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Prophenoloxidase from *Pieris rapae*: gene cloning, activity, and transcription in response to venom/calyx fluid from the endoparasitoid wasp *Cotesia glomerata*[*]

Jia-ying ZHU[†§1], Pu YANG[§2], Guo-xing WU[3]

(*[1]Key Laboratory of Forest Disaster Warning and Control of Yunnan Province and Key Laboratory of Southwest Mountain Forest Resources Conservation and Utilization of Ministry of Education, Southwest Forestry University, Kunming 650224, China*)

(*[2]Research Institute of Resource Insects, Chinese Academy of Forestry, Kunming 650224, China*)

(*[3]College of Plant Protection, Yunnan Agricultural University, Kunming 650201, China*)

[†]E-mail: jiayingzhu001@yahoo.com.cn

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Abstract: Prophenoloxidase (PPO) plays an important role in melanization, necessary for defense against intruding parasitoids. Parasitoids have evolved to inject maternal virulence factors into the host hemocoel to suppress hemolymph melanization for the successful development of their progeny. In this study, the full-length complementary DNA (cDNA) of a *Pieris rapae* PPO was cloned. Its cDNA contained a 2076-base pair (bp) open reading frame (ORF) encoding 691 amino acids (aa). Two putative copper-binding sites, a proteolytic activation site, three conserved hemocyanin domains, and a thiol ester motif were found in the deduced amino acid sequence. According to both multiple alignment and phylogenetic analysis, *P. rapae* PPO gene cloned here is a member of the lepidopteran PPO-2 family. Injection of *Cotesia glomerata* venom or calyx fluid resulted in reduction of *P. rapae* hemolymph phenoloxidase activity, demonstrating the ability to inhibit the host's melanization. Real-time reverse transcriptase polymerase chain reaction (RT-PCR) showed that transcripts of *P. rapae* PPO-2 in the haemocytes from larvae had not significantly changed following venom injection, suggesting that the regulation of PPO messenger RNA (mRNA) expression by venom was not employed by *C. glomerata* to cause failure of melanization in parasitized host. While decreased *P. rapae* PPO-2 gene expression was observed in the haemocytes after calyx fluid injection, no detectable transcriptional change was induced by parasitization, indicating that transcriptional down-regulation of PPO by calyx fluid might play a minor role involved in inhibiting the host's melanization.

Key words: Prophenoloxidase, Cloning, Venom, Calyx fluid, Gene expression, Parasitoid
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1 Introduction

Insects, lacking an acquired immune system, have an efficient innate immune system including an array of humoral and cellular immune responses to combat invading pathogens and parasites. Melanization is an important part of the immune defense involved in the process of sclerotization, pigmentation, and wound healing of the cuticle as well as in defence reactions (nodule formation, phagocytosis, and/or encapsulation) against invaders entering the insect's hemocoel (Ratcliffe *et al*., 1985; Sritunyalucksana and Söderhäll, 2000; Cerenius *et al*., 2008). The key enzyme involved in melanin formation is phenoloxidase (PO). PO generally exists in hemolymph, haemocytes, and/or cuticle of insects in its

[§] These two authors contributed equally to this work

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inactive form as a proenzyme, prophenoloxidase (PPO), which is converted to the active form via a serine protease cascade (Ashida, 1971; Ashida and Brey, 1995; Kopácek *et al*., 1995; Cerenius and Söderhäll, 2004). PPO activation cascade leads to the formation of cytotoxic molecules including quinones from phenolic compounds and reactive oxygen intermediates, which are important immune effectors vital against foreign intruders (Nappi and Christensen, 2005). In order to overcome insect defensive melanization, microorganisms and parasites depend to a large extent on regulations of PO activity and gene expressions that are associated with the PPO activating system (Nappi *et al*., 2009; Vazquez *et al*., 2009).

Endoparasitic wasps exhibit a unique form of immature development. They lay eggs into the body cavities of their hosts, consuming them during their own development, thereby almost ultimately causing the host's death (Kraaijeveld and Godfray, 2009). To ensure successfully development to maturity in the host hemocoel, parasitoids use a diversified range of virulence factors including polydnaviruses (PDVs), venom, virus-like particles (VLPs), teratocytes, ovarian proteins, and parasitoid larval secretions to deactivate their host's immune responses (Beckage and Gelman, 2004; Brodeur and Boivin, 2004; Moreau and Guillot, 2005; Pennacchio and Strand, 2006; Zhu *et al*., 2008; 2009a; 2009b; Kraaijeveld and Godfray, 2009). Inhibition of melanization and PO activity by parasitization has been frequently observed (Lavine and Beckage, 1995; Asgari *et al*., 2003; Colinet *et al*., 2009; Nappi *et al*., 2009). However, relatively little is known about the molecular mechanisms of the host's melanization disrupted by the parasitoid. Recently, several virulence proteins notably known to inhibit melanization have been characterized from parasitoid venom and PDVs (Asgari *et al*., 2003; Beck and Strand, 2007; Colinet *et al*., 2009; Zhu *et al*., 2011). These findings made a large contribution for explaining how parasitoids inhibit the host's melanization, and demonstrated that these virulence factors target the PPO activation cascade associated with the reduction in PO activity. Expression of PPO reduced by parasitization might be one of the strategies ascribed to decrease PO activity (Shelby *et al*., 2000), which is only investigated in *Tranosema rostrale*-*Choristoneura fumiferana* system (Doucet *et al*., 2008).

Cotesia glomerata is a common gregarious endoparasitoid attacking many species of lepidopterous young larvae as the host. It is an important larval parasitoid of the small white butterfly, *Pieris rapae*, which is heavily parasitized in fields of cruciferous vegetables (Laing and Levin, 1982). Its venom and calyx fluid impair the host's cellular and humoral immune defences, and its teratocytes possibly suppress the host's larval hemolymph PO activity (Kitano, 1974; 1982; Kitano and Nakatsuji, 1978; Kitano *et al*., 1990; Ockroy *et al*., 2002; Madanagopal and Kim, 2006). However, there is no report focused on the effect of *C. glomerata* venom or calyx fluid on *P. rapae* haemolymph PO activity. Here, complementary DNA (cDNA) of *P. rapae* PPO was cloned, and *P. rapae* PO activity and PPO transcription were investigated in response to *C. glomerata* venom or calyx fluid.

2 Materials and methods

2.1 Insects

C. glomerata colony was established by collecting cocoons or parasitized *P. rapae* larvae from cabbage fields in the suburbs of Kunming, Yunnan Province, China. Once emerged, wasps were held in a glass tube (20 cm×5 cm) and fed a 20% (v/v) honey solution. Its colony was maintained continuously on *P. rapae* larvae. Eggs of *P. rapae* on cabbage in the fields were collected and held in a growth chamber under a regime of 12-h light:12-h dark, (25±1) °C, and 60% relative humidity. Newly emerged larvae were reared on cabbage grown at the same conditions.

2.2 cDNA cloning of PPO

P. rapae larvae were surface sterilized in 95% ethanol and a small cut was made in a proleg. Hemolymph was collected in a 1.5-ml sterilized Eppendorf tube. After mixing by gently inverting the tube, haemocytes were collected by centrifuging the hemolymph at 250×*g* for 5 min at room temperature. The resulting haemocyte pellets were used for total RNA isolation. Total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. 3′ and 5′ RACE (rapid amplification of cDNA ends) cDNAs were synthesized with an SMART™ RACE cDNA amplification kit (Clontech, Mountain View,

CA, USA). Degenerated primer pairs (5′-GAR CTGTTYTAYTAYATGC-3′ and 5′-CACRTGNCC CATRTTRTG-3′) were designed based on the amino acid sequences of the two conserved copper-binding sites (QIFYYMH and HNMGHV) of other insect PPOs (Park *et al*., 1998). They were used to amplify part of the coding sequence of PPO from 3′ RACE cDNA. Polymerase chain reaction (PCR) was preceded by denaturation at 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min, and finishing with chain extension at 72 °C for 10 min. Based on this result, 3′ and 5′ gene-specific primers (5′-CGACCGTTACCATCCCATT CGAGCAGAC-3′ and 5′-TGGTTGGCAGTAAAT GGGTAGACGAAAT-3′) were designed, respectively. They were used to clone the 3′ and 5′ ends in conjunction with adapter primers for both ends provided in an SMART™ RACE cDNA amplification kit (Clontech). The RACE-PCR was carried as follows: 94 °C for 1 min; 30 s at 94 °C, 6 min at 68 °C, and 2 min at 72 °C for 30 cycles; extension at 72 °C for 10 min. PCR products were subjected to 0.01 g/ml agarose gel electrophoresis, purified, and cloned to pGEM-T® easy vector (Promega, San Luis Obispo, CA, USA) for sequencing.

2.3 Sequence analysis

The nucleotide and deduced amino acid sequences were analyzed using Genetyx Version 8.0 (Software Development, Tokyo, Japan). The signal peptide was analyzed by SigalP 3.0 Server (Dyrløv Bendtsen *et al*., 2004). The gene characteristic structures were predicted by Motif Scan (Hulo *et al*., 2008). The multiple sequence alignment was created with ClustalX (Version 1.83) program (Thompson *et al*., 1997). The phylogenetic analysis was conducted using molecular evolutionary genetics analysis 4 (MEGA4) with the neighbor-joining method and bootstrapping sampled 1000 times (Tamura *et al*., 2007).

2.4 Venom and calyx fluid preparation

Venom apparatus and ovaries were dissected from 4-d-old *C. glomerata* female wasps according to the method described by Rivers *et al*. (1993) and Yu *et al*. (2007). After several sequential washes, they were transferred to 0.5 ml sterilized Eppendorf tube containing 50 μl sterile phosphate buffered saline (PBS, pH 7.4). The samples were homogenized manually, and the extract was centrifuged at 12000×*g* at 4 °C for 10 min to discard cellular debris. The supernatant was collected and diluted in PBS to the concentration of 0.5 female equivalents of venom or calyx fluid (one female equivalent being defined as the supernatant from one venom reservoir or ovary in 1 μl PBS).

2.5 PO activity analysis

Each *P. rapae* larva of the 3rd instar was kept in a glass tube (5 cm×3 cm). One 4-d-old mated *C. glomerata* female wasp was introduced to each tube. Immediately after being attacked, the parasitoid wasp was removed from the tube. Similar aged host larvae were also injected with 0.5 female equivalents of venom or calyx fluid as described by Béliveau *et al*. (2000). The larvae were reared using cabbage leaves. Hemolymph collected from five larvae of each treatment was centrifuged at 10000×*g* for 5 min at 4 °C to precipitate insoluble material, and then used in PO activity assays. PO activity was assayed following the protocol of Fisher and Brady (1980) and Parkinson and Weaver (1999). Briefly, 5 μl hemolymph was added to 5 mmol/L L-3,4-dihydroxyphenylalanine (L-DOPA) in cacodylate buffer (10 mmol/L sodium cacodylate, 5 mmol/L $CaCl_2$; pH 7.0) and absorbance (490 nm) measured using a microplate reader (Bio-Rad, Hercules, CA, USA) for 30 min at room temperature. PO activity assays were performed on three separate collections of hemolymph at each of the time points. All data analysis was carried out by one-way analysis of variance (ANOVA). The acceptance level of statistical significance was $P<0.05$ in all instances.

2.6 Real-time reverse transcriptase polymerase chain reaction (RT-PCR)

Parasitization and injection of venom or calyx fluid were conducted as described above. Controls consisted of larvae non-parasitized and injected with equal volume of PBS. After 0, 12, 24, 48, 72, and 96 h treatments, haemocytes were collected. Their total RNA was isolated using TRIzol Reagent (Invitrogen). There were three replicates for each time point. Residual genomic DNA was removed using RNase-free DNase I (Promega). cDNAs were synthesized using a RevertAid™ first strand cDNA synthesis kit (Fermentas, Burlington, Canada), and then subjected to

real-time RT-PCR analysis. RT-PCR was performed in the SYBR® Premix (TaKaRa, Shiga, Japan) according to the manufacture's standard protocol. The PPO transcript was determined using specific primers (5′-CTTGACAGTCTTACATCCTCCCG-3′ and 5′-CCTCATCGCCTATTACGCCAAAC-3′). The 18S rRNA profile was used as internal control for normalization of input cDNA. The total RNA following DNase treatment was subjected to RT-PCR to assure that DNA removal has been successful. PCR conditions were as follows: 1 cycle of 94 °C for 3 min, followed by 40 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s. The relative mRNA level of PPO was normalized relative to the control using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001).

3 Results

3.1 cDNA cloning of *P. rapae* PPO

The cDNA of *P. rapae* PPO was 2397 base pairs (bp) in length, containing a 2076-bp of open reading frame (ORF), a 72-bp of 5′ untranslated region (UTR), and a 250-bp of 3′ UTR with polyadenylation signal sequences AATAAA and a poly(A) tail (GenBank accession No. HM210573) (Fig. 1). The deduced amino acid (aa) sequence revealed a protein of 691 aa with a predicted molecular weight of 79.15 kDa and a theoretical pI of 6.13. No putative signal peptide was predicted at the N-terminus. The proteolytic cleavage site forming PPO into PO was predicted to be between Arg51 and Leu52. Two conserved copper-binding sites, namely coppers A and B, were preserved at aa 201–245 and aa 346–413, which contained six histidine residues (aa 213, 217, 243, 366, 370, and 406). Three conserved hemocyanin domains were encoded by the polypeptide sequence: hemocyanin-N at aa 47–147, hemocyanin-M at aa 152–415, and hemocyanin-C at aa 423–679. A common central domain of tyrosinase was detected at aa 233–419. A highly conserved thiol ester motif (GCGWPQHM) was present at aa 584–591. Other possible functional sites were predicted, including 6 *N*-glycosylation sites, 2 cyclic adenosine monophosphate (cAMP)- and cyclic guanosine monophosphate (cGMP)-dependent protein kinase phosphorylation sites, 8 casein kinase II phosphorylation sites, 9 *N*-myristoylation sites, 12 protein kinase C phosphorylation sites, and 1 tyrosine kinase phosphorylation site.

Sequence comparison showed that the identity between the amino acid sequence of *P. rapae* PPO and those of *Plutella xylostella* PPO 1 and PPO 2, *Tenebrio molitor* PPO, *Musca domestica* PPO, and *Apis mellifera* PPO were 47%, 74%, 51%, 53%, and 50%, respectively (Fig. 2). The copper-binding regions were highly conserved among these species, and the histidine residues and the thiol ester motifs were present in all sequences analyzed.

A phylogeny tree was constructed by neighbour-joining distance method using amino acid sequences of PPO of *P. rapae* and PPOs of other Insecta and Crustacea (Fig. 3). The PPO of *P. rapae* together with PPOs of other insects placed far away from that of Crustacea. Seven main well-supported groups in the PPO sequences of Insecta analysis were identified. Dipteran and hymenopteran PPOs were separated into two distinct groups, respectively. PPOs from Coleoptera were clustered into the same group. Lepidopteran PPOs were subdivided into two distinct clades that separate PPO-1 from PPO-2. *P. rapae* PPO belonged to the PPO-2 clade, and was most closely related to *P. xylostella* and *C. fumiferana* PPO-2.

3.2 Effects of venom and calyx fluid on PO activity

Levels of PO activity of non-parasitized larvae increased as development progressed, while that in parasitized larvae only ascended gradually up to 48 h, followed by a gradual decrease during 72 to 96 h (Fig. 4). Parasitization had significant down-regulation effects on PO activity during 24 to 96 h. PBS-injected larvae were similar to non-parasitized larvae within the time observed. When compared to PBS injection, venom injection had significant effects on PO activity during 6–96 h. The PO activity of PBS-injected larvae was not statistically different from that of calyx fluid-injected larvae at 0–12 h. However, calyx fluid injection significantly reduced the PO activity throughout 24–96 h. From 48 to 96 h, calyx fluid injection induced a significantly higher decrease in PO activity than venom injection, and both venom and calyx fluid injections resulted in significantly lower levels of PO activity than parasitization during 24 to 96 h.

```
   1  GATTCTCACTTCACATTTACTGTGAAGTGGATTAATTAGTTTAAATATAAAGTGATAATAGTAGGCACAA   70
  71  AATGGCAAGTGTAGTAAAAGGGCTGTCTTTGCTCTTCGATAGGCCCAATGAGCCTATGGTGTCACCGAAA   140
       M  A  S  V  V  K  G  L  S  L  L  F  D  R  P  N  E  P  M  V  S  P  K
 141  GGAGACGATAATGCCATTTTCCAAATTTCCGAACAATTCTTGACTGACGACTACAAAAACAACGGTATTG   210
      G  D  D  N  A  I  F  Q  I  S  E  Q  F  L  T  D  D  Y  K  N  N  G  I  E
 211  AAATTAACAACCGTTTTGGGGAAAACGCATCGGAAATAGTTCCTCTAGAGAACCTACGTCGAGTGCCCAA   280
        I  N  N  R**F  G  E  N  A  S  E  I  V  P  L  E  N  L  R  R  V  P  K
 281  GCTTACGAAAGCAAGGCGCTTACCAAAGGATGCTGACTTCTCACTGTTCTTGCCAGCCCACAGGGAAATG   350
       L  T  K  A  R  R  L  P  K  D  A  D  F  S  L  F  L  P  A  H  R  E  M
 351  GCAGATGAAGTCGTTGACGAACTTATGGGTGTTCCTGAAAATCAATTGCAGCAGTTCCTGTCTACTTGTA   420
      A  D  E  V  V  D  E  L  M  G  V  P  E  N  Q  L  Q  Q  F  L  S  T  C  T
 421  CATATGCCCGTGTTAATATCAACCCTCAACTGTTCAACTATTGTTTCAGTGTTGCCTTAATGCATCGGCG   490
        Y  A  R  V  N  I  N  P  Q  L  F  N  Y  C  F  S  V  A  L  M  H  R  R
 491  TGACACCAGAAATGTCCCTATTGCAAACTTTGCAGAGACATTCCCTTCAAAATTTGTGGATTCTCAAGTT   560
       D  T  R  N  V  P  I  A  N  F  A  E  T  F  P  S  K  F  V  D  S  Q  V
 561  TTTGCTCAAGCCCGTGAGACAGCTGCGGTTGCTGCACAAGGAGCACCGCGCACTCCAATAATTATACCAA   630
      F  A  Q  A  R  E  T  A  A  V  A  A  Q  G  A  P  R  T  P  I  I  I  P  R
 631  GAGACTACACAGCAACAGACTTGGATGTAGAACATCGTTTAGCGTACTTCCGCGAAGATATTGGTGTTAA   700
        D  Y  T  A  T  D  L  D  V  E  H  R  L  A  Y  F  R  E  D  I  G  V  N
 701  CCTACACCACTGGCATTGGCATCTCGTCTACCCATTTACTGCCAACCAGAGGGAAATTGTTGCTAAAGAC   770
       L  H  H  W  H  W  H  L  V  Y  P  F  T  A  N  Q  R  E  I  V  A  K  D
 771  AGACGTGGAGAACTATTTTTCTACATGCACCAGCAAATTATTGCCAGGTACGATGGTGAGCGACTGAATA   840
      R  R  G  E  L  F  F  Y  M  H  Q  Q  I  I  A  R  Y  D  G  E  R  L  N  N
 841  ATTCTTTACCCAGAGTCAAAAAGTTCAATAACTTCAATGAGCCAATACCGGAAGCGTATTTCCCAAAACT   910
        S  L  P  R  V  K  K  F  N  N  F  N  E  P  I  P  E  A  Y  F  P  K  L
 911  TGACAGTCTTACATCCTCCCGGGGTTGGCCTCCACGACAGGCCAATATGCGATGGCAGGACTTGAATAGA   980
       D  S  L  T  S  S  R  G  W  P  P  R  Q  A  N  M  R  W  Q  D  L  N  R
 981  CCAGTTGATGGTCTTAATGTGACTATCGCGGATATGCAACGCTGGAGGCGGAACATCGAAGATGCTATTG   1050
      P  V  D  G  L  N  V  T  I  A  D  M  Q  R  W  R  R  N  I  E  D  A  I  A
1051  CCACCGGATTGGTGCAATTGCCCAACGGCTCGACCCGCCCCCTGGACATAGACACTCTCGGCAATATGGT   1120
        T  G  L  V  Q  L  P  N  G  S  T  R  P  L  D  I  D  T  L  G  N  M  V
1121  AGAAGCGAGTGTACTGTCTCCTAATATGGAGCTGTATGGCAGTTTGCATAACAACGGACACAGCTTCTCC   1190
       E  A  S  V  L  S  P  N  M  E  L  Y  G  S  L  H  N  N  G  H  S  F  S
1191  GCGTACATGCATGACCCAACACACAGATATCTGGAATCGTTTGGCGTAATAGGCGATGAGGCTACAACTA   1260
      A  Y  M  H  D  P  T  H  R  Y  L  E  S  F  G  V  I  G  D  E  A  T  T  M
1261  TGCGTGATCCATTCTTCTTCCGCTGGCACGCCTTTATTGACGACCTTTTCCAGAAATACAAGGAATCCGC   1330
        R  D  P  F  F  F  R  W  H  A  F  I  D  D  L  F  Q  K  Y  K  E  S  A
1331  TAATGTACGCCCATACACACGCTCCGAGTTGGAAAACCCAGGCGTTAGAGTAACATCTGCACGTATTGAT   1400
       N  V  R  P  Y  T  R  S  E  L  E  N  P  G  V  R  V  T  S  A  R  I  D
1401  AGCTCGGATGGCGCAACCAACGAATTGCATACATTTTGGATGTCAAGCGATGTTGACCTTTCTCGTGGCC   1470
      S  S  D  G  A  T  N  E  L  H  T  F  W  M  S  S  D  V  D  L  S  R  G  L
1471  TTGACTTCTCCAACAGAGGTCCTGTGTATGCCCGCTTCACTCATTTGAACCATCGCGATTTCCAATATAC   1540
        D  F  S  N  R  G  P  V  Y  A  R  F  T  H  L  N  H  R  D  F  Q  Y  T
1541  AATTGAAGTGGAAAATAATAGCAACGCCTCGCGTGGTACTGTCCGTATCTTTATTGCGCCGACAAACGAT   1610
       I  E  V  E  N  N  S  N  A  S  R  G  T  V  R  I  F  I  A  P  T  N  D
1611  GAACGTCAGCTCAGCTGGGCGCTCTCTGACCAGCGCAAGATGTTCATAGAGATGGACAAATTTGTTGTGC   1680
      E  R  Q  L  S  W  A  L  S  D  Q  R  K  M  F  I  E  M  D  K  F  V  V  P
1681  CATTGAATCGTGGCCGTAACGTCATAACCCGCCGTTCAACTGAGTCGACCGTTACCATCCCATTCGAGCA   1750
        L  N  R  G  R  N  V  I  T  R  R  S  T  E  S  T  V  T  I  P  F  E  Q
1751  GACTTTCCGTGATCTCGGCGCTCAGGGTTCAAATCCTAGGAACACAGATCTCGCGTCCTTCAACTACTGT   1820
       T  F  R  D  L  G  A  Q  G  S  N  P  R  N  T  D  L  A  S  F  N  Y  C
1821  GGTTGTGGCTGGCCCCAACACATGTTGGTGCCAAAGGGAACTGAGAGTGGCACACCATATCAATTGTTTG   1890
      G  C  G  W  P  Q  H  M  L  V  P  K  G  T  E  S  G  T  P  Y  Q  L  F  V
1891  TTATGGTTTCCAACTACGAACTGGATTCGGTGATCCAAGATGATGGTTCAGAAATGTCATGCAAACAAGC   1960
       M  V  S  N  Y  E  L  D  S  V  I  Q  D  D  G  S  E  M  S  C  K  Q  A
1961  TTCAAGCTTCTGCGGTATTAGAGACAAGTTATTCCCAGACAGACGAGCTATGGGATACCCCTTCGACCGG   2030
       S  S  F  C  G  I  R  D  K  L  F  P  D  R  R  A  M  G  Y  P  F  D  R
2031  CCATCAAGCTCTGCCACCAACATCCAGGACTTTATACTTCCCAACATGTTGCTTACTGATATCTCTATCA   2100
      P  S  S  S  A  T  N  I  Q  D  F  I  L  P  N  M  L  L  T  D  I  S  I  K
2101  AACTGCAGAACATTACTGAACCAAATCCACGTAACCCTACCAATTAAATTACGAAAATAAGCTTTTCCAA   2170
        L  Q  N  I  T  E  P  N  P  R  N  P  T  N  *
2171  TTGATAAAAGTGCTCTTAAAAGTAAATGTAACTTGCTCTATAGGTTTAAGCTGCTTTTATAAATTCAAAT   2240
2241  ATATAATAGATATCTTGATTAGTAGTAACTACAAAAATGAAAGGCTGAATAAGTTAATCTTGTAAATATT   2310
2311  GTACATAATTTATGTTAAGATATAATAGTGGCCCTTAGATAATAAAAAGCATATAGAAAAAAAAAAAAAA   2380
2381  AAAAAAAAAAAAAAAAA  2397
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Fig. 1 Nucleotide and deduced amino acid sequences of *Pieris rapae* PPO cDNA

Amino acids are represented by one letter abbreviations. Double asterisks (**) indicate the putative cleavage site. Copper-binding sites A and B are underlined and double underlined, respectively. The conserved histidine (H) residues of two copper-binding sites are shown in bold letter. The thiol ester-like motif is shaded in grey. The asterisk (*) indicates the stop codon. The potential polyadenylation signal (AATAAA) is boxed

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PraPPO2  : MASVVKGLSLLFDRPNEPMVSPKGDDNAIFQISEQFLTDDYKNNGIEINNRFGENASEIVPLENL :  65
PxylPPO1 : -MADKNNLLLFFDRPTEPCFMQKGDDKTVFQIPDNFYPEKYKKVGNQLADRFGTDAGRMVPVRNI :  64
PxylPPO2 : MSDVISSLQLLFDRPNESLITPKGEKKAVFQLTEQFLPKEYESNGLELNNRFGDDASEKIPLKNL :  65
TmolPPO  : -MASKKNILLLFDRPREPVFIAKGSKKAVFSVPNEYLADKYKPLGVALTNRFGEDADERIDVKKI :  64
MdomPPO  : -MTDKKNLLLLFDRPTEPVFMEKGKTSTVFDVPDKYLTKRYERLGNEIQSRFGEKAEQRVPVREI :  64
AmelPPO  : MASDKSGILYLFDRPSEPVYVPKGDNKVAFDIPPDYLPDRYRSVATQVFNRFGDDTESKLPVKAI :  65

PraPPO2  : RRVPKLTKARRLPKDADFSLFLPAHREMADEVVDELMGVPENQLQQFLSTCTYARVNINPQLFNY : 130
PxylPPO1 : A-LPDLSLPQQLPYHNQFSLFVPKHRRMAAKLIDIFMGMR--DVEDLQSVCSYCQLRINPYMFNY : 126
PxylPPO2 : SRIPKFTKARQLPVDADFSLFLPKHQQMADEVIEELMAVPTNQLQDFLSTCAFARVNLNPQLFNY : 130
TmolPPO  : S-IPPFGEILELSRDENFSLFIPKHRRIAGRLIDIFLGMR--NVDDLVSVAVYARDRVNPYLFNY : 126
MdomPPO  : S-LPDLRIPMSLGRDEQFSLFVPRHRRIAGRLIDIFVGMR--TVDDLLSVAVYARDRVNPYLFNH : 126
AmelPPO  : T-LPDLSIPMQLGRRQPFSLFIPAHRKIAARLIDIFMGMR--TYEDFLSVAVYCRDRLNPNLFIY : 127

PraPPO2  : CFSVALMHRRDTRNVPIANFAETFPSKFVDSQVFAQARETAAVAAQGAPRTPIIIPRDYTATDLD : 195
PxylPPO1 : CLSVAMLHRPDTKGLSPPTLVETFPDKFMDPKVFRRARETSTTAPAGD-RMPVLIPVNYTASDAE : 190
PxylPPO2 : GFSVALMHRPDTKNVPIPNFAETFPSKFLDSQVFSQAREAAAVIPQDIPRTPITIPRDYTATDLE : 195
TmolPPO  : ALSVAILHRPDTQDVDLPSFIESFPDKYVDAKVFAQAREQATVVPEGS-RAPIEIPKDYTASDLE : 190
MdomPPO  : ALSVALLHREDTKGLDLPSFAQNFPDKFVDSQVFRQVREEATVVPDGS-RMPIVVPRDYTASDLD : 190
AmelPPO  : ALSVAILHRPDTKDLPVPPLTEVFPDKYMDSGIFSRAREEANVVPEGA-RVPIEIPRDYTASDLD : 191

                            *   *                            *
PraPPO2  : VEHRLAYFREDIGVNLHHWHWHLVYPFTAN---QREIVAKDRRGELFFYMHQQIIARYDGERLNN : 257
PxylPPO1 : PEQRIAYFREDIGINLHHWHWHLVYPFEAAD---RAVVDKDRRGELLYYMHQQIIARYNAERLCN : 252
PxylPPO2 : EEHRLAYFREDVGINLHHWHWHLVYPFSATGPNARAIVAKDRRGELFFYMHQQIIARYNGERLNN : 260
TmolPPO  : EEHRLAYFREDLGINLHHWHWHLVYPFEAA----REVVAKNRRGELFYYMHQQIIARYNFERLCN : 251
MdomPPO  : PEHRLWYFREDMGINLHHWHWHLVYPFEAGD---RRIVEKDRRGELFYYMHQQVIARYNMERFSN : 252
AmelPPO  : VEHRVAYWREDIGINLHHWHWHLVYPFEGD----IRIVNKDRRGELFYYMHQQIMARYNCERLCN : 252

PraPPO2  : SLPRVKKFNNFNEPIPEAYFPKLDSLTSSRGWPPRQANMRWQDLNRPVDGLNVTIADMQRWRRNI : 322
PxylPPO1 : NLGFVTRYNDFRGPIAEGYFPKMDSQVASRAWPPRFSGTTIRDLDRPVDQIRSDVSEMETWRDRF : 317
PxylPPO2 : SLKRVKKFANWREPIPEAYFPKLDSLTSSRGWPPRQAGMSWQDLNRAADNLHVSISDMERWRRNV : 325
TmolPPO  : KLKRATRFNDFKQAIQEAYFPKLDSLVASRSWPARVGNQRLKDLNREVDQIKQDVDDLKRWSDRI : 316
MdomPPO  : NLARVTRFNNFREPIAEGYFPKMDSLVASRAWPPRFDNTSIKDLNRELDQINLDISDLERWRDRI : 317
AmelPPO  : RLGRVKRFINWHEPIPEAYFPKLDSLVASRTWPFRPSGTVLKDINRQVDELNFDIQDLERWRDRI : 317

                                                           *   *
PraPPO2  : EDAIATGLVQLPNGSTRPLD----IDTLGNMVEAS-VLSPNMELYGSLHNNGHSFSAYMHDPTHR : 382
PxylPPO1 : IQAIDSGTIVLPNGRTQRLDEETGIDVLANLMESS-IISRNRAYYGDLHNMGHVFISYAHDPDHR : 381
PxylPPO2 : EEAIATGQVRLPNGQQQPLD----IDMLGNMMESSPLLSPNPELYGAIHNNGHSFSAYMHDPTHR : 386
TmolPPO  : YAAIHQGSATDERGRKIELTENEGIDILGNMIESS-ILSPNRTFYGDMHNMGHVFISYVHDPDHR : 380
MdomPPO  : FEAIHQGFVVDASGNRIPLDERRGIDILGNMLEAS-IISPNQSVYGDFHNMGHVFISYAHDPDHR : 381
AmelPPO  : YEAIHTGSVINTRGERIQLTEKNGIDVLGNIMEAS-ILSPNQNVYGDLHNFGHVAISYIHDPDHR : 381

                                  *
PraPPO2  : YLESFGVIGDEATTMRDPFFFRWHAFIDDLFQKYKESANVRPYTRSELENPGVRVTSARIDSSDG : 447
PxylPPO1 : HLEQFGVMGDPATAMRDPIFYRWHAYVDDIFQRYTAT--LPAYTRERLDFPGIRVSSIAISGR-- : 442
PxylPPO2 : YLESFGVIADEATTMRDPFFYRWHAWVDDTFQKHKESAYVRPYTRSELENPGVMVQSARIETTGS : 451
TmolPPO  : HLESFGVMGDSATAMRDPIFYRWHSYIDDIFQEYKAT--LPRYTENQLNFPGVTVSKVEVQVQGG : 443
MdomPPO  : HLESFGVMGDSATAMRDPVFYRWHAYIDDIFQEHKTR--LTPYTLPQLQYDGISISGLQVSSEGG : 444
AmelPPO  : YLESFGVMGDSATAMRDPIFYRWHAFVDDVFQEHKNT--LPQYTVQQLDFPGIEIADIKLTTNQ- : 443

PraPPO2  : -ATNELHTFWMSSDVDLSRGLDFSNRGPVYARFTHLNHRDFQYTIEVENNSNASR-GTVRIFIAP : 510
PxylPPO1 : -TPNQFSTQWEQSSVNLARGLDFMPRGAVLARFTHLQHDEFEYTIECDNTTGQAAMGTVRIFLAP : 506
PxylPPO2 : NQANTLNTFWMQSDVDLSKGLDFSNRGPVYARFTHLNHRPFRYVINVNNAGSARR-ATVRVFLAP : 515
TmolPPO  : -SANTLNTFWQQSDVDMSRGMDFQPRGSVFVRFTHLQHQPFTYKITVKNSSNGNRKGTCRIFIAP : 507
MdomPPO  : -QPNVLSTFWQQSDVDLSRGMDFVPRGNVFARFTHLQHTPFTYTINVNNDSGAQRFGTVRIFTAP : 508
AmelPPO  : -QRNILNTFWTKSDVDLSRGLDFTPRGAVLARFTHLNHADFSYTIVINNRNNTSMKGTVRIFIGP : 507

PraPPO2  : TNDERQLSWALSDQRKMFIEMDKFVVPLNRGRNVITRRSTESTVTIPFEQTFRDLGAQGSNPRNT : 575
PxylPPO1 : TTDQAGNALNFEEQRRLMIELDKFTQGLRPGSNTIRRRSIDSSVTIPYERTFRDESQRPGDAGSA : 571
PxylPPO2 : KVDERNLPWSLNDQRKMFVEMDRFVVPLNGGANTITRQSTESSVTIPFEQTFRDLSAQGNDPRQQ : 580
TmolPPO  : KLDERGNPWLYRDQKNMFVELDKFTVNLKQGQNNITRASSQSSVTIPFERTFRNLDLNRP-QGGE : 571
MdomPPO  : KTDERGQPWLFRDQRLMMVELDKFVVQLNPGQNTIRRRSTESSVTIPFERTFRNLEVNRPAQGSP : 573
AmelPPO  : KEDERGLPFTFREQKNLMIELDKFPITLQPGKNTIEQKSTKSSVTIPFERTFRNLDENRP-IGGD : 571

PraPPO2  : DLASFNYCGCGWPQHMLVPKGTESGTPYQLFVMVSNYELDSVIQDDGSEMSCKQASSFCGIRDKL : 640
PxylPPO1 : QSADFDFCGCGWPHHMLIPKGTQQGWNCVLFCMITNWNEDRVEQD--TVGTCNDAASYCGIRDRR : 634
PxylPPO2 : GLAAFNFCGCGWPQHMLVPKGTEAGAQYQLFVMLSNYELDQVEQPDGRQISCKEASSFCGLRDRL : 645
TmolPPO  : ELAQFNFCGCGWTQHMLIPKGTPEGMPCQLFVMISNYEDDKVNQS--TEGVCNDAGSYCGIKDKL : 634
MdomPPO  : EELEFNFCGCGWPQHMLIPKGLPGGMRCELFVMVSNYEDDRVDQT--LVGACSDAASYCGVRDRL : 636
AmelPPO  : SLERFDFCGCGWPQHMLIPKGNKEGFAMELFVMVSDYKDDRVEQN--EPIGCKDASSYCGLRDRK : 634

PraPPO2  : FPDRRAMGYPFDR-PSSSATNIQDFI-LPNMLLTDISIKLQNITEPNPRNPTN-------- : 691
PxylPPO1 : YPDRKPMGFPFDR-PAPSTGSLGDFL-TPNMTVQNCSIRFTDAVRQRQQR----------- : 682
PxylPPO2 : YPDRRPMGFPFDR-PSRSVTNIEDFV-LPNMALVDVTIRLQNVTERNPRNVQQ-------- : 696
TmolPPO  : YPDRRSMGYPFDRMPRNGVDTLQQFL-TSNMRVQDVTIKFTNRTVRPKSRN---------- : 684
MdomPPO  : YPDRRAMGYPFDRLPRQGVDRLVQFL-TPNMSIVDVSIRHDANRVVMRQ------------ : 684
AmelPPO  : YPDARAMGYPFDRQPRAGVETLAQFL-TGNMAVTEVTVRFSD-TIVPRSRSGSISNTLTFM : 693
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Fig. 2 Multiple alignment of PPO amino acid sequences

The sequences compared are from PraPPO2, *Pieris rapae* PPO-2 (HM210573); PxylPPO1, *Plutella xylostella* PPO-1 (ADA60206); PxylPPO2, *P. xylostella* PPO-2 (ADA60207); TmolPPO, *Tenebrio molitor* PPO (BAA75470); MdomPPO, *Musca domestica* PPO (AAR84669); AmelPPO, *Apis mellifera* PPO (AAO72539). Black and grey shadings indicate different degrees of overall conservation for each site (black, identical; grey, conservative). Six conserved histidines are marked with asterisks (*). Thiol ester-like motif is boxed

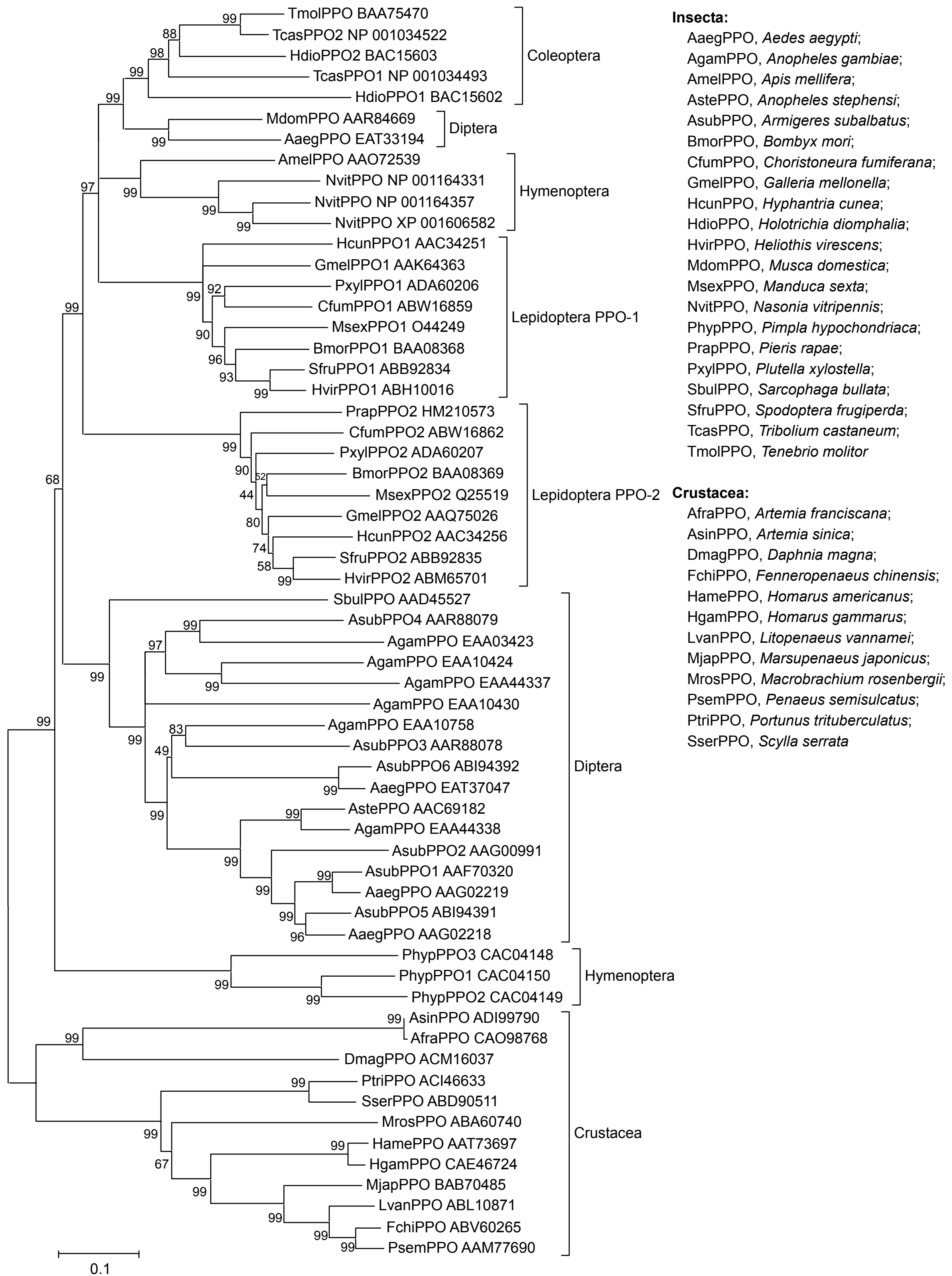

Fig. 3 Phylogenetic tree based on the neighbour-joining analysis of PPO amino acid sequences in different species of Insecta and Crustacea

The reliability of each branch was tested by 1000 bootstrap replications. Bootstrap values for each node are shown. GenBank accession numbers are listed for each PPO sequence

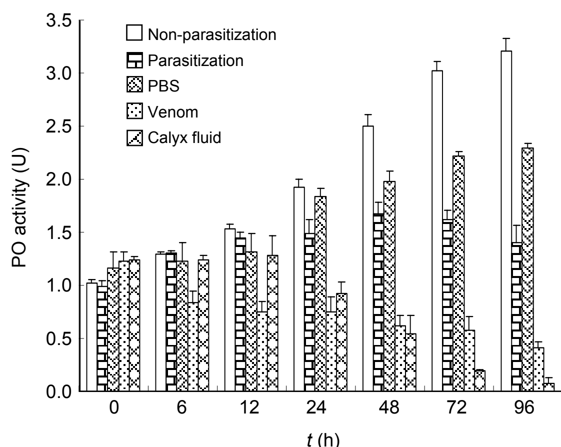

Fig. 4 PO activity profile in hemolymph of *Pieris rapae* after *Cotesia glomerata* parasitization and injection of *C. glomerata* venom or calyx fluid
Error bars represent standard deviation of three replications

3.3 Effects of venom and calyx fluid on PPO transcription

The relative mRNA profiles showed continuous increase in transcript levels of PPO in non-parasitized larvae with a steep rise between 24 and 96 h (Fig. 5). In contrast to non-parasitized larvae, PPO transcription in parasitized larvae did not significantly increase until 72 h after parasitization, and then the transcription significantly dropped down at 96 h. The transcription of PPO in PBS-injected larvae gradually rose up to 24 h after injection, and then kept at similar levels at between 48–96 h. In venom or calyx fluid-injected larvae, the PPO mRNA did not show differential expression compared to that of PBS-injected, non-parasitized, and parasitized larvae 12 h before treatment, and there were no significant differences between PBS and venom injection throughout 24–96 h. Significantly lower transcription levels were detected at 24–96 h, however, in calyx fluid-injected larvae. During this interval, the transcription levels in calyx fluid-injected larvae gradually decreased and reached the lowest level at 96 h.

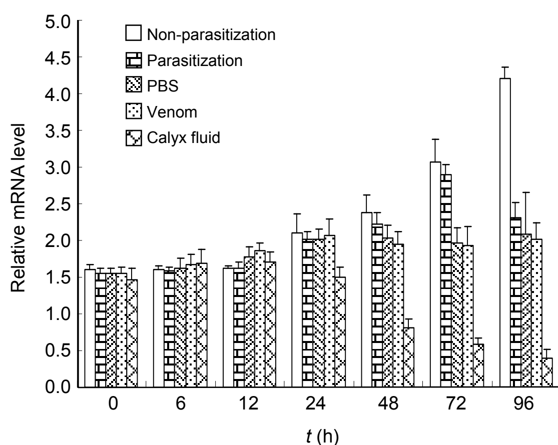

Fig. 5 PPO expression in haemocytes of *Pieris rapae* at different time intervals after *Cotesia glomerata* parasitization and injection of *C. glomerata* venom or calyx fluid
The levels of PPO transcription were analyzed by real-time RT-PCR using the SYBR® Green fluorescent probe. Error bars represent standard deviation of three replications

4 Discussion

Melanization reactions in insects as a common response to intruders occur due to changes in PO activity, an enzyme that is usually present in an inactive PPO form. As a potentially important immunity gene, PPO has been sequenced from a number of different insect species representative of Lepidoptera, Diptera, Coleoptera, and Hymenoptera (Park *et al.*, 1998; Cui *et al.*, 2000; Hartzer *et al.*, 2005; Lourenço *et al.*, 2005; Doucet *et al.*, 2008; Shelby and Popham, 2008). Two PPO isoforms have been characterized from several Lepidoptera such as *Manduca sexta*, *Bombyx mori*, *Galleria mellonella*, *Plodia interpunctella*, *Heliothis virescens*, and *Hyphantria cunea*, and in the case of some Diptera, many more PPOs have been characterized (Hartzer *et al.*, 2005). In this study, only one PPO cDNA cloned from *P. rapae*, and the protein deduced from it has a higher identity to PPO-2s reported in other lepidopteran species compared to other lepidopteran PPO-1s. Like in other lepidopteran insects PPO-2 from any species has a higher similarity to PPO-2s from other species than it does with PPO-1s from other species and its own PPO-1. The result presented here indicates that the protein encoded by *P. rapae* PPO gene cloned here is a PPO-2 representative. Signal peptide sequence present in the putative N-terminus has been noted in the case of few insect PPOs, and may be related to zymogen release via haemocyte lysis (Shelby and Popham, 2008). However, it was not detected in *P. rapae* PPO, as is the case in most arthropod PPOs (Olivares and Solano, 2009). PPOs lacking a signal

peptide necessary for secretion from endoplasmic reticulum are unlikely to have *N*-glycosylation associated with protein movement across the cell membrane (Hartzer *et al*., 2005). *N*-glycosylation has been demonstrated in *G. mellonella* PPO, and *P. interpunctella* PPO was a possible glycoprotein (Kopácek *et al*., 1995; Hartzer *et al*., 2005). Six potential N-linked glycosylation sites and other putative post-translational modifications, such as phosphorylation and myristoylation, which are usually involved in regulating translation, co-translation, and post-translation reactions, were present in *P. rapae* PPO, thus suggesting that it is released into the hemolymph by haemocyte rupture similar to PPOs of other insects lacking a signal peptide (Taft *et al*., 2001). A conserved N-terminal serine proteinase cleavage site for PPO activation by PPO-activating enzyme is predicted to locate at Arg50-Ser51 (Huang *et al*., 2001). Once pathogens and parasites invade the host, PPO would be cleaved and converted to the active PO by a serine protease cascade response (Söderhäll *et al*., 1994). Furthermore, as described for other insects, several highly conserved features, in particular two sites that code for copper-binding sites displaying the six canonical histidines involved in Cu^{2+} and oxygen binding, and a thiol ester-like motif, were present in *P. rapae* PPO (Jaenicke and Decker, 2004). These conserved features are common to PPO, suggesting a remarkable evolutionary conservation of the structure of this protein. Sequence comparison of PPOs suggests that copper-binding sites are present in a group of oxygen-transporting proteins of arthropods known as hemocyanins, suggesting that the origin of arthropod hemocyanins is ancient PPO-like proteins (Gaykema *et al*., 1984; Fujimoto *et al*., 1995; Burmester and Scheller, 1996). The domains of hemocyanin-N, hemocyanin-M, and hemocyanin-C were found in *P. rapae* PPO, indicating that this gene is a potential member of the hemocyanin gene family (Hughes, 1999). A thiol ester-like motif has been suggested to be responsible for binding to other macromolecules (Dodds and Day, 1996). The presence of a thiol ester motif in PPO may be related to the function of immobilization of invading organisms during the process of insect defense reactions (Sritunyalucksana *et al*., 1999; Lourenço *et al*., 2005). The phylogenic tree of the lepidopteran PPOs showed that they were divided into two groups, namely PPO-1 and PPO-2. The result is in good agreement with the previous findings that lepidopterans have two PPO genes (Cui *et al*., 2000; Hartzer *et al*., 2005; Lourenço *et al*., 2005; Shelby and Popham, 2008). The *P. rapae* PPO gene cloned here is obviously grouped into the PPO-2 clad, demonstrating that this gene is classified as PPO-2 type.

Partial or complete inhibition of hemolymph PO activity in host species attacked by parasitoid wasps has been reported from several systems (Stoltz and Cook, 1983; Beckage *et al*., 1990; Kitano *et al*., 1990; Beck *et al*., 2000; Shelby *et al*., 2000; Hartzer *et al*., 2005). The parasitoid associated virulence factors, such as PDVs, VLPs, venom, teratocytes, ovarian proteins, and parasitoid larval secretions, which are efficient to suppress host immune defense, are likely to do so with the regulation of host hemolymph melanization. For example, PDV of *Cotesia congregate* depressed hemolymph monophenoloxidase activity in *Manduca sexta* (Beckage *et al*., 1990), venom of *C. rubecula* inhibited melanization of *P. rapae* hemolymph (Asgari *et al*., 2003), and the feeding larvae produced factors of *Eulophus pennicornis* reduced host haemolymph PO activity (Richards and Edwards, 2003). In the *C. glomerata*-*P. rapae* system, teratocytes might contribute to the suppression of PO activity in the host hemolymph (Kitano *et al*., 1990). However, except for teratocyte, *C. glomerata* is also associated with venom and calyx fluid to knock out host's immune responses (Kitano, 1974; 1982; Kitano and Nakatsuji, 1978; Kitano *et al*., 1990; Ockroy *et al*., 2002; Madanagopal and Kim, 2006). Thus, *C. glomerata* venom and calyx fluid together may be involved in inhibiting the host's melanization. In order to test this hypothesis, it is important to investigate whether the PO activity is inhibited individually by *C. glomerata* venom and calyx fluid or not. In the present study, not only parasitism by *C. glomerata* was able to reduce the PO activity in *P. rapae* hemolymph, but also injection of *C. glomerata* venom or calyx fluid had the same effect, demonstrating that both venom and calyx fluid of this parasitoid are ascribed to the lack of melanization in the parasitized host. In addition, we found that both venom and calyx fluid injections induced significantly lower PO activity than parasitization.

This may be the result of significantly higher amounts of venom and calyx fluid manually injected into the host than that delivered by female wasp during oviposition (Cusson *et al*., 2000).

Parasitoid venom often mediates short-term immune suppression before PDV gene expression becomes effective (Webb and Luckhart, 1994). In accordance with this, at the first 6 h following injection of *C. glomerata* venom, *P. rapae* larvae showed a strong PO activity suppression. The venom induced a rapid decrease of PO activity, possibly due to its components exerting their action directly on the PPO activation cascade. In the same genus to *C. glomerata*, a venom protein (Vn50) from *Cotesia rubecula* inhibited melanization of *P. rapae* hemolymph by interfering with the PPO activation cascade (Asgari *et al*., 2003). Serpin from the venom of *Leptopilina boulardi* disrupted the activation pathway of PO in the *Drosophila* host (Colinet *et al*., 2009). Serine protease protein family involved in PPO signaling was best represented by *Nasonia vitripennis* venom constituents (de Graaf *et al*., 2010). Thus, regulation of PPO transcription by *C. glomerata* venom might not be the reason for PO activity reduction in *P. rapae* larvae. In the present study, *C. glomerata* venom injection had no observed effect on *P. rapae* PPO transcription.

In contrast to venom injection, PO activity in calyx fluid injected larvae was significantly lower than that detected in the controls until 24 h post treatment. Except for PDVs, the calyx fluid extract also contained non-viral proteins, ovarian proteins, which play a role in early and short-term immunosuppression (Webb and Luckhart, 1994). Thus, the results suggest that viral gene expression was required to inhibit melanization. Similarly, the delay in PO inhibition induced by PDVs observed here has been reported for other parasitic wasps, e.g., inhibition of PO activity in the hemolymph of *C. fumiferana* injected with *T. rostrale* calyx fluid was not elicited immediately following treatment, with maximum inhibition at 20 h after injection (Doucet and Cusson, 1996); inhibition of defensive melanization of *Heliothis virescens* increased after parasitization by *Campoletis sonorensis* in a time frame consistent with this wasp's CsIV expression (Shelby *et al*., 2000). The inhibitory effect of *C. glomerata* calyx fluid on *P. rapae* PO activity could be due to the down-regulation expression of PPO gene. A significant decrease in the transcript of PPO was found after 24 h injection with calyx fluid. However, there was no detectable PPO transcriptional difference between parasitized and non-parasitized larvae. Similarly, *C. fumiferana* injected with *T. rostrale* calyx fluid caused transient depressions in the titer of PPO mRNA levels, while *C. fumiferana* through natural parasitization by *T. rostrale* has no detectable impact on the abundance of PPO transcript (Doucet *et al*., 2008). As suggested by Doucet *et al*. (2008), significant reduction of PPO transcript resulting from calyx fluid injection could be due to the high PDV inoculum. In addition, there are several plausible reasons for PO activity reduced by calyx fluid, including depression in the activity and titer of PPO substrates, synthesis of PPO inhibitor, interference with the PPO-activating proteolytic cascade, and post-transcriptional disruption of PPO (Shelby and Webb, 1999; Shelby *et al*., 2000; Beck and Strand, 2007; Doucet *et al*., 2008).

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