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Proteomic analysis of parasitized *Plutella xylostella* larvae plasma

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ABSTRACT

Insects use their innate immunity to defend themselves against foreign invaders, such as microorganisms, nematodes and parasites. *Cotesia plutellae*, an endoparasitoid wasp that parasitizes the diamondback moth *Plutella xylostella*, uses several strategies to attack the host immune system, such as injection of viruses, venom, and serosal membrane-derived cells denoted teratocytes. However, the proteome profiles related to these immune deficiency systems have yet to be clearly defined. In this study, we investigate differences in protein expression patterns in parasitized *P. xylostella* larvae, with a view to identifying parasitism-specific factors. Using 2D polyacrylamide gel electrophoresis, proteins in the host plasma were assessed every 48 h after parasitism by *C. plutellae*. A large number of protein spots (350 in total) were detected, and approximately 50 spots were differentially expressed in the parasitized *P. xylostella* larvae every 48 h. In total, 26 potential candidates, including *P. xylostella* Serpin 2 (pxSerpin 2), translationally controlled tumor protein, signal transduction histidine kinase, apolipophorin-III, and fatty-acid binding protein were identified through quadrupole time-of-flight tandem mass spectrometry and sequence homology analysis. These proteins were classified into the following functional groups: immunity, signaling, lipid metabolism, energy metabolism, amino acid/nucleotide metabolism, and others. The pxSerpin 2 gene was cloned, and its expression profile investigated during the course of parasitism. Real-time PCR analysis of pxSerpin 2 revealed a poor correlation between the mRNA level and protein abundance. Our results clearly suggest that parasitism-specific proteins participate in suppression of the host immune response.

1. Introduction

The innate immune system of insects is highly efficient against foreign pathogens, microorganisms, and parasites. However, some endoparasitoid wasps effectively suppress the host's defense system and influence growth and development to meet their own needs (Strand and Pech, 1995; Beckage and Gelman, 2004). Several studies have shown that endoparasitoid wasps have dramatic effects on plasma proteins, metabolism, food consumption, pigmentation, and even the behavior of the host, and these effects often vary depending on the stage of parasitism (Beckage, 1985; Beckage and Kanost, 1993; Bae and Kim, 2004).

The diamondback moth, *Plutella xylostella*, a major pest of cruciferous crops, is highly resistant to *Bacillus thuringiensis* (Bt) toxin and several other types of synthetic insecticides (Talekar and Shelton, 1993). However, when parasitized by *Cotesia plutellae*, *P. xylostella* exhibits immunosuppression and a prolonged larval period, resulting in failure to initiate pupal metamorphosis (Bae and Kim, 2004; Ibrahim and Kim, 2006). Elucidation of this phenomenon, which may be beneficial for the crop industry, is thus a current focus of research.

The solitary endoparasitoid, *C. plutellae*, attacks *P. xylostella* during the larval stage. The wasp egg develops into larva in the host's hemocoel, and emerges from the host 7–8 days after parasitization (Shi et al., 2002; Takeda et al., 2006). The wasp evades the host's immune system using venom, polydnavirus, and embryonic factors, such as teratocytes, a specific cell type that originates from the embryonic serosal membrane (Webb and Luckhart, 1994; Luckhart and Webb, 1996; Jarlfors et al., 1997). Accordingly, we examined the plasma protein profiles of *P. xylostella* larvae at 2, 4, and 6 days after parasitization to distinguish the stages of parasitism.

Numerous investigators have identified a large number of differentially expressed genes related to parasitism. However, most employed mRNA-based approaches to measure message abundance, and there is a poor correlation between the mRNA level

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and protein abundance. Importantly, mRNA-based approaches cannot be used for fluids, such as plasma (Ideker et al., 2001; Vierstraete et al., 2004; Song et al., 2006). A proteomic approach would be more appropriate for protein/functional profiling.

In the present study, we identified 26 differentially expressed proteins in the plasma of parasitized *P. xylostella* larvae during larval development using two-dimensional polyacrylamide gel electrophoresis (2D PAGE) coupled with quadrupole time-of-flight tandem mass spectrometry (Q-TOF MS/MS), with a view to acquiring proteomic information on components of the innate immune system of insects.

2. Materials and methods

2.1. Insects and parasitization

P. xylostella larvae were reared on Oriental cabbage leaves at 25 ± 1 °C, 60% humidity, over a 16 h/8 h light/dark photoperiod in a plastic cage (35 cm in length, 25 cm in width, and 21 cm in height). Adult *P. xylostella* and wasps were provided with a cotton pad soaked in 10% and 40% sucrose solution, respectively. Adult *P. xylostella* were allowed to oviposit on fresh cabbage leaves. Late second instar larvae of *P. xylostella* were parasitized by *C. plutellae* at a density of 1:1 (host:wasp) for 24 h under previously described rearing conditions (Bae and Kim, 2004). Parasitized *P. xylostella* larvae were incubated and bled every 48 h (2 days (P2), 4 days (P4), and 6 days (P6) after parasitization). Parasitoid larvae emerged from the host 8–9 days after oviposition, and entered the cocoon stage. Adult *C. plutellae* were fed a 40% sucrose solution, and allowed to mate for 24 h before parasitization.

2.2. Protein preparation

P2, P4, and P6 *P. xylostella* larvae were bled into 45 μl of PBS (2.7 mM KCl, 2 mM KH_2PO_4, 137 mM NaCl, 10 mM Na_2HPO_4, pH 7.4), 2.5 μl of 20× protease inhibitor (Complete mini, Roche), and 2.5 μl of 65 mM dithiothreitol (DTT) on a concavity slide (electron microscopy sciences). Parasitization was confirmed by emergence of *C. plutellae* larva from that of host *P. xylostella*. The collected hemolymph was centrifuged for 5 min at 1200 × *g* (4 °C). Hemocytes and debris were discarded, and the supernatant desalted using the TCA/acetone precipitation method (Gorg et al., 1988). Protein pellets were centrifuged for 25 min at 18,000 × *g* (4 °C), washed three times with cold acetone, and dissolved in 7 M urea/2 M thiourea. Non-parasitized *P. xylostella* larvae at the same stage were designated C2, C4, C6, and pairwise compared.

2.3. 2D PAGE

Each protein (300 μg) was applied to an IPG strip (18 cm, linear pH 4–7) (Immobiline DryStrip, Amersham Biosciences) with rehydration buffer (DeStreak Rehydration Solution, Amersham Biosciences). Isoelectric focusing (IEF) was conducted for 80,000 V-h using the IPG-phor system (Amersham Biosciences). After IEF, strips were incubated in equilibration buffer (6 M urea, 50 mM Tris–HCl, 2% SDS and 30% glycerol) containing 13 mM DTT for 20 min, and alkylated for 20 min in equilibration buffer containing 2.5% iodoacetamide. Proteins were separated according to molecular mass by SDS-polyacrylamide gel electrophoresis on a 12% non-gradient gel using a Protein II xi cell system (Bio-Rad) (Pasquali et al., 1997). Gels were silver-stained to visualize protein spots (Mortz et al., 2001). Proteins displaying differential expression in parasitized larvae were excised from 2D gels, and processed for ESI-MS/MS.

2.4. Image analysis and statistical analysis

Gel images were captured using PowerLook 2100XL (UMAX), and analyzed with Progenesis software (Nonlinear dynamics) at the Korea Basic Science Institute (Seoul, Korea). The analysis included an automated spot detection and matching function, followed by manual designation of spots as landmarks for gel alignment. A master gel summarizing the spots that were detected at all times was constructed. Spot intensities were normalized and quantitative analysis performed during parasitism. The protein spots differentially regulated at least 1.5-fold in parasitized *P. xylostella* larvae were selected. Normalized volumes of these spot were subjected to statistical analysis with *t*-test for parasitization effect. 2D PAGE was repeated at least three times during three separate experiments to confirm the reproducibility of results. Gel-to-gel differences of three separate experiments were qualified statistically using Kruskal–Wallis test. The SAS statistical software (SAS v. 9.1) was used for *t*-test (Proc Ttest) and Kruskal–Wallis test (Proc Npar1way). In a quantitative assay, protein expression level in non-parasitized larvae was arbitrarily taken as 1.

2.5. In-gel digestion, Q-TOF MS/MS, and protein identification

Excised spots were destained to remove the remaining silver ions, and incubated in 200 mM ammonium bicarbonate. Gel pieces were dehydrated with 100 μl of acetonitrile, and dried in a vacuum centrifuge. Next, dried gel pieces were rehydrated with 20 μl of digestion solution (50 mM ammonium bicarbonate containing 0.2 μg of modified trypsin (Promega)) and incubated for 16 h at 37 °C. The digestion solution was collected and added to 50 μl of 50% acetonitrile, 0.1% trifluoroacetic acid. The peptide solution was desalted using a C18 nano-column (Millipore). MS/MS of peptides generated by in-gel digestion was performed with nano-ESI on a Q-TOF2 mass spectrometer (Micromass) (Shevchenko et al., 1996). To identify the proteins, all MS/MS spectra recorded for tryptic peptides derived from the spots were searched against protein sequences from NCBInr databases using the MASCOT search program (www.matrixscience.com, Matrix Science) or (http://dove.embl-heidelberg.de/Blast2/msblast.html) with MS-BLAST (Shevchenko et al., 2001).

2.6. In silico cloning and relative quantitative real-time PCR of *P. xylostella* Serpin 2 (pxSerpin 2)

Protein sequences of spots 271 and 272 were used as *in silico* cloning probes to identify full-length cDNA. Amino acid sequences were analyzed with a previously constructed expressed sequence tag (EST) database of immunized *P. xylostella* (Eum et al., 2007). One of the clones from the EST database (DDBJ/EMBL/GenBank accession No.: BP937849) matched the sequences of spots 271 and 272, and was subsequently identified as the full-length pxSerpin 2 gene (DDBJ/EMBL/GenBank accession No.: AB282640). Parasitized and normal larval samples were independently frozen in liquid nitrogen after 3, 6, 9, 12, 18, 24, 48, 72, 96, 120 and 144 h of parasitism and stored at −80 °C. For relative quantitative real-time PCR, total RNAs were isolated from time-dependent whole body samples using a SV Total RNA Isolation Kit (Promega). Total RNA was converted into cDNA using the high capacity cDNA Archive Kit (Applied Biosystems). The gene-specific primer sets used for pxSerpin 2 were 5′-GGCTG AGCTT CCATA CCAAG A-3′ and 5′-CGTTC TGGGC CACCT TCTC-3′. Real-time PCR was performed using the 7300 Real-Time PCR System (Applied Biosystems) in triplicate with SYBR Premix EX Taq (Takara) and Eukaryotic 18S rRNA Endogenous Control (Applied Biosystems) to produce a normalization control. Data were analyzed using the Sequence Detector