Journal of Environmental Analytical Chemistry

Liu et al., J Environ Anal Chem 2015, 2:3
DOI: 10.4172/2380-2391.1000144

Research article Open Access

# Characterization of Prophenoloxidase in Resisting Adverse Stresses in *Apis cerana cerana*

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## Abstract

Insect prophenoloxidases (PPOs) are a group of important innate immunity proteins and play an important role in melanin synthesis, defending the intruding microorganisms and parasites, healing wound and cuticle pigmentation. However, there is little study about immune-related defense mechanisms in *Apis cerana cerana*. Here we isolated a *PPO* gene from *A. cerana cerana* and evaluated the connection of *AccPPO* to immunity. *AccPPO* has an open reading frame of 2079 bp encoding a 693 amino acid protein. The genomic structure analysis showed that *AccPPO* is similar to that of *AmPPO*. AccPPO is also close to AmPPO in the evolutionary period. *AccPPO* expression could be detected in all the developmental stages of *A. cerana cerana* and was the highest in 15-day postemergence adults. In addition, we also found that *AccPPO* was induced by the infection of *Ascosphaera apis* and various oxidative stresses including 4, 16°C; UV light; and pesticides (acaricide, cyhalothrin, paraquat) treatments. These results revealed that AccPPO may play a critical role in resisting *Ascosphaera apis* and preventing *A. cerana cerana* from oxidative stresses.

**Keywords:** *Apis cerana cerana*; quantitative real-time PCR; *Ascosphaera apis*; Adverse stresses

## Introduction

Insect immunity is composed of cellular and humoral immunity. Cellular immunity is involved in circulating hemocytes that perform phagocytosis (of small bacteria) and encapsulation (around large parasites) [1]. While humoral immunity is induced through immunity proteins in hemolymph [2]. Insects depend on their innate immune system to protect against invasion by pathogens or parasites [3,4]. Insect prophenoloxidases (PPOs) are a group of important innate immunity proteins and are the zymogen of phenoloxidases (POs), which are generated by PPOs when insects are injuried and invaded by microbial or parasite [5]. Activated phenoloxidase (PO) can catalyse the formation of melanin during bacteria and parasite infections and melanin can encase and kill the invading pathogens [2,6]. POs, which catalyse the hydroxylation of monophenol to *o*-diphenol and its oxidation to *o*-quinone and *o*-quinone can generate in a nonenzymatic reaction [7,8], function in melanin synthesis and are necessary for defense against intruding microorganisms and parasites, wound healing and cuticle pigmentation [9].

As the type 3 copper proteins, insect PPOs have a pair of copper atoms in the active site pocket and both of the copper atoms are coordinated by three histidine residues [2,10]. In *Drosophila melanogaster*, three *PPO* genes have been identified: PPO1 (CG5779), PPO2 (CG8193) and PPO3 (CG2952) [11]. PPO1 and PPO2 are primarily expressed in crystal cells while PPO3 is mainly expressed in lamellocytes when *Drosophila melanogaster* was infected by parasite [12-14]. Nine *PPO* genes have been identified in *Anopheles gambiae* [15,16] while ten and three genes in *Aedes aegypti* and *Culex quinquefasciatus*, respectively [17]. In the honey bee *Apis mellifera*, the PPO cDNA was characterized [18].

Knockdown of *PPO4* and other *PPO* transcripts in *Anopheles dirus* increased the prevalence of *Plasmodium yoelii* infection and the intensity of oocyst and abolished the melanization of oocysts [19]. In *Aedes aegypti*, PPO1, PPO3, PPO5, and PPO8 were involved in response to microbial infection [20]. In *Culex pipiens pallens*, *PPOA3* could not only be involved in immune defense, but plays an important role in resistance, suggesting that *PPOA3* might be an insecticide-resistant associated gene [21]. Honey bee could be impacted when it was exposed to pesticides [22], which could also shorten the longevity of worker honey bee [23] and decrease the survival and weight of queen bees [24], and affect colony vitality [25]. There are also studies on *PPO* genes in other species, for example, *PPO* was induced in all studied tissues after the chanllenge of CpG oligodeoxynucleotide (ODN), *Aeromonas hydrophila* and white spot syndrome virus (WSSV) [26].

Although the functions of PPO proteins have been characterized in other species, there is no research regarding the expression of *PPO* gene when the organism is infected by parasite in the honey bee of *A. mellifera* and *A. cerana cerana*. The Chinese honeybee, *A. cerana cerana* is an importane indigenous species that plays an essential role in the balance of regional ecologies and agricultural economic development as the pollinator of flowering plants [27]. In addition, the survival environment is becoming more and more severe to *A. cerana cerana* due to the indiscriminate use of pesticides, infectious diseases and global warming [28]. It is important to characterize the resistant role of PPO protein in response to adverse environments in *A. cerana cerana*. Moreover, we also predicted that PPO might play an important role in resisting oxidative stresses. So we cloned and characterized the immune-related gene, *AccPPO*, from *A. cerana cerana* and analyzed the genomic structure of *AccPPO*. We also evaluated the expression profiles of *AccPPO* during different developmental stages using the quantitative real-time PCR (qRT-PCR) technology. At last, we investigated the transcripts of *AccPPO* in response to the infection of *Ascosphaera apis* and diverse oxidative stresses including 4, 16°C; UV light; and pesticides (acaricide, cyhalothrin, paraquat) treatments. Our results suggested that AccPPO might play an important role in defending the invasion of *Ascosphaera apis*. In addition, our data also first revealed that AccPPO may function in resisting oxidative stresses.

## Materials and Methods

### Insects and treatments

Colonies of *A. cerana cerana* at different developmental stages were collected from the experimental apiary of Shandong Agricultural

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**Received** June 01, 2015; **Accepted** June 23, 2015; **Published** June 29, 2015

**Citation:** Liu F, Yao P, Guo X, Liu Z, Guo X et al. (2015) Characterization of Prophenoloxidase in Resisting Adverse Stresses in *Apis cerana cerana*. J Environ Anal Chem 2: 144. doi:10.4172/2380-2391.1000144

University (Taian, China). The fourth (L4), fifth (L5), and sixth (L6) day instar larvae, white-eyed (Pw), pink-eyed (Pp), brown-eyed (Pb), dark-eyed (Pd) pupae and 1 day post-emergence adults (A1),15 day post-emergence (A15) and 30 day post-emergence adults (A30) were identified according to Yao et al. 2014. The worker honeybees of 15 day post-emergence adults were treated according to Yao et al. [29] with some modifications [30]. The larvae of *A. cerana cerana* were treated with *Ascosphaera apis* for 1, 2 and 3 days, respectively, which was cultured in the larvae food to $1 \times 10^6$ cfu/mL.

### Primers and PCR amplification conditions

The primers and PCR amplification conditions are listed in Tables 1 and 2, respectively.

### RNA extraction, cDNA synthesis and DNA preparation

Total RNA (TansGen Biotech, Beijing, China) was extracted using Trizol reagent and the RNA samples were digested by the RNase-free Dnase I to remove potential genomic DNA. First-strand cDNA was generated by the EasyScript cDNA Synthetic SuperMix (TransGen Biotech, Beijing, China) according to the manufacture's protocol. The EasyPure Genomic DNA Extraction Kit (TransGen Biotech, Beijing, China) was used to isolate the Genomic DNA according to the manufacturer's instructions.

### Identification of AccPPO

Primers PP1/PP2 were designed and synthesized (Biosune Biotechnological Company, Shanghai, China) based on the conserved region of *PPO* genes from other species to gain the internal region of *AccPPO* cDNA. Using the sequence of obtained fragment, the 3' rapid amplification of cDNA ends (RACE) of *AccPPO* was performed using primers 3P1R1/3P1R2. Then the partial cDNA sequence of *AccPPO* was obtained using primers QP1/QP2.

### Amplification of the genomic sequence of AccPPO

To obtain the genomic DNA sequence of *AccPPO*, primers N1/N2 were designed based on the obtained cDNA sequence of *AccPPO* and synthesized using the genomic DNA as the template. The PCR products were purified, cloned into pEASY-T3 vectors (TransGen Biotech, Beijing, China), and then transformed into competent *Escherichia coli* (*E. coli*) DH5α cells for sequence analysis.

### Bioinformatic analysis of AccPPO

The procedures to conduct bioinformatic analysis of AccPPO were performed as previously described [31] with some modifications.

### Transcriptional profiling by qRT-PCR

qRT-PCR was used to determine the expression level of *AccPPO* and primers TP1/TP2 were employed to amplify the a 137 bp fragment of *AccPPO*. qRT-PCR was performed in 25 μL reaction volume with the SYBR PrimeScript™ RT-PCR Kit (TaKaRa, Dalian, China) and a CFX96TM real-time PCR detection system (Bio-Rad). The amplification reaction protocol was performed as previous described. All samples were run in triplicate. The relative expression levels of *AccPPO* transcripts were determined using the $2^{-\Delta\Delta CT}$ method. Error bars denote the standard error of the mean (SEM) from three independent experiments. Significant differences were determined by Tukey HSD test using the SPSS software (Version 16.0, SPSS Inc). A p-value of 0.05 was used to determine statistical significance.

## Results

### Characterization of AccPPO

Based on the sequence information of *PPO* genes in *Apis mellifera*, *Apis dorsata*, *Apis florae*, *Bombus impatiens*, *Megachile rotundata*, the partial cDNA sequence of *AccPPO* was cloned using a combination of reverse transcription PCR (RT-PCR) and rapid amplification of cDNA ends (RACE). The partial cDNA sequence of *AccPPO* was 2293 bp in length and contained a 27-bp 5' untranslated region (UTR), a 184-bp 3' UTR and an open reading frame of 2079 bp. The newly cloned *AccPPO* gene was predicted to encode a protein of 693 amino acid residues with

| Abbreviation | Primer sequence (5′-3′) | Description |
|---|---|---|
| PP1 | CATTCGACATCAGAACTATCG | cDNA sequence primer, forward |
| PP2 | TTGCCATGTCAAGAGATATTC | cDNA sequence primer, reverse |
| 3P1R1 | GTGGATTGAGAGATAGAAAGTATCC | 3′ RACE forward primer, outer |
| 3P2R2 | CGTGCAGGTGTTGAAACTTTAG | 3′ RACE forward primer, inner |
| B26 | GACTCTAGACGACATCGA$(T)_{18}$ | 3′ RACE universal primer, outer |
| B25 | GACTCTAGACGACATCGA | 3′ RACE universal primer, inner |
| QP1 | CATTCGACATCAGAACTATCG | Full-length cDNA primer, forward |
| QP2 | TATTTTAACTATTCATTTATTTTTTTTTTATTTTATTACAATAGA | Full-length cDNA primer, reverse |
| *β*-s | TTATATGCCAACACTGTCCTTT | Standard control primer, forward |
| *β*-x | AGAATTGATCCACCAATCCA | Standard control primer, reverse |
| N1 | CATTCGACATCAGAACTATCG | Genomic sequence primer, forward |
| N2 | TATTTTAACTATTCATTTATTTTTTTTTTATTTTATTACAATAGA | Genomic sequence primer, reverse |
| TP1 | GGGTGATTCAGCAACAGCTATGAG | Real-time PCR primer, forward |
| TP2 | CTATTTCGATACCCGGGAAGTC | Real-time PCR primer, reverse |

**Table 1:** PCR primers in this study.

| Primes pair | Amplification conditions |
|---|---|
| PP1/PP2 | 10 min at 94°C, 40 s at 94°C, 40 s at 50°C, 150 s at 72°C for 35 cycles, 10 min at 72°C |
| 3P1/B26 | 10 min at 94°C, 40 s at 94°C, 40 s at 50°C, 40 s at 72°C for 28 cycles, 10 min at 72°C |
| 3P2/B25 | 10min at 94°C, 40 s at 94°C, 40 s at 53°C, 40 s at 72°C for 35 cycles, 10 min at 72°C |
| QP1/QP2 | 10 min at 94°C, 40 s at 94°C, 40 s at 51°C, 150 s at 72°C for 35 cycles, 10 min at 72°C |
| N1/N2 | 10 min at 94°C, 40 s at 94°C, 40 s at 52°C, 240 s at 72°C for 35 cycles, 10 min at 72°C |

**Table 2:** PCR amplification conditions.

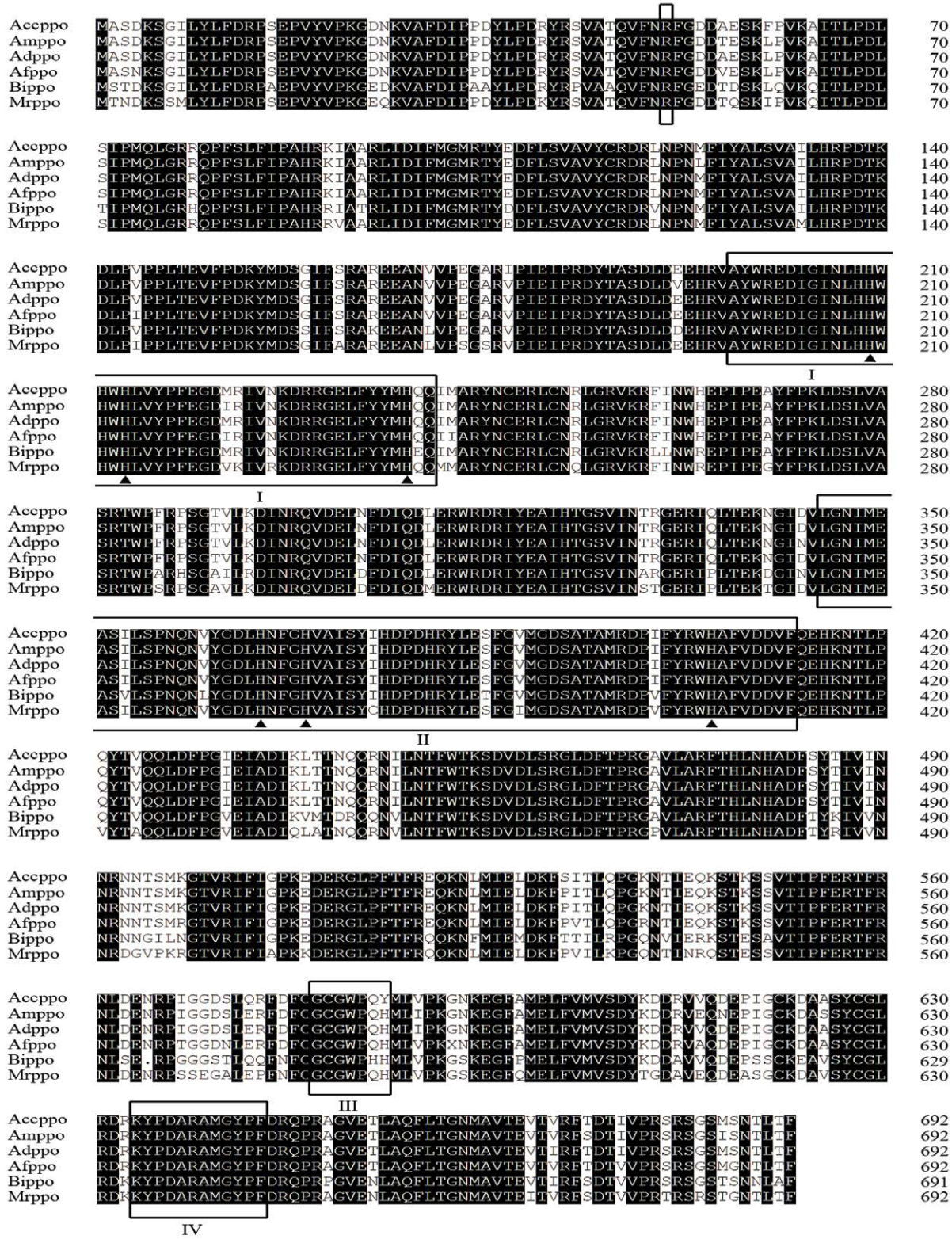


**Figure 1:** Molecular characterization of prophenoloxidases (PPOs) from various species. Comparison of the deduced amino acid sequences of AccPPO (prophenoloxidase, GenBank accession number: JX844653) from *A. cerana cerana*, AmPPO (prophenoloxidase, NP_001011627.1 from *Apis mellifera*, AdPPO (prophenoloxidase, XP_006616656.1) from *Apis dorsata*, AfPPO (prophenoloxidase, XP_003697839.1) from *Apis florae*, BiPPO (prophenoloxidase, XP_003484980.1) from *Bombus impatiens* and MrPPO (prophenoloxidase, XP_003707107.1) from *Megachile rotundata*. Identical amino acid residues are shaded in black. The predicted proteolytic cleavage site "R" is boxed. Conversed regions I, II, III and IV are also boxed. As shown in regions I and II, six histidine residues, which are considered to be ligands for the two copper atoms, are marked with ▲.

a putative molecular mass of 80.057 kDa and an isoelectric point of 6.71. Multiple sequence alignments showed that the predicted amino acid sequence of AccPPO shares 97.84%, 98.85%, 97.26%, 86.15%, and 86.44% similarities with the PPO proteins from *Apis mellifera*, *Apis dorsata*, *Apis florae*, *Bombus impatiens*, and *Megachile rotundata*, respectively. As shown in Figure 1, the predicted proteolytic cleavage site "R" is boxed and the predicted molecular weight of the active enzyme PO is 74.232 kDa. In addition, two conserved regions, the putative GCGWPQH/Y thiol ester site (region III) and the region IV are also boxed in (Figure 1). The regions I and II are two copper binding sites (CuA and CuB). In addition, there are also six histidine residues, which are considered to be ligands for the two copper atoms.

### Phylogenetic analysis of AccPPO

To further clarify the catalytic mechanism of AccPPO, the potential tertiary structure of AccPPO was constructed using SWISS-MODEL server, and the cysteines (His209, His213, His238, His365, His369 and His405) of catalytic cores were identified (Figure 2). To investigate the revolutionary relationship between AccPPO and its homologs, a phylogenetic tree was constructed and analyzed using MEGA (version 4.0) software. As shown in Figure 3, phylogenetic analysis revealed that AccPPO was closely related to the PPO protein from *A. mellifera*, which is in agreement with the relationship predicted from the multiple sequence alignment.

### Genomic structure analysis of AccPPO

To further investigate the properties of *AccPPO*, the genomic DNA sequence of AccPPO was obtained using the genomic DNA of *A. cerana cerana* for PCR amplification. The genomic DNA sequence of *AccPPO* (GenBank accession number: JX844654) was 3483 bp long, containing nine exons and eight introns, which is very similar to *AmPPO* (Figure 4).

### Expression profiles of AccPPO in different developmental stages

qRT-PCR was employed to analyse the mRNA accumulations of *AccPPO* during different developmental stages. As shown in Figure 5, the expression level of *AccPPO* could be detected in all the analysed developmental stages and it was expressed the highest in the 15-day postemergence adults.

### Expression patterns of AccPPO in response to oxidative stresses

As shown above, *AccPPO* was expressed the highest in the 15-day postemergence adults in different developmental stages and then 15-day postemergence adults were subjected to 4, 16°C; UV light; and pesticides (acaricide, cyhalothrin, paraquat) treatments. The expression level of *AccPPO* in larvae was induced by the infection of *Ascosphaera apis* (Figure 6A). As shown in (Figure 6B, 6C), when treated with cold stresses, the expression profiles of *AccPPO* was both induced under 4 and 16°C treatments and *AccPPO* was upregulated more obviously under 4°C treatment than that of 16°C treatment. At UV light treatment, the transcript of *AccPPO* was induced severely and reached 7.4-fold higher at 4 h in the treated groups compared with the control groups (Figure 6D). Under stress at acaricide, cyhalothrin and paraquat treatments, the expression levels of *AccPPO* were all induced by the above pesticides and reached their highest levels at 2.5 h, 1.0 h and 2.5 h, respectively (Figures. 6E, 6F, 6G).

## Discussion

Previous studies have shown that insect PPO plays an important role in immunity and the PO activity is essential to recover the damage caused by pathogen invasion and wounds [13]. There are also some researches about the function of PPO in resisting parasites in other species whereas there is little study on PPO in honey bee, particularly in Chinese honey bee (*A. cerana cerana*). To gain new insight into the functions of PPO in this important species, we identified and characterized a *PPO* gene from *A. cerana cerana* in this study. In addition, we analyzed the expression profiles of *AccPPO* during different developmental stages and we also investigated the transcripts of *AccPPO* during various environmental stresses. Based on our results, we predicted that AccPPO might play a critical role in immunity and resisting oxidative stresses.

Sequence analysis showed that AccPPO has the same length ORF as AmPPO and also had the high amino acids identity to AmPPO, suggesting AccPPO might play the same important role in *A. cerana cerana* as that in *A. mellifera*. In addition, we could also find that the copper binding sites CuA and CuB, the putative GCGWPQH/Y thiol ester site (region III) and the conserved region IV of AccPPO in *A. cerana cerana* were similar to those of AmPPO in *A. mellifera*, which further verified that AccPPO might function in immunity in *A. cerana cerana*. Genomic structure analysis revealed that *AccPPO* was very similar to *AmPPO* and phylogenetic analysis of AccPPO and its homologs also showed that PPO protein in *A. cerana cerana* was close to that in *A. mellifera*, suggesting that AccPPO has a conserved period of evolution [12,32].

It was not detected in pupae and expressed in other developmental stages in the lepidopteran *Hyphantria cunea* (*H. cunea*), which is not the same as that of *AccPPO* in *A. cerana cerana*. The *PPOA3* gene of *Culex pipiens pallens* is transcribed at all developmental stages [31]. As shown in Figure 5, *AccPPO* was present in all of the developmental stages including the 4th-, 5th-, and 6th-day instar larvae; white-eyed (Pw), pink-eyed (Pp), brown-eyed (Pb), and dark-eyed (Pd) pupae; 1-day postemergence adults; 15-day postemergence and 30-day postemergence adults, which is similar to that of *AmPPO* in *A. mellifera*, revealing that AccPPO may also play a critical role in all the developmental stages. Moreover, the highest expression profile of *AccPPO* was found in 15-day postemergence adults among all the developmental stages, which is consistent with that of *AmPPO* in *A. mellifer*. In addition, Zhou et al. [11,31] demonstrated that the expression level of *PPOA3* gene from *Culex pipiens pallens* was higher in adult stage than other stages, suggesting they may play a more important role in immunity in adult stage.

Lourenco et al. [12] demonstrated that AmPPO functions in adult exoskeleton melanization in the previous study and PO-mediated melanin synthesis plays a major role in immune defense in insects. Melanin synthesis plays an important role in preventing hemolymph loss and providing cytotoxic compounds (quinone) to encapsulate and eliminate opportunist-invading microorganisms at the wound site [32,33]. The expression profile of *PPO* was upregulated by after the stimulation of CpG ODN and WSSV in all studied tissues [11]. As shown in Figure 6, *AccPPO* was induced by the infection of *Ascosphaera apis*, revealing that AccPPO may function in preventing *A. cerana cerana* from the infection of *Ascosphaera apis* and play a major role in immunity, which is in agreement with previous studies. In addition, we could also surprisingly find that *AccPPO* was upregulated by some oxidative stresses including 4, 16°C; UV light; and pesticides (acaricide, cyhalothrin, paraquat) treatments, suggesting that AccPPO may also play an important role in resisting oxidative stresses.

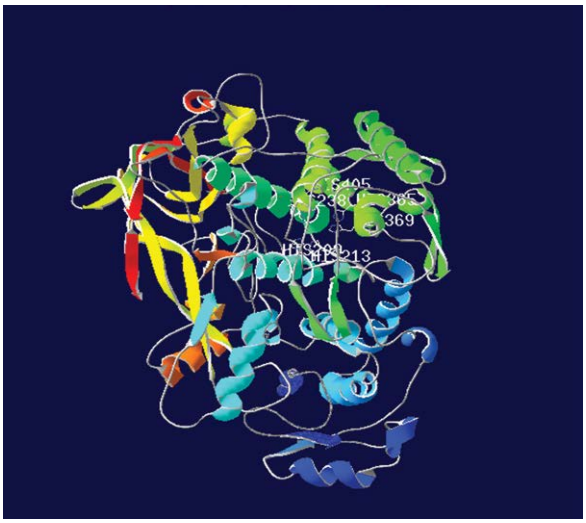


**Figure 2:** The tertiary structure of AccPPO. The structure was built using homology modeling in the SWISS-MODEL modeling environment. His209, His213, His238, His365, His369 and His405, which are found in the predicted catalytic active site, are shown.

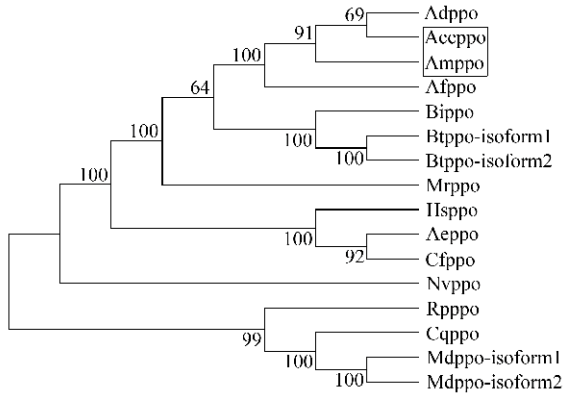


**Figure 3:** Phylogenetic analysis of *AccPPO* with other known PPO protein sequences. The protein sequences of various species are as follows: AccPPO (*A. cerana cerana*, GenBank accession number: JX844654) from *A. cerana cerana* AePPO (*Acromyrmex echinatior*, EGI66037.1), AdPPO (*A. dorsata*, XP_006616656.1), AfPPO (*A. florae*, XP_003697839.1), AmPPO (*A. mellifera*, NP_001011627.1), BiPPO (*B. impatiens*, XP_003484980.1), BtPPO isoform 1 (*Bombus terrestris*, XP_003400548.1), BtPPO isoform 2 (*Bombus terrestris*, XP_003400549.1), CfPPO (*Camponotus floridanus*, EFN74080.1), CqPPO (*Culex quinquefasciatus*, XP_001848965.1), HsPPO (*Harpegnathos saltator*, EFN88581.1), MrPPO (*M. rotundata*, XP_003707107.1), MdPPO isoform X1 (*Musca domestica*, XP_005174994.1), MdPPO isoform X2 (*Musca domestica*, XP_005174995.1), NvPPO (*Nasonia vitripennis*, XP_001606582.2), RpPPO (*Riptortus pedestris*, BAN20821.1).

In conclusion, we have identified an immune-related enzyme gene (*AccPPO*) from *A. cerana cerana* in this study. This enzyme possesses conserved functional domains of the PPO superfamilies and the genomic structure and phylogenetic tree of *AccPPO* are similar to that of *AmPPO*. The expression profile of *AccPPO* during different developmental stages was highest in 15-day postemergence adults. In addition, the expression level of AccPPO in response to the infection of *Ascosphaera apis* indicated that AccPPO may play a critical role in immune-related defense mechanisms. Very surprisingly, *AccPPO* was also induced by various environmental stresses. These findings provide solid evidence for the importance role of AccPPO in resisting the invasion of *Ascosphaera apis* and adverse environment to protect

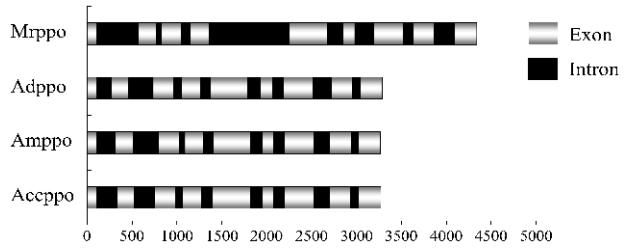


**Figure 4:** Genomic structure analysis of *AccPPO* from *A. cerana cerana* and its homologs, including *AmPPO* from *A. mellifera* (NP_001011627.1), *MrPPO* from *M. rotundata* (XP_003707107.1), and *AdPPO* from *A. dorsata* (XP_006616656.1).

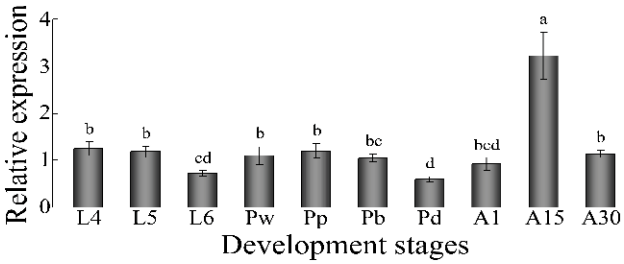


**Figure 5:** Expression patterns of *AccPPO* determined by real-time quantitative PCR. Expression profiles of *AccPPO* in all the developmental stages including larvae from the fourth to sixth instars (L4–L6), pupae (Pw, white-eyed pupae; Pp, pink-eyed pupae; Pb, brown-eyed pupae; and Pd, dark-eyed pupae), and adults (A1, 1 day postemergence; A15, 15 days postemergence; and A30, 30 days postemergence). The *β-actin* gene was used as an internal control. Histograms reveal the relative expression profiles of *AccTpx-3*. Each value is given as the mean (Kohgo et al.) of three replicates. The letters on the bar represent a significant difference at P<0.05, as determined by Tukey HSD test using the SPSS software (Version 16.0, SPSS Inc).

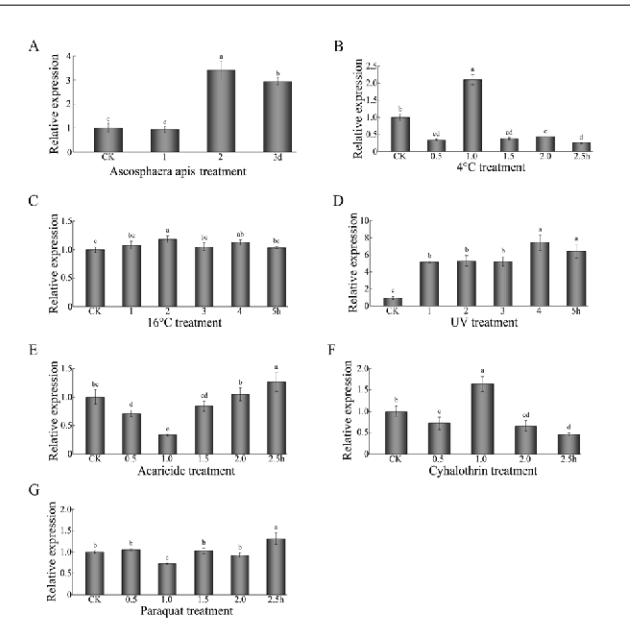


**Figure 6:** Effect of the infection of *Ascosphaera apis* and various oxidative stresses including 4, 16°C; UV light; and pesticides (acaricide, cyhalothrin, paraquat) treatments on *AccPPO* gene expression. The *β-actin* gene was used as an internal control. Histograms reveal the relative expression profiles of *AccTpx-3*. Each value is given as the mean of three replicates. The letters on the bar represent a significant difference at P<0.05 as determined by Tukey HSD test using the SPSS software (Version 16.0, SPSS Inc).

*A. cerana cerana* and enrich our understanding of PPO proteins in *A. cerana cerana*.

### Acknowledgments

This work was financially supported by the earmarked fund for the China Agriculture Research System (No. CARS-45), the National Natural Science Foundation of China (No. 31172275) and Shandong Province Agricultural Fine Varieties Breeding Projects (2014-2016).

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