玉米害虫专栏

Molecular cloning, sequence analysis and tissue expression of immulectin gene from the Asian corn borer, *Ostrinia furnacalis* (Lepidoptera: Pyralidae)*

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Abstract C-type lectin, a kind of pattern recognition molecule, recognizes the lipid A portion of lipopolysaccharide (LPS) and participates the cellular defense reaction in insects. In this paper, an immulectin (*Of*IML), C-type lectin with two carbohydrate-recognition domains, was cloned by reverse-transcription PCR (RT-PCR) and 3′/5′RACE from the larval hemocytes of corn borer, *Ostrinia furnacalis*. The cDNA of *Of*IML is 1 241 base pairs in length, and contains an open reading frame (ORF) of 924 nucleotides, which encoding a protein of 307 amino acids with a predicted molecularmass of approximately 34.65 ku. Alignment of *Of*IML with C-type lectins of other insects indicates that *Of*IML is a member of Lepidoptera immulectins. *Of*IML possesses two carbohydrate-recognition domains (CRDs), an amino-terminal domain, CRD1 (residues 1–135), and a carboxyl-terminal domain, CRD2 (residues 136–287). RT-PCR analyses shows that *Of*IML is expressed in cuticle, fat body, midgut, tracheae, and malpighian tubules, especially in hemocytes. The cDNA sequence has been deposited with GenBank under accession No. ABZ81710. *Of*IML is a kind of insect immunlectin, which contains two carbohydrate-recognition domains, and it possibly plays an important role during the immune reaction of *O. furnaclis* depending on its molecular structure and expression in tissues.

Key words immulectin, clone, sequence analysis, tissue expression, *Ostrinia furnacalis*

1 Introduction

Insects lack an adaptive immune system and mainly depend on the innate immune system, which is divided into cellular and humoral defense responses. Cellular defenses refer to hemocyte-mediated responses like phagocytosis, nodule formation, and encapsulation. Humoral defenses include a variety of antimicrobial peptides (AMPs), the cascades that regulate coagulation and melanization of hemolymph, prophenoloxidase (proPO) activation and the production of reactive intermediates of oxygen and nitrogen (Lavine and Strand, 2002; Shi and Yu, 2011).

Innate immune recognition is based on pattern recognition, which recognizes structures common among invading pathogens known as pathogen-associated molecular patterns (PAMPs). Molecules that recognize PAMPs are called pattern recognition receptors (PRRs) (Janeway, 1989; Medzhitov and Janeway, 1997). It has been reported that animal calcium-dependent (C-type) lectins, which contain one or more carbohydrate recognition domains (CRDs), function in pathogen recognition, cellular interactions, and innate immunity (Weis *et al*., 1998; Vasta *et al*., 1999). Multiple C-type lectin genes are also present in insects. More than 30 genes encoding C-type lectin domains have been found in

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Drosophila melanogaster, and two of them were reported to act as PRRs in hemocyte encapsulation and melanization. Five C-type lectins have been reported in the silkworm *Bombyx mori* with functions in innate immune responses (Shi and Yu, 2011). In the tobacco hornworm *Manduca sexta*, four C-type lectins named immulectins have been identified from plasma of bacteria challenged larvae and as PRRs they participate in proPO activation, defense against Gram-negative bacterial infection, and in hemocyte encapsulation and melanization (Yu *et al*., 1999, 2002, 2005, 2006; Yu and Kanost, 2000). Two C-type lectins have been reported in *Anopheles gambiae* to inhibit melanization of *Plasmodium berghei* ookinete and protect *A. gambiae* from Gram-negative bacterial infection (Osta *et al*., 2004; Schnitger *et al*., 2009). A C-type lectin was found in *Helicoverpa armigera* and was upregulated by bacteria, yeast and virus (Chai *et al*., 2008).

In this paper, we described the cloning, characterization and tissue expression of an immulectin gene (*Of*IML) from the Asian corn borer, *Ostrinia furnacalis*. The deduced amino acid sequence revealed that *Of*IML is a novel member of the C-type lectin superfamily, with a unique structural feature that consists of two different CRDs in tandem, a short and a long form. RT-PCR analyses shows that *Of*IML of the fifth instar larvae is expressed in all the tissues especially in hemocytes, but except brain.

2 Materials and methods

2.1 Insect culture

Ostrinia furnacalis were originally collected from cornfields in Jiangsu Province, China, and reared on an artificial diet as described previously (Hu *et al*., 2003). Larvae on the third day of the fifth instar were used in this study.

2.2 Collection of hemocytes and other tissues for total RNA

Hemocytes were collected from *O. furnacalis* larvae basically according to the modified procedure of Pech *et al*. (1994). Larvae were firstly sterilized with 75% ethanol, and then were bled onto parafilm by cutting away a caudal disc. 200 μL hemolymph was transferred into an eppendorf tube containing 1 mL Pringle's saline (Pringles, 1938). Then hemocytes were collected under a speed of 480×g at 4°C for 5 min, and washed once with Pringle's saline and collect again for further use. Other tissues of *O. furnacalis* larvae, including brain, cuticle, fat body, midgut, trachea and malpighian tubules, were dissected under ice-cold Pringle's saline, and stored at −80°C for further use.

2.3 Cloning of *Of*IML cDNA

Total RNA was isolated from hemocytes of the fifth instar *O. furnacalis* larvae using TRIzol reagent (Invitrogen), and then the first strand cDNA was prepared using reverse transcriptase AMV (Takara). A pair of degenerate primers LecF1 and LecR1 (Table 1) corresponding to the conserved regions of known C-type lectins from several insects was used to clone a fragment of *Of*IML (*Of*IML-1, Table 1). Polymerase Chain Reaction (PCR) reactions were performed in 25 μL total volumes using the following conditions: denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 48°C for 30 s and extension at 72°C for 1 min. A final extension time of 10 min was carried out at 72°C. The PCR product was cloned into the pMD18-T vector (Takara) and sequenced by the dideoxynucleotide method and gene-specific primers were designed depending on the partial cDNA sequence (LecF2, LecF3, LecR2 and LecR3 in Table 1). These primers were used to obtain overlapping PCR products using the hemocytes cDNA as a template. The 3′ and 5′ ends of the cDNA (*Of*IML-2 and *Of*IML-3) were obtained by 3 ′-and 5 ′-RACE (Rapid Amplification of cDNA Ends) with two gene-specific primers (Table 1) respectively according to the manufacturer's protocol (BD SMART™ RACE cDNA Amplification Kit, Clontech). The amplified PCR fragments from each reaction were subjected to electrophoresis in 1% agarose gels containing

Table 1 The primers used in the cDNA cloning of *Of*IML and RT-PCR analysis

PCR fragment	Name of primer	Type of primer	Nucleotide sequence (5 ′-3 ′)	Length of PCR fragment
*Of*IML-1	LecF1	F, DE	TTGTGTTDATHWYACDTCT	546 bp
	LecR1	R, DE	TGTANGCCCGC KMCCAVGTHC	
*Of*IML-2 (3 ′-RACE)	LecF2	F, GS	CCGAGCTCAACACGTCGAAGT	644 bp
	LecF3	F, GS	GAACTGGAGGCTGGTCCAAGT	
	3′-UPM	R, GS	CTAATACGACTCACTATAGGGCAAGCA-GTGGTATCAACGCAGAGT	
*Of*IML-3 (5 ′-RACE)	5 ′-UPM	F, GS	CTAATACGACTCACTATAGGGCAAGCA-GTGGTATCAACGCAGAGT	135 bp
	LecR2	R, GS	GCCAGGTTCGACCGAGTGTAT	
	LecR3	R, GS	CGCAAACCGACGGTGTCAGAT	
*Of*IML-4	LecF4	F, GS	GGAACACGCAACAGTCTTCTCG	925 bp
	LecR4	R, GS	CGCAAATGGCAGGGAAAGTGAC	

The RT-PCR fragment (*Of*IML-4) was used to confirm the assembled *Of*IML cDNA sequence.
UPM: Universal primer A mix; F: Forward; R: Reverse; DE: degenerate primer; GS: gene-specific primer.

ethidium bromide and purified using the E.Z.N.A DNA Gel Extraction kit (Omega). These PCR products were cloned into the pMD18-T vector and sequenced by the dideoxynucleotide method. Finally, the full length cDNA of *Of*IML was obtained by assembling the three overlapped fragments.

To confirm the assembled cDNA sequence from overlapping PCR products, the entire coding regions of *Of*IML (*Of*IML-4) were amplified by PCR reactions (LecF4 and LecR4 in Table 1). The PCR cycles were performed as follows: denaturation at 94°C for 30 s, annealing at 55°C for 30 s and elongation at 72°C for 1 min using Taq polymerase (Takara) for 30 cycles.

2.4 cDNA and protein sequence analyses

The sequence of *Of*IML cDNA was compared with other C-type lectins sequences deposited in the GenBank using the “BLAST-N” or “BLAST-X” tools at the National Center for Biotechnology Information (NCBI) web site. The amino acid sequence of *Of*IML was deduced from the corresponding cDNA sequence using the translation tool at the ExPASy Proteomics website (http://expasy.org/tools/dna.html). The transmembrane helix and the signal peptide were analyzed using TMHMM v.2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/) and SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/). The potential N-glycosylation and O-glycosylation sites were predicted using NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/) and NetOGlyc 3.1 (http://www.cbs.dtu.dk/services/Net-OGlyc/). Other protein sequence analysis tools used in this study, including MW, pI, and topology prediction, were obtained from the ExPASy Proteomics website (http://expasy.org/). Multiple sequence alignments of deduced amino acid sequences were made using Multiple Alignment software (http://www.ebi.ac.uk/clustalw/index.html). The phylogenetic tree was constructed by the Neighbor-Joining (NJ) method (Saitou and Nei, 1987) using software MEGA 3.1 based on the amino acid sequences of the known C-type lectins from insects. A bootstrap analysis was carried out, and the robustness of each cluster was verified in 100 replications.

2.5 RT-PCR analyses

Total RNA was isolated from hemocytes, brain, cuticle, midgut, fat body, trachea and

malpighian tubes of the fifth instar larvae with TRIzol reagent (Invitrogen), and reverse transcriptase AMV (Takara) was used to prepare the first strand cDNA. RT-PCR was performed in 25 μL reactions for 28 cycles using the following conditions: denaturation at 94°C for 30 s, annealing at 55°C for 30 s and elongation at 72°C for 1 min with the primers for the cDNA segment (LecF4 and LecR4 in Table 1). β-actin of *O. furnacalis* (actin-F: 5′- TGGTATGGGTCAGA AGGACTCGT -3′, actin-R: 5′- GCGGTGGTGG TGAAAGAGTAAC -3′) from different tissues were used to standardize the results. PCR products were analyzed on 1% agarose gel.

3 Results

3.1 Cloning and characterization of *Of*IML cDNA

The full-length cDNA sequence corresponding to the *Of*IML gene was obtained (GenBank accession No. ABZ81710) by PCR and 3′/5′ RACE. A fragment of about 550 bp was originally cloned from hemocytes using the forward and reverse degenerate primers, and then two overlapping fragments at both 3′ and 5′ directions with various lengths were obtained using combinations of gene-specific primers by 3′- and 5′- RACE respectively (Table 1). In total, 1 241 bp of the *Of*IML transcript were sequenced and determined to have an open reading frame (ORF) of 924 nucleotides, encoding a protein of 307 amino acids with a predicted molecular mass (MW) of approximately 34.65 ku and a pI of 6.63.

The deduced amino acid sequence of *Of*IML contains a 20-residue secretion signal peptide (Fig. 1). In *Of*IML, two potential N-linked glycosylation sites is present at Asn-121 and Asn-263. Analysis of the deduced amino acid sequence from the cDNA indicates that *Of*IML is a C-type lectin of the immulectin family. It is a member of a family of insect lectins that contain a unique domain structure with tandem C-type CRDs, an amino-terminal domain, CRD1 (residues 1–135), and a carboxyl-terminal domain, CRD2 (residues 136–287) (Fig. 1).

3.2 Sequence comparison with other proteins

Twelve C-type lectins amino acid sequences of insects were used to make homology comparison and phylogenetic analysis. Fig. 2 shows an alignment of five insect C-type lectins with tandem CRD structure. *Of*IML shows 41% identity to *M. sexta* immulectin-3 (*Ms*IML-3) and 40% to *M. sexta* immulectin-2 *(Ms*IML-2). It also shows 39% identify to LPS-binding lectin from *B. mori* (*Bm*LBL) and C-type lectin from *H. armigera* (*Ha*LEC). *Of*IML also shares 39% identify to *Hyphantria cunea* putative lectin (*Hc*LBL), and other C-type lectins followed by 37% identify to *M. sexta* immulectin-4 (*Ms*IML-4) and *Lonomia obliqua* lectin 3 (*Lo*LEC-3), 34% identify to *M. sexta* immulectin III (*Ms*IML-III), 30% identify to *B. mori* multi-binding protein (*Bm*MBP), 29% identify to *B. mori* immulectin (*Bm*IML) and 27% identify to *M. sexta* immunolectin-A (*Ms*IML-1). To investigate the evolutionary relationship between *Of*IML and other insect C-type lectins, phylogenetic analysis was performed (Fig. 3).

3.3 Tissue distribution of *Of*IML

Tissue-specific expression of *Of*IML was determined by RT-PCR analysis. *Of*IML was expressed in the all tested tissues, including the hemocytes, cuticle, fat body, midgut, tracheae, and the malpighian tubules, except brain (Fig.4, fragment of 925 bp). By using β-actin as a probe, *Of*IML mRNA was expressed at higher level in hemocytes than other tissues.

4 Discussion

C-type lectins are calcium-dependent carbohydrate binding proteins, and insect C-type lectins function as pattern recognition receptors participate in innate immunity and cell-cell interactions (Ao *et al*., 2007). In this report, we cloned the cDNA of a C-type lectin (*Of*IML) from *O. furnacalis* that belongs to the immulectin family. The insect immulectins include immulectin-1,

immulectin-2, immulectin-3 and immulectin-4 from *M. sexta* (Yu *et al*., 1999, 2005, 2006; Yu and

Fig. 1 Nucleotide and deduced amino acid sequences of *Of*IML

Nucleotide numbers are shown on the left of the nucleotide sequence, and the deduced amino acid sequence (one-letter abbreviations) is shown below the cDNA sequence. The numbers of the amino acid residues, starting from the first Met, are given to the right of each line. Residues in the predicted signal peptide (underlined) are assigned negative numbers whereas residues in the mature protein are assigned positive numbers. Two potential N-linked glycosylation sites are boxed, and the asterisk and double underlining denote the termination codon and cysteine residues, respectively. CRD1 (residues 1–135) and CRD2 (residues 136–287) are separated by oblique line.

Kanost, 2000), immulectin from *B. mori* (accession number: AY297159), and LPS-binding lectins from *B. mori* (Koizumi *et al*., 1999) and the fall webworm *H. cunea* (Shin *et al*., 1998). Some immulectins contain two CRDs. The amino-terminal CRD is a short form CRD stabilized by two disulphide bonds, whereas the carboxyl terminal CRD is a long form CRD, which contains three disulphide bonds (Yu and Kanost, 2000). The two CRD structure was also found in insect lectins include immulectin-1, immulectin-2 and immulectin-3 from *M. sexta* (Yu *et al*., 1999, 2005; Yu and Kanost, 2000), immulectin (accession number: AY297159) and LPS-binding lectin from *B. mori* (Koizumi *et al*., 1999), and a C-type lectin from *H. armigera* (Chai *et al*., 2008).

In *Of*IML, there are ten cysteine residues in total, and they are highly conserved in all other

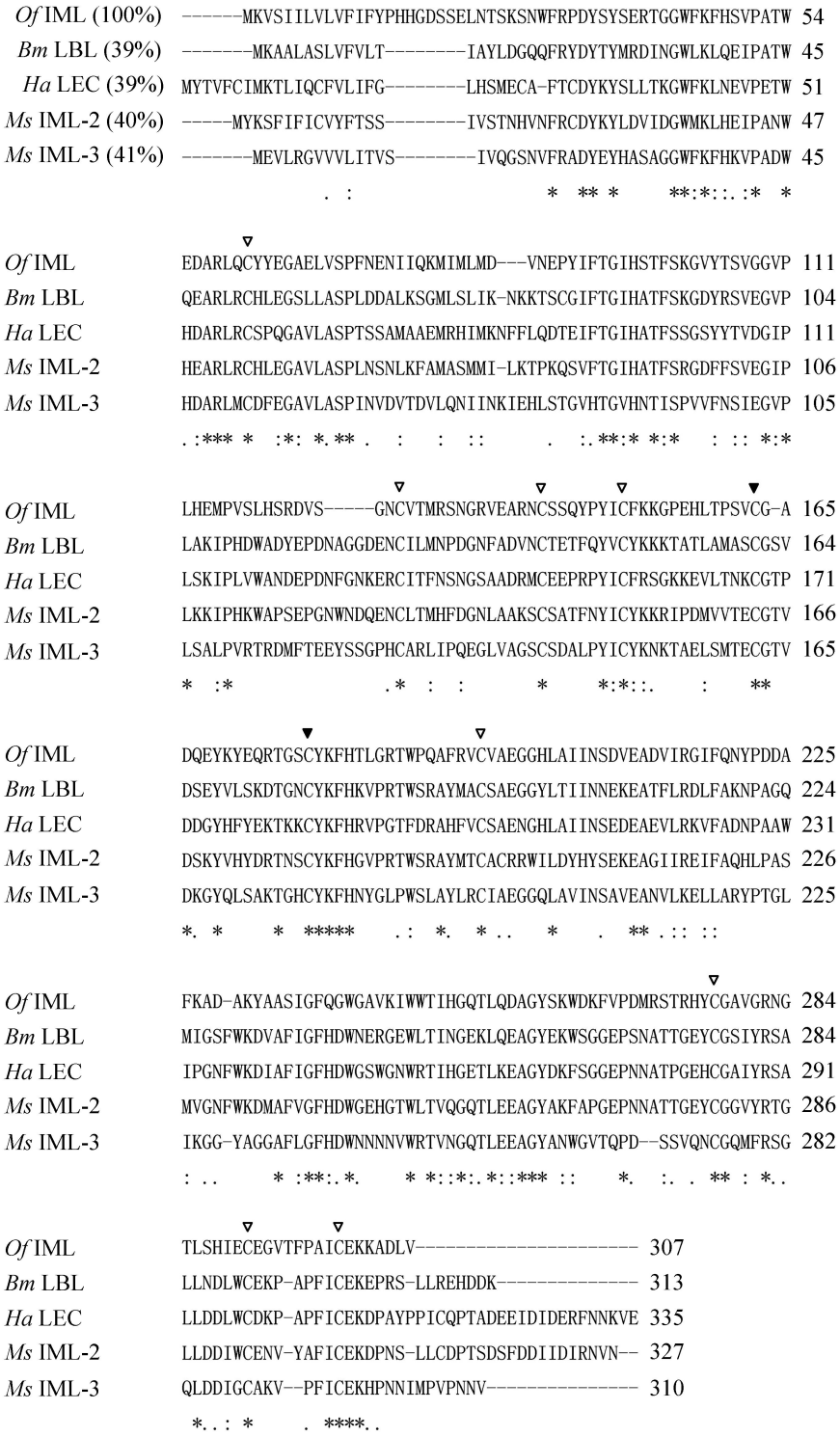

Fig. 2　Alignment of insect C-type lectins consisting of tandem CRDs

The polypeptide sequences of *Of*IML and four other insect lectins consisting of tandem CRDs are aligned. Residues conserved in all five lectins are marked with asterisks below the alignment. Invariant cysteine residues that define CRDs are marked with open triangles, whereas solid triangles indicate the extra two cysteines in the carboxyl-terminal long-form CRD. Identifies between *Of*IML and other insect lectins are shown in the parentheses. *Bm*LBL (*Bombyx mori*

lipopolysaccharide-binding protein, GenBank accession no. NM_001043591), *Ha*LEC (*Helicoverpa armigera* C-type lectin, DQ533877), *Ms*IML-2 (*Manduca sexta* immulectin-2, AF242202), *Ms*IML-3 (*Manduca sexta* immulectin-3, AY768811).

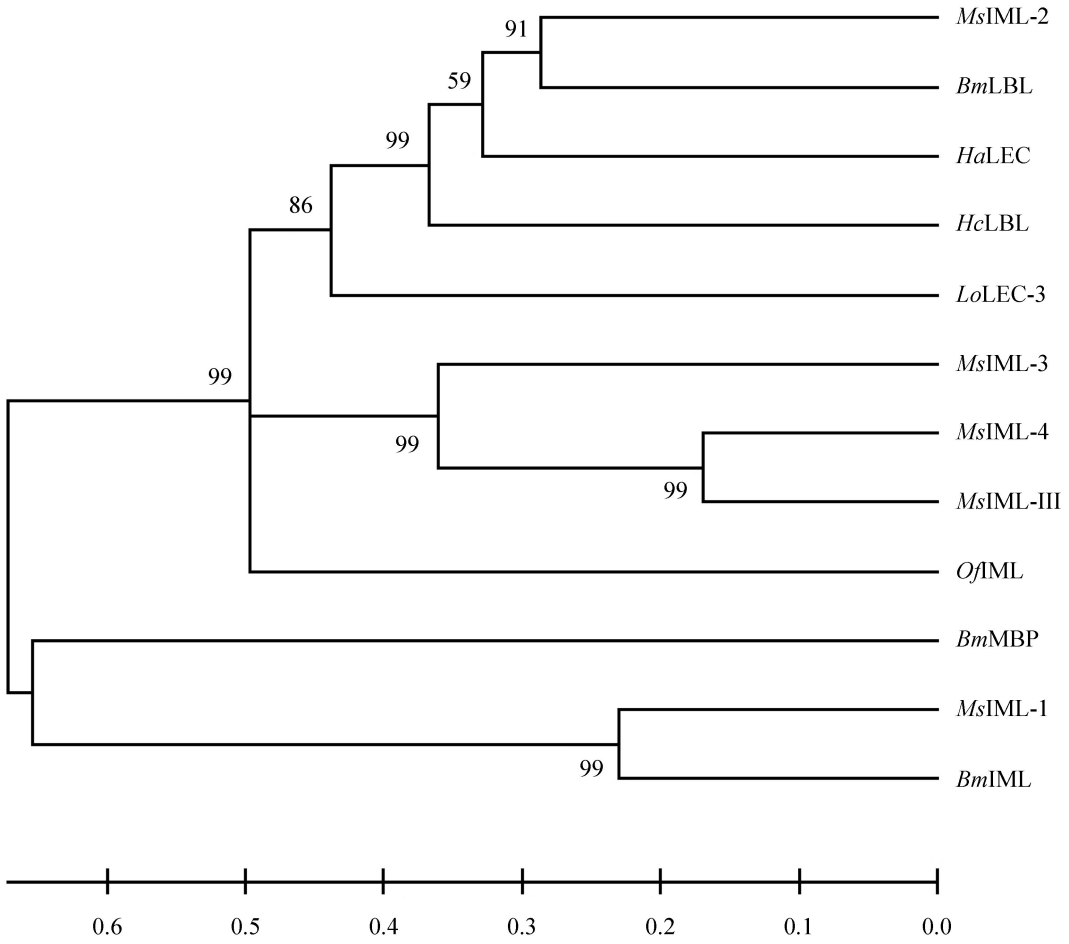

Fig. 3 Phylogenetic analysis of insect C-type lectins based on amino acid sequences

The number above or below individual branches represents the percentage of 1 000 bootstrap iterations supporting the branch. Whole sequences were aligned with BLAST program to generate the phylogenetic tree. *Ms*IML-1 (*M. sexta* immunolectin-A, GenBank accession no. AF053131), *Ms*IML-2 (*M. sexta* immulectin-2, AF242202), *Ms*IML-3 (*M. sexta* immulectin-3, AY768811), *Ms*IML-4 (*M. sexta* immulectin-4, AY768812), *Ms*IML-III (*M. sexta* for immulectin III, AM293329), *Bm*IML (*B. mori* immulectin, AY297159), *Bm*LBL (*B. mori* lipopolysaccharide binding protein, NM_001043591), *Hc*LBL (*Hyphantria cunea* putative lectin, AAD09286), *Bm*MBP (*B. mori* multi-binding protein, NM_001098314), *Ha*LEC (*Helicoverpa armigera* C-type lectin, DQ533877), *Lo*LEC-3 (*Lonomia obliqua* lectin 3, AY829836).

immulectins (Fig. 2). C-type lectins of insects have lower homology. *Of*IML shows 41% identity to *Ms*IML-3 and 40% to *Ms*IML-2 in amino acid sequence. It also shows 39% identify to *Bm*LBL and *Ha*LEC. *Ms*IML-1 and *H. cunea* lectin showed 27% overall identity in amino acid sequence, with 35% identity in the carboxyl-terminal CRD, but only 20% in the amino-terminal CRD (Yu *et al*., 1999). Among the four *M. sexta* immulectins, *Ms*IML-4 has 56% identity to *Ms*IML-3, 35% to *Ms*IML-2, but only 26% to *Ms*IML-1. *Ms*IML-4 also shows 36% and 33% identify to LPS-binding lectin from *B. mori* and *H. cunea*, respectively, but only 27% to *B. mori* immulectin (Yu *et al*., 2006).

RT-PCR analyses shows that *Of*IML is expressed in all the tissues of the fifth instar larvae especially in hemocytes, but except brain. The repeated analyses of tissue expression using

gene-specific primers LecF2 and LecR2 (504 bp) by RT-PCR showed the consistent results. And in *M. sexta*, all four immulectins are synthesized in the fat body and secreted into the hemolymph

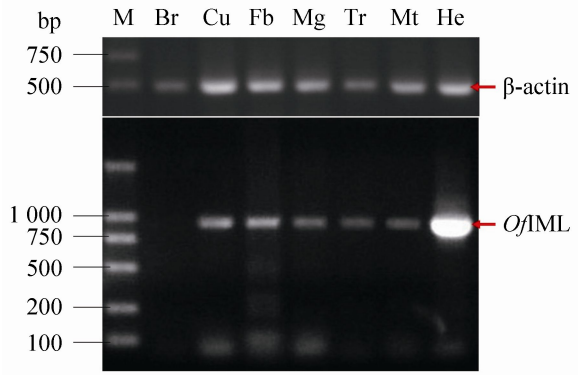

Fig. 4　Tissue specific expression of *Of*IML in *Ostrinia furnacalis* larvae by RT-PCR analysis

The uniformity of loading each lane in RT-PCR is assessed by using β-actin as a probe. Marker (M); Brain (Br); Cuticle (Cu); Fat body (Fb); Midgut (Mg); Trachea (Tr); Malpighian tubes (Mt); Hemocytes (He).

(Yu *et al*., 2005, 2006). *Ms*IML-1 is undetectable in hemolymph of naïve larvae, and its concentration remains low in hemolymph even after larvae are injected with bacteria (Yu *et al*., 1999). *Ms*IML-2 and *Ms*IML-3 are present in hemolymph of naïve larvae at a constitutive low concentration (Yu and Kanost, 2000; Yu *et al*., 2005). *Ms*IML-1 and *Ms*IML-2 stimulate prophenoloxidase activation in plasma when complexed with LPS. All four *Ms*IMLs apparently function as pattern recognition receptors involved in prophenoloxidase activation and encapsulation (Yu *et al*., 1999, 2006; Yu and Kanost, 2000, 2003). In future research, we will endeavor to understand *Of*IML' s functions in innate immunity of *O. furnacalis*, such as pattern recognition, encapsulation by hemocytes and melanization processes of foreign objects in hemolymph.

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亚洲玉米螟免疫凝集素基因的克隆、序列分析及组织表达

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2. 昆山科腾生物科技有限公司，苏州 215300；3. 苏州卓凯生物技术有限公司，苏州 215300）

摘 要 C 型凝集素作为模式识别分子可以识别部分脂多糖（LPS），进而参与昆虫细胞的防御反应。本文通过 RT-PCR 和 3′/5′RACE 技术从亚洲玉米螟 *Ostrinia furnacalis* 5 龄幼虫血细胞中克隆得到免疫凝集素基因（*Of*IML）。*Of*IML mRNA 全长为 1 241 bp，其中开放读码框（ORF）为 924 bp，编码 307 个氨基酸（aa），分子量约为 34.65 ku。与其它昆虫的 C 型凝集素比对分析结果显示，*Of*IML 属于鳞翅目免疫凝集素，并且含有一个独特的结构特征，即一前一后 2 个糖识别域，氨基末端（CRD1，aa#1-135）和羧基末端（CRD2，aa#136-287）。RT-PCR 检测 *Of*IML 在幼虫组织中的分布结果表明，其在血细胞、表皮、脂肪体、中肠、马氏管和气管中都有表达。*Of*IML GenBank 登录号为 ABZ81710。*Of*IML 是一种昆虫免疫凝集素，含有 2 个糖识别域，根据其分子结构及在组织分布中的结果显示可能在亚洲玉米螟的免疫反应中起重要作用。

关键词 免疫凝集素，克隆，序列分析，组织表达，亚洲玉米螟