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Proteome changes in the plasma of *Papilio xuthus* (Lepidoptera: Papilionidae): effect of parasitization by the endoparasitic wasp *Pteromalus puparum* (Hymenoptera: Pteromalidae)*

Jia-ying ZHU, Gong-yin YE†‡, Qi FANG, Cui HU

(*State Key Laboratory of Rice Biology, Institute of Insect Sciences, Zhejiang University, Hangzhou 310029, China*)

†E-mail: chu@zju.edu.cn

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Abstract: Although the biochemical dissection of parasitoid-host interactions is becoming well characterized, the molecular knowledge concerning them is minimal. In order to understand the molecular bases of the host immune response to parasitoid attack, we explored the response of *Papilio xuthus* parasitized by the endoparasitic wasp *Pteromalus puparum* using proteomic approach. By examining the differential expression of plasma proteins in the parasitized and unparasitized host pupae by two-dimensional (2D) electrophoresis, 16 proteins were found to vary in relation to parasitization compared with unparasitized control samples. All of them were submitted to identification by mass spectrometry coupled with a database search. The modulated proteins were found to fall into the following functional groups: humoral or cellular immunity, detoxification, energy metabolism, and others. This study contributes insights into the molecular mechanism of the relationships between parasitoids and their host insects.

Key words: Proteomics, Plasma, Parasitism, Immune response, *Pteromalus puparum*, *Papilio xuthus*
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INTRODUCTION

The insect's innate immune system comprises an array of cellular and humoral defensive responses that are highly efficient against attacks by pathogens or parasites (Lavine and Strand, 2002). However, as potent regulators of insect hosts, parasitic wasps can use an extensive array of immune-evasive or suppressive factors including venoms, polydnaviruses (PDVs), virus-like particles (VLPs), and ovarian fluids to suppress the host immune responses. These factors create a suitable microenvironment for successful completion of parasitoid offspring development in the hemocoel or at the external surface of host insects (Stettler *et al*., 1998; Turnbull and Webb, 2002; Asgari, 2006; 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; Asgari, 2006; Moreau and Guillot, 2005; Pennacchio and Strand, 2006), although little is known so far on the molecular basis of these alterations. Over the last several years, the mechanism of host response to parasitization at the molecular level has become a focal point of research. Some investigators are analyzing the host transcriptional response to parasitoid attack. A large number of differentially expressed genes related to parasitism have been identified by employing mRNA-based approaches such as microarray and amplified fragment length polymorphism (AFLP) to measure message abundance

‡ Corresponding author

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(Reineke and Löbmann, 2005; Wertheim *et al*., 2005; Barat-Houari *et al*., 2006; Schlenke *et al*., 2007; Carton *et al*., 2008). Despite increasingly available information on the alteration of host gene transcription caused by parasitism, little is known on the alterations of proteins (Dong *et al*., 1996; Nakamatsu *et al*., 2006; Salvador and Cônsoli, 2008). In addition, mRNA-based approaches displayed a poor correlation between the mRNA level and protein abundance, as such methods cannot either ensure that transcripts are actually translated, nor be used to analyze post-transcriptional fate of mRNA (Song *et al*., 2008). Recently, even several studies were involved in applying the powerful protein-based methods, proteomics, to investigate the host plasma changed by parasitoid (Kaeslin *et al*., 2005; Song *et al*., 2008; Nguyen *et al*., 2008); however, much further work is necessary. Moreover, in the investigations carried out at the gene or protein levels, the parasitoids were associated with PDVs or VLPs. No studies to date have demonstrated the regulation of host transcription or protein expression by venom alone. Venom is an important parasitoid maternal factor that can act alone or in conjunction with other factors (such as PDVs or VLPs) to affect the parasitoid-host intricate interaction. In addition, understanding how venom operates can help with the discovery of new biologically active molecules for their potential applications in medicine and agriculture (Beckage and Gelman, 2004; Moreau and Guillot, 2005; Pennacchio and Strand, 2006). To investigate the molecular mechanisms of parasitoid venom impact on the hosts is not only helpful to understand the parasitoid-host relationship, but is also invaluable to gain insights into the potential properties of this biological factor for future practical use.

In the present study, we investigated the host physiological changes at the protein level associated with envenomation by the endoparasitic wasp *Pteromalus puparum* (Hymenoptera: Pteromalidae). This wasp was chosen as a study model, as no other parasitoid-associated factors other than venom are found in the female reproductive organs (Cai *et al*., 2004; Zhu *et al*., 2008a; 2008b). Early studies revealed that its venom can inhibit cellular immune response, depress metabolic ability, disrupt development, and ultimately kill its host *Papilio xuthus* (Lepidoptera: Papilionidae) (Zhang *et al*., 2005). However, the exact mechanism by which *P. puparum* overcomes host defense reactions and regulates host physiology remains to be defined. We thus carried out a proteomics study to identify the proteomic changes in the plasma of *P. xuthus* pupae following parasitization by *P. puparum*, and found that a number of proteins associated with insect innate immunity were differentially expressed, providing some clues to the mechanisms of parasitoid-host interactions from a molecular prospective.

MATERIALS AND METHODS

Insects and parasitization

A colony of the pupal endoparasitoid, *P. puparum*, was maintained as described by Cai *et al*.(2004). *P. xuthus* larvae, of which pupae can be naturally parasitized by *P. puparum* (Hu, 1984), were collected from orange trees in Huangyan, Zhejiang, China, and reared on orange leaves until pupation in the laboratory. For parasitization experiments, newly pupated *P. xuthus* (within 2 h after pupation) was exposed to 3-d-old mated female wasps, which had no previous contact with hosts. One host pupa together with one mated female wasp was transferred into a glass container (18 mm×82 mm). In order to avoid superparasitism, the parasitoid was removed immediately after a single oviposition was observed. The parasitized and unparasitized pupae were then cultured at (25±1) °C under a photoperiod of 10 h:14 h (light:dark).

Protein preparation

Pupae were anaesthetized on ice 24 h after parasitization. To collect haemolymph, the pupal elytrum was pierced with a minute sterile pin under sterile conditions. Haemolymph from at least five pupae for each analysis was collected into an Eppendorf tube on ice. To obtain plasma, haemolymph was first centrifuged briefly at 300×*g* to dump the haemocytes and then the supernatant was centrifuged at 12000×*g* for 10 min at 4 °C. The supernatant was stored at –70 °C prior to use. Protein concentration in the plasma was measured with the DC Protein Assay (Bio-Rad, Hercules, CA, USA). Plasma volumes (parasitized/unparasitized) were adjusted in order to analyze the same amount protein of 500 μg for each set of experiments.

Two dimensional (2D) gel electrophoresis

Apparatus and chemicals were supplied by Bio-Rad (Hercules, CA, USA). Active rehydration and isoelectric focusing (IEF) were carried out on a PROTEIN® IEF Cell at 17 °C according to the manufacture's guide. 50 μl of plasma was dissolved in 300 μl rehydration solution including 7 mol/L urea, 2 mol/L thiourea, 4% (w/v) 3-[(3-cholamidopropyl) dimethylammonio]propanesulfonic acid (CHAPS), 0.5% (v/v) Triton X-100, 65 mmol/L dithiothreitol (DTT), 0.5% (v/v) Bio-Lyte, and 0.001% (w/v) bromophenol blue. Samples were loaded on linear immobilized pH gradient (IPG) strips (ReadyStrips IPG Strips, pH range 3~10, 17 cm). The active rehydration of the IPG strips was performed at 50 V for 12 h. Then, IEF was carried out following the four steps: 250 V for 30 min, 250~1000 V for 30 min, 1000~10000 V for 3 h, and at 10000 V to 80000 V·h. The current was limited to 50 μA per strip. The strips were equilibrated for 15 min in 50 mmol/L Tris-HCl buffer (pH 8.8) containing 6 mol/L urea, 4% (w/v) sodium dodecyl sulfate (SDS), 30% (v/v) glycerol, and 3% (w/v) DTT. This step was repeated using the same buffer containing 4% (v/v) iodoacetamide in place of 3% (w/v) DTT. The 2D gel electrophoresis was performed at 17 °C at 25 mA per gel under denaturing conditions (SDS polyacrylamide gel electropheresis (SDS-PAGE), 12% (w/v) separate gel with 5% (w/v) stacking gel) on a PROTEIN II xi apparatus. The gels were stained with 0.1% (w/v) Coomassie blue (R-250) in 40% (v/v) methanol and 10% (v/v) acetic acid, and then destained with the same solution without Coomassie blue. For image analysis, the gels were scanned on GS-800 image analysis software (Bio-Rad) calibrated densitometer and analyzed with the PDQUEST software V 7.1.0. Gel images were normalized so that the total density in each analyzed gel was made equal. After background subtraction (filtered image), the spots were automatically detected even though verification by visual inspection was still needed. To define the differential expression of proteins, the spot volume density values for the three gel images of parasitized and unparasitized samples were calculated using the PDQUEST software. Spot volumes (spot optical intensities) were analyzed by *t*-test at $P<0.05$ for treatment effect using the DPS package (Version 8.01 for Windows) (Tang and Feng, 2007).

Protein digestion and mass spectrometer (MS) identification

Protein spots of interest were excised with a scalpel and subjected to tryptic digestion (Shevchenko *et al*., 1996). The resulting peptide fragments were analyzed on a ThermoFinnigan LCQ Deca XP plus ion trap mass spectrometer system (Finnigan, San Jose, CA, USA) with a Protana nanospray ion source interfaced to a self-packed 15 cm×150 μm i.d. Phenomenex Jupiter 10-μm C18 RP capillary column (Phenomenex, Torrence, CA, USA) with some parameter modifications as described in Pellitteri-Hahn *et al*.(2006). Solvent A was 0.1% (v/v) formic acid in H_2O_2 and solvent B was 0.1% (v/v) formic acid in acetonitrile. The protein digest injected into the microcapillary column was resolved at the rate of 1 μl/min, by the following gradient conditions: 0→7 min 97% A, 7→58 min 3%→40% B, 58→64 min 40%→90% B, 64→70 min 90% B, and 70→75 min 90%→3% B. The resulting MS/MS spectra were analyzed by database searching using the SEQUEST search algorithm against the National center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/). Peptides were considered as being positively identified if they passed the following criteria: charge +1, Xcorr ≥1.9; charge +2, Xcorr ≥2.2; charge +3, Xcorr ≥3.75; and DelCN ≥0.1.

RESULTS AND DISCUSSION

As proteomics emerged as a powerful method for gaining insights into different physiological changes at the cellular level, some investigators are trying to apply this technique to study insect adaptation and immune response (Sharma *et al*., 2004; Kaeslin *et al*., 2005; Untalan *et al*., 2005; Francis *et al*., 2006). To our knowledge, only three studies have been published on the proteomics of host response to parasitization by parasitic wasps (Kaeslin *et al*., 2005; Song *et al*., 2008; Nguyen *et al*., 2008). The present study explored proteome changes in the plasma of an insect after parasitization by a parasitoid devoid of symbiotic viruses. Proteins resolved in each gel (each containing five pooled host pupal plasmas) were collected 24 h after parasitization for parasitized pupae and at the equivalent time for unparasitized

pupae, and the three replicated gels were analyzed for each condition. This time point was chosen because previous work indicated that the highest differential protein expression in haemolymph was occurred at 24 h post-parasitization (Zhang, 2005). When protein from pooled pupal plasma was separated by 2D SDS-PAGE gel and visualized by Coomassie blue staining, distinct differential spot intensity patterns were observed. Sixteen protein spots (Figs.1 and 2) were found to be significantly (P<0.05) modulated in parasitized pupae, compared with the expression level in the control. Fig.3 provided the expression level for these proteins. It can be seen that 8 spots were elevated (spots 2, 3, 6, 8, 9, 12, 13, and 14), 5 showed decreased expression (spots 1, 5, 7, 11, and 15), 1 disappeared (spot 4), and 2 newly occurred (spots 10 and 16) after parasitization. In order to know the functions of each of these proteins, all 16 spots were excised from the gels and submitted to the identification by MS analysis and database searches. We were thus able to identify 15 of the 16 spots in the database (Table 1), the only unidentified one being spot 12. The identified proteins included important insect immune proteins, enzymes, cell structural, signal transduction, and detoxification proteins. According to their functional roles, these proteins were divided into several groups.

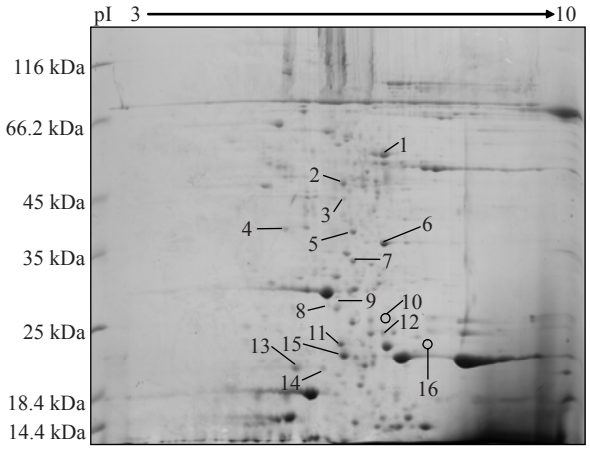

Fig.1 Two dimensional (2D) gel electrophoresis of haemolymph plasma collected from *P. xuthus* pupae unparasitized by *P. puparum*. Plasma was resolved in a linear pI range of 3 to 10 and 12% SDS-PAGE. The gels were stained with Coomassie blue. The numbers in the pictures indicate the spots which were differentially expressed after parasitization and were further processed by LC/MS-MS. Information about each spot can be found in Table 1

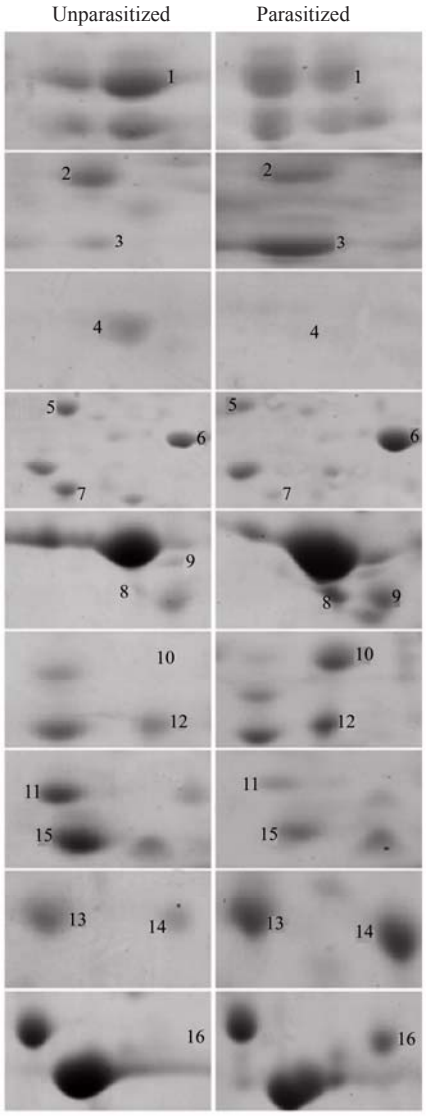

Fig.2 Differential expressions of selected *P. xuthus* plasma proteins in relation to parasitization status. Spot numbers indicated are the same as those in Fig.1

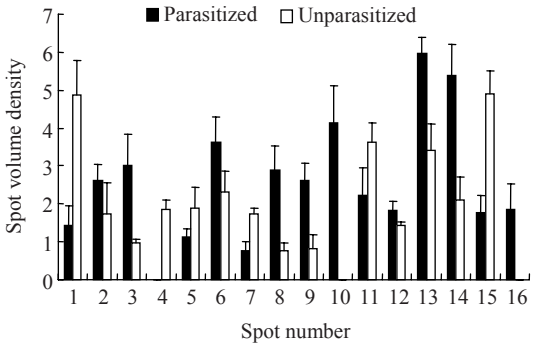

Fig.3 Quantification of the differential expressions of proteins in the plasma of *P. xuthus*. Spots volume densities were quantified by scanning the optical density intensities on the gels. Values are shown as mean±*SD*

Table 1 Identification of differentially expressed proteins in the plasma of *P. xuthus* after parasitization by *P. puparum*

Spot No.[a]	Protein identification[b]	Species origin	M_w[c]	pI[d]	GI accession No.[e]	Identified peptide[f]
1	TPA_inf: HDC08545	*Drosophila melanogaster*	42624.39	9.02	41617708	QIHLDAMQTGAER
2	GD21841	*Drosophila simulans*	48988.76	5.38	195579860	TLEQIDVIK; TLEQIDVIKR
3	Arginine kinase	*Bombyx mori*	39991.31	5.87	82658675	EMYDGIAELIK; GTFYPLTG MSK; ASVHIKLPK; EHTEA EGGVYDISNK; GTRGEHTE AEGGVYDISNKR; GLTEYD AVK; MGLTEYDAVK; RMG LTEYDAVK
4	Leucine-rich transmembrane protein	*Aedes aegypti*	82940.84	5.93	108878943	SLEVLYLGMNK
5,7	Masquerade-like serine proteinase homolog	*Bombyx mori*	45937.83	4.96	25992174	CFATGWGK; CFATGWGKDK
6	Hydroxyacyl dehydrogenase subunit B/thiolase	*Lysiphlebus testaceipes*	50320.92	9.30	67043781	VLDIAGLSMK
8	Thiol peroxiredoxin	*Bombyx mori*	21916.03	6.09	38260562	DISLSDYKGK; DISLSDYQGK; KQGGLGPM; QGGLGPM; GLFIIDDKQNLR; NIPLLSDK
9	AF161261_1 prophenoloxidase	*Sarcophaga bullata*	79796.77	6.15	5579390	GTCRIFLCPK
10	PRDX1_DROME peroxiredoxin 1		21737.93	5.52	27734441	GLFIIDDKQNLR
11	TPA_inf: HDC13282	*Drosophila melanogaster*	21429.26	9.10	41618846	ASNFINEIAAK
12	No identified					
13	Retinoid x receptor (rxr)	*Aedes aegypti*	53983.09	8.88	108884373	AIILFNPDIR
14	Actin-depolymerizing factor 1	*Bombyx mori*	17009.18	6.17	95103010	YIQATDLSEASQEAVEEK
15	Tyrosine-protein kinase	*Aedes aegypti*	237063.39	5.97	108876293	IFWNVPHLLGIK
16	PREDICTED: similar to retinoblastoma-binding protein 2 (RBBP-2)	*Apis mellifera*	181791.55	5.33	66504706	MAGFNTKEGKK

[a]Spot numbers are indicated in Figs.1 and 2; [b]Proteins were identified by searching the NCBI non-redundant database with peptide fragmentation data using the SEQUEST search algorithm; [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

Humoral immune proteins

A masquerade-like serine proteinase homolog (MasSPH) (spots 5 and 7) and a prophenoloxidase (proPO) (spot 9) were identified as under-expressed and up-regulated after parasitization, respectively. These proteins are vital proteins associated with melanization (Amparyup *et al*., 2007). As a key defense mechanism against intruding organisms, melanization is the result of the proPO activation cascade regulated by serine proteinases, which leads to the formation of melanin and other toxic phenolic compounds (Ashida and Brey, 1998; Vass and Nappi, 2000). Among the known mechanisms to fend off parasitoid, defensive melanization is perhaps the most ubiquitous and best characterized among humoral response (Doucet *et al*., 2008). Thus a reduced melanization caused by the inhibition of phenoloxidase (PO) activity and proPO-activating enzyme serine protease (PPAE) is often observed of parasitization (Lavine and Beckage, 1995; Asgari, 2006). PO is present in the plasma fraction of the haemolymph or in the haemocytes of insects. It is activated from its precursor, proPO, by a proPO-activating proteinase (PAP), also known as PPAE, via a serine protease cascade (Gupta *et al*., 2005; Amparyup *et al*., 2007). MasSPH has been found to be required for PAP regulation in *Manduca sexta* (Amparyup *et al*., 2007). Our results suggest that the transcription or translation of proPO and MasSPH can be altered by parasitization. Studies on other parasitoid-host systems have found that the transcription of melanization-related genes such as proPO, serine proteases,

and serpins is regulated by parasitization (Wertheim *et al*., 2005; Doucet *et al*., 2008). In accordance with these studies, we found that the expressions of proPO and MasSPH were altered following parasitization, suggesting that disruption of melanization would be a way for *P. puparum* to modulate *P. xuthus* immune reaction.

Cellular immune proteins

Three protein spots modulated by parasitization contained proteins involved in cellular regulation: a leucine-rich transmembrane protein (LRTMP) (spot 4), an actin-depolymerizing factor (ADF) (spot 14), a tyrosine-protein kinase (TPK) (spot 15), and a homolog of the retinoblastoma-binding protein 2 similar (RBBP-2) (spot 16). LRTMP is a group of leucine-rich repeat proteins (LRPs), which share a common structural motif containing 20~29 asparagine and leucine residues (Chen *et al*., 2006). LRPs have been implicated in the control of cell growth, collagen deposition, and the activation and inactivation of cytokines and growth factors (Shimizu-Hirota *et al*., 2004; Liu *et al*., 2005). ADFs are part of the ADF/cofilin group of small (15~22 kDa) actin-binding proteins that include cofilin, destrin, depactin, and actophorin and are ubiquitous in all eukaryotes (Carlier, 1998; Yeoh *et al*., 2002). They are essential for cell viability, playing critical roles in accelerating actin filament turnover during cell locomotion, cytokinesis, and other forms of cell motility (Yeoh *et al*., 2002; Gurniak *et al*., 2005). Mutants have been characterized in several organisms and are associated with lethality, arrest in cell proliferation, and a disorganized actin cytoskeleton (Gunsalus *et al*., 1995). TPKs, both receptor and non-receptor types such as the Src family kinases, are important components of signaling pathways and play major roles in cell growth, proliferation, cell cycle, and cell survival (Wang and Yu, 2005; Ahmad *et al*., 2007). RBBPs are thought to act as retinoblastoma protein modulating factors, and contain highly conserved RBBP motifs. They participate in several processes of cell growth and differentiation, including cell cycle regulation and control of gene expression (Roesch *et al*., 2005; Klose *et al*., 2007). Modulation of the proteins described above should lead to changes in the regulation of the cell cytoskeleton dynamics and/or arrangement. Cellular immunity mediated by blood cells plays a major role in the defense against parasitoids. Disruption of host cellular defenses by parasitoids through the way of modifying haemocyte behavior, abundance and morphology has been well documented (Stettler *et al*., 1998), while little is known about the molecular mechanism of such modifications. In the present study, the proteins with special roles in insect cellular immunity regulated by parasitization provide us an opportunity for understanding the molecular bases of parasitic wasps overcoming hosts, cellular defense reaction.

Detoxification proteins

Cellular stress such as phagocytosis and melanotic encapsulation can induce the generation of reactive oxygen species (ROS) such as superoxide anions, hydrogen peroxide, and hydroxyl radicals that damage nucleic acids, proteins, lipids, and membranes (Müller, 2004; de Morais Guedes *et al*., 2005). In order to counter these deleterious events, cells use several protective systems that either repair the various types of damage (for instance, by using DNA repair enzymes) or inactivate the ROS (de Morais Guedes *et al*., 2005). There are three different kinds of primary antioxidant enzymes that act directly on ROS: superoxide dismutases, catalases, and peroxidases (Corona and Robinson, 2006). Two proteins belonging to peroxidases, thiol peroxiredoxin (Tpx) (spot 8) and peroxiredoxin (Prx) 1 (spot 10), were identified as strongly induced after parasitization in this work. Tpx is known to eliminate H_2O_2 and alkyl hydroperoxidases with the use of a thiol-reducing equivalent (Munks *et al*., 2005). The antioxidant activity of Prx is due to a reduction of hydroperoxide, with thioredoxin as an immediate hydrogen donor (Rodriguez *et al*., 2000). Perturbation in the expression of detoxification proteins has been found in insect subjected to an immune challenge, exposed to a temperature stress or to viral infections (de Morais Guedes *et al*., 2005; Lee *et al*., 2005). Using 2D gel analysis, Nguyen *et al*.(2008) reported an increase in such types of proteins in the aphid *Macrosiphum euphorbiae* challenged by the parasitoids *Aphidius nigripes* or *A. ervi*. Altered expression of detoxification-related proteins following parasitization could be the result from the host response to the physiological injury brought about during the process of oviposition.

Energy metabolism proteins

Three proteins involved in energy metabolism showed induction upon parasitic stress. They included arginine kinase (AK) (spot 3), hydroxyacyl dehydrogenase subunit B/thiolase (HDSB/T) (spot 6), and retinoid X receptor (RXR) (spot 13). AK and RXR were up-regulated by parasitization, while HDSB/T was down-regulated. AK is a phosphagen kinase that catalyses the reversible transfer of high-energy phosphate from arginine phosphate (AP) to adenosine diphosphate (ADP) to form adenosine triphosphate (ATP) (Kucharski and Maleszka, 1998). AK thus provides an energy reservoir and facilitates energy transport in cells. RXR is involved in the transcriptional regulation of genes, which controls lipid metabolism through the retinoic acid signaling pathway (Mansfield *et al*., 1998). HDSB/T is an enzyme playing a special role in fatty acid metabolism (Beis *et al*., 1980). As these proteins are responsible for energy metabolism, their abnormal expression would likely result in an energy metabolism disorder, a strategy evolved by *P. puparum* to divert resources for its progeny development.

Others

Three proteins that showed alterations in expression after parasitism showed sequence similarity to TPA_inf: HDC08545 (spot 1), GD21841 (spot 2) and TPA_inf: HDC13282 (spot 11) of *Drosophila*. There is no function reported for these proteins. Moreover, one protein (spot 12) has not been identified.

CONCLUSION

In conclusion, this work provides a proteomic approach to explore the molecular mechanisms underling the parasitoid-host interaction by identifying the proteins affected during parasitization. Several different functional classes of proteins were affected by parasitization such as immune response, antioxidant, and energy metabolism. The present results here provide a better understanding of the toxic properties of parasitoid venom as well as the molecular biology of how the parasitoid regulates the physiology of its host. In Further studies, we will be focused on some of these proteins to examine their exact roles in host innate immunity to defense the parasitoid.

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