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Molecular Characterization and Bioinformatics Analysis of a Prophenoloxidase-1 (PPO1) in *Plutella xylostella*

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ABSTRACT: Phenoloxidase (PO) is an important enzyme in insect life, which is involved in important physical functions, such as defensive encapsulation and melanization of foreign organisms and wound healing. In this study, we obtained a cDNA sequence of 2838 bp with 2049 open reading frames encoding 682 amino acids. The protein sequence deduced from the cDNA has high homology with the known PPO1 sequences of other lepidopterous insects. There were three conserved regions, including the two copper-binding sites characteristic of arthropod PPOs. The whole *PxPPO1* DNA was also obtained with 7202 bp when the five fragments were stitched together and the overlapping sequences were deleted. The *PxPPO1* DNA consists of 11 introns and 12 exons, and the homology is 99.9% when the exons are compared with the above cDNA. Moreover, the gene expression levels were also determined by semiquantitative polymerase chain reaction (PCR), Western blotting, and real-time quantitative PCR; the results indicated that *PxPPO1* transcripts in the eggs and the fourth instar larvae were more abundant, followed by the second and the third instar larvae, prepupae, and pupa.

KEYWORDS: prophenoloxidase, intron, exon, temporal expression, *Plutella xylostella*

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Introduction

Phenoloxidase (PO) (EC 1.14.18.1) is a copper oxidase that catalyzes two distinct reactions of melanin synthesis—the hydroxylation of monophenol and the oxidation of *o*-diphenol to the corresponding *o*-quinone.[1,2] *O*-quinones are subsequently converted to melanin or react with proteins forming protein-catechol complexes, which may be important biochemical reactions, especially in the recognition and melanization of foreign organisms during immune responses.[3] In insects, many studies have indicated that PO might be involved in some important biochemical processes, such as defensive encapsulation and melanization of foreign organisms and wound healing.[4] In contrast, some RNAi studies have clearly shown that laccase, but not PO, is the very enzyme having the major function in cuticle sclerotization and pigmentation during insect development.[5,6]

The molecular biological studies during the past 20 years have drastically promoted our understanding of the molecular mechanisms of insect PPO activation and melanization pathways.[7,8] It has been assumed that each reaction step of melanization synthesis could produce many toxic intermediates, including semiquinones, dopaquinone, indolequinones, and reactive oxygen species.[9] It has been known that these responses are mediated through activation of serine proteinase cascades. However, in normal physiological conditions, they are controlled by serine protease inhibitors (serpins) to suppress PPO activation spatially and temporally and to avoid the deleterious effects on the host by the toxic intermediates.[10,11] Serpins contain a reactive center loop close to the carboxyl-terminus, which interact with the active serine of target proteases and trap the protease in an inactive state.[10,12] Because the recombinant serpins could inhibit prophenoloxidase-activating proteinase (PAP) activity and suppress PPO activation finally,[13,14] PAPs have at least one clip domain at the N-terminal side of the serine protease domain. For the activation of PO, PAPs sometimes require an additional cofactor, serine proteinase homologs; the serine residue at the active center is replaced by other amino acids.

The diamondback moth, *Plutella xylostella* (L.), is a worldwide insect pest of cruciferous vegetables in the tropical and subtropical regions, which cost $ 4–5 billion per year for its management throughout the world.[15] Previously, we cloned and characterized the cDNA for *PxPPO2* (GQ149238).[16] In this article, we cloned the *PxPPO1* cDNA, carried out its bioinformatic analysis, and analyzed the *PxPPO1* temporal expression in *P. xylostella* using semiquantitative PCR (sPCR), Western blotting, and real-time quantitative PCR (qPCR). Besides, the characterization of introns and exons in *PxPPO1* was also

investigated. These results would shed light on a further study of the PPO gene structure and function in *P. xylostella*.

Materials and Methods

Experimental insects. *P. xylostella* was originally obtained from Tai'an City, Shandong, China, and maintained in this laboratory for eight years. The larvae were fed on cabbage plants (*Brassica oleracea*) and the adults were fed a 10% (W/V) honey solution and allowed to lay eggs on radish seedlings (*Raphanus sativus* L.). The larvae and adults were maintained at 25 ± 1°C and an relative humidity (RH) of 60%–70% with a photoperiod of 14:10 hours light: darkness (L:D).

RNA extraction and cDNA synthesis. According to the manufacturer's instructions, total RNA was extracted from the fourth instar larvae using Trizol Reagent (Invitrogen, www.invitrogen.com). Using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, www.fermentas.com), cDNA was cloned with Oligo(dT)$_{18}$ primer.

Conserved region clone and cDNA sequence. Degenerate primers were designed according to PPO1 conserved amino acid sequences in GenBank with the following accession nos: *Bombyx mori* PPO (AAG09304), *Choristoneura fumiferana* PPO (ABW16859), *Galleria mellonella* PPO (AAK64363), *Hyphantria cunea* PPO (AAC34251), *Manduca sexta* PPO (AAC05796), and *Spodoptera frugiperda* PPO (ABB92834).

The forward primer was 5′-GGCSTACTTCCGCGARGACAT-3′ (where S = C or G and R = A or G) and the reverse primer was 5′-GCNGTCGCNGAGTCDCCCATCA-3′ (where N = A or C or G or T and G = A or G or T). PCR was carried out on T-Gradient Thermoblock (Biometra); the reaction conditions were as follows: 94°C with 4 minutes for denaturation, followed by 35 cycles at 94°C with 1 minute for denaturation, at 60°C with 1 minute for annealing, at 72°C with 1 minute for extension, and at 72°C with a final 10-minute extension. The obtained PCR products were tested by 1.5% agarose gel electrophoresis, purified with TIANgel Midi Purification Kit (Tiangen), ligated into the pMD18-T vector (TaKaRa), and transformed into competent cells of *Escherichia coli* strain DH5α. The positive clones were selected and sequenced on ABI PRISM 3730 by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd.

Rrapid amplification of cDNA ends clone. The 5′-gene-specific primer (GSP; 5′-GCAGGTTGATGCCGATGTCC-3′) and 3′-GSP(5′-CGACCACAGACACTTGG AACA-3′) were prepared according to the cloned sequence mentioned above. The 3′and 5′ ends of the gene amplification were performed with RACE (rapid amplification of cDNA ends) kit (TaKaRa). Both the RACE PCR products were tested, purified, ligated, and sequenced as mentioned above, respectively.

Genomic DNA extraction. Total gDNA was extracted from *P. xylostella* larvae by using E.Z.N.A.™ Insect DNA Kit (Omega Co.) following the manufacturer's instructions. All the utensils were autoclaved or baked at 180°C for 8–10 hours in the process of extraction. The extracted gDNA from *P. xylostella* was validated in order by 0.8% agarose gel, ethidium bromide staining, and gel imaging detection. The RNA was digested completely, and the gDNA was usable for the next experiments.

Cloning the PxPPO1 conserved fragment. Primers were prepared according to the conserved amino acid sequences of PPO1 from the *PxPPO1* cDNA (GU199189) and the known lepidopterous insects' PPO1 in NCBI GenBank. The forward (PS-F) and reverse (PS-R) primers were 5′-ATGGCGGACAAAAACAACT-3′ and 5′-CTCCATCTCTCCCACTCTACC-3′, respectively.

According to the manufacturer's protocol, PCR was carried out within 1 μg of gDNA of *P. xylostella* with the third instar larvae as template using LA Taq Kit (TaKaRa), under the conditions of 94°C with 5 minutes for predenaturation, 30 seconds at 94°C for denaturation, 45 seconds at 55°C–65°C for annealing, at 72°C with 4 minutes for extension followed by 35 cycles, and at 72°C with a final 10-minute extension.

The 5 μL PCR products with 1 μL loading buffer were separated on 0.8% agarose gel, using gel electrophoresis apparatus. According to the detection results, the best temperature for annealing was 65°C. Next, the new PCR was done and its products were tested by 1% agarose gel, purified with TIANgel Midi Purification Kit (Tiangen), then cloned into the pMD18-T vector (TaKaRa), and the plasmid was used to transform *E. coli* DH5α competent cells. Positive clones were sequenced by Shanghai Sangon Biol. Co., this is the initial fragment, named A.

Fragments amplification, stitching, and validation. The whole *PxPPO1* gene sequence was cloned using Genome Walking Kit (TaKaRa). First, three specific primers (SPs) with higher annealing temperature at the same amplifying direction were designed (Table 1). Second, a thermal asymmetric interlaced PCR (TAIL-PCR) was carried out, following the manufacture's protocol, using the three primers

Table 1. Primers used for fragment A downstream and upstream amplification.

NAME	PRIMER (5′→3′)
SP1-P	CGCAGTATCTAACGGACCTTTGT
SP2-P	TCATCGTTGTGCAGATGCGTGAA
SP3-P	TGCGTCGAGAACTGGTTCGGAGT
SP1-C	TGCGGCTGATGATGGACGACTC
SP2-C	CAAATCCCTTACCAAGTGTCGG
SP3-C	CTTCGTTATTTCTTTCTGGGAT
SP1-F	TGTAATGAGGTCCCACAGAAGT
SP2-F	TCCTGGGCATCAGAATCTTTGT
SP3-F	CATTTCGGCTTACTAACACCCT
PR-F	GGAATGAAGATAGGGTGAGTT
PR-R	TCCGTGTATTATAGCCAAAGG

combined with four SPs within lower annealing temperatures. The amplification direction is from 5′ to 3′ with the primer order, SP1, SP2, and SP3 (Supplementary Fig. 1). Third, PCR products 5 µL of first, second and third circles, respectively, were separated on 1% agarose gel, purified with TIANgel Midi Purification Kit (Tiangen), then cloned into the pMD18-T vector, and the plasmid was used to transform *E. coli* DH5α competent cells. Positive clones were sequenced by Shanghai Sangon Biol. Co. (China), four fragments were obtained, namely, B, C, D, and E.

The produced five fragments were used to do sequence alignment by NCBI BLASTn program. And, all the fragments were stitched together as designing pattern (Supplementary Fig. 2) using the BioEdit and DNAstar software. To further validate the proper of the whole *PxPPO1* gene sequence, two primers was designed also to clone a new fragment, which one is in the upstream of the fragment, JY-F: 5′-CCAGGAGTGGACGATTATTG-3′, the other is in the downstream interval 5000 bp with the upstream primer, JY-R: 5′-GAAGGGTTGAGGGGGTTAGA-3′.

Bioinformatics analysis of PxPPO1 cDNA. The cDNA was assembled into a consensus sequence containing the complete open reading frame (ORF) with ORF Finder. The *PxPPO1* protein sequence was analyzed by ExPASy, SignalP, and Compute pI/MW (http://us.expasy.org/tools/). The *PxPPO1* (GenBank accession no. ACZ97553) alignment was performed by ClustalW (version 2.0) online. Phylogenetic tree and its analysis were performed using the method of our previous study.[16]

Bioinformatics analysis of PxPPO1 genomic DNA. The online software EMBOSS Needle (http://www.ebi.ac.uk/Tools/services/web) was used to analyze the splicing rules of mRNA gene. The softwares Genomatix (http://www.genomatix.de) and TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html) were used to analyze the potential transcription factor binding sites of the gene introns and the existing situation of CAAT box, TATA box, and GC box in the introns. The software Winstar was used to analyze the adenine thymine (AT) proportion in the introns. The software EMBOSS CpGPlot (http://www.ebi.ac.uk/Tools/em-boss/cpgplot/index.html) and RepeatMasker were used to predict the CpG island and repetitive sequence, respectively.

Temporal expression of PxPPO1. sPCR and real-time qPCR were carried out to determine temporal expression of *PxPPO1* during the development of *P. xylostella*. Total RNA ~900 ng from ~70 mg of the eggs, the first, second, third, and fourth instar larvae, prepupae, pupae, and adults of their mid-stage, respectively, were used as templates for 10 µL first-strand cDNA synthesis using FastQuant RT Kit (with gDNase; Tiangen). Then, these cDNA were used as templates for the next PCR amplification and detection. GAPDH (GenBank accession no. AJ489521) was used as housekeeping gene for normalization of equal sample loading. The primers used for GAPDH were 5′-CAGTGCCGATGCACCTATGTTC-3′ (forward) and 5′-AAGTTGTCGTTGAGG GAGATGCC-3′ (reverse).[17]

Semiquantitative PCR. The primers used for *PxPPO1* were 5′-CCGTGGTGGACAAGGACAGG-3′ (forward) and 5′-GGTCGTGAGCGTAGG AGATGA-3′ (reverse). The reaction conditions were as follows: 0.2 µL first-strand cDNA, 2.5 µL 10× reaction buffer, 0.5 µL dNTPs (10 mM each), 1 unit *Taq* polymerase (TransGen, Beijing, China), 15 pmol of each *PxPPO1* primer, and 15 pmol of each GAPDH primer in a total volume of 25 µL. Cycling conditions were 94°C for 4 minutes, and then by 30 cycles of 94°C, 60°C, and 72°C for 30 seconds each, followed by 72°C for 10 minutes on T-Gradient Thermoblock. PCR products were tested by 1.5% agarose gel electrophoresis and stained by ethidium bromide. There are three replicates in sPCR test.

Real-time qPCR. The primers used for *PxPPO1* were 5′-CGTCCATCATCAGCCGCAACC-3′ (forward) and 5′-TCTCCCATCACGCC GAATT-3′ (reverse). qPCR was performed with iQ™5 (Bio-Rad) with three replicates, using 1.2 µL cDNA in a final volume of 25 µL with 0.5 pmol of each *PxPPO1* or GAPDH primer and 12.5 µL of SYBR® Premix Ex Taq™ (2× conc.; TaKaRa). qPCR conditions were as follows: initial denaturation at 94°C for 30 seconds, followed by 40 cycles of denaturation at 94°C for 15 seconds, 60°C for 20 seconds, and 72°C for 30 seconds. The melting curve analysis and C_T value calculating methods were referenced in the previous studies.[16,18]

Western blot analyses. The protein from the eggs, the first, second, third, and fourth instar larvae, pupae, and adults of *P. xylostella* were extracted using Trizol lysate (Roche, Shanghai, China) as the reference.[19] Twenty micro-liter protein samples of the seven different development stages were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The protein concentrations were tested before applied to Western blotting using the BCA Protein Assay Kit (Beyotime, www.beyotime.com) following the manufacturer's protocol with four replicates. For Western blot analysis, the proteins were transferred onto polyvinylidene fluoride (PVDF) membrane (0.45 µm Merck Millipore, Shanghai, China); after blocked with 5% skim milk in TBS-T (0.05% Tween-20 in TBS buffer), the membranes were incubated overnight at 4°C with a rabbit antibody against *M. sexta* PPO in a 1:5000 dilution; and the next day, after washing with TBS-Tween (0.05%), for 1.5 hours with a goat anti-rabbit IgG (H + L) (Beyotime) secondary antibody in a 1:8000 dilution. The blots were developed with an ECL system.

Statistical analyses. The obtained data are expressed as mean ± standard deviation (SD). One-way analysis of variance was used for tests of significance of differences between groups. Statistical calculations were carried out using SPSS 16.0 software.

Results

PxPPO1 cDNA cloning and alignment. The *PxPPO1* cDNA was produced and registered (accession no. GU199189) in the GenBank. The deduced ORF sequence starts from the

145th nucleotide with 2049 bp encoding a protein of 682 amino acids (Fig. 1). The 3′ UTR extends from the 2194th nucleotide with 645 nucleotides in length, and a polyadenylation signal (AATAAA) was predicted, locating at 14 bp upstream from the poly(A)$^+$ tail. The calculated molecular mass is about 78.56 kDa, and the isoelectric point is 6.43. The proteolytic cleavage site, DRFG, that is important for PPO activation locates at ~50 residues from the N-terminus (box and arrow in Fig. 1). An active enzyme with 72.65 kDa would be generated by cleaving this site.

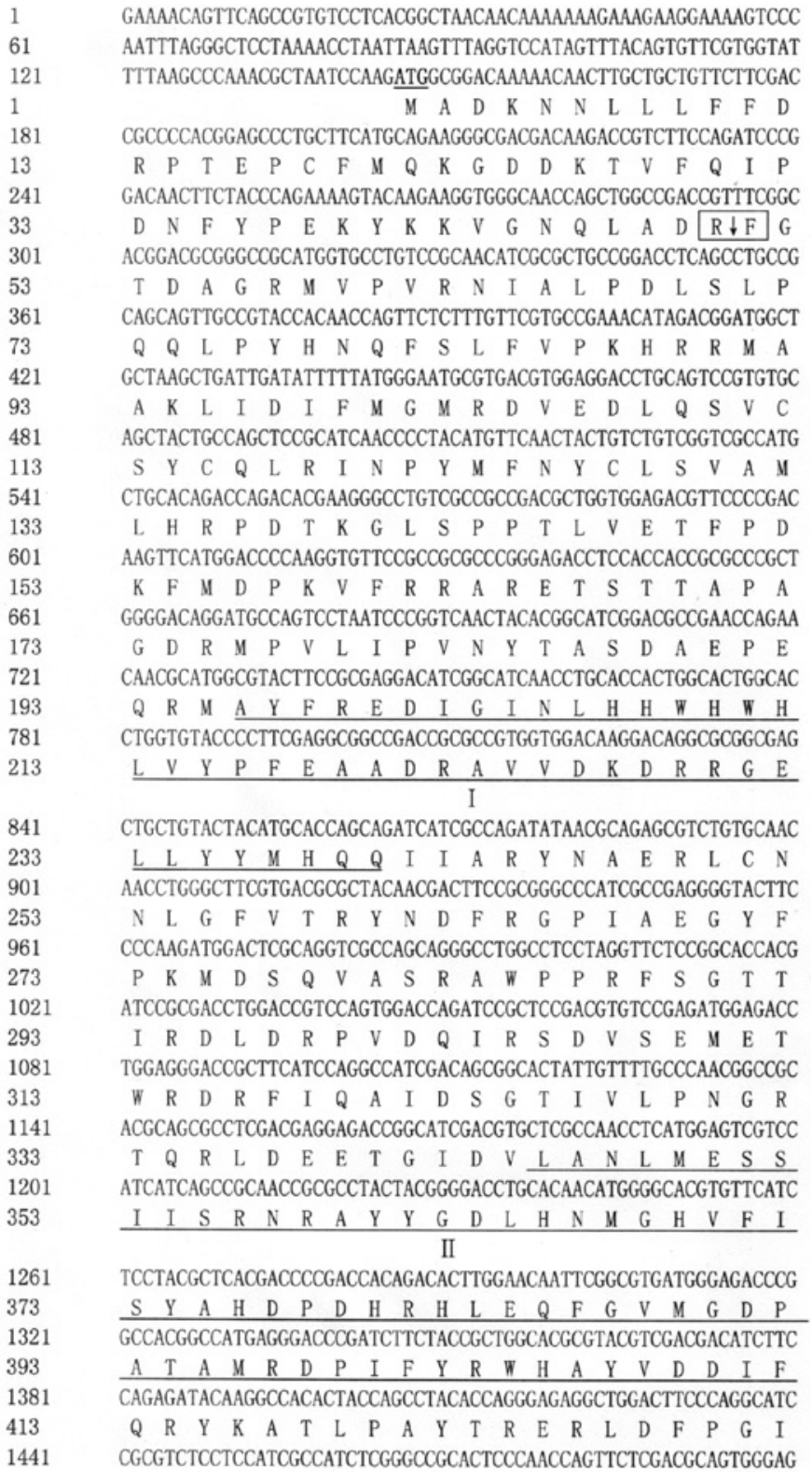

Figure 1. (*Continued*)

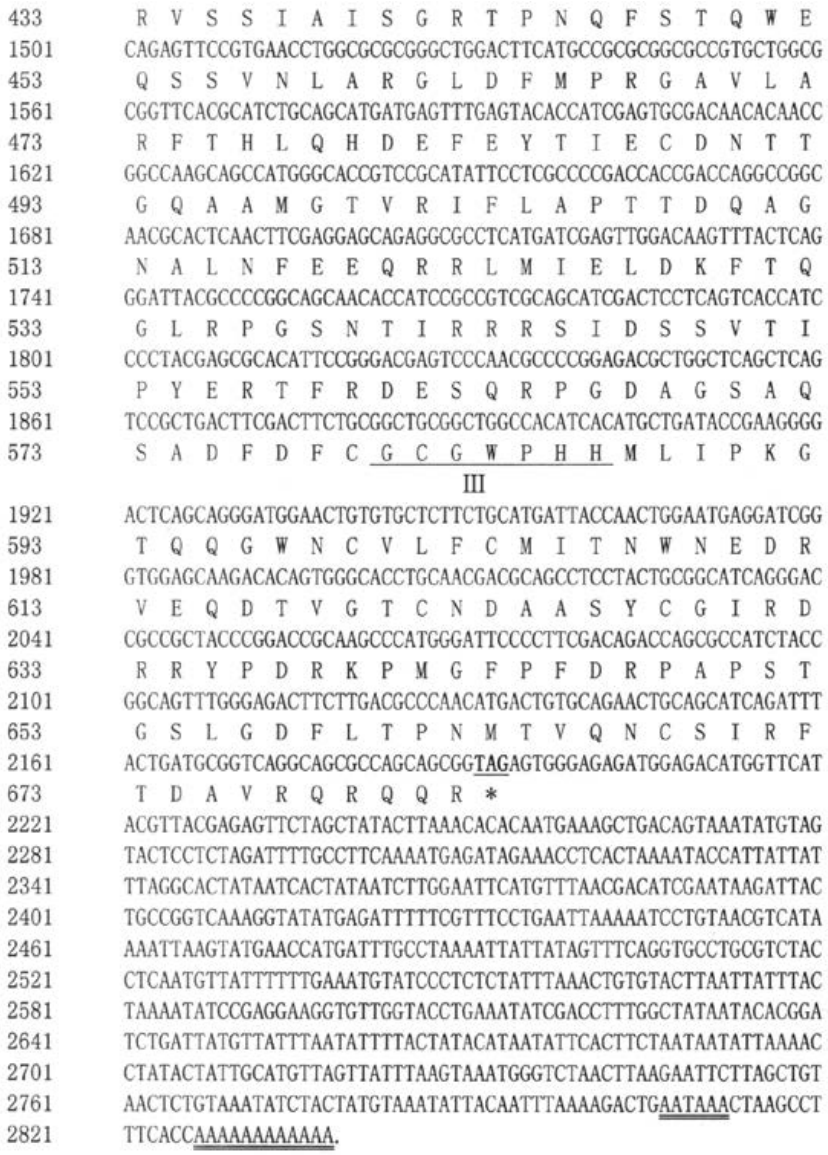

Figure 1. Nucleotide and deduced amino acid sequence of *P. xylostella* PPO1. The sequence was deposited in GenBank (GenBank accession no. GU199189). Possible polyadenylation signal (AATAAA) and poly(A)$^+$ tail were doubly underlined. Conserved motifs were underlined and numbered. Note in region I: copper-binding site A; region II: copper-binding site B; region III: thiolester region-like motif. Potential cleavage site for the activation to PO are boxed and shown with arrows. *Stop codon.

The *PxPPO1* sequence was aligned with the PPOs of other insects by ClustalW; the results showed that the *PxPPO1* sequence has a high homology with the PPO1 of other lepidopterous insects. High sequence identity was found with *C. fumiferana* PPO1 (74.6%), *B. mori* PPO1 (74.5%), *Heliothis virescens* PPO1 (73.3%), *S. frugiperda* PPO1 (73.1%), *Plodia interpunctella* PPO1 (72.5%), and *M. sexta* PPO1 (70.5%). However, the homology between *PxPPO1* and *PxPPO2* (GQ149238) was only 45.3%. Similar to all insect PPOs, a putative thiolester site and Cu^A and Cu^B binding regions, with six histidine residues, existed in *PxPPO1* also (Supplementary Fig. 3). The phylogenetic tree showed the evolution of *PxPPO1* in Supplementary Figure 4.

Whole PxPPO1 DNA producing and validation. According to genome walking method, five fragments were obtained (Supplementary Fig. 5), validated, stitched together, and reconstructed the whole *PxPPO1* gene sequence of 7532 bp. At last, the whole *PxPPO1* sequence is 7202 bp when the overlapping area of the fragments is deleted. To further validate the proper of the entire sequence, another PCR and BLAST alignment were carried out; the results showed that the new fragment (name JY2) was 5000 bp, which was identical with the template. The stitching sequence was validated successfully (Supplementary Fig. 6).

Bioinformatics analysis of PxPPO1 DNA. The results of BLAST and EMBOSS Needle alignment showed that there were 11 introns and 12 exons in the *PxPPO1* DNA sequence,

in which the exons encoded 682 amino acids (Supplementary Table 1). The absent black lines were introns, and the red lines were exons (Fig. 2A); furthermore, all the exons and introns were schematic and labeled (Fig. 2B). The homology is 99.9% when aligned all the exons with *PxPPO1* mRNA (GU199189). The intron's number was similar to that of 12 introns in *BmPPO1* when retrieved in NCBI using EMBOSS Needle (Fig. 2C). Additionally, 11 exon–intron splicing junction motifs were also defined in *PxPPO1* (Supplementary Table 2).

The analysis results of the softwares Genomatix and TFSEARCH indicated that there was one CAAT box in the second, fourth, fifth, and seventh intron, one TATA box in the second, third, and sixth intron, two TATA box in the seventh intron, and there was no GC box and CpG island in any intron. RepeatMasker software predicted the gene duplication sequences, the results showed that there were two simple repeats with 47 bp in the gene, which were 0.62% of the gene sequence.

Temporal expression of PxPPO1 transcripts. The *PxPPO1* expression levels in different stages were detected as shown when compared with the GAPDH control (Fig. 3A). The *PxPPO1* transcripts were much abundant in the eggs and the fourth instar larvae, followed by the second or third instar larvae or pupa. However, the *PxPPO1* transcripts were absent in the first instar larvae or adults. Direct immunoblotting results showed that antibody to *M. sexta* PPO could be recognized by *P. xylostella* PPO in different developments (Fig. 3B). The lower band was PPO1 with 78 kD, and the upper was PPO2 with 80 kD. In order to further compare the temporal expression of PPO in *P. xylostella*, the Image-Pro Plus software was used to analyze the mean gray value. The total values of the whole body PPO (PPO1 and PPO2) in the different developments were 1.86, 3.98, 3.85, 5.54, 2.86, and 0.57 for the first, second, third, and fourth larvae, pupae, and adult, respectively, and the protein concentrations were 0.57, 1.15, 1.62, 4.65, 2.95, 3.10, and 2.01 mg/mL for the egg, the first, second, third, and fourth larvae, pupae, and adult, respectively. The result also indicated that the PPO1 was more abundant than PPO2 in pupae, while the PPO2 was more abundant than PPO1 in the adult; such interesting findings need to be further studied.

The *PxPPO1* expression model was also performed by qPCR, which showed that *PxPPO1* was highly abundant in eggs and the fourth instar larvae, with 21.78-fold and 19.51-fold compared with that of the adult stage, respectively. In the first instar larvae, the *PxPPO1* expression was 6.44-fold (Fig. 3C). In summary, the results of the *PxPPO1* expression pattern could be verified mutually through the sPCR, immunoblotting, and qPCR tests.

Discussion

There are three conserved regions in PPO proteins, referred to as regions I, II, and III. Regions I and II correspond to the Cu^A and Cu^B binding sites, respectively. The copper-binding sites are highly conserved oxygen-binding regions, which is common to all invertebrate PPOs and other associate proteins, including the hemocyanins from lower arthropods. Region III is a thiolester motif (GCGWPHH) characteristic of α2-macroglobulin and C3 and C4 complement proteins, with

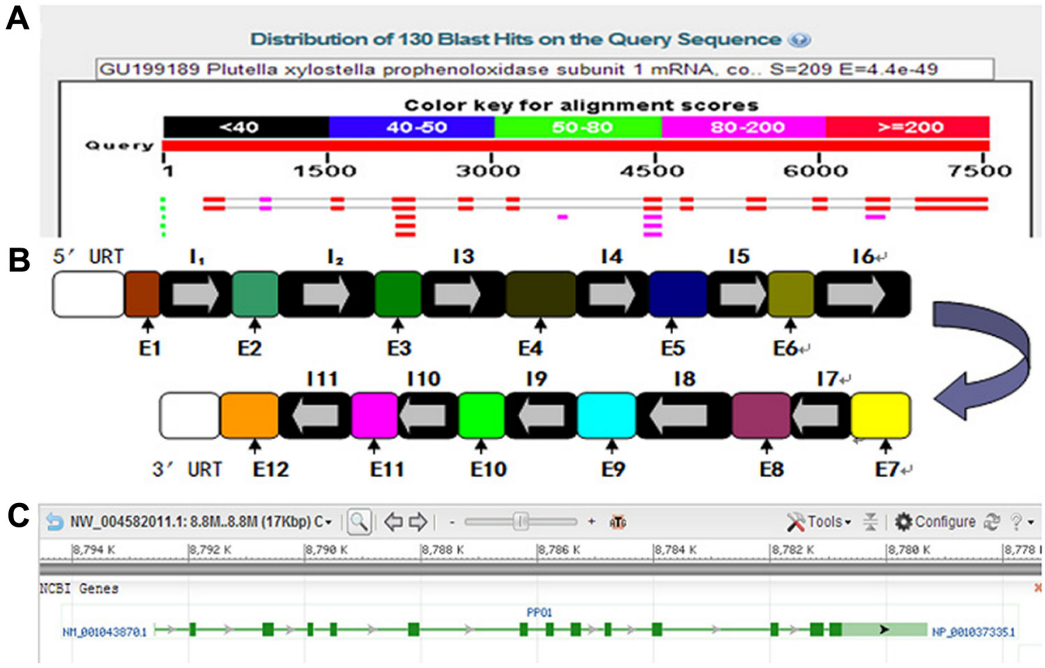

Figure 2. The situation of exons and introns. (**A**) Sequencing results by BLAST. (**B**) The schematic arrangement of exons and introns, $I_{1–11}$ means the intron I_1–I_{11}; $E_{1–12}$ means the exon E_1–E_{12}. (**C**) The retrieval results of *B. mori* PPO1 from NCBI.

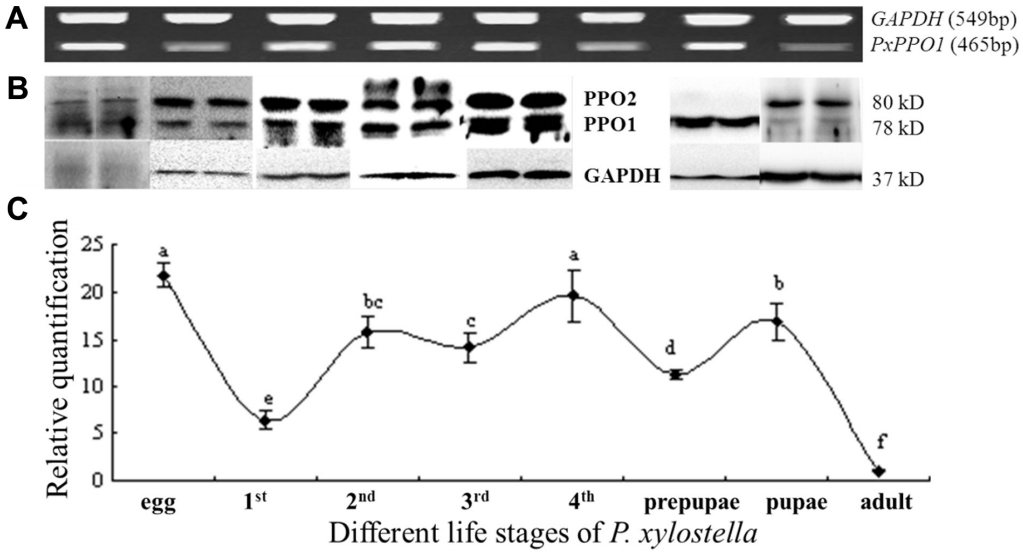

Figure 3. Temporal expression of *PxPPO1* transcripts: (**A**) sPCR analysis; (**B**) immunoblotting analysis, antibodies of rabbit antiserum to *M. sexta* PPO were used by Western blotting; and (**C**) qPCR analysis, the methods used as the reference.[16]

function in macromolecule binding.[20] The PPO thiolester motif could be correlated to the immobilization function of invading organisms while insect defense reactions occurred.[21] As reported, there is no N-terminal signal sequence at most of the insect PPOs, and two copper-binding sites are present in the active site of the enzyme. There is an increase in the transcript level of PPO following parasitic invasion, and insects having impaired machinery to produce this enzyme show reduced ability to suppress pathogen attack by melanizing and encapsulating them.[16,22]

This study found 11 introns and 12 exons in *PxPPO1*; however, of the introns, only I_5 demonstrates any identity with sequences in NCBI and shows partial identity to microsatellite pxy001 (GenBank: DQ649107.1) of *P. xylostella*. Although the diamondback moth genome has been sequenced successfully,[23] there are still some unnamed sequences in the genome due to the restriction of the sequencing method. The further sequence of the BLAST alignment results showed that the *PxPPO1* gene was numbered with ACZ97553.1, located at scaffold 134 but with lower identity. When the scaffold 134 was drawn from the genome, some unknown sequences were also found with the marking N. Thus, it was deduced that the sequenced fragments in this study could fill the unknown N sequence in the scaffold 134 of genome.

In this study, the *PxPPO1* was a fluctuant expression during *P. xylostella* ontogenesis, which was similar to that of the expression of *PxPPO2*.[16] In *Apis mellifera*, the PPO transcriptional levels in adults and older pupae are higher than that of younger pupae and larvae.[24] In *A. gambiae*, six PPO gene expressions have been assessed using sPCR during its development.[25] In *H. cunea*, there are no PPO mRNAs in pupae, although expression has been detected in other developing stages.[26] These genes showed distinct temporal expression profiles, but with uncoordinate expressions. Here, we just compared the expression levels of the *PxPPO1* transcripts in the whole body at different developmental stages; whether *PxPPO1* involves in some responses of *P. xylostella* developmental is still unknown.

In summary, this study presented *PxPPO1* cDNA sequence. Like other lepidopterous PPO1, the protein sequence deduced from cDNA has three conserved regions, including the two copper-binding sites. The *PxPPO1* DNA was 7202 bp, consists of 11 introns and 12 exons. Moreover, the expression of the *PxPPO1* transcripts in the eggs and the fourth instar larvae were more abundant, followed by the second and the third instar larvae in *P. xylostella*. These findings would provide a better understanding of the *PxPPO1* gene.

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Author Contributions

Supervised the project: C-BX. Performed experiments and analyzed the data: M-HJ and X-LZ. Participated in the figures preparation and discussed results: G-YL, X-ZC, and Z-GL. Wrote the manuscript: C-BX, M-HJ, and X-LZ. All the authors reviewed and approved the manuscript.

Supplementary Materials

Supplementary figure 1. Schematic of SP designed for genome walking.

Supplementary figure 2. Schematic diagram of amplified five fragments.

Supplementary figure 3. Multiple sequence alignment of the two copper-binding site (Cu^A and Cu^B) regions of arthropod PPOs.

Supplementary figure 4. Neighbor-joining dendrogram showing the relationship among the known arthropod PPOs using a crustacean (*Scylla serrata*) PPO as an outgroup. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed.

Supplementary figure 5. Gene fragments amplification.

Supplementary figure 6. PCR amplification of gene fragments JY2 (DL5000 marker).

Supplementary table 1. Exons location and length.

Supplementary table 2. Exons–introns junction sequences in *P. xylostella*.

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