cDNA Cloning and Transcriptional Expression of a Peritrophic-Like Gene in the Hessian Fly, *Mayetiola destructor* [Say]

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One of the well-studied components of the insect gut is the peritrophic matrix (PM). This semipermeable structure primarily functions in digestion, and protection against invasive microorganisms and mechanical damage. We report the cDNA cloning and transcriptional profiles of a peritrophic-Like gene (designated *MdePERIAT*) in the Hessian fly *Mayetiola destructor*. The predicted amino acid sequence of *MdePERIAT* revealed a putative secretion signal peptide at its amino terminus, similarity to peritrophins from other insects including dipterans, and the presence of two chitin binding domains each containing six cysteine residues. Quantitative expression analysis of *MdePERIAT* mRNA in different larval tissues revealed the transcript to be predominantly present in the midgut (597.9-fold) compared to other tissues assayed including salivary glands and fat bodies. Spatial expression patterns during development showed a peak expression of *MdePERIAT* in the feeding second-instar (14.6-fold) and a decline in expression in the pupal and adult stages. Transcription profiling of *MdePERIAT* during compatible (larvae on susceptible plants) and incompatible (larvae on resistant plants) interactions with wheat revealed a greater level (1.7-fold) of *MdePERIAT* transcript in larvae on resistant plants in the initial time point examined. However, *MdePERIAT* expression declined in larvae on resistant plants at the later time points. Arch. Insect Biochem. Physiol. 64:19–29, 2007. © 2006 Wiley-Liss, Inc.

Keywords: Hessian fly, wheat; peritrophic matrix; gene expression; *MdePERIAT*; midgut

INTRODUCTION

The peritrophic matrix (PM) is an important structure within the gut of insects. It was originally described as a “membraneous sac” that aided in digestion by surrounding the ingested food in the gut lumen (Balbiani, 1890). In its ability to compartmentalize the gut, the PM has several functions including digestion and protection of the midgut epithelia from invasive microorganisms and abrasion (Tellam, 1996; Lehane, 1997). The porosity of these structures partially aid in the regulatory movement of digestive enzymes from the midgut epithelia into the gut lumen and also to prevent entry of larger molecules from the gut lumen into the midgut epithelia (Lehane, 1997). The PM is an acellular structure primarily composed of peritrophins (proteins), chitin, and proteoglycans (Peters, 1992; Tellam, 1996; Tellam et al., 1999). However, the relative concentrations of each com-

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Grant sponsor: USDA-ARS CRIS Project; Grant number: 3602–22000–014–000.

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Received 20 April 2006; Accepted 8 September 2006

© 2006 Wiley-Liss, Inc.
DOI: 10.1002/arch.20157
Published online in Wiley InterScience (www.interscience.wiley.com)
ponent can fluctuate between developmental stages, different species, and also vary within a specific life stage (Tellam, 1996).

There are two different types of PMs in insects based on their mode of synthesis in the gut lumen (Wigglesworth, 1930; Tellam, 1996). The most common is type I, which is synthesized by the midgut epithelial cells usually in response to the event of feeding. Type II PM is a tubular structure that is synthesized by the cardiae at the anterior portion of the midgut. The latter type of PM has been extensively studied because they are produced prior to food ingestion and, therefore, are easier to extract from the gut. Some of the best-examined insects for Type I and Type II PMs are members of Lepidoptera (Spence, 1991) and Diptera (Peters, 1992), respectively.

The Hessian fly interacts with wheat in two modes. In compatible interactions, larvae on susceptible plants are able to establish a sustained feeding site, and complete their development successfully. During these interactions, the wheat plants are stunted, develop dark green foliage (Cartwright et al., 1959) with enhanced leaf sheath permeability (Shukle et al., 1992), ultimately leading to adverse effects on yield. In incompatible interactions, larvae on resistant plants are unable to establish a feeding site, and die within a period of 5-days post hatch (Painter, 1930). The wheat plants during incompatible interactions undergo little or no physiological stress (Williams et al., 2002) and yield normally.

The deployment of resistant wheat is the most effective means of protecting wheat from damage caused by Hessian fly. This resistance is expressed as larval antibiosis (larvae die four to five days after infesting plants) and is controlled primarily by single dominant to partially dominant genes (Gallun, 1977). For some resistance genes, virulence in the insect is controlled by non-allelic recessive genes and operates in a gene-for-gene manner with resistance in wheat (Hatchett and Gallun, 1970; Gallun, 1978; Formusoh et al., 1996; Zantoko and Shukle, 1997). As in other gene-for-gene specificities, the deployment of resistance (R) genes in wheat results in the appearance of new genotypes (biotypes) that can survive on formerly resistant wheat. Thus, there is a need to identify potential targets in the insect for transgenic resistance, which when pyramided with native R genes could serve to enhance the durability of resistance in wheat. Because alimentary processes are basic to Hessian fly biology, the larval midgut PM may be one of the potential targets. The objectives of the current study were to reveal the tissue and developmental specificities of MdesPERI-A1 as well as its expression during the interactions of larvae with susceptible and resistant wheat.

MATERIALS AND METHODS

Insect and Plant Material

Biotype I. of the Hessian fly was used in the current study. To date, sixteen biotypes (designated GP and A to O) have been identified (Ratcliffe et al., 1994). Biotype I. (larvae on resistant plants on resistance genes H3, H5, H6, H7H8) was selected from a Posey County, Indiana, field collection made in 1986. Wheat cultivars used in the study were “Newton” (carries no Hessian fly resistance gene) and “Iris” (carries resistance gene H9). For compatible interactions, Biotype I. was reared on Newton wheat in growth chambers at 20°C with a 12-h photoperiod. Instars (1st, 2nd, and 3rd) of Hessian fly larvae and pupae were collected by dissecting the crown portions of infested wheat seedlings in water to remove them from the plants, and then immediately flash-frozen them in liquid nitrogen. Adults were collected after emergence. Larvae, pupae, and adults were stored at −80°C until RNA was isolated.

Tissue Dissections and RNA Isolation

Two hundred midguts were dissected from four-day post hatch 1st-instar larvae and eight-day post hatch early 2nd-instar larvae as described earlier (Grover et al., 1988; Mittapalli et al., 2005). Briefly, midguts were dissected in ice-cold Schneider’s insect medium (Sigma-Aldrich, St. Louis, MO) by first pinching off the posterior tip of the larvae and then gently compressing the body, commencing
from the anterior end. Salivary gland and fat bodies were dissected from the remaining larval carcass. Midgut, salivary glands, and fat body were collected in 100 µl of ice-cold Schneider’s medium contained in a 1.5-ml microcentrifuge tube. All the collected tissues were immediately flash-frozen in liquid nitrogen and stored at -80°C until RNA was isolated. Total RNA from tissues and from developmental stages were extracted using the RNAqueous®-4PCR kit from Ambion (Austin, TX) following the manufacturer’s protocol. Also, total RNA from Biotype I larvae on Newton plants (compatible interaction) and from Biotype I larvae on Iris plants (incompatible interaction) was isolated from one, two, three, and four days post-hatch larvae using the same protocol.

**Construction of Midgut cDNA Libraries**

A cDNA library was constructed from total RNA isolated from midguts using the “SMART™” cDNA library construction kit from BD Biosciences (Palo Alto, CA), following the manufacturer’s protocol with one modification. All the PCR fragments obtained were cloned directly into the PCR®4-TOPO® vector included in a TOPO TA cloning® for sequencing kit (Invitrogen, Carlsbad, CA) rather than cloning into the original phage vector. Plasmid DNA was isolated using a Qiagen BioRobot 3000 and sequenced from a single end using a sequencing oligomer designed to a specific region of the cloning vector.

**Characterization of MdesPERI-A1**

Annotations and sequence similarity analyses were done using BLAST programs (Altschul et al., 1990) on the National Center for Biotechnology Information (Bethesda, MD) website (http://www.ncbi.nlm.nih.gov/). Conserved domain search was done using blastp and rpsblast tools on the NCBI website. Analysis for secretion signal peptides was performed using SignalP v1.1 (Center for Biological Sequence Analysis, Technical University of Denmark, http://www.cbs.dtu.dk/services/SignalP/ ) and PSORT II analysis (Prediction of Protein Sorting Signals and Localization Sites in Amino Acid Sequences, http://psort.hiroshima-u.ac.jp/). The protein structure of MdesPERI-A1 was predicted using the PSIPRED protein structure prediction server at http://bioinf.cs.ucl.ac.uk/psipred/ (Jones, 1999).

**Quantitative Real-Time PCR**

To quantitatively measure the level of the Hessian fly peritrophin transcript in different tissues, during development and in compatible and incompatible interactions with wheat, qRT-PCR was performed using total RNA extracted as described above. The software Primer Express from Applied Biosystems (Foster City, CA) was used to design real-time primers used in this study. The entire (relative) expression analysis was performed using a Hessian fly ubiquitin as an internal standard, which in our evaluation has shown constant expression in Hessian fly.

Quantification of gene expression, displayed as relative expression value (REV), was based on the Relative Standard Curve method (User Bulletin no. 2: ABI Prism 7700 Sequence Detection System http://docs.appliedbiosystems.com/pebidocs/04303859.pdf). In brief, to calculate the REV, first the target quantities were calculated using serial dilutions of a cDNA sample containing the target sequence. The threshold cycle (Ct) value for each dilution was plotted against the log of its concentration, and Ct values for the experimental samples were plotted onto this dilution series standard curve. Target quantities were calculated from separate standard curves generated for each experiment. REVs were then determined by dividing the target quantities of the gene of interest with the target quantity obtained for ubiquitin. PCR cycling parameters included 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 sec and 60°C for 1 min. The primer sequences used in this study are listed in Table 1.

**Statistical Analysis**

For calculations of significance, the logs of the REVs for each gene were analyzed by ANOVA
(Analysis of Variance) using the PROC MIXED procedure of SAS (SAS Institute Inc. SAS/STAT User’s Guide, Version 9.1). For expression analysis in tissues and developmental stages, the statistical model included treatment and interaction between treatments whereas for the analysis of expression in different interactions (compatible and incompatible), the statistical model included treatment, time points, and interaction between treatments and time points as fixed effects. Biological replicates were included as a random effect in the analysis model. Treatment differences at each time point were evaluated using orthogonal contrasts and considered statistically significant if the $P$ value associated with the contrast was $P < 0.05$.

Relative fold change in tissues and during development was determined by taking the sample that showed the lowest level of expression known as the calibrator sample (Pfafii, 2001). Hence, the fold changes in the midgut and fat body tissues for all the genes assessed were calculated relative to the salivary gland tissue, which showed the lowest level of expression for all the transcripts. Similarly, during development the basal expression level obtained in adults was used to determine the fold changes in other developmental stages. The fold change in gene expression during compatible and incompatible interactions was assessed by dividing the REV for larvae on resistant plants by the REV for larvae on susceptible plants for all the four times points examined (1-, 2-, 3-, and 4-day post hatch). The standard error represented the variance in three technical replicates for the tissue/development expression analysis, and two biological replicates (two technical replicates within each) for the interaction study. Technical replicates involved the same samples replicated in the setup of the qRT-PCR plate to correct for errors during pipetting, whereas biological replicates involved experimental replicates separated spatially and/or temporally to correct for experimental error.

RESULTS

Characterization of MdesPERI-A1

A cDNA clone containing the entire coding region of an insect peritrophin-A was identified from a larval midgut cDNA library of the Hessian fly. The length of the cDNA and deduced amino acid sequences of the Hessian fly peritrophin-A was in agreement with other insect peritrophin-A sequences (Fig. 1A). The open reading frame (orf) for MdesPERI-A1 was constituted by 726 bases (242 amino acid residues). The deduced amino acid sequence of MdesPERI-A1 showed a high degree of similarity with other insect peritrophins including dipteran species (Drosophila melanogaster 65% and Anopheles gambiae 60% identity). Two closely spaced chitin binding peritrophin-A domains spanned the protein sequence. These domains were present downstream of a putative signal peptide at the amino-terminus indicating that it was secreted (Fig. 1A). The secretory signal peptide was constituted by 21 amino acid residues. Other characteristic features of MdesPERI-A1 that classified it as a peritrophin-A protein included the six conserved cysteine residues (Fig. 1A) and several aromatic residues within each of the chitin-binding domains. Additionally, the deduced amino acid sequence of MdesPERI-A1 was devoid of any transmembrane domains and also functional or structural domains characteristic of serine proteases or chitinases.

The predicted secondary structure obtained for MdesPERI-A1 revealed the presence of an alpha-helix and a beta-strand within the deduced secretory signal peptide (Fig. 1B). The first chitin-binding peritrophin-A domain was characterized by the presence of three beta-strands towards the center of the domain, intermittent coiled regions, and an absence of alpha helices. However, the second chitin-bind-
Fig. 1. Characterization of the Hessian fly peritrophin. A: Nucleotide and deduced amino acid sequences of MdesPERI-A1. The start codon (ATG) is boxed and the stop codon (TGA) is shown as an asterisk. An "AATAAA" sequence matching the consensus polyadenylation signal in other species is underlined (bold wavy-line). A predicted secondary signal peptide is double underlined and the most probable cleavage site is indicated by an arrow. Two closely spaced chitin-binding domains are shown. B: Predicted secondary structure of MdesPERI-A1. The top shows the predicted protein components corresponding to the deduced amino acid sequence (lower line). Cylinders, arrows, and solid black lines represent alpha-helices, beta-strands, and coils, respectively.
ing peritrophin-A domain of MdesPERI-A1 constituted five uniformly scattered beta-strands, intermittent coiled regions, and a single alpha-helix towards the carboxyl-terminus (Fig. 1B).

Comparison of MdesPERI-A1 Domains

A homology comparison for each of the two chitin-binding peritrophin-A domains of the Hessian fly peritrophin with peritrophin-like proteins from other members of Diptera that had similar chitin-binding peritrophin-A domains revealed similarity at the amino acid level. This analysis included the two chitin binding peritrophin-A domains of a peritrophin-A from D. melanogaster (AF030895), A. gambiae (AAR02439), and a peritrophin-A like amino acid sequence that contained only a single chitin-binding peritrophin-A domain from C. cephalotes felis (AAM21354). The first chitin-binding peritrophin-A domain of MdesPERI-A1 showed greater similarity to the single chitin-binding peritrophin-A domain of C. felis (27% identity) than it showed to the first chitin-binding peritrophin-A domain of D. melanogaster (20% identity, Fig. 2). The second chitin-binding peritrophin-A domain of MdesPERI-A1 showed greater similarity to the second chitin-binding peritrophin-A domain of A. gambiae (31% identity) than to the single chitin-binding peritrophin-A domain of C. felis (20% identity, Fig. 2).

MdesPERI-A1 Expression in Tissues

To determine if the MdesPERI-A1 transcript was predominantly expressed in the midgut tissue relative to other tissues, we performed an expression analysis. Using total RNA extracted from midgut, fat body, and salivary gland tissues, we determined the level of expression by qRT-PCR. Results indicated the expression of MdesPERI-A1 was greatest in the midgut and the least in the salivary gland samples (Fig. 3). A 597.9-fold difference between the midgut and salivary gland tissues, a 153-fold difference between the midgut and fat body tissues, and a 3.9-fold difference between the fat body and salivary gland tissues (P < 0.05) were revealed.

MdesPERI-A1 Expression During Development

Expression analysis during the developmental stages of Hessian fly was also assessed with qRT-PCR. Results revealed levels of the MdesPERI-A1 transcript to be highly expressed in the three larval instars (Fig. 4), with the peak in expression occurring in the feeding 2nd-larval instar. The greatest difference in MdesPERI-A1 expression during de-

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Fig. 2. Comparison of MdesPERI-A1. The two chitin-binding peritrophin-A domains of MdesPERI-A1 were compared at the amino acid level with similar domains of peritrophin-like proteins from members of Diptera. Shown is the similarity of MdesPERI-A1 with sequences from Drosophila melanogaster (AF030895), Anopheles gambiae (AAR02439), and C. cephalotes felis (AAV41248). Identical residues among all taxa are highlighted in black and identical residues in two of the three taxa are highlighted in gray. Gaps in the alignment are indicated by dashes.
Development was observed between the feeding 2nd-instar and the adult samples, which corresponded to a 146-fold change (Fig. 4). A significant difference ($P < 0.05$) in the expression of *MdesPERI-A1* was observed between all other developmental stages excluding the expression level between 1st- and 3rd-instars ($P > 0.05$).

**Differential Expression of MdesPERI-A1 During Compatible and Incompatible Interactions**

To assess the expression profile of *MdesPERI-A1* in larvae on susceptible plants versus larvae on resistant plants, total RNA was extracted from 1st-instar larvae at 1-, 2-, 3-, and 4-days post hatch. Results obtained revealed the expression profile of *MdesPERI-A1* during these interactions (Fig. 5). The level of *MdesPERI-A1* transcript was significantly greater ($P < 0.05$) in 1-day post-hatch larvae on resistant plants compared to similar aged larvae on susceptible plants. In the next time point assayed (2-day post hatch), there was no significant difference ($P > 0.05$) observed in *MdesPERI-A1* expression between both types of larvae. However, from 3-day post-hatch onwards, there was a decline in the *MdesPERI-A1* transcript in larvae on resistant plants but a steady increase in similar aged larvae on susceptible plants (Fig. 5A). A 1.7-fold increase in expression was revealed in 1-day post-hatch larvae on resistant plants compared to larvae on susceptible plants (Fig. 5B). Further, a 1.9-fold decrease in expression in 4-day post-hatch larvae on resistant plants compared to larvae on susceptible plants was calculated.

**DISCUSSION**

An EST project of the Hessian fly midgut undertaken in our laboratory revealed a diverse set of transcripts that could be involved in various physiological processes during its interactions with wheat. To date, there have been no reports of a structural gene expressed in the Hessian fly midgut, which may be important for its development and serve as a target for its control. We report here the identification, characterization, and expression analysis of a putative peritrophin-A gene in larval Hessian fly during interaction with its host. The deduced amino acid sequence suggested that MdesPERI-A1 is a secreted protein by the presence
of a signal peptide at its amino-terminus. Similar to other dipteran peritrophin-A proteins, MdesPERI-A1 was characterized by two closely spaced chitin-binding peritrophin-A domains that contained several conserved amino acid residues including six cysteines (Figs. 1, 2). An interesting feature of MdesPERI-A1 was the difference revealed between the two chitin-binding peritrophin-A domains. A significant variation in the homology of the deduced amino acid sequence constituting the two chitin-binding peritrophin-A domains and in the predicted secondary structure was observed (Fig. 2). These results suggest the chitin-binding peritrophin-A domains of MdesPERI-A1 share greater similarity with domains in other insect peritrophin-like proteins than the domains within MdesPERI-A1. Similar findings for two of the three “peritrophin-like” proteins (PL1, PL2) and one of the two “mucin/peritrophin-like” proteins (MPL1) in the cat flea (*Ctenocephalides felis*) were reported by Gaines et al., (2003).

Temporal expression patterns of MdesPERI-A1 in midgut, salivary glands, and fat body suggested a primary role of MdesPERI-A1 as a component of the PM in larval Hessian fly. Several studies have revealed a differential expression pattern of peritrophins or peritrophin-like proteins in various insect tissues (Wijffels et al., 2001; Gaines et al., 2003; Shao et al., 2005; Du et al., 2005). Expression analysis of Cb-peritrophin-15 in the Old World screwworm fly (*Chrysomya bezziana*) indicated it was highly tissue specific in expression. Cb-peritrophin-15 was reported to be expressed only in cardiæ and not in the non-gut-associated tissues such as crop, salivary glands, fat body, Malphigian tubules, or tracheae (Wijffels et al., 2001). However, Gaines et al. (2003) revealed that both peritrophin-like (PL1, PL2, and PL3) and mucin/peritrophin-like (MPL1 and MPL2) mRNAs in C. *felis* were expressed in the hindgut and Malphigian tubules. More recently, the transcript of a peritrophin-like protein Fc-peritrophin was observed to be expressed in hemocytes, heart, stomach, intestines, and gills in the fleshy prawn *Fenneropenaeus chinensis* (Du et al., 2005).

Similarly, the Hessian fly peritrophin-like transcript also showed a distinct expression profile during development (Fig. 4). MdesPERI-A1 mRNA was most abundant in larvae, in particular, the feeding 2nd instar. This trend in expression further suggests a function of MdesPERI-A1 in the PM as the 3rd
instar is non-feeding, pupae diapause, and adults do not feed (Shukle, 2003). These results are in line with those of Wijffels et al. (2001), who showed larval PMs contained the predominant levels of Cb-peritrophin-15, while the adults completely lacked, its expression both in cardiae and PMs. However, in the case of C. felis, Pl 1 and Pl 2 were expressed only in adults, whereas Pl 3 was highly expressed in all three larval stages (Gaines et al., 2003). Thus, while there are differences in expression during development, the characteristic tissue- and life stage-specificity of MdesPERI-A1 in Hessian fly correlate with the transcriptional expression of peritrophins in other insect species (Tellam et al., 1999; Wijffels et al., 2001; Gaines et al., 2003).

Expression analysis of MdesPERI-A1 during compatible and incompatible Hessian fly/wheat interactions revealed a different expression profile of MdesPERI-A1. It has been reported that in resistant wheat seedlings, the mRNA of a lectin-like Hessian fly response gene (Hfr-1) is up-regulated during larval infestation and returns to normal pre-infestation levels after the infesting larvae have died at 5-day post-hatch (Williams et al., 2002). This group further observed the peak expression of Hfr-1 between 24–64 h post-larval feeding. The data obtained in the current study with Hessian fly larvae correlates with the expression profile obtained for Hfr-1 in resistant wheat seedlings.

Plant lectins have been well documented to play an important role in insect resistance (Peumans et al., 2000). A primary criterion adopted by these proteins is to bind to the PM of feeding larvae. Mannose-binding snowdrop lectin (Galanthus nivalis agglutinin, GNA) effectively targets the midgut of herbivorous insect species such as the green leafhopper, Nephrotettix virescens and brown plant hopper, Nilaparvata lugens (Foissac et al., 2000). More recently, studies on GNA in aphid predators (Chrysoperla carnea, Adalia bipunctata, and Coccinella septempunctata) revealed the protein to directly affect longevity by binding to glycoproteins in the larval midguts (Hogervorst et al., 2006). Additionally, jasmonate-regulated wheat protein (JRP-32) was found to contain a jacalin-related lectin domain that could potentially be required for plant defense against diseases and insect pests (Wang and Ma, 2005). Similar gene inductions have also been reported during wound-induced responses in tomato and potato (Pena-Cortes et al., 1993) and soybean (Staswick, 1990). Further, the effect of wheat germ agglutinin (WGA) has profound effects on the PM formation and its structure in various lepidopteron larvae (Hopkins and Harper, 2001). The subsequent decrease in expression of MdesPERI-A1 observed at days 3 and 4 post-hatch could be due to a decline/cessation in feeding and also general distress of larvae on resistant plants.

ACKNOWLEDGMENTS

This is a joint contribution of the USDA-ARS and Purdue University. This article represents the results of research only. Mention of a commercial or proprietary product does not constitute an endorsement by the USDA for its use.

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