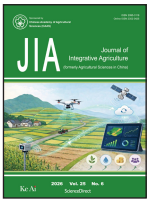
Journal of Integrative Agriculture 2026, 25(6): 2485–2495

Available online at ScienceDirect

Journal of Integrative Agriculture



RESEARCH ARTICLE

PxGalectin-4 with single carbohydrate recognition domain involved in the immunity of *Plutella xylostella* against entomopathogenic fungus *Isaria cicadae*

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Highlights

- PxGalectin-4 directly bound microbes and pathogen-associated molecular patterns, and agglutinated microbial cells.
- PxGalectin-4 promoted nodulation and encapsulation of hemocytes, and activated prophenoloxidases.
- PxGalectin-4 possessed a direct antimicrobial activity *in vitro* and *in vivo*.
- Knockdown of *PxGalectin-4* enhanced the susceptibility of *Plutella xylostella* to fungi.

Abstract

The diamondback moth, *Plutella xylostella* represents a worldwide threat to Brassicaceae crops and has developed substantial resistance to conventional insecticides. Entomopathogenic fungi (EPF) have emerged as environmentally sustainable alternatives to chemical insecticides. Since insect immunity constitutes the primary defense against fungal pathogens, understanding these mechanisms could advance biocontrol strategies. Nevertheless, research on the immune functions of galectins in insects remains limited. This study identifies a Galectin-4 homolog in *P. xylostella* (PxGalectin-4) and systematically examines its immunological functions against an EPF *Isaria cicadae* infection. The open reading frame of *PxGalectin-4* encoded 338 amino acids with a carbohydrate recognition domain (CRD). *PxGalectin-4* expression exhibited peak levels in late-instar larval stages and fat body, and increased significantly following *I. cicadae* challenge. Functional characterization demonstrated that recombinant PxGalectin-4 (rPxGalectin-4) directly bound cells and cell wall components of microbes, and displayed Ca^{2+}-dependent microbial agglutination. Additionally, rPxGalectin-4 enhanced hemocyte-mediated immune responses by promoting nodulation and encapsulation, and increased phenoloxidase activity of hemolymph. Knockdown of *PxGalectin-4* significantly increased the susceptibility of *P. xylostella* larvae to *I. cicadae* infection. In conclusion, PxGalectin-4 serves a vital immune function in *P. xylostella* defense against *I.cicadae*, and presents a potential target for novel pest control strategies.

Keywords: galectin, immunity, biocontrol, *Plutella xylostella*, entomopathogenic fungi, *Isaria cicadae*

1. Introduction

Insects, representing the most abundant and diverse terrestrial fauna, encounter numerous pathogens, including bacteria, fungi, and viruses (Wang and St Leger 2006). These organisms possess an innate immune system that provides protection against invading pathogens (Hoffmann 2003; Zhang *et al.* 2021). The insect immune system integrates cellular and humoral immunity components. This comprehensive defense system implements multiple strategies: phagocytosis, agglutination, encapsulation, melanization, and the production of antimicrobial peptides (AMPs) (Lemaitre and Hoffmann 2007; Lin *et al.* 2020). The innate immune response is activated when host pattern recognition receptors (PRRs) detect conserved

Received 3 March 2025; Received in revised form 14 May 2025; Accepted 9 June 2025; Available online 5 July 2025
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doi: 10.1016/j.jia.2025.07.002

pathogen-associated molecular patterns (PAMPs), including peptidoglycan (PGN), lipopolysaccharides (LPS), β-1,3-Glucan and related components (Lu *et al.* 2020). Currently, researchers have identified numerous PRRs in insects, including peptidoglycan recognition proteins, β-1,3-glucan recognition proteins, Gram-negative bacteria binding proteins, scavenger receptors, C-type lectins and galectins (Yu *et al.* 2002; Xia *et al.* 2015; Wang *et al.* 2019; Sheehan *et al.* 2020).

Galectins comprise a family of β-galactosides-specific lectin characterized by at least one conserved carbohydrate recognition domain (CRD) (Yang *et al.* 2008; Ayona *et al.* 2020). These evolutionarily conserved proteins exist in vertebrates, invertebrates, and fungi (Lin *et al.* 2020). Vertebrate systems contain more than 16 identified galectins, categorized into three types based on their structures: prototype galectins, chimeric galectins, and tandem repeat galectins (Arthur *et al.* 2015; Liu and Stowell 2023). Mammalian galectins serve crucial functions in various biological processes, including development, immune response, and infection (Kamhawi *et al.* 2004; Vasta 2012; Liu and Stowell 2023). In invertebrates, mounting evidence over recent decades indicates galectins' immunological significance. For example, *Litopenaeus vannamei galectin* expression increased following bacterial exposure, demonstrating a vital role in Pacific white shrimp immunity against bacterial infection (Cha *et al.* 2015). *CgGalectin-9* from the Pacific oyster *Crassostrea gigas* exhibited binding to various microorganisms and participated in hemocyte autophagy (Yang *et al.* 2023). In Diptera insects, *Drosophila galectin* appeared abundantly in embryo, larva, and adult stages, with expression in hemocytes and circulating phagocytic cells, suggesting roles in both developmental processes and immune defense (Pace *et al.* 2002). *Aedes aegypti* galectin-6 inhibited Cry11Aa from binding to toxin receptors, reducing Cry11Aa toxicity (Hu *et al.* 2020). In Lepidoptera insects, *Bombyx mori* BmGalectin4 showed high expression in eggs and demonstrated bacterial cell agglutination capabilities (Rao *et al.* 2016). *Spodoptera litura* galectins potentially functioned as PRRs and modulated insect immune responses to pathogens (Zhong *et al.* 2025). Nevertheless, the immunological functions of insect galectins remain largely unexplored.

The diamondback moth, *Plutella xylostella* (L.) is a significant pest of cruciferous crops that has developed resistance to numerous insecticide classes (Li *et al.* 2016). Entomopathogenic fungi (EPF) have emerged as environmentally sustainable alternatives to chemical insecticides for pest biocontrol, particularly in addressing insecticide-resistant pests (Litwin *et al.* 2020; Li *et al.* 2022). Among these, *Isaria cicadae* is an EPF that naturally infects larvae of Lepidoptera and Homoptera insects (Peng *et al.* 2020). While genome-wide analysis has identified four *galectins* in *P. xylostella* genome (Xia *et al.* 2015), their specific functions remain largely undefined. Understanding the immune roles of these galectins could enhance the development of more effective biocontrol strategies against this pest.

This study characterized the immune function of Galectin-4 from *P. xylostella* (PxGalectin-4). The research analyzed the *PxGalectin-4* sequence through bioinformatics tools. Expression patterns of *PxGalectin-4* were examined across developmental stages, tissues, and in response to pathogen exposures using reverse transcriptase quantitative PCR (RT-qPCR). The study also included the expression and purification of recombinant PxGalectin-4 protein. Further investigation of PxGalectin-4's immunological function utilized *in vitro* and *in vivo* assays.

2. Materials and methods

2.1. Insect and microorganisms

The larvae of *P. xylostella* were maintained on bok choy (*Brassica rapa* Chinensis) under controlled conditions of 25°C, 75% relative humidity, and a 16 h L:8 h D photoperiod. Adult moths received 10% (w/v) honey solution as a nutritional supplement.

Isaria cicadae, an entomopathogenic fungus, was cultured on potato dextrose agar (PDA) at 28°C in complete darkness. *Escherichia coli* (Gram-negative) and *Staphylococcus aureus* (Gram-positive) were cultured in Luria-Bertani (LB) broth at 37°C.

2.2. Sequence analysis of *PxGalectin-4*

SMART (https://smart.embl.de/) and SignalP 5.0 (https://services.healthtech.dtu.dk/services/SignalP-5.0/) were used to predict the conserved domains and signal peptide of PxGalectin-4, respectively. MIB2 (http://combio.life.nctu.edu.tw/MIB2/) predicted the Ca^{2+} binding sites. A phylogenetic tree of galectins was constructed using MEGA 7, employing the neighbor-joining (NJ) method with 1,000 replications bootstrap. The Swiss Model Workspace (https://swissmodel.expasy.org/) predicted the three-dimensional structure of PxGalectin-4.

2.3. Expression patterns of *PxGalectin-4* in developmental stages and tissues

The expression levels of *PxGalectin-4* across developmental stages (eggs, first to fourth instar larvae, pupae and adults), and tissues (malpighian tubule, epidermis, midgut, fat body and hemocytes) of *P. xylostella* were analyzed using RT-qPCR. Total RNA was extracted and reverse-transcribed into cDNA using PrimeScript RT Master Mix (Takara, Dalian, China). PCR amplification was performed on the Bio-Rad CFX96 system (Hercules, CA, USA) with the following conditions: denaturation at 95°C for 30 s, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. Each reaction mixture contained 2 μL of cDNA, 0.4 μL of each primer (10 μmol), 7.2 μL of ddH_2O and 10 μL SYBR qPCR Master Mix (Vazyme, Nanjing, China). *Actin* served as the reference gene for normalization, and the primers are detailed in Appendix A. The $2^{-\Delta\Delta Ct}$ method was utilized to determine the relative expression level of *PxGalectin-4*.

2.4. Expression profiles of *PxGalectin-4* after microbial challenge

Aliquots of 1 μL *S. aureus* (1.0×10^6 cells mL^{-1}), *E. coli* (1.0×10^6 cells mL^{-1}) and *I. cicadae* (1.0×10^7 cells mL^{-1}) were injected into the third instar larvae, respectively. Sterile water served as a control. After injection, the larvae were maintained under

the previously described conditions for 24 h. *PxGalectin-4* expression level was evaluated by RT-qPCR following the method described above.

2.5. Expression and purification of recombinant PxGalectin-4 (rPxGalectin-4)

The sequences encoding PxGalectin-4 were artificially synthesized *via* HUABIO (Hangzhou, China). The synthesized fragment underwent digestion with *Bam*HI/*Xho*I and was subsequently ligated into the pET-30a(+) vector. After confirmation by DNA sequencing, the plasmid was transformed into *E. coli* BL21(DE3) cells. rPxGalectin-4 was expressed following induction with isopropyl-β-D-thiogalactoside (IPTG), and purified using Nickel-Nitrilotriacetic acid (Ni-NTA) affinity column. The purified rPxGalectin-4 was analyzed using 12% SDS-PAGE, and then utilized for generating rabbit anti-rPxGalectin-4 antiserum for subsequent immunological studies.

2.6. Western blotting analysis

After electrophoresis on 12% SDS-PAGE gel, the rPxGalectin-4 was transferred onto a polyvinylidene difluoride membrane. Following blockage with blocking buffer for 1 h, the membrane was incubated at 37°C with rabbit anti-rPxGalectin-4 antiserum (1:3,000) for 1 h. After three washes with Tris-buffered saline with 0.1% Tween 20 (TBST), the membrane was incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (1:10,000) for 1 h at 37°C. Following three additional washes with TBST, the protein bands were visualized using ECL staining reagents (Shandong SparkJade, Jinan, China).

2.7. Microbes binding assay

The binding activity of rPxGalectin-4 to microbes was assessed according to a previously established method (Li *et al.* 2022). A total of 30 μg of rPxGalectin-4 was combined with 200 μL bacterial cells (1×10^8 cells mL^{-1}) or fungal cells (1×10^7 cells mL^{-1}), and incubated at room temperature (RT) for 1 h. Bovine serum albumin (BSA) served as a control. After incubation, cells were collected, washed three times with PBS, and eluted with 5×protein loading solution. Both the washed and eluted samples were analyzed using Western blotting assay to determine the binding interactions between rPxGalectin-4 and the microbial cells.

2.8. Binding of rPxGalectin-4 with PAMPs

PAMPs binding ability of rPxGalectin-4 was evaluated using a modified method from Lim *et al.* 2023. In brief, 0.1 μg μL^{-1} of PGN, β-1,3-glucan, and LPS was coated at 4°C on a 96-well plate overnight. After three washes with PBST (PBS containing 0.3% Tween 20), the plate was blocked with 5% BSA in PBS and incubated at 37°C for 1 h. Subsequently, increasing concentrations of rPxGalectin-4 (0–20 μg mL^{-1}) were added and incubated at RT for 3 h, with BSA as a control. Following washing, the wells were incubated with rabbit anti-rPxGalectin-4 antiserum (1:1,000) and HRP-conjugated goat anti-rabbit IgG antibody (1:4,000) at 37°C for 1 h, respectively. After washing, tetramethylbenzidine solution was added and incubated at RT for 15 min in darkness. The reaction was terminated by adding 1 mol L^{-1} H_2SO_4, and absorbance at 450 nm (OD_{450}) was measured using a Multiskan GO Microplate Spectrophotometer (Thermo Fisher Scientific, Waltham, USA).

2.9. Microbes agglutination assay

Microbial agglutination was examined microscopically following the method of Li *et al.* (2022). A total of 10 μL of *I. cicadae* (1×10^6 cells mL^{-1}) and *E. coli* or *S. aureus* (1×10^7 cells mL^{-1}) were mixed with 10 μL PBS containing 75 ng rPxGalectin-4, 1 mmol L^{-1} $CaCl_2$ with 75 ng rPxGalectin-4, and 1 mmol L^{-1} $CaCl_2$ with 75 ng rPxGalectin-4 plus 1 mmol L^{-1} EDTA, respectively. BSA served as a control. After incubation at RT for 2 h, the samples were analyzed using an Eclipse Ti microscope (Nikon, Tokyo, Japan).

2.10. *In vitro* antimicrobial activity assay

Various concentrations of rPxGalectin-4 were added to 10 μL of microbial suspension (1.0×10^5 cells mL^{-1}) and incubated for 12 h. Sterile water and BSA served as blank and protein controls, respectively. The mixtures were plated on LB (for bacterium) or PDA (for fungus) plates. Following incubation at 37°C for 24 h (for bacterium) or 28°C for 48 h (for fungus), colonies were enumerated and the inhibition rate was calculated using the equation:

Inhibition rate (%)=(Average $colonies_{blank}$–$Colonies_{protein}$)/Average $colonies_{blank}$×100

2.11. *In vivo* microbial clearance assay

Microbial cells (*E. coli* and *S. aureus* at 1×10^7 cells mL^{-1}, and *I. cicadae* at 1×10^6 cells mL^{-1}) were incubated with 150 μg mL^{-1} of rPxGalectin-4 or BSA (control) at room temperature for 1 h. After three PBS rinses, the mixtures were administered to third instar larvae. Following 2 h incubation, larval hemolymph was collected and plated on LB (for bacterium) or PDA (for fungus) plates. Colonies were subsequently enumerated after incubation.

2.12. Phenoloxidase (PO) activity assay

PO activity assay was conducted following an established method (Sumathipala and Jiang 2010). Hemolymph was collected from healthy third instar larvae and diluted with buffer E (20 mmol L^{-1} Tris-HCl, pH 8.0, 1 mmol L^{-1} $CaCl_2$, 0.001% Tween 20). The diluted hemolymph (5 μL) was combined with 500 ng of rPxGalectin-4 or BSA. Buffer E served as a control. Following incubation at RT for 1 h, 200 μL of 2 mmol L^{-1} dopamine was added, and OD_{490} was measured. One unit of enzyme activity (U) is defined as PO activity producing 0.001 absorption units per min.

2.13. RNA interference of *PxGalectin-4*

RNA interference (RNAi) assay was conducted following an established method (Li *et al.* 2022). Double-stranded RNA (dsRNA) targeting *PxGalectin-4* was synthesized using the T7 RiboMAX Express RNAi System (Promega, Madison, USA) with primers listed in Appendix A, while dsRNA targeting green fluorescence protein (ds*GFP*) functioned

as a control. Third instar larvae each received 1 μL of 2 μg μL^{-1} ds*PxGalectin-4* or ds*GFP*. After injection, larvae were maintained under the specified conditions. The effect of dsRNA treatment on *PxGalectin-4* expression was evaluated by RT-qPCR at 12, 24, 36 and 48 h. To assess whether *PxGalectin-4* regulates AMPs genes expressions, transcript levels of three AMPs genes (*Gloverin*, *Moricin* and *Cecropin*) were analyzed. After *PxGalectin-4* silencing for 12 h, the larvae were injected with or without 1 μL of 50 μg mL^{-1} rPxGalectin-4. Subsequently, the expression levels of three AMPs genes were examined after 12 h.

2.14. Effect of rPxGalectin-4 injection on AMPs genes expressions

The expressions of AMPs genes of *P. xylostella* following rPxGalectin-4 injection were analyzed according to a previous method (Liu *et al.* 2023). Third instar larvae were administered 0.5 μg of either rPxGalectin-4 or BSA (control). Twenty-four hours post-injection, the larvae were immersed in a spore suspension of *I. cicadae* (1×10^8 cells mL^{-1}) for 10 s. Subsequently, the larvae were maintained for an additional 24 h. The transcript levels of three AMPs genes were then analyzed using RT-qPCR.

2.15. Bioassay of ds*PxGalectin-4* treated insects to infection

Following the previously described method, third instar larvae were microinjected with ds*PxGalectin-4*. ds*GFP* served as a control. Twelve hours post-injection, the larvae were injected with or without 1 μL of 50 μg mL^{-1} rPxGalectin-4 or GFP. After 12 h, the larvae were immersed in a spore suspension of *I. cicadae* (1×10^8 cells mL^{-1}) for 10 s. Subsequently, larval survival of *P. xylostella* was monitored every 12 h.

2.16. Nodulation of hemocytes assay

The nodulation of hemocytes assay was conducted according to an established protocol (Liu *et al.* 2022). In brief, fresh hemolymph from *P. xylostella* larvae was collected into anticoagulant. Hemocytes were isolated and resuspended in 150 μg mL^{-1} of rPxGalectin-4 or BSA (control) in L15 (10% FBS) medium, and incubated at 25°C for 1 h. The preparations were then co-incubated with fluorescein isothiocyanate (FITC) labeled *I. cicadae* spores (1×10^8 cells mL^{-1}) at 25°C for 2 h. Subsequently, 4′,6-diamidino-2-phenylindole (DAPI) was added and stained for 20 min in darkness. Hemocyte nodulation was observed using a confocal laser scanning microscope (CLSM) (Carl Zeiss, Jena, Germany).

2.17. Encapsulation of hemocytes assay

Encapsulation of hemocytes was conducted following previously described methods (Li *et al.* 2021). The rPxGalectin-4 was incubated with Ni-NTA agarose beads (Sangon Biotech, Shanghai, China) containing 10 mmol L^{-1} $CaCl_2$ at 25°C for 4 h, using BSA as a control. After incubation, the rPxGalectin-4-coated beads were washed twice with PBS and resuspended in fresh PBS. Fresh hemocytes were then incubated at 25°C with the treated agarose beads for 2 h. Following two additional washes, the samples were examined under an Eclipse Ti microscope (Nikon, Tokyo, Japan).

2.18. Statistical analysis

Shapiro-Wilk test was used to assess data normality. Statistical analyses were performed using Data Processing System (DPS 7.05), employing Student's *t*-test for pairwise comparisons and Tukey's HSD test for multiple comparisons. Statistical significance was defined as $P<0.05$.

3. Results

3.1. Identification and sequence characterization of *PxGalectin-4*

The open reading frame of *PxGalectin-4* (XM_011552475) comprised 1,017 bp encoding 338 amino acid residues with 55 Ca^{2+} binding sites (Fig. 1-A). The PxGalectin-4 sequence contained neither signal peptide nor transmembrane region. PxGalectin-4 contained one CRD domain in the N-terminus with a conserved β-galactoside binding motif WGxEE, classifying it as a member of the prototype group of galectins.

As illustrated in Fig. 1-B, the phylogenetic analysis revealed evolutionary relationships among insect galectins, which segregated into six clusters: Lepidoptera, Orthoptera, Isoptera, Hymenoptera, Diptera and Coleoptera. PxGalectin-4 demonstrated close phylogenetic relationships with Lepidoptera galectins.

The tertiary structure of PxGalectin-4 protein consisted primarily of α helix (7.1%), β turn (8.58%), extended strand (19.23%) and random coil (65.09%) (Fig. 1-C). The CRD domain of PxGalectin-4 spans positions V_{11} to E_{144}, exhibiting high structural similarity with human galectin-4 and chicken galectin-8 N-terminal domain.

3.2. Expression profiles of *PxGalectin-4*

PxGalectin-4 expression was detected throughout all developmental stages (Fig. 2-A), with peak levels observed in third and fourth larval stages and adults (13.19-, 14.20- and 16.00-fold higher than first larvae, respectively). Tissue distribution analysis revealed ubiquitous *PxGalectin-4* expression (Fig. 2-B), with notably elevated levels in fat body and hemocytes (941.55- and 177.26-fold higher than epidermis). Immune challenge assays demonstrated significant upregulation of *PxGalectin-4* following exposure to *I. cicadae* (2.39-fold), *E. coli* (1.86-fold), and *S. aureus* (3.56-fold) compared to control (Fig. 2-C). After *I. cicadae* infection, *PxGalectin-4* expression increased beginning at 12 h post infection and continued to rise through 36 h post infection (Fig. 2-D).

3.3. Expression and purification of rPxGalectin-4

The gene encoding *PxGalectin-4* was successfully inserted into the pET-30a(+) vector and expressed in *E. coli* BL21(DE3) cells. The rPxGalectin-4 was purified using Ni-NTA affinity column, resulting in a distinct protein band, as verified by SDS-PAGE and Western blotting (lane 5; Fig. 3-A; Appendix C). Western blotting analysis confirmed the identity of the purified protein through specific reactivity with anti-PxGalectin-4 antibody (lane 1; Fig. 3-B).

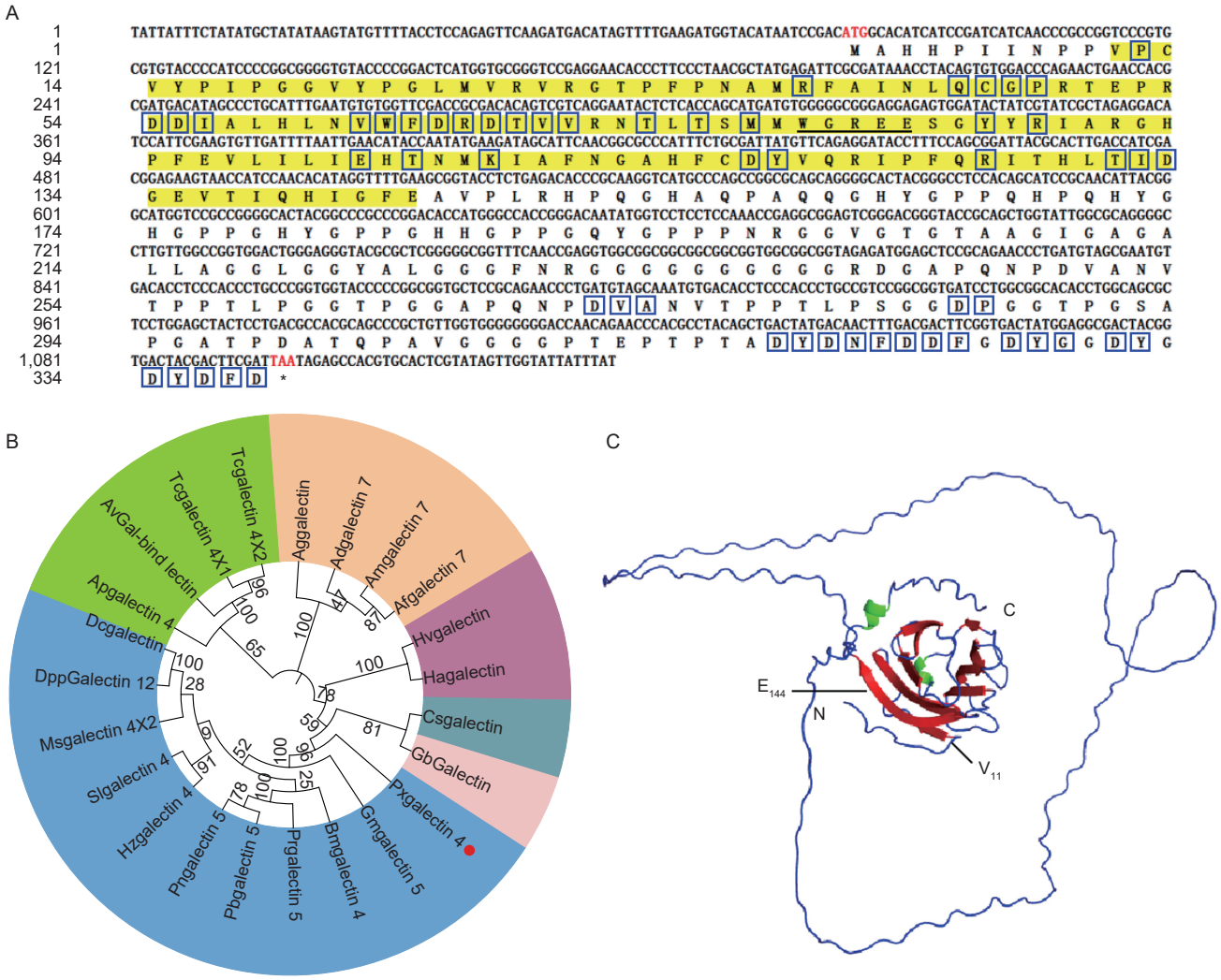


Fig. 1 Sequence, phylogenetic analysis and structural characterization of PxGalectin-4. A, nucleotide and deduced amino acid sequences of *PxGalectin-4*. The start codon and the stop codon are highlighted in red. N-terminal CRD domain is shaded in yellow. The Ca^{2+} binding sites are boxed. WGxEE motif is underlined. B, phylogenetic tree of PxGalectin-4 and related galectins. The GenBank accession numbers of insect galectins are listed in Appendix B. The phylogenetic tree was constructed using the neighbor-joining method based on amino acid sequences. Blue, Lepidoptera; Green, Coleoptera; Orange, Diptera; Purple, Hymenoptera; Pink, Orthoptera; Bluish green, Isoptera. C, tertiary structure of PxGalectin-4. C-terminus and N-terminus, and CRD domain are indicated. α-helices, β-sheets and random coils are depicted in green, red and blue, respectively.

3.4. Binding of rPxGalectin-4 to microorganisms and PAMPs

As anticipated, none of the tested bacteria cells or fungal spores exhibited binding to the BSA control protein (lanes 3, 5 and 7; Fig. 4-B). In contrast, rPxGalectin-4 demonstrated strong affinity to *I. cicadae* spores, *E. coli* and *S.aureus* cells (lanes 3, 5 and 7; Fig. 4-A). These findings demonstrated that rPxGalectin-4 could directly bind to microbes.

To examine the binding capacity of rPxGalectin-4 to PAMPs, three distinct PAMPs (LPS, PGN, and β-1,3-glucan) of microbes were selected for ELISA assay (Fig. 4-C). The rPxGalectin-4 exhibited direct binding to all tested sugars in a concentration-dependent manner.

3.5. Microbial agglutination

Under both Ca^{2+}-supplemented and Ca^{2+}-free conditions, the control protein BSA did not induce agglutination in *I. cicadae*, *E. coli* and *S. aureus* (BSA and Ca^{2+}+BSA; Fig. 5). However, the cells of examined microorganisms exhibited marked agglutination upon addition of rPxGalectin-4 (rPxGalectin-4; Fig. 5). Furthermore, the presence of Ca^{2+} significantly enhanced the agglutination of microbial cells (Ca^{2+}+rPxGalectin-4; Fig. 5). The agglutination induced by rPxGalectin-4 was inhibited in the presence of 1 mmol L^{-1} EDTA (Ca^{2+}+rPxGalectin-4+EDTA; Fig. 5). These results indicated that rPxGalectin-4 can agglutinate microbial cells in a calcium ion-dependent manner.

3.6. rPxGalectin-4 possesses a direct antimicrobial activity *in vitro* and *in vivo*

As shown in Fig. 6-A–C, compared with the control (BSA), rPxGalectin-4 exhibited stronger antimicrobial activity against *I. cicadae*, *E. coli* and *S. aureus* across all tested concentrations. Among the microbes, rPxGalectin-4 demonstrated the highest

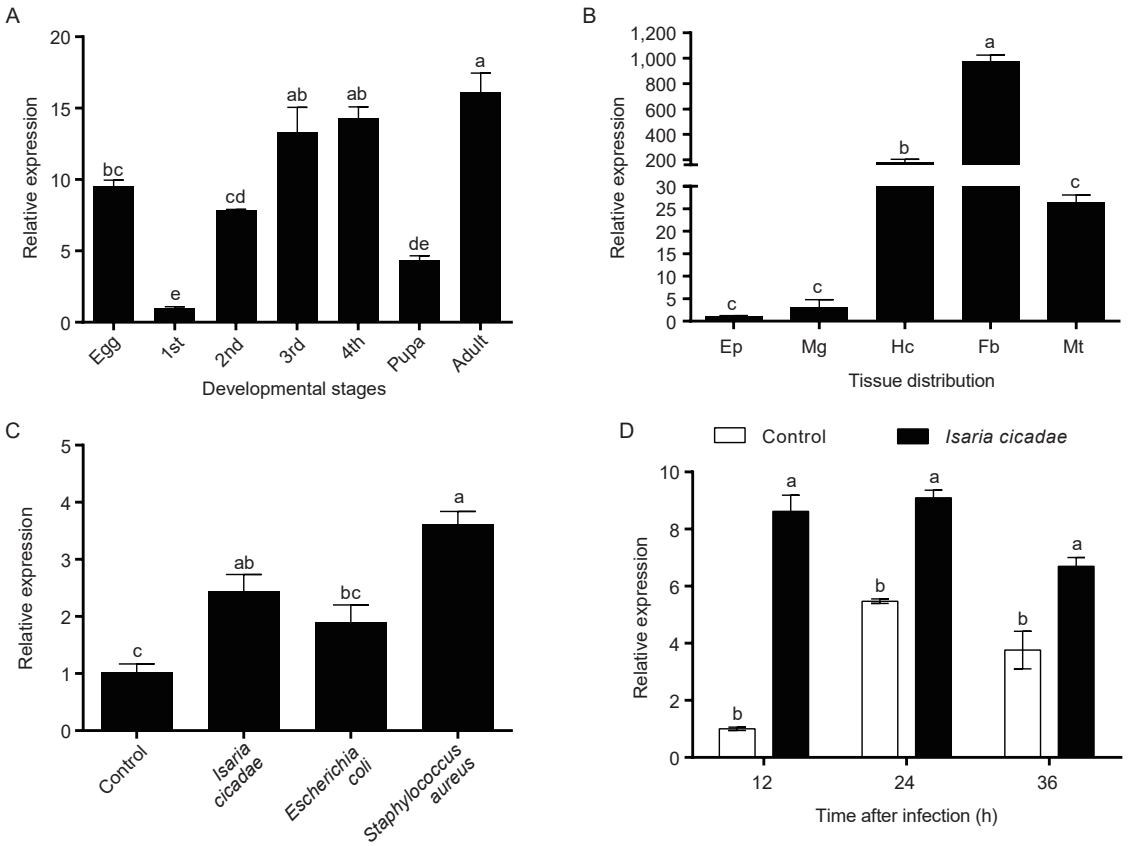


Fig. 2 Expression patterns of *PxGalectin-4*. A, developmental stage-specific expression. B, tissue-specific distribution. Ep, epidermis; Mg, midgut; Hc, hemocytes; Fb, fat body; Mt, malpighian tubule. C, response to microbial challenges. D, temporal expression following *Isaria cicade* infection. Control, ddH_2O. Data are presented as mean±standard error of the mean (SEM) (n=3). Significant differences (P<0.05) are denoted by different letters above the bars.

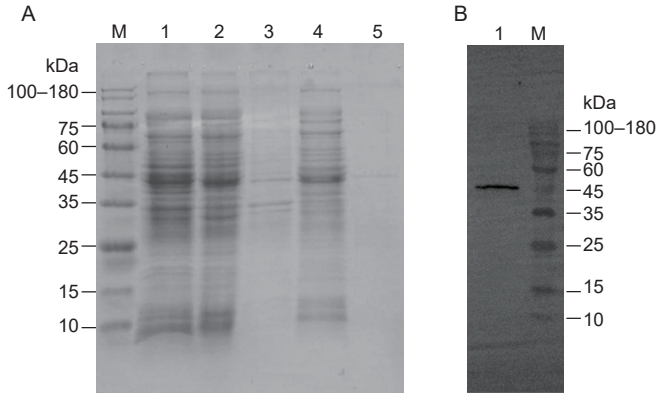


Fig. 3 Expression, purification, and Western blotting confirmation of rPxGalectin-4. A, SDS-PAGE analysis of rPxGalectin-4 expression. Line 1, uninduced bacterial culture; line 2, bacterial culture following IPTG induction; line 3, IPTG-induced precipitate; line 4, IPTG-induced supernatant; line 5, the purified rPxGalectin-4. B, Western blotting analysis of the purified rPxGalectin-4. Line 1, rPxGalectin-4; M, protein marker.

antimicrobial activity against *I. cicadae*, achieving an inhibition rate of 86.12 % at 500 μg mL^{-1}.

The *in vivo* microbial clearance activity of rPxGalectin-4 was subsequently evaluated. The microbial count in the hemolymph of *P. xylostella* larvae injected with rPxGalectin-4 decreased significantly compared to the control. Quantitative assessment revealed that the remaining microbial counts were 51.5 % (*S. aureus*), 17.5 % (*E. coli*), and 5.5 % (*I. cicadae*) of the control, respectively (Fig. 6-D). These findings demonstrated that rPxGalectin-4 significantly enhances the clearance of microorganisms in *P. xylostella*.

3.7. rPxGalectin-4 increases PO activity of hemolymph

To investigate whether rPxGalectin-4 could activate prophenoloxidases, the PO activity of hemolymph was measured (Fig. 7). Compared to the control, the PO activity of hemolymph treated with BSA showed no significant difference. However, the addition of rPxGalectin-4 to the hemolymph significantly enhanced PO activity. This observation suggested that PxGalectin-4 is involved in the PO activation pathway.

3.8. Bioassay of ds*PxGalectin-4* treated insects to fungal infection

RT-qPCR results demonstrated that the injection of ds*PxGalectin-4* resulted in a significant decrease in *PxGalectin-4* expression at 24 and 36 h (Fig. 8-A). The transcription level of *PxGalectin-4* decreased by 16.25 and 45.64% at 24 and 36 h, respectively, compared to dsGFP injection.

At 24 h post-*PxGalectin-4* silencing, the expression

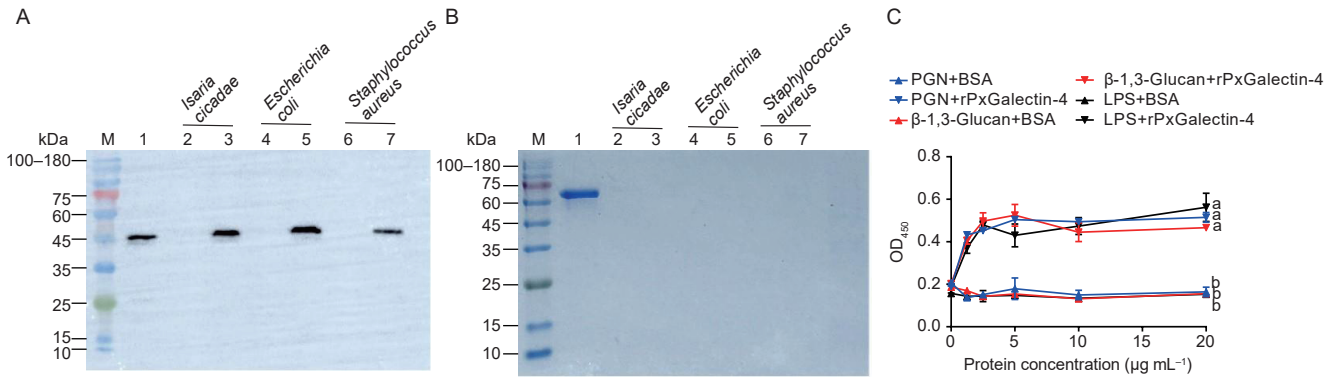


Fig. 4 Binding of rPxGalectin-4 to microorganisms and pathogen-associated molecular patterns (PAMPs). A, microbial binding activity of rPxGalectin-4. B, microbial binding activity of bovine serum albumin (BSA). Lane 1, rPxGalectin-4 for Fig. 4-A and BSA for Fig. 4-B; lanes 2 , 4 and 6, washed solutions; lanes 3, 5 and 7, eluted solutions; M, protein marker. C, PAMP-binding capacity. Data are presented as mean±SEM (n=5). Significant differences (P<0.05) are indicated by different letters.

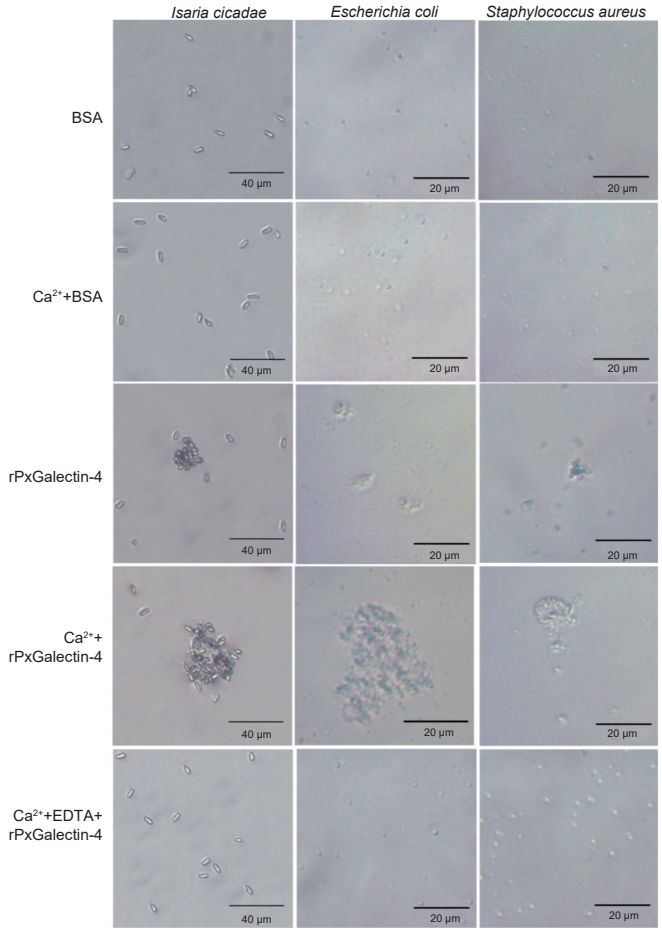


Fig. 5 Agglutination of microbes by rPxGalectin-4. Ca^{2+} indicates 1 mmol L^{-1} of $CaCl_2$. Bovine serum albumin (BSA) served as the control.

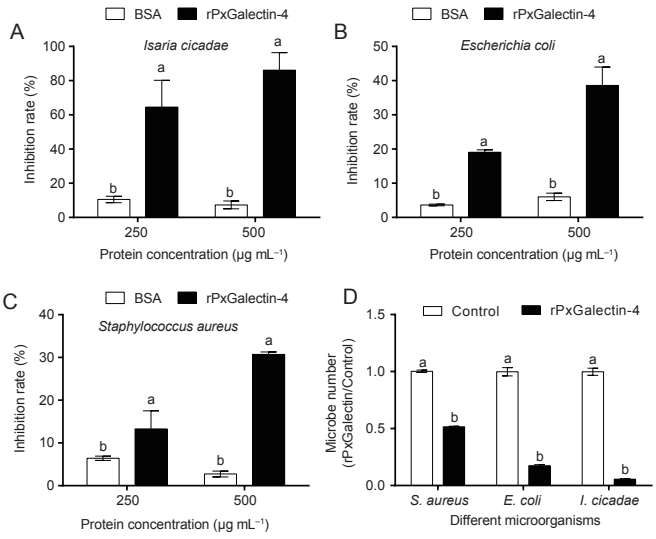


Fig. 6 Antimicrobial activity of rPxGalectin-4. A, B and C, *in vitro* antimicrobial activity assay. D, *in vivo* microbial clearance assay. Data are presented as mean±SEM (n=5). Bovine serum albumin (BSA) served as the control. Significant differences (P<0.05) are indicated by different letters above the bars.

of *Gloverin*, *Moricin*, and *Cecropin* showed significant downregulation compared to the ds*GFP* control group (Fig. 8-B). The addition of rPxGalectin-4 protein reversed these knock down effects, with increased expression levels of the three AMPs genes compared to the group without rPxGalectin-4. Moreover, following *I. cicadae* infection, larvae injected with rPxGalectin-4 exhibited significantly higher expression levels of *Gloverin*, *Moricin* and *Cecropin* compared to those injected with BSA (Fig. 8-C). These results indicated that PxGalectin-4 may influence *P. xylostella* immunity through regulation of AMPs expressions.

The survival rates of larvae treated with ds*PxGalectin-4* were monitored following *I. cicadae* infection. The ds*PxGalectin-4*-treated group showed significantly reduced larval survival compared to the ds*GFP*-injected group after *I. cicadae* infection (Fig. 8-D). At 48 h post-infection, the larval survival rate in the ds*PxGalectin-4*-treated group decreased to 24%, substantially lower than the ds*GFP*-treated group. However, supplementation with rPxGalectin-4 protein improved the survival of ds*PxGalectin-4*-treated larvae.

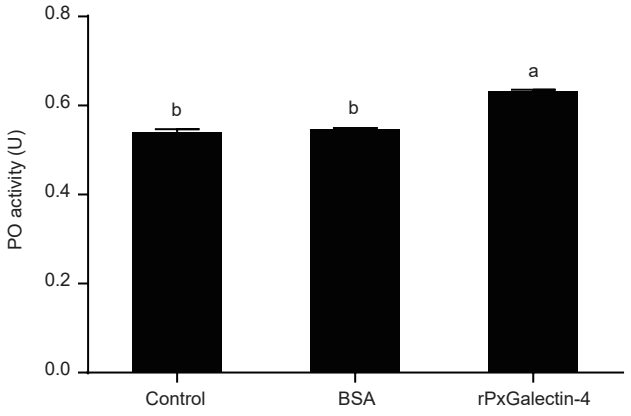


Fig. 7 Enhancement of hemolymph phenoloxidase (PO) activity by rPxGalectin-4. Buffer E served as the control. BSA, bovine serum albumin. Data are presented as mean±SEM (n=5). Different letters above the bars indicate statistically significant differences (P<0.05).

3.9. rPxGalectin-4 promotes nodulation and encapsulation of hemocytes

Nodulation and encapsulation assays were conducted to examine the cellular immune functions of rPxGalectin-4. The control group (BSA) showed no nodulation of hemocytes or *I. cicadae* spores (Fig. 9-A), while distinct nodulation was observed in the presence of rPxGalectin-4 (Fig. 9-B).

In vitro encapsulation was assessed using rPxGalectin-4-coated agarose beads (Fig. 9-C and D). These beads demonstrated significantly enhanced hemocyte encapsulation compared to BSA-coated agarose beads, indicating rPxGalectin-4's ability to promote hemocyte encapsulation. The dark coloration of encapsulated beads indicated the occurrence of melanization (Fig. 9-D).

4. Discussion

While insect lectins in innate immunity have been extensively studied (Xia *et al.* 2018), the function of galectin, a lectin family member, remains poorly understood in insects. The genome of *P. xylostella* contains four *galectins* genes: *Galectin-3-like*, *Galectin-4*, *Galectin-5* and *Galectin-8* (Xia *et al.* 2015). This investigation examined the molecular characterization and immune function of PxGalectin-4 in *P. xylostella*, contributing new perspectives on galectins' role in insect immunity.

PxGalectin-4 protein sequence demonstrates significant homology with other insect galectins. Phylogenetic analysis reveals that insect galectins form 6 distinct clades, with PxGalectin-4 showing close relation to Lepidoptera galectins. In galectins, the β-galactoside binding sites are typically characterized by two conserved motifs: HxNPR and WGxEE (Yang *et al.* 2023). Notably, PxGalectin-4 exhibits a distinct

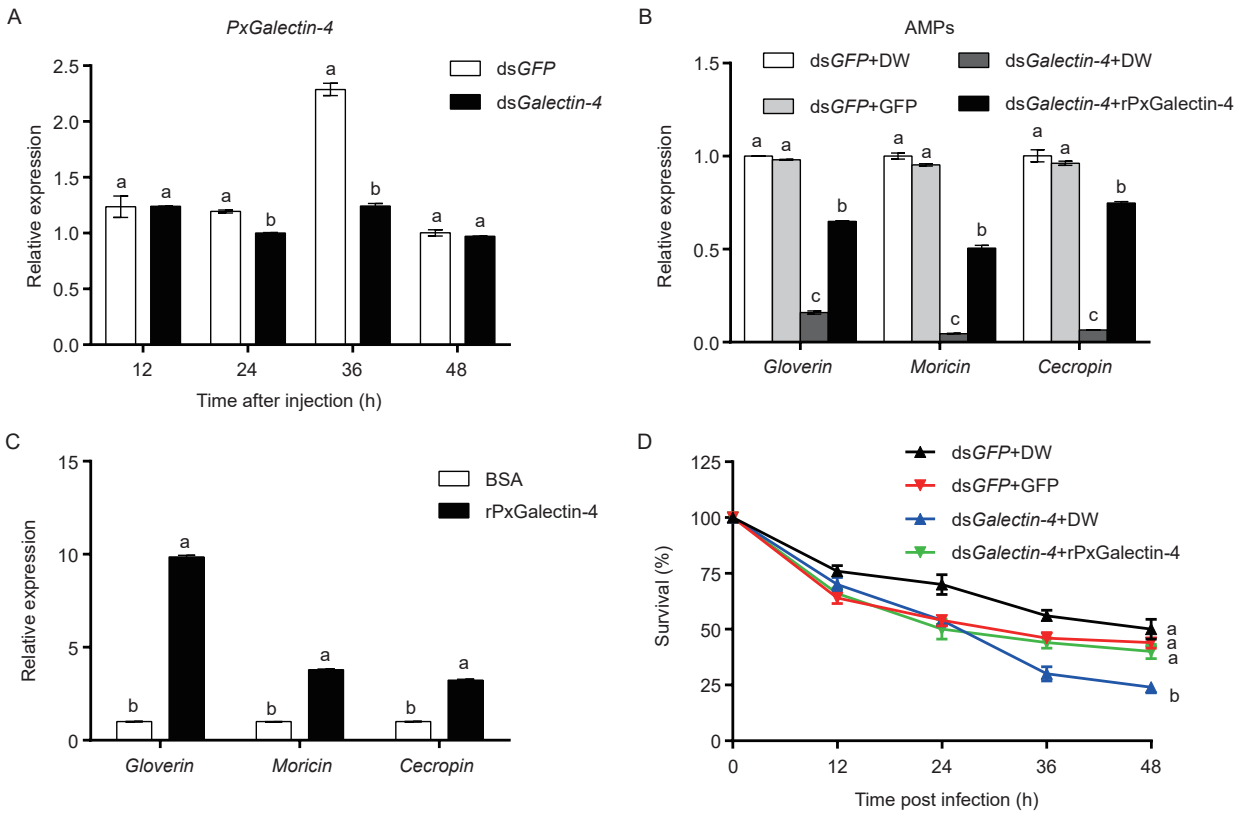


Fig. 8 Bioassay of ds*PxGalectin-4*-treated insects following *Isaria cicadae* infection. A, temporal expression of *PxGalectin-4* after ds*PxGalectin-4* injection. B, influence of *PxGalectin-4* silencing on antimicrobial peptides (AMPs) genes expressions. DW, diethylpyrocarbonate-treated water. C, influence of rPxGalectin-4 injection on AMPs genes expressions. Bovine serum albumin (BSA) served as the control. D, survival analysis of dsRNA-treated larvae following fungal infection. Data are presented as mean±SEM (n=3 for A–C; n=10 per each replicate for D). Significant differences (P<0.05) are indicated by different letters above the bars.

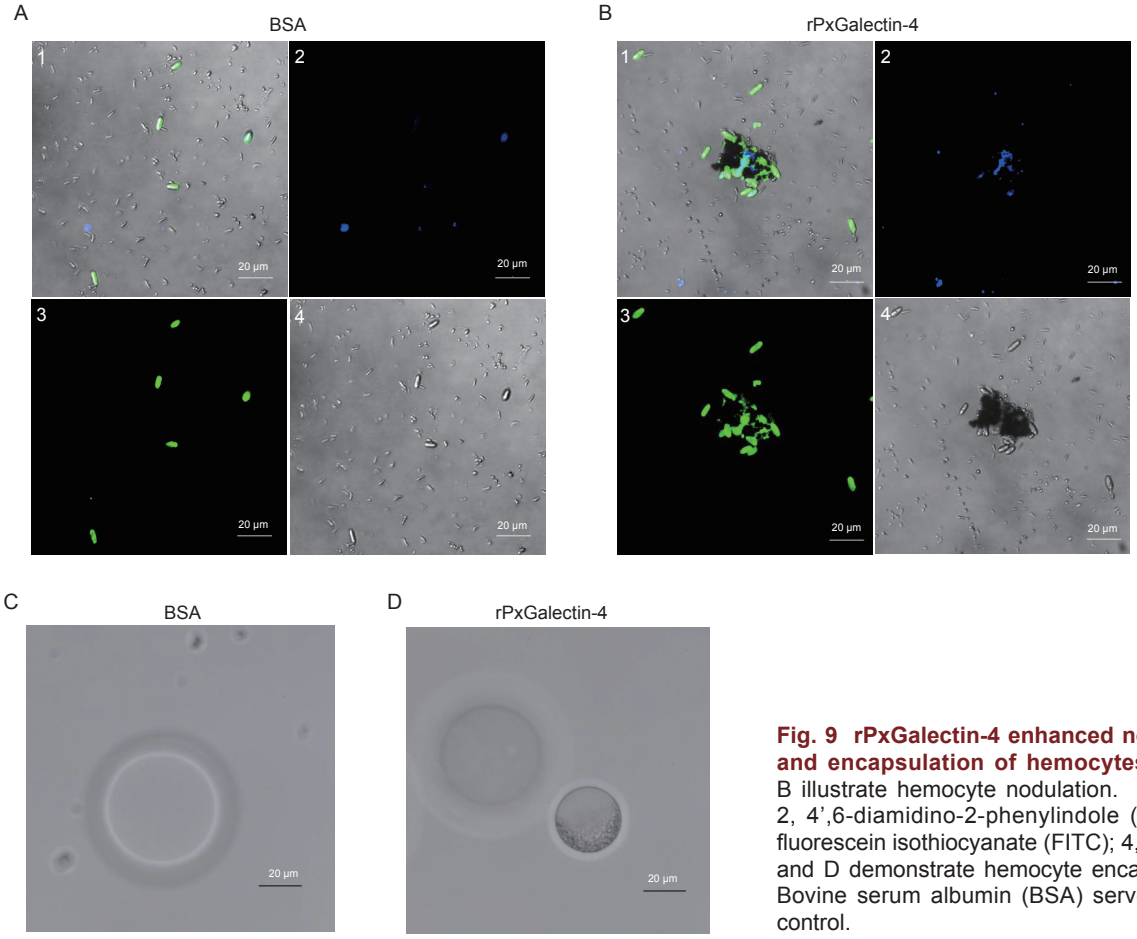


Fig. 9 rPxGalectin-4 enhanced nodulation and encapsulation of hemocytes. A and B illustrate hemocyte nodulation. 1, merge; 2, 4',6-diamidino-2-phenylindole (DAPI); 3, fluorescein isothiocyanate (FITC); 4, bright. C and D demonstrate hemocyte encapsulation. Bovine serum albumin (BSA) served as the control.

structural feature, containing only the WGxEE motif while lacking the HxNPR motif. BmGalectin4 of *B. mori* contains the two conserved motifs and dual CRDs (Rao *et al.* 2016). In contrast, PxGalectin-4 contains one CRD domain, classifying it as a member of the prototype group of galectins. The structural homology and conserved evolutionary characteristics of PxGalectin-4 substantiate its classification within the galectin family.

Galectins are a class of animal lectins demonstrating diverse biological functions. To understand the immune role of *PxGalectin-4*, its transcriptional profile was examined across developmental stages and tissues. *PxGalectin-4* showed ubiquitous expression throughout all developmental stages, with the highest levels observed in the third larvae, fourth larvae and adult stages. BmGalectin4 protein was detectable in larvae and eggs, with the highest abundance in eggs (Rao *et al.* 2016). These findings suggest that Galectin-4 serves different functions at various stages, including development, reproduction and embryogenesis. While *PxGalectin-4* was detectable in all examined tissues, its expression predominantly occurred in the fat body and hemocytes, which constitute key immune-response tissues in insects (Gillespie *et al.* 1997). PRRs are present in the insect hemolymph, secreted by either the fat body or hemocytes. Western blotting analysis confirmed PxGalectin-4 presence in hemolymph (Appendix D). However, BmGalectin4 protein remained undetectable in larval plasma, indicating it is not a PRR in hemolymph (Rao *et al.* 2016). Furthermore, the *PxGalectin-4* expression significantly increased following microbial challenge, similar to *Galectin-4* in *Procambarus clarkii* (Luo *et al.* 2023). These findings suggest that *PxGalectin-4* participates in immune defense against microbial infection.

Traditionally, galectins were known to function intracellularly through interactions with endogenous glycans (Liu and Stowell 2023). Recent evidence reveals their extracellular roles as PRRs that identify microbial surface glycans and modulate immunity (Vasta 2012; Lin *et al.* 2020). In this study, unlike most immulectins, PxGalectin-4 lacked a signal peptide, suggesting potential secretion into extracellular space through direct plasma membrane translocation (Luo *et al.* 2023). Similarly, BmGalectin4 lacked a signal peptide (Rao *et al.* 2016). Functional analysis showed that rPxGalectin-4 demonstrates broad-spectrum binding activity towards various pathogenic glycans, including LPS, PGN, and β-1,3-glucan. This binding profile corresponds with that of *P. xylostella* C-type lectin (Li *et al.* 2021), *B. mori* Galectin4 and C-type lectin-S2 (Rao *et al.* 2016; Shahzad *et al.* 2017), but differs from the diverse binding specificities observed in five C-type lectins from *Armigeres subalbatus* (Shi *et al.* 2014). The *A. subalbatus* lectins exhibited preferential binding to PGN and zymosan over LTA, while maintaining universal LPS recognition.

Galectins have demonstrated the capability to directly interact with various pathogens, including parasites, viruses, fungi and bacteria (Vasta 2009; Luo *et al.* 2023).

rPxGalectin-4 exhibited direct binding to the tested microbes, suggesting its function in microbial recognition within the *P. xylostella* immune system. Similarly, rBmGalectin4 demonstrated binding to bacterial cells (Rao *et al.* 2016). However, SDS completely disrupted the binding of rBmGalectin4 to *E. coli*. While C-type lectins are typically characterized as Ca^{2+}-dependent carbohydrate-binding proteins, galectins have traditionally been considered Ca^{2+}-independent lectins (Hughes 2001). Notably, our analysis identified Ca^{2+} binding sites in PxGalectin-4. This discovery was corroborated by the observation that rPxGalectin-4 exhibited Ca^{2+}-dependent agglutinating activity against *I. cicadae*, *E. coli* and *S. aureus*, although the underlying mechanism requires further investigation. Galectin 2 of *Argopecten irradians* also demonstrated strong agglutinating activity against bacterial cells (Song *et al.* 2011). These findings collectively indicate that PxGalectin-4 functions as a PRR in the *P. xylostella* immune system, potentially facilitating pathogen recognition and immune activation through specific binding to PAMPs.

To further understand the *in vivo* immune role of PxGalectin-4, the study examined the effects of *PxGalectin-4* silencing on the response to *I. cicadae* infection. Injection of *PxGalectin-4* specific dsRNA effectively reduced its expression. Although few studies have investigated galectins' role in modulating AMPs expression (Luo *et al.* 2023), this research revealed that silencing *PxGalecctin* caused a significant decrease in the expression of *Gloverin*, *Moricin*, and *Cecropin*. Conversely, *in vivo* injection of rPxGalectin-4 protein significantly enhanced the expression of these genes. Further investigation demonstrated that suppression of *PxGalectin-4* expression resulted in increased mortality of *P. xylostella* following *I. cicadae* infection. Moreover, rPxGalectin-4, similar to other lectins, showed broad spectrum antimicrobial activity against *E. coli*, *S. aureus* and *I. cicadae in vitro* and *in vivo*, indicating its direct antimicrobial function. In contrast, rBmGalectin4 lacked antibacterial activity against *E. coli* and *S. aureus* (Rao *et al.* 2016). These results suggest that PxGalectin-4 serves a dual role in *P. xylostella* humoral immunity: regulating AMPs expressions and directly eliminating microbes, emphasizing its significance in insect immunity.

Insect immunity encompasses a coordinated interaction of cellular and humoral responses. The cellular response includes nodulation, encapsulation and phagocytosis of invading microbes by hemocytes (Lavine and Strand 2002). Research has shown that immulectins and galectins mediate cellular immunity in response to microbes (Rao *et al.* 2016). Nodulation involves the formation of multicellular hemocyte aggregates around microorganisms, embedded in extracellular material (Eleftherianos *et al.* 2021). The present study revealed that rPxGalectin-4 aggregated hemocytes around fungal spores. Encapsulation, another essential immune mechanism, involves hemocytes attaching to and enveloping large foreign particles. For instance, C-type lectin IML-10 of *Ostrinia furnacalis* bound to hemocytes, enhancing their aggregation and encapsulation efficiency (Song *et al.* 2020). Research has shown that galectin from the shrimp *Fenneropenaeus merguiensis* enhances hemocyte-mediated encapsulation of pathogenic invaders (Praparatana *et al.* 2022). In this study, rPxGalectin-4 demonstrated the ability to promote hemocyte encapsulation against agarose beads.

5. Conclusion

This study identified and characterized PxGalectin-4 and demonstrated its diverse immunological functions. *PxGalectin-4* expression exhibited significant upregulation in response to microbial challenges. rPxGalectin-4 functioned as a PRR by directly binding to microbes and PAMPs, and agglutinated fungal and bacterial cells. The protein displayed direct antimicrobial activity *in vitro* and *in vivo*. Additionally, rPxGalectin-4 enhanced nodulation and encapsulation of hemocytes, and increased PO activity in hemolymph. Moreover, ds*PxGalectin-4* injection led to decreased expression of AMPs and increased mortality of *P. xylostella* following infection with the fungal pathogen *I. cicadae*. These findings establish PxGalectin-4 as a crucial component in *P. xylostella* humoral and cellular immunity against fungal infection, suggesting its potential application in the development of novel pest control strategies.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (32372522), the Natural Science Foundation of Anhui Province, China (2108085MC104), and the Natural Science Research Project for Universities in Anhui Province, China (2022AH050929).

Declaration of competing interest

The authors declare that they have no conflict of interest.

Declaration of generative AI and AI-assisted technologies in the writing process

The authors declare that they did not use AI in the preparation and writing of this manuscript.

Ethical statements

All applicable international, national and institutional guidelines for the care and use of animals were followed.

Appendices associated with this paper are available at https://doi.org/10.1016/j.jia.2025.07.002

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