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A teratocyte-specific serpin from the endoparasitoid wasp *Cotesia vestalis* inhibits the prophenoloxidase-activating system of its host *Plutella xylostella*

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Abstract

Many endoparasitoids adopt several parasitic factors, such as venom, polydnavirus and teratocytes, to suppress the immune response of their associated hosts including melanization for successful parasitism. A teratocyte-specific expressed serpin gene, designated as *CvT-serpin6*, was identified from the parasitoid *Cotesia vestalis*. The immunoblot result suggested that CvT-serpin6 was secreted into extracellular space. qPCR results showed that *CvT-serpin6* was mainly transcribed at later stages of parasitism, and the transcriptional abundance of *CvT-serpin6* in teratocytes was significantly increased in response to the challenge of bacteria. Inhibitory assay indicated that recombinant CvT-serpin6 (rCvT-serpin6) could inhibit the activation of *Plutella xylostella* prophenoloxidase and ultimately resulted in the inhibition of melanization in *P. xylostella* haemolymph. Furthermore, we confirmed that rCvT-serpin6 could form SDS-stable complexes with activated *Px*PAP1 and *Px*PAP3 in a dose-dependent manner. Altogether, our results further shed insight into the molecular mechanisms that teratocytes involved in controlling host immune response.

KEYWORDS
CvT-serpin6, melanization, prophenoloxidase activation, teratocytes

INTRODUCTION

Insects rely on innate immune system, comprising cellular and humoral immunity, to defend the invaders (Medzhitov, 2007; Strand, 2008). The melanization reaction, as one of conserved humoral immunity (Cerenius et al., 2008), is regulated by the prophenoloxidase-activating system (proPO system) (Cerenius & Söderhäll, 2004). After infection or wounding, the proPO system is initiated with the sequential activation of a series of serine proteinases, and finally resulting in the activation of proPO into active PO. The active PO catalyses the oxygenation of monophenols to o-diphenols, and further oxidates o-diphenols to o-quinones, which forms melanin by polymerization (Cerenius & Söderhäll, 2004; Kanost & Jiang, 2015). During the process of melanization, some cytotoxic elements are produced to kill the invaders. However, they are also harmful to the host at the same time (Nappi et al., 2009). The melanin localizes to the surface of pathogens

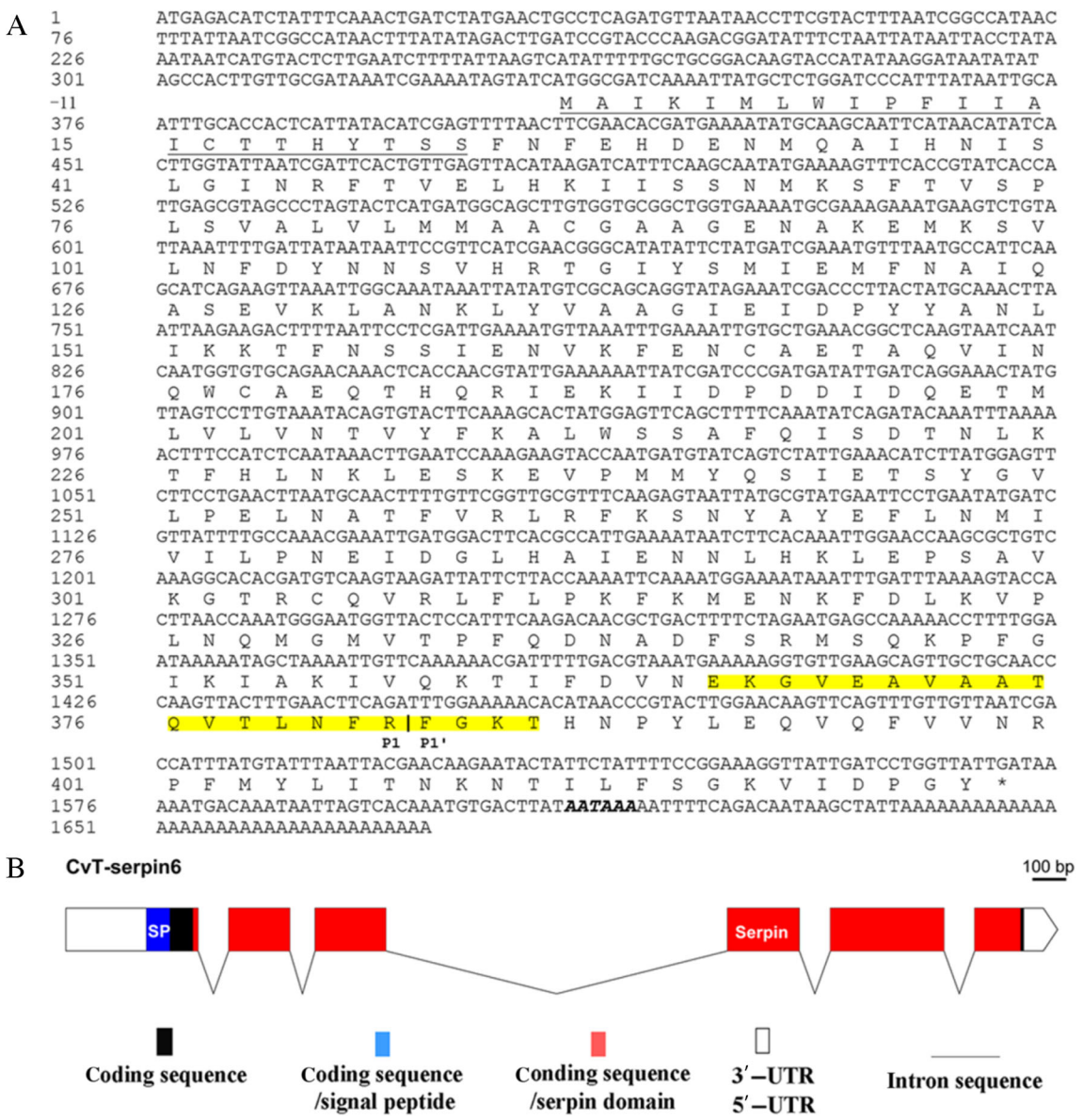

FIGURE 1 The nucleotide sequences of *CvT-serpin6* with its encoding amino acid sequences and structural analysis. (a) Sequence features of *CvT-serpin6* and its encoding amino acid. Black italic indicated polyadenylate signalling site. The predicted signal peptide was underlined. The asterisk represented the stop codon. Yellow shadow indicated the amino acid residues of the reactive central loop. The P1-P1′ position indicated the cleavage site. (b) Gene structure of *CvT-serpin6*. The organization of *CvT-serpin6* in *Cotesia vestalis* genome included 3′-UTR, 5′-UTR, signal peptide sequence, coding sequence (6 exons) and 5 introns

and parasites to prevent them spreading inside the host. It is obvious that the PO-mediated melanization needs to be tightly controlled for the survival of their hosts (Carton et al., 2008). Actually, the proPO system is precisely regulated by a guild of serine proteinase inhibitors (serpins) (M. Li et al., 2018; Meekins et al., 2017). Serpins are a superfamily of proteins, comprising 350 ~ 400 amino acids (aa) with a reactive central loop (RCL) at the C-terminus, in which the scissile bond P1-P1′ determines the specificity of targeted serine proteases exists (Gettins, 2002; Law et al., 2006). It has been known that serpins regulate the PO-mediated melanization by forming complexes with serine proteases in the PO cascade (Chu et al., 2015; He et al., 2017).

Endoparasitoid are hymenopteran parasitic wasps that complete their development inside their host hemocoel. As invaders, endoparasitoids have to face the immune response of their hosts when their eggs are injected into the body cavity of their hosts (Carton et al., 2008; Schmidt et al., 2001). Endoparasitoids have evolved different strategies to regulate their host immune response (Falabella, 2018; Pennacchio & Strand, 2006; Schmidt et al., 2001). Three parasitic factors, including polydnavirus (PDVs), venom and teratocytes, have been adopted by wasps to impede the host immunity (Beckage & Gelman, 2004; Laurino et al., 2016; Salvia et al., 2017, 2018, 2019). Moreover, there is a further parasitic factor that must be taken into account, ovarian proteins (Salvia et al., 2021). Previous studies have shown that maternal factors (such as PDVs and venom), which are injected into host at oviposition, involved in regulating the PO cascade and the molecular mechanisms were largely elucidated

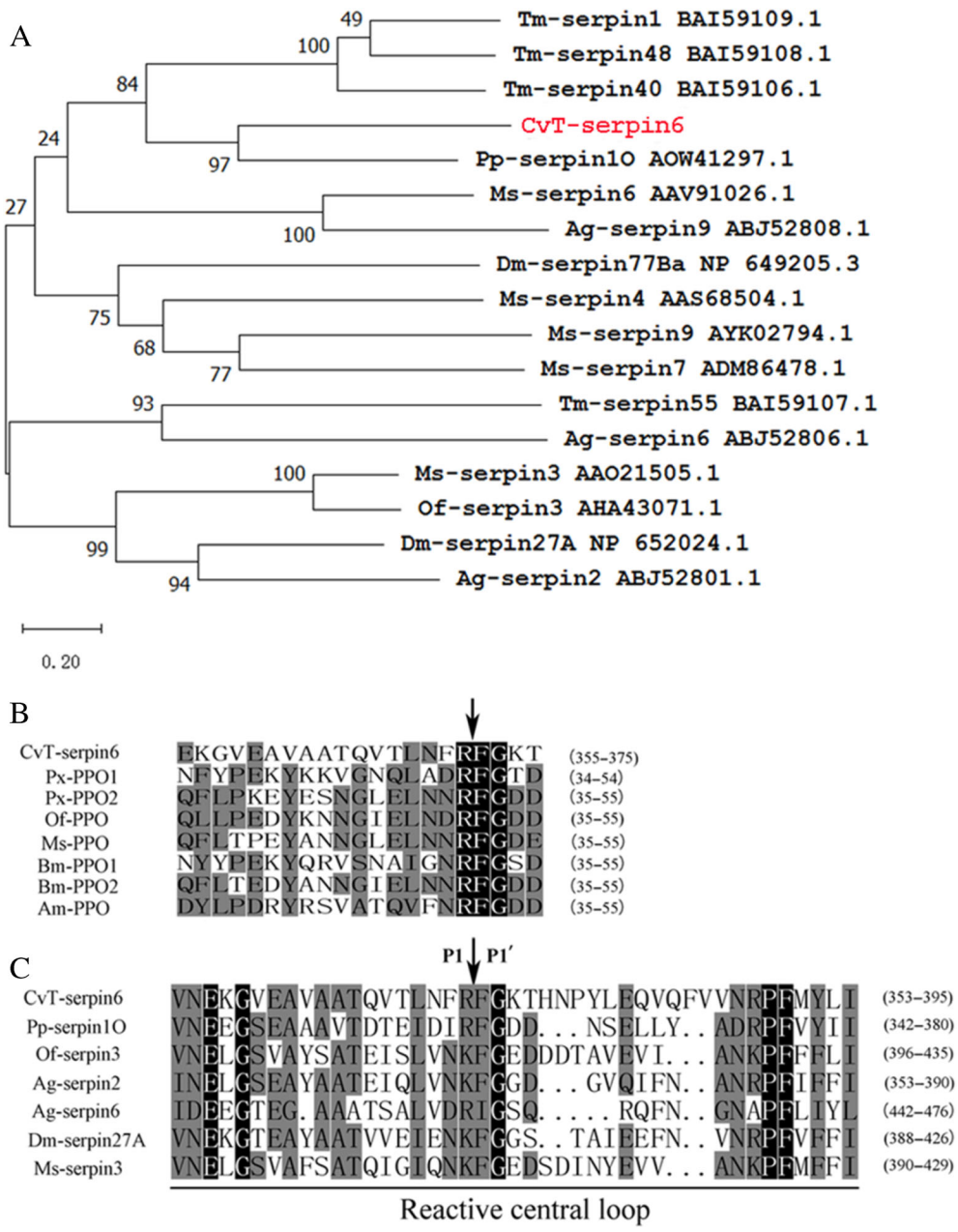

FIGURE 2 Phylogenetic tree of CvT-serpin6 and sequence comparison. (a) Phylogenetic analysis of CvT-serpin6. The maximum likelihood method was used and the bootstrap was set as 10,000. . (b) Sequence comparison between the activation cleavage sites of prophenoloxidases and the reactive central loop (RCL) of CvT-serpin6. (c) Sequence comparison of RCLs of the serpins from other insects involved in melanization with CvT-serpin6. Ag, *Anopheles gambiae*; Am, *Apis mellifera*; Bm, *Bombyx mori*; Dm, *Drosophila melanogaster*; Ms, *Manduca sexta*; Of, *Ostrinia furnacalis*; Pp, *Pteromalus puparum*; Px, *Plutella xylostella*; Tm, *Tenebrio molitor*

(Asgari, Zareie, et al., 2003; Asgari, Zhang, et al., 2003; Beck & Strand, 2007; Lu et al., 2008, 2010; Thomas & Asgari, 2011; Z. H. Wang et al., 2020; Yan et al., 2017; Zhang et al., 2004). For instance, Egf1.0 and Egf1.5 encoded by *Microplitis demolitor* bracovirus (MdBV) inhibit the melanization of *Manduca sexta* haemolymph by inhibiting the activities of prophenoloxidase-activating proteinases (PAPs) (Beck & Strand, 2007; Lu et al., 2010). Serpin proteins were also found in venoms of other endo and ecto parasitoids (Colinet et al., 2014; Laurino et al., 2016; Lin et al., 2019; Yan et al., 2017). A serpin from the venom of the endoparasitoid wasp *Pteromalus puparum* can suppress the PO cascade by forming complexes with the haemolymph proteinase 8 (PrHP8) and prophenoloxidase-activating proteinase 1 (PrPAP1) of its host *Pieris rapae* (Yan et al., 2017). As for teratocytes, specialized cells dissociated from the embryonic serosal membrane of some endoparasitoid wasps during the parasitism, more attention has been focused on the regulation of host development and nutrition metabolism (Ali et al., 2013; Basio & Kim, 2005; Dahlman et al., 2003; Falabella et al., 2005; Gopalapillai et al., 2005; Qin et al., 2000; Strand, 2014; Z. Z. Wang et al., 2018), and only fewer studies clarified the molecular mechanisms underlying

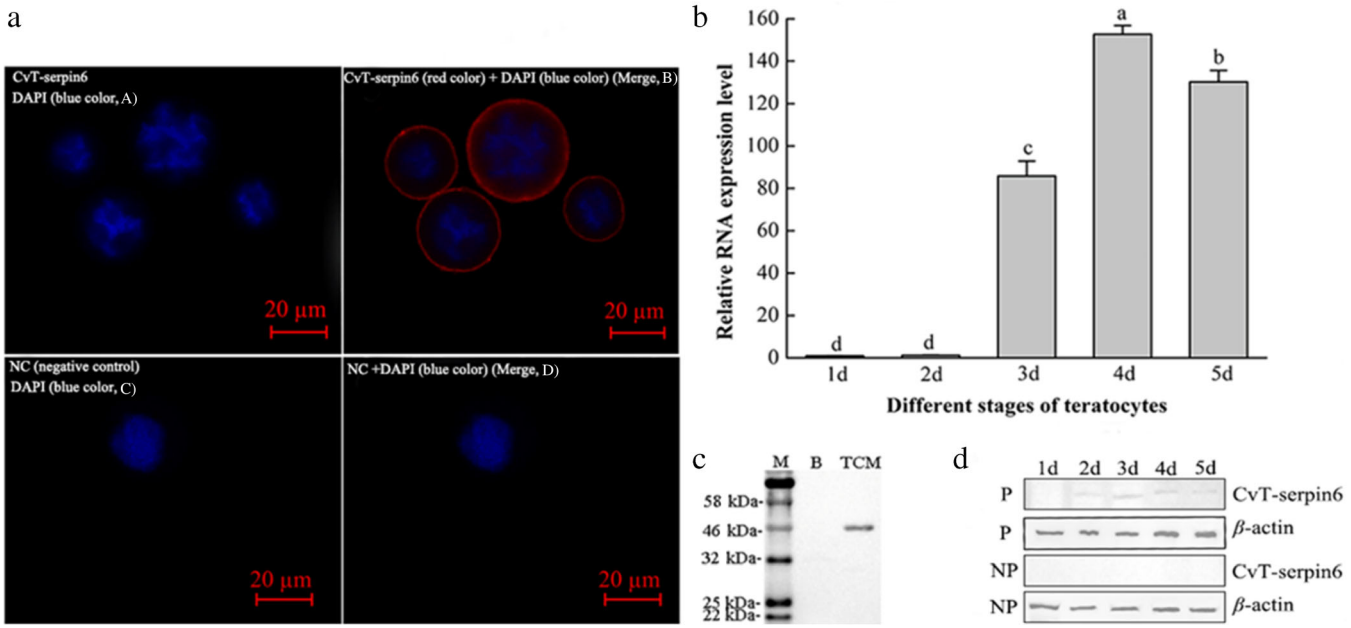

FIGURE 3 The expression of *CvT-serpin6* in teratocytes and the secretion of CvT-serpin6 by teratocytes. (a) Immunohischemistry of CvT-serpin6 in teratocytes. Blue represented nuclei stained with DAPI. Red indicated the secondary antibody labelled with Alexa Fluor 594. A and C showed the results of teratocytes probed with or without first antibody anti-serpin6, respectively. A and C showed the DAPI staining of nuclei. B and D showed the merged results. Scale bar: 20 μm. (b) The expressional pattern of CvT-serpin6 in different developmental stages of teratocytes. Each sample was repeated three times. Bars labelled with different letters were significantly different (one-way analysis of variance followed by Tukey-test, $p < 0.05$). (c) Detection of CvT-serpin6 in teratocytes culture medium (TCM). SDS-PAGE of TCM was followed by immunoblotting using antibody against CvT-serpin6. Lane: M, protein marker. (d) Detection of CvT-serpin6 in cell-free haemolymph of *Plutella xylostella* parasitized by *C. vestalis*. NP, non-parasitized host larvae by *C. vestalis*; P, parasitized host larvae by *C. vestalis*. β-Actin was used as an internal reference

the host immune regulation of teratocytes (Gao et al., 2016; Gu et al., 2019; Kitano et al., 1990). In our recent published work, a small serine proteinase inhibitor containing a cysteine-rich domain from *Cotesia vestalis* teratocytes, which was similar to the MdBV Egfs was found to inhibit melanization by suppressing the activity of *Plutella xylostella* PAP3 (Gu et al., 2019).

C. vestalis (Haliday) is a dominant endoparasitoid wasp of *P. xylostella* (L.), a worldwide destructive pest of brassica crops (Shi et al., 2008). Three parasitic factors including PDVs, venom and teratocytes are adopted to modulate host physiological processes in this host-parasitoid system. Herein, *CvT-serpin6* specifically expressed in teratocytes was identified and characterized. The scissile bond site Arg (P1)-Phe (P1′) of CvT-serpin6 was similar to the cleavage site of *P. xylostella* prophenoloxidase (PPO) activation. Our results revealed that CvT-serpin6 was secreted into the hemocoel of *P. xylsotella* larvae and inhibited PPO activation by forming SDS-stable complexes with the activated *Px*PAP1 or activated *Px*PAP3.

RESULTS

Characterization of CvT-serpin6

We obtained the full-length cDNA sequence of *CvT-serpin6* based on the *C. vestalis* genome sequence. *CvT-serpin6* was located at scaffold30_6, including six exons and five introns. The cDNA of *CvT-serpin6* comprised 1579 bp nucleotides with a 239 bp 5′-untranslated region (5′-UTR), a 1239 bp open reading frame (ORF) and a 101 bp 3′-UTR including a poly (A) tail (Figure 1a).

The deduced protein of CvT-serpin6 consists of 412 aa residues and contains a 23 aa signal peptide, which indicates CvT-serpin6 might be secreted into the extracellular space. Our results showed that there is an RCL from Glu^{365} to Thr^{385} including a predicted cleavage site of Arg (P1)-Phe (P1′) at the C-terminus of the CvT-serpin6 protein sequence (Figure 1b). The P1 usually determines the specificity of its target proteinase. The calculated molecular mass of the mature CvT-serpin6 is 44.3 kDa, and the calculated isoelectric point is 7.09.

Sequence comparison and phylogenetic analysis of CvT-serpin6

The phylogenetic analysis showed that CvT-serpin6 was clustered into one clade with one splicing isoform of *P. puparum* serpin, serpin 1O (Figure 2a). We further compared the sequence of CvT-serpin6 with serpins from other insects that have been experimentally verified to regulate the proPO activation cascade by inhibiting PAPs. Results suggested that the residues, Arg (R)-Phe (F) at P1-P1′ site of the scissile bond in CvT-serpin6 is the same as the

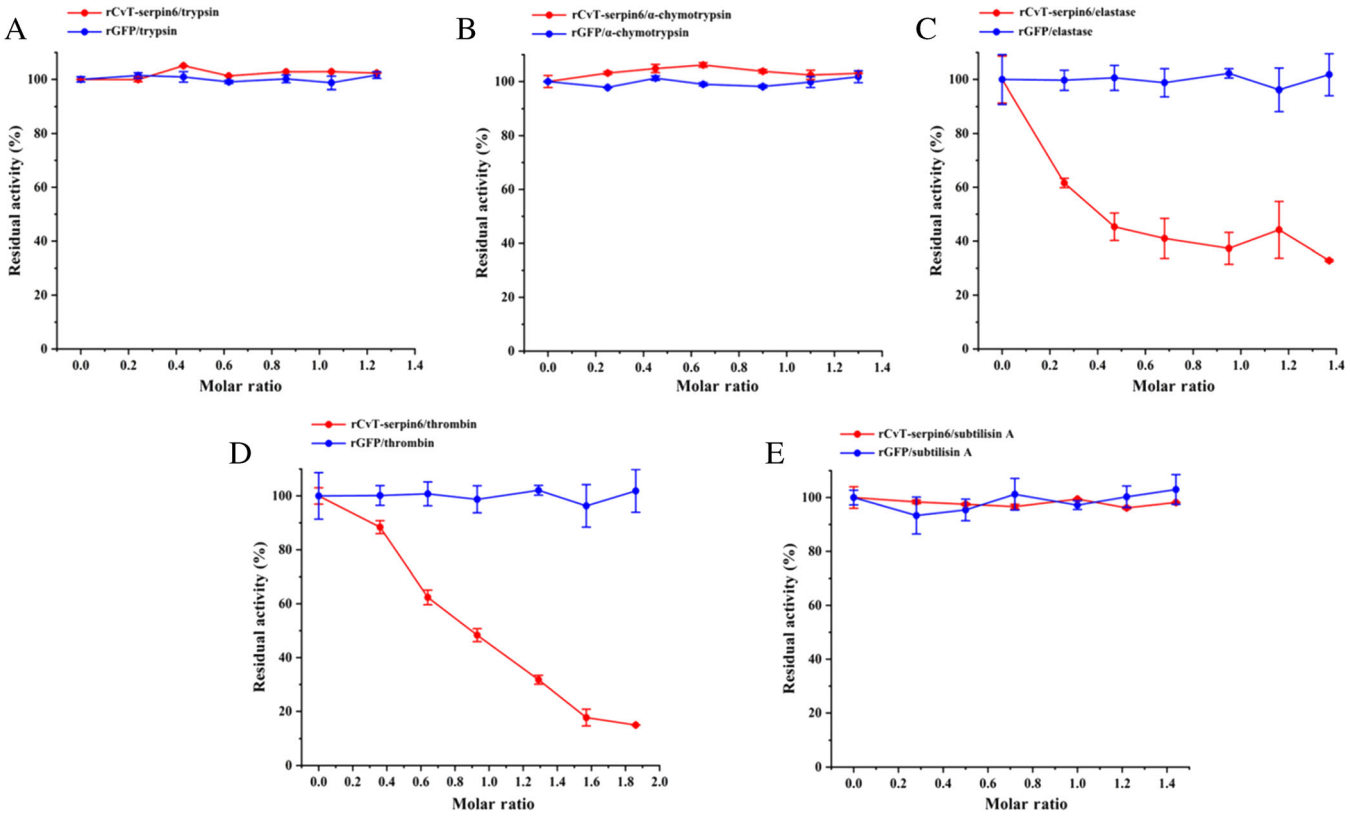

FIGURE 4 Effects of rCvT-serpin6 on the activity of the commercial serine protease. (a), (b), (c), (d) and (e) represents the inhibitory effect on trypsin, α-chymotrypsin, elastase, thrombin and subtilisin A, respectively. Each enzyme was reacted with rCvT-serpin6 or rGFP as a negative control of the different concentrations for 10 min at room temperature in total 50 μl Tris–HCl buffer. Specific chromogenic substrates for above serine proteinases were used to determine the activities of residual enzymes, respectively. OD405 was monitored with a microplate reader. One unit of the amidase activity was defined as 0.001 ΔA405/min. The inhibitory effects of rCvT-serpin6 were plotted as the residual activity of the proteases against the concentrations of rCvT-serpin6. Error bars represent the mean ± standard error ($N = 3$)

P. puparum serpin 1O and *Anopheles gambiae* Ag-serpin6 (Figure 2c). In addition, the P1-P1′ residues (RF) of CvT-serpin6 are identical with the activation cleavage site (RF) of the PPOs in lepidopteran insects, including *P. xylostella* (Figure 2b), which further suggests that CvT-serpin6 might regulate the PPO activation by interacting with *P. xylostella* PAPs.

Expression, localization and secretion of CvT-serpin6

The immunolocalization of CvT-serpin6 in teratocytes indicated that CvT-serpin6 accumulated on the cellular plasma membrane (Figure 3a-A), which was probably ready for the secretion of CvT-serpin6 into extracellular space (Figure 3a). In addition, we observed a 46 kDa specific band of immune reaction for CvT-serpin6 in TCM (Figure 3c). qPCR results suggested that CvT-serpin6 had lower transcriptional level in teratocytes at 1- and 2-day-old teratocytes. The transcritptional level of CvT-serpin6 in 4-day-old teratocytes reached to the maximum, which increased by 152-fold compared with those in 1-day-old teratocytes (Figure 3b). When teratocytes were five days old, the transcriptional level of CvT-serpin6 was decreased (Figure 3b). Meanwhile, detection of CvT-serpin6 in the haemolymph of parasitized *P. xylostella* indicated that there was a ~46 kDa specific band when teratocytes were 2-, 3-, 4- and 5-day-old and no band was observed in 1-day-old teratocytes. Additionally, the specific band from 3- and 4-day-old teratocytes was stronger than those in 2- and 5-day-old teratocytes (Figure 3d). Altogether, these results demonstrated that CvT-serpin6 could be transcribed and secreted into *P. xylostella* hemocoel by teratocytes during parasitism, and the concentration of CvT-serpin6 reached the maximum level in the host haemolymph when the teratocytes were 3- and 4-day-old.

Inhibitory activity of rCvT-serpin6 on serine proteases

We obtained totally 8.5 mg rCvT-serpin6 with 6 × His-tag at the C-terminus, and the anti-6 × His antibodies recognized a ~46 kDa band (Figure S1). To confirm the inhibitory activities, five commercial serine proteases were used to conduct Bio activity assays and the results suggested that rCvT-serpin6 can inhibit the activity of elastase (Figure 4c) and thrombin (Figure 4d), but had no inhibitory activities against trypsin (Figure 4a), α-chymotrypsin (Figure 4b) and subtilisin A (Figure 4e). It could inhibit elastase and thrombin by 85.1% and 67.1%, when the volume of recombinant CvT-serpin6 reached to 12 μl.

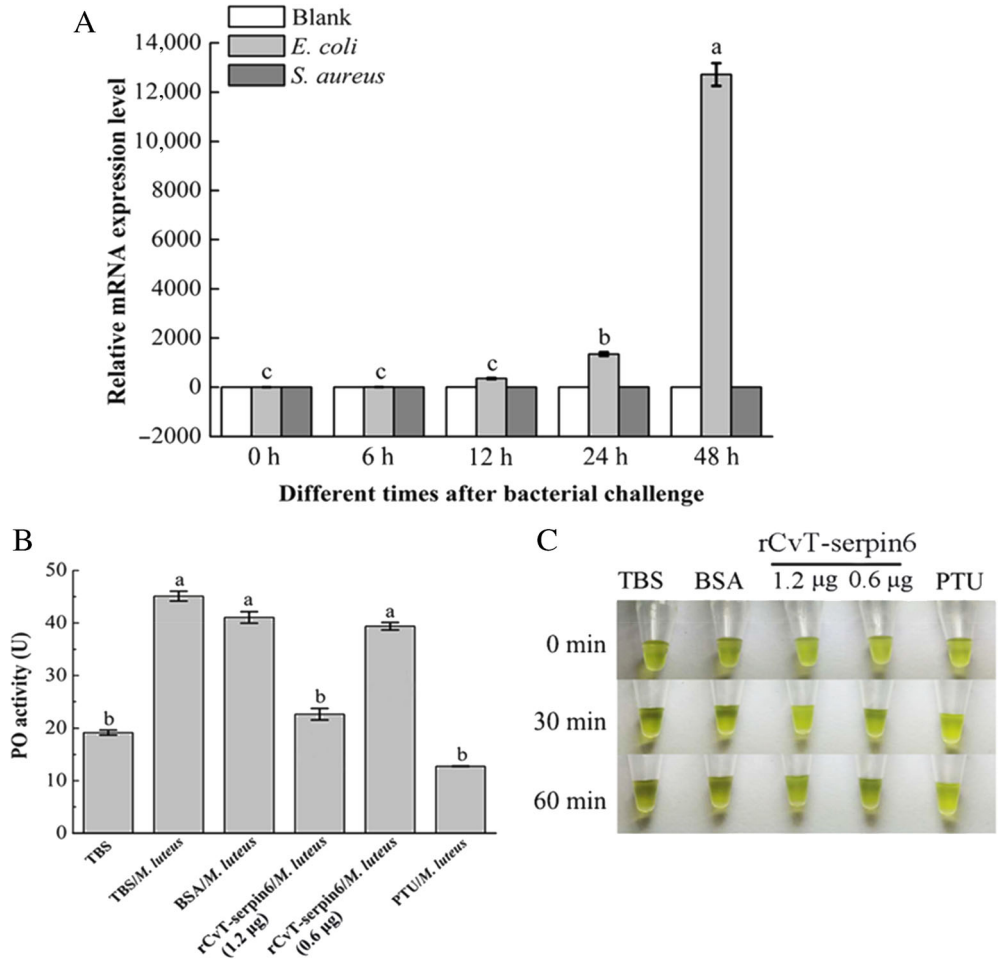

FIGURE 5 CvT-serpin6 involved in the immune response of melanization by inhibiting the prophenoloxidase (PPO) activation of *Plutella xylostella*. (a) Expression pattern of *CvT-serpin6* in teratocytes cultured in vitro after microbial challenges. Teratocytes cultured in vitro were induced with the inactivated *Escherichia coli* or *Staphylococcus aureus* for 0, 6, 12, 24 and 48 h for qPCR. Error bars represent mean ± standard error ($N = 3$). Different letters above the bars are significantly different (one-way analysis of variance [ANOVA] followed by Tukey-test, $p < 0.05$). (b) Inhibition of the PPO activation of *P. xylostella* haemolymph by rCvT-serpin6. TBS, blank; bovine serum albumin (BSA), negative control; phenylthiourea (PTU), positive control; inactivated *Micrococcus luteus* was an elicitor. Cell-free haemolymph was mixed with sterilized TBS, sterilized TBS plus *M. luteus*, BSA plus *M. luteus*, rCvT-serpin6 plus *M. luteus*, rCvT-serpin6 plus *M. luteus* and PTU plus *M. luteus*, respectively. The mixtures were reacted at room temperature for 45 min and then were added with L-dopamine as a substrate. Phenoloxidase (PO) activity was measured by monitoring OD490 and one unit of the PO activity was defined as 0.001 ΔA490/min. Error bars represent the mean ± standard error ($N = 3$). The data were analysed using one-way analysis of variance (one-way ANOVA, Tukey-test, $p < 0.05$). Significant differences were indicated with different letters. (c) Inhibition of the spontaneous melanization of *P. xylostella* haemolymph by rCvT-serpin6. Cell-free haemolymph of non-parasitized *P. xylostella* larvae was incubated with sterilized TBS, BSA as negative control, rCvT-serpin6 (1.2 μg/μl), rCvT-serpin6 (0.6 μg/μl) and PTU as a positive control in total 20 μl TBS buffer at room temperature. The photographs were taken at 0 min, 30 min and 60 min

Increased transcriptional level in response to immune challenge

We detected the fluctuation of the transcriptional level of *CvT-serpin6* in cultured teratocytes in response to the challenges of two different bacteria, Gram-positive *Staphylococcus aureus* and Gram-negative *Escherichia coli*. The results showed that the transcriptional level of *CvT-serpin6* began to increase at 24 h after challenge by *E. coli* and reached to the maximum of transcriptional level at 48 h (Figure 5a). However, the transcriptional level of *CvT-serpin6* in teratocytes treated with *S. aureus* showed no significant change (Figure 5a).

Inhibition of *P. xylosltella* PPO activation by rCvT-serpin6

Based on the result of sequence comparison of the scissile bond of CvT-serpin6 and the activation cleavage site of *P. xylostella* PPO, we conducted the inhibitory assay of rCvT-serpin6 on the PPO activation of *P. xylsotella*. The results showed that the PPO activation of *P. xylostella* haemolymph was almost completely inhibited when 1.2 μg of rCvT-serpin6 plus *Micrococcus luteus* was incubated with cell-free haemolymph, and slightly inhibited by 0.6 μg of rCvT-serpin6 (Figure 5b). Consequently, we observed that the spontaneous

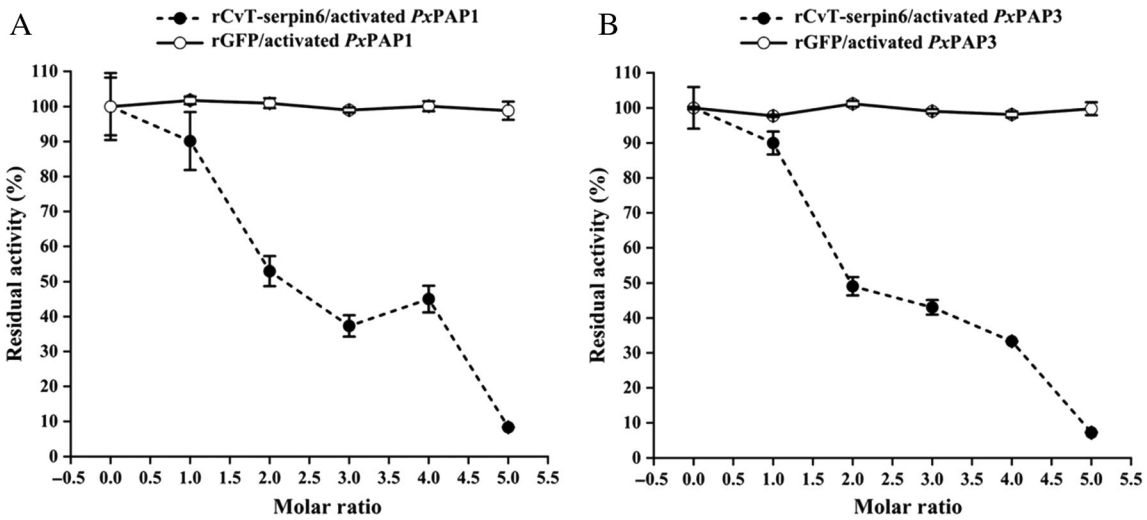

FIGURE 6 Inhibition of the activated *Px*PAP1 and *Px*PAP3 by rCvT-serpin6. (a) Inhibition of the activated *Px*PAP1 by rCvT-serpin6. (b) Inhibition of the activated *Px*PAP3 by rCvT-serpin6. Purified rCvT-serpin6 or rGFP as a negative control was incubated with Factor Xa-activated *Px*PAP1 or *Px*PAP3 at various molar ratios for 30 min at room temperature. The amidase activities of the activated *Px*PAP1 and *Px*PAP3 were measured using the substrate, acetyl-Ile-Glu-Ala-Arg-*p*-nitroanilide (IEARpNA). The OD405 was monitored using a microplate reader and one unit of the amidase activity was defined as 0.001 ΔA405/min. The residual IEARase activities of the activated *Px*PAP1 or *Px*PAP3 was plotted as mean ± standard error ($N = 3$) against the corresponding molar ratios of rCvT-serpin6 and the activated *Px*PAP1 or *Px*PAP3

melanization of *P. xylostella* haemolymph was substantially inhibited after addition of 1.2 μg rCvT-serpin6 to the cell-free plasma and almost not affected by lower concentrations of CvT-serpin6 (0.6 μg) (Figure 5c). Our result showed that the PPO activation of *P. xylostella* was inhibited by the teratocyte-secreted proteins (TSPs) (Figure S5). We also identified the CvT-TIL that inhibited PPO activation of *P. xylostella* in our recently published work (Gu et al., 2019). In comparison with CvT-TIL, CvT-serpin6 has stronger inhibitory ability on the PPO activation of *P. xylostella* (Figure S6).

Inhibition of rCvT-serpin6 on activated *Px*PAP1 and *Px*PAP3

The recombinant *Px*proPAP1$_{Xa}$ was obtained and the SDS-PAGE analysis indicated that the band of the recombinant *Px*proPAP1$_{Xa}$ was 41.5 kDa (Figure S4). The activated *Px*PAP1 was about 29 kDa (Figure S2) and its activity was confirmed with the increased IEARase activity after activation (Figure S2). After adding of the rCvT-serpin6 into the activated *Px*PAP1 or *Px*PAP3, our results showed that the amidase activities decreased as molar ratio of rCvT-serpin6: *Px*PAP1 or *Px*PAP3 increased (Figure 6a,b).

CvT-serpin6 forms complexes with activated *Px*PAP1 or *Px*PAP3

To investigate whether CvT-serpin6 could directly interact with the activated *Px*PAP1 or *Px*PAP3 by forming complexes, which is the typical characteristic of interaction between serpin and its target protease. We mixed rCvT-serpin6 with Factor Xa-activated *Px*PAP1 or *Px*PAP3 and the complexes were detected by western blot using antibodies against 6 × His or CvT-serpin6. When *Px*proPAP1 or *Px*proPAP3 was incubated with Factor Xa, anti-His antibody recognized a ~29 kDa-activated *Px*PAP1 (Figure 7a) or ~32 kDa-activated *Px*PAP3 (Figure 7c), respectively. However, after addition of rCvT-serpin6, we detected a new immunoreactive band at ~74 kDa for rCvT-serpin6-activated *Px*PAP1 complex (Figure 7a,b) or ~ 77 kDa for rCvT-serpin6-activated *Px*PAP3 complex (Figure 7c,d) and the intensity of ~29 kDa-activated *Px*PAP1 or ~ 32 kDa-activated *Px*PAP3 decreased using antibodies against 6 × His or CvT-serpin6 (Figure 7a, c). Meanwhile, some non-specific bands were also observed, which possibly due to the degradation of rCvT-serpin6 or rCvT-serpin6-activated *Px*PAP1/*Px*PAP3 complex by Factor Xa.

DISCUSSION

Parasitoid wasps inhibit host melanization by recruiting virulence proteins derived from parasitic factors (Yang et al., 2018). Some virulence proteins from venom and PDV were reported to play an important role in suppressing the host melanization immune response (Beck & Strand, 2007; Colinet et al., 2009; Z. H. Wang et al., 2020; Yan et al., 2017). Our result showed that the PPO activation of *P. xylostella* was inhibited by the TSPs. Actually, some research studies demonstrated that teratocytes from wasps can involve in inhibition of host melanization (Kitano et al., 1990; Tanaka & Wago, 1990; Webb & Luckhart, 1996) and transcripts of serpin genes also detected in parasitoid teratocytes (Ali et al., 2015; Burke & Strand, 2014; Gao et al., 2016). In our published work, a trypsin inhibitor-like protein, which contains the cysteine-rich domain, was identified. This protein is the only known virulence protein from teratocytes inhibiting the

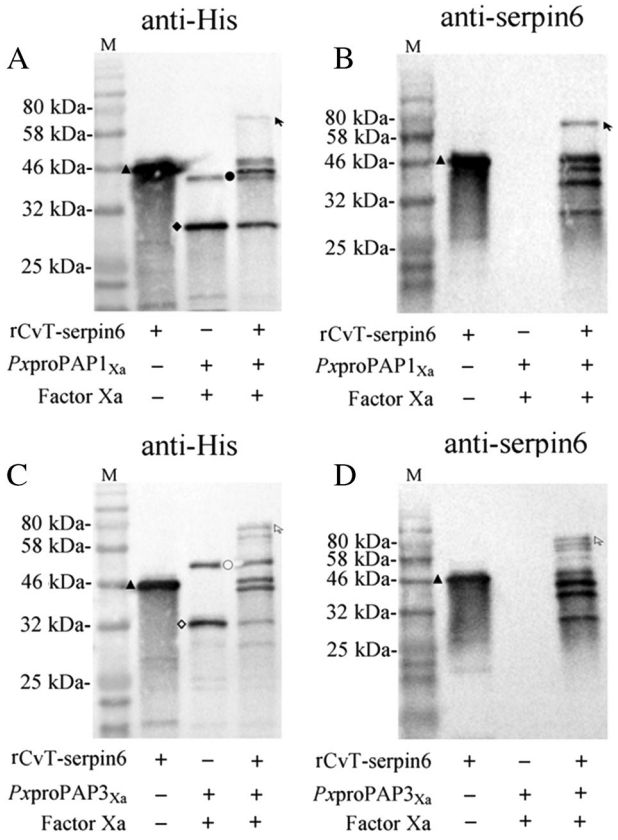

FIGURE 7 SDS-stable complexes formation between rCvT-serpin6 and *Px*PAP1 or *Px*PAP3. Purified recombinant *Px*proPAP1$_{Xa}$ and *Px*proPAP3$_{Xa}$ were activated by Factor Xa as mentioned above and mixed with the purified rCvT-serpin6 at molar ratio of 1:1, respectively. The mixtures were incubated at room temperature for 30 min and then subjected to 12% SDS-PAGE. In control sample, the mixtures contained no Factor Xa. The immunoblot analysis were conducted using antiserum against His (a, c) or rCvT-serpin6 (b, d). Complexes bands were detected using Pierce™ ECL Western Blotting Substrate and imaged by ChemiDoc MP Imaging System. Solid arrows, rCvT-serpin6/activated *Px*PAP1; hollow arrow, rCvT-serpin6/activated *Px*PAP3 complex; triangle, rCvT-serpin6; solid circle, *Px*proPAP1$_{Xa}$; hollow circle, *Px*proPAP3$_{Xa}$; solid diamond, activated *Px*PAP1; hollow diamond, activated *Px*PAP3

melanization (Gu et al., 2019). In this paper, a serpin gene, designated as CvT-serpin6, was found during the process of analysing the transcriptome data of *C. vestalis* teratocytes (Gao et al., 2016) and its function was further investigated.

The cloned sequence of CvT-serpin6 was blasted to *C. vestalis* genome and the location in *C. vestalis* genome was confirmed (Shi et al., 2019). The transcript of CvT-serpin6 was analysed, and exhibited that *CvT-serpin6* was not transcribed in *C. vestalis* venom gland (Gao et al., 2016; Zhao et al., 2017). Meanwhile, PDVs of *C. vestalis* lack gene family of serpins in their genomes (Chen et al., 2011; X. Q. Ye et al., 2018). Therefore, CvT-serpin6 gene was specifically transcribed in teratocytes and qPCR also showed that CvT-serpin6 was mainly expressed in the later developmental stages of teratocytes. Through analysis of the *CvT-serpin6* sequence, a signal peptide in the protein sequence encoded by *CvT-serpin6* was predicted, which led us to speculate that CvT-serpin6 secreted into *P. xylostella* hemocoel by teratocytes through a signal peptide. CvT-serpin6 was secreted into the extracellular space at the later stages of parasitism according to immunoblot. Phylogenetic analysis of CvT-serpin6 revealed that CvT-serpin6 was clustered with serpin1O from *P. puparum* venom, which has been proved to suppress its host melanization by forming complexes with *Pr*HP8 and *Pr*PAP1 (Yan et al., 2017). We conducted further sequence comparison and suggested that CvT-serpin6 had similar composition of aa residues at the position of P1-P1′ (Arg-Phe/Lys-Phe/Arg-Ile) in the predicted RCL region with other serpins inhibiting PAPs from other insects (Abraham et al., 2005; An et al., 2011; Chu et al., 2015; De Gregorio et al., 2002; He et al., 2017; Yan et al., 2017; Zhu et al., 2003). Meanwhile, the P1-P1′ (Arg-Phe) of CvT-serpin6 resembled the activation cleavage site of lepidopteran PPOs (Arg-Phe) (Chu et al., 2015; Chu et al., 2017). The scissile bond (P1-P1′) of serpins usually determine the specificity of their target proteases (Gettins, 2002; Irving et al., 2000). Considering this result, we thus assumed that CvT-serpin6 can function as a PAP inhibitor to regulate the melanization reaction by inhibiting the PPO activation of the host *P. xylostella* haemolymph, which would guarantee the successful parasitism and the development of wasp in the host hemocoel.

Previous studies have reported that the transcriptional levels of serpins were upregulated after bacterial challenge if the serpins had involved in immune regulation (Abraham et al., 2005; Chu et al., 2015; B. Li et al., 2017; Meekins et al., 2017; Suwanchaichinda et al., 2013; L. Wang et al., 2019; Zhu et al., 2003). In our study, the transcriptional level of CvT-serpin6 in teratocytes could be induced by *E. coli* but not by *S. aureus*. Our results suggested CvT-serpin6 might play an important role in host immune response, which might be only present in Gram-negative bacteria infection. The basal level of *P. xylostella* haemolymph PO activity was low, similar to other lepidopteran insects, such as *M. sexta* and increased significantly after challenge (Gu et al., 2019; Tong & Kanost, 2005). Spontaneous melanization of larval plasma typically occurred and the colour of haemolymph changed to dark brown or blackish and continued to darken up with increasing time (Suwanchaichinda et al., 2013). Our results demonstrated the PPO activation of *P. xylostella* haemolymph and melanization were inhibited by rCvT-serpin6 of high concentration, consistent with the above hypothesis based on the sequence comparison that CvT-serpin6 might inhibit the PPO activation as a PAP inhibitor.

Firstly, we tested the inhibitory activities of purified rCvT-serpin6 against selected serine proteases, and the results indicated CvT-serpin6 inhibited the activities of elastase and thrombin, but not trypsin, α-chymotrypsin and subtilisin A, so we obtained active rCvT-serpin6. Meanwhile, we obtained the activated *Px*PAP1 and *Px*PAP3 in vitro and tested their inhibitory activities of CvT-serpin6 on them. As *Px*proPAP1 and *Px*proPAP3 were expressed as zymogens, we produced recombinant r*Px*proPAP1$_{Xa}$ and r*Px*proPAP3$_{Xa}$ in *Sf9* cells to allow their activation by commercial Factor Xa (Chu et al., 2017; Y. Wang et al., 2014). Our results confirmed that CvT-serpin6 inhibited the activities of activated *Px*PAP1 and *Px*PAP3 in a dose-dependent manner. Meanwhile, rCvT-serpin6 could form SDS-stable

complexes of serpin-protease with them. During the inhibitory interaction between serpin and its target protease, the target protease formed a covalent acyl-intermediate by attacking the scissile bond P1-P1′ in the RCL, which results in a large conformational change in the serpin (Gettins, 2002). Subsequently, the RCL inserts into a β-sheet and the active centre of the protease distorted, which leads to the formation of a covalent complex between serpin and proteinase (Whisstock & Bottomley, 2006; S. Ye et al., 2001). The covalent serpin-protease complex is SDS-stable and could be analysed by SDS-PAGE (Osterwalder et al., 2004).

In summary, we identified an exclusively expressed serpin gene, namely, CvT-serpin6, containing a serpin domain from *C. vestalis* teratocytes. CvT-serpin6 protein was secreted into the extracellular, and formed complexes with its host-activated *Px*PAP1 and *Px*PAP3 to inhibit the PPO activation, that results in inhibition of the host melanization. Previously, a small serine proteinase inhibitor, CvT-TIL, we identified (Gu et al., 2019) contained a cysteine-rich domain, similar to the Egfs encoded by bracovirus MdBV (Beck & Strand, 2007), which was also involved in the melanization by only inhibiting activated *Px*PAP3. In comparison with CvT-TIL, the inhibitory ability of CvT-serpin6 on the PPO activation of *P. xylostella* is significantly stronger. In addition, CvT-serpin6 inhibited *P. xylostella* PPO activation by interacting with activated *Px*PAP1 or *Px*PAP3, while CvT-TIL only interacted with *Px*PAP3. A new host melanization inhibitor released by teratocytes was identified in this study, and our results would shed light on the mechanism by which parasitoid teratocytes suppressed host immunity. Some cytotoxic elements produced during melanization were harmful to *C. vestalis* larva, which resides in the hemocoel of host *P. xylostella*, so CvT-serpin6 was favourable to successful parasitism of *C. vestalis* by controlling melanization of *P. xylostella* haemolymph.

EXPERIMENTAL PROCEDURES

Insect rearing

P. xylostella and its endoparasitoid, *C. vestalis* were maintained as previously described (Shi et al., 2014). *P. xylostella* larvae were reared on cabbage at 25°C, 60%–65% RH with a photoperiod of 14 h light: 10 h dark and adult female *C. vestalis* parasitize third instar *P. xylostella* larvae to rear *C. vestalis*. Adult *P. xylostella* and *C. vestalis* were fed with 20% (w/v) honey solution. For experiments, one individual of 3rd instar *P. xylostella* larva was parasitized by exposing to a mated female wasp within a glass tube.

Teratocytes collection, incubation and induction

Teratocytes were collected following previously described steps (Gao et al., 2016). Briefly, parasitized *P. xylostella* larvae were torn and agitated to totally release haemolymph in Serum-Free Medium (HyClone, Logan, UT, USA) plus ampicillin and kanamycin, each at 100 μg/L after surface sterilization using 70% ethanol. The released haemolymph rested for 30 min to ensure that haemocytes attached to the bottom of the culture dish, while the larger teratocytes remained non-adhesive. The teratocytes were then transferred to another dish with fresh medium using a pipette. Teratocytes were washed five times with medium in this manner, and then transferred to a centrifuge tube. After centrifuging the cells at 500 *g* for 5 min at room temperature, the supernatant was discarded and the pellet of teratocytes was used for further study. The teratocytes collected at 60 h after oviposition were designated as 1-day-old, for teratocytes dissociated from embryonic serous membrane about 2-day post-oviposition at room temperature. Teratocytes of different days, 1-, 2-, 3-, 4- and 5-day post-hatching, were collected and used for qPCR.

To determine whether CvT-serpin6 was secreted, 3-day-old teratocytes were collected from 200 individuals of parasitized *P. xylostella* larvae and cultured in 50 μl Serum-Free Medium plus ampicillin and kanamycin, each at 100 μg/L at room temperature. After 36 h, the supernatant that was teratocytes culture medium (TCM), was collected by centrifuging at 650 *g* for 5 min at 4°C and stored for immunoblot.

To determine whether CvT-serpin6 involved in immune response, 3-day-old teratocytes were collected under sterile condition from fifty parasitized *P. xylostella* larvae and placed into 96-well plates containing Serum-Free Medium plus ampicillin and kanamycin. *E. coli* (ATCC69925) or *S. aureus* (ATCC2592) obtained from American Type Culture Collection was cultured in Luria-Bertani medium by picking a single colony from the cultured plate at 37°C overnight. The cultures were centrifuged at 3500 rpm for 5 min and the pellet was resuspended with sterile tris-buffer solution (TBS, 50 mM Tris–HCl, pH 7.4). A quantity of 2 μl heat-treated resuspended *E. coli* ($OD_{600} = 0.2$) or *S. aureus* ($OD_{600} = 0.2$) and sterile TBS as a control were added into the TCM. Teratocytes were collected at 0, 6, 12, 24 and 48 h after co-culture for qPCR analysis.

Haemolymph collection

The abdominal prolegs of *P. xylostella* larvae surface sterilized by 70% ethanol were cut to release haemolymph on ice using forceps. The haemolymph was collected into the chilled sterile centrifuge tubes using capillaries and diluted two times with the anticoagulant buffer (4 mM sodium chloride, 40 mM potassium chloride, 8 mM EDTA, 9.5 mM citric acid, 27 mM sodium citrate, 5% sucrose, pH 6.8). For melanization assays, four instar non-parasitized *P. xylostella* was used for collecting haemolymph, and cell-free plasma was obtained by centrifugation at 5500 *g* for 10 min at 4°C.

To determine whether CvT-serpin6 was secreted into hemocoel of *P. xylostella* by teratocytes, *P. xylostella* haemolymph was collected as the method previously described (Gu et al., 2019). The haemolymph of the parasitized *P. xylostella* when teratocytes were 1-, 2-, 3-, 4- and 5-day-old and the non-parasitized *P. xylostella* were collected separately as stated in the above methods and centrifuged at 5500 *g* for 10 min at 4°C. The supernatant containing no haemocytes was stored at −80°C and used for immunoblot.

Gene cloning and sequence analysis

Total RNAs were isolated from the collected teratocytes following the steps described by High Pure RNA isolation kit (Roche, Mannheim, Germany) and the quality and concentrations of total RNAs were determined by NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Complementary DNAs (cDNAs) were synthesized using SMART RACE cDNA Amplification kit (Clontech, San Francisco, CA, USA) following the manufacturer's instructions. The *CvT-serpin6*-specific primers were designed based on the sequence of CvT-serpin6 from transcriptome data obtained by Gao et al. to conduct the PCR of the rapid amplification of cDNA ends (RACE) (Gao et al., 2016). The amplified PCR products were sent to TSINGKE Biological Technology for sequencing. We further designed the specific primers based on the correct sequence for amplifying the ORF of *CvT-serpin6*. The amplified products were ligated into pMD-19 vectors (Takara, Osaka, Japan) and then transformed into competent cells of *E. coli* DH5α purchased from Sangon Biotech. The positive clone was confirmed by sequencing and stored in 15% glycerol at −80°C for further study.

The sequence of *CvT-serpin6* and its deduced amino acid sequence were analysed by DNASTAR software 5.02 (Madison, WISC, USA) and National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/). The signal peptide was predicted by the SignalP 5.0 server (http://www.cbs.dtu.dk/services/SignalP/). Sequence alignment was conducted using DNAMAN6.0 (Lynnon Biosoft, USA). The gene structure of *CvT-serpin6* was pictured using the website, the Exon-Intron Graphic Maker (http://www.wormweb.org/exonintron). The cleavage site P1-P1′ in amino acid sequence of CvT-serpin6 was predicted by MEROPS, the Peptidase Database (https://www.ebi.ac.uk/merops/). The phylogenetic tree was constructed by the maximum likelihood method with 10,000 bootstrap replicates in MEGA X 10.2 software and embellished in Figtree v1.4.4 software. All serpin sequences used in the sequence alignment and the phylogenetic tree were downloaded from NCBI and listed in Table S2.

To investigate the transcriptional level of *CvT-serpin6* in the different developmental stages of teratocytes, the first-strand cDNAs were synthesized following the instructions of the ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan). The specific primers for CvT-serpin6 gene were designed and 18S rRNA gene of *C. vestalis* was used as an internal control (Gao et al., 2016). The qPCR reactions were carried out on the iCycleriQTM Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The SYBR Green Real-time PCR Master Mix (Toyobo, Osaka, Japan) was used in the reaction system. The cycling conditions were as follows: 95°C for 30 s, 40 cycles of 95°C for 5 s and 60°C for 34 s. Three independent biological repeats were performed and the relative mRNA transcriptional level was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

Recombinant protein expression, purification and antibody production

For protein expression of the recombinant CvT-serpin6 in *E. coli*, the cDNA fragment encoding mature CvT-serpin6 protein was amplified and ligated into the expression vector pET-28a with the restriction enzyme sites, *Bam*HI and *Xho*I (Promega, Madison, WI, USA). The primers used are listed in Table S1. The recombinant pET-28a-CvT-serpin6 plasmid was transformed into the competent *E. coli* DH5α cell (Sangon, Shanghai, China) and the positive clone was verified by double enzyme digestion and sequencing. The correct recombinant pET-28a-CvT-serpin6 plasmid was then transformed into *E. coli* BL21 (DE3) cell (Transgen Biotech, Beijing, China) after confirmation, which was then cultured in Luria-Bertani (LB) culture medium containing 50 μg/μl Kanamycin (Sangon, Shanghai, China). The culture was induced with 0.2 mM Isopropyl β-D-Thiogalactoside (IPTG) (Sangon, Shanghai, China) at 22°C when optical density (OD) at 600 nm reached 0.5, and then was harvested after overnight and ultrasonically lysed. The supernatant was collected for protein purification. The rCvT-serpin6 was purified according to the manufacturer's protocol of the HisTALON™ Gravity Columns Purification Kit (Clontech, San Francisco, CA, USA). The protein concentration was determined by the Bradford method (Bradford, 1976). The recombinant GFP (rGFP) as a control was expressed and purified according to the above procedures. A quantity of 5 mg of the purified rCvT-serpin6 was sent to Anclonal Technology (Wuhan, China) to produce antibodies. The residual protein was stored in liquid nitrogen for further use.

*Px*proPAP1 and *Px*proPAP3 exist in haemolymph as zymogens, which should be activated to regulate PO cascade (Ji et al., 2003). To obtain the active *Px*PAP1 and *Px*PAP3, the *Px*proPAP1 (*Px*proPAP1$_{Xa}$) and *Px*proPAP3 (*Px*proPAP3$_{Xa}$) mutants were produced by changing the cleavage activation site NGDR^{126} of *Px*proPAP1 to IEGR^{126} and QGNR^{170} of *Px*proPAP3 to IEGR^{170} to allow the activation by the commercial bovine Factor Xa (New England Biolabs, Ipswich, MA, USA) (Figure S3). The recombinant *Px*proPAP1$_{Xa}$ was produced using *sf9* cells as the methods recently reported by Gu et al. (Gu et al., 2019). The recombinant *Px*proPAP3$_{Xa}$ was obtained in our recently published work (Gu et al., 2019).

Bioactivity of rCvT-serpin6

Five serine proteases, trypsin from bovine pancreas (Sigma, Saint Louis, MO, USA), α-chymotrypsin from bovine pancreas (Sigma, Saint Louis, MO, USA), elastase from porcine pancreas (Sigma, Saint Louis, MO, USA), thrombin from human plasma (Sigma, Saint Louis, MO, USA) and subtilisin A from *Bacillus licheniformis* (Sigma, Saint Louis, MO, USA), were used in Bioactivities assay of rCvT-serpin6, according to the methods previously reported (Gu et al., 2019). A total of 10 μl each enzyme (0.21 nM trypsin, 0.20 nM α-chymotrypsin, 0.14 nM thrombin, 0.18 nM subtilisin A and 0.19 nM elastase) was reacted with rCvT-serpin6 (1 mg/ml) of different concentrations (0 nM, 0.05 nM, 0.09 nM, 0.13 nM, 0.18 nM, 0.22 nM, 0.26 nM) for 10 min at room temperature in total 50 μl Tris–HCl buffer containing 0.1 M NaCl and 1 mM $CaCl_2$, pH 8.0. Specific chromogenic substrates for above serine proteinases that were Nα-Benzoyl-L-arginine 4-nitroanilide hydrochloride (Sigma B3133), N-Succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide (Sigma S7388), N-Succinyl-Ala-Ala-Pro-Leu *p*-nitroanilide (Sigma S8511), N-Succinyl-Ala-Ala-Pro-Leu *p*-nitroanilide

(Sigma S8511) and Z-Gly-Gly-Leu *p*-nitroanilide (Sigma C3022) were used to determine the activities of residual enzymes, respectively. OD_{405} was monitored with a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). One unit of the amidase activity was defined as 0.001 ΔA_{405}/min.

Immunolocalization of CvT-serpin6

The collected teratocytes were fixed with 4% paraformaldehyde for 20 min, and then washed three times with 1 × PBST. The fixed teratocytes were incubated with 1: 500 diluted CvT-serpin6 polyclonal antibodies for overnight at 4°C after block with 1% bovine serum albumin (Sangon, Shanghai, China) for 2 h. The teratocytes were then incubated with 1: 1000 diluted Alexa Fluor 594-conjugated AffiniPure Goat Anti-Rabbit IgG (H + L) (Thermo Fisher Scientific, Waltham, MA, USA) in dark at room temperature for 2 h after washing three times. Finally, the teratocytes were placed on slides, sealed with DAPI Fluoromount-GTM (MyBioSource, San Diego, CA, USA) and photographed with Laser scanning confocal microscope, Zeiss LSM800 Airyscan (Zeiss, Oberkochen, Germany).

Melanization assays

The inhibitory assay of PPO activation by rCvT-serpin6 was conducted according to the method previously described with minor modification (Tong & Kanost, 2005). Inactivated *M. luteus* was usually used as an elicitor of PPO activation in previous studies (Suwanchaichinda et al., 2013; Yang et al., 2018). A total of 2 μl cell-free haemolymph was mixed with 12 μl sterilized TBS, 10 μl sterilized TBS plus 2 μl *M. luteus* (0.5 μg/μl), 10 μl bovine serum albumin (BSA, 1 μg/μl) (Roche, USA) as a negative control plus 2 μl *M. luteus* (0.5 μg/μl), 10 μl rCvT-serpin6 (1.2 μg/μl) plus 2 μl *M. luteus* (0.5 μg/μl), 10 μl rCvT-serpin6 (0.6 μg/μl) plus 2 μl *M. luteus* (0.5 μg/μl), 10 μl saturated phenylthiourea (PTU) plus 2 μl *M. luteus* (0.5 μg/μl) and 10 μl CvT-TIL (0.45 μg/μl) plus 2 μl *M. luteus* (0.5 μg/μl) as a positive control, respectively. The mixtures were reacted at room temperature for 45 min and then were added with 200 μl L-dopamine (2 mM in 0.1 M Tris–HCl, pH 6.5) as a substrate. PO activity was measured by monitoring OD_{490} and one unit of PO activity was defined as 0.001 ΔA_{490}/min. To test spontaneous melanization, 2 μl cell-free haemolymph of non-parasitized *P. xylostella* larvae was incubated with 10 μl sterilized TBS, 10 μl BSA as a negative control, 10 μl rCvT-serpin6 (1.2 μg/μl), 10 μl rCvT-serpin6 (0.6 μg/μl) and 10 μl PTU as a positive control in total 20 μl TBS buffer at room temperature, respectively. The processes were photographed every 15 min for total 60 min.

Inhibition of amidase activity by rCvT-serpin6

The activated *Px*PAP3 were obtained during our previously published work (Gu et al., 2019). To activate the recombinant *Px*proPAP1$_{Xa}$, 0.5 μg recombinant *Px*proPAP1$_{Xa}$ were mixed with 0.5 μl Factor Xa (1 μg/μl) in total 20 μl reaction buffer (20 mM Tris–HCl, pH 8.0, 150 mM NaCl, 2 mM $CaCl_2$, pH 8.0) and incubated at 37°C for 6 h, respectively. The reaction mixtures were subjected to 12% SDS-PAGE and then immunoblot analysis was conducted. The amidase activities of activated *Px*PAP1 and *Px*PAP3 were measured using the substrate, acetyl-Ile-Glu-Ala-Arg-*p*-nitroanilide (IEAR*p*NA) (Gupta et al., 2005) and the OD_{405} was monitored using a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). One unit of the amidase activity was defined as 0.001 ΔA_{405}/min. To investigate the effect of rCvT-serpin6 on activated *Px*PAPs, the rCvT-serpin6 was incubated with the Factor Xa-activated *Px*proPAP1$_{Xa}$ and *Px*proPAP3$_{Xa}$ at different molar ratios. The residual amidase activities of the activated *Px*PAP1 and *Px*PAP3 were measured and calculated as above.

Detection of SDS-stable CvT-serpin6-*Px*PAP1 or -*Px*PAP3 complexes

Purified recombinant *Px*proPAP1$_{Xa}$ (0.5 μg) and *Px*proPAP3$_{Xa}$ (0.5 μg) were activated by Factor Xa as the method mentioned above and mixed with purified rCvT-serpin6 at molar ratio of 1:1, respectively. The mixtures were incubated at room temperature for 30 min and then subjected to 12% SDS-PAGE. In control samples, the mixtures contained no Factor Xa. The immunoblot analysis was conducted with the mouse anti-6 × His-tag monoclonal antibody (1: 2000) or the rabbit anti-CvT-serpin6 polyclonal antibody (1: 500) as primary antibodies. The secondary antibody was horseradish peroxidase (HRP)-conjugated anti-rabbit/mouse IgG (1: 2000) and complexes bands were detected using Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific, Waltham, MA, USA) and imaged by ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA, USA).

Western blot

Protein samples were denaturized by boiling for 10 min after adding 1 × SDS sample buffer, separated by 12% SDS-PAGE and then transferred onto polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA) using the semi-dry transfer membranes (Bio-Rad, Hercules, CA, USA). The PVDF membranes were blocked with 3% BSA and washed using 1 × TBST buffer. The anti-6 × His-tag mouse monoclonal antibody (1: 2000) purchased from Abcam was used as a primary antibody for detection of the recombinant protein with His-tag and the polyclonal antibody against rCvT-serpin6 (1: 500) was incubated with the membrane as a primary antibody for detection of CvT-serpin6 in TCM and parasitized *P. xylostella* haemolymph. β-actin mouse monoclonal antibody (1: 2000) (ABclonal Tech., Wuhan, China) was used as an internal reference (Gao et al., 2016). The secondary antibodies for both were HRP-conjugated anti-rabbit/mouse IgG (1: 2000) purchased from Abcam (Cambridge, UK). CvT-serpin15 protein bands on membranes were detected using Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific, Waltham, MA, USA) and imaged by ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA, USA).

Statistical analysis

All statistical analyses were conducted using SPSS 19.0 software (SPSS, Chicago, IL, USA) and figures were plotted using Origin 8.0 software (OriginLab, Northampton, MA, USA). One-way analysis of variance with Tukey-test was used to determine the significant difference between groups and $p < 0.05$ was set as significant threshold.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Min Shi, Xuexin Chen and Qijuan Gu designed the study. Min Shi, Qijuan Gu and Zhizhi Wang wrote the manuscript. Qijuan Gu and Zhiwei Wu performed experiments. Qijuan Gu analysed data with the help of Yuenan Zhou and Jianhua Huang. All authors read and approved the final manuscript.

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 in the online version of the article at the publisher's website.

Figure S1. Prokaryotic expression and purification of *CvT-serpin6*. (A) SDS-PAGE of rCvT-serpin6. M, protein marker; lane 1, non-induced *E. coli*; lane 2, induced *E. coli*; lane 3, supernatant; lane 4, inclusion body; lane 5, purified rCvT-serpin6. (B) Western blot with His-tag antibody. M, protein marker; lane 6, purified rCvT-serpin6. (C) SDS-PAGE of rGFP. lane 7, non-induced *E. coli*; lane 8, induced *E. coli*; lane 9, purified rGFP. (D) Western blot with His-tag antibody. lane 10, non-induced *E. coli*; lane 11, induced *E. coli*; lane 12, purified rGFP.

Figure S2. Activation of *Px*proPAP1$_{Xa}$ by Factor Xa. (A) Immunoblot analysis of the activating *Px*proPAP1$_{Xa}$ in vitro. *Px*proPAP1$_{Xa}$ was incubated with Factor Xa at 37°C for 6 h to generate activated *Px*PAP1. SDS-PAGE of protein samples followed by immunoblotting using an antibody against His-tag. • represents *Px*proPAP1$_{Xa}$. * indicates activated *Px*PAP1. (B) Amidase activity of activated *Px*PAP1. The amidase activity of activated *Px*PAP1 was reflected by monitoring OD changes at 405 nm using IEARpNA as substrate. Error bars are means ± S.E. (n=3). Bars labeled with different letters were significantly different (one-way ANOVA followed by Tukey-test, $p < 0.05$).

Figure S3. Mutations of activation site in *Px*proPAP1 and *Px*proPAP3. The *Px*proPAP1(*Px*proPAP1$_{Xa}$) and *Px*proPAP3 (*Px*proPAP3$_{Xa}$) mutants were produced by changing the cleavage activation site NGDR^{126} of *Px*proPAP1 to IEGR^{126} and QGNR^{170} of *Px*proPAP3 to IEGR^{170} to allow activation by commercial bovine Factor Xa.

Figure S4. SDS-PAGE analysis of *Px*proPAP1$_{Xa}$. The recombinant *Px*proPAP1$_{Xa}$ was analyzed by SDS-PAGE. M, protein marker.

Figure S5. Inhibition of teratocytes secretory proteins (TSPs) on PPO activation of *P. xylostella* hemolymph. Cell-free hemolymph was mixed with sterilized TBS, sterilized TBS plus *M. luteus*, TPS plus *M. luteus*, PTU plus *M. luteus*, and Medium plus *M. luteus*, respectively. The mixtures were reacted at room temperature for 45 min and then were added with L-dopamine as a substrate. PO activity was measured by monitoring OD490 and one unit of PO activity was defined as 0.001 ΔA490/min. TBS, Blank; Medium, negative control; PTU, positive control; Inactivated *M. luteus* is an elicitor. Error bars represent the mean ± SE ($N = 3$). The data was conducted one-way analysis of variance (one-way ANOVA, Tukey-test, $p < 0.05$). Significant differences were indicated with different letter.

Figure S6. Inhibition of PPO activation by rCvT-TIL and rCvT-serpin6 under the same concentration. Cell-free hemolymph was mixed with sterilized TBS, sterilized TBS plus *M. luteus*, BSA plus *M. luteus*, PTU plus *M. luteus*, rCvT-serpin6 plus *M. luteus* and CvT-TIL plus *M. luteus*, respectively. The mixtures were reacted at room temperature for 45 min and then were added with L-dopamine as a substrate. PO activity was measured by monitoring OD490 and one unit of PO activity was defined as 0.001 ΔA490/min. Error bars represent the mean ± SE ($N = 3$). The data was analyzed using one-way analysis of variance (one-way ANOVA, Tukey-test, $p < 0.05$). Significant differences were indicated with different letters.

Table S1. Primers sequences used in this paper.

Table S2. Information of sequenes for phylogenetic tree and comparison.

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