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Gene cloning and expression patterns of two prophenoloxidases from *Catantops pinguis* (Orthoptera: Catantopidae)

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Abstract

In insect, fat body plays major roles in insect innate immunity. Phenoloxidase (PO) is an important component in insect innate immunity and is necessary for acclimatization. In our study, two prophenoloxidase (PPO) subunits were obtained from fat body of *Catantops pinguis* (Stål). The full-length cDNA sequence of one PPO (*CpPPO*1) consisted of 2347 bp with an open reading frame (ORF) of 2187 bp encoding 728 amino acids, while the other subunit (*CpPPO*2) had a full length of 2445 bp, encoding 691 amino acids. Both the *PPO* gene products are predicted to possess all the structural features of other PPO members, including two putative tyrosinase copper-binding motifs with six highly conserved histidine residues and a thiolester-like motif. Tissue distribution analysis showed that both *PPO* mRNAs were abundantly expressed in the fat body among 11 tissues examined, and they were transiently up-regulated after *Escherichia coli* infection, consistent with them being immune-responsive genes. Total levels of *CpPPO*1 and *CpPPO*2 mRNA transcripts were much higher in first instar larvae and adults. A much higher transcript level of *CpPPO*1 was detected in several months, while there were extremely high mRNA expression levels of *CpPPO*2 in January, July, October, and December. The above results suggested that PPO from fat body might also bring significant function during the processes of development and acclimatization for *C. pinguis*.

Keywords: prophenoloxidase, cloning, expression pattern, *Catantops pinguis*

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Introduction

Lacking an adaptive immune system like that of vertebrates, insects rely exclusively on their effective innate immune response system to eliminate invading pathogens (Eleftherianos & Revenis, 2011). Fat body as well as hemolymph are important tissues in insect that are involved in defense responses against pathogens (Bao *et al.*, 2010). Most of hemolymph proteins are synthesized in fat body, like antimicrobial peptides (AMP) and other humoral response molecules essential for insects' immunity (Ferrandon *et al.*, 2007; Arrese & Soulages, 2010; Bao *et al.*, 2010; Tian *et al.*, 2010).

Melanization, involving the rapid synthesis and deposition of melanin at a site of infection or injury, is an important component of the innate immune response in insects (Cerenius *et al.*, 2008; Eleftherianos & Revenis, 2011). The melanin can physically shield an intruder and therefore efficiently restrain its growth, and it seems more importantly that highly reactive and toxic quinone substances and other short-lived reaction intermediates are also produced during melanin formation that kill intruding pathogens (Cerenius & Söderhäll, 2004; Nappi & Christensen, 2005; Jiravanichpaisal *et al.*, 2006;

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Table 1. Primer sequences used for amplification of CpPPO1 and CpPPO2.

Primer	Direction[1]	Type[2]	Sequence[3] (5′–3′)	Purpose
CpPPO1F	F	D	5′-CCTBCAYCAYTGGCAYTGGCA-3′	RT–PCR
CpPPO1R	R	D	5′-GGCCABCCRCANCCRCAG-3′	RT–PCR
CpPPO2F	F	D	5′-CACCAYTRKCAYTGGCA-3′	RT–PCR
CpPPO2R	R	D	5′-GGCCANCCRCANYYRCAG-3′	RT–PCR
CpPPO1RA	R	G	5′-GCTTCATAGATGCGGTCTCTCC-3′	5′-RACE
CpPPO1RB	R	G	5′-GTCCCGTAGAGTTGTAAATGCA-3′	5′-RACE
CpPPO1FA	F	G	5′-CTCCAAGAGCAGGAACTGTCAG-3′	3′-RACE
CpPPO1FB	F	G	5′-TGCGAGACCAGAGATTGCTC-3′	3′-RACE
CpPPO2RA	R	G	5′-CCTTGATTGGCTCGTATAAACTC-3′	5′-RACE
CpPPO2RB	R	G	5′-CTCCTCTGCGATCTTTCCTCAC-3′	5′-RACE
CpPPO2FA	F	G	5′-CAGCGCAAATCAACGGAGTC-3′	3′-RACE
CpPPO2FB	F	G	5′-TCCGTGATCTCACAACAAACC-3′	3′-RACE
CpPPO1PF	F	G	5′-CCAAGAGCACAAGAAAGTTATTCC-3′	Probe
CpPPO1PR	R	G	5′-GTCAAAAGGGTAGCCCATGC-3′	Probe
CpPPO2PF	F	G	5′-CGACTGCCTTTGGATTATACTGC-3′	Probe
CpPPO2PR	R	G	5′-TCCAAGTGTCTGTTATCAGGGTC-3′	Probe
CpActinPF	F	G	5′- CTCAACCCGAAGGCTAACC-3′	Probe
CpActinPR	R	G	5′-TGGAGTTGTACACGGTCTCG-3′	Probe

[1] F: forward, R: reverse.
[2] D: degenerate, G: gene-specific primer.
[3] *B*: T/C/G; *K*: G/T; *S*: C/G; *Y*: C/T; *M*: A/C; *V*: A/C/G; *N*: A/C/T/G; *R*: A/G; *W*: A/T; *D*: A/T/G.

Cerenius *et al.*, 2008). Phenoloxidase (PO) is a key enzyme in melanin biosynthesis, which catalyzes the oxidation of monophenols and *o*-diphenols to quinones, leading to the formation of melanin (Söderhäll & Cerenius, 1998; Nappi & Ottaviani, 2000; Sugumaran, 2002; Cerenius & Söderhäll, 2004; Christensen *et al.*, 2005; Eleftherianos & Revenis, 2011).

Generally, PO is present as a zymogen, prophenoloxidase (PPO). The PPO cascade is activated after limited proteolysis, once it is triggered by certain microbial cell wall components, such as peptidoglycans, lipopolysaccharides, and β-1,3-glucans (Ashida & Brey, 1998; Cerenius *et al.*, 2008). Previous researches have shown that, besides in hemocytes, PPO mRNA is also transcribed in fat body (Cui *et al.*, 2000; Shelby& Popham, 2008; Feng *et al.*, 2011). In arthropods, the number of PPO genes varies among different species and several PPO genes have been cloned and used to further investigate gene function (Cerenius *et al.*, 2003; Tang *et al.*, 2006; Liu *et al.*, 2007; Amparyup *et al.*, 2009). In most crustaceans, only one PPO gene has been reported, while in insects, there are multiple PPO subunits (Jiang *et al.*, 1997; Asano & Ashida, 2001; Sezaki *et al.*, 2001; Taft *et al.*, 2001; Christophides *et al.*, 2004; Kim *et al.*, 2005; Doucet *et al.*, 2008; Li *et al.*, 2009). However, there is even little attention devoted to PPO from orthopteran, let alone the PPO expression patterns from fat body of orthopteran.

Catantops pinguis (Stål) are worldwide pests in agriculture. They have spread in temperate zones and subtropical zones, and are common in Africa and south and east Asia. They bring about considerable damage to crops, such as Gramineae, Leguminosae, and Compositae, because of their complex feeding habits. In the present study, two *PPO* cDNA sequences from fat body of *C. pinguis* were cloned and sequenced. The two *PPOs* were characterized in terms of their tissue distribution and expression patterns in response to infection by *Escherichia coli* strain DH 5α. Furthermore, *PPO* mRNA transcripts from fat body in different developmental stages and different months were detected. These results suggest that *PPO* is essential for locust survival in response to challenge by *E. coli* and for its growth.

Materials and methods

Insect rearing and tissue isolation

C. pinguis were obtained from the tea plantation of Hangzhou in Zhejiang province, China. They were reared at 25 ± 1°C, under a photoperiod of 14/10 h (light/dark) and 65–70% relative humidity in an artificial climatic chamber. Locusts were kept in a group of 200 individuals in each large cage (55 × 55 × 55 cm), and fed daily with fresh wheat seedlings supplemented with bran. The larval instars were determined by molting.

About 600 eggs of *C. pinguis* per batch were used for hatching. Fat bodies were dissected from different developmental stages in a sterile operation. Eleven tissues including fat body were isolated from other adults for tissue analysis. Wild-caught adults at the beginning, middle, and end of each month were also used for fat bodies dissections. All tissue samples were stored at −80°C until further use.

RNA isolation, cDNA synthesis, and polymerase chain reaction (PCR)

Total RNA was extracted from the fat body using the Trizol reagent (Invitrogen), and contaminating genomic DNA was eliminated with RNase-free DNase (Promega). The purified mRNA was used to synthesize first-strand cDNA using AMV reverse transcriptase (Takara) according to the manufacturer's protocol. First-strand cDNA was generated in a 25-μl reaction volume containing 1 μg total RNA, 2 pM oligo(dT_{18}), 20 U RNase inhibitor, and 5 U AMV reverse transcriptase.

Four degenerate primers (table 1) for PCR were designed based on the highly conserved sequences of the conserved regions of entomic PPO1 and PPO2 gene sequences, respectively, from *Tribolium castaneum* (NM_001039404, NM_001039433), *Tenebrio molitor* (AB020738), *Anopheles gambiae* (XM_312089, XM_316323), *Aedes aegypti* (XM_001648918, AF292113), *Drosophila melanogaster* (AB055857), *Bombyx mori* (NM_001043870, NM_001044069), *Apis mellifera*

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(NM_001011627), *Holotrichia diomphalia* (AB079665), and *Manduca sexta* (O44249) (http://www.ncbi. nlm.nih.gov) using the Primer 5 software. PCR amplification of the target cDNA fragments was performed in reaction mixtures containing 1 µl of the fat body cDNA, 10 pmol of forward and reverse primers, 1 U of Platinum Taq High Fidelity DNA polymerase, 10 × high-fidelity PCR buffer, and sterile H_2O to a final volume of 25 µl. The PCRs were performed as follows: an initial denaturation step at 94°C for 5 min, followed by three cycles of denaturation at 94°C for 30 s, annealing at 45°C for 30 s, and elongation at 72°C for 90 s, and then 28 cycles of denaturation at 94°C for 30 s, annealing at 48°C for 30 s, and elongation at 72°C for 90 s, followed by a 10 min extension at 72°C and cooling to 12°C. PCR products were analyzed by 1.0% agarose gel electrophoresis and each fragment corresponding to the expected size of approximately 1100 bp was purified and subcloned into the pMD18-T simple vector (Takara). After being transformed into *E. coli* DH 5α competent cells, positive clones were sequenced, and partial cDNA sequences of *CpPPO*1 and *CpPPO*2 were obtained using the pair of degenerate primers.

Rapid amplification of cDNA ends (RACE)

To obtain 5′- and 3′-end sequences, 1 µg of purified total RNA from the fat body was used for the preparation of 3′-RACE-Ready and 5′-RACE-Ready cDNA templates using the BD SMART RACE cDNA Amplification Kit (Clontech, USA) according to the manufacturer's protocol. Two internal gene-specific primers for each 3′-RACE and 5′-RACE PCR (table 1) were designed according to the gene segments obtained. PCR mixtures were used and the primary PCR amplification profiles were similar to the program described before, while the annealing temperature was set at 55°C. After agarose gel electrophoresis, the RACE products were purified and cloned into the pMD18-T vector (Takara). Positive colonies were selected and sequenced completely.

Sequences analysis and phylogenetic tree construction

Similarity searches of cDNA and deduced amino acid sequences were performed using the BLASTN and BLASTP programs (http://www.ncbi.m.nih.gov/blast). Protein sequences analyses (such as putative signal peptide and *N*-glycosylation sites) were performed with various online software, such as SignalP3.0 Server and NetNGlyc (http://www.cbs.dtu.dk/services/). A multiple-sequence alignment was performed using the ClustalW program (Vector NTI 11.0, Invitrogen) and phylogenetic analysis was performed using maximum likelihood method analysis with the Mega 5 software.

Tissue distribution analysis

Total RNA was isolated from the fat body, epidermis, muscle, Malpighian tubule, heart, ovary, testis, trachea, midgut, hindgut, and brain. After eliminating contaminated DNA, total RNA was used for cDNA synthesis.

Expression patterns of *CpPPO*1 and *CpPPO*2 were analyzed by semi-quantitative using reverse transcription–PCR (RT–PCR) combined with oligonucleotide probe hybridization. Gene-specific primers for *CpPPO*1 and *CpPPO*2 (CpPPO1-PF, CpPPO1-PR, CpPPO2-PF, and CpPPO2-PR; table 1) were designed. β-Actin was used as an internal control. PCRs were performed as described above. The PCR products were subsequently blotted onto Hybond-N^+ membranes (Amersham, UK) with downward capillary action in 20 × SSC transfer buffer (3 M NaCl, 0.3 M $NaC_6H_5O_7{\cdot}2H_2O$, pH 7.0) overnight. Suitable cDNA fragments (*CpPPO*1, 700 bp; *CpPPO*2, 614 bp) (fig. 1a, b) were labeled with DIG-High Prime and used as probes. Membranes were pre-hybridized at 42°C for 0.5 h and incubated in the DIG-high prime-labeled probes for 6 h in hybridization buffer containing 50% formamide, 5 × Denhardt's solution, and 0.1% SDS. After hybridization, the membranes were washed in 2 × SSC and 0.5 × SSC twice. Then, the hybridized probes were immunodetected with anti-digoxigenin-AP Fab fragments and visualized with the colorimetric substrates NBT/BCIP.

Bacteria challenge and expression profiles of CpPPO1 and CpPPO2

The insect pathogenic bacterium *E. coli* strain DH 5α was cultured on an Amp^- LB plate and incubated at 37°C overnight. A single colony was picked and placed in LB media and incubated overnight in a shaking incubator at 37°C and 254 rpm. Bacterial cultures were then centrifuged (5000 rpm, 4°C) and the pellets were resuspended in 0.2 M PBS buffer Phosphate-Buffered Saline. Bacterial density in the PBS buffer was adjusted to obtain an optical density (OD) of 0.6. Five-day-old adults were chosen for immune challenging analysis by *E. coli*. Each was injected with 2 µl bacterial suspension. Ten locusts from each treatment were sampled at 0, 6, 12, 24, 36, and 48 h after the immune challenge began. Total RNA from the fat body dissected from the samples was isolated and cDNA was synthesized. A semi-quantitative determination of *CpPPO*1 and *CpPPO*2 was carried out as described above.

Temporal expression analysis of CpPPO1 and CpPPO2 transcripts

Total RNA extracted from fat bodies in different stages, including the first, second, third, fourth, and fifth instar larvae and adults, and fat bodies from adults caught in different months were prepared for cDNA synthesis. Temporal expression levels of *CpPPO*1 and *CpPPO*2 transcripts were assessed by semi-quantitative detection, as described above.

Results

Characterization of PPO1 and PPO2 from C. pinguis

Two distinct homologous PPO genes of *C. pinguis* were cloned using RT–PCR and RACE. The full-length cDNA of *CpPPO*1 is 2347 bp, including an open reading frame (ORF) of 2187 bp encoding a predicted protein of 728 amino acid residues with a predicted molecular mass of approximately 84.26 kDa and a theoretical pI of 6.91. Translation initiation occurred at an ATG codon, while termination occurred at a TGA codon. A single polyadenylation signal was located in the 3′-UTR, beginning at 2358 bp. Possible *N*-glycosylation sites were predicted at positions 25 and 595 (fig. 1a). The complete sequence of *CpPPO*2 was 2445 bp, including an ORF of 2076 bp encoding a predicted protein of 691 amino acid residues with a predicted molecular mass of approximately 79.66 kDa and a theoretical pI of 6.27. Translation initiation occurred at an ATG codon, while termination occurred at a

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Fig. 1. Continued

TAA codon. A single polyadenylation signal was located in the 3′-UTR, beginning at 2453 bp. Possible *N*-glycosylation sites were predicted at positions 83, 293, 463, 543, and 660 (fig. 1b). According to the Signal P3.0 Server on line, the N-terminus of deduced PPO protein assumes the characters as a pupative signal peptide including 21 amino acid residues (fig. 1a), but no sufficient data to support the result at present.

Multiple sequence alignment and phylogenetic analysis

Alignment of PPO proteins revealed that both CpPPO protein sequences contained three highly conserved features: two highly conserved copper-binding regions, six histidine residues, and a thiol-ester motif (fig. 2). For CpPPO1, six conserved histidine residues, which are likely to bind two copper ions, were present at conserved sites within the two copper-binding sites: copper A (H243, H247, and H272) and copper B (H399, H403, and H439) (fig. 1a). For CpPPO2, six conserved histidine residues were present at the two copper-binding regions: copper A (H212, H216, and H241) and copper B (H369, H373, and H409) (fig. 1b). The highly conserved thiol ester motif (GCGWPQHM) was present in both CpPPO1 and CpPPO2 at Gly612 to Met619 and Gly584 to Met591 (fig. 1a, b), respectively. A BLASTX analysis showed that the two proteins exhibited high amino acid sequence identity (50–89%, 50–93%, respectively) with those of other insect species (tables 2 and 3).

The evolutionary relationship among CpPPO1 and CpPPO2 amino acid sequences with other arthropod PPOs was assessed by the maximum likelihood method, using Mega 5 (fig. 3). There are 73 PPO sequences in the tree that form two different groups including the crustacean PPOs and the insect PPOs. The insect PPOs group can also be separated into five distinct clades: diptera PPO, coleopteran PPO, Lepidoptera PPO, hymenoptera PPO, and orthoptera PPO. According to the dendogram, CpPPO1 and CpPPO2 branched within the clade of PPOs of orthoptera, and separately from other insect orders. Within orthoptera, only two PPO gene sequences were obtained for each insect species. CpPPO1 and CpPPO2 showed exceptionally high homology with LmPPO1 and LmPPO2, respectively. The phylogenetic analysis also showed

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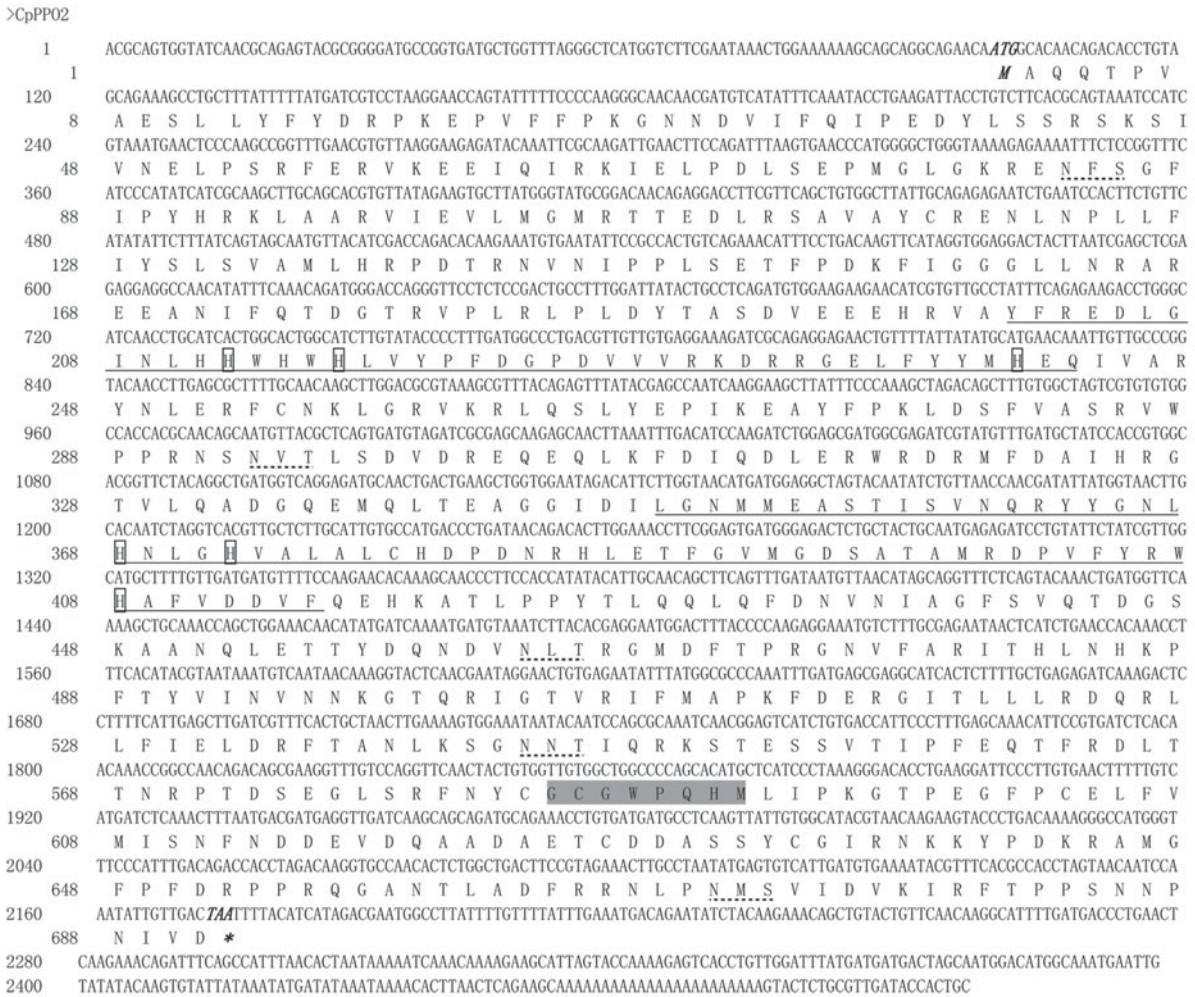

Fig. 1. Nucleotide and deduced amino acid sequences of *CpPPO*. (a) Nucleotide and deduced amino acid sequences of *CpPPO*1. (b) Nucleotide and deduced amino acid sequences of *CpPPO*2. Translation initiation and termination sites are indicated in bold and italic font. The putative conserved copper-binding regions are underlined and the conserved histidine residues are boxed. The completely conserved thiol ester motif region (GCGWPQHM) is indicated in grey shading. The *N*-glycosylation sites are indicated with a dotted curve. Polyadenylation signal sites are indicated. Two allele-specific primers are marked by arrowheads. An arrow indicates a possible cleavage site for signal peptidase and the signal peptide is double underlined.

that PPOs from hemiptera were more closely related to PPOs from orthoptera than from coleoptera (fig. 3).

Expression analysis of CpPPO1 and CpPPO2 in different tissues

Total RNA was extracted from the fat body, epidermis, muscle, Malpighian tubule, heart, ovary, testis, trachea, mid-gut, hindgut, and brain of adults. The expression patterns of *CpPPO*1 and *CpPPO*2 were assessed by semi-quantitative analysis (fig. 4a). The *CpPPO*1 mRNA transcript was expressed in all the examined tissues except the epidermis, muscle, ovary, and brain, and the highest expression level was detected in the fat body. The *CpPPO*2 mRNA was transcribed in all the examined tissues except the ovary and a high expression level was detected in the fat body, while a very low level was observed in the mid-gut. In comparison, a higher expression level of *CpPPO*2 was observed in the same tissue.

Bacterial challenge and expression profiles of CpPPO1 and CpPPO2

To understand the influence of *E. coli* on locust immunity, expression levels of *CpPPO*1 and *CpPPO*2 mRNA were determined from 0 (untreated, CK), 6, 12, 24, 36, and 48 h after *E. coli* injection in adults (fig. 4b). The expression level of *CpPPO*1 was low at 0, 6, and 12 h after *E. coli* injection, then significantly up-regulated at 24 h, and total two peaks of *CpPPO*1 transcripts at 24 and 48 h after injection were observed. The transcript level of *CpPPO*2 mRNA was significantly increased at 24 and 48 h after *E. coli* injection, while a slight decrease was seen at 36 h after treatment.

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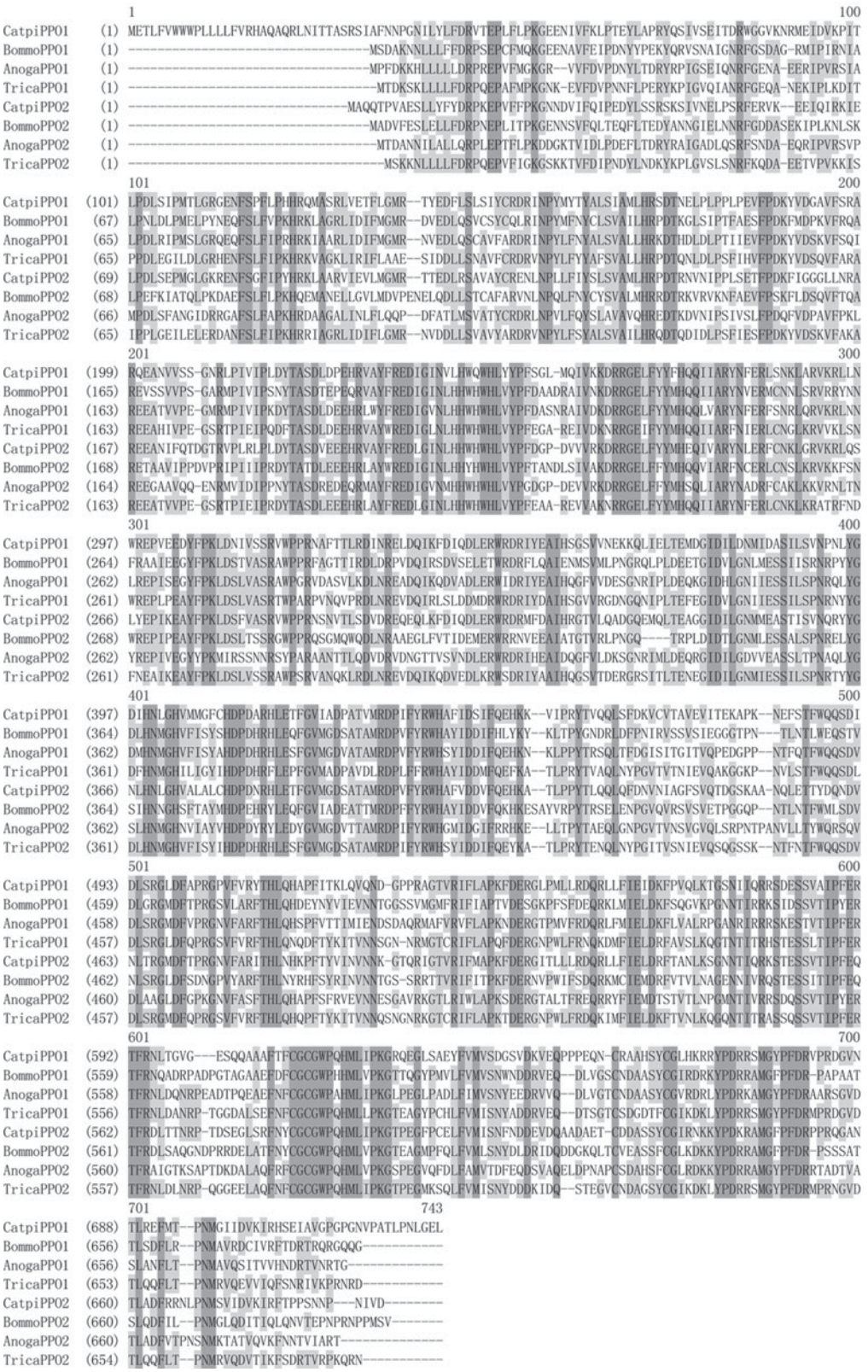

Fig. 2. Multiple amino acid sequence alignment of CpPPOs with those of other insects. Alignments of CatpiPPO1, CatpiPPO2 (FJ598047), AnogaPPO1 (XM_312089), SarbuPPO1 (AF161260), TricaPPO1 (NM_001039404), BommoPPO1 (NM_001043870), ChofuPPO1 (EU046570), PieraPPO1 (HM776513), AnogaPPO2 (XM_316323), SarbuPPO2 (AF161261), BommoPPO2 (NM_001044069), TricaPPO2 (NM_001039433), ChofuPPO2 (EU046573), CamflPPO (GL435066), ApimePPOA3 (NM_001011627) and PieraPPO2 (HM235814) proteins were performed using the Vector NTI 11.0 software.

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Table 2. The related information of PPO gene family and the identity (%) to CpPPO1.

Gene family	Species	Number of amino acid	GenBank number	Identity (%) to CpPPO1
PPO1	*Catantops pinguis*	728		–
PPO1	*Locusta migratoria*	697	FJ771025	89
PPOCG8193	*Harpegnathos saltator*	695	GL446009	59
PPOA3	*Acyrthosiphon pisum*	703	XM_001949272	59
PPOA3	*Apis mellifera*	693	NM_001011627	59
PPO	*Musca domestica*	684	AY494738	59
PPO1	*Anopheles gambiae*	683	XM_312089	59
PPO	*Culex quinquefasciatus*	681	XM_001848913	59
PPO1	*Sarcophag bullata*	685	AF161260	58
PPO	*Aedes aegypti*	681	XM_001648918	58
PPO2	*Catantops pinguis*	691	FJ598047	57
PPO2	*Locusta migratoria*	691	FJ771024	57
PPO2	*Tribolium castaneum*	683	NM_001039433	57
PPO	*Tenebrio molitor*	684	AB020738	57
PPO1	*Tribolium castaneum*	682	NM_001039404	57
PPOCG8193	*Camponotus floridanus*	713	GL435066	56
PPO2	*Holotrichia diomphalia*	684	AB079665	54
PPOA3	*Drosophila melanogaster*	670	AB055857	53
PPO	*Galleria mellonella*	683	AF336289	52
PPO1	*Heliothis virescens*	684	DQ841706	52
PPO	*Plodia interpunctella*	681	AY665397	52
PPO1	*Plutella xylostella*	682	GU214206	51
PPO1	*Spodoptera frugiperda*	684	DQ289581	51
PPO1	*Bombyx mori*	685	NM_001043870	51
PPO1	*Choristoneura fumiferana*	684	EU046570	51
PPO1	*Bombyx mandarina*	685	EU569724	51
PPO1	*Holotrichia diomphalia*	684	AB079664	50
PPO1	*Manduca sexta*	685	AF003253	50
PPO1	*Pieris rapae*	682	HM776513	50
PPO	*Hyphantria cunea*	681	HCU86875	50
PPO2	*Plutella xylostella*	696	GU214207	50

Temporal expression analysis of CpPPO1 and CpPPO2 transcripts

The developmental expression profiles of *CpPPO*1 and *CpPPO*2 were investigated. Both *CpPPO*1 and *CpPPO*2 mRNAs were constitutively transcribed during different developmental stages, from the first instar nymphae (fig. 4c). High transcript levels of *CpPPO*1 mRNA were measured in the first instar and the earlier stages of second instar larvae, while the transcript was undetectable at day 6 of second instar larvae, and days 2 and 5 of fourth instar larvae. Then, the *CpPPO*1 mRNA transcript level increased significantly at day 8 of fourth instar larvae and stayed high in fifth instar larvae before their molt. The *CpPPO*1 mRNA transcript level was significantly decreased in day 1 adults and remained at a low level. The *CpPPO*2 mRNA was persistently transcribed during all the developmental stages examined. High expression levels of *CpPPO*2 were detected in the earlier stages of first, second, third, and fourth instar larvae, the later stages of fifth instar larvae and adults. However, the transcript level of *CpPPO*2 mRNA was extremely low at day 8 of fourth instar larvae.

As *C. pinguis* are overwintering insects, we also investigated the expression profiles of *CpPPO*1 and *CpPPO*2 in different months of the year (fig. 4d). A high transcript level of *CpPPO*1 was detected in a couple of months from January to July along with December, relatively, except in early April. Expression of *CpPPO*1 mRNA decreased from August and remained low until later November, and an extremely low level was detected in early November. *CpPPO*2 mRNA was abundantly expressed during the whole year, with extremely high levels in January, early May, late July, late October, and December.

Discussion

The fat body is the most prominent organ in insects. It plays major roles in the intermediary metabolism, in the energy storage, in the innate immunity and is responsible for the synthesis and supply of hemolymph compounds (Antonova *et al.*, 2009; Cardoso *et al.*, 2010; Martins *et al.*, 2011; Roy & Raikhel, 2011). The fat body can regulate the concentration in the hemolymph substances and participate in insect homeostasis (Haunerland & Shirk, 1995; Arrese & Soulages, 2010; Martins *et al.*, 2011).

In the present research, we report two homologous PPO genes from fat body of *C. pinguis* (Stål), which were designated *CpPPO*1 and *CpPPO*2. Like other insect PPO genes, both *CpPPO*1 and *CpPPO*2 contain two putative tyrosinase copper-binding motifs with six highly conserved histidine residues and a highly conserved thiol ester-like motif (GCGWPQHM), whose function has not been reported. Although it was predicted that there is a pupative signal peptide, consisting of 21 amino acids, in *CpPPO*1 by signal P3.0 server, there was no further work to support the result at present, in addition, no hydrophobic signal peptide has been reported in other arthropods to date (Aspan *et al.*, 1995; Fujimoto *et al.*, 1995; Cho *et al.*, 1998; Lee *et al.*, 1998; Cui *et al.*, 2000; Ling & Yu, 2005; Shelby & Popham, 2008; Tsao *et al.*, 2009; Feng *et al.*, 2011). Probably, further researches should be carried out concerning the structure and function of PPO1 protein.

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Table 3. The related information of PPO gene family and the identity (%) to CpPPO2.

Gene family	Species	Number of amino acid	GenBank number	Identity (%) to CpPPO2
PPO2	*Catantops pinguis*	691	FJ598047	–
PPO2	*Locusta migratoria*	691	FJ771024	93
PPOA3	*Apis mellifera*	693	NM_001011627	60
PPO	*Musca domestica*	684	AY494738	59
PPOCG8193	*Harpegnathos saltator*	695	GL446009	59
PPO	*Sarcophag bullata*	685	AF161260	59
PPO2	*Holotrichia diomphalia*	684	AB079665	59
PPO1	*Locusta migratoria*	697	FJ771025	57
PPO2	*Tribolium castaneum*	683	NM_001039433	57
PPOA3	*Acyrthosiphon pisum*	703	XM_001949272	57
PPOCG8193	*Camponotus floridanus*	713	GL435066	57
PPO	*Tenebrio molitor*	684	AB020738	57
PPO	*Aedes aegypti*	681	XM_001648918	56
PPO1	*Anopheles gambiae*	683	XM_312089	56
PPO	*Culex quinquefasciatus*	681	XM_001848913	55
PPO1	*Spodoptera frugiperda*	684	DQ289581	55
PPO1	*Bombyx mori*	685	NM_001043870	55
PPOA3	*Drosophila melanogaster*	670	AB055857	55
PPO1	*Tribolium castaneum*	682	NM_001039404	54
PPO	*Nasonia vitripennis*	994	XM_001606532	54
PPO1	*Choristoneura fumiferana*	684	EU046570	54
PPO1	*Bombyx mandarina*	685	EU569724	54
PPO	*Plodia interpunctella*	681	AY665397	54
PPO1	*Heliothis virescens*	684	DQ841706	54
PPO1	*Manduca sexta*	685	AF003253	54
PPO1	*Pieris rapae*	682	HM776513	54
PPO	*Galleria mellonella*	683	AF336289	53
PPO1	*Plutella xylostella*	682	GU214206	53
PPO1	*Holotrichia diomphalia*	684	AB079664	53
PPO	*Hyphantria cunea*	681	HCU86875	52
PPO2	*Plutella xylostella*	696	GU214207	52
PPO2	*Bombyx mori*	693	NM_001044069	51
PPO2	*Bombyx mandarina*	693	EU047703	51
PPO2	*Galleria mellonella*	692	AY371489	51
PPO2	*Bombyx mori*	693	AF178461	51
PPO1	*Sarcophag bullata*	685	AF161260	50
PPO2	*Spodoptera exigua*	693	EF684939	50
PPO2	*Heliothis virescens*	696	EF044308	50
PPO2	*Helicoverpa armigera*	698	DQ114946	50
PPO2	*Biston betularia*	691	GU953227	50
PPO	*Ostrinia furnacalis*	692	DQ333883	50
PPO2	*Pieris rapae*	691	HM235814	50

Multiple alignments of the deduced proteins of the two genes *CpPPO*1 and *CpPPO*2 showed a high relevant identity of 89% and 93% with LmPPO1 (FJ771025) (table 2) and LmPPO2 (FJ771024) (table 3), but they shared less identity, 57%, with each other. Previous studies have revealed that the PPOs from one species are less homologous to each other than to PPOs from other species, suggesting that duplication and divergence of the *PPO* genes occurred prior to the emergence of these distinct insect species (Cui *et al*., 2000). Similarly, *PPO*1 and the ancestor of other *PPO*s in *A. gambiae* may have duplicated and diverged before the separation of Diptera and Lepidoptera (Müller *et al*., 1999).

Previous studies have shown that, besides in hemolymph, *PPOs* are also transcribed in insect fat body and other tissues. Our study showed that both *CpPPO*1 and *CpPPO*2 transcripts are also abundantly expressed in the fat body. *CpPPO*1 and *CpPPO*2 mRNA were also transcribed in other tissues, including the Malpighian tubule, heart, testis, trachea, midgut, and hindgut. A knockdown experiment with the *Drosophila* melanization proteases (MP1 and MP2) showed that melanization is crucial for the innate immune response against bacterial and fungal infections (Tang *et al*., 2006). In *Pacifastacus leniusculus*, RNA interference-mediated depletion of PPO leads to increased bacterial growth, lower phagocytosis, lower PO activity, lower nodule formation, and higher mortality when infected with a highly pathogenic *bacterium, Aeromonas hydrophila*. However, RNA interference of the aninhibitor of PPO results in lower bacterial growth, increased phagocytosis, increased nodule formation, higher PO activity, and delayed mortality (Liu *et al*., 2007). These studies further demonstrate that the PPO-activating system plays an essential role in invertebrate defense against diverse pathogens (Cerenius & Söderhäll, 2004). In our study, *CpPPO*1 and *CpPPO*2 transcript levels are transiently and significantly influenced after *E. coli* infection. However, apparently contradictory results have also been reported: studies on *Drosophila* and *A. gambiae* revealed that PO activity was redundant in response to several bacterial and fungal pathogens (Leclerc *et al*., 2006; Schnitger *et al*., 2007).

Insect fat body has been demonstrated to synthesize various AMPs and PPO mRNA transcript was also detected

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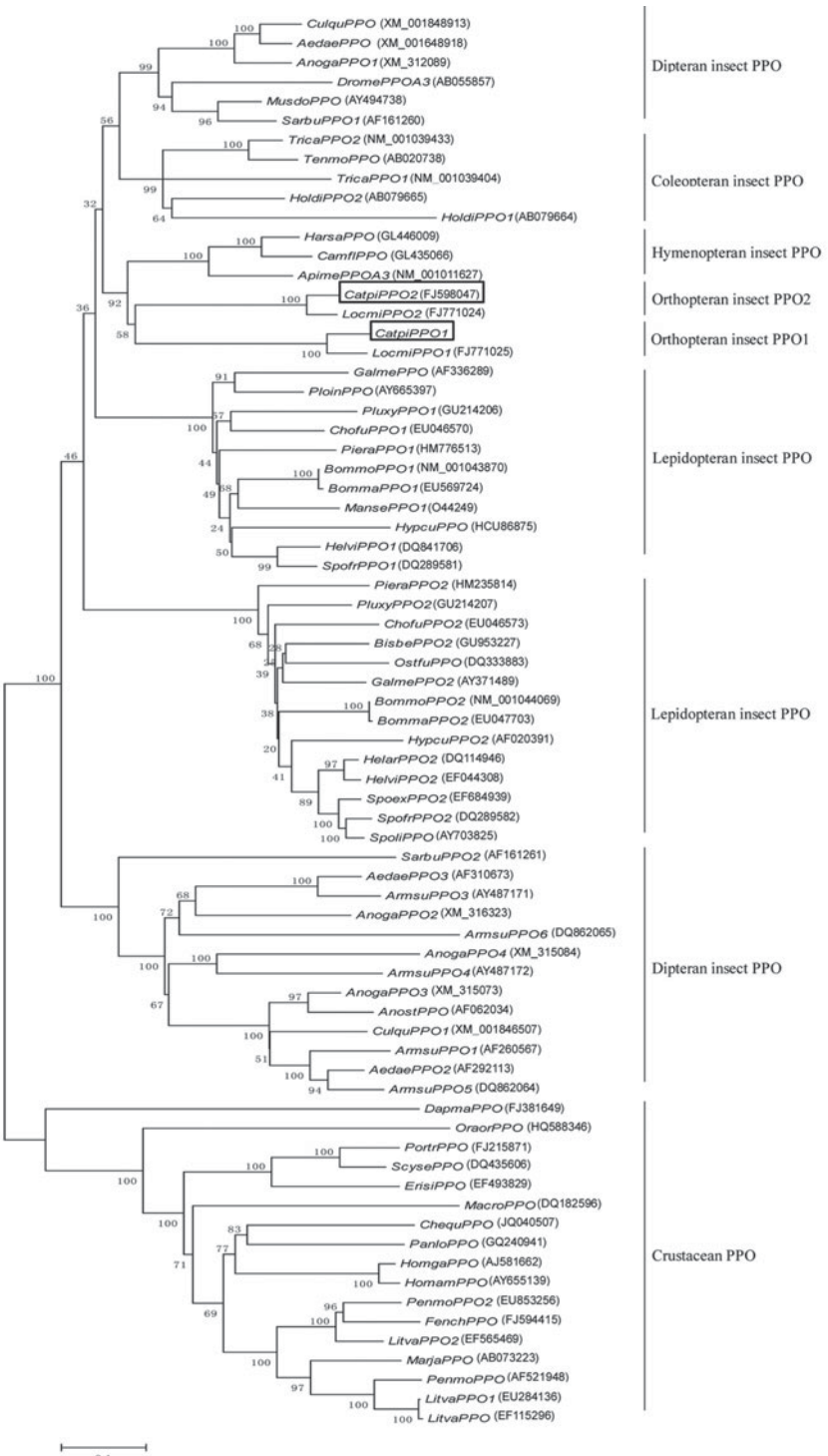

Fig. 3. Phylogenetic relationships of CpPPO1 and CpPPO2 with PPOs from other insects. The phylogenetic tree was constructed on the basis of known amino acid sequences using a maximum likelihood method analysis and the Mega 5 software. A bootstrap analysis was carried out and the robustness of each cluster was verified using 100 replicates. Values at the cluster branches indicate the results of the bootstrap analysis. Insect PPOs: *Locusta migratoria* (*LocmiPPO*1: FJ771025; *LocmiPPO*2: FJ771024); *Catantops pinguis* (*CatpiPPO*1; *CatpiPPO*2: FJ598047); *Bombyx mori* (*BommoPPO*1: NM_001043870; *BommoPPO*2: NM_001044069); *Bombyx mandarina* (*BommaPPO*1: EU569724; *BommaPPO*2: EU047703); *M. sexta* (*MansePPO*: O44249); *H. cunea* (*HypcuPPO*: HCU86875; *HypcuPPO*2: AF020391); *Heliothis virescens* (*HelviPPO*1: DQ841706; *HelviPPO*2: EF044308); *Spodoptera frugiperda* (*SpofrPPO*1: DQ289581; *SpofrPPO*2: DQ289582); *Pieris rapae* (*PieraPPO*1: HM776513; *PieraPPO*2: HM235814); *Plutella xylostella* (*PluxyPPO*1: GU214206; *PluxyPPO*2: GU214207); *Choristoneura fumiferana* (*ChofuPPO*1: EU046570;

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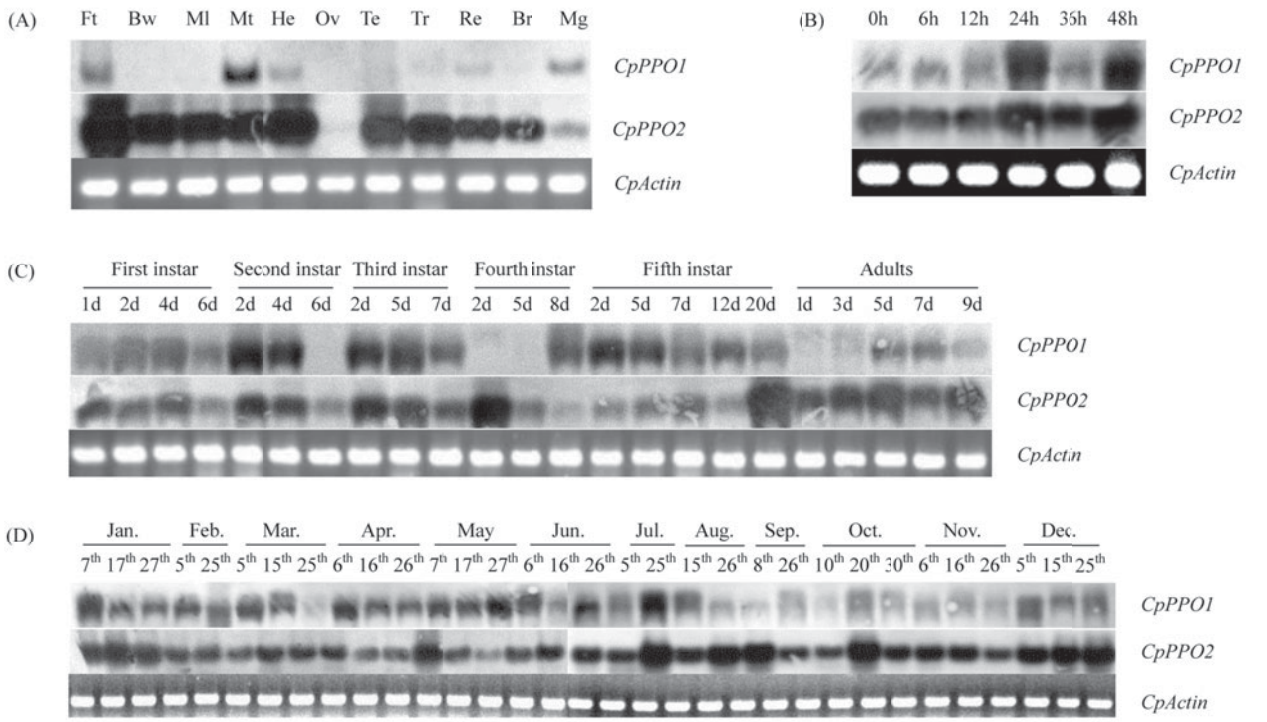

Fig. 4. Expression patterns analysis of *CpPPO*1 and *CpPPO*2 by semi-quantitative RT–PCR combined with oligonucleotide probe hybridization. (a) Tissue distribution analysis of *CpPPO*1 and *CpPPO*2: fat body (Fb), epidermis (Ep), muscle (Mu), Malpighian tubule (Mt), heart (H), ovary (O), testis (Te), trachea (Tr), midgut (Mg), hindgut (Hg), and brain (Br). β-Actin was used as a control housekeeping gene to indicate and standardize the amount of cDNA template in each RT–PCR. Specific probes were labeled with digoxin and used for hybridization and detection of target gene fragments. (b) Effect of bacterial challenge on expression of *CpPPO*1 and *CpPPO*2 transcripts. Fat bodies were collected from adult *C. pinguis* at 0, 6, 12, 24, 36, and 48 h after infection by *E. coli*. (c) *CpPPO*1 and *CpPPO*2 transcripts in different developmental stages. Fat bodies were collected from 1st instar larvae to adults of *C. pinguis*. (d) *CpPPO*1 and *CpPPO*2 mRNA expression patterns in *C. pinguis* fat bodies collected from different months. Fat bodies were collected from adults from January to December.

in it. However, there is little report involving PPO expression pattern in fat body.

For many insect species, there are several distinct PPO genes, and each may have different physiological functions or cooperative interactions (Jiang *et al.*, 1997; Müller *et al.*, 1999; Cerenius & Söderhäll, 2004). It had been indicated that PPO genes in *A. gambiae* are differentially expressed in different developmental stages (embryo to adult) (Müller *et al.*, 1999). *C. pinguis* undergoes several ecdyses, from eggs to the adults, and the interval duration tends to be diverse. Semi-quantitative analysis revealed that both *CpPPO*1 and *CpPPO*2 mRNAs were constitutively transcribed during different developmental stages, from first instar nymphae. High transcript levels of the two *PPO* genes were detected in first instar and second instar larvae, and high expression levels of *PPO*2 were observed in adults. In contrast, high expression levels of *PPO*1 and *PPO*2 mRNA were detected in mid-instar larvae, but not in pupae, adults, or eggs in *Hyphantria cunea* (Park *et al.*, 1997). Intriguingly, this seems to be distinct from the situation in *A. gambiae*, where the *PPO* transcript was

*ChofuPPO*2: EU046573); *Galleria mellonella* (*GalmePPO*: AF336289; *GalmePPO*2: AY371489); *Plodia interpunctella* (*PloinPPO*: AY665397); *Ostrinia furnacalis* (*OstfuPPO*: DQ333883); *Helicoverpa armigera* (*HelarPPO*2: DQ114946); *Spodoptera exigua* (*SpoexPPO*2: EF684939); *Spodoptera litura* (*SpoliPPO*: AY703825); *Biston betularia* (*BisbePPO*2: GU953227); *Culex quinquefasciatus* (*CulquPPO*: XM_001848913; *CulquPPO*1: XM_001846507); *A. aegypti* (*AedaePPO*: XM_001648918; *AedaePPO*2: AF292113; *AedaePPO*3: AF310673); *D. melanogaster* (*DromePPOA*3: AB055857); *A. gambiae* (*AnogaPPO*1: XM_312089; *AnogaPPO*2: XM_316323; *AnogaPPO*3: XM_315073; *AnogaPPO*4: XM_315084); *Musca domestica* (*MusdoPPO*: AY494738); *Armigeres subalbatus* (*ArmsuPPO*1: AF260567; *ArmsuPPO*3: AY487171; *ArmsuPPO*4: AY487172; *ArmsuPPO*5: DQ862064; *ArmsuPPO*6: DQ862065); *Anopheles stephensi* (*AnostPPO*: AF062034); *Sarcophaga bullata* (*SarbuPPO*1: AF161260; *SarbuPPO*2: AF161261); *T. castaneum* (*TricaPPO*1: NM_001039404; *TricaPPO*2: NM_001039433); *T. molitor* (*TenmoPPO*: AB020738); *H. diomphalia* (*HoldiPPO*1: AB079664; *HoldiPPO*2: AB079665); *Harpegnathos saltator* (*HarsaPPO*: GL446009); *Camponotus floridanus* (*CamflPPO*: GL435066); *A. mellifera* (*ApimePPOA*3: NM_001011627). Crustacean PPOs: *Daphnia magna* (*DapmaPPO*: FJ381649); *Oratosquilla oratoria* (*OraorPPO*: HQ588346); *Litopenaeus vannamei* (*LitvaPPO*: EF115296; *LitvaPPO*1: EU284136; *LitvaPPO*2: EF565469); *Marsupenaeus japonicus* (*MarjaPPO*: AB073223); *Cherax quadricarinatus* (*ChequPPO*: JQ040507); *Penaeus monodon* (*PenmoPPO*: AF521948; *PenmoPPO*2: EU853256); *Eriocheir sinensis* (*ErisiPPO*: EF493829); *Portunus trituberculatus* (*PortrPPO*: FJ215871); *Homarus gammarus* (*HomgaPPO*: AJ581662); *Homarus americanus* (*HomamPPO*: AY655139); *Fenneropenaeus chinensis* (*FenchPPO*: FJ594415); *Macrobrachium rosenbergii* (*MacroPPO*: DQ182596); *Scylla serrata* (*ScysePPO*: DQ435606); *Panulirus longipes* (*PanloPPO*: GQ240941).

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highly expressed in eggs, but at a low level in other developmental stages (Lee *et al*., 1998). In contrast, real-time RT–PCR showed higher amounts of *AmPPO* transcripts in adults and older pupae than in younger pupae and larvae, suggesting a function of *AmPPO* in adult exoskeleton melanization and differentiation (Lourença *et al*., 2005). In *B. mori*, there was a gender difference in the developmental changes in *PPO* mRNA level, because the high expression level falls in different stages between females and males (Yamamoto *et al*., 2000). This differentiation of PPO transcription during the developmental stages suggests a role of PPO in insect growth and development.

In the wild, insects often suffer when faced with poor weather conditions (Robb & Forbes, 2006). As wintering insects, the adults of *C. pinguis* were collected in the field to investigate the changing trends of their *PPO* mRNA transcripts and PO activity in different months. Seasonal differences between *PPO*1 and *PPO*2 mRNA transcripts were obvious: high transcript levels of *CpPPO*1 were detected in couple of months from January to July, along with December, except in early April and an extremely low level was detected in the early November, while the *CpPPO*2 mRNA was abundantly expressed in January, early May, late July, late October, and December. The variation of *PPO* mRNA transcripts during different months may be related to changing climate factors, such as temperature and humidity, which can dramatically influence the development and survival of many insects (Abril *et al*., 2010; Sgolastra *et al*., 2010; Tamiru *et al*., 2011). Moreover, food composition is another important factor playing a vital role in insect immunity (Alaux *et al*., 2010; Fellous & Lazzaro, 2010).

In this study, we present evidence supporting an important role for PPO in *C. pinguis* immunity and growth. Interestingly, according to current signal P3.0 server, the deduced CpPPO1 protein sequence contains a pupative signal peptide, which has not been reported in other insects. Clearly, further research is required to determine the structure and function of CpPPO1. Both *CpPPO*1 and *CpPPO*2 are immune-responsive genes, and their expression is tissue-specific. Distinct expression levels of *CpPPO*1 and *CpPPO*2 were detected in locusts from different developmental stages and different months. Further studies are needed to ensure the influence of certain environmental factors individually. Nevertheless, it seems clear that both *CpPPO*1 and *CpPPO*2 are important components of the *C. pinguis* immune defense system.

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