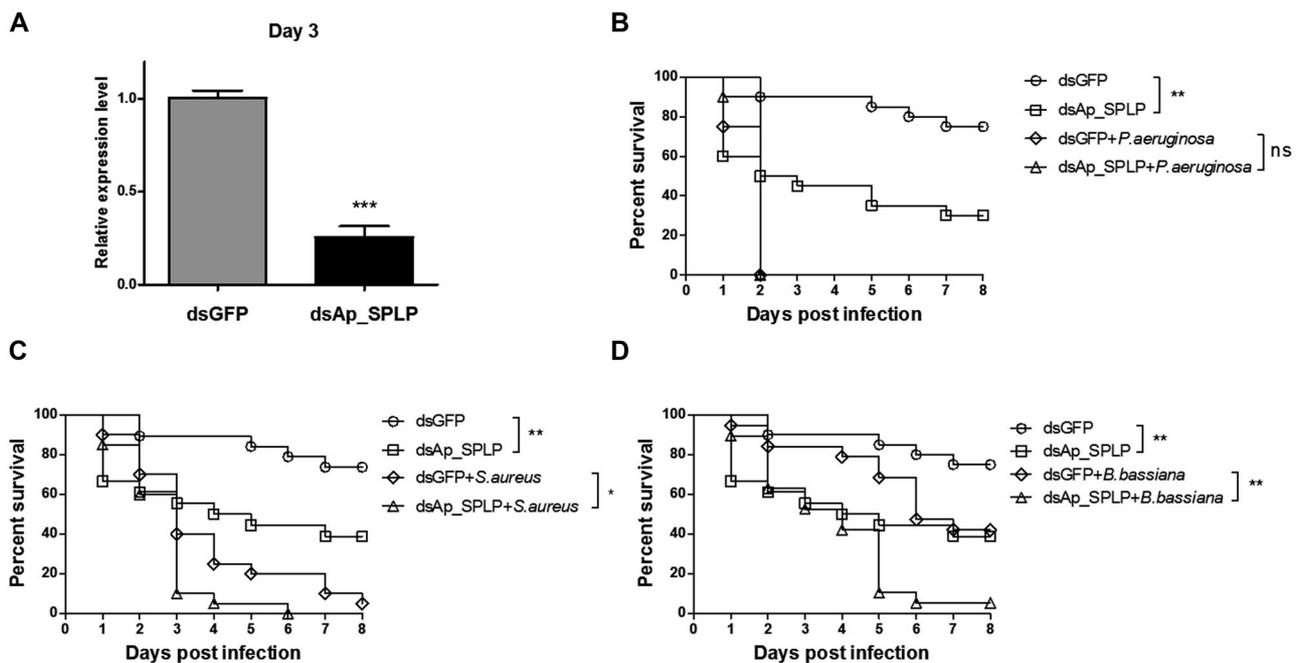
To determine whether Ap\_SPLP and Ap\_VP respond to microbial challenge in the pea aphid, we first measured the mRNA levels of Ap\_SPLP and Ap\_VP after bacterial and fungal infections. Our results showed that Gram-positive bacterium *S. aureus* and fungus *B. bassiana* infections induced up-regulation 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 ( $\pm$  SEM) of three independent experiments. The statistical differences between the control group and infection groups were denoted with asterisks. \* $P < 0.05$ ; \*\* $P < 0.01$ .

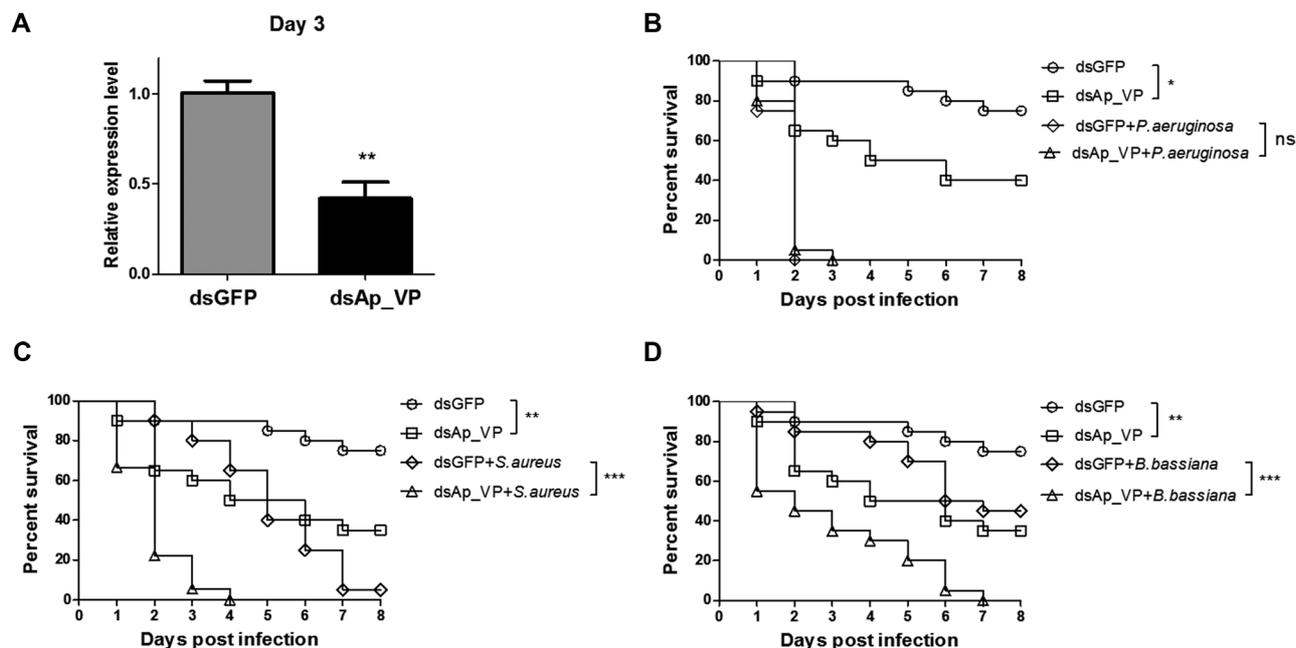


**Fig. 3** Knockdown of expression of *Ap\_SPLP* by RNA interference (A) and its effect on survival of pea aphids after *Pseudomonas aeruginosa* (B), *Staphylococcus aureus* (C) and *Beauveria bassiana* (D) infections. Relative expression level of *Ap\_SPLP* was examined by quantitative real-time polymerase 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.

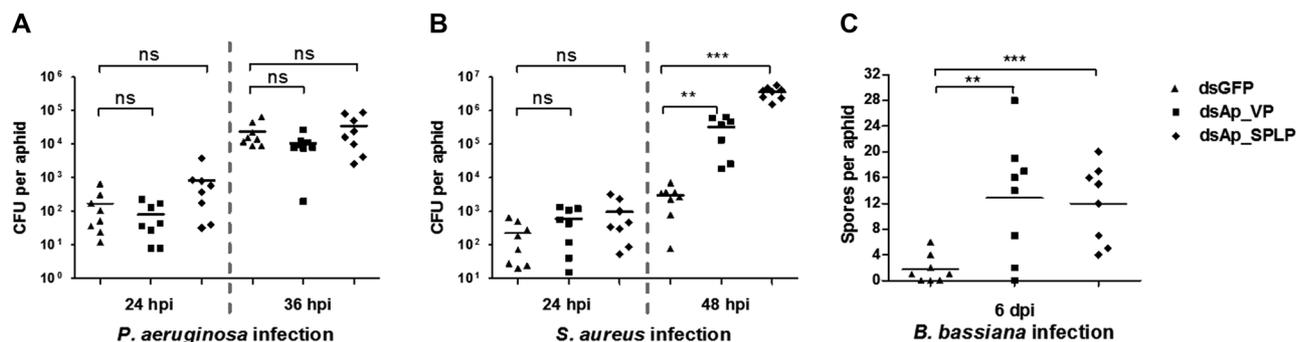
*Knockdown of Ap\_SPLP and Ap\_VP caused higher susceptibility to S. aureus and B. bassiana infections*

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

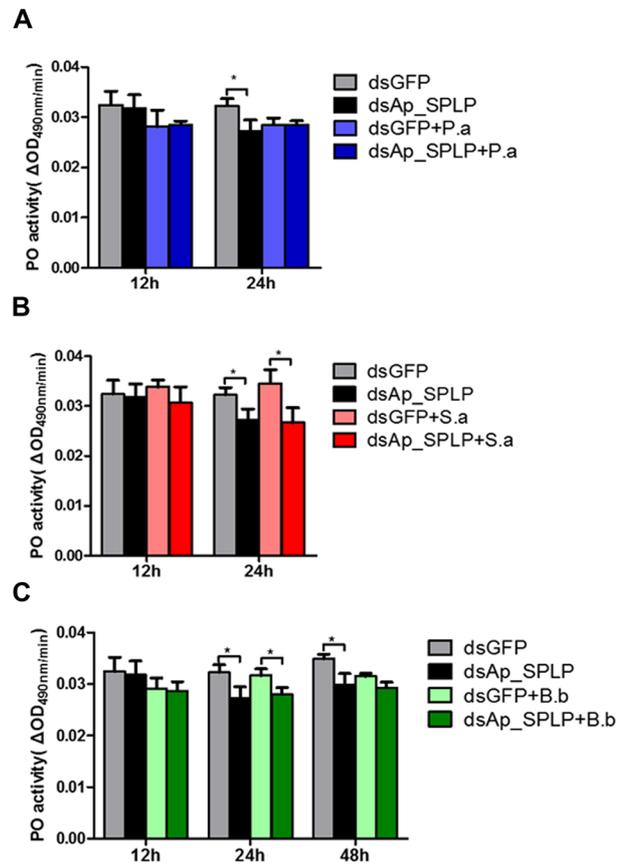
inside the aphids. CFU counting indicated that knockdown of *Ap\_SPLP* and *Ap\_VP* did not affect the growth of *P. aeruginosa* in the aphids (Fig. 5A). On the contrary, knockdown of *Ap\_SPLP* and *Ap\_VP* resulted in higher loads of *S. aureus* and *B. bassiana* inside the aphids (Fig. 5B and 5C). 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*.

## Discussion

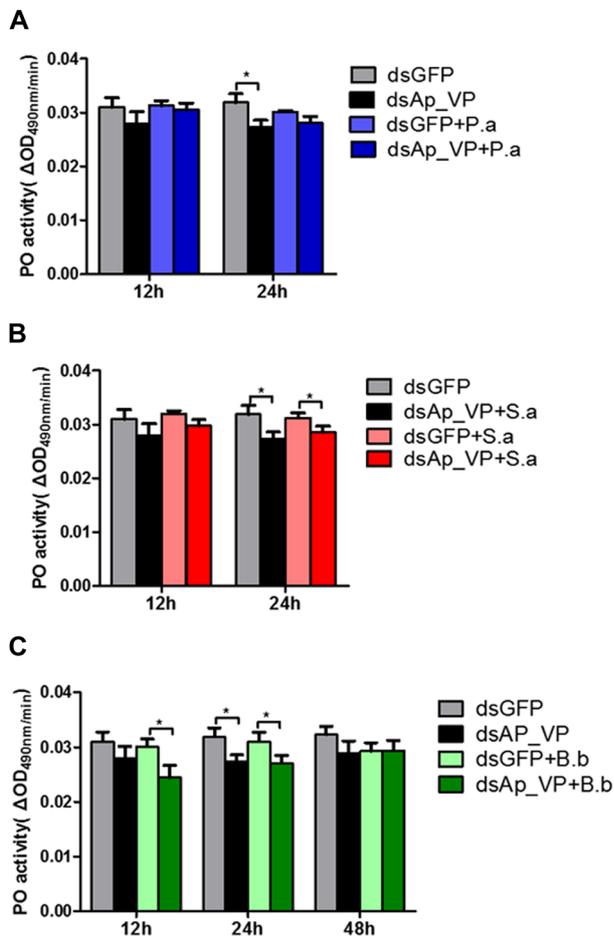
PO-mediated melanization plays a critical role in the insect immune system (Cerenius *et al.*, 2008; Nappi *et al.*, 2009; Yassine *et al.*, 2012; Binggeli *et al.*, 2014). We recently showed that PO is required in the pea aphid for survival against microbial infections (Xu *et al.*, 2019). The activation 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



**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 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\_SPLP* groups were denoted with asterisks. \* $P < 0.05$ . Three independent biological replicates were performed for each treatment.

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 helices 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-helices 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

This work was financially supported by the National Natural Science Foundation of China grant 31772530 and Natural Science Foundation of Shaanxi Province (2017JM3003) to ZL. The authors thank Dr. Haobo Jiang in Oklahoma State University for his critical reading and suggestions on the manuscript.

## Disclosure

The authors declare no conflicts of interest.

## References

- Altincicek, B., Braak, B., Laughton, A.M., Udekwu, K.I. and Gerardo, N.M. (2011) *Escherichia coli* K-12 pathogenicity in the pea aphid, *Acyrtosiphon pisum*, reveals reduced antibacterial defense in aphids. *Developmental and Comparative Immunology*, 35, 1091–1097.
- An, C., Budd, A., Kanost, M.R. and Michel, K. (2011) Characterization of a regulatory unit that controls melanization and affects longevity of mosquitoes. *Cellular and Molecular Life Sciences*, 68, 1929–1939.
- An, C.J., Zhang, M.M., Chu, Y. and Zhao, Z.W. (2013) Serine protease MP2 activates prophenoloxidase in the melanization immune response of *Drosophila melanogaster*. *PLoS ONE*, 8, e79533.

- Binggeli, O., Neyen, C., Poidevin, M. and Lemaitre, B. (2014) Prophenoloxidase activation is required for survival to microbial infections in *Drosophila*. *PLoS Pathogens*, 10, e1004067.
- Buchon, N., Poidevin, M., Kwon, H.M., Guillou, A., Sottas, V., Lee, B.L. *et al.* (2009) A single modular serine protease integrates signals from pattern-recognition receptors upstream of the *Drosophila* Toll pathway. *Proceedings of the National Academy of Sciences USA*, 106, 12442–12447.
- Cao, X.L., He, Y., Hu, Y.X., Zhang, X.F., Wang, Y., Zou, Z., *et al.* (2015) Sequence conservation, phylogenetic relationships, and expression profiles of nondigestive serine proteases and serine protease homologs in *Manduca sexta*. *Insect Biochemistry and Molecular Biology*, 62, 51–63.
- Cerenius, L., Lee, B.L. and Söderhäll, K. (2008) The proPO-system: pros and cons for its role in invertebrate immunity. *Trends in Immunology*, 29, 263–271.
- Gerardo, N.M., Altincicek, B., Anselme, C., Atamian, H., Barribeau, S.M., de Vos, M., *et al.* (2010) Immunity and other defenses in pea aphids, *Acyrtosiphon pisum*. *Genome Biology*, 11, R21.
- Issa, N., Guillaumot, N., Lauret, E., Matt, N., Schaeffer-Reiss, C., Van Dorsselaer, A., *et al.* (2018) The circulating protease persephone is an immune sensor for microbial proteolytic activities upstream of the *Drosophila* Toll pathway. *Molecular Cell*, 69, 539–550.e6.
- Jiang, H.B., and Kanost, M.R. (2000) The clip-domain family of serine proteinases in arthropods. *Insect Biochemistry and Molecular Biology*, 30, 95–105.
- Jiang, H.B., Wang, Y. and Kanost, M.R. (1998) Pro-phenol oxidase activating proteinase from an insect, *Manduca sexta*: A bacteria-inducible protein similar to *Drosophila* easter. *Proceedings of the National Academy of Sciences USA*, 95, 12220–12225.
- Jiang, H.B., Wang, Y., Yu, X.Q. and Kanost, M.R. (2003) Prophenoloxidase-activating proteinase-2 from hemolymph of *Manduca sexta*. A bacteria-inducible serine proteinase containing two clip domains. *Journal of Biological Chemistry*, 278, 3552–3561.
- Kan, H., Kim, C.H., Kwon, H.M., Park, J.W., Roh, K.B., Lee, H., *et al.* (2008) Molecular control of phenoloxidase-induced melanin synthesis in an insect. *Journal of Biological Chemistry*, 283, 25316–25323.
- Kanost, M.R. and Jiang, H.B. (2015) Clip-domain serine proteases as immune factors in insect hemolymph. *Current Opinion in Insect Science*, 11, 47–55.
- Kellenberger, C., Leone, P., Coquet, L., Jouenne, T., Reichhart, J.M. and Roussel, A. (2011) Structure-function analysis of grass clip serine protease involved in *Drosophila* Toll pathway activation. *Journal of Biological Chemistry*, 286, 12300–12307.
- Laughton, A.M., Garcia, J.R., Altincicek, B., Strand, M.R. and Gerardo, N.M. (2011) Characterisation of immune responses in the pea aphid, *Acyrtosiphon pisum*. *Journal of Insect Physiology*, 57, 830–839.
- Muta, T., Hashimoto, R., Miyata, T., Nishimura, H., Toh, Y. and Iwanaga, S. (1990) Proclotting enzyme from horseshoe crab hemocytes. cDNA cloning, disulfide locations, and sub-cellular localization. *Journal of Biological Chemistry*, 265, 22426–22433.
- Nakabachi, A., Shigenobu, S., Sakazume, N., Shiraki, T., Hayashizaki, Y., Carninci, P., *et al.* (2005) Transcriptome analysis of the aphid bacteriocyte, the symbiotic host cell that harbors an endocellular mutualistic bacterium, *Buchnera*. *Proceedings of the National Academy of Sciences USA*, 102, 5477–5482.
- Nappi, A., Poirié, M. and Carton, Y. (2009) The role of melanization and cytotoxic by-products in the cellular immune responses of *Drosophila* against parasitic wasps. *Advances in Parasitology*, 70, 99–121.
- Patterson, R.A., Juarez, M.T., Hermann, A., Sasik, R., Hardiman, G. and McGinnis, W. (2013) Serine proteolytic pathway activation reveals an expanded ensemble of wound response genes in *Drosophila*. *PLoS ONE*, 8, e61773.
- Roh, K.B., Kim, C.H., Lee, H., Kwon, H.M., Park, J.W., Ryu, J.H., *et al.* (2009) Proteolytic cascade for the activation of the insect Toll pathway induced by the fungal cell wall component. *Journal of Biological Chemistry*, 284, 19474–19481.
- Veillard, F., Troxler, L., and Reichhart, J.M. (2016) *Drosophila melanogaster* clip-domain serine proteases: Structure, function and regulation. *Biochimie*, 122, 255–269.
- Volz, J., Osta, M.A., Kafatos, F.C. and Müller, H.M. (2005) The roles of two clip domain serine proteases in innate immune responses of the malaria vector *Anopheles gambiae*. *Journal of Biological Chemistry*, 280, 40161–40168.
- Xu, L., Ma, L., Wang, W., Li, L. and Lu, Z. (2019) Phenoloxidases are required for the pea aphid's defence against bacterial and fungal infection. *Insect Molecular Biology*, 28, 176–186.
- Yassine, H., Kamareddine, L. and Osta M.A. (2012) The mosquito melanization response is implicated in defense against the entomopathogenic fungus *Beauveria bassiana*. *PLoS Pathogens*, 8, e1003029.
- Zhang, X., An, C.J., Sprigg, K. and Michel, K. (2016) CLIPB8 is part of the prophenoloxidase activation system in *Anopheles gambiae* mosquitoes. *Insect Biochemistry and Molecular Biology*, 71, 106–115.

Manuscript received January 15, 2019

Final version received March 1, 2019

Accepted March 14, 2019

## 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  $\mu$ L of hemolymph collected from the aphids (as described in the Methods) were mixed with 15  $\mu$ L of phosphate-buffered saline in Eppendorf tubes. The samples were placed at room temperature for 2 h and photographed.