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Two major cuticular proteins are required for assembly of horizontal laminae and vertical pore canals in rigid cuticle of *Tribolium castaneum*

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ABSTRACT

The insect exoskeleton is composed of cuticle primarily formed from structural cuticular proteins (CPs) and the polysaccharide chitin. Two CPs, TcCPR27 and TcCPR18, are major proteins present in the elytron (highly sclerotized and pigmented modified forewing) as well as the pronotum (dorsal sclerite of the prothorax) and ventral abdominal cuticle of the red flour beetle, *Tribolium castaneum*. Both CPs belong to the CPR family, which includes proteins that have an amino acid sequence motif known as the Rebers & Riddiford (R&R) consensus sequence. Injection of double-stranded RNA (dsRNA) for *TcCPR27* and *TcCPR18* resulted in insects with shorter, wrinkled, warped and less rigid elytra than those from control insects. To gain a more comprehensive understanding of the roles of CPs in cuticle assembly, we analyzed for the precise localization of TcCPR27 and the ultrastructural architecture of cuticle in TcCPR27- and TcCPR18-deficient elytra. Transmission electron microscopic analysis combined with immunodetection using gold-labeled secondary antibody revealed that TcCPR27 is present in dorsal elytral procuticle both in the horizontal laminae and in vertical pore canals. dsRNA-mediated RNA interference (RNAi) of *TcCPR27* resulted in abnormal electron-lucent laminae and pore canals in elytra except for the boundary between these two structures in which electron-dense molecule(s) apparently accumulated. Insects subjected to RNAi for *TcCPR18* also had disorganized laminae and pore canals in the procuticle of elytra. Similar ultrastructural defects were also observed in other body wall regions with rigid cuticle such as the thorax and legs of adult *T. castaneum*. TcCPR27 and TcCPR18 are required for proper formation of the horizontal chitinous laminae and vertical pore canals that are critical for formation and stabilization of rigid adult cuticle.

1. Introduction

Insect cuticle or exoskeleton is a complex biocomposite material consisting of three major morphologically distinct layers, the waterproofing envelope, the protein-rich epicuticle and the chitin/protein-rich procuticle (Locke, 2001; Moussian, 2010; Moussian et al., 2006). It provides a physical barrier against water loss and protects against physical damage, irradiation, xenobiotics and pathogens. Structural cuticular proteins (CPs) and the polysaccharide chitin are the major components of the exo- and endocuticular layers that comprise the procuticle. The former layer is formed before molting (pre-molt), whereas the latter is mainly deposited after completion of the molting process (post-molt). During cuticle maturation and tanning (sclerotization and pigmentation), CPs are post-translationally modified and cross-linked by quinones or quinone methides produced by the oxidation of *N*-acylcatechols catalyzed by laccase 2 (Arakane et al., 2005; Hopkins and Kramer, 1992). This vital chemical process is required to stabilize and harden the cuticle, protecting insects from microbial, physical and environmental stresses. Although the factors contributing to the synthesis of cuticles with differing mechanical properties are not well understood, appropriate combinations and degrees of cross-linking of CPs as well as dehydration are required for determining the physical properties of the exoskeleton (Lomakin et al., 2011).

Bioinformatics searches of fully sequenced and annotated genomes of several insect species such as the honey bee, *Apis melifera* (Honeybee Genome Sequencing Consortium, 2006), the fruit fly,

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Drosophila melanogaster (Karouzou et al., 2007) and the red flour beetle, *Tribolium castaneum* (Dittmer et al., 2012; Richards et al., 2008) indicate that there is a large number of genes encoding CP-like proteins in insect genomes. Indeed, more than 200 putative *CP* genes have been identified in the malaria mosquito, *Anopheles gambiae* (Cornman et al., 2008) and the silkworm, *Bombyx mori* (Futahashi et al., 2008). *T. castaneum* has 108 genes encoding CP-like proteins (Dittmer et al., 2012; Richards et al., 2008).

Many of the insect structural CPs have been classified into 12 different families as defined by unique amino acid sequence motifs characteristic of each of the families (Willis, 2010; Ioannidou et al., 2014). The largest family is the CPR family whose members contain the Rebers & Riddiford (R&R) Consensus (Rebers and Riddiford, 1988). When amino acid sequences of proteins belonging to the CPR family are aligned, they fall into three groups denoted as RR-1, RR-2 and RR-3 based on sequence similarity (Andersen, 1998, 2000; Karouzou et al., 2007). CPR proteins containing the RR-1 motif have been found primarily in soft and flexible cuticles, while proteins with the RR-2 motif have been found mostly in rigid cuticles (Willis et al., 2005). CPR proteins with the RR-3 motif have been identified only in a few species (Andersen, 2000; Futahashi et al., 2008; Willis, 2010). Transcriptional regulation of *CP* gene expression appears to be regulated by developmental and hormonal cues (Ali et al., 2013; Charles, 2010). Togawa et al. (2008) analyzed the temporal expression patterns of 152 *CPR* genes in *A. gambiae* by using real-time PCR. These were grouped into 21 clusters based on expression profiles. Interestingly, in *B. mori* larvae, several *CP*-like genes were primarily expressed in internal tissues (e.g. ovary, brain and posterior silk gland) rather than in the epidermis, and a few of the transcripts were detected only in the internal organs (Futahashi et al., 2008).

Although it is generally believed that CPs expressed at different developmental stages or in different body regions assemble a cuticle with appropriate mechanical properties such as rigidity or flexibility (Cox and Willis, 1985; Willis et al., 2005), the precise location of CPs within a cuticle is still not well determined. Very recently, Vannini et al. (2014) analyzed the expression of AgamCPF3 and AgamCPLCG3/4 proteins in *A. gambiae* and localized them using electron microscopic immunocytochemistry. The *AgamCPF3* gene was highly expressed at the pharate adult stage, whereas maximal levels of transcripts of *AgamCPLCG3/4* genes were detected in the young adult right after adult eclosion. The temporal expression of these three genes appears to be correlated with the locations of their products because the former is predominantly localized in the exocuticle, while the latter two are restricted to the endocuticle.

We previously identified two abundant cuticular proteins, TcCPR27 and TcCPR18, in elytra whose dorsal cuticle becomes highly sclerotized and pigmented to protect the underlying soft hindwings and dorsal abdominal portions of the *T. castaneum* adult (Arakane et al., 2012). These two proteins are members of the CPR family that contains the RR-2 motif. TcCPR27 and TcCPR18 proteins are abundant in rigid cuticles found in the elytron, pronotum and ventral abdomen but are absent or very minor in soft and flexible cuticles present in the dorsal abdomen and hindwing of *T. castaneum* (Arakane et al., 2012; Dittmer et al., 2012). dsRNA-mediated gene silencing (RNAi) of these proteins resulted in phenotypes with malformed and weakened elytra. In particular, the elytra of TcCPR27-deficient adults were shorter, wrinkled, warped, fenestrated and less rigid than those of control insects, and the adults eventually died from dehydration approximately one week after eclosion. Those results revealed that these two major CPRs are structural proteins essential for formation and stabilization of the rigid cuticle of *T. castaneum* adults. However, ultrastructural changes in the cuticle after depletion of these proteins by RNAi have not been investigated so far. In this study we performed RNAi of these two *CPR* genes in *T. castaneum* and analyzed the localization of TcCPR27 in cuticle by immunogold-histochemistry as well as by transmission electron microscopy (TEM) of the ultrastructure of rigid cuticle from both TcCPR27- and TcCPR18-deficient adults.

2. Materials and methods

2.1. Insects

T. castaneum (GA-1 strain) was used for this study. Insects were reared on organic flour at 30 °C at 50% relative humidity (Beeman and Stuart, 1990). Under this rearing condition, adult eclosion occurs 5 days after pupation.

2.2. RNA interference

dsRNAs for *TcCPR27* and *TcCPR18* were synthesized as described previously (Arakane et al., 2012). One hundred ng of dsRNA was injected into late-stage larvae (a mixture of penultimate instar and last instar larvae), after which the phenotypes and morphology of the adult cuticle were analyzed approximately 13–17 days later when the insects had reached the pharate adult stage. dsRNA for the *T. castaneum vermilion* gene (ds*TcVer*) was injected as a negative control (Arakane et al., 2009; Lorenzen et al., 2002). Total RNA was isolated from whole insects (5 d-old pupae) after RNAi of *TcCPR27*, *TcCPR18* and *TcCPR27/18* (co-injection) by using the RNeasy Mini kit, and then treated with DNase I (Qiagen). Three insects were pooled for each RNA extraction. One μg of total RNA was used to prepare cDNA using SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The detail of condition of real-time PCR was described in supplemental information. The total RNA was independently isolated for each of the three replications and significant differences were analyzed using the Student *t*-test. To estimate knockdown levels of the targeted proteins, soluble and insoluble proteins were extracted from elytra ($n = 5$) in cold phosphate-buffered saline (PBS) and analyzed 15% SDS-PAGE as described previously (Arakane et al., 2012).

2.3. Transmission electron microscopy

Five day-old pupae that had been injected with dsRNA at the late larval stage of development were collected and fixed in a mixture of 0.1% glutaraldehyde and 4% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 24 h at room temperature. Samples were rinsed three times for 15 min with 0.1 M sodium cacodylate buffer, and then dehydrated in a progressive ethanol gradient of 50, 60, 70, 80, 90, 95 and 100% for 15 min each. The tissues were infiltrated in LR white resin (Electron Microscopy Sciences, PA, USA) (2:1 ethanol:resin for 4 h, 1:1 ethanol:resin for 4 h, 1:2 ethanol:resin for 4 h and 100% resin overnight). The tissues were vacuum-infiltrated for 2 h, embedded in gelatin capsules (Electron Microscopy Sciences), and then polymerized at 55 °C for 12 h followed by ultrathin sectioning. Ultrathin sections (~90 nm) were stained with 4% aqueous uranyl acetate for 10 min and then imaged using a transmission electron microscope (JEM-1400, JEOL).

2.4. Immunogold labeling

To determine the precise location of the TcCPR27 protein in the rigid adult cuticle of *T. castaneum*, we performed immunogold labeling for TcCPR27. First, we extracted TcCPR27 protein from elytra of 5 d-old pupae and purified by Ni-NTA chromatography