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Gene Expression and Regulation of Wax Moth Transferrin by PAMPs and Heavy Metals

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Abstract: A complete mRNA sequence of transferrin from the wax moth, *Galleria mellonella*, was obtained, and compared with those of other species. We previously reported that the sequence was most similar to those of *Manduca sexta* and *Bombyx mori*. As in other moths, *G. mellonella* transferrin had only one iron-binding site at its N-terminal region. Semi-qRT PCR was conducted to investigate tissue-specific distribution and transcriptional regulation of the wax moth transferrin mRNA. Larval muscle and fat body contained larger quantity of mRNA than other tested tissues. In this study, it was observed that iron and cadmium regulated transferrin transcription, and this regulation pattern was tissue specific. Iron up-regulated transferrin mRNA level in fat body, while suppressed it in the Malpighian tubules and silk glands. Cadmium decreased the mRNA level in fat body, muscle, and Malpighian tubules, but significantly increased the mRNA level in silk glands. In addition, the mRNA expression was induced by all tested pathogen-associated molecular patterns (PAMPs) including LPS, lipoteichoic acid (LTA), glucan, and even chitin.

Keywords: transferrin, wax moth, cDNA cloning, pathogen-associated molecular pattern, PAMP, iron, cadmium

INTRODUCTION

Transferrin is a glycoprotein that binds and transports iron. In vertebrates, transferrin family shows a close structural relationship (Weinberg, 1993; Aisen, 1998). The molecular mass of the protein is about 80 kDa with two ferric-binding regions, N- and C-terminal lobe, respectively. However, insect transferrin can be classified into two groups (Bartfeld et al., 1990; Jamroz et al., 1993; Gasdaska et al., 1996; Yoshiga et al., 1997, 1999; Thompson et al., 2003; do Nascimento et al., 2004; Han et al., 2004). One group has N-terminal lobe only, even though the size of the protein is not significantly smaller compared to its vertebrate counterparts. Transferrins of the insects such as *Manduca sexta*, *Galleria mellonella*, *Drosophila melanogaster*, and *Aedes aegypti*, are the members of this group. However, the proteins of another insect group, including *Blaberus discoidalis* and *Mastotermes darwiniensis*, have two ferric-binding lobes like vertebrate transferrins. We previously sequenced wax moth transferrin mRNA, and compared it with other transferrins (Han et al., 2004). In the study, we showed the protein had only one ferric binding region like that of other moths.

Recently, several reports showed the possibility of transferrin as an immune-relevant protein in that the protein was up-regulated by bacterial infection or fungal treatment (Yoshiga et al., 1997; Seitz et al., 2003; Thompson et al., 2003). There is also a traditional view that transferrin carries out its antibacterial activity by withholding iron from bacteria (Weinberg, 1993). Therefore, it is an acceptable assumption that Nichol et al. (2002) even considered the protein as an antibiotic agent. However, it has not been demonstrated whether insect transferrin has direct antibacterial activity.

Pathogen-associated molecular patterns (PAMPs) are found in microbial membranes, cell walls, proteins, and nucleotide structures, and can be viewed as molecular 'signatures' of the invading pathogen by hosts (Medzhitov et al., 1997). In this study, we selected four kinds of PAMPs: lipopolysaccharide (LPS) from Gram-negative bacteria, lipoteichoic acid (LTA) from Gram-positive bacteria, β-1,3-glucan from fungi and yeast, and chitin (poly-[1→4]-β-*N*-acetyl-D-glucosamine) from the exoskeletons of invertebrates. These were selected because bacteria and fungi are common

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pathogens for insects, and chitin can be introduced into insect hemocoels by parasitoids such as wasp larvae.

Since transferrin is a well-known iron-binding protein, the interactions between the protein and metal ion are mainly focused on the relationship to iron (McKnight et al., 1980; Law, 2002; Nichol et al., 2002). In the case of cadmium, many studies are taking notice of the effects of cadmium on iron accumulation and metabolism in mammalian fetal growth (Petering et al., 1979; Whelton et al., 1993; Crowe et al., 1997).

Even though there are many reports on the regulation of transferrin by external stresses, most results focused on mammalian species rather than insects. In this study, we address the transcriptional regulation of *G. mellonella* transferrin by PAMPs and cadmium, a metal ion in relation to insect immune responses.

MATERIALS AND METHODS

Insect

G. mellonella larvae were reared on an artificial diet mixed with their natural diet, bee wax, as described previously (Han et al., 2003, 2004). The insects were kept in darkness at 32±1°C and 75±5% relative humidity. Except for egg samples, the fourth instar larvae were used in all experiments to avoid the hormonal fluctuation occurring when they enter the pupal stage.

PAMP and heavy metal treatment

G. mellonella larvae were immunized by injection of PAMPs in PBS, and treated with metal ions mixed with the artificial diet. Four selected PAMPs, LPS from *Escherichia coli* (O111:B4), LTA from *Streptococcus faecalis,* β-1,3-glucan from *Saccharomyces cerevisiae*, and chitin from crab shells, were purchased from Sigma, and 2 μg of each reagent in PBS was dorsolaterally injected into the fourth instar larvae. LPS, LTA, and β-1,3-glucan were individually suspended in PBS, and the β-1,3-glucan was also sonicated to reduce its particle size (Selvaraj et al., 2005). The chitin solution was prepared according to the method of Cuesta et al. (2003). Purified chitin powder was suspended in PBS, sonicated, and sterilized by exposure to ultraviolet light for 1 h. To investigate the effect on transferrin mRNA level by metal ions, the larvae were administered an artificial diet containing 12.5 mM $FeCl_3$ or 12.5 mM $CdCl_2$ for 48 h, respectively, as previously described by Kim et al. (2001).

RNA Isolation and Semi-qRT PCR

Insect tissues were carefully dissected and collected in cold diethyl pyrocarbonate (DEPC)-treated deionized water, in preparation for study using semi-quantitative reverse transcription polymerase chain reaction (semi-qRT PCR). Whole body samples were prepared by freezing in liquid nitrogen, then grinding in a mortar. Total RNA was then extracted from larval tissues or whole bodies using the easy-BLUE™ reagent (iNtRON, Sungnam, Korea), a commercially available guanidinium-phenol-based solution. cDNA was synthesized from the purified total RNA by M-MLV reverse transcriptase (Bioneer, Daejeon, Korea), and used as a template for semi-qRT PCR. Table 1 shows the primers used for semi-qRT PCR. To avoid errors from internal control fluctuation, the optimization of the cDNA concentration was confirmed by PCR amplification of two different internal controls, actin and S7e. We followed the procedure and used the primer sequences of Lee et al. (2003) to amplify and quantitate wax moth actin. S7e, a ribosomal protein, was used to confirm the validity of actin as an internal control. A complete S7e mRNA was newly sequenced for this study and deposited in GenBank (accession no. **DQ137845**).

The number of PCR amplification cycles required for half of the maximum amplification of each protein was determined by running the PCR reactions for cycles varying from 20 to 40. The intensity of the amplified product from each cycle was checked on 1% agarose gel with EtBr, and analyzed using the software, Quantity One™ ver. 4.5.0 (Bio-Rad). All primers used in this study were synthesized by Bioneer Inc. (Seoul, Korea). PCR experiments were conducted using Ex Taq™ polymerase (Takara, Japan) on GeneAmp® 2700 thermocycler.

RESULTS

Tissue specific distribution of wax moth transferrin

Figure 1 shows wax moth transferrin had only one ferric binding region like that of other moths (A) and multiple sequence alignment of the transferrin mRNA (Han et al., 2004) was conducted to compare various transferrins such as *Manduca sexta*, *Blaberus discoidalis*, and *Homo sapiens* (B). Expression of *G. mellonella* transferrin in larval tissues was studied by semi-qRT PCR (Fig. 2). The tissues were carefully prepared, and the expression levels of transferrin mRNA from fat body, muscle, Malpighian tubules, silk glands, hemocytes, midgut, integument, and eggs were compared. Transferrin mRNA was highly expressed in larval fat body and muscle. Less expression was detected in Malpighian tubules, silk gland, and hemocytes. No transferrin mRNA was found in the midgut and integument. Considering that gut and integument are the main routes through which most external molecules could be introduced into insect bodies, the lack of mRNA in the two tissues is notable. The transferrin mRNA was also found in eggs.

Effect of metal ions and PAMPs on expression of wax moth transferrin

Two metal ions, iron and cadmium, were administrated to

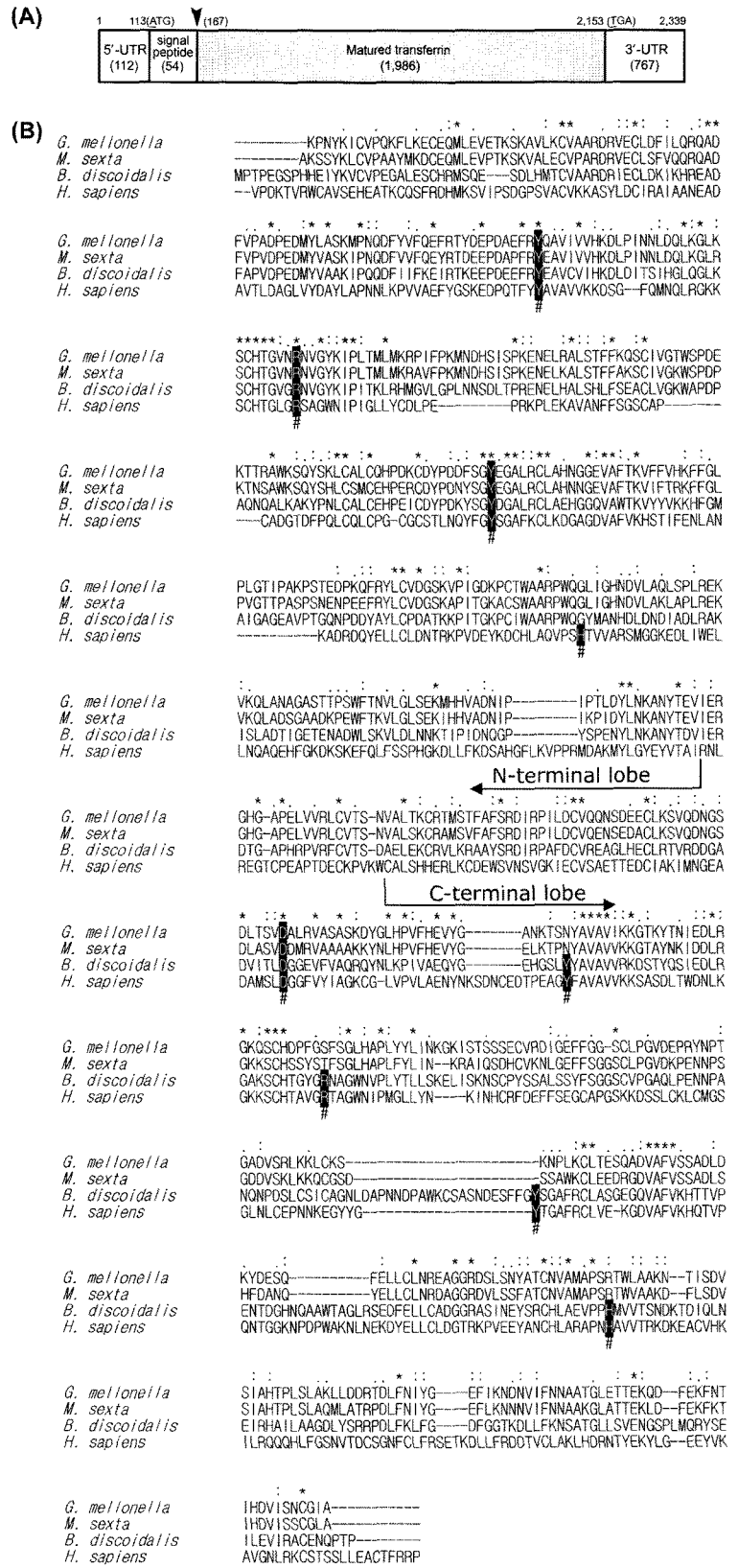

Fig. 1. G. *mellonella* transferrin mRNA was sequenced and analyzed. A schematic illustrations of G. *mellonella* transferrin mRNA is presented (A). Multiple sequence alignment (B) was conducted to compare various transferrins: G. *mellonella* (**AY364430**), *Manduca sexta* (**M62802**), *Blaberus discoidalis* (**L05340**), and *Homo sapiens* (**NM_001063**). Signal peptide sequences of each protein was excluded, and only the regions of mature proteins were compared. The # symbols indicate positions of potential iron-binding residues of *H. sapiens*. The amino acids identical to the iron-binding residues of human transferrin are black-shadowed.

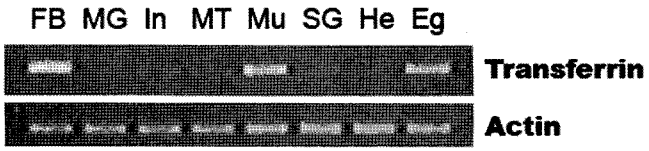

Fig. 2. Tissue-specific distribution of *G. mellonella* transferrin mRNA. Tissues were prepared from larval body except for egg. FB, fat body; MG, mid gut; In, integument; MT, Malpighian tubule; Mu, muscle; SG, silk gland; He, hemocyte; Eg, egg. Similar results were observed from two individual experiments.

G. mellonella larvae. Transferrin mRNA was slightly induced by iron in fat body, whereas it was down-regulated in muscle, Malpighian tubules, and silk gland. Cadmium decreased the mRNA level of the protein in fat body, muscle, and Malpighian tubules, but it distinctly increased the level in silk gland (Fig. 3).

The four selected PAMPs, LPS, LTA, β-1,3-glucan, and chitin, were suspended in PBS and injected into the fourth instar larvae. In this study, the expression of the mRNA from whole body was up-regulated by the treatment of PAMPs (Fig. 4). The regulation was confirmed using two different internal controls, actin and a ribosomal protein S7e.

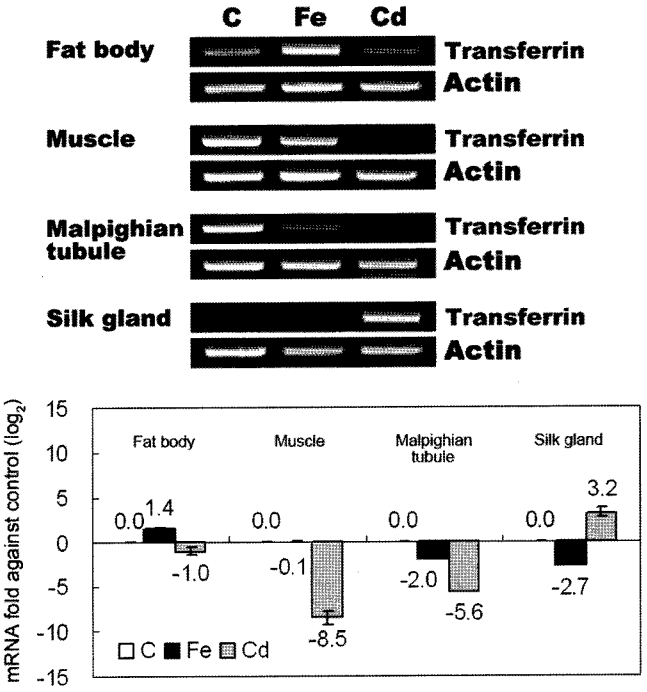

Fig. 3. Iron and cadmium were administrated to wax moth larvae for 48 hours. Semi-qRT PCR was conducted with total RNA prepared from each tissue. Transferrin mRNA levels were normalized to the levels of actin, and compared to the PBS treated control. The relative expression ratio was log transformed (base=2), and analyzed. Mean±SD (n=3) values are presented.

DISCUSSION

We previously described the wax moth ferritin, an iron storage protein, in response to heavy metal (Choi et al., 2006a, b). In the same species, we also described the transferrin mRNA sequence, and showed the sequence was very similar to those of other moths (Han et al., 2004). The wax moth transferrin has only one iron-binding site at its N-terminal region, even though there is no evidence on the function for the C-terminal region. The C-terminal region of insect transferrin has been considered as a nonfunctional area because of several deletions in its amino acid sequence compared to mammalian transferrins. However, recent studies show other possibilities in that transferrin is considered not only an iron carrier, but also an important immune-relevant protein in various species (Csorba et al., 1973; Westerhausen et al., 1977; Oria et al., 1988; Yoshiga et al., 1997; Seitz et al., 2003; Stafford et al., 2003). The C-terminal region of the group containing only one iron-binding lobe may play certain roles in insect immune responses. It is interesting that transferrin C-terminal lobe in humans is responsible for binding to bacterial transferrin receptors (Alcantara et al., 1993; Cornelissen et al., 1994).

The insect fat body is a major site of synthesis for many hemolymph proteins, including transferrin (Bartfeld et al., 1990; Kanost et al., 1990). Ampasala et al. (2004) reported that the transferrin mRNA was highly expressed in fat body of spruce budworm, *Choristoneura fumiferana*. The mRNA had also been detected in fat bodies of other insects such as *M. sexta* (Bartfeld et al., 1990) and *B. discoidalis* (Jamroz et al., 1993). In this study, we found that the fat body and muscle have the highest level of *G. mellonella* transferrin mRNA compared to other tissues (Fig. 2). Intriguingly, no transferrin mRNA was found in *G. mellonella* midgut and integument, duplicating the result reported for *C. fumiferana* (Ampasala et al., 2004).

Transferrin has intensively been studied in vertebrates where it binds and transports iron (Aisen, 1998), and there are several reports that the mRNA level of the protein is down-regulated by iron treatment (McKnight et al., 1980; Yoshiga et al., 1999; Ampasala et al., 2004). In *G. mellonella*, iron regulated transferrin transcription tissue-specifically: up-regulating it in fat body, while suppressing it in Malpighian tubules and silk glands (Fig. 3).

When cadmium is introduced into mammalian intestines, it can lead to iron deficiency, causing an increase in the level of transferrin mRNA (Crowe et al., 1997; Bárány et al., 2005). However, mRNA was decreased by cadmium in an Antarctic icefish (Carginale et al., 2002). In this study, the level of *G. mellonella* transferrin mRNA was suppressed by cadmium in fat body, muscle, and Malpighian tubules, but was significantly induced in silk glands (Fig. 3).

There are many reports about transferrin mRNA induction by injection of bacteria or fungus treatment (Yoshiga et al., 1997, 1999; Thompson et al., 2003; Ampasala et al., 2004).

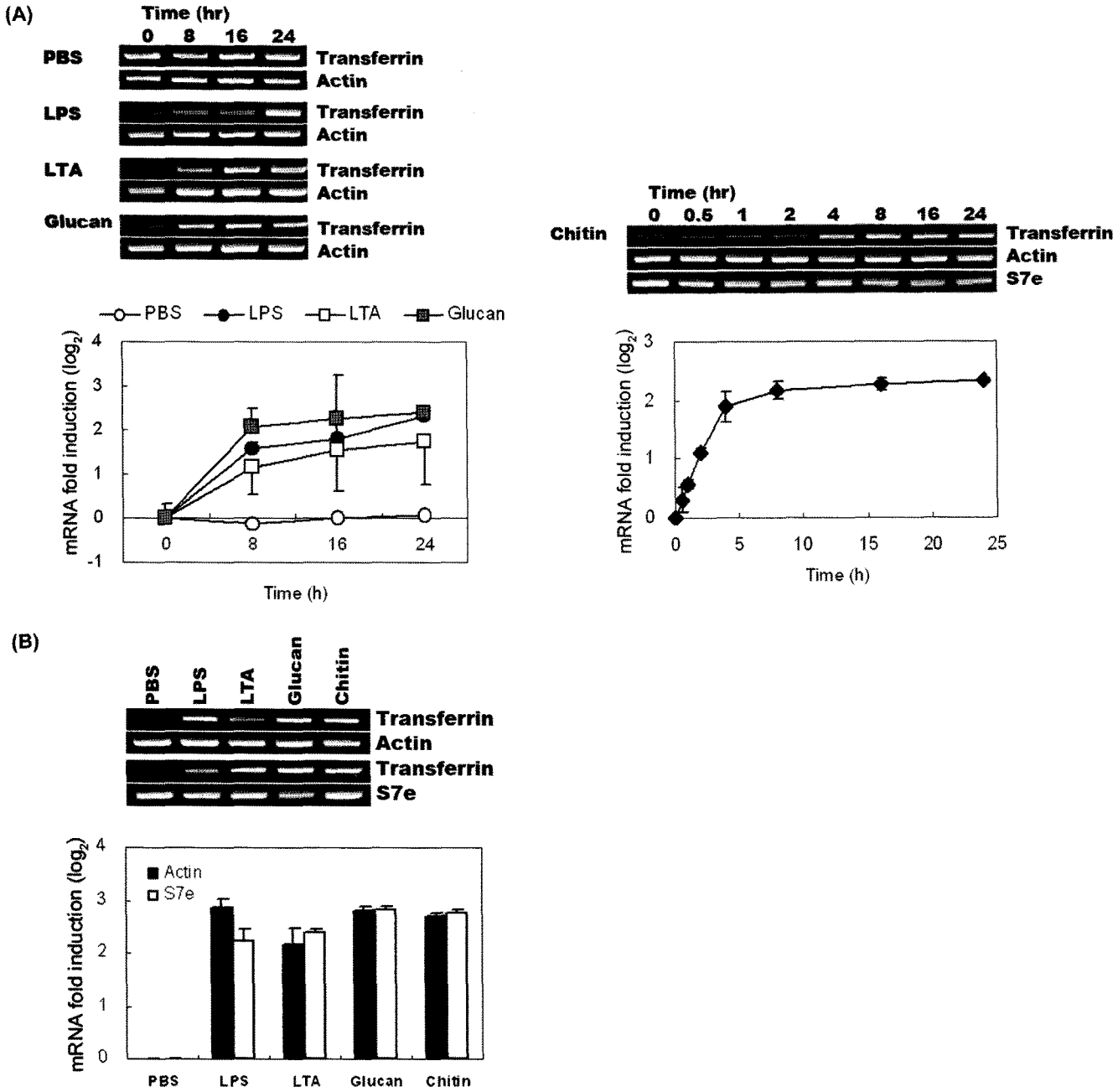

Fig. 4. Transcriptional regulation of *G. mellonella* transferrin. Total RNA was prepared from whole larval bodies, and time-dependant regulation of transferring mRNA was observed using semi-qRT PCR (A). Transferrin mRNA induction by various PAMP treatments for 16 hours was double-checked using two house keeping genes (B). The expression level was normalized to actin or S7e, respectively. Each transferrin level in PBS, LPS, LTA, glucan and chitin represents relative value respectively to the level at 0 hr as a control. The log-transformed expression ratio was compared against each control. Mean±SD (n=3) values are presented.

These results provide evidence for transferrin as an immune-relevant protein. However, the studies of insect transferrin gene regulation by these pathogens are not sufficient, and their results sometimes conflict. For example, there are two contrary reports on the fungal effect on insect transferrin transcription. Fungal infection reduced the mRNA level in the spruce budworm (Ampasala et al., 2004), while it increased the level in a termite (Thompson et al., 2003). These ambiguous results could perhaps be avoided by treatment with specific types of molecules rather than crude bacteria or fungi. Seitz et al. (2003), for example, used LPS to identify immunorelevant genes from the wax moth.

In this study, we used LPS, LTA, β-1,3-glucan, and chitin to investigate the effect of PAMPs on the expression of transferrin mRNA. Wax moth transferrin mRNA level was increased by all treated PAMPs. This broad range of response suggests that the wax moth transferrin is one of the proteins which are regulated by transcription factors and

Table 1. Primer sequences designed for semi-qRT PCR

Protein	Accession no.	Primer sequence	Expected band size (bp)
Actin	Not deposited	F: 5'-GTAACGAGAGGTTCCGTTGCCCAG-3' R: 5'-GGGGCCGGACTCGTCGTATTCTTG-3'	350
S7e	**DQ137845**	F: 5'-CTCTGACCTTAAGGCGCAAT-3' R: 5'-CGTGGCCTCTTTTGTTTGTT-3'	252
Transferrin	**AY364430**	F: 5'-ACTGGCCGGTTGTATCTACG-3' R: 5'-GTCATCCGGATAGTCGCATT-3'	619

thus indirectly reacted to foreign molecules. The protein would be one of the second reactants in immune response rather it is a direct target of certain unique foreign molecules. This assumption could partly be supported by the presence of κB-like sites in the transferrins from a fruit fly, *D. melanogaster*, and a mosquito, *A. aegypti* (Adams et al., 2000; Harizanova et al., 2005). We also found more κB-like sites and GATA sites in the transferrin promoter region from other insect species (data not shown). The GATA sequence is known as an important regulatory motif in immune-relevant proteins (Yamakawa et al., 1999; Senger et al., 2004).

Two different pathways are described for the role of pattern-recognition proteins in the innate immunity of *D. melanogaster* (Tanji et al., 2005). Toll signaling is mainly for cellular response by the recognition of the surface molecules of fungi and gram-positive bacteria. On the other hand, the proteins belonging to Imd/Relish pathway recognize and bind the molecular patterns of gram-negative bacteria. The products of this pathway are various antimicrobial peptides for humoral response. However, both of these pathways cannot be generalized to whole classes of microorganisms, and they sometimes cross-reacted to each other pathway. Through complex cascades, the both pathways finally activate NF-κB homologues (Hultmark 2003; Tanji et al., 2005).

Dorsal, Dif, and Relish are well-known κB-like transcription factors in insects. In addition, there is one other important immune reaction in insects, the prophenoloxidase (proPO) cascade (Marmaras et al., 1996). Once proPO is activated by PAMPs, it generates peptides and sticky proteins, and mediates many defense reactions in arthropods for example aggregation, melanization, and sclerotization. Melanization in this process is also related to the activation of NF-κB (Englaro et al., 1999).

The κB-like transcription factor binding motifs are found in the promoter region of some insect transferrin (Adams et al., 2000; Harizanova et al., 2005). We analyzed several insect transferrin genes, and found κB-like motifs and also GATA motifs in the genes (data not shown). GATA motif is known to regulate immune genes with κB-like motif (Kadalayil et al., 1997; Petersen et al., 1999; Tingvall et al., 2001; Lee et al., 2004). From the above, it could be assumed that the κB-like and GATA sites of the transferrin gene will probably regulate the transcription of transferrin.

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