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Insights into the venom protein components of *Microplitis mediator*, an endoparasitoid wasp

Zhe Lin[a,1], Rui-Juan Wang[b,1], Yang Cheng[a,d], Jie Du[a,d], Olga Volovych[a], Li-Bin Han[a], Jian-Cheng Li[c], Yang Hu[a], Zi-Yun Lu[c], Zhiqiang Lu[b,**], Zhen Zou[a,d,*]

[a] State Key Laboratory of Integrated Management of Pest Insects and Rodents, Institute of Zoology, Chinese Academy of Sciences, Beijing, China
[b] Department of Entomology, College of Plant Protection, Northwest A&F University, Yangling, Shaanxi, China
[c] Institute of Plant Protection of Hebei Academy of Agriculture and Forestry Sciences, Baoding, China
[d] University of Chinese Academy of Sciences, Beijing, China

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ABSTRACT

Endoparasitoid wasps deliver a variety of maternal factors, such as venom proteins, viruses, and virus-like particles, from their venom and calyx fluid into hosts and thereby regulate the hosts' immune response, metabolism and development. The endoparasitoid, *Microplitis mediator*, is used as an important biological agent for controlling the devastating pest *Helicoverpa armigera*. In this study, using an integrated transcriptomic and proteomic analysis approach, we identified 75 putative venom proteins in *M. mediator*. The identified venom components were consistent with other known parasitoid wasps' venom proteins, including metalloproteases, serine protease inhibitors, and glycoside hydrolase family 18 enzymes. The metalloprotease and serpin family showed extensive gene duplications in venom apparatus. Isobaric tags for relative and absolute quantitation (iTRAQ) based quantitative proteomics revealed 521 proteins that were differentially expressed at 6 h and 24 h post-parasitism, including 10 wasp venom proteins that were released into the host hemolymph. Further analysis indicated that 511 differentially expressed proteins (DEP) from the host are primarily involved in the immune response, material metabolism, and extracellular matrix receptor interaction. Taken together, our results on parasitoid wasp venoms have the potential to enhance the application of endoparasitoid wasps for controlling insect pest.

1. Introduction

The cotton bollworm, *Helicoverpa armigera*, is distributed worldwide and causes significant losses in agricultural production annually (Wu and Guo, 2005). Its natural enemy *Microplitis mediator* is a solitary endoparasitoid wasp and used as an important biological control agent (Guo et al., 2009; Luo et al., 2014; Slovak, 1985). Parasitoid wasps deliver maternal virulence factors into their hosts by oviposition, thus altering their host's physiology after parasitism to support the development of wasp eggs (Moreau and Asgari, 2015; Pennacchio and Strand, 2006; Strand and Pech, 1995). In numerous parasitoid wasps, venom is the main source of factors manipulating host physiological processes including immune responses (Moreau and Asgari, 2015). Recently, high-throughput transcriptomic and proteomic approaches was used to investigate the venom composition of many species, including *Nasonia vitripennis*, *Chelonus inanitus*, *Leptopilina boulardi*, *Leptopilina heterotoma*, *Hyposoter didymator*, *Microplitis demolitor*, *Aphidius ervi*, *Pteromalus puparum*, and *Toxoneuron nigriceps* (Burke and Strand, 2014; Colinet et al., 2013, 2014; De Graaf et al., 2010; Doremus et al., 2013; Goecks et al., 2013; Laurino et al., 2016; Vincent et al., 2010; Yan et al., 2016).

Host immunity is the major obstacle for the successful survival and development of koinobiont endoparasitoid wasp larvae (Harvey et al., 2013; Pennacchio and Strand, 2006). Many species have efficient

Abbreviations: iTRAQ, isobaric tags for relative and absolute quantitation; DEP, differentially expressed protein; ECM-receptor interaction, extracellular matrix receptor interaction; serpin, serine protease inhibitor; GH18 enzyme, glycoside hydrolase family 18 enzyme

* Corresponding author. State Key Laboratory of Integrated Management of Pest Insects and Rodents, Institute of Zoology, Chinese Academy of Sciences, Beijing, China.
** Corresponding author.
E-mail addresses: zhiqiang.lu@nwsuaf.edu.cn (Z. Lu), zouzhen@ioz.ac.cn (Z. Zou).
[1] These authors contributed equally to this work.

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cellular and humoral immune mechanisms that protect them from invasion by microbial pathogens and parasites (Cheng et al., 2018; Lavine and Strand, 2002; Wang et al., 2015; Xing et al., 2017). However, some endoparasitoids may utilize their venom to suppress host cellular responses, thereby protecting their offspring during the early stages of its development. P4, an immune suppressor in the venom of *L. boulardi*, contains a Rho GTPase activating protein (Rho-GAP) domain, induces an abnormal shape of lamellocytes, and prevents encapsulation (Labrosse et al., 2005a, 2005b). Similarly, calreticulin in *Cotesia rubecula* venom inhibits capsule formation by compromising the spread of host hemocytes (Zhang et al., 2006). In *Ganaspis sp. 1* (G1, a parasitoid of *Drosophila*), the sarco reticulum Ca^{2+}-ATPase pump acts as a virulence factor by suppressing cytoplasmic calcium signaling in host plasmatocytes (Mortimer et al., 2013).

Apart from damaging cellular immunity, venom proteins could also alter the host insect's humoral responses mainly through inhibition of melanization in arthropods. Prophenoloxidase (PPO) is a key enzyme in melanization, and its activation depends on PPO activating proteases (PAPs) that are negatively regulated by serine protease inhibitors (serpins) (Li et al., 2016; Yang et al., 2017a; Yuan et al., 2017). LbSPNy, a serpin identified in the venom gland of *L. boulardi* ISy strain, could significantly inhibit the activation of *Drosophila yakuba* PPO (Colinet et al., 2009). Likewise, a clip-domain serine protease homolog (SPH) named Vn50, a venom protein from the parasitoid *C. rubecula*, impairs the enzymatic activation of PPO by PAP, resulting in the suppression of host hemolymph melanization (Asgari et al., 2003). Similarly, *P. puparum* venom decreases the expression level of antimicrobial genes and impairs the antimicrobial activity of its host *Pieris rapae* (Fang et al., 2016).

In this study, by using an integrated transcriptome and proteome analysis, we identified the putative venom proteins in *M. mediator*. Through isobaric tags for relative and absolute quantitation (iTRAQ) based quantitative proteomic analysis, we compared the protein expression profiles during the early phase post-parasitism. The data we report in this study could contribute to obtaining a comprehensive view on the evolution of venoms among hymenopteran parasitoids and help develop better biological control strategies to mitigate insect pests.

2. Materials and methods

2.1. Experimental insects

Larvae of *H. armigera* were reared on artificial diet at 28 °C, 70% relative humidity (RH), and under a 14/10 h (light/dark) photoperiod (Xiong et al., 2015). Adult insects were kept under the same conditions and provided with a 10% honey solution. The *M. mediator* wasps were maintained in laboratory cultures at 26 °C with a 14/10 h photoperiod and 60% RH (Wang et al., 2015). Adult wasps were provided routinely with 10% sucrose and water. Wasps were allowed to parasitize second instar *H. armigera* larvae and produce a single offspring per host.

2.2. Transmission electron microscopy

The venom apparatus was dissected from the abdomen in PBS solution, and fixed in glutaraldehyde solution for 3 h at 4 °C. After processing with 1% osmium tetroxide for 3 h, the sample was dehydrated using acetone, embedded in Epon SPURR resin, and sectioned. Ultrathin sections were examined using an electron microscope JEM-1230 at 80 kV.

2.3. Transcriptomic analysis of M. mediator

Three tissue samples were collected from adult female wasps, in addition to whole bodies. The whole body (WB) was separated into three parts: the venom apparatus (VA), the ovary (OV), and the remaining tissue, which was designated the carcass (CA). Three replicates for each sample were dissected on different dates to provide biological replication. We dissected venom apparatuses from 400 wasps, ovaries from 100 wasps, and used 15 female carcasses and 15 female whole bodies. To generate 12 RNA libraries, total RNA was extracted from 4 samples with 3 replicates using an RNeasy mini kit (Qiagen). RNA integrity was checked using an Agilent 2100 Bioanalyzer (Agilent Technologies). A total of 5 μg RNA from each sample was used to isolate mRNA using oligo (dT) magnetic beads (Invitrogen). Then the Paired-end RNA-seq libraries were prepared according to the manufacturer's protocol and sequenced using the Illumina HiSeq™ 4000 platform (Illumina). Raw sequence data ($Q < 20$) from each library was filtered to remove adapters and low quality reads. The resulting clean reads were assembled to produce unigenes using Trinity (ver. r2013-02-25) (Haas et al., 2013). The transcripts were produced from contigs that were at least 300 bp in length and were reduced by removing redundant sequences (≥90% identity) using CD-Hit software (ver. 4.5.4) (Grabherr et al., 2011). Blast2GO was used to annotate genes from the Trinity transcripts. We performed a BLASTX search against the NCBI non-redundant (NR) protein database with e-values ≤ 10^{-5}. Transcript expression levels were estimated from the fragments per kilobase of transcript per million fragments mapped (FPKM) values generated by the RSEM software package. The p values were adjusted using the Benjamini-Hochberg method. Differentially expressed genes between whole body library and other libraries were compared to identify genes with significant changes (fold change > 2 or < 0.5, $p < 0.05$, and average FKPM > 5) (Anders and Huber, 2010). FPKM values were hierarchically clustered using the Heatmap2 package in R, the Pearson correlation based metric, and the average linkage clustering method.

2.4. Proteomic analysis of venom apparatuses and venom reservoirs

Proteomic analysis was performed on 50 independently dissected venom apparatuses (VA) and venom reservoirs (VR). The samples were stored on ice in PBS containing complete protease inhibitor cocktail (Roche), and 0.5 mM EDTA. Samples containing large amounts of venom proteins were centrifuged at 500 × g for 5 min at 4 °C to remove cells and tissue debris. Supernatants were transferred to new tubes and mixed with lysis buffer on ice. Sample proteins were resolved on 4–15% gradient gels (Bio-Rad) and stained with Coomassie blue R-250. Each gel lane was treated using trypsin to digest proteins in the gel. A total of 10 μL supernatant was collected by infusion using the UltiMate 3000 nanoLC system (Dionex and applied to an Acclaim PepMap100 C18 column (Thermo). The peptides were eluted onto a Venusil XBP C18 column (Agela Technologies) and then subjected to nanoelectrospray ionization followed by MS/MS in a Q-Exactive mass spectrometer (Thermo) linked to the high-performance liquid chromatography (HPLC) system. Intact peptides were detected using an orbitrap mass analyzer at a resolution of 70,000 and 17,500 for the MS and MS/MS analysis, respectively. Proteins were identified using MASCOT software (version 2.3.0.2; Matrix Science) and our own *M. mediator* transcriptome database (NCBI database accession number PRJNA396461). The data were selected based on criteria (FDR lower than 1% confidence). Putative venom proteins had to correspond to at least two peptides.

2.5. iTRAQ analysis

iTRAQ analysis of *H. armigera* hemolymph proteins was performed on five samples: second instar *H. armigera* larvae (N0), second instar *H. armigera* larvae at 6 h post-parasitism (P6), second instar *H. armigera* larvae at 24 h post-parasitism (P24), and the corresponding larvae with naïve no parasitism at 6 h (N6) and 24 h (N24). We used about 200 larvae. 200 μL hemolymph was collected from each sample and subject to centrifugation. In each sample, 100 μg total protein was digested with trypsin (Promega). Then the peptides were labeled with the 8-plex iTRAQ reagent (Applied Biosystems). Labeled peptides were mixed and

loaded onto a 4.6 × 250 mm Ultremex SCX column (Phenomenex) using an LC-20AB HPLC system (Shimadzu). The peptides were eluted with a gradient of buffer. Collected fractions were desalted using a Strata X C 18 column (Phenomenex). The samples were analyzed using MS/MS in a Q-Exactive mass spectrometer (Thermo Fisher Scientific) coupled to a micro-flow HPLC system. Raw data were converted into MGF files using Proteome Discoverer software (version 1.2; Thermo Fisher Scientific). Proteins were identified using MASCOT software (version 2.3.0.2; Matrix Science) with our own *H. armigera* (NCBI database accession number PRJNA264881) and *M. mediator* (NCBI database accession number PRJNA396461) transcriptome databases with false discovery rate (FDR) less than 1% confidence. DEPs were analyzed using unique peptide and normalized on protein median, for significant up-regulation ($q < 0.01$ and fold-change > 1.2) and significant down-regulation ($q < 0.01$ and fold-change < 0.83). KEGG analysis was performed to identify enriched pathways and metabolic networks using the online KEGG Orthology-Based Annotation System (KOBAS) tool.

2.6. Data availability

All raw sequence data and the final *M. mediator* transcriptome described in this study are available under the BioProject accession number PRJNA396461. Illumina sequence reads have been deposited in the NCBI SRA database under the following accession numbers (whole body: Sample SRS2395955, Experiment SRX3049256, Reads SRR5885339, SRR5885340, SRR5885341; ovary: Sample SRS2395957, Experiment SRX3049258, Reads SRR5885434, SRR5885444, SRR5885445; venom apparatus: Sample SRS2395958, Experiment SRX3049259, Reads SRR5885438, SRR5885439, SRR5885440; carcass: Sample SRS2395959, Experiment SRX3049260, Reads SRR5885441, SRR5885442, SRR5885443). Unigenes of *M. mediator* are available under the NCBI TSA accession numbers associated with the same BioProject. The mass spectrometry proteomics data was deposited to the ProteomeXchange Consortium via the PRIDE (Vizcaino et al., 2016) partner repository with the dataset identifier PXD007905.

3. Results

3.1. Identification of putative venom proteins of M. mediator

3.1.1. Overview of the M. mediator venom apparatus

The *M. mediator* venom apparatus organization is typical of Hymenoptera and consists of three parts, with a branched tubular venom glands connecting to venom reservoir. The canal-shaped venom duct (VD) is located between the reservoir and the ovipositor (OP). Parasitoid wasp venom glands secrete venom into the lumen (Fig. S1A). Transmission electron microscopy (TEM) demonstrated that the venom glands comprised of cells filled with numerous secretory vesicles (SV) and an extensive endoplasmic reticulum (ER) with intracellular canals to release secretions (Fig. S1B). Intracellular canals are found in many parasitoid species and described as vesicular organelles (VOs). Similar to other hymenopteran insects, the *M. mediator* venom apparatus is specifically adapted for secretion and can synthesize and inject numerous proteins into the host hemocoel as the eggs are deposited. SDS-PAGE (4–15%) of *M. mediator* ground venom apparatus and fluid from venom reservoir reveals that most of the major bands observed in the venom apparatus were also detected in the venom reservoir (Fig. S1C). Protein bands from the replicates were consistent. These results demonstrated that the venom contained numerous proteins ranging in size from 10 to 200 kDa, with the majority ranging from 35 to 100 kDa.

3.1.2. Integrated analysis of transcriptomic and proteomic data to find venom components

Four types of samples were collected to prepare RNA-sequencing libraries under three independent experimental conditions: female whole bodies, venom apparatuses, ovaries, and carcasses. In total, 12 libraries were sequenced using the HiSeq 4000 platform (Illumina). About 411.89 million raw paired-end reads were produced. After removing the adaptor and filtering out low quality sequences, approximately 399.39 million reads were reserved for further analysis. Because there is no reference genome available for *M. mediator*, the clean reads were assembled *de novo* into 102,541 contigs using Trinity. After filtering the redundant contigs using CD-Hit, the final set of 18,883 unigenes with N_{50} equal to 2653 bp was generated. Based on BLASTX searches (e-values $\leq 10^{-5}$) of the NR protein database, about 10,731 unigenes of *M. mediator* were annotated.

To identify the venom proteins in *M. mediator*, nanoLC-MS/MS proteomic analysis was performed on *M. mediator* venom apparatus and venom reservoir samples. The proteomic analysis provided a total of 288,482 spectra with 15,268 tryptic peptides in venom apparatus proteome, and 64,067 spectra with 2301 tryptic peptides in venom reservoir proteome. Based on the *M. mediator* unigenes obtained by the transcriptomic analysis, 2267 and 313 proteins were found in the venom apparatus and venom reservoir respectively (Table S1 and Data S1). Most of the venom apparatus proteins (2,264) were differentially expressed in the venom apparatus relative to the other three samples. Hierarchical clustering was performed for all venom apparatus proteins, which created two discrete clusters. Cluster I contained 1077 transcripts, which were highly expressed in the venom apparatus. In contrast, cluster II contained 1187 transcripts with the highest transcript expression levels in carcasses (Fig. 1A). In parasitoid wasps, venom proteins are usually expected to have signal peptides. Consistent

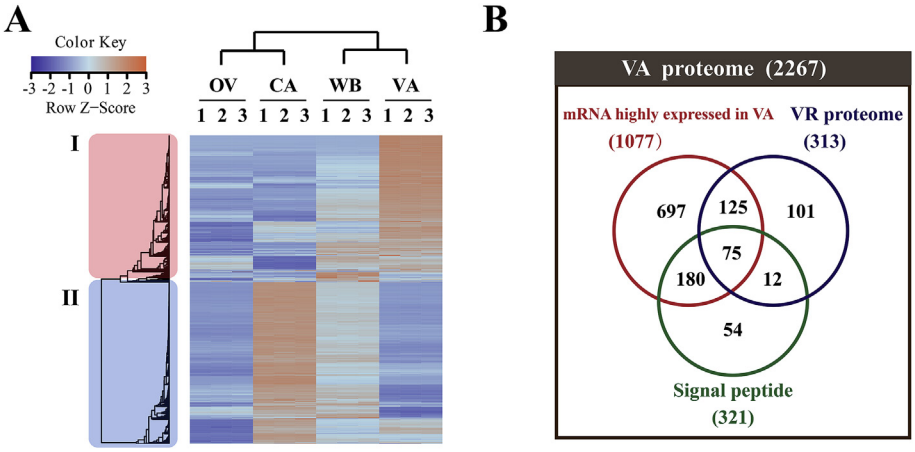

Fig. 1. Identification of putative venom proteins from *M. mediator*. **(A)** Hierarchical clustering analysis of the venom apparatus genes from four transcriptomes: venom apparatus (VA), carcass (CA, or remainder after dissecting venom apparatus and ovary), ovary (OV), and whole body (WB). Two discrete clusters identified from the gene dendrogram, are shown in red (Cluster I) and blue (Cluster II). Clusters I and II contained genes that were highly expressed in venom apparatuses and carcasses respectively. 1–3 represent replicates. **(B)** Venn diagram showing putative venom proteins. The red circle indicates genes highly expressed in the venom apparatus. The blue circle indicates proteins identified from venom reservoirs proteome analysis. The green circle indicates transcripts containing signal peptides. Numbers in the overlapping regions are shown in circles. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Venom proteins identified from *M. mediator* venom reservoirs proteome with known functions. The putative venom proteins of *M. mediator* marked with bold font, which indicated them highly expressed in the venom apparatus and contained signal peptides. The venom proteins from other parasitoid wasps have been reported: *Microctonus aethiopoides* (Mae) (Crawford et al., 2008); *Microctonus hyperodae* (Mh) (Crawford et al., 2008); *Chelonus inanitus* (Ci) (Vincent et al., 2010), *Nasonia vitripennis* (Nv) (De Graaf et al., 2010), *Leptopilina boulardi* (Lb) (Colinet et al., 2013; Goecks et al., 2013), *Leptopilina heterotoma* (Lh) (Colinet et al., 2013; Goecks et al., 2013), *Hyposoter didymator* (Hd) (Doremus et al., 2013), *Microplitis demolitor* (Md) (Burke and Strand, 2014), *Aphidius ervi* (Ae) (Colinet et al., 2014); and *Pteromalus puparum* (Pp) (Yan et al., 2016); *Toxoneuron nigriceps* (Tn) (Laurino et al., 2016); *Cotesia rubecula* (Cr); *Pimpla turionellae* (Pt); *Eulophus pennicornis* (Ep); P*impla hypochondriaca* (Ph); *Chelonus sp. near curvimaculatus* (*C. sp.*).

Protein name	Asscession number	Putative function	Species and references
Enzymes			
Metalloprotease	MmV11, MmV12, MmV26, **MmV29**, **MmV69**, MmV94, MmV139, MmV146, MmV165, **MmV189**, MmV221, **MmV223**, **MmV270**, MmV275, MmV277, MmV367, **MmV374**, **MmV391**, MmV531, MmV733	Toxicity towards the host, manipulation of host development (Ep)	Ph (Parkinson et al., 2002a), Ep (Price et al., 2009), Ci, Md, Lh, Nv, Hd, Mae, Mh, Ae, Lb, Pp, Tn
Serine proteases and serine protease homologs	**MmV113**, **MmV168**, MmV207, **MmV670**, **MmV1121**	Inhibition of melanization (Cr)	Cr (Asgari et al., 2003), Ci, Nv, Ph (Parkinson et al., 2002b), Pp, Ae, Hd, Tn
Glycoside hydrolase family 18 enzyme	**MmV234**, **MmV664**, MmV1759	Chitinolytic	C. sp. (Krishnan et al., 1994), Ci, Nv, Mae, Mh, Ae
γ-glutamyl transpeptidase	MmV713, MmV1205	Induce apoptosis (Ae)	Ae (Falabella et al., 2007), Nv
Superoxide dismutase	MmV360, **MmV953**, **MmV2036**, **MmV2172**	Inhibition phenoloxidase activity (Lb)	Lb (Colinet et al., 2011), Lh, Pp, Hd
Phenoloxidase	MmV57	Melanization (Ph)	Ph (Parkinson et al., 2001)
Acid phosphatase	**MmV502**	Release of hydrocarbons	Pp, Ph, Lb, Lh, Nv
Phospholipase	MmV298, **MmV1329**	Cytolytic	Pt (Uckan et al., 2006)
Cathepsin	MmV414	Associated with lysosomes	Mae, Mh, Tn
Yellow-e3-like protein	**MmV193**		Ci
Hyaluronidase	**MmV240**		Ci, Pp, Hd
Angiotensin	**MmV259**		Nv, Hd, Mae, Mh, Ci
Protease inhibitors			
Serpin	**MmV24, MmV112,** MmV226, **MmV237, MmV245, MmV506, MmV813,** MmV823, **MmV1086, MmV2098**	Inhibition of melanization (Lb, Pp)	Lb (Colinet et al., 2009), Pp (Yan et al., 2017), Ae, Lh, Hd, Md
Cysteine-rich protease inhibitor	MmV604, MmV2142, **MmV2186**	Protease inhibitor	Nv, Ph (Parkinson et al., 2004), Lb
Neurotoxin-like/Paralytic factors			
Icarapin	**MmV969**	Similar to the allergen	Mae, Mh
Others			
RhoGAP	MmV1531, MmV2008	Deformation of host hemocytes, suppression of encapsulation (Lb)	Lb (Labrosse et al., 2005b), Lh
Calreticulin	**MmV30**	Inhibition of host hemocyte spreading behavior (Cr)	Cr (Zhang et al., 2006), Hd, Nv, Lb, Lh, Pp, Mh, Mae, Tn
Ci-48a	MmV222, MmV319, MmV389	Similar to the lethal G0193 isoform	Ci, Md, Tn
SERCA	MmV38	Regulate host calcium levels and inhibit cellular immunity (Lb)	Lb (Mortimer et al., 2013)

with this, 321 venom proteins in the venom apparatus and 87 venom proteins in the venom reservoir included secretory signal peptides. Finally, 75 venom components were defined as putative venom proteins in *M. mediator* through an integrated transcriptomic and proteomic approach of venom reservoir and were expressed robustly in the venom apparatus and had a signal peptide for secretion (Fig. 1B, Table S1).

3.2. Similarity comparison of *M. mediator* putative venom proteins

After gene annotation, we found that *M. mediator* venom proteins have homologs detected in other parasitoid wasps. The resulting proteins with known functions were classified into four groups: enzymes, protease inhibitors, neurotoxin-like/paralytic factors, and others (Table 1). The enzymes group contained 42 venom proteins from 12 different families. The protease inhibitors contained 13 venom proteins from 2 families. The most abundant groups were metalloproteases and serpins, which contained 20 and 10 members respectively.

3.2.1. Metalloproteases

The metalloproteases are among the most diverse proteases and they need a metal ion, usually zinc, for their catalytic activity. Around half of the known metalloproteases contain consensus HEXXH as the metal binding site. They are involved in a variety of physiological reactions (Giebeler and Zigrino, 2016; Nishiwaki et al., 2000; Rawlings and Barrett, 1995). Metalloproteases have been reported as venom components in almost all parasitoid wasps (Table 1). Our integrative analysis of transcriptomic and proteomic data suggested that metalloproteases represent the most abundant group of enzymes in the *M. mediator* venom apparatus. In total, 25 metalloproteases were identified from the *M. mediator* venom apparatus proteome. Based on phylogenetic analysis, 52 metalloproteases from 8 different insect species were divided into five separate groups (Fig. 2A). Group I consisted of M12B proteinase, which contained a pro-peptide, a catalytic M12B domain (Pfam: PF01421) and an additional disintegrin-like domain with molecular masses ranging from 40 to 60 kDa (Fig. S2). The M12B proteinase, also referred to as adamalysin or reprolysin. MmV189, MmV374, MmV165, MmV531 and Ph-Rep1, which was the first reported venom metalloprotease from *Pimpla hypochondriaca* (GenBank: CAD21587.1), were present in Group I (Parkinson et al., 2002a). Among these 4 members, MmV189 and MmV374 had signal peptides. Except MmV374, the other three metalloproteases contained the conserved catalytic motif for metal-binding. The amino acid sequences in MmV189 and MmV531 were the same as in Ph-Rep1 (HELGH), and the motif sequence was HEIAH in MmV165. MmV189 was designated as venom regulatory factor-1 (VRF1), plays a critical role in regulating the egg encapsulation rate in their host (Lin et al., 2018). Group V proteins are also a subfamily of M12B domain, but they lacked the disintegrin-like domain. Three venom metalloproteases from *Eulophus pennicornis* (GenBank: B5AJT2.1, B5AJT3.1, B5AJT4.1) were present in this group. EpMP3, the reprolysin-like molecule has been reported to induce mortality and block metamorphosis of the host *Lacanobia oleracae* (Price et al., 2009). Group II consisted M13 proteinase (Pfam: PF05649

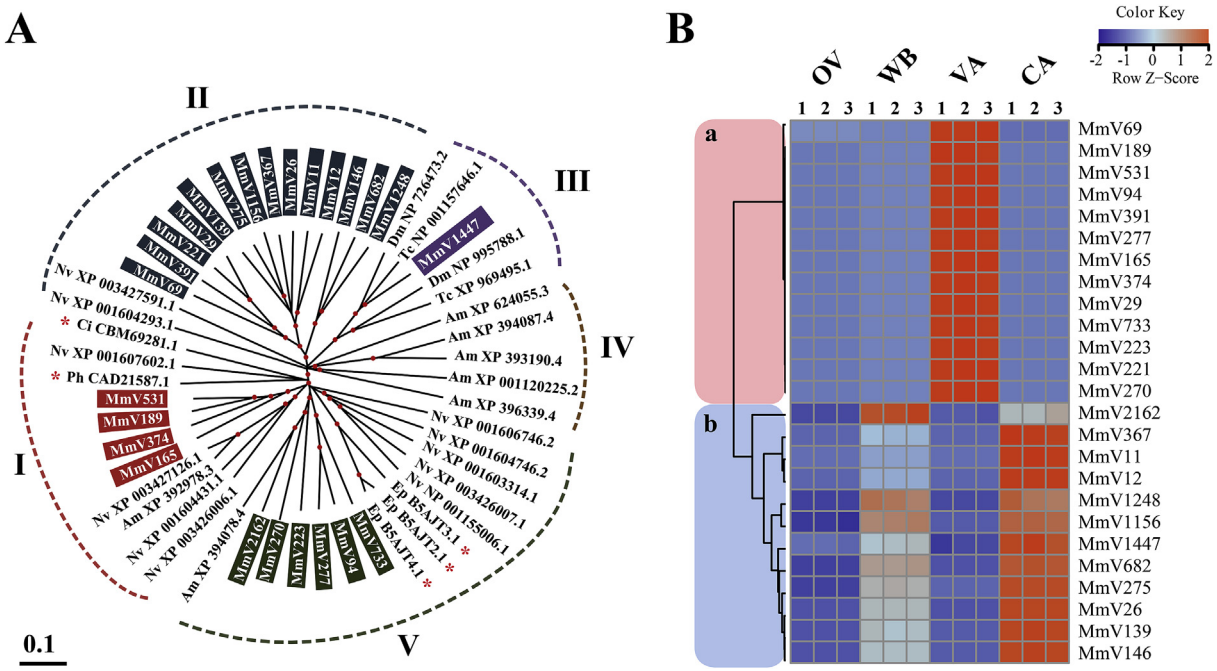

Fig. 2. Phylogenetic relationships of metalloproteases and their expression profiles in *M. mediator*. **(A)** Phylogenetic relationships among the metalloproteases from *M. mediator* (Mm), *E. pennicornis* (Ep), *P. hypochondriaca* (Ph), *C. inanitus* (Ci), *N. vitripennis* (Nv), *A. mellifera* (Am), *D. melanogaster* (Dm) and *Tribolium castaneum* (Tc). Accession numbers of genes are listed after the abbreviations of generic names. The phylogenetic tree was built using the neighbor-joining method. Red dots at the nodes denote bootstrap values greater than 800 from 1000 trials. *, venom metalloproteases from other parasitoid wasps. **(B)** Expression profiles of *M. mediator* metalloproteases for each locus in three tissues VA (venom apparatus), OV (ovary), and CA (the remainder after dissecting venom apparatus and ovary), and WB (whole body) based on transcriptomic analysis. 1–3 represent replicates. Color codes on the left represent their cluster group. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

and PF01431) domain with molecular masses ranging from 50 to 90 kDa. M13 proteinase is also named as neprilysin-like metalloprotease. Group III proteins belong to the M10 proteinase family and these contained a pro-peptide, peptidoglycan binding-like domain (Pfam: PF01471), an N-terminal catalytic M10 domain, (Pfam: PF00413) and four hemopexin domains (Pfam: PF00045) (Fig. S2). This group included only one *M. mediator* transcript (MmV1447). All of the members in group IV were from *Apis mellifera*. Hierarchical clustering analysis of metalloproteases expression from whole bodies, ovaries, venom apparatuses, and carcasses yielded two discrete clusters (Fig. 2B). Most members of M12B proteinase (Groups I and V) are highly expressed in the venom apparatuses.

3.2.2. Serine protease inhibitors

Serine protease inhibitors (serpins) are a superfamily of functionally diverse proteins and evolved to inhibit serine proteases (SPs) mediated processes and maintain homeostasis (Kanost, 1999; Kanost and Jiang, 2015; Zou et al., 2009). To date, serpins have been identified in animals, plants, bacteria and viruses. Serpins from parasitoid wasp venoms have been found in *L. boulardi*, *P. puparum*, *M. demolitor*, and other parasitoid wasps (Table 1). Typical mature serpins contain 350-450 amino acid residues and the conserved tertiary structure. They act as baits for their cognate proteases through an exposed C-terminal reactive site loop (RSL) (Yang et al., 2017b). Analysis of venom apparatus proteome yielded 10 serpins. Among these, 8 serpins had a signal peptide for secretion, except MmV226 and MmV823. Phylogenetic analysis was performed using 42 known serpin sequences from 12 different insect species (Fig. 3A). The 10 venom serpins of *M. mediator* belong to a hymenopteran specific clade likely arising as a result of duplication (Fig. 3A). In the phylogenetic tree, LbSPNy (GenBank: ACQ83466.1) and PpS1V (GenBank: AOW41297.1) were located in the same clade with *M. mediator* venom serpins. LbSPNy identified in the venom gland of *L. boulardi* ISy strain could significantly inhibit the activation of host's prophenoloxidase (PPO) cascade (Colinet et al., 2009). PpS1V, a venom serpin of *P. puparum*, could suppress PPO activation by forming complexes with host hemolymph proteinases (Yan et al., 2017). The *M. mediator* venom serpins are more abundant in the venom apparatus compared to other tissues (Fig. 3B).

3.2.3. Glycoside hydrolase family 18 enzymes

The glycoside hydrolases (GHs) are a widespread group of enzymes that hydrolyse the glycosidic bonds of glycans. Glycoside hydrolase family 18 (GH18) enzymes cleave chitin which are the second most abundant polysaccharides in nature and comprise linear β-(1,4)-N-acetylglucosamine residues (Gooday, 1999; Pesch et al., 2016; Zhang et al., 2002). GH18 enzymes are venom proteins and have been identified in *C. inanitus*, *Chelonus sp.* near *curvimaculatus*, *A. ervi*, and other parasitoid wasps (Table 1). MmV234, MmV664 and MmV1759 with the glycosyl hydrolases family 18 domain (PF00704) were identified from the *M. mediator* venom apparatus proteome. Both MmV234 and MmV664 possess a signal peptide. The phylogenetic analysis classified 16 GH18 enzymes from 10 different insects into three groups (Fig. 4A). MmV1759 and two reported venom proteins, the *C. sp.* venom chitinase (GenBank: AAA61639.1) and the Ci-45 venom chitinase (GenBank: CBM69270.1), were present in Group I (Krishnan et al., 1994; Vincent et al., 2010). This group consisted of insect chitinases with four highly conserved regions. The second conserved region is FDG(L/F)DLDWE(Y/F)P, which is near catalytic site of the protein with the signature residue glutamate (E) (Fan et al., 2018; Zhang et al., 2002). This region is also conserved in MmV1759 with the consensus of FDGLDLDWEFP. However, MmV1759 is different from venom chitinase *C. sp.* and teratocyte released chitinase from *T. nigriceps* (GenBank: AAX69085.1). It does not contain the chitin-binding Peritrophin-A domain (CBM 14, Pfam: PF01607). MmV234 belongs to Group II, which consisted of imaginal disc growth factors (IDGFs)-like proteins. MmV664 belongs to Group III chitinase-like proteins. The *M. mediator* venom GH18 enzymes are not

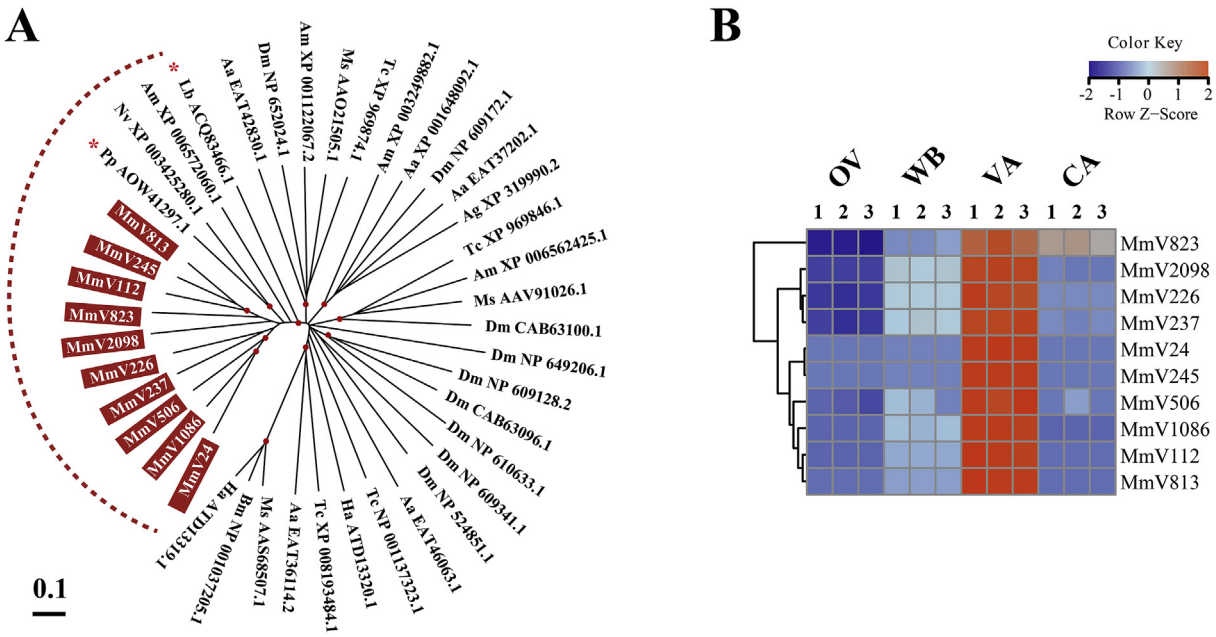

Fig. 3. Phylogenetic relationships of serpins and their expression profiles in *M. mediator*. **(A)** Phylogenetic relationships among the serpins from *M. mediator* (Mm), *P. puparum* (Pp), *L. boulardi* (Lb), *N. vitripennis* (Nv), *A. mellifera* (Am), *D. melanogaster* (Dm), *Aedes aegypti* (Aa), *Anopheles gambiae* (Ag), *H. armigera* (Ha), *Manduca sexta* (Ms), *Bombyx mori* (Bm), and *T. castaneum* (Tc). Accession numbers of genes are listed after the abbreviations of generic names. The phylogenetic tree of amino acid sequences was built using the neighbor-joining method. Red dots at the nodes denote bootstrap values greater than 800 from 1000 trials. *, venom serpins from other parasitoid wasps. **(B)** Expression profiles of *M. mediator* serpins for each locus in three tissues VA (venom apparatus), OV (ovary), and CA (the remainder after dissecting venom apparatus and ovary), and WB (whole body) based on transcriptomic analysis. 1–3 represent replicates. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

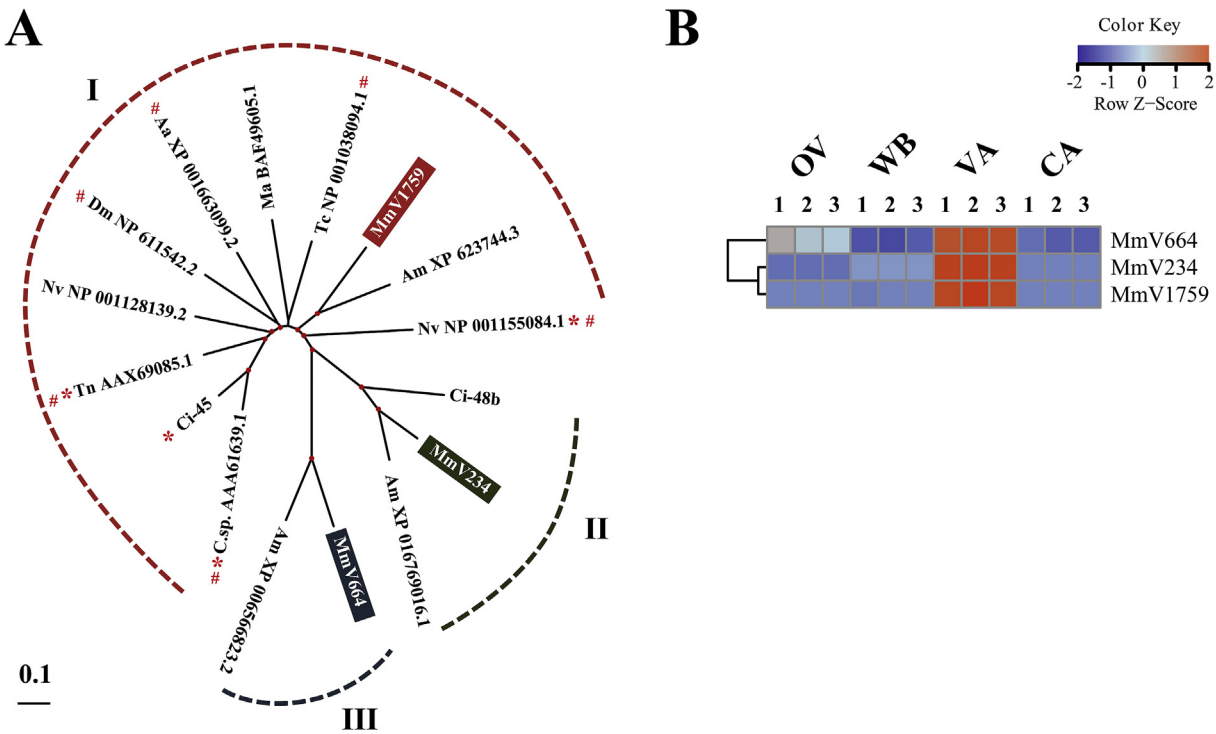

Fig. 4. Phylogenetic relationships of GH18 enzymes and their expression profiles in *M. mediator*. **(A)** The phylogenetic relationships among the GH18 enzymes from *M. mediator* (Mm), *C. inanitus* (Ci), *Chelonus sp.* (*C. sp.*), *T. nigriceps* (Tn), *N. vitripennis* (Nv), *A. mellifera* (Am), *D. melanogaster* (Dm), *A. aegypti* (Aa), *Monochamus alternatus* (Ma), and *T. castaneum* (Tc). Accession numbers of genes are listed after the abbreviations of generic names. The phylogenetic tree of amino acid sequences was built using the neighbor-joining method. Red dots at the nodes denote bootstrap values greater than 800 from 1000 trials. *, venom GH18 enzymes from other parasitoid wasps. #, GH18 enzymes containing chitin-binding Peritrophin-A domain. **(B)** Expression profiles of *M. mediator* GH18 enzymes for each locus in three tissues VA (venom apparatus), OV (ovary), and CA (the remainder after dissecting venom apparatus and ovary), and WB (whole body) based on transcriptomic analysis. 1–3 represent replicates. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

more close to those from honey bee than parasitoid wasp in phylogenetic analysis. The mRNA abundance of *M. mediator* venom GH18 enzymes is higher in the venom apparatus than in the other tissues (Fig. 4B).

3.3. Parasitism alters protein levels in H. armigera hemolymph

3.3.1. Identification and enrichment analysis of differentially expressed proteins

To examine the dynamic changes of host hemolymph protein levels in the early phase post-parasitism, iTRAQ-coupled LC-MS/MS was used to compare the protein expression profiles of the 6 h and 24 h post-parasitism groups (P6 and P24) and the no parasitism groups (N0, N6, and N24). A total of 2108 proteins were identified from 309,664 unique spectra and 7985 unique peptides. Among 521 differentially expressed proteins (DEPs), 10 were identified from the *M. mediator* venom apparatus including calreticulin (MmV30), heat shock protein 90 (MmV21), Ras-like GTP-binding protein Rho1 (MmV802), neuroserpin (MmV112), leukocyte elastase inhibitor-like (MmV24), Rho GTPase-activating protein (MmV2008), protein p26 (MmV102), and three metalloproteases (MmV189, MmV12, MmV165) (Table S2).

We also performed hierarchical clustering of 511 DEPs from *H. armigera* hemolymph and divided them into two discrete clusters. Using the dendrogram, we found that N0, N6, and N24 formed a no parasitism group, whereas P6 and P24 formed a post-parasitism group (Table S2). Cluster i included 346 proteins that were up-regulated in the post-parasitism groups, whereas cluster ii included 165 proteins that were highly expressed in the no parasitism groups (Fig. 5A). To explore the potential functions of these proteins, we used the KOBAS database to determine which pathways were enriched in the clusters i and ii. Three major functional categories were found among the DEPs, and the functional categories were involved in immune response, material metabolism and extracellular matrix receptor interaction (ECM-receptor interaction) (Fig. 5B and Table S3). These results demonstrate that the parasitism caused significant changes in the host hemolymph.

3.3.2. Protein levels were significantly up-regulated at 6h post-parasitism

Heatmap revealed the changes in protein abundance observed at 6h post-parasitism (Fig. 5A and Table S2). The abundance of 200 proteins showed high expression level in hemolymph at 6h post-parasitism but remarkably decreased at 24h post-parasitism. Among them, only 86 were identified in both N6 and N24, containing 59 up-regulated and 27 down-regulated at N24. Further analysis identified 10 proteins belong to *M. mediator* venom components, and all members were up-regulated at 6h versus 24h post-parasitism (fold-change > 1.2). These results suggested that the changes of host hemolymph and venom components would be more readily detected in the early stage of post-parasitism.

In-depth functional analysis revealed that proteins belonging to carbohydrate and fatty acid metabolism pathways showed specific trends (Fig. 6). Glycolysis plays an important role in converting glucose to pyruvate for producing energy. After parasitism, several key enzymes in glycolysis increased significantly at P6, with glucose-6-phosphate isomerase (Ha_Unigene31404) increasing 2.57 fold, pyruvate kinase (Ha_Unigene30179) increasing 2.07 fold, and phosphoglucomutase (Ha_Unigene30910) increasing 1.86 fold (Fig. 6B). While these enzymes showed no significant change at N6 stage (Fig. 6A). From 6h to 24h, the level of these proteins slightly increased in the no parasitism group N24, and changed in the opposite direction in the parasitism group P24 (Fig. 6A and B). Additionally, isocitrate dehydrogenase (Ha_Unigene26688) and malate dehydrogenase (Ha_Unigene24288) in citrate cycle, 6-phosphogluconate dehydrogenase (Ha_Unigene28992) and transketolase (Ha_Unigene32047) in pentose phosphate pathway, glycogen phosphorylase (Ha_Unigene34580) in glycogenolysis showed similar patterns of change (Fig. 6A and B).

Both enoyl-CoA hydratase (Ha_Unigene22621) and 3-hydroxyacyl-CoA dehydrogenase (Ha_Unigene23459) are involved in fatty acid metabolic processes. The abundance of these two enzymes increased 1.74 fold and 3.32 fold 6h post-parasitism (Fig. 6D), but decreased remarkably at 24h (Fig. 6D). However, in the no parasitism group, no significant change between the samples were observed (Fig. 6C).

4. Discussion

For successful parasitism, endoparasitoid wasps lay their eggs into the host hemocoel and accompany maternal venom and calyx fluid. This study reports the identification of the putative venom proteins from the endoparasitoid wasp *M. mediator* from integrated transcriptomic and proteomic analysis. The data obtained from iTRAQ-based quantitative proteomics reveals that the parasitism causes significant changes in the host hemolymph at the early phase post oviposition.

Parasitoid wasps are a large and diversified group of insects on earth

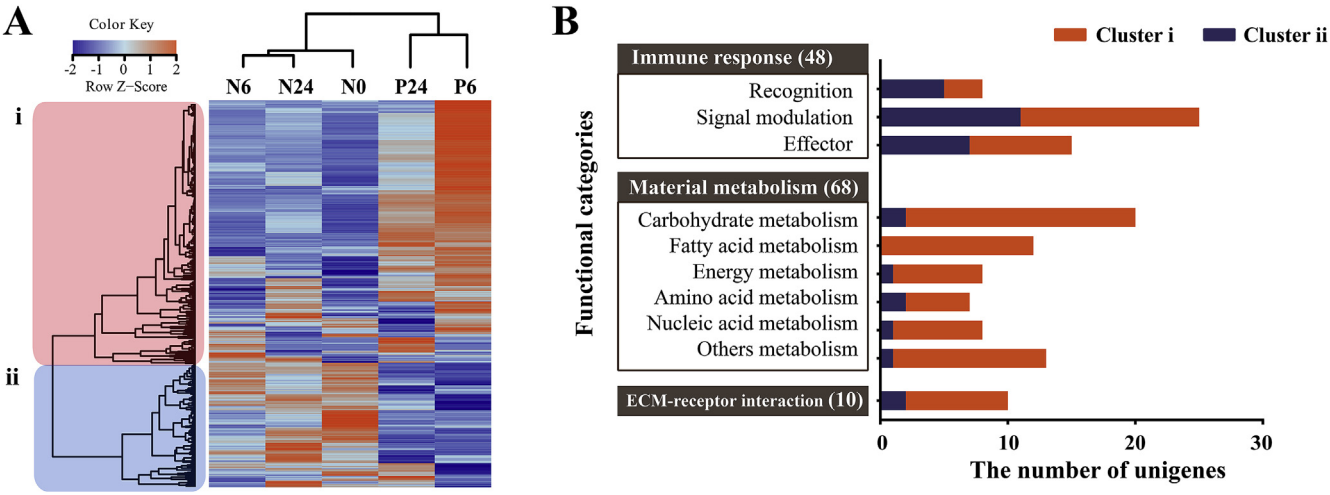

Fig. 5. iTRAQ analysis of *H. armigera* hemolymph proteins post-parasitism. **(A)** Hierarchical cluster analysis of differentially expressed proteins from five samples: second instar *H. armigera* larvae (N0), second instar *H. armigera* larvae at 6 h post-parasitism (P6), second instar *H. armigera* larvae at 24 h post-parasitism (P24), and the corresponding larvae with no parasitism at 6 h (N6) and 24 h (N24). The protein levels were significantly changed as up-regulation ($q < 0.01$ and fold-change > 1.2) and down-regulation ($q < 0.01$ and fold-change < 0.83). Two discrete clusters identified from the protein dendrogram, are shown in red (Cluster i) and blue (Cluster ii). **(B)** The functional categories enrichment analysis of the DEPs from Cluster i and Cluster ii. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

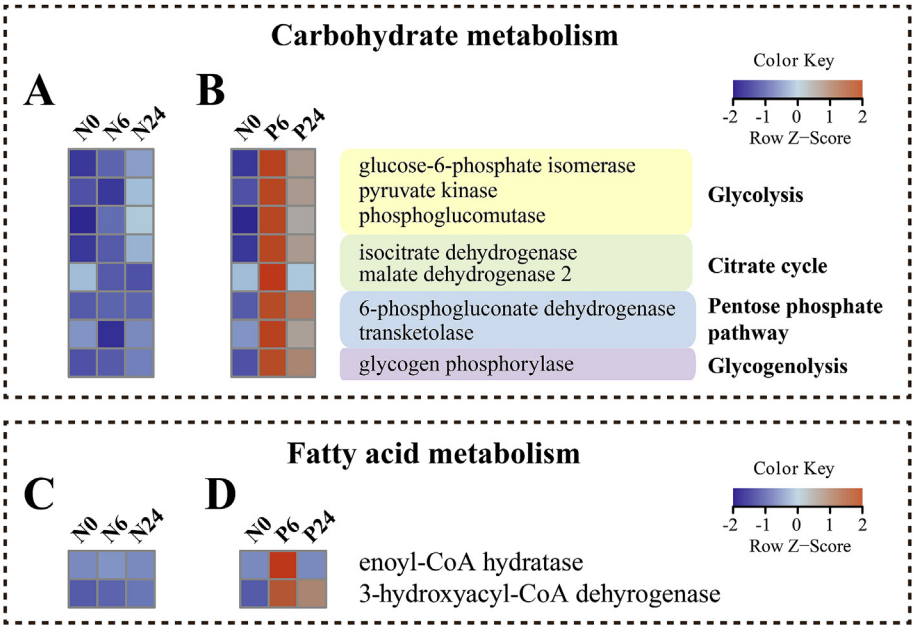

Fig. 6. Heatmaps of the DEPs associated with carbohydrate and fatty acid metabolism pathways in no parasitism group and post-parasitism group. The protein expression profiles of DEPs generated by iTRAQ analysis related to **(A)** carbohydrate metabolism in no parasitism (N0, N6, and N24) groups, **(B)** carbohydrate metabolism in post-parasitism (N0, P6, and P24) groups, **(C)** fatty acid metabolism in no parasitism (N0, N6, and N24) groups, **(D)** fatty acid metabolism in post-parasitism (N0, P6, and P24) groups. The red and blue colors reflect high and low protein levels. The names of enzymes and pathways were shown on the right. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

(Pennacchio and Strand, 2006). They share a conserved organ, venom apparatus. Ectoparasitoid wasp venoms could induce paralysis and alter the development, metabolism, and immune responses of their hosts (Libersat and Gal, 2014; Moreau and Asgari, 2015; Mrinalini et al., 2015; Price et al., 2009; Rivers et al., 2002). Endoparasitoid wasp venom proteins can regulate host physiology and immune responses, often in concert with other virulence factors, including polydnaviruses (PDV) and virus-like particles (VLPs) (Asgari, 2006; Asgari and Rivers, 2011; Lu et al., 2010). However, they are not essential for parasitism in some ichneumonid wasps, such as *H. didymator* and *Campoletis sonorensis* (Doremus et al., 2013; Kroemer and Webb, 2005). In *M. mediator,* the venom and calyx fluid are the first batch of substances that are injected into their host hemocoel. In addition to the maternal factors impairing host cellular immunity, *M. mediator* parasitism within 24 h induced the differential expression of 521 proteins in the hemolymph of *H. armigera*. Among these proteins, 10 were derived from *M. mediator*, and further identified from venom reservoir proteome. In some host-parasitoid systems, viral transcripts are detected from a few hours to several days post parasitism (Asgari et al., 1997; Beckage and Gelman, 2004; Hepat et al., 2013; Le et al., 2003). However, MmBV viral proteins were not identified among the DEPs. It is possible due to experimental condition or that MmBV viral protein are not accumulated in such a short temporal window in *M. mediator* and *H. armigera* system.

NanoLC-MS/MS was performed to examine the components of venom apparatus and venom reservoir in *M. mediator*. Fewer proteins were found in the venom reservoir (313) than in the venom apparatus (2,267). In total, 62 proteins identified from the *M. mediator* venom reservoirs proteome were homologous to venom proteins from other parasitoid wasps, and 31 proteins among them were highly expressed in the venom apparatus and had signal peptides for secretion. There are multiple metalloproteases and serpins identified from venom apparatus transcript and protein profiling data. Among them, 7 metalloproteases and 8 serpins were identified as the putative venom proteins. This indicates that both families could undergo extensive gene duplications in *M. mediator*. The reprolysin-like metalloprotease genes from *M. demolitor* and the serpin genes from *P. puparum*, were also documented as duplicated parasitoid venom proteins (Burke and Strand, 2014; Yan et al., 2017). Several reports speculated that venom proteins diversified into multigene families were recruited through gene duplication from non-venom proteins via the 'birth and death' model (Casewell et al., 2013; Nei et al., 1997). In this evolution model, genes were expressed in both ancestral venom glands and other tissues. As a highly specialized organ, venom gland produces a small number of proteins at high levels. Under the force of positive selection, some venom genes expanded by gene duplication, but others became pseudogenes (Hargreaves et al., 2014; Wong and Belov, 2012).

Insects mount an efficient cellular and humoral immune mechanism to defend against invading microorganisms and parasites. As a result, parasitoid wasps have evolved strategies for evading or suppressing their hosts' defense. A key host immune mechanism involves encapsulating wasp egg or larva in layers of hemocytes (Anderl et al., 2016; Mortimer et al., 2012, 2013; Schlenke et al., 2007). The capsules are often melanized due to the proteolytic activation of PPO (Lu et al., 2014; Yan et al., 2017). iTRAQ analysis revealed that 48 DEPs in host hemolymph post parasitism were involved in the immune response, and were enriched in the melanization cascade. During HearNPV infection, the *H. armigera* serpin-9 plays an important role in regulating the melanization cascade by directly inhibiting the target protease cSP6, which activates PPO purified from hemolymph (Yuan et al., 2017). iTRAQ based quantitative proteomics suggested that the melanization pathway was suppressed in *H. armigera* post parasitism through downregulation of cSP6.

Another major functional category was involved in material metabolism through iTRAQ analysis, which contained 68 DEPs in host hemolymph post-parasitism. Among them, 61 DEPs belonged to cluster i, which included enzymes related to carbohydrate, fatty acid, energy, amino acid, and nucleic acids metabolism that were highly expressed in the post-parasitism groups. The reason might be due to the developing parasitoid wasps compete with their hosts for nutrients. For benefit wasp offspring development, parasitoids use virulence factors to regulate the material metabolism in ways to prepare the host tissues as a suitable environment and promote resources availability to wasp offspring (Asgari and Rivers, 2011; Beckage and Gelman, 2004; Pennacchio and Strand, 2006). Numerous studies have reported evidence that the venom and calyx fluid can change the amount of lipids, proteins, and carbohydrates in host insect hemolymph (Asgari and Rivers, 2011; Martinson et al., 2014; Mrinalini et al., 2015; Strand and Burke, 2015). The venom acid phosphatase (MmV502) is a putative venom protein of *M. mediator*, and has been identified from several other parasitoid wasps (Table 1). In *P. hypochondriaca*, the venom acid phosphatase might be essential to release hydrocarbons and provide nutrients for wasps (Dani et al., 2005).

In conclusion, our study reported 75 putative venom proteins from *M. mediator,* including metalloproteases and serpins that show extensive gene duplications. We present the significant change in protein levels host hemolymph at 6 h and 24 h post-parasitism. These proteins are involved in immune response, and other interaction three major functional categories. The venom proteins contribute toward a better understanding of the evolution of venom functions and play promising functional roles in the biological control of agricultural pests.

Author contributions

Experimental design: ZL, ZZ and ZQL. Performed experiments: ZL, YC, RJW, JD, OV, LBH, and YH. Contributed reagents and materials: JCL and ZYL. Writing-original draft: ZL and ZZ. Writing-review and editing: ZL, ZZ and ZQL.

Founding

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2018.12.013.

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