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Apriona germari Larval Cuticle Protein Genes: Genomic Structure of Three Cuticle Protein Genes and cDNA Cloning of a Novel Cuticle Protein

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In a previous study, three larval cuticle protein genes were cloned from the mulberry longicorn beetle, *Apriona germari* (Comp. Biochem. Physiol. B 136, 803-811, 2003). In the present study, the genomic structures of these three larval cuticle protein genes (AgLCP9.2, AgLCP12.6 and AgLCP12.3) were elucidated. All three cuticle protein genes consist of one intron and two exons. Southern blot analysis of genomic DNA suggested that three cuticle protein genes are a single copy gene. In addition, a novel larval cuticle protein gene, AgLCP10.6, was cloned from *A. germari* in this study. The AgLCP10.6 cDNA contains an ORF of 300 nucleotides that are capable of encoding a 100-amino acid polypeptide with a predicted molecular mass of 10.6 kDa. The amino acid sequence deduced from the AgLCP10.6 cDNA contained a type-specific consensus sequence identifiable in other insect cuticle proteins and is most homologous to *Drosophila melanogaster* cuticle protein ACP65A (51% protein sequence identity). Northern blot analysis revealed that AgLCP10.6 showed epidermis-specific expression.

Key words: *Apriona germari*, cDNA cloning, Cuticle protein, Gene structure, Insect, Mulberry longicorn beetle, mRNA expression

Introduction

The insect cuticle undergoes drastic morphological changes during development from larva to pupa to adult. The insect cuticle is a complex extracellular structure composed mainly of chitin and proteins that are synthesized and secreted by epidermal cells. Cuticle proteins, the major components of insect integument, are being studied for the mechanisms of gene regulation during molting and metamorphosis (Marcu and Locke, 1998; Shofuda *et al.*, 1999; Togawa *et al.*, 2001). The growth and morphogenesis in insects are strictly dependent on the capability to remodel chitin-containing structures, such as the cuticles of the epidermis and trachea and the peritrophic matrices lining the midgut epithelium (Merzendorfer and Zimoch, 2003).

A number of insect cuticle proteins have been identified and characterized in various species (Snyder *et al.*, 1982; Rebers and Riddiford, 1988; Charles *et al.*, 1992; Binger and Willis, 1994; Jensen *et al.*, 1997; Nakato *et al.*, 1997; Dotson *et al.*, 1998; Rondot *et al.*, 1998; Shofuda *et al.*, 1999; Togawa *et al.*, 2001; Sawada *et al.*, 2003; Kim *et al.*, 2003; Kim *et al.*, 2005a, b). Many insect cuticle proteins include a 35-36 amino acid motif of G-x(7)-[DEN]-G-x(6)-[FY]-x-A-[DGN]-x(2,3)-G-[FY]-x-[AP], known as the R&R consensus sequence (Rebers and Riddiford, 1988). The revised consensus has now been observed in a wide range of insect cuticle proteins (Andersen *et al.*, 1995). In the silkworm, *Bombyx mori*, four larval cuticle proteins (LCPs), designated as LCP17, LCP18, LCP22 and LCP30, are well characterized. The synthesis and deposition of cuticle proteins are regulated stage-dependently and are governed by ecdysteroid and juvenile hormone (Nakato *et al.*, 1994, 1997; Braquart *et al.*, 1996; Hiruma *et al.*, 1997; Kramer and Wolbert, 1998; Shofuda *et al.*, 1999). In coleopteran insects, the cuticle protein genes, CP22 and CP23, were first reported in *Tenebrio molitor* (Rondot *et al.*, 1998). Recently, our previous result showed that three larval cuticle proteins are differ-

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entially expressed in the epidermis after larval ecdysis in the mulberry longicorn beetle, *Apriona germari* (Kim *et al.*, 2003).

With the aim of furthering the understanding the cuticle protein genes in coleopteran insects, we cloned a cDNA encoding a novel larval cuticle protein from *A. germari* (Coleoptera: Cerambycidae), which feeds on mulberry trees by tunneling inside the stem and ingesting the living wood (Yoon and Mah, 1999). In this study, the cDNA cloning, sequencing and mRNA expression of an *A. germari* larval cuticle protein (AgLCP10.6) are described. We also report the genomic structure of three previously reported larval cuticle protein genes from *A. germari*.

Materials and Methods

Insects

The mulberry longicorn beetle, *Apriona germari* (Coleoptera: Cerambycidae), was reared on an artificial diet as described previously (Yoon and Mah, 1999). Larvae are maintained at 25°C and 60% humidity with a 14 h : 10 h light/dark photoperiod.

Genomic DNA isolation and polymerase chain reaction (PCR)

Genomic DNA was extracted from the fat body of *A. germari* using the Wizard™ Genomic DNA Purification Kit according to the manufacturer's instructions (Promega, Madison, WI, USA). The gene specific primers used for amplification of the genomic DNA that encodes the cutcle protein were as follows: for AgLCP9.2, 5'-ATGAAAGTGATCATCGCCCTCGCCGCC-3' (forward primer) and 5'-TTACGATGGGATGTGGGCTCCAACG-3' (reverse primer); for AgLCP12.6, 5'-ATGATGAAACTGGTAATATTCTCCGCC-3' (forward primer) and 5'-CTATAGTACCCTGGCTTCAGGTAATG-3' (reverse primer); and for AgLCP12.3, 5'-ATGTACAAGTTAACGGTAATAGTTGC-3' (forward primer) and 5'-TTAGCTAGTGAGCTCCCCGCTGGG-3' (reverse primer). After a 35-cycle amplification (94°C for 30 s; 48°C for 40 s; 72°C for 2 min), the PCR products were purified with AccuPrep® PCR Purification Kit according to manufacturer's instructions (Bioneer, Korea). The purified PCR products were analyzed by 1.0% agarose gel electrophoresis. The PCR products for sequencing were cloned into pGem-T vector (Promega). The construct was transformed into *Escherichia coli* TOP10F' cells (Invitrogen, Carlsbad, CA, USA).

Southern blot analysis

Genomic DNA from *A. germari* was digested with *Hin*dIII or *Eco*RV and electrophoresed on a 1.0% agarose gel. The DNA from the gel was transferred onto a nylon blotting membrane (Schleicher & Schuell, Dassel, Germany) and hybridized at 42°C with a probe in a hybridization buffer containing 5 × SSC, 5 × Denhardt's solution, 0.5% SDS, and 100-μg/ml denatured salmon sperm DNA. The cuticle protein cDNA clones were labeled with [α-^{32}P]dCTP (Amersham, Arlington Heights, IL, USA) using the Prime-It II Random Primer Labeling Kit (Stratagene, La Jolla, CA, USA) and were used as a probe for hybridization. After hybridization, the membrane filters were washed three times for 30 min each in 0.1% SDS and 0.2 × SSC (1 × SSC contains 0.15 M NaCl and 0.015 M sodium citrate) at 65°C with high stringency and then exposed to autoradiography film.

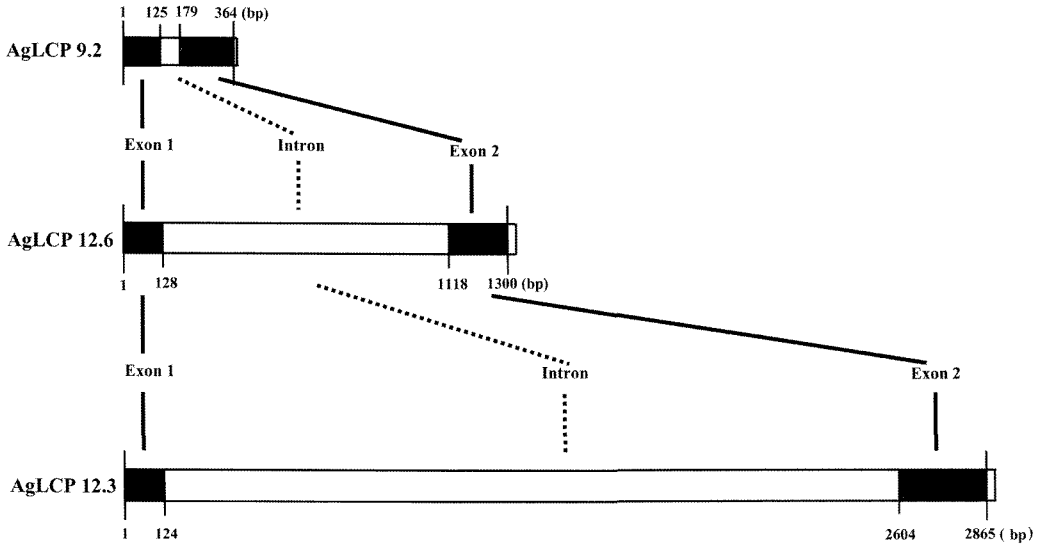
cDNA library screening, nucleotide sequencing and data analysis

The clone containing the cDNA insert was selected from the expressed sequence tags (ESTs), which were generated from a cDNA library constructed using whole bodies of *A. germari* larvae (Kim *et al.*, 2001). The plasmid DNA was extracted using the Wizard mini-preparation kit (Promega). The nucleotide sequence was determined using a BigDyeTerminator cycle sequencing kit in an automated DNA sequencer (model 310 Genetic Analyzer; Perkin-Elmer Applied Biosystems, Foster City, CA, USA). The sequences were compared using the DNASIS and BLAST programs provided by the NCBI (http://www.ncbi.nlm.nih.gov/BLAST). MacVector (ver. 6.5, Oxford Molecular Ltd., Oxford, UK) was used to align the amino acid sequences of insect cuticle proteins.

RNA isolation and Northern blot analysis

Sixth instar *A. germari* larvae were dissected on ice under a stereo-microscope (Zeiss, Jena, Germany), and individual samples of the fat bodies, gut and epidermis were collected and washed twice with PBS (140 mM NaCl, 27 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4). Total RNA was isolated from the fat bodies, gut and epidermis of *A. germari* larvae using the Total RNA Extraction Kit (Promega). Total RNA (5 μg/lane) from *A. germari* was separated by 1.0% formaldehyde agarose gel electrophoresis, transferred onto a nylon blotting membrane (Schleicher & Schuell), and hybridized at 42°C with a probe in a hybridization buffer containing 5 × SSC, 5 × Denhardt's solution, 0.5% SDS, and 100 -μg/ml denatured salmon sperm DNA. The probe used to detect the cuticle protein gene transcripts was AgLCP10.6 cDNA, cloned in this study and labeled with [α-^{32}P]dCTP (Amersham) using the Prime-It II Random Primer Labeling Kit (Stratagene). After hybridization, the procedures for washing of the membrane filter and exposing to autora-

(A)



(B)

Cuticle type	Exon	Length of exon (bp)	Position in gene	Sequence at exon - intron junction
AgLCP 9.2	1	125	1 - 125	ATGAAAGTGATC ····· CTACAACTACGCgtaagtccgt M K V I ····· Y N Y A
	2	186	179 - 364	tgttacagATACGACACCAGC ····· CACATCCCATCGtaa Y D T S ····· H I P S Stop
AgLCP 12.6	1	128	1 - 128	ATGATGAAACTG ····· TACCAGTTTAAgtaagtattca M M K L ····· Z Q F N
	2	182	1118 - 1300	atttgcagCTACGAAACCGAG ····· GCCAGGGTACTAtag Y E T E ····· P R G L Stop
AgLCP 12.3	1	124	1 - 124	ATGTACAAGTTA ····· TTTCACTACAGgtataaaattc M Y K L ····· F H Y S
	2	262	2604 - 2865	atttgcagCTATGAAACCGGA ····· GAGCTCACTAGCtaa Y E T E ····· E L T S Stop

Fig. 1. Genomic structure of AgLCP genes. (A) Schematic drawing of the genomic structure of AgLCP9.2, AgLCP12.6 and AgLCP12.3. Solid and open boxes represent exons and introns, respectively. Numbers indicate position in the genomic sequences. (B) Lengths of exons and exon/intron boundaries.

diography film were performed as in the Southern blot analysis.

Results and Discussion

Genomic organization of three larval cuticle proteins in *A. germari*

Three larval cuticle protein cDNAs were cloned from *A. germari* in our previous study (Kim *et al.*, 2003). To identify the genomic structure of the three cuticle proteins in this study, primer sets based on the sequences of the cuticle protein cDNAs were designed, and each band was amplified from *A. germari* genomic DNA using these primer sets. The PCR products were cloned and sequenced. Genomic PCR product sequences were 100% identical with the cuticle protein cDNAs in both size and sequence. The organization of the cuticle protein genes is illustrated

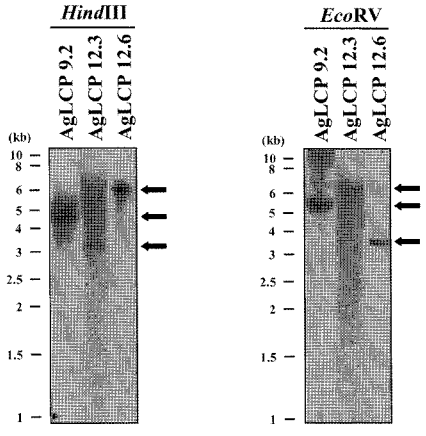


Fig. 2. Copy number of AgLCP genes. Southern blot analysis for AgLCP9.2, AgLCP12.3 and AgLCP12.6 genes was performed using *A. germari* genomic DNA. Genomic DNA was digested with two restriction enzymes, *Hind*III (left panel) or *Eco*RV (right panel) and hybridized with radiolabeled AgLCP cDNA. Size markers are shown on the left. Solid arrows indicate hybridized bands.

(A)

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-24                                                    GGCAATCAACAAGTACAATCCAAA
  1  ATGAAAGTATTCATCGCTCTTGCTGCCTTGGCTGCTGTATGTATTGCAGCTCCTCCAGAT
  1  M  K  V  F  I  A  L  A  A  L  A  A  V  C  I  A  A  P  P  D
 61  GCCGTAGTTCTGAAGAACGAACTTGATAACATTGGTGTTGATGGATACTCATTCAGTGTT
 21  A  V  V  L  K  N  E  L  D  N  I  G  V  D  G  Y  S  F  S  V
121  GAGACTAGCGATGGCTTGAACCGCGTTGAGCAAGGTGTAGTCAATAATTTGGGAAGCGAG
 41  E  T  S  D  G  L  N  R  V  E  Q  G  V  V  N  N  L  G  S  E
181  AACGAAAGCTTGAGAGTCACAGGAAGTATCAGCTGGGTGGATCTTGAGGGTACCCCATAC
 61  N  E  S  L  R  V  T  G  S  I  S  W  V  D  L  E  G  T  P  Y
241  AAGCTTGACTTCGTTGCTGACGAGAACGGTTTCCAACCCGTTGGAGCTCACCTTCCTAAA
 82  K  L  D  F  V  A  D  E  N  G  F  Q  P  V  G  A  H  L  P  K
301  TAAAACTTTAATCTTGACTGGATGGCTAGACCTCGACTGTAATATATACATTGGAAGCAT
101  *
361  ACCTTGTACATATTTTGGTAAGTTGTGAATAAAGTATAAGTATAAAAAAAAAAAAAAAA
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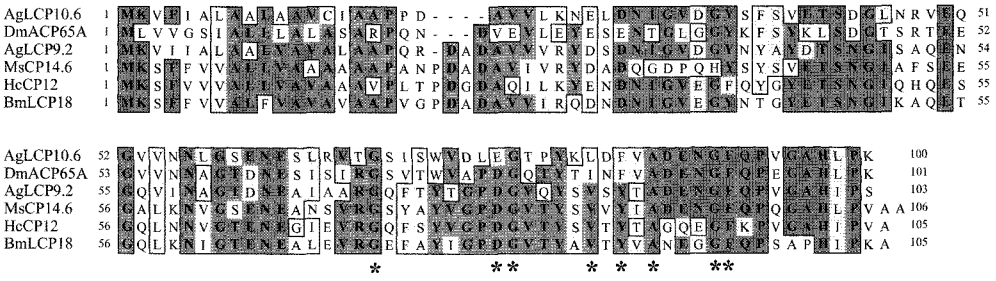
(B)



(C)

Percent similarity

		1	2	3	4	5	6
AgLCP10.6	1		65	67	60	62	61
DmACP65A	2	51		65	63	62	58
AgLCP9.2	3	49	49		67	74	73
MsCP14.6	4	44	42	54		71	72
HcCP12	5	43	40	59	59		82
BmLCP18	6	42	38	60	60	65	

Percent identity

Fig. 3. Sequence of AgLCP10.6 cDNA. (A) The nucleotide and deduced amino acid sequences of AgLCP10.6 cDNA. The start codon of ATG is boxed, and the termination codon is shown by an asterisk. The putative polyadenylation signal is underlined. The GenBank accession number is AY769984. (B, C) Multiple sequence alignment (B) and pairwise identity (C) of the deduced protein sequence of AgLCP10.6 cDNA with related insect cuticle protein sequences. The identical residues are shown in solid boxes. Asterisks indicate the cuticle protein consensus sequence of G-------(D/E)G----(V/L/I)-(Y/F)-A---G(Y/F). The abbreviations and GenBank accession numbers for the aligned sequences are: AgLCP10.6, *A. germari* larval cuticle protein LCP10.6 (this study, GenBank accession no. AY769984); DmACP65A, *Drosophila melanogaster* cuticle protein ACP65A (AAB88068); AgLCP9.2, *A. germari* larval protein cuticle LCP9.2 (AAL16187); MsCP14.6, *Manduca sexta* cuticle protein CP14.6 (Q94984); HcCP12, *Hyalophora cecropia* cuticle protein CP12 (AAA85640); and BmLCP18, *Bombyx mori* larval cuticle protein LCP18 (AB012081).

in Fig. 1A. Comparison of the genomic sequence with the sequence of the cDNA revealed one intron and two exons in the three cuticle protein genes. The genomic DNA size from translation start codon to stop codon was 364 bp for AgLCP9.2, 1300 bp for AgLCP12.6 and 2865 bp for AgLCP12.3, which indicates that the size of genomic DNA was dependent on the intron. The exon-intron boundaries are listed in Fig. 1B. The sequences at the exon-intron

boundaries conformed to consensus eukaryotic splice sites, including an invariant GT at the intron 5' boundary and an invariant AG at its 3' boundary. In this study, the genomic structure of AgLCPs is identical in all three AgLCPs, containing one intron and two exons. Previous studies in wild silkmoths showed that *Antheraea yamamai* cuticle protein (AyCP12) gene consists of one intron and two exons (Kim *et al.*, 2005b), but the *A. pernyi* cuticle protein (AyCP13) gene is intron-less (Kim *et al.*, 2005a), showing various structures of cuticle protein genes in insects.

The copy number of the three cuticle protein genes in the *A. germari* genome was determined by Southern blot analysis. The genomic DNA was digested with restriction enzymes that do not cut within the cuticle protein genes, blotted, and then hybridized with the cuticle protein cDNA. A single hybridizing band was detected with each enzyme, indicating that the three cuticle protein genes are present as a single copy (Fig. 2). This result suggests that the cuticle protein genes are encoded by a single copy gene in *A. germari*, as have been demonstrated in *Hyalophora cecropia* (Binger and Willis, 1994) and *Calpodes ethlius* (Marcu and Locke, 1998).

Cloning, sequencing and analysis of a novel cuticle protein cDNA in *A. germari*

Searching the *A. germari* ESTs, a cDNA was identified that had high homology with previously reported cuticle protein genes. The cDNA clone, including the full-length open reading frame (ORF), was sequenced and characterized. The cuticle protein cDNA contains an ORF of 300 nucleotides that are capable of encoding a 100-amino acid polypeptide with a predicted molecular mass of 10.6 kDa and pI of 4.06 (Fig. 3A). Thus, we designated this AgLCP10.6. This cDNA sequence has been deposited in GenBank under the accession number AY769984.

A comparison of the deduced amino acid sequence of AgLCP10.6 with that of other cuticle protein sequences is shown in Fig. 3B. Alignment of the AgLCP10.6 sequence with those of cuticle proteins from several insect species indicates the extent of identity that exists. AgLCP10.6 contained the cuticle protein consensus sequence of G------(D/E)G----(V/L/I)-(Y/F)-A---G(Y/F), present in a hydrophilic region near the C-terminal end. This consensus sequence was conserved among cuticle protein sequences from dipteran, lepidopteran, coleopteran and orthopteran species (Andersen *et al.*, 1995; Kim *et al.*, 2003; Kim *et al.*, 2005a, b), suggesting that AgLCP10.6 is a putative member of the insect cuticle protein family.

The identity and similarity of the deduced amino acid sequence of AgLCP10.6 with that of other insect cuticle protein sequences are shown in Fig. 3C. Among the known cuticle protein sequences, AgLCP10.6 was most similar to *Drosophila melanogaster* cuticle protein ACP65A (Charles *et al.*, 1997) (51% protein sequence identity). AgLCP10.6 also had 49% protein sequence identity with AgLCP9.2 (Kim *et al.*, 2003). However, AgLCP10.6 showed lower protein sequence identity to the coleopteran insect *T. molitor* proteins CP22 and CP23 (Rondot *et al.*, 1998) than to several lepidopteran and dipteran cuticle proteins, as has been shown for AgLCPs (Kim *et al.*, 2003).

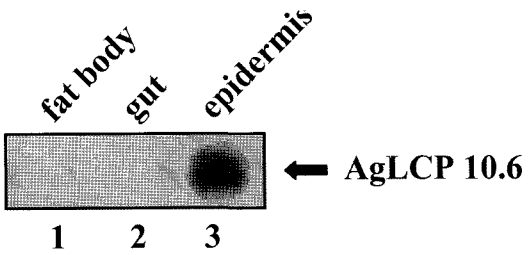


Fig. 4. Tissue-specific expression of AgLCP10.6 mRNA. Total RNA was isolated from the fat body (lane 1), gut (lane 2) and epidermis (lane 3) of *A. germari* larvae. The RNAs were separated by 1.0% formaldehyde agarose gel electrophoresis, transferred on to a nylon membrane, and hybridized with radiolabelled AgLCP10.6 cDNA. Transcripts of AgLCP10.6 are indicated on the right side of the panel by an arrow.

Expression of AgLCP10.6 mRNA in *A. germari*

To confirm the tissue-specific expression of AgLCP10.6 at the transcriptional level, Northern blot analysis was performed using mRNA prepared from fat bodies, gut and epidermis of *A. germari* larvae (Fig. 4). A hybridization signal was detected as a single band of mRNA from the epidermis, indicating that AgLCP10.6 is expressed only in the epidermis. This result is consistent with previous reports that the cuticle protein gene is tissue-specifically expressed in the epidermis (Rebers *et al.*, 1997; Nakato *et al.*, 1997; Shofuda *et al.*, 1999; Togawa *et al.*, 2001; Kim *et al.*, 2003; Sawada *et al.*, 2003; Kim *et al.*, 2005a, b).

In conclusion, we cloned and characterized a novel larval cuticle protein gene (AgLCP10.6) from *A. germari*. We also found that three larval cuticle protein genes (AgLCP9.2, AgLCP12.6 and AgLCP12.3) consist of one intron and two exons. We hope that the molecular characterization of cuticle proteins in *A. germari* in this study will expand the understanding of cuticle proteins in coleopteran insects.

Acknowledgements

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