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Novel cuticular proteins revealed by the honey bee genome

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Abstract

With the completion of the honey bee genome project, a transition is now occurring from the acquisition of gene sequence to understanding the role and context of gene products within the genome. Here we annotated and characterised a cluster of three genes in a GC-rich 11 kb genomic region on the linkage group 4 encoding highly hydrophobic polypeptides (named apidermins; APD 1–3) containing both sequence motifs characteristic of cuticular proteins and distinctly novel features. Five amino acids, Ala, Gly, Leu, Pro and Val, account for 74–86% of their respective sequences with Ala being the most abundant residue (at least 30% of each peptide). A conserved tetra-peptide AAPA/V is found in all three proteins, but none has the ′R and R′ signature implicated in chitin binding. Two proteins, APD-1 and APD-2, contain an arginine-rich motif RERR in short non-hydrophobic stretches near the N-terminal of mature proteins and in both proteins tryptophan is the C-terminal residue. All three genes are spliced and highly expressed in a defined spatio-temporal pattern. *apd-1* is expressed in the exoskeletal epidermis, but only during a restricted period of a few days of late pupal and early adult life when the cuticle becomes dark. APD2 appears to be a protein of ''internal'' cuticles and is expressed in the tracheas, oesophagus and stomach, and also in the embryo. The expression of *apd-3* partly overlaps with both *apd-1* and *apd-3*, but *apd-3* also is uniquely associated with non-pigmented cuticles such as the eye cover and external cuticle of white pupae. This study expands the collection of genes encoding cuticular proteins by three novel and well characterised members.

Keywords: *Apis mellifera*; Trachea; Genome annotation; Flexible cuticle; Hydrophobic proteins

1. Introduction

Insect cuticles are complex composite materials of proteins and chitin with remarkable biological properties that were optimised during the successful evolution of this group of animals (Andersen, 2005; Willis et al., 2005). Insect growth and morphogenesis are strictly dependent on the capability to remodel chitin-containing structures, namely the cuticles of the epidermis and trachea and the peritrophic matrices lining the midgut epithelium (Merzendorfer and Zimoch, 2003). Not surprisingly, insect cuticle has proved to be a difficult experimental material and in spite of extensive research remains only partially characterised. In recent years, the completion of a few insect genome projects brought a new dimension to this field of research. As a result, a significant number of sequences encoding putative cuticular proteins have been identified and added to the cuticular proteome database (Magkrioti et al., 2004). Comparison of the amino acid sequences indicates some degree of similarity between various proteins, but prominent differences are often apparent (Andersen et al., 1995; Willis et al., 2005) underscoring the complexity of the insect protective shell and/or scaffolding structures.

Cuticle of the exoskeleton is made of two main layers, an outer thin epicuticle, rich in lipids and proteins, and a thicker procuticle containing proteins and chitin. Cuticles undergo drastic changes in architecture and composition during metamorphosis and the synthesis and deposition of cuticular proteins are governed by ecdysterone and juvenile hormone. Some of these changes are associated with complex biochemical processes such as extensive sclerotisation, pigmentation and crosslinking. At the molecular level, the behaviour of epidermal cells producing cuticular

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structure is controlled by a complex interconnected network that links growth and development with cuticle proteins expression, chitin biosynthesis and degradation. For example, recent studies in *Drosophila* reveal that tracheal cuticle pattern is regulated by a complex genetic network via a RhoA-dependent formin DAAM, a protein that controls the tracheal cuticle pattern through organising actin cytoskeleton (Matusek et al., 2006). Since the physical properties of cuticle are largely determined by the proteins deposited into both layers the characterisation of genes encoding cuticular proteins is an important step towards the full description of the insect cuticle.

Here we used the honey bee genome assembly (Honey Bee Genome Consortium, 2006; Robinson et al., 2006) to annotate a cluster of three genes encoding small proteins with sequence characteristics resembling motifs found in cuticular proteins. These genes reside in a genomic region with a relatively high GC content and are fully supported by transcriptional data (ESTs, northern blots and RT-PCR). All three predicted proteins, designated apidermins 1–3 (APD 1–3) have putative leader peptides and contain at least one hydrophobic tetra-peptide (AAPA/V) found predominantly in proteins of hard cuticles (Andersen et al., 1995; Willis et al., 2005). Both the sequence analysis and expression patterns suggest a distinct role for each protein. We show that *apd1* is highly expressed in the epidermis that produces pigmented cuticle and its temporal pattern of expression is restricted to late pupal and early adult development and coincides with the process of sclerotisation (Thompson and Hepburn, 1978; Andersen et al., 1981; Andersen, 2005). The temporal onset and restricted expression of *apd1* suggest that APD-1 may play an important role in the maturation of the adult cuticle. In contrast, *apd-2* is found in the internal cuticles of the trachea and stomach, whereas *apd-3* is expressed more broadly and appears to be associated with non-pigmented cuticles. It partially overlaps with *apd-1* and *apd-2*, but also is uniquely expressed in the transparent eye integument and during pre-imaginal developmental stages that do not express *apd-1*.

Our results illustrate the effectiveness of genomic annotations combined with quality cDNA/EST libraries and detailed spatio-temporal expressional profiling in functional tagging of predicted orphan genes. The approach used in this study adds new members to a fast growing cuticular protein database and broadens our understanding of both cuticle structure and evolution.

2. Materials and methods

2.1. Insects and tissue dissections

Adult honey bees were collected either at the hive entrance or from small queenless cages kept in a laboratory incubator at 32 °C and 80% humidity. Newly emerged bees, pupae and embryos were removed from brood frames and kept on ice until dissection. The age of pupae was determined according to Cameron (1962) and Rembold et al. (1980). Pieces of cuticles with the attached epidermal layer were carefully removed from different body parts in bee Ringer (Bicker, 1995) under a stereo microscope making sure that no other tissues were left behind. All fragments were collected in 1.5 ml Eppendorf tubes containing 100–500 μl of Trizol (Invitrogen). RNA was extracted as described elsewhere (Kucharski and Maleszka, 2005).

For microscopic examination the pieces of integument were fixed overnight in 2.5% glutaraldehyde in PBS. After three washes in PBS the fixed samples were dehydrated in a series of ethanol solutions, cleared in xylene, infiltrated and embedded in paraplast X-TRA (Oxford Labware). Eight micron sections were mounted on coated slides and photos were taken using a digital camera attached to a Zeiss microscope.

2.2. Molecular techniques

Northern blots, PCR amplification and other molecular methods are described in Kucharski and Maleszka (2002, 2005). Primers used for PCR amplification of apidermins and the expected sizes of the amplicons: APD1: F GAAGTACATGATCGTCCTTG, R CTGGTCCAAC CACAGCCGCAGCT (242 bp). APD2: F CGAGTTC CAAGAAGCTCAAGTTA, R GGTGCAAGAGGTA CGGCTGCCAA (153 bp). APD3: F GCGGATCGAG CAATCATGAAGTA, R CCAGCAGCTGGTCCAG CAACCA (245 bp).

The honey bee genomic and EST sequences are available via BeeBase (http://racerx00.tamu.edu/bee_resources.html) and GenBank (http://www.ncbi.nlm.nih.gov).

The name Apidermin is meant to indicate that these genes have been identified in *Apis* and are expressed in epidermal cells.

3. Results

3.1. Apidermins and the underlying genomic landscape

Apidermin-1 (APD-1) was initially discovered by microarray hybridization as a differently expressed gene in the heads of newly emerged and older bees and described as a ligand-gated ion channel LGIC (Kucharski and Maleszka, 2002). However, the corresponding cDNA (EST_127) used for microarray printing turned out to be a chimera composed of two ligated transcripts encoding LGIC and a small orphan protein. The differential expression was subsequently associated with a subset of this sequence encoding a small protein with unknown function. Following the release of the honey bee genome assembly we used the coding sequence of APD-1 to fully annotate the underlying genomic landscape. As shown in Fig. 1 *apd-1* is a member of a small cluster of genes on linkage group 4.18. In addition to *apd-1*, two other genes encoding small orphan proteins have been uncovered in this region