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Phenoloxidases are required for the pea aphid's defence against bacterial and fungal infection

L. Xu*, L. Ma*, W. Wang*, L. Li* and Z. Lu*†

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

Abstract

The pea aphid, *Acyrthosiphon pisum*, has an incomplete immune system compared to those of other insect species; some conserved components and pathways in other species are missing in its genome. As a core component of the insect immune system, *prophenoloxidase* (*PPO*) genes are retained in the pea aphid. Early studies have also shown the presence of phenoloxidase activity in specific tissues or cells in the pea aphid and suggested its involvement in response to immune challenges. In this study, we knocked down the expression of *PPO* genes in the pea aphid using double-stranded RNA-based interference, and quantitative PCR analysis and an enzyme activity assay confirmed our success in the *PPO* gene knockdown. In bacterial and fungal infection experiments, we observed that the knockdown of *PPO* resulted in more live bacterial cells and fungal spores in the body of the aphids and higher mortality of the aphids after infection. Our study provides evidence supporting a critical role of PPO in the defence of the pea aphid.

Keywords: pea aphid, phenoloxidase, bacteria, fungi, defence, RNA interference.

Introduction

Phenoloxidase (PO) belongs to the type 3 copper protein family (Decker *et al*., 2007) and catalyses the hydroxylation of monophenols and the oxidation of the derived *o*-diphenols, resulting in the formation of *o*-quinones, which further polymerize to form melanin (Nappi and Christensen, 2005). Reactive intermediates generated in PO-catalysed melanization have been shown to directly kill bacteria, fungi, viruses, and parasitic wasps (Zhao *et al*., 2007, 2011). Melanization is a general immune response in arthropods (Cerenius *et al*., 2008). For instance, melanin is deposited on the surface of endoparasitic wasp eggs inside the host insects and forms a capsule to retard the development of the eggs (Nappi and Christensen, 2005; Beck and Strand, 2007).

PO is present as a zymogen (prophenoloxidase, PPO) and is activated through a serine protease cascade upon recognition of pathogen-associated molecular patterns such as lipopolysaccharides and peptidoglycans from bacteria and β-1,3-glucans from fungi by pattern recognition proteins (Cerenius and Söderhäll, 2004). The PPO activation pathway is regulated by factors from the hosts or the invaders (Cerenius *et al*., 2008). In *Drosophila melanogaster*, PPO1 and PPO2 play an essential role in survival after infection by Gram-positive bacteria and fungi (Binggeli *et al*., 2014). In the model beetle, *Tribolium castaneum*, RNA interference (RNAi)-mediated gene knockdown revealed that PPO1 and PPO2 contribute towards both antibacterial and antifungal defence (Yokoi *et al*., 2015). In a mosquito, it has been shown that PPO activation and melanization are involved in the antifungal response (Yassine *et al*., 2012; Wang *et al*., 2017), as well as the antiviral response (Rodriguez-Andres *et al*., 2012). These studies underline the importance of PO-mediated melanization in insect immune defence.

The pea aphid, *Acyrthosiphon pisum*, has an incomplete immune system compared to other insect species; ie, some conserved immune components and pathways in other species are missing in the pea aphid genome. For instance, peptidoglycan recognition proteins, antimicrobial peptides (AMPs) and Immune Deficiency (IMD), or Dredd and Relish of the IMD pathways that mediates AMP transcription, are absent in the pea aphid based on genome annotation (Gerardo *et al*., 2010). Experimental

Correspondence: Zhiqiang Lu, Department of Entomology, College of Plant Protection, Northwest A&F University, Yangling, Shaanxi 712100, China. Tel.: + 86 29 8709 1997; e-mail: zhiqiang.lu@nwsuaf.edu.cn

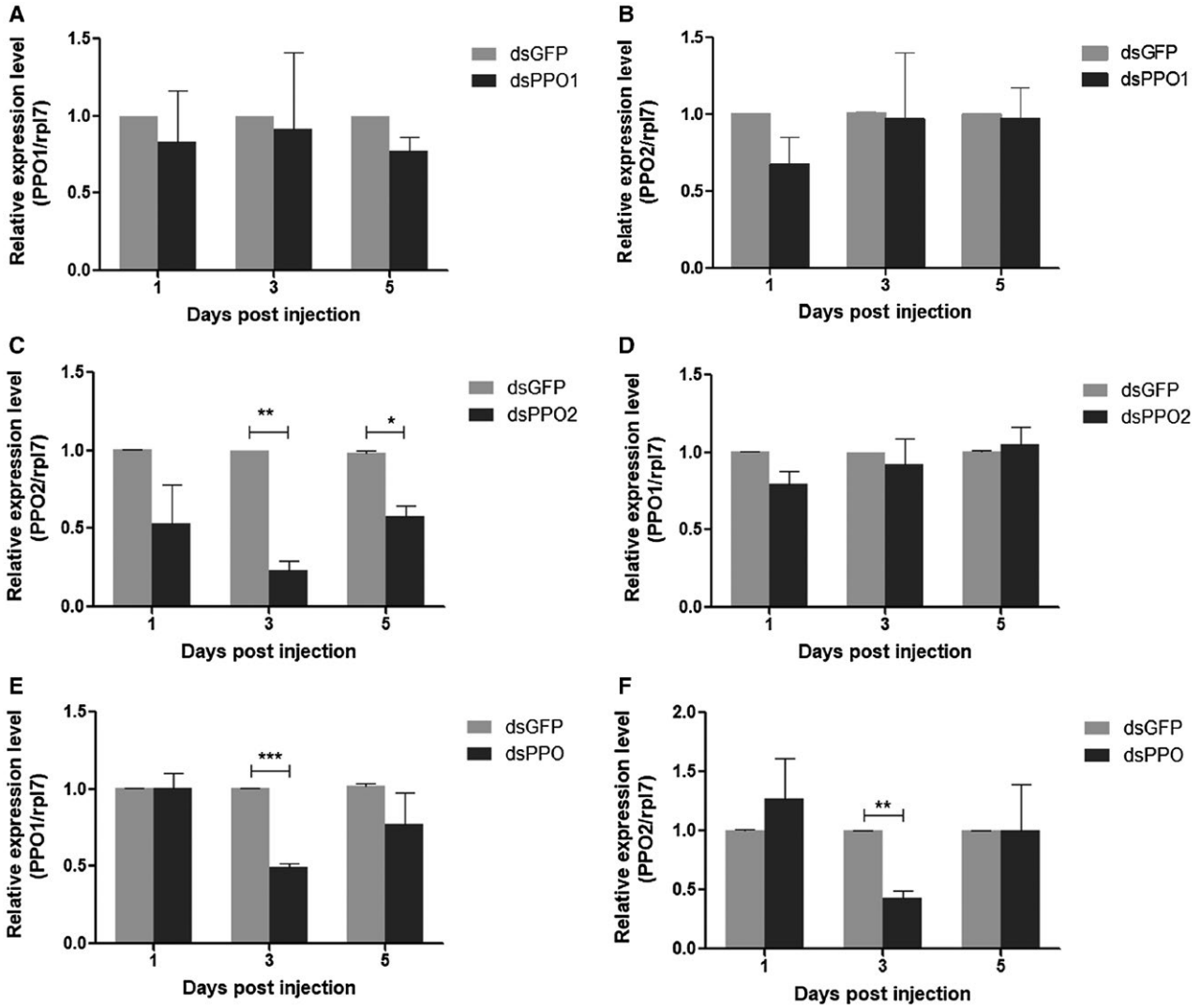

Figure 1. Efficiency of RNA interference-mediated knockdown of pea aphid prophenoloxidase 1 (PPO1), PPO2 and PPO. The relative expression levels were analysed by quantitative real-time PCR postinjection of double-stranded PPO1 (dsPPO1) (A, B), dsPPO2 (C, D) and dsPPO (E, F) in the aphids. Graphs display the mean value ± SEM in messenger RNA levels relative to the double-stranded green fluorescent protein (dsGFP)-injected group. Asterisks denote differences between an experimental group and the dsGFP group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

data further confirmed that no AMP was produced, and consequently, no antimicrobial activity was detected after bacterial infection in the pea aphid (Gerardo *et al.*, 2010; Laughton *et al.*, 2011). The pea aphid encodes two PPO genes (Gerardo *et al.*, 2010). Other studies found that a particular type of haemocyte was PO-positive, and that melanotic capsules formed around foreign objects in the pea aphid (Laughton *et al.*, 2011; Schmitz *et al.*, 2012). This evidence suggested that PO might be involved in the pea aphid's immune responses to microbial infection. To fully evaluate the contribution of PO to the pea aphid's defence against microbial infection, we knocked down the expression of *PPOs* and investigated the PO activity, aphid survival, and viability of bacteria and fungi in the aphids after infection.

Results

Knockdown of PPO expression by RNAi

The expression of pea aphid *PPO1* and *PPO2* was knockdowned by double-strand RNA. We first checked the efficiency of the knockdown of these two genes. The relative expression level was measured by quantitative real-time PCR (qPCR) conducted at days 1, 3 and 5 after injection of double-stranded RNA (dsRNA) into the aphids. We injected dsRNA targeted to different regions of the *PPO1* gene; however, none of them decreased the expression of *PPO1* or *PPO2* (Fig. 1A, B). dsPPO2 decreased *PPO2* expression significantly at days 3 and 5 postinjection (Fig. 1C), with no effect upon the expression of *PPO1* (Fig. 1D), indicating its specificity in silencing

PPO2. When we injected dsPPO, which targets a common region of *PPO1* and *PPO2*, the expression of *PPO1* and *PPO2* decreased approximately 50% on the third day (Fig. 1E, F), suggesting that *PPO1* and *PPO2* expression was knocked down simultaneously. Therefore, in the following experiments, we used dsPPO2 to knock down the expression of PPO2, and used dsPPO to knock down the expression of both PPO1 and PPO2.

Melanization of the haemolymph from PPO-knockdown aphids

We next examined spontaneous melanization of the haemolymph from the aphids in which the *PPO* expression was knocked down. As shown in Fig. 2, in 2 h, melanin synthesized spontaneously in the dsGFP (Green Fluorescent Protein)-injected pea aphid haemolymph sample, whereas only slight melanin synthesized in the dsPPO2-injected sample, and slighter melanin synthesized in the dsPPO-injected sample. These results suggest that the knockdown of *PPO* expression impedes haemolymph melanization in pea aphids.

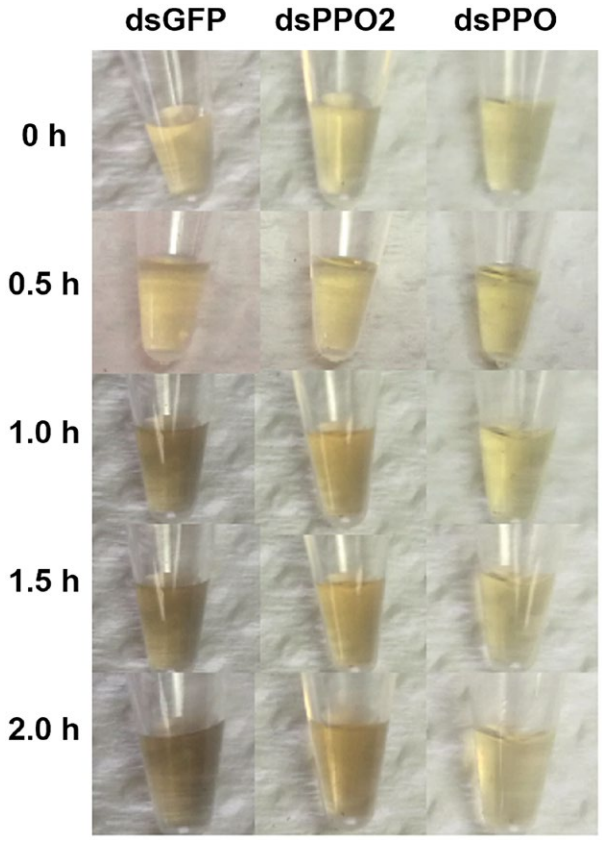

Figure 2. Spontaneous melanization of the haemolymph from prophenoloxidase (PPO)-knockdown aphids. Haemolymph was collected from the aphids injected with dsGFP, dsPPO2 and dsPPO. Melanization was recorded by photography at indicated time points. [Colour figure can be viewed at wileyonlinelibrary.com]

Haemolymph PO activity in the pea aphid after the knockdown of PPO expression

Because PO catalyses melanization, we next measured PO activity in the haemolymph from *PPO*-knockdown aphids after they were challenged by *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Beauveria bassiana* (Figs 3–5). Our results showed that neither bacterial infection nor fungal infection affected PO activity in pea aphids. Second, the knockdown of *PPO2* decreased PO activity but not significantly. However, the knockdown of *PPO* caused a decrease in PO activity in both unchallenged and challenged haemolymph samples.

Bacterial and fungal loads in the PPO-knockdown aphids

We then investigated the propagation of bacteria and fungi inside the aphids when *PPO* expression was knocked down. When the aphids were infected with *P. aeruginosa*, the knockdown of *PPO2* did not affect the growth of the bacterium in the aphids (Fig. 6A), whereas the knockdown of *PPO* resulted in a higher bacterial load in the aphids 36 h postinfection (Fig. 6B). In the *S. aureus*-challenged aphids, the knockdown of either *PPO2* or *PPO* resulted in higher bacterial loads 48 h postinfection (Fig. 7). When the aphids were challenged with *B. bassiana*, more fungal spores were found in the *PPO*-knockdown aphids (Fig. 8). These results indicate that PPO blocks the growth of bacteria and fungi invading the pea aphids.

Survival of PPO-knockdown aphids after bacterial and fungal infection

Finally, we monitored the survival of aphids after *PPO* knockdown and infection. Under the uninfected conditions, the knockdown of either *PPO2* or *PPO* caused the death of the aphids. The knockdown of *PPO2* and *PPO* did not affect the survival of the aphids infected by *P. aeruginosa* (Fig. 9). In the *S. aureus*-infected groups, the knockdown of either *PPO2* or *PPO* resulted in the aphids being more susceptible to infection (Fig. 10), whereas in the fungal-infected groups, the knockdown of *PPO* caused the aphids to be more susceptible (Fig. 11).

Discussion

The PPO pathway is a conserved immune response in invertebrates (Cerenius *et al*., 2008). In the pea aphid, previous studies have shown that PO activity is present in whole body crude enzyme solution (Odonbayar *et al*., 2016), in the salivary glands (Cherqui and Tjallingii, 2000) and in a specific type of haemocyte (Laughton *et al*., 2011). It has also been suggested that PO might be involved in defence against infection (Laughton *et al*., 2011; Schmitz *et al*., 2012). In this study, we present supporting evidence of the contribution of PO to the

defence of the pea aphid against bacterial and fungal infections. dsRNA-based knockdown of *PPO* genes expression demonstrated that decreased PO activity resulted in more bacterial and fungal cells growing inside the aphids and higher death rates of the aphids after infection, particularly by the Gram-positive bacterium *S. aureus* and the fungus *B. bassiana*. Our study highlights the significant role of PO in the pea aphid immune system, particularly in the circumstance that the antimicrobial peptide pathway is absent. However, further study is needed to investigate PPO distribution in the tissues and its activation and regulation in the pea aphid.

In addition to the role in defence against infection, insect PO plays a role as a detoxifying factor to sequester host plant-derived phenolics, such as L-3,4-dihydroxyphenylalanine (L-DOPA) (Wu *et al*., 2015). In fact, the pea aphid PO is involved in the assimilation of L-DOPA from the broad bean for wound healing and UVA radiation protection (Zhang *et al*., 2016). In the grain aphid *Sitobion avenae*, it was shown that laccase 1, a member of the multicopper oxidase family, is involved in the detoxification of phenolic compounds from the wheat host plant (Zhang *et al*., 2018). These studies imply a potential role of PO in the interaction between the aphid and plant. A study in the grain aphid suggested that PPO in the aphid saliva triggers certain defence response in wheat (Ma *et al*., 2010).

In conclusion, we knocked down the expression of the pea aphid *PPO* genes and showed that PO activity is responsible for the counteraction of bacterial and fungal infection and the survival of the infected aphids. Our work provides clues for the dissection of the pea aphid immune system, along with reinforcing the critical role of PO in the insect immune responses to infections.

Experimental procedures

Aphid rearing

The *A. pisum* strain used in this study was collected originally from Yunnan Province, China. The colony was maintained on *Vicia faba* (broad bean) seedlings in a laboratory artificial climate chamber under the following conditions: a temperature of 20 ± 1 ℃, a photoperiod of 16L:8D and a relative humidity of 70 ± 5%. Some healthy female aphids were placed on broad bean seedlings and allowed to produce progeny for 2 days. Then, the adults were removed; the nymphs continued to be raised on broad bean seedlings until they became wingless adult aphids. These adult aphids were used in the following experiments.

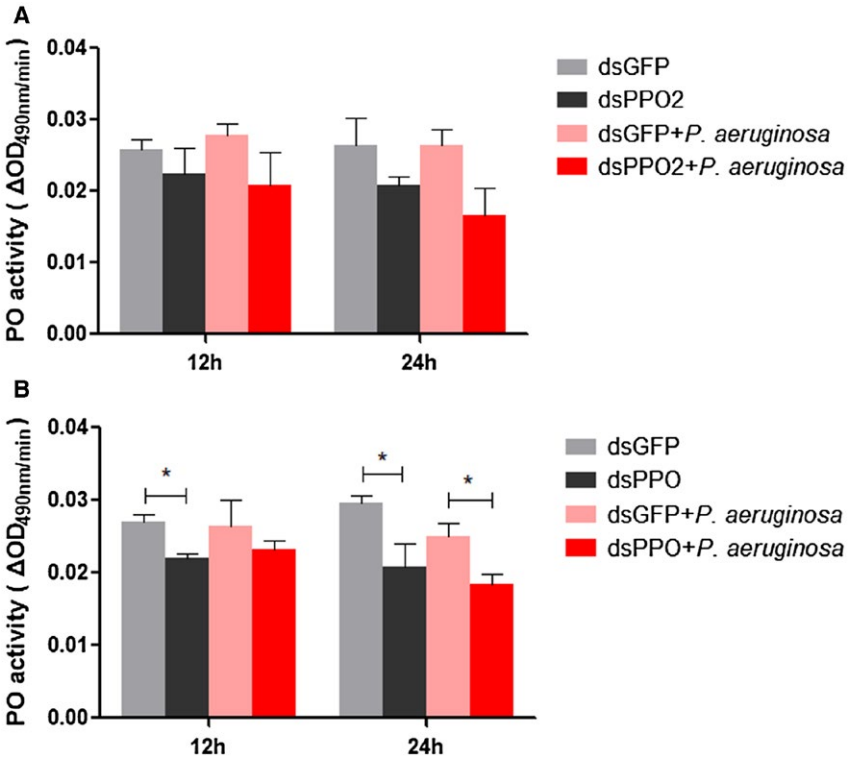

Figure 3. Phenoloxidase (PO) activity in the haemolymph from dsPPO2 (A) and dsPPO (B) injected-aphids infected by *Pseudomonas aeruginosa*. Forty-eight hours after double-stranded RNA injection, the aphids were challenged with *P. aeruginosa*. Haemolymph samples were collected at 12 and 24 h postinfection from at least five aphids from each group and subjected to activity assays. Each value is given as the mean ± SEM of three replicates. Asterisks denote differences (*, $P < 0.05$) between the indicated groups. [Colour figure can be viewed at wileyonlinelibrary.com]

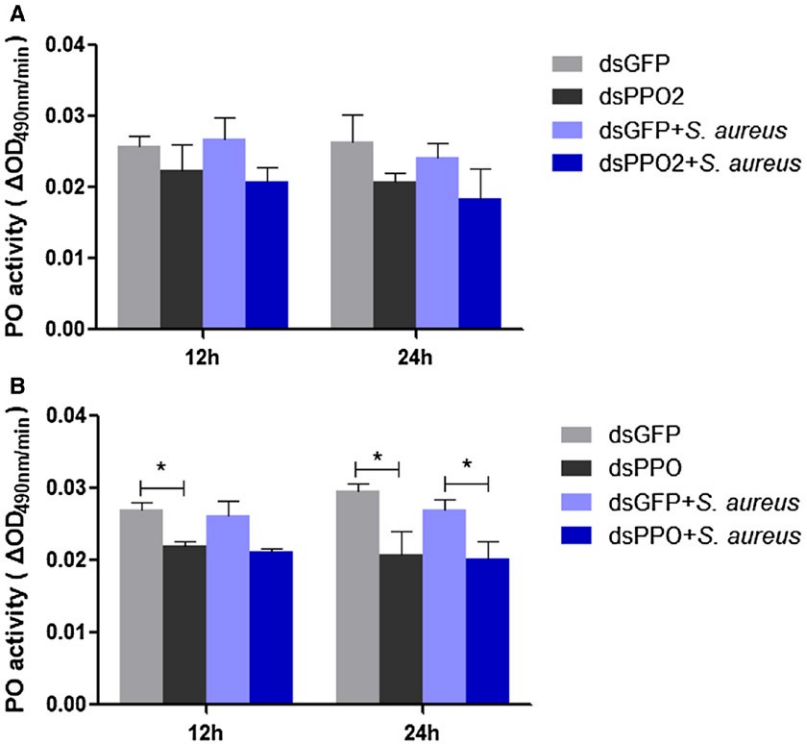

Figure 4. Phenoloxidase (PO) activity in the haemolymph from dsPPO2 (A) and dsPPO (B) injected-aphids infected by *Staphylococcus aureus*. Forty-eight hours after double-stranded RNA injection, the aphids were challenged with *S. aureus*. Haemolymph samples were collected at 12 and 24 h postinfection from at least five aphids from each group and subjected to activity assays. Each value is given as the mean ± SEM of three replicates. Asterisks denote differences (*, $P < 0.05$) between the indicated groups. [Colour figure can be viewed at wileyonlinelibrary.com]

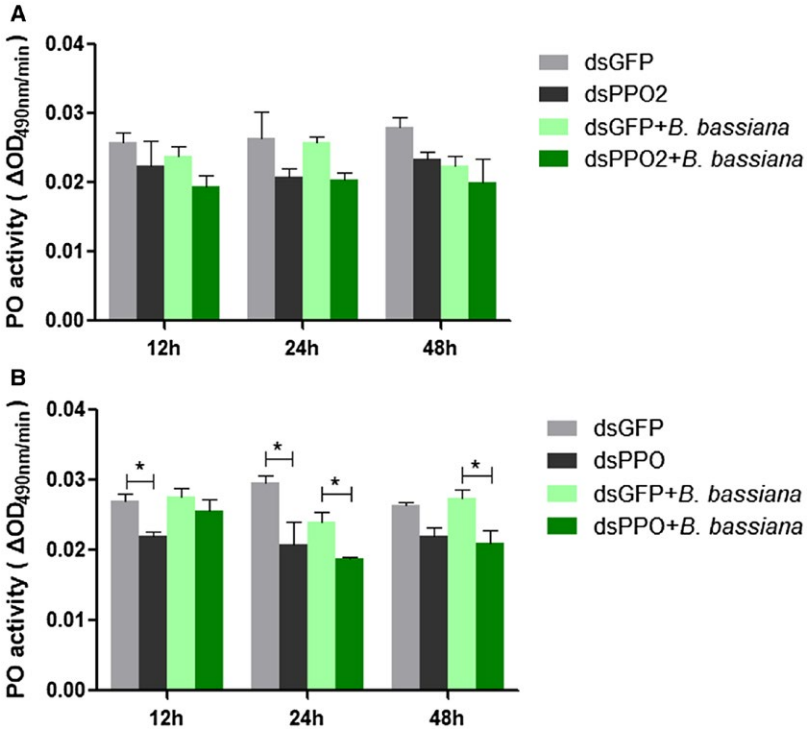

Figure 5. Phenoloxidase (PO) activity in the haemolymph from dsPPO2 (A) and dsPPO (B) injected-aphids infected by *Beauveria bassiana*. Forty-eight hours after double-stranded RNA injection, the aphids were challenged with *B. bassiana*. Haemolymph samples were collected at 12, 24 and 48 h postinfection from at least five aphids from each group and subjected to activity assays. Each value is given as the mean ± SEM of three replicates. Asterisks denote differences (*, $P < 0.05$) between the indicated groups. [Colour figure can be viewed at wileyonlinelibrary.com]

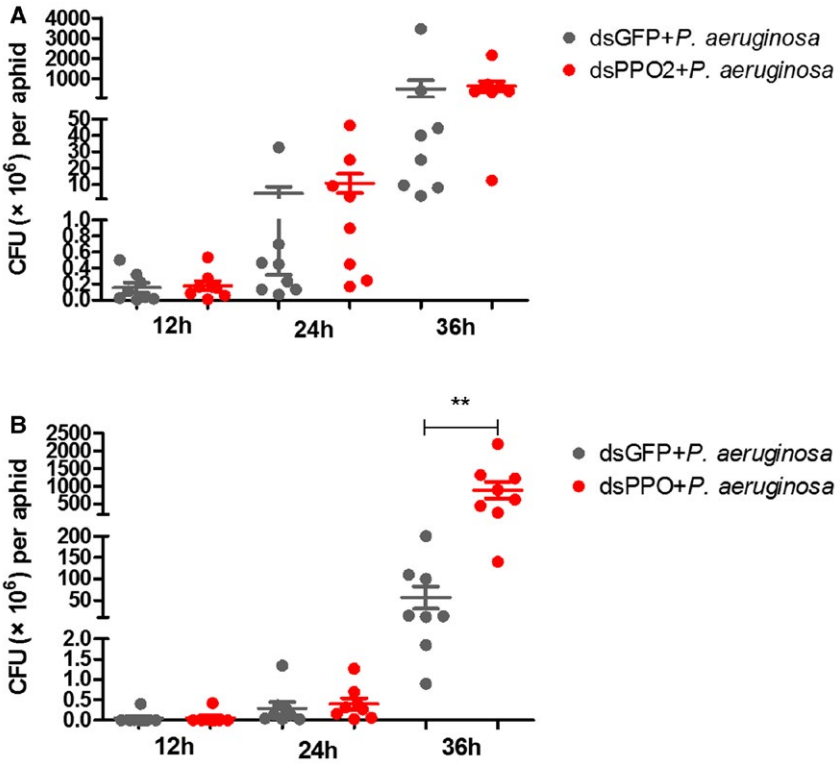

Figure 6. Colony forming units (CFU) of *Pseudomonas aeruginosa* in the dsPPO2 (A) and dsPPO (B) injected-aphids. Forty-eight hours after double-stranded RNA injection, the aphids were challenged with *P. aeruginosa*. Aphids from dsGFP-, dsPPO2- and dsPPO-injected groups were selected at 12, 24 and 36 h postinfection and homogenized individually. The homogenates were spread on Luria–Bertani plates, and the colonies were counted when visible. Each dot in the graph represents an individual aphid. The horizontal bars indicate mean values, and the vertical bars indicate the SEM of the replicates. Asterisks denote differences (**, $P < 0.01$) between the indicated groups. [Colour figure can be viewed at wileyonlinelibrary.com]

Infections of pea aphids with bacteria and fungus

For bacterial infections, *P. aeruginosa* and *S. aureus* were cultured to logarithmic phase at an optical density (OD_{600nm}) of approximately 1 and harvested by centrifugation. *P. aeruginosa* and *S. aureus* cells were resuspended in a sterile 0.85% NaCl solution at 10^9 and 2×10^{11} colony forming units (CFU)/ml. The adult aphids were placed on ice. The end of a capillary tube with an ~4-mm-long tip was sealed with Parafilm (Sigma, Saint Louis, MO, USA). The tip was dipped in the bacteria preparations and then immediately pricked dorsolaterally through the abdominal wall into the aphid to approximately 1 mm in depth. The aphids of the control group were treated with sterile 0.85% NaCl solution (Altincicek *et al.*, 2011).

For *B. bassiana* infections, *B. bassiana* was cultivated on a potato dextrose agar (PDA) plate and cultured for 5–7 days at 28 ℃ until the plate was full of white hyphae and conidia. Then, the mycelium was picked with a pipette tip and placed in an Eppendorf tube (Eppendorf, Hamburg, Germany) with 1 ml sterile H_2O; the mycelium was mixed, then transferred to an Eppendorf tube filled with absorbent cotton and centrifuged for 5 min at 8000 *g* to isolate hyphae and spores. We added 0.05% Tween-20 (Sigma, Saint Louis, MO, USA), counted the fungal spores using a haemocytometer under an inverted microscope and diluted them to 2×10^7 spores/ml with 0.05% Tween-20.

RNA extraction and cDNA synthesis

The total RNA was extracted from the pea aphid using Tripure Isolation Reagent (Roche, Basel, Switzerland) and purified using a Direct-Zol RNA Miniprep kit (Zymo Research, Irvine, CA, USA). cDNA synthesis was performed according to the manufacturer's recommended procedure using a First Strand cDNA Synthesis kit (Roche).

qPCR

qPCR was used to measure the expression levels of *PPO1* and *PPO2* in *A. pisum* after RNAi. The messenger RNA sequences of the pea aphid *PPO1* (accession number: XM_001949272.3) and *PPO2* (accession number: XM_001951102.3) genes were retrieved from the National Center for Biotechnology Information website (https://www.ncbi.nlm.nih.gov/). Primer Premier 5.0

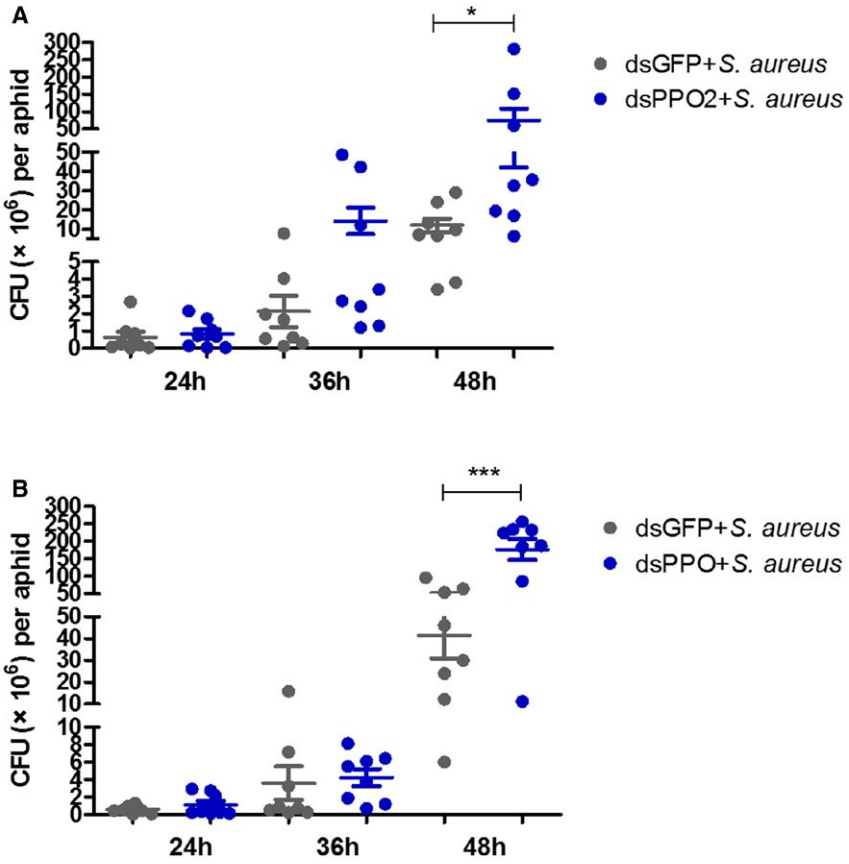

Figure 7. Colony forming units (CFU) of *Staphylococcus aureus* in the dsPPO2 (A) and dsPPO (B) injected-aphids. Forty-eight hours after double-stranded RNA injection, the aphids were challenged with *S. aureus*. Aphids from dsGFP-, dsPPO2- and dsPPO-injected groups were selected at 24, 36 and 48 h postinfection and homogenized individually. The homogenates were spread on Luria–Bertani plates, and the colonies were counted when visible. Each dot in the graph represents an individual aphid. The horizontal bars indicate mean values, and the vertical bars indicate the SEM of the replicates. Asterisks denote differences (*, $P < 0.05$; ***, $P < 0.001$) between the indicated groups. [Colour figure can be viewed at wileyonlinelibrary.com]

Table 1. Primers used in this study

Primer names	Primer sequences	Product size (bp)
Quantitative real-time PCR		
PPO1-F	GCTATTGTGGTATTCGTAA	191
PPO1-R	CTGTTGGCTTCCTATTCTGT	
PPO2-F	CACTGTCCGTAGCATTGAT	179
PPO2-R	GGCAGAATAATCGTGAGGTA	
Rpl7-F	TTGAAGAGCGTAAGGGAACTG	76
Rpl7-R	TATTGGTGATTGGAATGCGTTG	
dsRNA synthesis		
dsGFP-F	*GTGTTCAATGCTTTTCCCGT	100
dsGFP-R	*CAATGTTGTGGCGAATTTTG	
dsPPO1-F	*GAATCAGTTGAGTAACAGTCT	415
dsPPO1-R	*GGCTAACGACAGTAATTCATC	
dsPPO2-F	*GGGAACCAGTAATAACAACAAG	403
dsPPO2-R	*GGTCGAATAGTCAATGTAGGT	
dsPPO-F	*CAGAGGGTTCCAGTGTCAAT	453
dsPPO-R	*CCCCTAACGACTGTTCGTGT	

*These are preceded by the T7 adaptor TAATACGACTCACTATAGGG for dsRNA synthesis.

[a]dsRNA, double-stranded RNA; F, forward; R, reverse; PPO, prophenoloxidase; Rpl7, ribosomal protein L7.

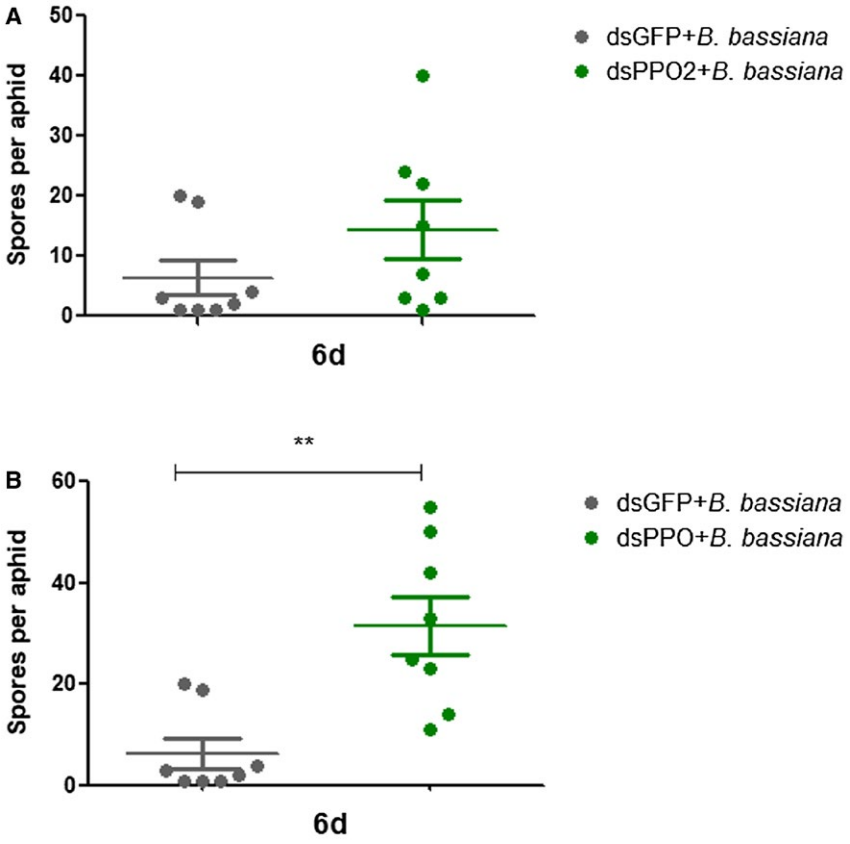

Figure 8. Spore numbers of *Beauveria bassiana* in the dsPPO2 (A) and dsPPO (B) injected-aphids. Forty-eight hours after double-stranded RNA injection, the aphids were challenged with *B. bassiana*. Aphids from dsGFP-, dsPPO2- and dsPPO-injected groups were selected at 6 days postinfection and homogenized individually. The homogenates were spread on potato dextrose agar plates, and the spores were counted after 5 days of incubation. Each dot in the graph represents an individual aphid. The horizontal bars indicate mean values, and the vertical bars indicate the SEM of the replicates. Asterisks denote differences (**, $P < 0.01$) between the indicated groups. [Colour figure can be viewed at wileyonlinelibrary.com]

software (PREMIER Biosoft, Palo Alto, CA, USA) was used to design the sequences used for PCR reactions (Table 1). qPCR was performed on a Rotor Q thermocycler (Qiagen, Hilden, Germany) using FS Essential DNA Green Master mix (Roche) under the following conditions: 95 ℃ for 10 min, followed by 40 cycles at 95 ℃ for 10 s, 54 ℃ for 20 s and 72 ℃ for 20 s. Melting curves (65–95 ℃) were performed to confirm the identity of the PCR product. The pea aphid *ribosomal protein L7* gene was used as the endogenous reference (Nakabachi *et al*., 2005). The results were calculated using a relative quantitative method ($2^{-\Delta\Delta Ct}$, 2 to the power of minus Delta Delta CT). All qPCR analyses were performed with three biological replicates.

dsRNA synthesis and RNAi

The primers used for dsRNA synthesis were designed at https://www.flyrnai.org/cgi-bin/RNAi_find_primers.pl. Using the synthesized cDNA as a template, the *PPO1* and *PPO2* fragments were amplified by PCR using primers with a T7 promoter sequence (Table 1). The resulting PCR products were purified using a Gel Extraction kit (Omega, Norcross, GA, USA) and used as the templates for synthesis of dsRNA. dsRNA was synthesized following the procedure of the T7 RiboMAX™ Express RNAi System (Promega, Madison, WI, USA). The quality of the dsRNA was examined using ultraviolet spectrophotometry and agarose gel electrophoresis. GFP dsRNA was cloned from the pGLO vector and used as the control. The dsRNA was diluted to 8 μg/ml and stored at −80 ℃. The adult pea aphids were CO_2-anaesthetized and injected with 46 nanolitres of dsRNA at the dorsal site of the abdomen using a Nanoject III micro-injector (Drummond Scientific, Broomall, PA, USA) with glass capillaries prepared on a Micropipette Puller (Sutter Instrument Co., Novato, CA, USA). The aphids were then transferred to broad bean seedlings and collected at each time point for RNA preparation or bacterial and fungal infections. RNAi efficiency was determined by measuring the *PPO1* and *PPO2* expression levels.

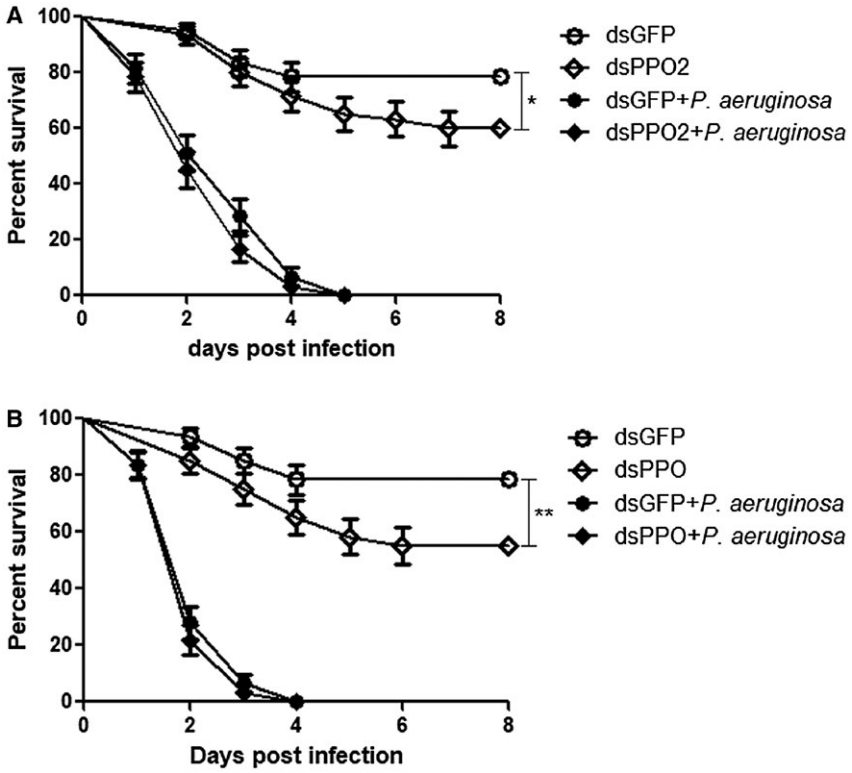

Figure 9. Survival of the dsPPO2 (A) and dsPPO (B) injected-aphids after *Pseudomonas aeruginosa* infection. Forty-eight hours after double-stranded RNA injection, the pea aphids were infected. The survival of aphids was recorded every day, and the curves between different groups were analysed with a log-rank (Mantel–Cox) test. *, $P < 0.05$; **, $P < 0.01$. The bars indicate the SEM of three replicates. Note: each group was composed of 30 aphids.

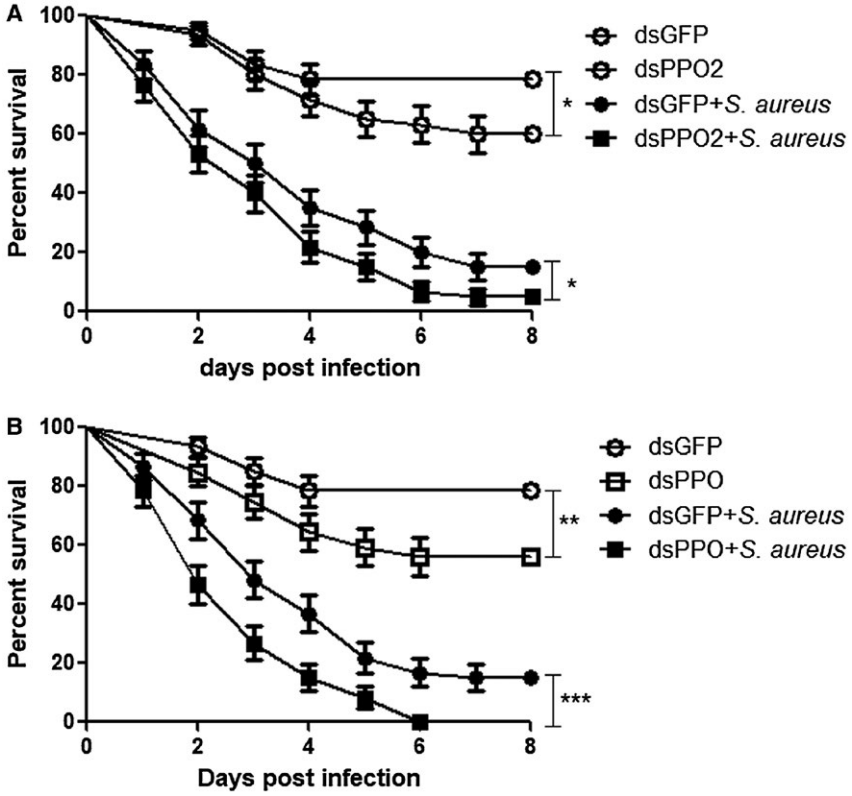

Figure 10. Survival of the dsPPO2 (A) and dsPPO (B) injected-aphids after *Staphylococcus aureus* infection. Forty-eight hours after double-stranded RNA injection, the pea aphids were infected. The survival of the aphids was recorded every day, and the curves between different groups were analysed with a log-rank (Mantel–Cox) test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. The bars indicate the SEM of three replicates. Note: each group was composed of 30 aphids.

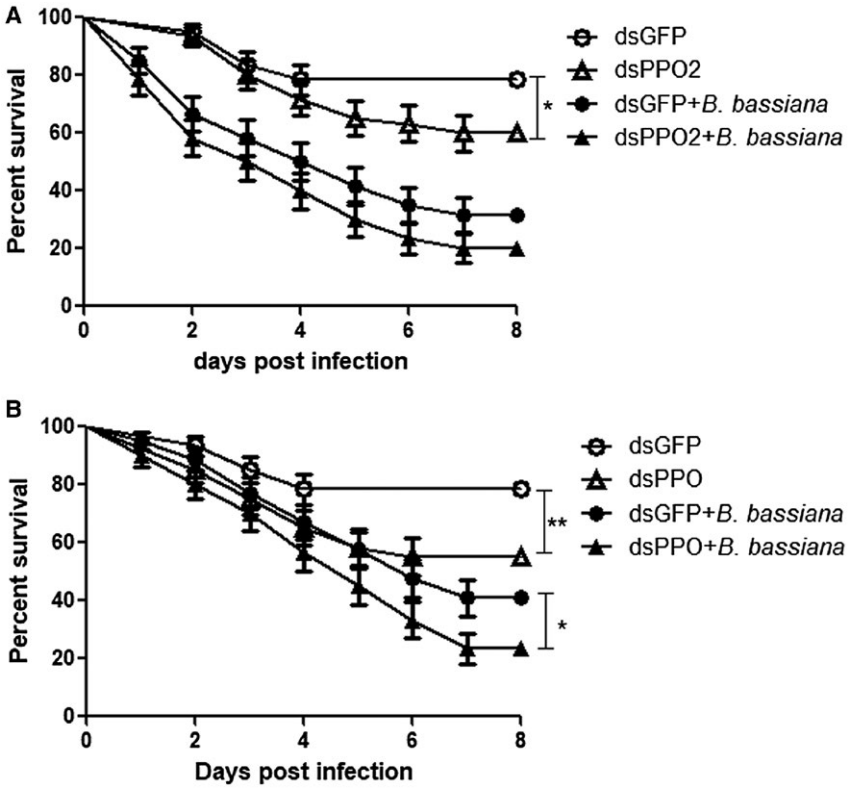

Figure 11. Survival of the dsPPO2 (A) and dsPPO (B) injected-aphids after *Beauveria bassiana* infection. Forty-eight hours after double-stranded RNA injection, the pea aphids were infected. The survival of the aphids was recorded every day, and the curves between different groups were analysed with a log-rank (Mantel–Cox) test. *, $P < 0.05$; **, $P < 0.01$. The bars indicate the SEM of three replicates. Note: each group was composed of 30 aphids.

Aphids' survival rate, bacterial CFU, and fungal spore count after RNAi

Forty-eight hours after dsRNA injection, the pea aphids were infected with bacteria and fungus as described. The survival rates of aphids were recorded every 24 h for 8 days.

To determine bacterial CFU, the aphids were surface-sterilized with 75% ethanol and then washed twice with 0.85% NaCl solution until the ethanol was completely clear. Each aphid was then ruptured in 200 µl sterilized 0.85% NaCl solution. After diluting the mixture to a suitable concentration, 10 µl of the mixture was evenly spread on Luria–Bertani plates and cultured at 37 ℃.

For fungal spore counting, the aphid samples were obtained as above; 200 µl of the mixture was evenly spread on a PDA plate. The fungal spores were counted after a 5-day incubation at 28 ℃.

Phenoloxidase activity assay

Forty-eight hours after dsRNA injection, the aphids were infected as described above. The aphids were decapitated, put in an Eppendorf tube filled with absorbent cotton and then centrifuged at 800 *g* for 10 min to collect haemolymph. Two µl of haemolymph and 100 µl of 2 mM dopamine (in 50 mM sodium phosphate, pH 6.5) were mixed in each well of a 96-well plate, and the absorbance at 490 nm was immediately measured on a microplate reader (Tecan Pro200, Tecan, Männedorf, Switzerland). The absorbance was measured every 30 s for 30 min, and the maximum slope was read optimally. PO activity was shown as the maximum slope, which was defined as the increase of absorbance at 490 nm per minute (Jiang *et al*., 2003). Activity assays were independently repeated using haemolymph samples from three biological replicates.

Melanization assay of the aphid haemolymph

The pea aphids were treated as in the '*Phenoloxidase activity assay*' section, and the haemolymph was collected on the third day after RNAi. Five µl of haemolymph and 15 µl phosphate-buffered saline were mixed in an Eppendorf tube and placed at room temperature for 2 h to observe melanization.

Statistical analyses

All the data in this experiment were plotted using PRISM 5.0 (GraphPad Software Inc., La Jolla, CA, USA); the control and treatment groups were compared using Student's *t*-tests for data analysis. A log-rank (Mantel–Cox) test was used to analyse the survival curves.

Acknowledgements

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