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RESEARCH ARTICLE

CRISPR/Cas9-mediated knockout of *serpin15* impacts reproduction and immunity in *Plutella xylostella* Linnaeus

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Highlights

- *Serpin15* plays a crucial role in the reproductive regulation and immunity of *Plutella xylostella*.
- The CRISPR/Cas9-mediated knockout of *serpin15* in *P. xylostella* reduces both the oviposition rate and embryonic hatching rate of the offspring.
- *Serpin15* enhances the immunity of *P. xylostella* by inhibiting the activity of phenoloxidase (PO).

Abstract

Plutella xylostella represents a significant agricultural pest affecting cruciferous crops globally. The extensive use of synthetic insecticides has resulted in environmental contamination and resistance development, necessitating research into environmentally sustainable biopesticides. Serine protease inhibitors (serpins) serve essential functions in melanization during innate immunity, reproduction, and metamorphic development. Through proteomic analyses conducted across developmental stages of *P. xylostella*, *serpin15* was identified as a crucial member of the typical inhibited serpin family, though its precise function remained undetermined. RT-qPCR analyses of gene expression patterns across tissues and developmental stages demonstrated that the *serpin15* gene exhibits high expression in male adult gonads and reaches maximum levels in hemolymph. The *serpin15* mRNA levels showed dynamic regulation in the midgut following *Serratia marcescens* (PS-1) infection, characterized by an initial decline followed by upregulation. CRISPR/Cas9-mediated knockout of *serpin15* in homozygous lines led to decreased oviposition and embryonic hatching rates in offspring. Functional analyses confirmed that *serpin15* inhibits phenoloxidase activity, while exogenous supplementation with recombinant serpin15 protein effectively suppressed hemolymph melanization, establishing its regulatory role in countering PS-1 through immune melanization. These findings demonstrate *serpin15*'s dual functionality in regulating both fecundity and immunity against PS-1 in *P. xylostella*. This research establishes a theoretical foundation for developing biocontrol strategies targeting insect immune and developmental systems.

Keywords: *Plutella xylostella*, serine protease inhibitors, CRISPR/Cas9, melanization

1. Introduction

The diamondback moth (DBM), *Plutella xylostella* (Lepidoptera: Plutellidae), is a major agricultural pest affecting cruciferous vegetables, with significant presence in southern North America, South America, Southeast Asia, South Africa, and southeastern Australia (Zalucki *et al.* 2012). The extensive pesticide resistance developed by *P. xylostella* has rendered conventional chemical insecticides ineffective in protecting these crops from substantial yield losses. Key challenges in managing *P. xylostella* include pesticide residues, pest resurgence, and resistance to biopesticides (Furlong *et al.* 2013; Li *et al.* 2016). This situation necessitates the development of environmentally sustainable control methods for *P. xylostella*. Through evolutionary processes, insects have developed innate

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immunity, comprising both humoral and cellular responses to combat pathogens (Lemaitre and Hoffmann 2007; Mongelli *et al.* 2022). Beyond these responses, intracellular mechanisms including apoptosis, RNA interference (RNAi), and melanization serve essential functions in insect defense (Briscoe and Thérond 2013). Extensive research has examined immunity and development in various insects, including *Drosophila melanogaster* (Watanabe *et al.* 2006; Homma *et al.* 2022; Karanja *et al.* 2022; Shi *et al.* 2022), *Bombyx mori* (Wheeler *et al.* 2000; Xu *et al.* 2017; Li *et al.* 2021; Zhang *et al.* 2022), and *P. xylostella* (Moller *et al.* 2001; Gasteiger 2003; Hashimoto *et al.* 2003; Waterhouse *et al.* 2018). However, the precise mechanisms through which functional genes contribute to insect immunity and development remain incompletely understood.

Insect immune responses are intrinsically linked to developmental stages, as their capacity for foreign invasion resistance varies throughout development (Bao *et al.* 2024). The Toll pathway in *D. melanogaster*, for example, controls both antimicrobial peptide expression and dorsal-ventral axis formation (Hashimoto *et al.* 1988; Armenteros *et al.* 2019). Similarly, serpin27a in *D. melanogaster* regulates the phenoloxidase cascade pathway through PPAE modulation and the Toll signaling pathway (Jang *et al.* 2006). Furthermore, cytochrome P450 serves an essential function in regulating mitochondrial hormone synthesis, which can trigger antimicrobial peptide expression. Thus, elucidating the molecular functions of these genes may offer crucial insights for developing effective strategies to control *P. xylostella*.

Serine protease inhibitors (serpins), recognized as the largest family of protease inhibitors, demonstrate diverse physiological functions in arthropods (Meekins *et al.* 2017; Shakeel *et al.* 2019). These inhibitors regulate antimicrobial peptide expression within the insect innate immune system by modulating serine protease cascades. For instance, *serpin1J* inhibits hemolymph proteinase 8 (HP8), an activator of proSpätle, thereby influencing the Toll pathway and antimicrobial peptide expression in *Manduca sexta* (An *et al.* 2011). In *P. xylostella*, Destruxin A challenge upregulates s*erpin2/4/5* in larval fat body and hemolymph, while RNAi-mediated silencing of these genes amplifies antimicrobial peptides (cecropin 1 and cecropin E) expression and increases host mortality post-infection (Han *et al.* 2014) and miR-8 from *P. xylostella* positively regulates the transcript levels of the *serpin27*, enabling upregulation of antimicrobial peptides, such as gloverin, that are controlled by the Toll pathway and activation of proteolytic cascades (Etebari and Asgari 2013). Furthermore, serpins regulate melanization processes in insect hemolymph by inhibiting serine protease cascades. Studies have identified several serpins, including *serpin1J*, *serpin3*, *serpin6*, and *serpin7* in *M. sexta*, as well as *serpin 27A* in *D. melanogaster*, which function as inhibitors of prophenoloxidase activating proteinases (PAPs), thus controlling the melanization process (Ligoxygakis 2002; Jiang *et al.* 2003; Zhu *et al.* 2003; Nappi *et al.* 2005; Zou and Jiang 2005; Christen *et al.* 2012; Suwanchaichinda *et al.* 2013). Beyond their immune functions, *serpin 27A* participates in dorsal-ventral axis formation, while the accessory gland protein 76 (Acp76) serpin is essential for hormone transport, highlighting their significant roles in insect reproduction and development (Wolfner *et al.* 1997; Ligoxygakis *et al.* 2003; Garrett *et al.* 2009). The expression of specific immune-responsive serpins increases rapidly in response to pathogen infection or injury, functioning as acute phase proteins (Shin *et al.* 1998; Danielli *et al.* 2005).

Furthermore, serpins play a vital role in host-pathogen interactions. For example, LbSPNy inhibits the melanization of insect hemolymph, thereby protecting parasitic wasp eggs from the host immune system in *Leptopilina boulardi* (Colinet *et al.* 2009, 2013). The *H. armigera serpin5* and *serpin9* regulate melanization by directly inhibiting their target proteases cSP4 and cSP6, respectively. Significantly, cSP6 activates prophenoloxidase (PPO) purified from hemolymph and *serpin5/9*-depleted insects exhibited elevated phenoloxidase (PO) activities and demonstrated increased resistance to baculovirus infection (Yuan *et al.* 2017). Four *P. xylostella* serpin genes were activated, and the PPO1 gene was suppressed by Bt8010. Inhibition of the two PPO proteins was observed at 18 h post-infection. Feeding Bt8010-infected larvae recombinant PPOs enhanced their survival (Lin *et al.* 2020). Additionally, serpins present in insect saliva can mediate clotting and anti-inflammatory responses in mammalian blood. For instance, Alboserpin in the saliva of *Aedes albopictus* affects anticoagulation by inhibiting Factor Xa activity (Stark and James 1998; Calvo *et al.* 2011). Given their diverse functions, serpins of *P. xylostella* may serve as cross-regulatory factors in various aspects, particularly in immunity and development, by regulating melanization, the Toll pathway, or other signaling pathways (Lin *et al.* 2017).

To investigate the relationship between insect immunity and development, we employed bioinformatics to correlate transcriptomic data with proteomic data based on the genome of *P. xylostella*. This research was based on the determination and assessment of transcriptomic and proteomic data at different developmental stages of *P. xylostella*. Our analysis identified a serpin, designated *serpin15*, which exhibits high expression in the hemolymph (He) and fat body (FB) of fourth-instar larvae (Zhang *et al.* 2022). Here, we preliminarily determined the functions of the serpin15 protein in regulating the immunity and reproduction of *P. xylostella* using CRISPR/Cas9 technology. The serpin15 protein was found to participate in the reproductive mating process of *P. xylostella*, as well as in the hatching rates of eggs and embryos of offspring, and in the immune response of *P. xylostella* against PS-1. This research advances our understanding of the functional roles of serpins in arthropods.

2. Materials and methods

2.1. Insects and pathogens

The experimental population of the diamondback moth (*P. xylostella*) was initially collected in 2017 from a cabbage field at the Dafeng Research Station of the Institute of Plant Protection, Guangdong Academy of Agricultural Sciences, China. The population has been continuously reared for over ten generations under laboratory conditions (temperature:

(25±1)°C, photoperiod of 16 h L:8 h D, relative humidity of (65±5)%. The larvae were sustained on an artificial diet, while the adults were provided with a 10% honey solution (Waterhouse *et al.* 2018).

Serratia marcescens PS-1 was obtained from the State Key Laboratory of Green Pesticide, South China Agricultural University and stored at –80°C with a glycerol concentration of 20–25%.

2.2. The expression pattern of *serpin15*

Various developmental stages were analyzed, including egg mixture (0 to 66 h after laying), larval mixture (0 to 144 h after hatching), female pupae mixture (0 to 96 h after pupation), male pupae mixture (0 to 96 h after pupation), female adults (0 h after eclosion), and male adults (0 h after eclosion). Multiple tissues were collected as samples, including the midgut, hemocytes, fat body, testis and epidermis of larvae (132 h after hatching), the testis of prepupae (168 h after hatching) as well as the female and male gonads of adults (0 h after eclosion). For PS-1 infection, larvae (84 h after hatching) underwent 6 h of starvation before infection. The PS-1 strain was cultured in LB medium at 120 r min^{-1} and 30°C for 20 h. After culture, the medium was centrifuged at 8,000 r min^{-1} for 5 min to collect bacteria, which were then resuspended in phosphate-buffered saline (PBS) to achieve a concentration of 10^6 CFU mL^{-1} for infection. A total of 1 mL bacterial solution was evenly distributed on the surface of an artificial diet (7 cm×10 cm) and fed to the larvae. Twenty-four hours post-treatment, the larvae received fresh diet (without bacteria). Control groups were treated with PBS. The midgut, fat body, and hemocytes of surviving larvae were collected at 0, 18, 24, 30, and 36 h after infection for analysis.

The methods for total RNA isolation and RT-qPCR were adapted from previous studies (Zhang *et al.* 2022). Total RNA of samples was extracted using Total RNA Kit II (OMEGA, Norcross, GA, USA) and cDNA was prepared from 2 μg of total RNA using Prime script RT Master Kit according to the manufacturer's instructions. RT-qPCR was performed on CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using TB Green® Premix *Ex Taq*™ II (Tli RNaseH Plus, TaKaRa,Tokyo, Japan). The reaction program consisted of initial denaturation at 95°C (30 s), followed by 39 cycles at 95°C (5 s) and 55°C (30 s), with melt curve analysis performed according to manufacturer's instructions. The experiments were conducted in triplicate with three biological repeats and the data referenced to internal control ribosomal protein S13 (RPS13) using comparative CT methods. The primers used for RT-qPCR are presented in Appendix A.

2.3. Bioinformatics analysis of serpin15

The 3D structure of serpin15 was modeled using SWISS-MODEL (Gasteiger 2003). The molecular weight (Mw) and isoelectric point (pI) of serpin15 were determined with the Compute pI/Mw tool (Moller *et al.* 2001). The transmembrane structure of serpin15 was assessed using TMHMM. The signal peptide of serpin15 was evaluated with SignalIP (Waterhouse *et al.* 2018). The domain of serpin15 was analyzed using SMART (Letunic *et al.* 2020) and InterPro (Blum *et al.* 2020). Phylogenetic analysis was conducted with MEGA11 (Tamura *et al.* 2021) and refined using iTOL (Letunic and Bork 2006). Serpin protein sequences used for phylogenetic tree construction were obtained from the following species: *B. mori* (Bm), *D. melanogaster* (Dm), *Tenebrio molitor* (Tm), *Manduca sexta* (Ms), *Homo sapiens* (Ha), *Anopheles gambiae* (Ag), *P. xylostella* (Px).

2.4. The construction of *P. xylostella serpin15*-knock-out mutants

sgRNA design and synthesis The sgRNA targeting *serpin15* was designed using CRISPR (Concordet and Haeussler 2018). The design process comprised the following steps: Step 1: Enter the exon sequence of *Serpin15*; Step 2: Select the genome of the diamondback moth (DBM, GCF_000330985.1); Step 3: Select the protospacer adjacent motif type (20bp-NGG-sp Cas9). The primers for the sgRNA template are presented in Appendix A. Subsequently, the sgRNA was synthesized using the HiScribe T7 Quick High Yield RNA Synthesis Kit (NEB, E2050S) based on the template.

Embryo injection A mixture containing 100 ng sgRNA and 50 ng Cas9-N-NLS (GenCrispr, Z03468) was prepared in a 20 μL reaction and incubated at 37°C for 10 min. The prepared mixture was injected into eggs (1 nL per egg) within 20 min after oviposition, using the FemtoJet 4x (Eppendorf, Germany).

Screening of homozygous mutants and verification Adults carrying the initial mutated gene and wild-type adults were designated as generation 0 (G_0). The progeny of G_0 were classified as generation 1 (G_1), comprising heterozygous mutants. These heterozygous mutants were screened and crossed to obtain generation 2 (G_2), which included homozygous mutants. Subsequently, appropriate homozygous mutants were selected for propagation to establish stable *serpin15*-knockout mutants. Throughout this process, pupae were reared individually until eclosion, after which adults were paired for mating. Heterozygous or homozygous genotypes were identified through DNA sequencing. Genomic DNA extraction from adults and target fragment amplification utilized RAPA3G PCR Mix (A0609, Haigene, China). The primers used for target site detection and off-target effect verification are listed in Appendix A. Furthermore, four samples (W-FM, W-M, Mt-FM, Mt-M) were collected from wild-type and *serpin15*-knockout mutants to verify the knockout of serpin15 at the protein level *via* Western blot analysis. Protein extraction was conducted using tissue total protein lysis buffer (C500028, Sangon, China). Anti-alpha tubulin (ab52866, Abcam, Cambridge, MA, USA) served as the reference primary antibody, while a rabbit polyclonal antibody produced by ABclonal was used for serpin15. The secondary antibody employed was Goat anti-Rabbit IgG H&L (HRP) (ab6721, Abcam).

2.5. The statistics of oviposition rate and embryonic hatching rate

The pupae of wild-type and *serpin15*-knockout mutants were reared individually until eclosion. Adults were paired for mating 24 h after eclosion, and males were removed

following mating. Egg oviposition was quantified after 72 h. Eggs laid within the initial 24 h were collected and maintained under optimal conditions (25°C, 70% relative humidity), and embryonic hatching rates were calculated on the second and fifth days. The mating combinations included: wild-type females with wild-type males (group A); wild-type females with mutant males (group B); mutant females with wild-type males (group C); and mutant females with mutant males (group D). Each group comprised 30 pairs, with three biological replicates performed. Statistical significance was evaluated using the Kruskal-Wallis test (α=0.05). Different lowercase letters indicate significant differences among groups (P<0.05).

2.6. The survival analysis of *P. xylostella* after PS-1 infection

Larvae (84 h post-hatching) of both wild-type and *serpin15*-knockout mutants, subjected to 6 h of starvation, were infected with PS-1 at concentrations of 10^5, 10^6, 10^7, 10^8, 10^9 CFU mL^{-1}, as previously described. Survivors were enumerated at 0, 12, 24, 36, 48, and 60 h post-infection. Survival curves were analyzed using the Kaplan-Meier method, and statistical significance was assessed using the Log Rank (Mantel-Cox) test. Additionally, Cox proportional-hazards model analysis was conducted using the "Forward: Likelihood ratio" method.

2.7 PO activity determination and melanization assay

PO activity determination The midgut, fat body and hemolymph of wild-type and *serpin15*-knockout mutants were collected at 0, 18, 24, 30, 36 h post PS-1 infection. The control groups of both wild and mutant larvae were treated with PBS. PO activity was determined using the Polyphenol Oxidase Activity Assay Kit (PPO-1-Y; Cominbio, Suzhou, China).

Melanization assay Hemolymph samples from mutants were collected 24 h post-PS-1 infection. Serpin15 recombinant protein (10 and 20 μg) was added to the hemolymph samples, and the mixture was incubated at 25°C for 2 h. Microscopic observations were conducted. The methods for producing serpin15 recombinant protein followed protocols from previous studies (Zhang *et al.* 2022).

2.8. Statistical analysis

Data are presented as mean±standard error of the mean (SEM). Statistical analyses were conducted using SPSS Statistics 26 software (IBM Corp, USA). Significant differences between groups were determined through one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) post hoc test. A P-value<0.05 was considered statistically significant. Figure captions specify the statistical tests employed. Figures were generated using Origin 2022 software.

3. Results

3.1. The immune and developmental association of *serpin15*

To investigate the immune and developmental association of *serpin15*, expression patterns were analyzed across developmental stages, tissues, and following PS-1 infection using RT-qPCR. *Serpin15* showed high expression in larvae but low levels in eggs, pupae and adults. The expression of *serpin15* in male adults was significantly higher than in females (five-fold), while pupal expression showed no significant sex-based difference (Fig. 1-A). Among larval tissues, *serpin15* expression was the highest in hemocytes, followed by the fat body and epidermis, with minimal expression in the midgut (Fig. 1-B). During the pupal stage, critical for reproductive organ development, tissues were collected from testes of *P. xylostella* at fourth-instar larval, prepupal, pupal, and adult stages, and from ovaries at pupal and adult stages. Analysis revealed consistently high *serpin15* expression in larval and prepupal testes compared to adult stage, with significant expression also detected in pupal ovaries, though lower than in testes. These findings support *serpin15*'s role in reproductive development in both sexes (Fig. 1-C). Following PS-1 infection in *P. xylostella*, *serpin15* expression decreased in the midgut at 18 h post-infection but increased significantly at 24 and 36 h. Conversely, expression decreased significantly in fat body and hemocytes at 30 and 36 h post-infection (Fig. 1-C). These results indicate serpin15's potential significance in immune responses and developmental processes.

3.2. The structure of serpin15

After confirming the immune and developmental association of *serpin15*, functional predictions were made through sequence analysis. The serpin15 transcript spans 1,391 bp, encoding 401 amino acids (aa), with a predicted molecular weight of 44.121 kDa. The protein lacks a transmembrane structure but contains a signal peptide (1–20 aa), indicating its secretory nature (Fig. 2-A). As a member of the serpin superfamily, serpin15 features three β-sheets and nine α-helices, including a characteristic reactive center loop (P1-P1': Arg-Leu) (Fig. 2-B). The high sequence conservation of serpin15 among Lepidoptera (Fig. 2-C) suggests functional similarity with proteins in other Lepidopteran species. Based on its conserved structure, serpin15 likely functions in the prophenoloxidase (PO) cascade as a serine protease (SP) inhibitor regulating melanization.

3.3. The fecundity deficiency of *serpin15*-knockout mutants

Using the CRISPR/Cas9 system, we generated DBM *serpin15*-knockout mutants. Homozygous mutants were obtained by G_2 (Fig. 3-A). The deletion of a nucleotide (guanine) caused premature termination of serpin15 translation in the *Serpin15*-knockout mutants (Fig. 3-B and C). Western blot analysis confirmed that serpin15 is located on an autosome and was successfully knocked out in the mutants (Fig. 3-D), with the full-page blot image provided in Appendix B.

Multiple observational and physiological experiments demonstrated no significant differences in appearance or developmental duration between wild-type organisms and *serpin15*-knockout mutants. However, the mutants showed notable deficiencies in specific capabilities. The total egg production of *serpin15*-knockout mutants (Group D) decreased

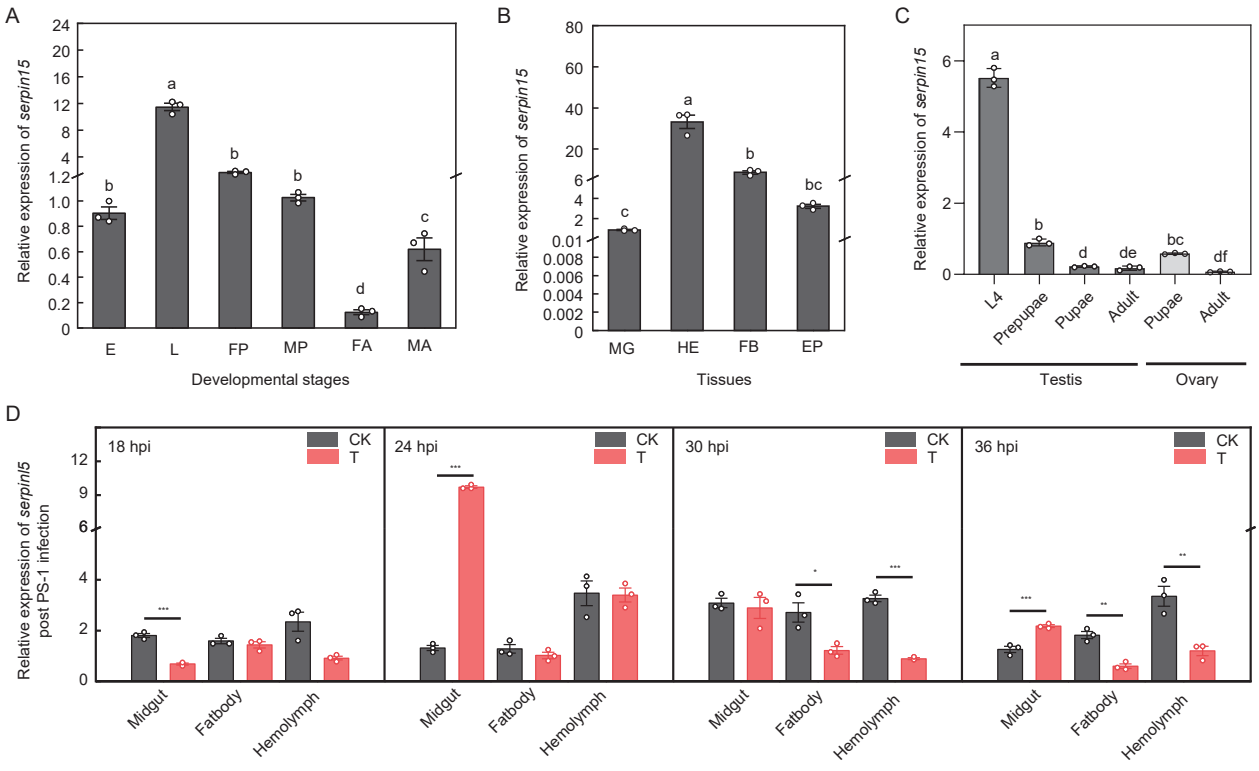


Fig. 1 Expression patterns of *serpin15* validated by RT-qPCR. A, expression of *serpin15* during the developmental stages of *Plutella xylostella*. E, eggs; L, larvae; FP, female pupae; MP, male pupae; FA, female adults; MA, male adults. B, expression of *serpin15* in different tissues of *P. xylostella*. MG, midgut; HE, hemocyte; FB, fat body; EP, epidermis. C, expression of *serpin15* in gonad of *P. xylostella*. D, expression of *serpin15* in *P. xylostella* post-PS-1 infection. CK, control check groups treated with PBS; T, treatment groups infected with PS-1. hpi, hours post infection. Data are presented as mean±SEM from three independent experiments. Different lowercase letters indicate significant differences among different samples using LSD and Duncan analysis (α=0.05). Statistical significance between control check groups and treatment groups was determined using two-tailed Student's *t*-tests (α=0.05; *, P<0.05; **, P<0.01; ***, P<0.001).

significantly by 41.27% compared to wild-type organisms (Group A) (Fig. 4-A). Egg production data from various combinations of female and male organisms (Groups B and C) demonstrated gender differences in *serpin15* function (Fig. 4-A). Groups B and C showed reduced egg production by 28.91 and 28.35%, respectively, compared to Group A; additionally, Group D's egg production was 17.39 and 18.02% lower than Groups B and C. No significant difference emerged between Groups B and C. These results indicate that *serpin15* absence in either males or females reduces egg production, with a more substantial decrease when *serpin15* is absent in both sexes. Notably, adult reproductive system morphology showed no apparent differences between wild-type organisms and *serpin15*-knockout mutants (Fig. 4-B). Both wild-type organisms and mutants completed oviposition within 72 h, indicating no oviposition delay in mutants.

The embryonic hatching rate of *serpin15*-knockout mutants (Group D) decreased significantly by 48.41 and 27.23% compared to the wild type (Group A) at 2 days and in total (Fig. 4-C). Unlike egg production, no significant differences in hatching rates occurred between Groups A and B, Groups B and C, or Groups B and D (Fig. 4-C and D). Group C's hatching rate was 23.26 and 12.78% lower than Group A, while Group D showed a decrease of 32.78 and 16.56% compared to Group C at 2 days and in total. These results suggest that *serpin15* absence leads to reduced embryonic hatching rates, particularly during early stages.

3.4. The immune deficiency of *serpin15*-knockout mutants to PS-1

Beyond fecundity deficiency, an immune deficiency to PS-1 was identified in *serpin15*-knockout mutants (Fig. 5-C). Kaplan-Meier analysis revealed survival curves for both wild-type and mutant strains following PS-1 infection (Fig. 5-A). Significant differences were observed in both dose (P=6.318E-89) and strain (P=4.045E-07) as independent factors. Comparing wild-type and mutant strains at specific doses, significant differences emerged only in groups treated with doses of 10^6 (P=4.187E-04), 10^8 (P=0.005), 10^9 (P=0.036) CFU mL^{-1}. Subsequently, a treatment dose of 10^6 CFU mL^{-1} was selected for further experiments. Cox proportional-hazards model analysis assessed survival rates of wild-type and mutant strains after PS-1 infection (Fig. 5-B). Results indicated that the mortality hazard of wild-type strains was significantly lower than mutants (P=1.064E-04, hazard ratio=0.689, 95% confidence interval=0.582–0.837). This indicates reduced immunity to PS-1 infection in mutants lacking *serpin15*.

3.5. The regulation of melanization by *serpin15*

Based on hypothesized functions of *serpin15*, we measured

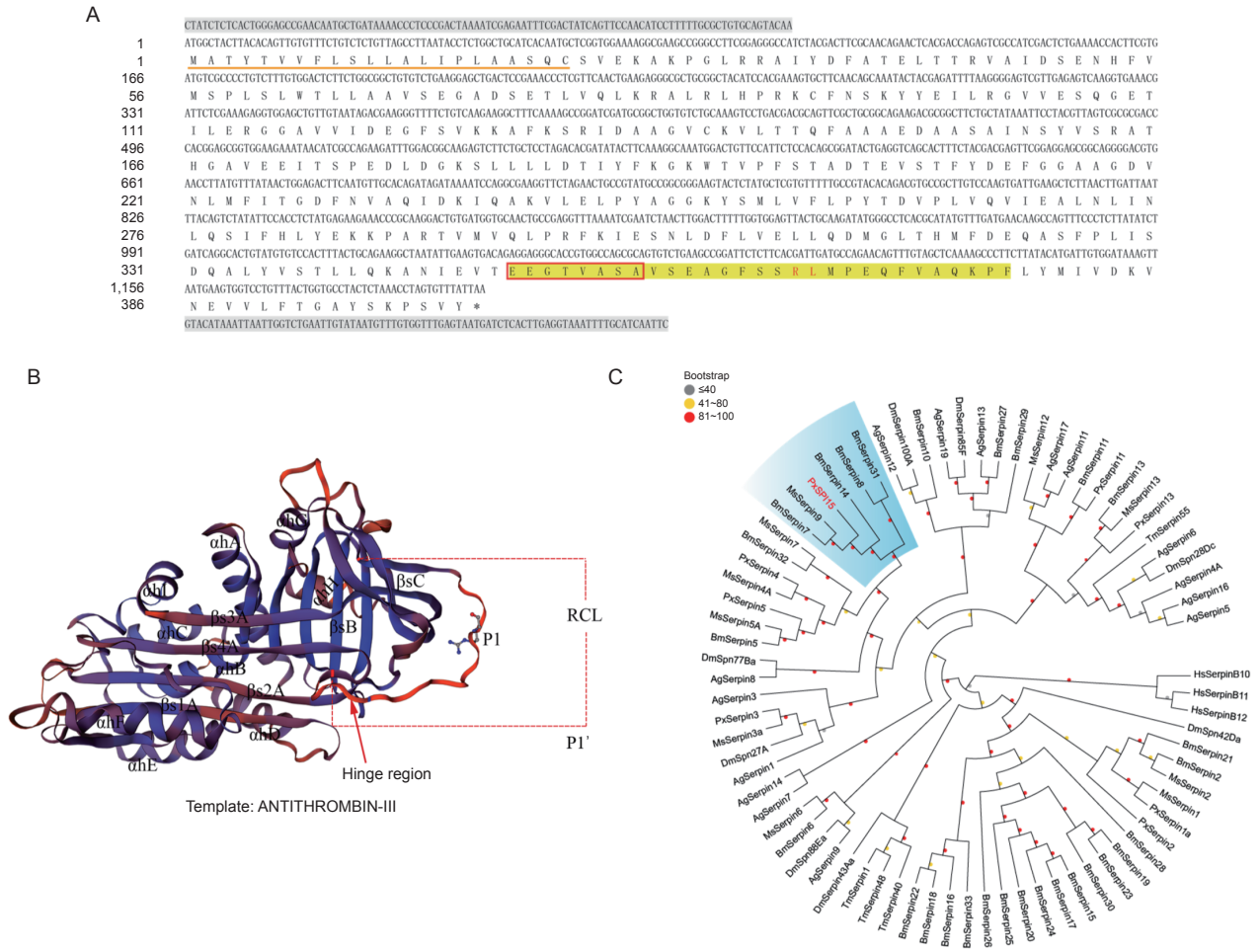


Fig. 2 The sequence analysis of serpin15. A, mRNA and protein sequences of serpin15. The 5′ untranslated region (UTR) and 3′ UTR are highlighted in grey, the reactive center loop (RCL) is highlighted in yellow, and the hinge region is marked with a red box. The P1 and P1′ residues are highlighted in red. B, three-dimensional structural model of serpin15, where αh denotes alpha helices and βs denotes beta sheets. RCL indicates the reactive center loop, while P1 and P1′ represent the amino acid residues of the scissile bond. C, phylogenetic analysis of serpin15, with the gene of interest highlighted in red font. Bm, *Bombyx mori*; Dm, *Drosophila melanogaster*; Tm, *Tenebrio molitor;* Ms, *Manduca sexta*; Ha, *Homo sapiens*; Ag, *Anopheles gambiae*; Px, *Plutella xylostella.*

PO activity in the midgut, fat body and hemolymph (Fig. 6). The PO activity of Group W-T was significantly higher than Group W-CK in all three tissues at 24 and 36 hours post infection (hpi), indicating PO cascade activation following PS-1 infection. Group Mt-CK showed significantly higher PO activity than Group W-CK in all tissues at 30 and 36 hpi. Notably, the increase in PO activity after PS-1 infection was significantly greater in mutants compared to wild types across all groups. These results demonstrate that *serpin15* inhibits PO activity and plays a crucial role in the PO cascade following PS-1 infection. RNAi experiments further validated this finding — knockdown of *serpin15* led to significantly increased PO activity in the hemolymph (Appendix C). *In vitro*, addition of serpin15 recombinant protein effectively inhibited melanization in *P. xylostella* hemolymph (Fig. 6-D).

4. Discussion

Serine protease inhibitors (serpins) regulate serine protease activity and mediate essential biological processes, including coagulation, fibrinolysis, programmed cell death, development, and inflammation (Wei *et al.* 2024). However, the immunological and developmental functions of serpins in *P. xylostella* remain largely unexplored. This study generated *serpin15*-knockout homozygous mutants using CRISPR/Cas9 technology. Phenotypic analysis revealed significantly reduced fecundity and embryonic hatching rates in the mutants. Comparative infection assays with PS-1 demonstrated decreased resistance in mutants, indicating compromised immune defenses. These findings establish *serpin15* as a dual-function gene regulating reproductive fitness and antibacterial immunity in *P. xylostella*, providing valuable insights for targeted pest control strategies.

Tissue-specific gene expression patterns frequently correlate with genes' functional roles in biological systems (Mantica *et al.* 2024). Using RT-qPCR, we systematically examined *serpin15* expression across developmental stages, tissues, and PS-1 challenged conditions in *P. xylostella*. *Serpin15* showed high expression during larval stages but minimal expression in eggs, pupae, and adults, suggesting

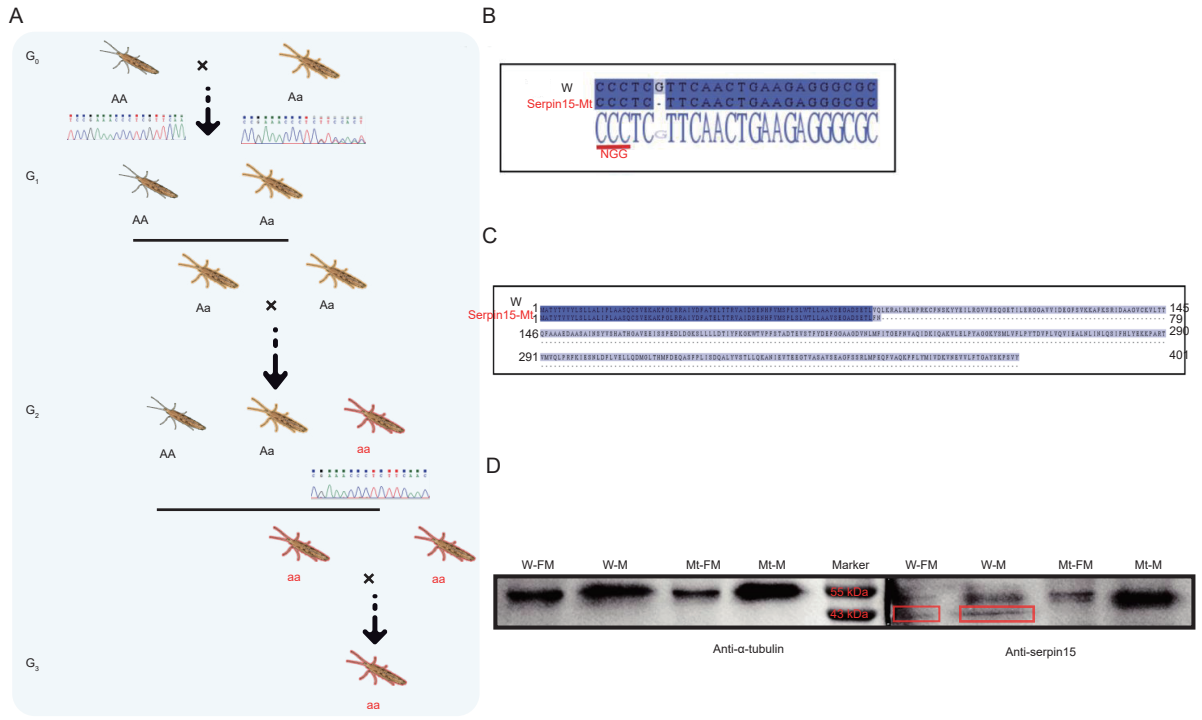


Fig. 3 The screening and verification processes for *Plutella xylostella serpin15*-knockout mutants. A, the procedures for homozygote screening. G, generation; AA, the homozygous genotype of wild-type; Aa, the hemizygous genotype of *serpin15*-knockout mutants; aa, the homozygous genotype of *serpin15*-knockout mutants. B, alignment of DNA sequences between the wild-type and Serpin15-knockout mutants. W, wild type; *serpin15*-Mt, *serpin15*-knockout mutants; NGG, the characteristic sequences of protospacer adjacent motif. C, protein sequences alignment between wild-type and *serpin15*-knockout mutants. D, verification of *serpin15* knockout *via* Western blot analysis. W-FM, the female of wild-type; W-M, the male of wild-type; Mt-FM, the female of *serpin15*-knockout mutants; Mt-M, the male of *serpin15*-knockout mutants. The serpin15 protein is highlighted within the red box.

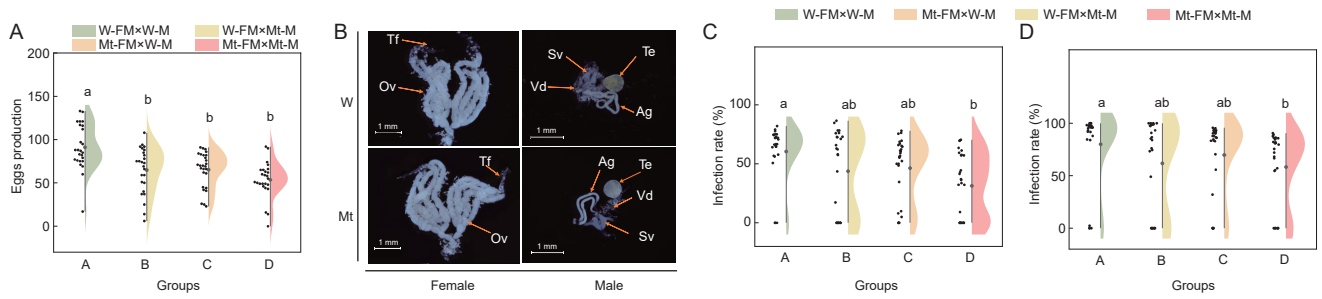


Fig. 4 Egg production and reproductive system morphology in different strains of *Plutella xylostella*. A, total egg production of wild-type and *serpin15*-knockout mutants. W-FM, female wild-type; W-M, male wild-type; Mt-FM, female *serpin15*-knockout mutants; Mt-M, male *serpin15*-knockout mutants. B, morphology of the reproductive system in wild-type and *serpin15*-knockout mutants. Tf, terminal filament; Ov, ovariole; Te, testis; Ag, accessory glands; Sv, seminal vesicle; Vd, vas deferens. C, hatching rate of wild-type and *serpin15*-knockout mutants over two days. D, total hatching rate of wild-type and *serpin15*-knockout mutants. Different lowercase letters indicate significant differences among groups using the Kruskal-Wallis test (α=0.05, P<0.05).

involvement in developmental regulation. Furthermore, *serpin15* exhibits high expression in larval and pupal testes and pupal ovaries, demonstrating male bias during larval stages and female bias during pupal stages. This indicates its sex-specific role in reproduction, particularly in sexual reproductive systems. This finding aligns with the conserved function of serpins in reproductive regulation across insect species (Meekins *et al.* 2017).

The secretions of male accessory glands play a vital role in fertility by influencing oocyte production and oviposition in females (Peng *et al.* 2025). The serpin family comprises essential components of seminal fluid and accessory gland secretions. In mammals, murine SERPINE2 inhibits cholesterol efflux-a critical event triggering sperm capacitation-thereby blocking serum albumin-induced *in vitro* sperm capacitation and preventing sperm-oocyte binding (Lu *et al.* 2011). Similar regulatory mechanisms involving broad physiological responses exist in insects. The male accessory

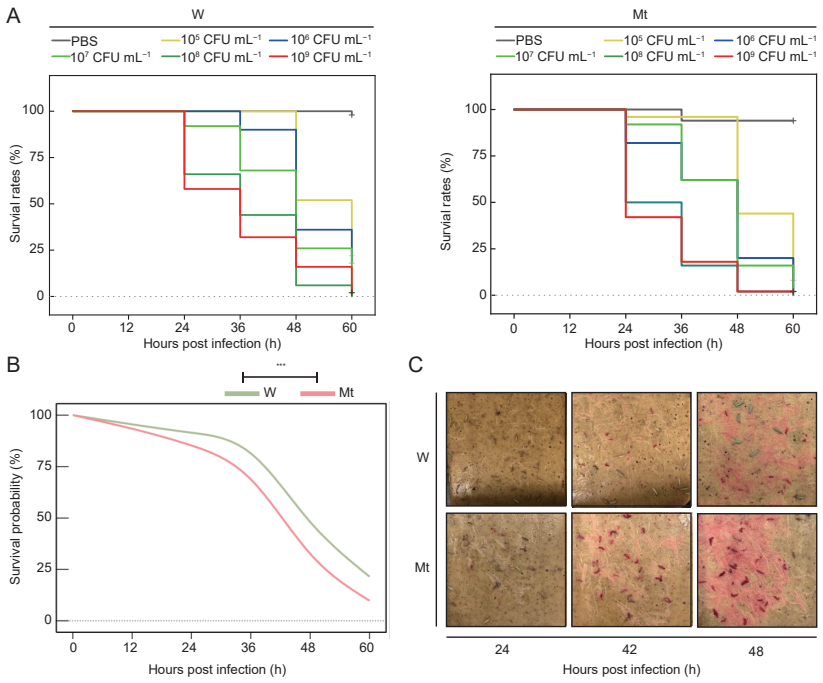


Fig. 5 The infection efficiency of PS-1 across different *Plutella xylostella* strains. A, the survival rates of wild-type and *serpin15*-knockout mutants following PS-1 infection. B, Cox proportional-hazards survival analysis of wild-type *vs*. serpin15-knockout mutants post-PS-1 infection. The logistic analysis employed the forward stepwise likelihood ratio method (confidence interval=95%; ***, P<0.001). C, a comparative analysis of infection manifestation between wild-type (WT) strains and *serpin15*-knockout mutants (Mt).

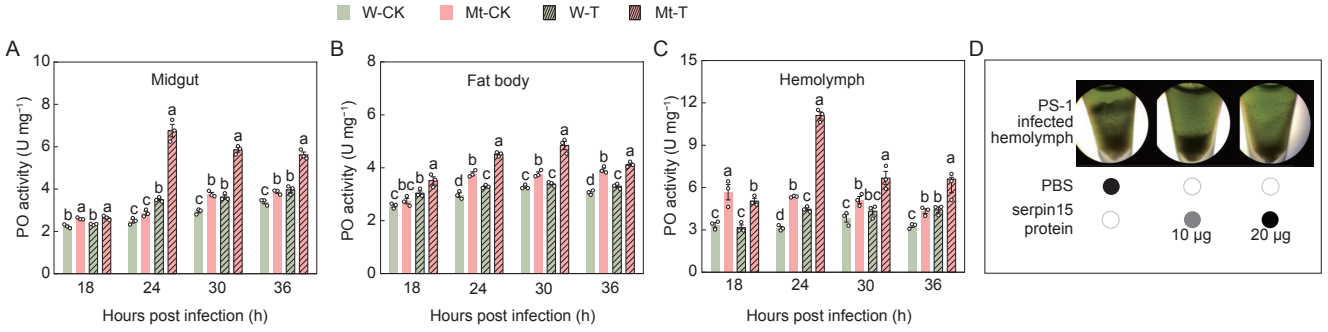


Fig. 6 The regulation of melanization by *serpin15*. A, phenoloxidase activity in the midgut following PS-1 infection. W-CK, control groups treated with PBS; W-T, treatment groups of wild-type specimens infected with PS-1; Mt-CK, control groups of the *serpin15*-knockout mutants treated with PBS; Mt-T, treatment groups comprising *serpin15*-knockout mutants infected with PS-1. B, phenoloxidase (PO) activity in the fat body following PS-1 infection. C, PO activity in the hemolymph after PS-1 infection. Different lowercase letters indicate significant differences among groups using LSD and Duncan analysis (α=0.05, P<0.05). D, melanization phenomenon observed in hemolymph after treatment with serpin15 protein.

gland secretions of *D. melanogaster* contain at least nine serpin proteins, including seven putative non-inhibitory serpins (e.g., *Spn76A*) and two inhibitory serpins (*Spn28B* and *Spn28F*). Most of these serpins possess signal peptides, suggesting their potential roles as seminal components transferred to the female reproductive tract during mating (Owji *et al.* 2018). These accessory gland proteins induce substantial post-mating physiological and behavioral changes in females, including morphological alterations in the female reproductive tract (Suwanchaichinda *et al.* 2013) and proper sperm storage and utilization (Christen *et al.* 2012; Mongelli *et al.* 2022). Although most accessory gland serpins may not function through protease inhibition, they participate in hormone binding or regulate female fecundity post-mating. This study demonstrates that silencing the *serpin15* gene impairs reproductive performance in adult *P. xylostella*. The deficiency of serpin15 protein in either sex results in significantly reduced oviposition. This indicates that serpin15 functions in both males and females to support fecundity, with a synergistic effect when absent in both sexes. Notably, the absence of morphological differences in adult reproductive organs and unchanged oviposition timing (completed within 72 h) suggests that serpin15 is not involved in the structural development of reproductive organs or oviposition behavior,

but rather in processes related to gamete formation, maturation, or interaction-such as sperm-egg recognition or ovarian follicle development, which directly influence the quantity of viable eggs produced. The research suggests that male *serpin15* deficiency reduces sperm motility and lifespan, whereas female deficiency impairs sperm maintenance and longevity, collectively resulting in diminished female fertility. Given the limited research on serpin functions in insect development, these findings provide novel evidence for the involvement of serpin family members in insect developmental processes.

Serpins play a critical role in embryonic development. In *Drosophila*, *serpin27A* regulates the Toll signaling cascade by inhibiting Easter protease activity, thereby ensuring proper formation of the embryonic dorsoventral axis. The absence of *serpin27A* can result in embryonic polarity disorders such as ventralization phenotypes due to excessive Easter protease activation (Mueller *et al.* 2007). In *B.mori*, serpin18 effectively inhibits fibroin protease activity; excessive sericin proteins in the silk gland lumen induce apoptosis. However, *serpin18* downregulates silk protein expression in the lumen, preventing apoptosis and enabling *B.mori* to progress normally to pupation (Ram and Wolfner 2007). This study demonstrated significantly higher rates of unhatched eggs in mutant lineages compared to wild-type self-cross progeny. Female-specific *serpin15* deficiency caused a greater reduction in hatchability than male-specific deficiency, while combined deficiency in both sexes resulted in the most severe hatchability impairment. The expression profile of serpin15 provides important evidence for its role in reproductive development. The consistent high expression in larval and prepupal testes, combined with significant expression in pupal ovaries, corresponds with the dynamic developmental processes of reproductive organs during these stages-testicular fusion and sperm maturation in larvae/prepupae, and ovarian maturation in pupae. Additionally, RHS8 and RHS9 from the tick *Rhipicephalus haemaphysaloides*, whose ovarian expression increases from feeding to oviposition and whose knockdown affects oocyte maturation, fecundity, and hatchability respectively, support the importance of serpins in embryonic and ovarian development (Xu *et al.* 2020a, b). The pronounced reduction in embryonic hatching rates in serpin15-knockout mutants, particularly at early stages (2 days), adds complexity to its function. The 48.41% decrease in 2-day hatching rate in Group D, with male-specific knockout (Group C) showing greater impairment than female-specific knockout (Group B), while combined deficiency in both sexes resulted in the most severe hatchability impairment. This suggests serpin15 functions in both males and females to support fecundity, with a synergistic effect when absent in both sexes. These findings correlate with previous reports: while gene silencing of different types produces similar outcomes in reproductive capacity, distinct differences exist. Collectively, this research identifies serpin15 as a key regulator of reproductive fitness in *P. xylostella*, with dual roles in fecundity and embryonic development.

Additionally, microbial invasion triggers immune recognition and defense responses in hosts, typically characterized by upregulation of gene expression upon immune stimulation (Lin *et al.* 2020). Following PS-1 infection, *serpin15* expression in the midgut showed an initial decrease followed by an increase, while expression in the fat body and hemocytes decreased significantly, indicating that *serpin15* participates in the immune stress response of *P. xylostella*. In *M. sexta*, *serpin2*, *serpin3*, and *serpin6* exhibit low expression in larval hemocytes under normal conditions but are highly upregulated after pathogen stimulation (Gan *et al.* 2001; Hashimoto *et al.* 2003; Guo *et al.* 2015). Bacteria and yeast induce significant increases in protein levels of serpin4 and serpin5 in *M. sexta* hemocytes (Tong *et al.* 2005). In *Antheraea pernyi*, Ap*serpin6* mRNA levels increase significantly after treatment with *Enterococcus pernyi*, *Beauveria bassiana*, or *Antheraea pernyi nucleopolyhedrovirus* compared to controls. In *B. mori*, *serpin3* and *serpin2* demonstrate the highest expression in the fat body and are upregulated following exposure to bacteria, fungi, and viruses. After BmNPV infection, transcription levels of Bm*serpin*3 and Bm*serpin*2 increase in the hemocytes, midgut, and fat body of *B. mori* larvae. Similarly, in *B. mori*, *serpin32* (Wang *et al.* 2019) and *serpin4* (Qie *et al.* 2023) exhibit elevated transcription levels in the fat body under immune stimulation by various pathogens. These findings demonstrate that serpins respond to immune challenges across diverse insect species.

Serpins play a critical role in regulating melanization during insect innate immunity. When pathogens invade, serine proteases are activated, converting PPO to active PO. While PO-mediated melanin deposition is essential for pathogen elimination, excessive melanization can be toxic to the host. Serpins maintain homeostasis by negatively regulating serine proteases, thereby controlling melanization intensity (Yuan *et al.* 2017). Analysis of *serpin15*, a serpin gene in *P. xylostella*, demonstrated that CRISPR/Cas9-mediated knockout led to doubled PO activity following PS-1 infection compared to wild-type, correlating with decreased survival rates. Addition of recombinant serpin15 effectively inhibited hemolymph melanization *in vitro*, confirming its role in PO activity regulation. However, *in vivo* regulatory mechanisms may differ from *in vitro* observations. The evolutionary conservation of this function is demonstrated by *Drosophila serpin27A*, which similarly regulates PO-driven melanization (De Gregorio *et al.* 2002). Similar patterns exist in *Helicoverpa armigera serpin5/9* and *B.mori serpin5/9/19*, which inhibit PO activity during viral infection (Yuan *et al.* 2017; Chen *et al.* 2019). These findings establish serpins as key regulators of immunoprotective melanization, maintaining balance between pathogen elimination and host survival.

5. Conclusion

This study presents compelling evidence for the dual function of *serpin15* in coordinating immunity and reproduction in *P. xylostella*. The serpin15 protein serves as an essential regulator in both oviposition and embryonic hatching processes. These discoveries illuminate the multifunctional nature of serine protease inhibitors while providing theoretical foundations for developing novel pesticides and biological control strategies targeting *serpin15*.

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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.

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