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Two atypical gram-negative bacteria-binding proteins are involved in the antibacterial response in the pea aphid (*Acyrthosiphon pisum*)

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Abstract

The activation of immune pathways is triggered by the recognition of pathogens by pattern recognition receptors (PRRs). Gram-negative bacteria-binding proteins (GNBPs)/β-1,3-glucan recognition proteins (βGRPs) are a conserved family of PRRs in insects. Two GNBPs are predicted in the genome database of pea aphids; however, little is known about their functions in the aphid immune system. Here, we show that pea aphid GNBPs possess domain architectures and sequence features distinct from those of typical GNBPs/βGRPs and that their expression is induced by bacterial infection. Knockdown of their expression by dsRNA resulted in lower phenoloxidase activity, higher bacterial loads and higher mortality in aphids after infection. Our data suggest that these two atypical GNBPs are involved in the antibacterial response in the pea aphid, likely acting as PRRs in the prophenoloxidase pathway.

Keywords: pea aphid, gram-negative bacteria-binding protein, phenoloxidase, RNA interference, antibacterial response.

1. Introduction

Insects rely on the innate immune system for protection from pathogens. The first step in mounting an immune response is the sensing of pathogens and the recognition of pathogens as a danger (Gillespie *et al*., 1997). Pathogen recognition in insects is achieved through the interaction of pathogen-associated molecular patterns (PAMPs, such as peptidoglycans and β-glucans) and pattern recognition receptors (PRRs) (Welchman *et al*., 2009). Several families of PRRs, including peptidoglycan recognition proteins (PGRPs), gram-negative bacteria-binding proteins (GNBPs), and scavenger receptors (SRs), have been identified in a variety of insect species (Wang *et al*., 2019; Lu *et al*., 2020). Intriguingly, the pea aphid genome encodes two GNBPs and two β-galactoside binding lectins (galectins) but no other PRRs (Gerardo *et al*., 2010).

GNBPs and β-1,3-glucan recognition proteins (βGRPs) belong to the same PRR family that recognizes β-1,3-glucan, a surface component of fungi and bacteria. Typically, GNBPs/βGRPs consist of a carbohydrate-binding module (CBM) at the N-terminus and a glucanase-like domain (Glu) in the C-terminus (Rao *et al*., 2018). The CBM interacts with microbial polysaccharides and the Glu domain interacts with downstream proteases, triggering immune pathways (Mishima *et al*., 2009; Kanagawa *et al*., 2011; Dai *et al*., 2013; Takahashi *et al*., 2014; Takahashi *et al*., 2015). GNBPs recognize bacterial and fungal pathogens, leading to the activation of immune signalling pathways in insects (Lee *et al*., 1996; Kim *et al*., 2000; Warr *et al*., 2008). Specifically, in *Drosophila*, GNBP1 and peptidoglycan-recognition protein-SA (PGRP-SA) jointly activate the Toll pathway in response to gram-positive bacterial infections (Gobert *et al*., 2003; Pili-Floury *et al*., 2004), whereas GNBP3 is required for Toll pathway activation in response to fungal infections (Gottar *et al*., 2006). The tobacco hornworm *Manduca sexta* βGRP2 binds to laminarin, a water-soluble polysaccharide that consists of β-(1-3)-glucan with β-(1-6)-linkages, and stimulates prophenoloxidase (proPO) activation (Jiang *et al*., 2004). GNBPs have also been found to be involved directly in the cuticular antifungal defence of *Reticulitermes* subterranean termites (Hamilton and Bulmer, 2012).

The pea aphid (*Acyrthosiphon pisum*) has reduced and limited immune responses. Genes encoding antibacterial peptides, PGRPs, and key components of the immune deficiency (IMD) pathway are missing in its genome

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(Gerardo *et al*., 2010). However, we have proved that phenoloxidase (PO) and the JNK (Jun N-terminal kinase) pathway are required for the pea aphid defence against bacterial and fungal infections (Xu *et al*., 2019; Ma *et al*., 2020). To date, no PRR has been functionally studied in aphids. Therefore, in this study, we analysed, for the first time, the expression of two putative GNBPs (GNBP1: XM_001944438.5 and GNBP2: XM_029486299.1) in the pea aphid after bacterial infection. We further knocked down their expression by RNA interference (RNAi) and examined PO activity and bacterial loads in and survival of the aphids.

2. Results

2.1 *Pea aphid GNBP1 and GNBP2 are atypical members of the GNBP family*

Typically, insect GNBPs contain a CBM at the N-terminus and a Glu domain at the C-terminus. This can also be observed in the fruit fly GNBP3 (DROME_GNBP3; Fig. 1A). GNBPs from the pea aphid, green peach aphid, yellow sugarcane aphid, cotton aphid, and corn aphid contain six tandem cell wall integrity and stress response component (WSC) domains in their N-termini. GNBPs of bed bug, stink bug, and amphioxus (*Branchiostoma belcheri*) have four, three, and two tandem WSC domains, respectively, in their N-termini. Interestingly, the whitefly has three GNBPs; one contains nine, one contains six, and one contains three tandem WSC domains.

We compared the sequences of the Glu domain in GNBP/βGRP from amphioxus (*B. belcheri*) and other insects (Fig. 1B). Only the amphioxus GNBP (BRABE_GNBP) has the four critical residues (W, E, D, and E) that are required for glucanase activity (indicated by black dots above the residues in Fig. 1B; Rao *et al*., 2014). Among the 10 conserved amino acid residues in GNBP/βGRP (indicated by arrows; Hughes, 2012), eight are common among different species (indicated by blue arrows in Fig. 1B). Interestingly, in the amphioxus and pea aphid GNBPs, two sites are altered (indicated by red arrows in Fig. 1B). Lysine (K) replaces aspartate (D) and, notably, proline (P) is replaced by serine (S) in the pea aphid GNBP2. These mutations may impair their function as PRRs. Phylogenetic analysis also revealed that pea aphid GNBPs formed a distinct group.

2.2 *Expression of GNBPs in the pea aphid is induced by bacterial infection*

We first examined whether the pea aphid *GNBP1* and *GNBP2* responded to infection. Using qPCR, we found that *GNBP1* expression increased at 6 and 12 h after infection with *Escherichia coli* (Fig. 2A). *GNBP2* expression increased at 6 h after infection with *E. coli* and *Staphylococcus aureus* (Fig. 2B). This result suggests that GNBP1 and GNBP2 might be involved in the defence against infection in pea aphids.

2.3 *Knockdown of pea aphid GNBP genes*

To investigate the roles of GNBP1 and GNBP2 in the defence of pea aphids, we knocked down their expression using RNAi. By injecting gene-specific dsRNA, the expression of *GNBP1* and *GNBP2* was silenced already 3 or 4 days post-injection, respectively (Fig. 3A, B). The silencing of *GNBP1* had no effect on the expression of *GNBP2* and *vice versa* (data not shown). By sequential injection of dsRNA, we successfully knocked down the expression of *GNBP1* and *GNBP2* (Fig. 3C, D).

2.4 *Knockdown of GNBP genes resulted in decreased haemolymph PO activity in the aphid*

Next, we measured PO activity in the haemolymph of GNBP-knockdown aphids. Overall, when a single (Fig. 4A, B) or both (Fig. 4C, D) GNBP genes were knocked down, PO activity decreased under both infected and uninfected conditions. These results imply that GNBPs play a role in the PO pathway.

2.5 *Knockdown of GNBP genes resulted in higher bacterial loads in the aphids*

We further examined bacterial proliferation in the *GNBP*-knockdown aphids. The number of *E. coli* cells at 24 and 36 h post-infection was significantly higher in the *GNBP1*-knockdown aphids than in the ds-green fluorescent protein (GFP)-injected aphids (Fig. 5A). In addition, the number of *S. aureus* cells post-infection was slightly higher in the *GNBP1*-knockdown aphids than in the control aphids (Fig. 5B). The knockdown of *GNBP2* resulted in higher *S. aureus* loads 24 h post infection (Fig. 5B) but had no effect on *E. coli* loads (Fig. 5A). The numbers of *E. coli* (Fig. 5C) and *S. aureus* (Fig. 5D) cells were higher in aphids with knockdown of both GNBP genes than in the control aphids. These results suggest that GNBPs participate in controlling the proliferation of bacteria in pea aphids.

2.6 *Knockdown of GNBP genes increased susceptibility to bacterial infection in the aphid*

Finally, we monitored the survival of *GNBP*-knockdown aphids after bacterial infection. Under uninfected conditions, the *GNBP2*-knockdown aphids showed significantly higher mortality than did dsGFP-injected aphids (Fig. 6A, B). When infected with *E. coli*, both *GNBP1* and *GNBP2* single knockdown aphids showed higher mortality than that of the controls (Fig. 6A). When infected with *S. aureus*, *GNBP1*-knockdown aphids showed significantly higher mortality than did the control aphids, whereas the mortality of *GNBP2*-knockdown aphids was only slightly higher than

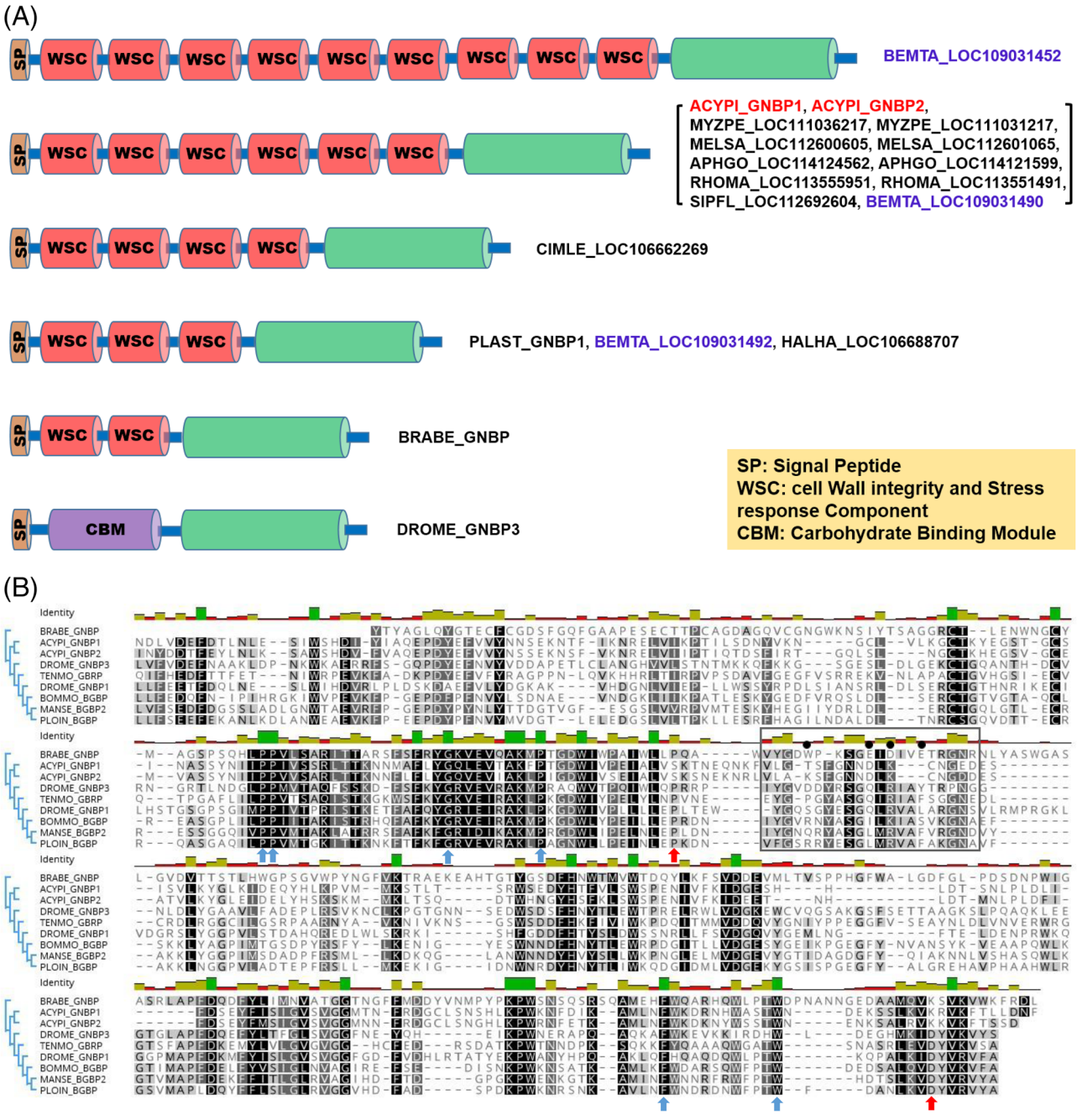

Figure 1. Domain architecture of GNBPs (A) and alignment of sequences of the glucanase-like domain in GNBPs and βGRPs (B). Species: ACYPI, *Acyrthosiphon pisum*; APHGO, *Aphis gossypii*; BEMTA, *Bemisia tabaci*; BOMMO, *Bombyx mori*; BRABE, *Branchiostoma belcheri*; CIMLE, *Cimex lectularius*; DROME, *Drosophila melanogaster*; HALHA, *Halyomorpha halys*; MANSE, *Manduca sexta*; MELSA, *Melanaphis sacchari*; MYZPE, *Myzus persicae*; PLAST, *Plautia stali*; PLOIN, *Plodia interpunctella*; RHOMA, *Rhopalosiphum maidis*; SIPFL, *Sipha flava*; TENMO, *Tenebrio molitor*. Access numbers: ACYPI_GNBP1, XP_001944473.2; ACYPI_GNBP2, XP_029342159.1; BOMMO_BGBP, Q9NL89; BRABE_GNBP, AFR24264.1; DROME_GNBP1, Q9NHB0; DROME_GNBP3, Q9NHA8; MANSE_BGBP2, Q8ISB6; PLAST_GNBP1, BBE08129.1; PLOIN_BGBP, Q8MU95; TENMO_BGRP, Q76DI2. The black lines boxed the glucanase activity region and the four critical residues (W, E, D, and E) are indicated by black dots above the residues. The 10 conserved amino acid residues in GNBP/βGRP are indicated by arrows, blue arrows indicate eight conserved residues in all of the species, and red arrows indicate variations. [Colour figure can be viewed at wileyonlinelibrary.com].

that of the controls (Fig. 6B). In the groups of aphids in which both GNBPs were knocked down, higher mortality was found under infection with *E. coli* (Fig. 6C) and *S. aureus* (Fig. 6D) than in the control groups. These results indicate that GNBPs contribute to defence against bacterial infection in pea aphids.

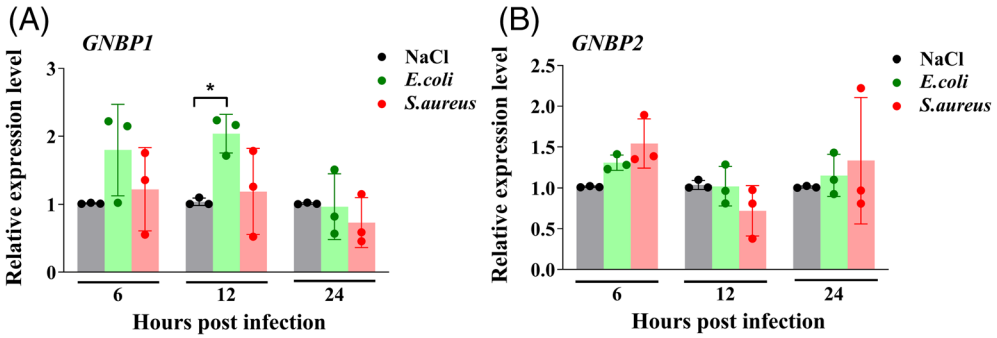

Figure 2. Bacterial infections induced expression of *GNBPs* in the pea aphids. The expression levels of *GNBP1* (A) and *GNBP2* (B) were detected 6, 12, and 24 h post infection by qPCR analysis, with sterilized 0.85% NaCl solution as the control, and *Rpl7* was used as the reference gene. All samples were analysed in triplicate from three independent experiments. One-way ANOVA with a Bonferroni test was used for significance analysis, asterisks indicate significance of the difference between the compared groups. * indicates $P < 0.05$. [Colour figure can be viewed at wileyonlinelibrary.com].

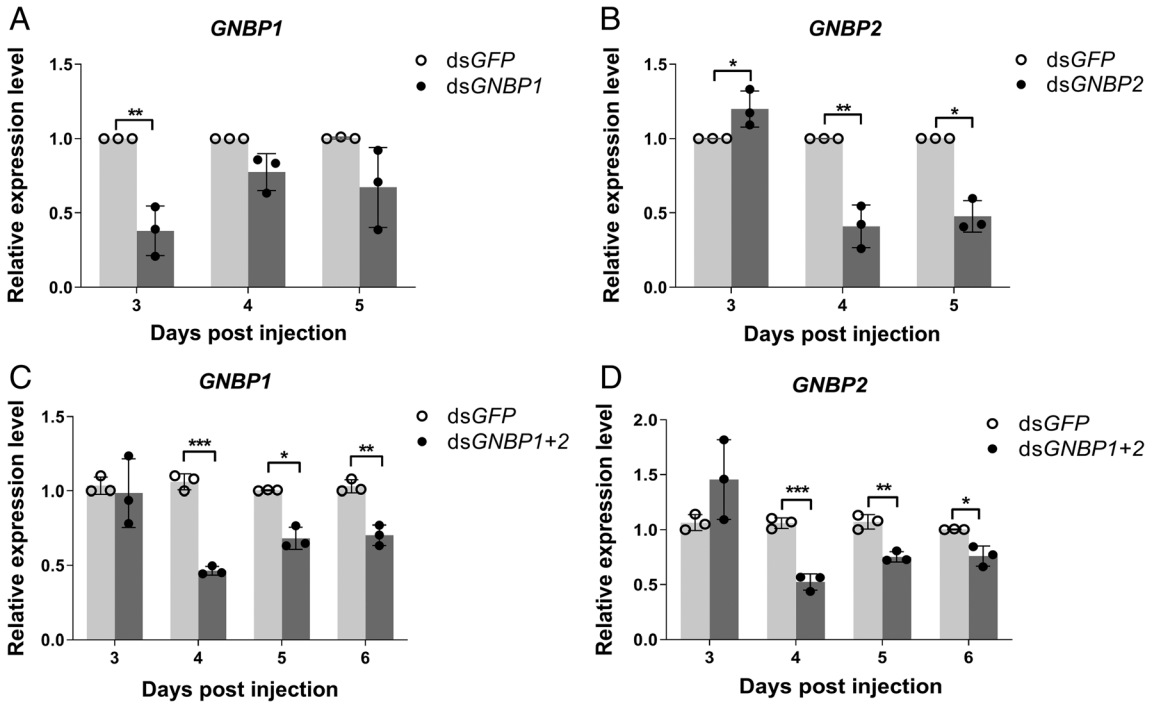

Figure 3. Efficiency of knock-down of expression of *GNBPs* by RNA interference. Relative expression of *GNBP1* after injection of dsGNBP1 (A), expression of *GNBP2* after injection of dsGNBP2 (B), expression of GNBP1 after injection of dsGNBP1 + 2 (C), expression of GNBP2 after injection of dsGNBP1 + 2 (D) was measured by qPCR. In the control group, the aphids were injected with dsGFP. The *Rpl7* was used as the reference gene. Paired *t* test was used for significance analysis, asterisks show significance of the difference between the compared groups. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

3. Discussion

As pattern recognition receptors, GNBPs/βGRPs sense bacterial and fungal pathogens. This sensing leads to the activation of defence pathways and immune responses (Lee *et al*., 1996; Kim *et al*., 2000; Warr *et al*., 2008; Takahashi *et al*., 2014; Manniello *et al*., 2021). In this study, we preliminarily investigated the function of two GNBPs in pea aphid defence against bacterial infection. Our results demonstrated that these GNBPs are upregulated in response to infection. Furthermore, the knockdown of their expression resulting in decreased haemolymph PO activity, increased bacterial loads, and increased mortality in the aphids. Our findings suggest that these two GNBPs participate in the defence against bacterial infection through the proPO pathway. Considering the fact that the antimicrobial peptide pathway is absent in the pea aphid immune system (Gerardo *et al*., 2010), our current study reinforces the critical role of the proPO pathway in the pea aphid pathogen defence system (Xu *et al*., 2019).

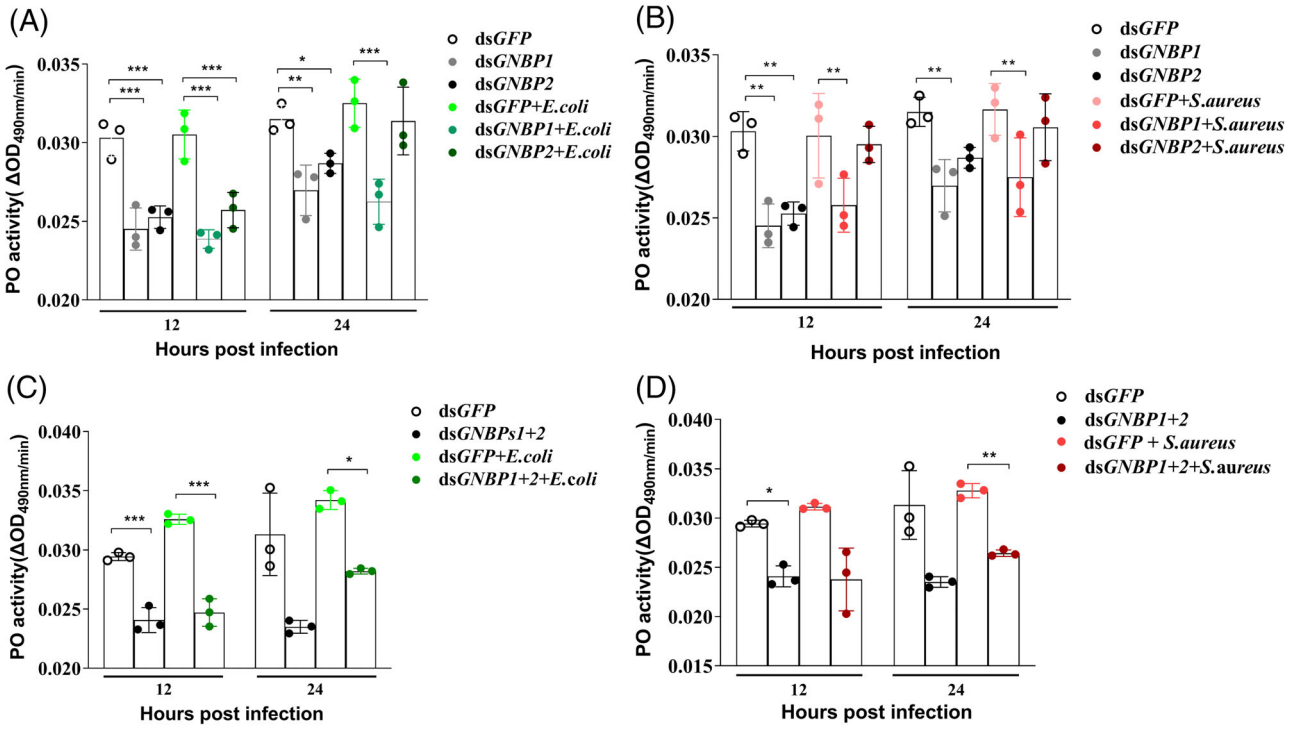

Figure 4. Knock-down of expression of *GNBPs* decreased phenoloxidase (PO) activity in the pea aphids. PO activity in the *GNBP1* or *GNBP2* knocked down aphids infected with *E. coli* (A) and *S. aureus* (B), and in the *GNBP1* + *2* knocked down aphids infected with *E. coli* (C) and *S. aureus* (D). The data are shown as the mean ± standard deviation from at least three independent experiments. One-way ANOVA with a Bonferroni test was used for significance analysis. Asterisks indicate significance of the difference between the compared groups. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. [Colour figure can be viewed at wileyonlinelibrary.com].

Compared with typical GNBPs/βGRPs, aphid GNBPs/βGRPs have six tandem WSC domains instead of the CBM domain in the N-termini. Other hemipteran insects, such as whiteflies, sting bugs, and bed bugs, also have tandem WSC domains in the N-terminus (Fig. 1A). Biochemical and structural studies have revealed that the CBM domain in *Drosophila* GNBP3 recognizes and binds to long chain β-1,3-glucan (Mishima *et al*., 2009). This binding induces the formation of the βGRP-glucan complex, which serves as a platform for the recruitment of downstream proteases to initiate the activation of the proPO pathway in *Manduca* (Dai *et al*., 2013; Takahashi *et al*., 2014). In hemipteran GNBPs, tandem WSC domains are replaced by the CBM domain. The WSC domain was first identified in the Wsc proteins, which are putative receptors in the stress response in yeasts (Lodder *et al*., 1999). Further studies have indicated that the WSC domain is required for the clustering of Wsc1 under stress conditions (Heinisch *et al*., 2010). The WSC domain is conserved from fungal to mammalian cells (Andersson *et al*., 2013; Ponting *et al*., 1999). Recent studies on WSC domain-containing fungal proteins have shown that the WSC domain can bind to β-1,3-glucan (Oide *et al*., 2019; Wawra *et al*., 2019). Therefore, it is likely that the tandem WSC domains in the atypical GNBPs are capable of binding to β-1,3-glucan, but this speculation needs to be further validated by biochemical evidence.

GNBP-3 recognizes fungal β-1,3-glucan and recruits modular serine proteases (Buchon *et al*., 2009; Roh *et al*., 2009). This triggers the auto-activation of modular serine proteases, resulting in the activation of the Toll pathways in *Drosophila* and the beetle *Tenebrio molitor* (Buchon *et al*., 2009; Roh *et al*., 2009). Similarly, *Manduca* βGRP2 recognizes β-1,3-glucan, leading to the auto-activation of the proHP14 pathway and the activation of the proPO pathway (Wang and Jiang, 2006). Biochemical studies have revealed that the interaction between the Glu domain of βGRP2 and the low-density lipoprotein receptor class A (LA) repeats of proHP14 mediates βGRP2/HP14 interaction, leading to HP14 zymogen auto-activation (Takahashi *et al*., 2015). In the pea aphid genome database, we found a homologue (XP_016659387.1) of *Manduca* HP14 and modular serine proteases and compared their sequences (Fig. S1). The four LA repeats and the serine protease activity triad (H-D-S) in these proteins were highly conserved. Therefore, we propose that GNBPs interact with this modular serine protease, resulting in the activation of proPO

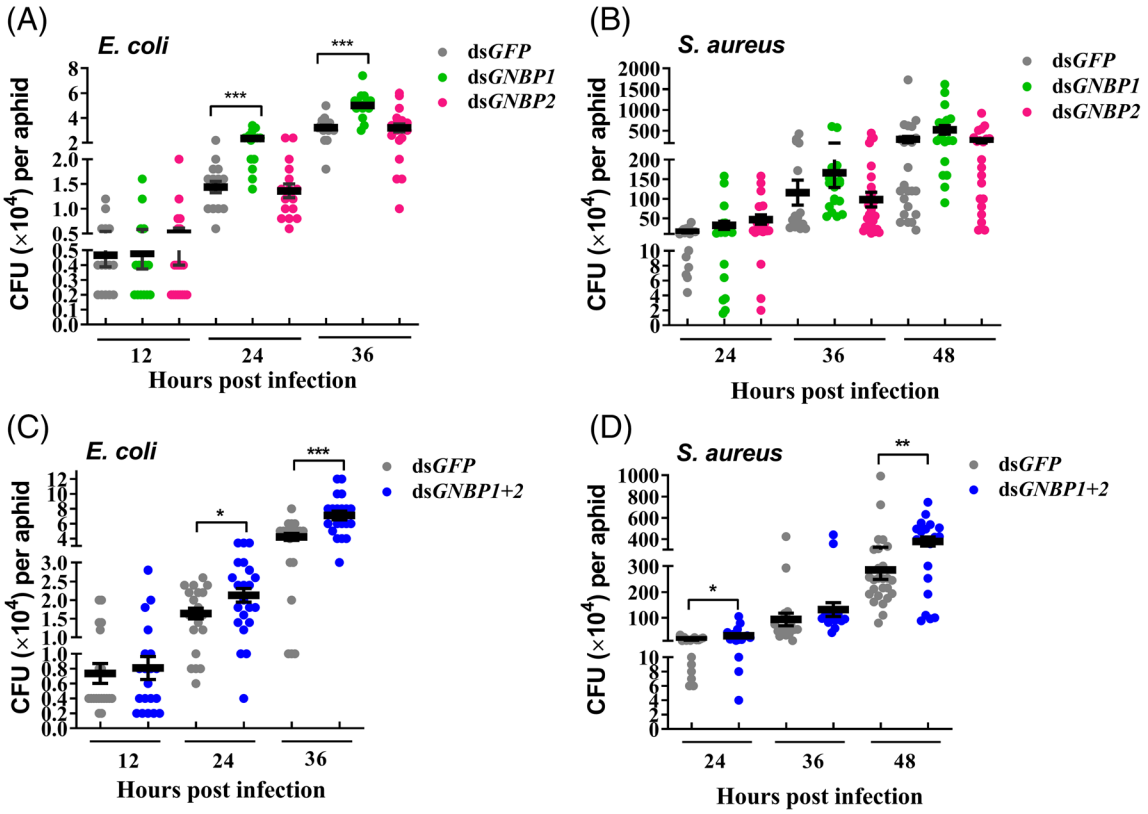

Figure 5. Bacterial loads in the GNBPs knocked down aphids after infection. (A) and (B) show *E. coli* and *S. aureus* loads respectively in the *GNBP1* or *GNBP2* knocked down aphids. (C) and (D) show *E. coli* and *S. aureus* loads respectively in the *GNBP1* + *2* knocked down aphids. Each dot represents the CFU obtained from an individual aphid. One-way ANOVA with a Bonferroni test was used for significance analysis in (A) and (B); paired *t* test was used for significance analysis in (C) and (D). Asterisks indicate significance of the difference between the compared groups. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. [Colour figure can be viewed at wileyonlinelibrary.com].

pathway in aphids. Little is known about how the invading pathogens are recognized and how proPO pathway is activated, and further investigation is required to elucidate the events involved these processes.

In summary, we identified two atypical GNBPs in the pea aphid that were induced in response to bacterial infection. After the knockdown of their expression, PO activity decreased and bacterial load increased, resulting in increased aphid mortality. These findings collectively suggest that these two GNBPs might function as PRRs in the proPO activation pathway.

4. Experimental procedures

4.1 Aphid rearing

Pea aphids, *Acyrthosiphon pisum* Harris (Aphidinae: Macrosiphini), were originally captured in Yunnan Province, China. The colonies were derived from a single parthenogenetic female and were maintained on broad beans (*Vicia faba*) in growth chambers at 21 ± 1 °C and 70 ± 5% relative humidity under a 16 h light/8 h dark photoperiod.

4.2 Protein sequence, domain identification, and phylogenetic analysis

The nucleotide and amino acid sequences of the pea aphid GNBPs were retrieved from the NCBI database (https://www.ncbi.nlm.nih.gov/). Homologues of pea aphid GNBPs were searched using the BlastP program in the NCBI database. Domains were predicted by searching InterPro (http://www.ebi.ac.uk/interpro/search/sequence/). Sequences of GH16 domains in selected GNBP homologues were compared using ClustalX, and a phylogenetic tree was constructed using the UPGMA method in the Geneious 10 package (Auckland, New Zealand).

4.3 Infection of aphids with bacteria

E. coli (DH5α) and *S. aureus* (ATCC43300) were cultured in Luria-Bertani (LB) broth at 37 °C, and their growth was monitored by measuring the absorbance of the culture at 600 nm until the optical density reached approximately 1. Cells were harvested by centrifugation at 8,000 rpm for 15 min, washed three times 0.85% NaCl, and resuspended in sterile 0.85% NaCl at a final concentration of 10^{10} and 2×10^{11} colony forming units (CFU)/ml for *E. coli* and *S. aureus*, respectively. The newly emerged adult aphids were anaesthetized with CO_2, cold-immobilized, and infected with

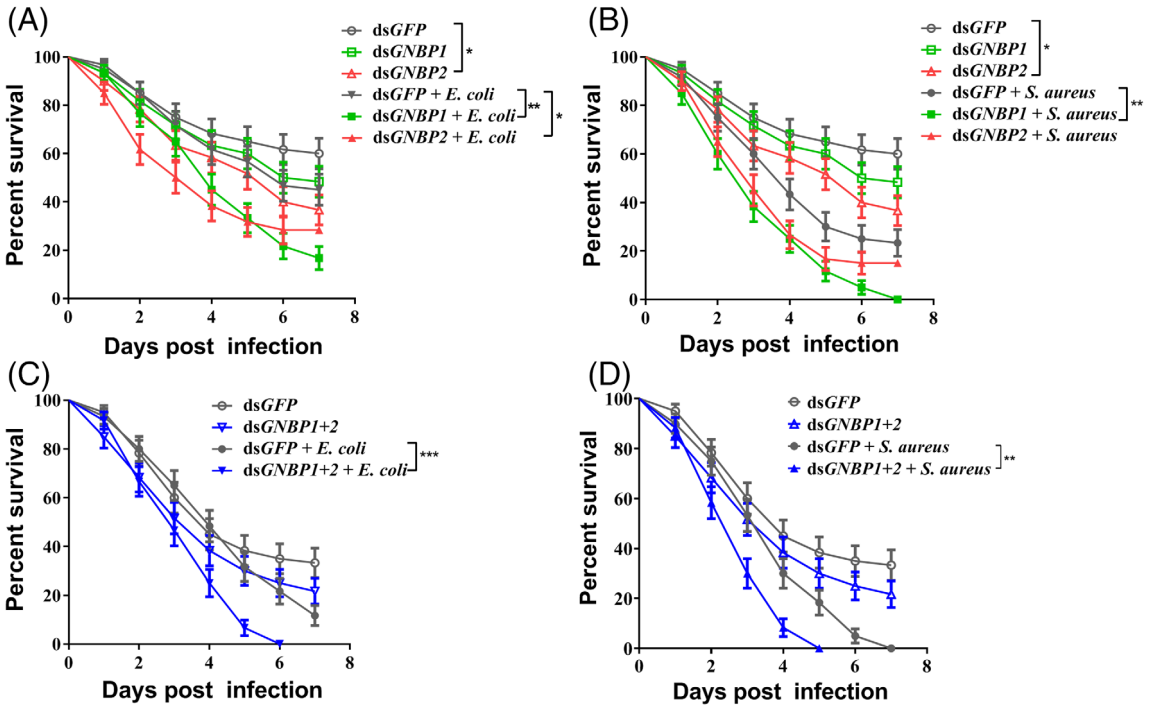

Figure 6. Survival of GNBPs knocked down aphids after infection. (A) and (B) show survival of the *GNBP1* or *GNBP2* knocked down aphids after infection with *E. coli* and *S. aureus*, respectively. (C) and (D) show survival of the *GNBP1 + 2* knocked down aphids after infection with *E. coli* and *S. aureus*, respectively. The data show mean ± error from three independent experiments and the curves were compared by Log-rank (Mantel-Cox) method. N = 20. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. [Colour figure can be viewed at wileyonlinelibrary.com].

bacteria using a capillary. The end of the capillary tube with a ~4 mm long tip was sealed with Parafilm. The tip was dipped in the bacterial preparations and then immediately inserted dorsolaterally through the abdominal wall into the aphid to approximately 1 mm in depth. The aphids in the control group were treated with sterile 0.85% NaCl solution.

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

Total RNA from five aphids in each group was extracted using TriPure (Roche, Mannheim, Germany), following the manufacturer's instructions. One microgram of total RNA was used for reverse transcription PCR using the First Strand cDNA Synthesis kit (Roche) according to the manufacturer's protocol. qPCR was carried out using specific primers for the pea aphid GNBP1 and GNBP2 (Table 1). The pea aphid ribosomal protein L7 gene (*Rpl7*) was used as an endogenous reference for normalization (Nakabachi *et al*., 2005). qPCR was performed on a Rotor Q thermocycler (Qiagen, Hilden, Germany) using a 20 µl reaction mixture containing 2 µl cDNA, 10 µl SYBR FAST Universal Green Mix (KAPA, Cape Town, South Africa), 1 µl of each primer (10 pmol/ml), and 6 µl ultrapure water. The reaction conditions are as follows: an initial denaturing step for 5 min at 95 °C, followed by 40 cycles of heating at 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 20 s. Samples from three independent experiments were analysed in triplicate. The comparative CT method ($2^{-\Delta\Delta CT}$ method) was used to calculate expression levels.

Table 1. Sequence of primers used in this study

Name	Sequence (5′–3′)	Product size (bp)
Rpl7-F	TTGAAGAGCGTAAGGGAACTG	76 bp
Rpl7-R	TATTGGTGATTGGAATGCGTTG	
GNBP1-F	TCCAGCTGGGCCGTATACTT	132 bp
GNBP1-R	GCCGACTTTGCTGGTCTTTG	
GNBP2-F	TCCTTATGCGGCACTTGTATC	132 bp
GNBP2-R	AGACCACCGCATGTATCTTTAG	
dsGNBP1-F	[a]CTCGCCTAACACCCAAACAT	413 bp
dsGNBP1-R	[a]ACTTGGTGATTGGCTTCCAC	
dsGNBP2-F	[a]CACAAAGTGTTCGGGTGATG	483 bp
dsGNBP2-R	[a]GGTTCCTGTGCAACGAAAAT	
dsGFP-F	[a]GTGTTCAATGCTTTTCCCGT	356 bp
dsGFP-R	[a]CAATGTTGTGGCGAATTTTG	

[a]T7 adaptor TAATACGACTCACTATAGGG for dsRNA synthesis).

4.5 *dsRNA preparation and RNAi*

Sense and antisense RNAs for the pea aphid *GNBP1*, *GNBP2*, and *GFP* were prepared following the Promega RiboMax™ T7 system protocol (Promega, Madison, WI, USA); the primers used are listed in Table 1. The synthesized dsRNA was diluted with nuclease-free water to a final concentration of 10 µg/µl. For the knockdown of *GNBP1* or *GNBP2*, adult aphids were injected with 1 µg of dsGNBP1 or dsGNBP2 dorsolaterally through the abdomen. We attempted to knock down *GNBP1* and *GNBP2* simultaneously by injecting a dsGNBP1 and dsGNBP2 mixture, but this attempt was not successful (data not shown). Alternatively, we first

injected 0.5 μg dsGNBP2, then injected 0.7 μg dsGNBP1 the following day. After injection, the aphids were transferred to fresh broad bean seedlings and collected at different time points for RNA preparation. qPCR was used to evaluate RNAi efficiency.

4.6 Aphid survival and bacterial load after RNAi

The aphids were infected with bacteria as described above 2 days after injection of dsGNBP1 or dsGNBP2 and 3 days after the first injection for knock down of both *GNBP1* and *GNBP2*. Twenty aphids in each group were used in survival assays in the following week at 1-day intervals. The bacterial loads in the aphids were determined as previously described (Xu *et al*., 2019). Briefly, at least 15 aphids at each time point were selected and surface-sterilized by rapid washing in 70% ethanol. They were then rinsed twice using sterilized 0.85% NaCl. The aphids were individually homogenized in 0.2 ml sterilized 0.85% NaCl and diluted to a suitable concentration at which it was easy for counting the colonies on the plates. Ten microliters of the homogenate was evenly spread on an LB plate, and the bacterial colonies were counted after overnight culturing at 37 °C.

4.7 Phenoloxidase (PO) activity assay

After infection, the aphids were decapitated and placed in an Eppendorf tube filled with absorbent cotton and then centrifuged at 800*g* for 10 min to collect the haemolymph. Two microliters of the haemolymph and 100 μl of 2 mM dopamine were mixed in each well of a 96-well plate. The optical density was read at 490 nm using a microplate reader (Tecan Pro200; Tecan, Männedorf, Switzerland) every 30 s for 30 min. PO activity was determined by the maximum slope, which was defined as the increase in absorbance at 490 nm/min (Xu *et al*., 2019). Activity assays were repeated using haemolymph samples from three biological replicates.

4.8 Data analysis

All data were plotted using Prism 5.0 (GraphPad Software Inc., La Jolla, CA, USA). The control and treatment groups were compared using paired *t*-tests for significance analysis. Multiple group comparison was analysed by one-way ANOVA test with Bonferroni correction. A log-rank (Mantel-Cox) test was used to analyse survival curves.

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Data availability statement

The data that supports the findings of this study are available in the supplementary material of this article.

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Supporting Information

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

Figure S1: Sequence alignment of modular serine proteases. The low-density lipoprotein receptor class A (LA) domains are underlined and serine protease catalytic triad H-D-S are indicated by arrowheads. Access numbers: Ms-HP14, AAR29602.1; Dm_MSP, NP_536776.2; Tm_MSP, BAG14264.1; Ap_MSP, XP_016659387.1.

File S1: Position of primers on GNBP1 and GNBP2.

