Journal of Zhejiang University-SCIENCE B (Biomedicine & Biotechnology)
ISSN 1673-1581 (Print); ISSN 1862-1783 (Online)
www.zju.edu.cn/jzus; www.springerlink.com
E-mail: jzus@zju.edu.cn

Proteome changes in the plasma of *Pieris rapae* parasitized by the endoparasitoid wasp *Pteromalus puparum**

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Received Apr. 28, 2010; Revision accepted July 26, 2010; Crosschecked Jan. 7, 2011

Abstract: Parasitism by the endoparasitoid wasp *Pteromalus puparum* causes alterations in the plasma proteins of *Pieris rapae*. Analysis of plasma proteins using a proteomic approach showed that seven proteins were differentially expressed in the host pupae after 24-h parasitism. They were masquerade-like serine proteinase homolog (MSPH), enolase (Eno), bilin-binding protein (BBP), imaginal disc growth factor (IDGF), ornithine decarboxylase (ODC), cellular retinoic acid binding protein (CRABP), and one unknown function protein. The full length cDNA sequences of MSPH, Eno, and BBP were successfully cloned using rapid amplification of cDNA ends-polymerase chain reaction (RACE-PCR). Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis indicated that the transcript levels of MSPH and BBP in the fat bodies of host pupae were inducible in response to the parasitism and their variations were consistent with translational changes of these genes after parasitism, while the transcript levels of Eno and IDGF were not affected by parasitism. This study will contribute to the better understanding of the molecular bases of parasitoid-induced host alterations associated with innate immune responses, detoxification, and energy metabolism.

Key words: Proteomics, Plasma, Parasitism, Gene cloning, *Pteromalus puparum*, *Pieris rapae*
doi:10.1631/jzus.B1000158 **Document code:** A **CLC number:** Q96; Q51

1 Introduction

Host-parasitoid interactions represent integrating adaptations of considerable complexity involving the host's immune capacity to recognize and destroy the parasitoids, and the latter's ability to successfully invade the host and to circumvent its immune response (Carton and Nappi, 2001). Typically, parasitoids achieve this regulation by using an extensive array of immune-evasive or suppressive factors including venom, polydnaviruses (PDVs), virus-like particles (VLPs), teratocytes, ovarian proteins, and larval secretions (Beckage and Gelman, 2004; Schmidt, 2006). The destructive or subversive functions of these virulent factors on host physiology have been well documented (Stettler *et al*., 1998; Beckage and Gelman, 2004; Moreau and Guillot, 2005; Asgari, 2006; Pennacchio and Strand, 2006), while little is known concerning the molecular dissection of host-parasitoid interactions.

Pteromalus puparum (Hymenoptera: Pteromalidae) is a gregarious pupal endoparasitoid with a wide host range that prefers to parasitize the pupae of certain butterfly species (Dweck, 2009). It is the most predominant pupal parasitoid of *Pieris rapae* (Lepidoptera: Pieridae) with a parasitizing rate >90% in fields of cruciferous vegetables in China (Hu, 1984;

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* Project supported by the National Basic Research Program (973) of China (No. 2006CB102005), the National Natural Science Foundation of China (Nos. 30571251 and 30971959), the Zhejiang Provincial Natural Science Foundation of China (No. Z3090191), and the Program for New Century Excellent Talents in University of the Ministry of Education of China (No. NCET-05-0513)

Takagi, 1985). This parasitoid has evolved a unique means to manipulate its hosts, as no parasitoid associated factors other than venom are found in the female reproductive organ (Zhu *et al*., 2008a; 2008b; 2009a; 2009b). Its venom is able to alternate the morphological structures, population composition, and adhesive properties of host hemocytes, suppress a host's hemocyte-mediated immune reactions, induce endocrine changes, depress metabolic ability, disrupt development, and ultimately kill its host *P. rapae* (Cai *et al*., 2004; Zhang *et al*., 2005; Zhu *et al*., 2008a; 2008b; 2009a; 2009b). However, the actual role of *P. puparum* venom in overcoming *P. rapae* immune reactions to the benefit of the developing parasitoid larva remains to be elucidated. Here we investigated the plasma protein changes of *P. rapae* in response to parasitization by *P. puparum* using a proteomic approach, and found that a number of proteins, which were associated with cytoskeleton, detoxification, and energy mobilization involved in the immune response, were differentially expressed due to parasitization.

2 Materials and methods

2.1 Insect rearing, parasitization, and protein preparation

P. rapae was reared on cabbage (*Brassica oleracea* var. *capitata*) plants in a greenhouse throughout the year without any insecticide spray, and *P. puparum* was maintained on *P. rapae* pupae in a laboratory at (25±1) °C under a photoperiod of 10 h: 14 h (light:dark) as described by Cai *et al*. (2004). The parasitization was conducted following the methods described by Zhu *et al*. (2009b). Briefly, newly pupated hosts (within 2 h after pupation) were exposed to 2-d-old mated female wasps which had no previous contact with hosts. One host pupa and one mated female wasp were paired and transferred into a glass tube container (18 mm×82 mm). To avoid superparasitism, the parasitoid was removed immediately after a single oviposition was observed. At 24 h after treatment, hemolymph from at least five pupae for each analysis was collected, and was centrifuged briefly at 300×*g* to pellet the hemocytes as well as the eggs oviposited by *P. puparum*. Then the supernatant was centrifuged at 12000×*g* for 10 min at 4 °C to obtain plasma. Its protein content was adjusted to 500 μg for each set of experiments using a Bio-Rad detergent compatible (DC) protein assay kit (Bio-Rad, Hercules, CA, USA). Then plasma samples were stored at −70 °C prior to use.

2.2 Proteomic analysis

Proteomic analysis was performed according to Zhu *et al*. (2009a). Each 50 μl aliquot of sample containing 500 μg of plasma protein diluted in 300 μl rehydration solution (7 mol/L urea, 2 mol/L thiourea, 0.04 g/ml 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate (CHAPS), 0.5% Triton X-100, 65 mmol/L DL-dithiothreitol (DTT), 0.5% Bio-Lyte, and 10 μg/ml bromophenol blue) was loaded on each linear immobilized pH gradient (IPG) gel (ReadyStrips IPG Strips, pH range 3–10, 17 cm) for active rehydration at 50 V for 12 h. The first dimension was carried out for 80000 V·h. After isoelectric focusing the gels were equilibrated for 15 min with equilibration solution (50 mmol/L Tris-HCl (pH 8.8), 6 mol/L urea, 0.04 g/ml sodium dodecyl sulfate (SDS), 30% glycerol, and 0.03 g/ml DTT), and were incubated for another 15 min with equilibration solution using the same buffer containing 4% iodoacetamide in place of 0.03 g/ml DTT. For the second dimension, the gels were embedded in agarose across the top of a 0.05 g/ml stacking gel which overlaid a 0.12 g/ml polyacrylamide slab gel. The gels were stained with Coomassie blue. The gel images were scanned using GS-800 image analysis software (Bio-Rad) and analyzed with PDQuest software Version 7.1.0. The spot volume density values of differentially expressed protein spots were analyzed by Student's *t*-test at $P<0.05$ for treatment effect using the data processing system (DPS) package (Version 8.01 for Windows) (Tang and Feng, 2007). Protein spots of interest were excised from a gel following a tryptic digestion (Shevchenko *et al*., 1996) and analyzed on a Thermo Finnigan LCQ Deca XP Plus ion trap mass spectrometer system (Finnigan, San Jose, CA, USA). The mass spectra were subjected to a sequence database search with the SEQUEST search algorithm against the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/).

2.3 Gene cloning

Total RNA was isolated from the fat body of *P. rapae* using a homogenizer and TRIzol regent

(Invitrogen, Carlsbad, CA, USA) according to the manufacture's instruction. The rapid amplification of cDNA ends (RACE) was then performed using SMART™ RACE cDNA amplification kit (Clontech, CA, USA) to clone the full cDNA sequence of the differentially expressed proteins in the plasma of *P. rapae* induced by *P. puparum* parasitism. Briefly, cDNA was synthesized according to the manufacturer's instructions, priming by CDSIII/3′ polymerase chain reaction (PCR) and the SMART II A oligonucleotide primers for 3′ and 5′ RACEs, respectively. For 3′ and 5′ RACEs, their primers shown in Table 1 were designed based on the peptide sequences obtained by mass analysis and corresponding to the 3′ end fragments, respectively. The PCR amplification was carried out using Advantage 2 PCR kit (Clontech). The PCR products were separated in a 0.01 g/ml agarose gel electrophoresis, and target fragments were extracted and inserted into pGEM®-T-easy vector (Promega, San Luis Obispo, CA, USA), and then sequenced according to the dideoxy method with the CEQ Dye Terminator using 3730 DNA analyzer (Applied Biosystems, Foster City, CA, USA). The cDNA sequences and deduced amino acid sequences were compared with the sequences from the NCBI database using basic local alignment search tool (BLAST) program. Multi-sequence alignment was conducted by ClustalX Version 1.83 (Thompson *et al*., 1997).

2.4 Reverse transcriptase (RT)-PCR

Transcript levels of the proteins differentially expressed between non-parasitized and parasitized *P. rapae* pupae were verified using RT-PCR. In each sampling time, the total RNAs of the fat bodies from non-parasitized and parasitized hosts were isolated using TRIzol reagent (Invitrogen). The concentration of each RNA sample was adjusted to be the same in all samples, and treated with RQ1 RNase-Free DNase (Promega, Madison, WI, USA) to eliminate any remaining DNA. cDNA, as the template for PCR, was synthesized using a RevertAid™ first strand cDNA synthesis kit (Fermentas, St. Leon-Rot, Germany), and primers are shown in Table 1. The PCR reaction consisted of 3 min at 94 °C, 30 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min, and a final extension at 72 °C for 10 min. The *18S RNA* gene of *P. rapae* was used as a reference gene.

3 Results

3.1 Differentially expressed proteins in the plasma induced by parasitism

A comparison of the two-dimensional electrophoresis gels between parasitized *P. rapae* plasma and non-parasitized controls showed that seven proteins were significantly ($P<0.05$) differentially expressed after parasitization by *P. puparum* (Fig. 1). Five of these proteins were up-regulated with an intensity 1.8, 2.6, 2.2, 1.7, and 2.3 times greater than those in control (Spots 2, 3, 5, 6, and 7), one was down-regulated 0.65 times the intensity (Spot 4), while one was represented by a new spot (Spot 1) (Fig. 2).

Table 1 Primers used in the full length cDNA cloning and RT-PCR

Gene	Primer sequence
3′ RACE	
MSPH	5′-CCNTAYCAAGAYMGNACNGTCAARGARAT-3′
Eno	5′-GCKTGYAACTGYCTSCTSCTGAAGGT-3′
BBP	5′-TGYGGATGGGCTGARTACNCCTGAAG-3′
5′ RACE	
MSPH	5′-CACTGGCTCGATCTTGAGTATGGCAACC-3′
Eno	5′-CCGATTTGATTGACCTTCAGGAGGAGAC-3′
BBP	5′-GTTCTCCCTGGTGACGCCTCCGTATGTG-3′
RT-PCR	
MSPH	F: 5′-AACCAGACGCTAGACATTGAG-3′; R: 5′-CCAGACCACGAAGGAGATTTA-3′
Eno	F: 5′-AGAATCCTAAGTCAAACCCTA-3′; R: 5′-CAGGAGGAGACAGTTACAAGC-3′
BBP	F: 5′-GCTGGCTCCCGTTCAAGAATC-3′; R: 5′-CCTGGTGACGCCTCCGTATGT-3′
18S RNA	F: 5′-CAGTGATGGGATGAGTGCTTT-3′; R:5′-TACGCTATTGGAGCTGGAATT-3′

RACE: rapid amplification of cDNA ends; *MSPH*: masquerade-like serine proteinase homolog; *Eno*: enolase; *BBP*: bilin-binding protein; F: forward; R: reverse

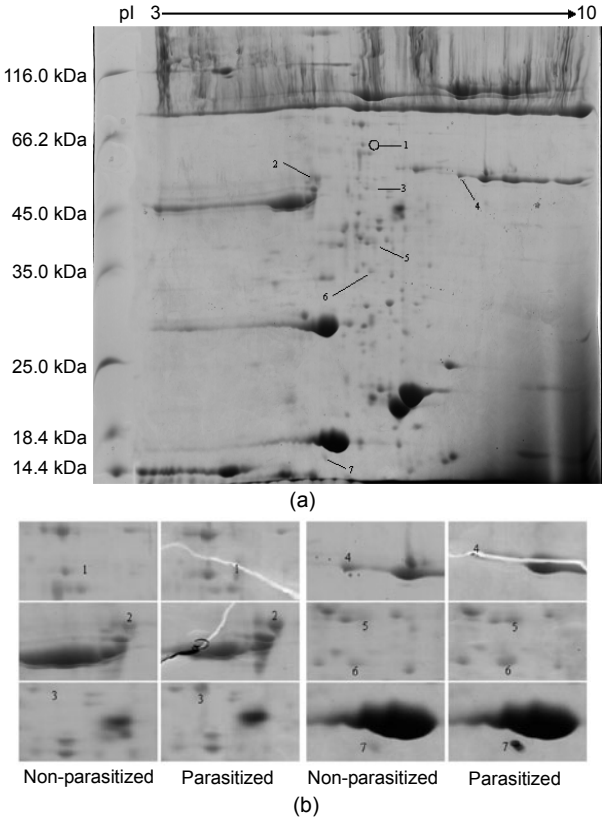

Fig. 1 Two-dimensional gel map of *Pieris rapae* plasma proteins

(a) Plasma from the non-parasitized pupae. The numbers on the gel indicate the spots which were differentially expressed after parasitization and were applied to introduction to liquid chromatography/mass spectrometry/mass spectrometry (LC-MS/MS); (b) Gel areas of differentially expressed proteins related to parasitization

All these differentially expressed proteins were excised for identification by mass spectral analysis. The proteins were identified as the following: masquerade-like serine proteinase homolog (MSPH), enolase (Eno), bilin-binding protein (BBP), imaginal disc growth factor (IDGF), ornithine decarboxylase (ODC), cellular retinoic acid binding protein (CRABP), and one unknown protein (Table 2).

3.2 cDNA clone and sequence analysis

Peptide sequences of the differentially expressed proteins obtained from the mass spectral analysis were used to design 3′ RACE degenerate primers for

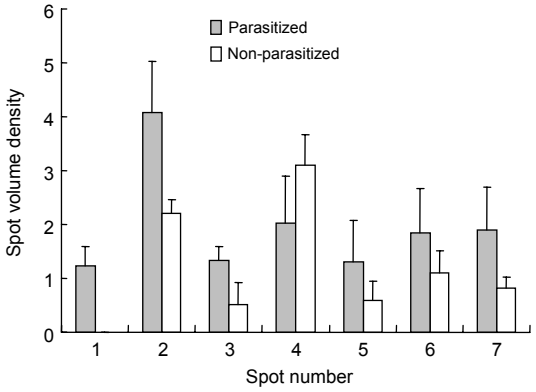

Fig. 2 Quantification of differentially expressed proteins in *Pieris rapae*

Spot volume density was quantified by scanning the optical density (OD) intensities on the gels. Values are shown as mean±standard deviation (SD)

Table 2 List of differentially expressed proteins identified by LC-MS/MS in plasma of *Pieris rapae* after parasitized by *Pteromalus puparum*

Spot No.[a]	Protein identification[b]	Species origin	M_W (Da)[c]	pI[d]	GI[e]	Identified peptide[f]
1	PREDICTED: similar to CG12283-PA	*Apis mellifera*	69740.98	6.90	66499167	YAEATIGENASIVCR
2	MSPH	*Bombyx mori*	45937.83	4.96	25992174	EIYPYQDRTVKEIVIHK; TVKEIVIHKDFNK
3	Eno	*Aedes aegypti*	46621.15	6.28	108882996	ACNCXLLK
4	IDGF	*Pieris rapae*	48086.77	8.10	47607477	EADYSAPTYKPQNR; IVVGIATYAR; KTFGTTLVDEK; KTFGTTLVDEKEAEHR; MVPLNENLDVDR; TEGLLSYPEVCAK; TFGTTLVDEK; TFGTTLVDEKEAEHR; VLLSVGGDADKEEEQK; YNLLLESPQAR
5	ODC	*Aedes aegypti*	46522.29	6.61	108884773	KSKVNLGSK
6	BBP	*Pieris brassicae*	21305.95	6.59	434995	CGWAEYTPEGK; EYFIEGTAYPVGDSK; LVYSDFSEAACK; NYIIGYYCK
7	CRABP	*Plutella xylostella*	14705.64	6.35	49532918	AANAVTPTVELKK

[a] Spot numbers are indicated in Figs. 1 and 2; [b] Proteins were identified by searching the National Center for Biotechnology Information (NCBI) non-redundant database with peptide fragmentation data using the SEQUEST search algorithm. MSPH: masquerade-like serine proteinase homolog; Eno: enolase; IDGF: imaginal disc growth factor; ODC: ornithine decarboxylase; BBP: bilin-binding protein; CRABP: cellular retinoic acid binding protein; [c] Theoretical molecular mass of the protein; [d] Theoretical isoelectric point of the protein; [e] Prefix "GI" refers to protein entry code of the NCBI; [f] Sequence information obtained from peptide fragmentation data

cloning the 3′ end fragments, and then the 5′ end fragments were cloned by 5′ RACE. The full length cDNAs of MSPH, Eno, and BBP were successfully obtained. The complete cDNA of MSPH was 1380 bp in length and contained a 1245-bp open reading frame (ORF) with 78 bp of 3′ untranslated region (UTR) and 57 bp of 5′ UTR. The putative protein of 415 amino acids (aa) has a calculated molecular weight and pI of 45.22 kDa and 5.55, respectively (GenBank accession No. FJ882067). A 17-aa putative signal peptide (MMNKLLVLIAVLSLAYC) rich in hydrophobic residues was found at the N-terminus of the deduced amino acid sequence. The deduced amino acid sequence of MSPH displayed identity with those of *Bombyx mori* (64%), *Holotrichia diomphalia* (42%), and *Tenebrio molitor* (36%) (Fig. 3). The full length cDNA of Eno was 1670 bp, which consisted of a 255-bp 5′ UTR, an ORF encoding 434 aa with a calculated molecular weight and pI of 47.11 kDa and 6.03, respectively, and a 113-bp 3′ UTR (GenBank accession No. FJ882068). No signal peptide was predicted in the deduced amino acid sequence. The sequence of the predicted protein revealed the highest level of overall identity (77%–90%) with Enos of other insects, *B. mori*, *Aedes aegypti*, and *Blattella germanica* (Fig. 4). The full cDNA sequence of BBP was 854 bp, comprising of a putative ORF of 570 bp encoding a 434-aa protein with a predicted molecular mass of 21.31 kDa and a pI of 7.25, flanked by a 5′ UTR of 233 bp and a 3′ UTR of 52 bp (GenBank accession No. FJ882066). The hydropathy profile of the deduced amino acid sequence of BBP suggested that its N-terminal amino acid sequence was followed by a cluster of 15 hydrophobic amino acid indicating a signal peptide (MQYLIVLALVAAASA). The amino acid sequence of BBP showed a high identity with those of *Pieris brassicae* (94%) and *Papilio xuthus* (41%) (Fig. 5).

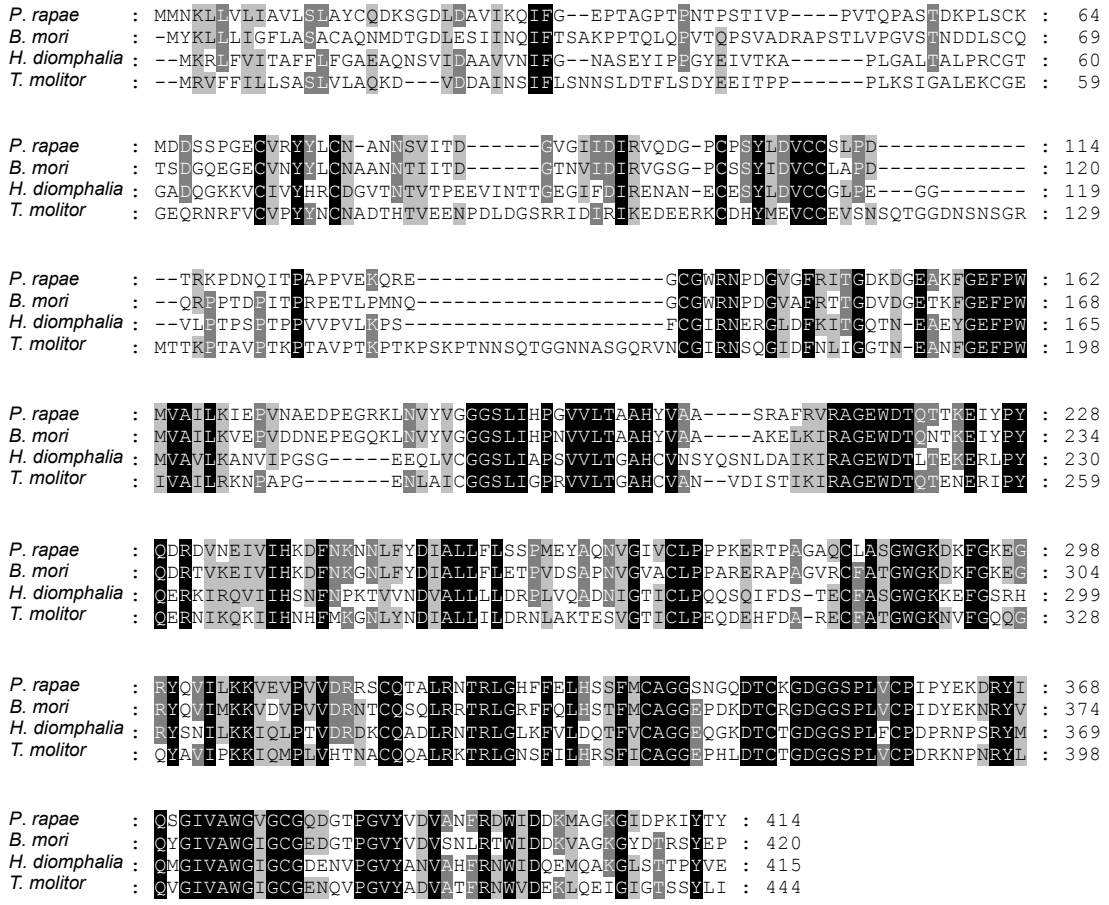

Fig. 3 Multiple alignments of MSPH protein sequences from *Pieris rapae*, *Bombyx mori*, *Holotrichia diomphalia*, and *Tenebrio molitor*

The amino acid sequences shown in the alignments are *B. mori* (AAN77090), *H. diomphalia* (CAC12665), and *T. molitor* (BAC15605). Identical amino acids are shaded in black and similar amino acids are shaded in grey

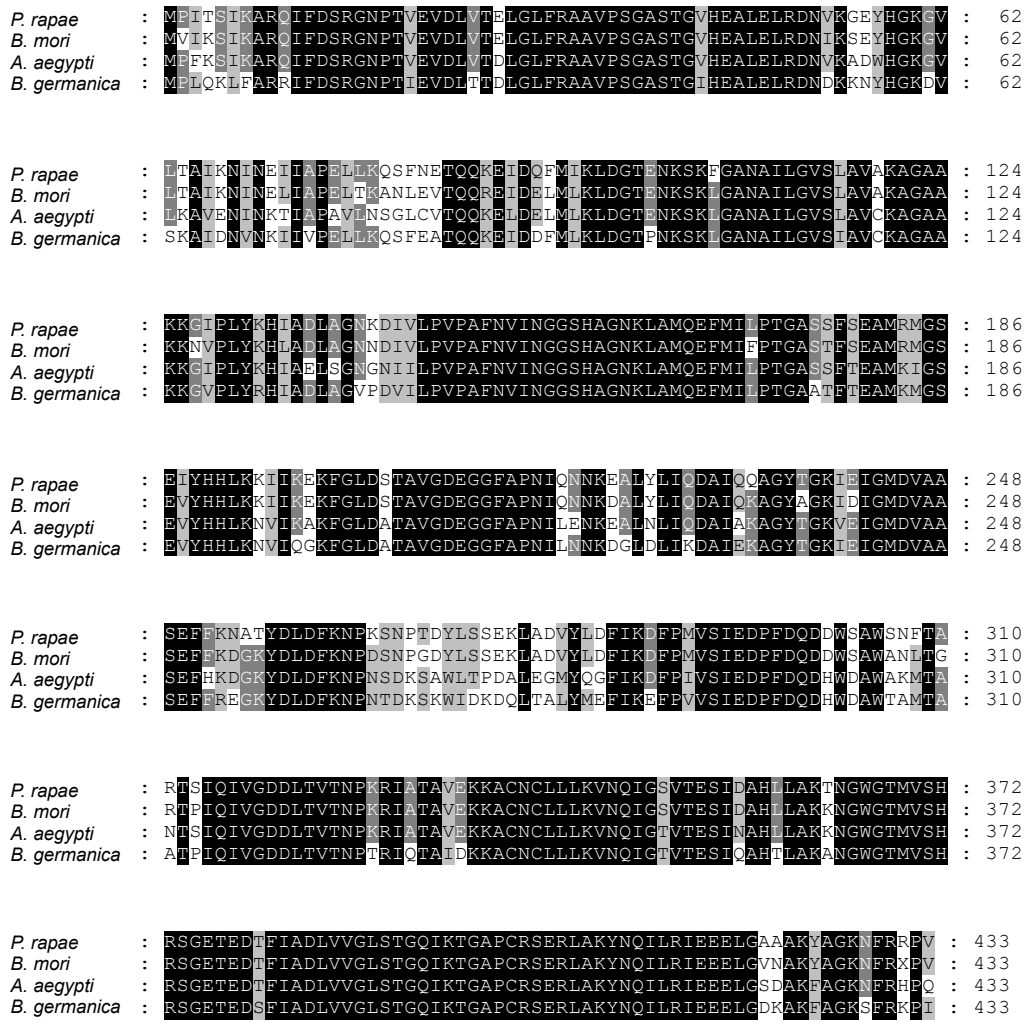

Fig. 4 Multiple alignments of Eno protein sequences from *Pieris rapae*, *Bombyx mori*, *Aedes aegypti*, and *Blattella germanica*

The amino acid sequences shown in the alignments are *B. mori* (NP_001091831), *A. aegypti* (XP_001653750), and *B. germanica* (ABC96322). Identical amino acids are shaded in black and similar amino acids are shaded in grey

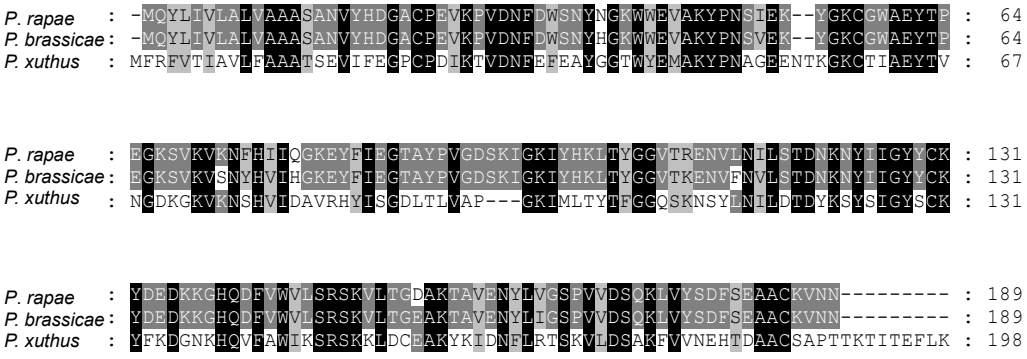

Fig. 5 Multiple alignments of BBP protein sequences from *Pieris rapae*, *Pieris brassicae*, and *Papilio xuthus*

The amino acid sequences shown in the alignments are *P. brassicae* (P09464) and *P. xuthus* (BAG12758). Identical amino acids are shaded in black and similar amino acids are shaded in grey

3.3 Regulation at mRNA level of differentially expressed protein by parasitism

To assess the mRNA expression profile of differentially expressed proteins in *P. puparum* parasitized versus non-parasitized hosts pupae, total RNAs were extracted from the *P. rapae* pupae fat bodies at 0, 4, 8, 12, 16, 20, 24, 36, and 48 h post parasitization. We employed a semi-quantitative PCR method to obtain the expression profile of these genes during these time intervals (Fig. 6). During the first 12 h following parasitization, the transcript level of *MSPH* in the parasitized fat body was similar to that in the non-parasitized control at the same sampling times. However, the transcript level of *MSPH* was significantly greater after 12 h post parasitization in the parasitized fat bodies compared to non-parasitized controls. Parasitization did not affect the *Eno* and *IDGF* transcript levels throughout the investigation period. As to the *BBP*, no changes in transcript levels were observed in the first 8 h following parasitization. They were, however, up-regulated from 12 to 48 h.

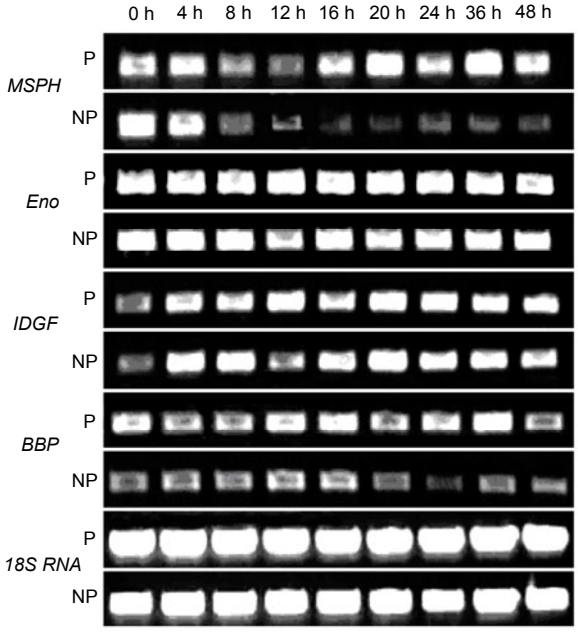

Fig. 6 Expressions of *MSPH*, *Eno*, *IDGF*, and *BBP* in fat bodies of *Pieris rapae* pupae at different time points after parasitization (P) by *Pteromalus puparum* and in control non-parasitized (NP) pupae

RNA samples were obtained from fat bodies of the hosts at 0, 4, 8, 12, 16, 20, 24, 36, and 48 h after parasitization. The gene expression was standardized with that of the *18S RNA* expression level

4 Discussion

Parasitoids successfully develop in or out of the bodies of their hosts, depending on their ability to effectively overcome the host's immune response and regulate its physiology to benefit their own development. Regulation of a host's immunity and physiology was shown to target the host hormones or the hemolymph protein content by the active factors such as venom, PDVs, and VLPs produced and released by parasitoids (Pennacchio and Strand, 2006). In several host-parasitoid systems, alteration of the amino acid and protein compositions of the host hemolymph has been extensively described (e.g., the amount of total proteins in the hemolymph of *Pseudaletia separate* increased when *Euplectrus* sp. near *plathypenae* venom was injected; free amino acid levels and total soluble protein titers of the 3rd instar boll weevil *Anthonomus grandis* were changed by the venom of *Bracon mellitor*; and, the nutrients that *Cotesia kariyai* larvae require for their growth increased in the hemolymph of the *Pseudaletia separata* during the 2nd stadium of the parasitoid larva) (Guerra *et al*., 1993; Coustau *et al*., 1996; Nakamatsu *et al*., 2001; Richards and Edwards, 2001; Nakamatsu and Tanaka, 2003; Consoli and Vinson, 2004). Thus, although this phenomenon has been well documented in several parasitoid-host systems, few studies have been carried out to identify the differentially expressed proteins induced by parasitism (Brandt *et al*., 1996; Kaeslin *et al*., 2005; Reineke and Löbmann, 2005; Mahadav *et al*., 2008; Nguyen *et al*., 2008; Song *et al*., 2008). In this study, by comparing the two-dimensional electrophoresis patterns of *P. puparum* parasitized and non-parasitized *P. rapae* plasma, seven proteins were found to be differentially expressed and were subjected to mass spectral analysis for identification. The results indicated that these proteins were related to immunity, cytoskeleton, cell detoxification, and energy metabolism.

The humoral immune response is one of the major ways for hosts to defend themselves against invasion, and is mainly exhibited as hemolymph melanization, a biological reaction process involving prophenoloxidase, prophenoloxidase activating enzyme, and other serine proteases (Ashida and Brey, 1998; Vass and Nappi, 2000). In order to overcome the host's humoral immune response, a parasitoid

needs to block the host's hemolymph melanization. Some investigations have found that host prophenoloxidase activating enzyme and phenoloxidase activities can be decreased by parasitization (Lavine and Beckage, 1995; Asgari, 2006). Mature phenoloxidase circulating in the plasma originates from prophenoloxidase activated by its activating enzyme, while MSPH is a key regulator for prophenoloxidase activating enzyme (Gupta *et al*., 2005; Amparyup *et al*., 2007). In this study, MSPH in the plasma of *P. rapae* was significantly up-regulated by *P. puparum* parasitization, suggesting that this wasp could disrupt the host's hemolymph melanization. However, this needs to be experimentally confirmed. IDGF and ODC are closely associated to cell differentiation and growth (Zhang *et al*., 2006), and their expression levels change under stress (Untalan *et al*., 2005; Francis *et al*., 2006). CRABP is involved in lipid metabolism, and its abnormal expression will result in energy metabolism disorder and immune response reduction (Mansfield *et al*., 1998). Eno is not only a key factor for controlling energy metabolism, but also is an immunosuppressive factor involved in phosphoenolpyruvate synthesis (Veiga-Malta *et al*., 2004). The abnormal expressions of these proteins, to some extent, may explain the effects caused by *P. puparum* venom, such as causing haemocyte death, inhibiting haemocyte spreading and encapsulation, and inducing the changes of hemolymph nutrients of *P. rapae* (Cai *et al*., 2004; Zhang *et al*., 2005).

The full length gene sequences of *MSPH*, *Eno*, and *BBP* were successfully cloned using the RACE technique. Protein differential expression can be ascribed by the alteration of its gene transcription and translation. After the genes of *MSPH*, *Eno*, and *BBP* of *P. rapae* were cloned, and the *IDGE* gene of *P. rapae* was retrieved from the NCBI database, the transcription levels of these genes affected by *P. puparum* parasitization were investigated using semi-quantitative RT-PCR analysis. The results showed that *MSPH* and *BBP* gene transcription levels were elevated by parasitization, which was consistent with their protein content increased after parasitization. However, the gene transcription levels of *Eno* and *IDGF* were not impacted by parasitization. The differential expressions of *Eno* and *IDGF* in the plasma of *P. rapae* may be the result of their translation level altered by *P. puparum* parasitization. For example, the storage protein and juvenile hormone esterase transcription levels of *Heliothis virescens* were not affected by *Campoletis sonorens* parasitization, but the translation levels of these two genes were changed after parasitization, eventually leading to the titers of these two proteins being altered (Shelby and Webb, 1997); the growth-blocking peptide gene transcription level of *Pseudaletia separata* did not change after parasitization by *Cotesia kariyai*, but its translation level was up-regulated, ultimately blocking the host's growth and development (Hayakawa *et al*., 1998). It would be helpful in this regard to study the effect of *P. puparum* parasitism on the translation of the differential expression proteins of *P. rapae*. Protein expression of the full-length cDNA cloned in this study would be useful to produce antibodies needed for such an investigation.

Acknowledgements

We thank the Research Center for Proteome Analysis located at the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences for performing proteomic mass spectrometry analyses. We are also grateful to the anonymous reviewers for valuable comments on the manuscript and English revisions.

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