

FUNCTIONAL GENOMICS AND DYNAMIC ASSEMBLY OF CUTICULAR PROTEINS
ANALOGOUS TO PERITROPHINS AND KNICKKOPF INTO THE PROCUTICLE OF
TRIBOLIUM CASTANEUM

by

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Abstract

The exoskeleton of insects, the cuticle, functions as a support structure and a physical barrier that protects insects from mechanical damage and dehydration. The exoskeleton is mainly made of chitin and proteins, some cross-linked to one another into certain patterns to form the rigid and resistant cuticle. In previous studies from our laboratory, cuticular proteins analogous to peritrophins (CPAPs) and Knickkopf (Knk) were identified and characterized mainly at the pharate adult stage during insect development. However, the dynamic assembly of both CPAP and Knk into the cuticle and the functions of the CPAPs are still not fully understood. Our study is to investigate how these cuticular proteins are assembled into the cuticle during different developmental stages and carry out their functional characterizations in the red flour beetle, *Tribolium castaneum*. RNA interference (RNAi) experiments that resulted in down-regulation of transcripts for CPAP 1-C, CPAP1-H, CPAP 1-J, CPAP 3-C and Knk genes resulted in molting defects. Confocal and transmission electron microscopic analysis examined protein expression at twelve stages of development, as well as the span from young larva through adult day 3 stages. The results suggested that the CPAP 3-C protein is present in the lower part of endocuticle in the so-called assembly zone and it was not distributed throughout the procuticle with chitin. Down-regulation of CPAP 3-C transcripts revealed a disorganized assembly zone; however, no loss of chitin content or the laminar architecture of the procuticle was found. Knk protein was present throughout the procuticle and some of the protein was found inside of the epithelial cells.

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Dedication

This work is dedicated to my friends, who have given me the care, guidance and encouragement when I am away from home.

Chapter 1 - Introduction

Insect Cuticle

Chitin

Chitin is the second abundant polysaccharide after cellulose in nature. It is a major component of the cell walls of fungi and exoskeletons of most arthropods and nematodes (Merzendorfer, 2006). Chitin is a long chain polysaccharide, which has a chemical structure of β -1, 4-linked N-acetylglucosamine; it also may contain some deacetylated residues (Rudall, 1963). However, it was not found in tobacco hornworm cuticle (Kramer et al., 1995). Chitin in the cuticle is secreted by a membrane bound protein chitin synthase-A. Adjacent chitin chains are linked by hydrogen bonds between CO-NH groups and tend to form chitin microfibrils. Many such microfibrils running parallel to each other form a thin layer called lamina. In some arthropods, such as crab, during cuticle synthesis, these thin layers may be laid down on top of one another at a slightly different angle to form a twisted lamellate cuticle; this is called a helicoidal or twisted plywood structure (Chapman, 1982; Ifuku et al., 2011), which is predicted to account for various properties, such as stress tolerance. However, this structure is not present in the cuticle of *T. castaneum*, which has only stacks of parallel arrays of laminae (Noh, personal communication). In insects, chitin has been found in the cuticle-forming tissues, such as elytron, body wall and trachea, as well as the membrane of the peritrophic matrix (Kramer and Muthukrishnan, 2005). Chitin together with lipids, proteins and catecholamine undergoes certain modifications to assemble into cuticle in order to contribute as the physical barrier. For instance, it protects the insect from mechanical and

biological damage and dehydration. The mechanism of protein and chitin cross-linking, however, remains poorly understood.

Proteins

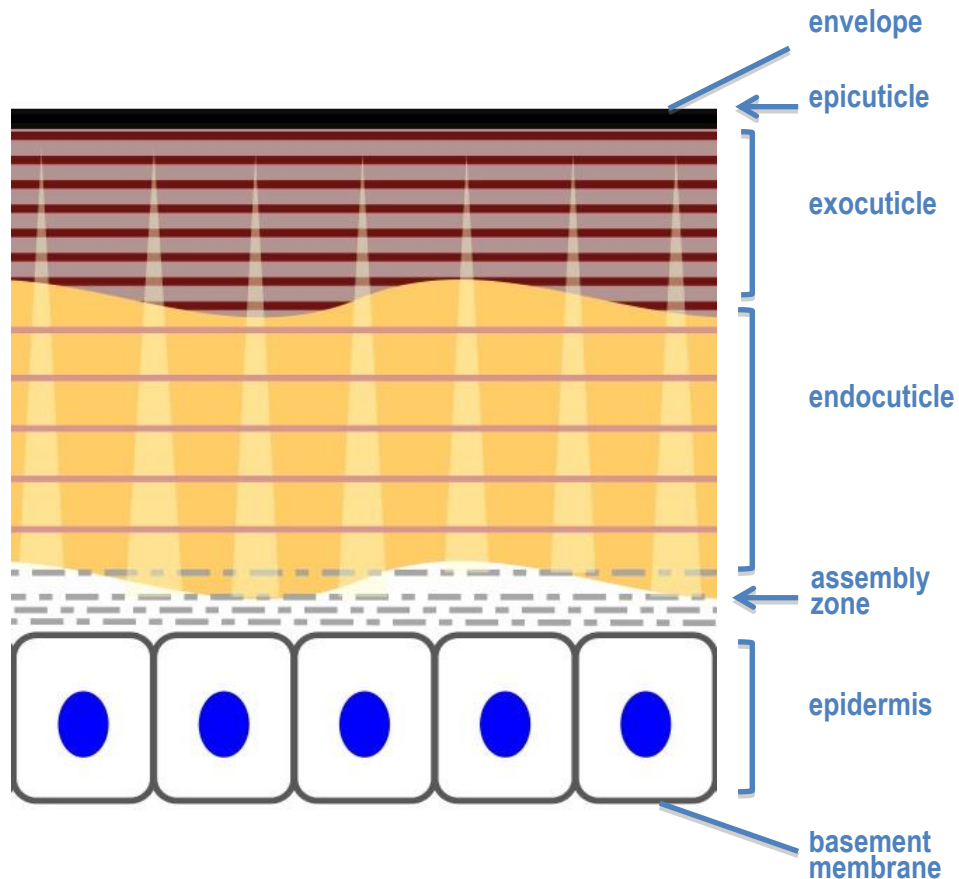
Cuticular proteins are reported and predicted to assist in cuticle organization due to the possibility of chitin binding. One group of cuticular proteins contains the **Rebers-Riddiford** consensus sequence (RR), $G_{X8}G_{X7}Y_XA_XE_XGY_{X7}P_{X2}P$ (Rebers and Riddiford, 1988). Another group contains one or more chitin binding peritrophin-A domains (ChtBD2), $CX_{13-20}CX_{5-6}CX_{9-19}CX_{10-14}CX_{4-14}$ (Tellam et al., 1999). Cuticular proteins with ChtBD 2 domains were identified in the midgut-lining membrane and denoted as peritrophic matrix proteins or PMPs (Elvin et al., 1996; Schröder et al., 1998), and later a family of proteins was extracted from cuticle-forming tissues and given the name **Cuticular Proteins Analogous to Peritrophins or CPAPs (Jasrapuria et al., 2010).**

Basic structure of insect cuticle

Insect cuticle is also known as exoskeleton or external skin consists of five different layers, including the epidermis, assembly zone, procuticle, epicuticle and envelope (Fig 1.1). A single layer of epidermal cells continuously secretes cuticle during development. The assembly zone (above the epidermis), also known as Schmidt's layer, may play a role in packing proteins with chitin in a certain way, which later either are transported to the procuticle or remain to stay in this region. The procuticle (above assembly zone) is a chitin- and protein-rich region of cuticle. Chitin microfibers are embedded in a protein matrix and in some species also a mineral-enriched component such as calcium carbonate

(Chen et al, 2008). During cuticle development, the microfibrils of chitin can be oriented in different angles into thin layers stacked as a twisted plywood structure as the procuticle is built up (Ifuku et al., 2011). The procuticle can be differentiated into highly sclerotized and rigid exocuticle (outer layer), and pale and flexible endocuticle (inner layer). The epicuticle is localized immediately above the procuticle, and consists of cuticulin and lipoproteins. The outermost layer is the envelope that is a waxy layer preventing water loss as well as infection. The thickness of the cuticle may also be varied among different species or even various regions of the same insect.

A



B

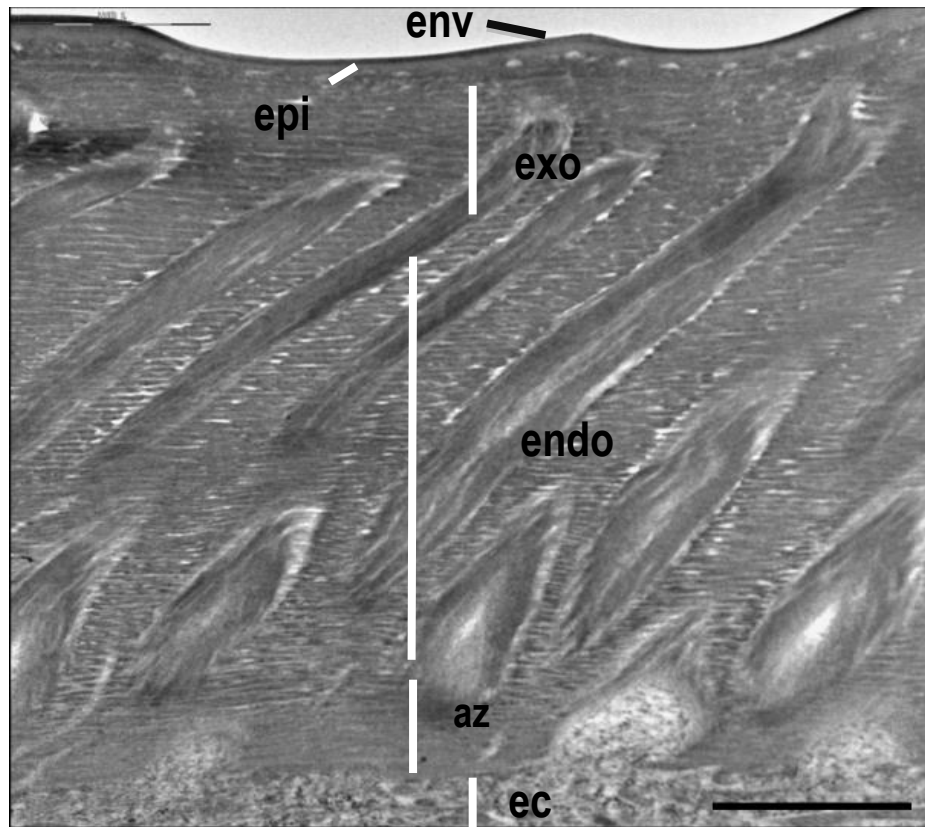


Figure 1.1 Structure of insect cuticle.

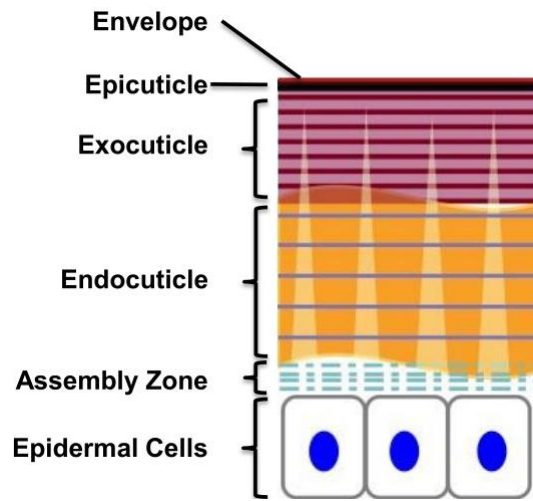
(A) Diagrammatic representation of structure and (B) transmission electron microscopic analysis of *T. castaneum* pupal elytral cuticle. The cuticle consists of multiple layers including the outermost envelope (env), waxy epicuticle (epi), protein-chitin rich exocuticle (exo) and endocuticle (endo), assembly zone (az), and epidermal cell (ec) layer. Scale bar = 1 μ m.

Molting

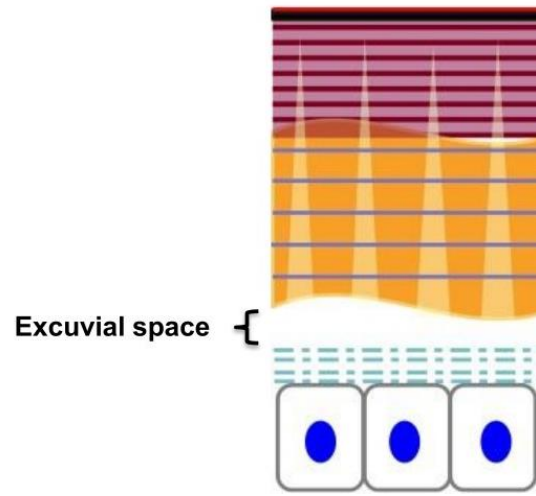
Molting is the periodic process that allows insects to expand their body size, and it may take days to complete. An insect's growth immediately occurs right after egg hatch. Depending on species, insects have various life stages throughout their growth and development, while each stage or juvenile instar ends up with molting. Molting must occur within a safe condition; therefore, the new exoskeleton has to be constructed underneath the old one. In other words, replacement takes place by a new exoskeleton before casting off the old inelastic exoskeleton. In addition, molting is a complex process that is triggered by hormones, such as ecdysone. When an insect's growth is limited with space for body expansion, this stimulates a physical separation of the old exoskeleton to detach from the epidermis, called apolysis (Jenkin and Hinton, 1966). Epidermal cells increase inactive molting fluid secretion to fill in the exuvial space. Molting fluid actively digests the old cuticle following formation of outer epicuticle (the cuticulin layer) of newly formed cuticle. Synthesis of new epicuticle was postulated to protect the new cuticle from degradation. However, Chaudhari et al. disproved this dogma in 2011. Knickkopf (Knk) protein in *T. castaneum* has been reported to not only protect newly formed cuticle from degradation by molting fluid, also to help chitin to organize in new cuticle (Chaudhari et al., 2011). Other cuticular proteins possibly also are involved in cuticle assembly with similar functions as Knk. Molting fluid consists of multiple chitinolytic enzymes and proteinases. Chitinases are a group of proteins (family-18 glycosylhydrolases) that insects use to degrade the polysaccharide chitin into smaller sugar molecules, soluble and insoluble oligosaccharides (Coutinho and Henrissat, 1999, Kramer, 1985, Kramer and Muthukrishnan, 1997, Arakane et al., 2003). The chitinases

digest the unsclerotized old cuticle (endocuticle), and the products of digestion (chitin and amino acids) can pass through the epicuticle and be recycled by the epidermal cells. However, chitinases have little or no effect on the highly sclerotized old cuticle (exocuticle) (Chapman, 1982). The process of actual shedding of the old cuticle or emergence is named ecdysis. The insect eventually breaks out via the ecdysial lines – cuticle splits (Chapman, 1982). Newly formed cuticle expands, contiguously develops and sclerotizes in the next few days.

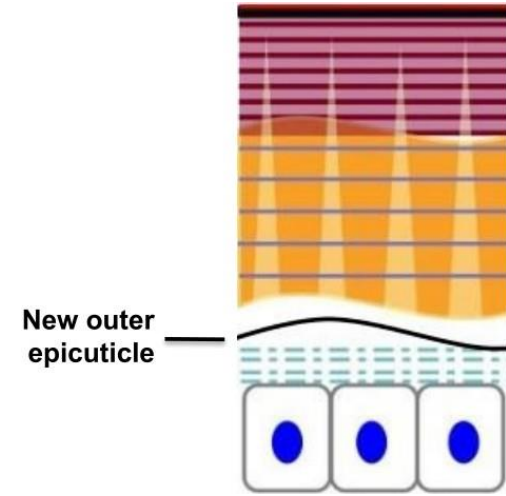
A



B



C



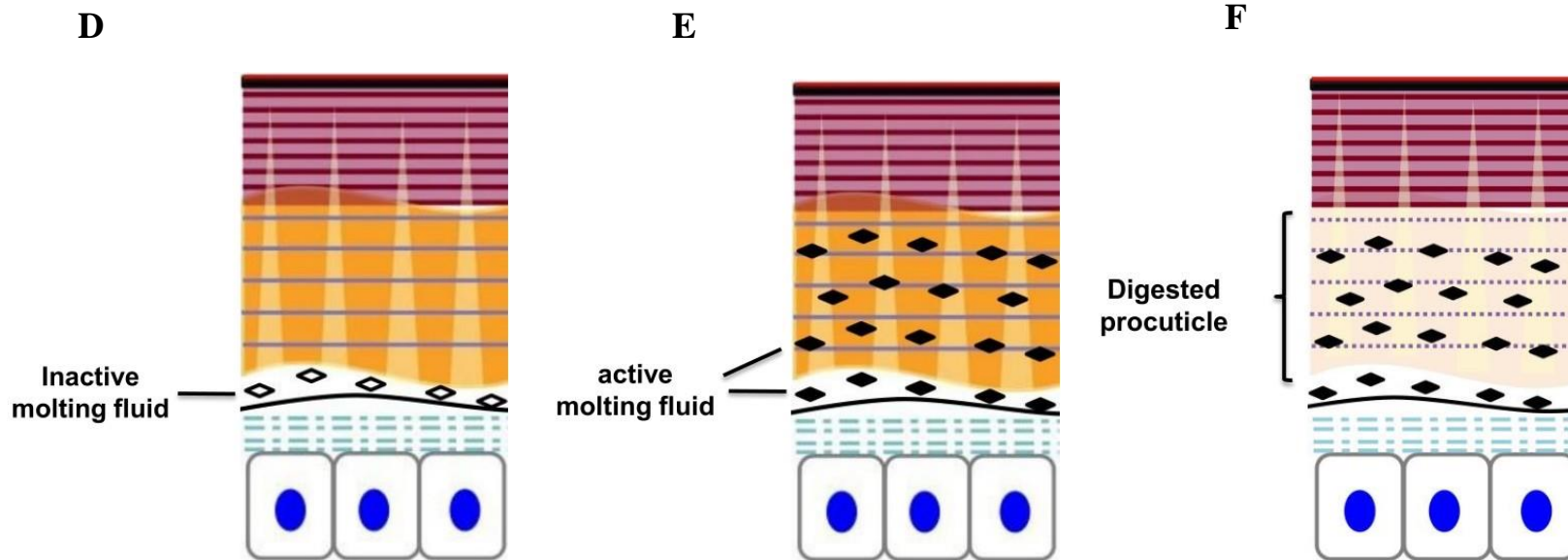


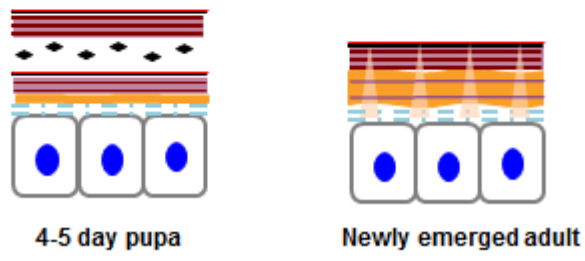
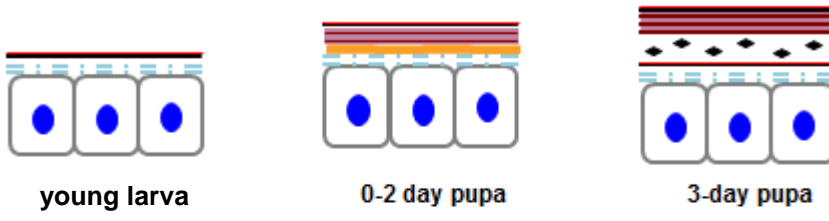
Figure 1.2 Diagrammatic representation of cuticle changes in molting.

(A) The mature insect cuticle has multiple layers. (B) The molting process is initiated as the endocuticle separates from the epidermis, so apolytic (exuvial) space forms and molting fluid fills in the space, but is inactive (hollow symbols) (D). New cuticle starts to form underneath the old procuticle (C). Later, molting fluid actively (solid symbols) digests the old cuticle, while Knk protects newly formed cuticle from degradation. (F) Endocuticle of old procuticle breaks down to peptides, amino acids and sugar molecules, which are reabsorbed and recycled as building blocks for new cuticle synthesis.

Cuticle formation

The formation of new cuticle is immediately after apolytic space appears. Meanwhile the molting fluid accumulates in this space. Patches of cuticulin are secreted and deposited at the margins of apical side of epidermal cells, which becomes the outer layer of epicuticle. It is the first layer of new cuticle formed during the molting process. The outer layer of epicuticle is very thin, contains proteins with lipids that probably are stabilized from the beginning due to sclerotization mechanisms (Locke, 1976; Locke and Krishnan, 1971). Once the outer layer of epicuticle is complete, inner epicuticle starts to form as a continuous layer above the apical epidermis. A monolayer of wax is secreted probably by the oenocytes and seals the new cuticle from its surface. Deposition of procuticle occurs as epicuticle is completed, and the region where new cuticle deposition is taking place is called the assembly zone or Schmidt's layer that is predicted to play a central role in packing proteins and chitin microfibers. Different regions of insect cuticle may function differently depending on the thickness, elasticity, and color. For instance, in *T.castaneum* modified forewings (elytron) are highly sclerotized to be responsible as physical barrier. However, in *Schistocerca*, the base of the wing and wing-hinge contains resilin (rubber-like protein) that probably lacks cross-linking and provides the ability of elasticity during flight. The properties of insect cuticle are also determined by the process of sclerotization or tanning. Sclerotization was first introduced by Mark Pryor in 1940. It is a process utilized by insects to stabilize cuticle by incorporating phenolic components with functional cuticular proteins (Hopkins and Kramer, 1992; Anderson, 2010; Gorman, 2010; Anderson, 2005). Sclerotization continues after ecdysis. Transmission electron microscopy showed that synthesis of elytral cuticle in *T. castaneum* probably ends around

3 days after adult eclosion (Arakane and Noh, unpublished data). Adult elytral procuticle has significant growth on pupa day-4. During adult days 1~3, one newly formed cuticle layer is added to the inner procuticle every day.



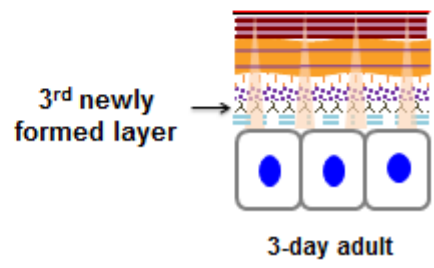
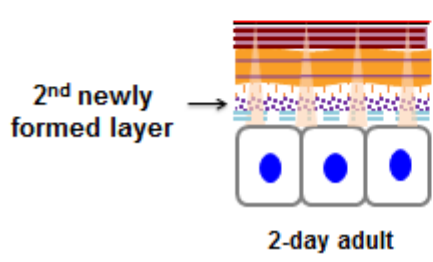
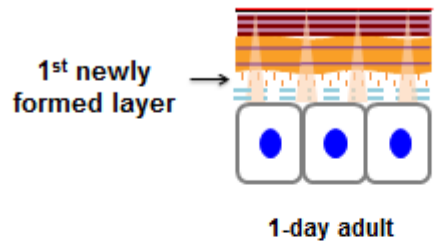


Figure 1.3 Insect cuticle formation.

(A) Newly formed pupal cuticle on young larva. (B) During the first two days after larval-pupal molt, cuticle grows in thickness, as it darkens or sclerotizes. (C) On 3rd day of pupal period, there is already a very thin layer of adult cuticle, and molting fluid starts to fill the exuvial space, digesting the old pupal procuticle. (D) Adult cuticle keeps growing on pupal days 4~5. Remaining pupal cuticle is essentially the sclerotized exocuticle that is indigestible. (E-H) Adult cuticle keeps growing after emergence. A morphologically distinct layer of procuticle is added between day 1 and day 3.

Outline of this thesis

The literature review (Chapter 1) includes the previous studies and available knowledge of the cuticular proteins with chitin-binding domains in insects. In **Chapter 2**, previous studies on Cuticular Protein Analogous to Peritrophins (CPAPs) provided the evidence that down-regulating the transcripts of selected genes, *TcCPAP1-C*, *TcCPAP1-H*, *TcCPAP1-J* and *TcCPAP3-C*, resulted in death during the pharate adult stage when cuticle growth is rapid. Therefore, these CPAPs proteins are predicted to have essential functions in insect exoskeleton deposition during metamorphosis. This chapter provides information on the localization and turnover of the cuticular protein, CPAP3-C. A detailed time-course study of CPAP3-C protein indicates that it appears in the early stages of new cuticle formation and disappears prior to its turnover at the next molt during development of *T. castaneum*. **Chapter 3** introduces a dynamic study of the chitin binding/organizing protein Knickkopf (Knk) during cuticle formation. This chapter provides information on the localization and turnover through the developmental period young larva to adult day 3. **Chapter 4** includes a general conclusions of the completed research and future directions for the project.

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**Chapter 2 - Functional characterization of selected Cuticular
Proteins Analogous to Peritrophins: TcCPAP1-C, 1-H, 1-J & 3-
C that help to assemble insect cuticle.**

Abstract

Molting to either the next life stage or the next juvenile instar requires shedding of the old cuticle and, more importantly, synthesizing new cuticle or exoskeleton. Formation of the new cuticle is a process in which chitin microfibrils embedded in the protein matrix are arranged and modified into a complex cuticle in a poorly understood process. The newly synthesized cuticle is responsible for widely varying mechanical properties of the organs according to its localization within the body plan. A dramatic example of differing cuticular properties can be seen between the elytron, a hardened forewing that is hard and thick, and the hindwing that is thin and flexible. Chitin-binding cuticular proteins are thought to confer the ability to bind to chitin. Functional characterizations of these proteins, therefore, is crucial in a study of insect physiology as well as in the innovation of pest insect control strategies and the design of new biomaterials. A family of proteins with the chitin-binding peritrophin-A motif (ChtBD2) (Tellam et al., 1979) was identified in *T. castaneum* recently by Jasrapuria et al. (2010). These proteins were expressed in cuticle forming tissues, but not in the midgut, which gives rise to the peritrophins that are constituents of the peritrophic matrix were named “Cuticular Proteins Analogous to Peritrophins” An extensive RNAi study of CPAPs reported cuticle defects and mortality for several of these genes (Jasrapuria, 2012; Jasrapuria et al., 2012). This study is focused on the functional characterization of four CPAPs including TcCPAP1-C, TcCPAP1-H, TcCPAP1-J and TcCPAP3-C as well as their localization and turnover in the developing cuticle in *T. castaneum*. The expression profile of *CPAP* genes showed that all of these genes are expressed in cuticle-forming tissues, such as tracheae, elytron and hindgut. CPAP proteins can be divided into CPAP1 and CPAP3 groupings based on the number of ChtBD2 repeats (CPAP1 = 1 ChtBD2 and CPAP3 = 3 ChtBD domains). RNAi studies

revealed that down-regulating *TcCPAP1-C*, *TcCPAP1-H*, *TcCPAP1-J* or *TcCPAP3-C* causes lethality at the pharate adult stage. Immunohistochemistry and confocal microscopic analysis at multiple developmental stages of wild type insects shows that TcCPAP3-C protein is confined to the assembly zone of the procuticle near the apical plasma membrane of the epidermal cell. These results provide experimental support for the notion that there is specialization in the functions of several CPAP proteins in *T. castaneum* and provide a biological rationale for the conservation of orthologs of these genes within CPAP families of proteins in insect species of different orders. Many of these proteins serve essential and non-redundant functions in maintaining the structural integrity of the cuticle.

Introduction

At specific points of their life span, insects have to routinely undergo a molting process, casting off their old outer exoskeletons, in order to let the organisms develop further. Molting includes digestion of the inner procuticle (endocuticle), sloughing off outer procuticle (exocuticle) and waxy layer, recycling of the digested products (amino acids and sugar), and construction of the new cuticle (Chang, 1993). Molting takes place in response to hormonal changes, such as 20-hydroxyecdysone and juvenile hormone. As the molting process begins, the inner procuticle (endocuticle) detaches from the epidermis, the exuvial space appears and is later filled with molting fluid (Jenkin and Hinton, 1966). The epidermal cells secrete the first layer of the new cuticle (outer epicuticle) to cover the epidermis. The newly synthesized cuticle continues to grow while the assortment of molting fluid enzymes degrades the old cuticle. Cuticle biogenesis is a process during which chitin microfibrils complex with proteins, especially chitin-binding proteins that are predicted to have the ability to bind to chitin and become arranged in the form of a laminar cuticle. Jasrapuria et al. (2010) have identified a family of cuticular proteins in *T. castaneum*, which contain one or three repeats of the peritrophin-A domain or chitin-binding type 2 domain (ChtBD2), named **C**uticular **P**roteins **A**nalogous to **P**eritrophins (CPAPs). They have been further subdivided into two subfamilies, CPAP1 with 10 members that have one ChtBD2 domain and CPAP3 subfamily with seven members containing 3 ChtBD2 domains. Both families of proteins are expressed only in cuticle-forming tissues, such as tracheae, body wall epidermal cells, elytra and linings of fore- and hindgut. RNA interference studies showed that down-regulation of transcripts for the genes, *TcCPAP1-C*, *TcCPAP1-H*, *TcCPAP1-J* and *TcCPAP3-C*, causes lethality

at the pharate adult stage, whereas RNAi of the other *CPAP* genes exhibited cuticle abnormalities and/or joint defects without significant mortality (Jasrapuria et al., 2011). We report here that the TcCPAP1-H protein is present throughout the procuticle and co-localizes with chitin. This indicates that TcCPAP1-H protein may be responsible for maintenance of the chitin level because knocking down transcripts of *TcCPAP1-H* reduces chitin content (Jasrapuria et al., 2011). In addition, TcCPAP1-H may help to organize the chitin into laminae in the procuticle. On the other hand, TcCPAP3-C protein is present primarily in the assembly zone and is predicted to play a role in proper cuticle organization.

Materials and Methods

Insect culture Results

Red flour beetle, *T. castaneum* GA-1 strain was used for all experiments. Insects were reared in wheat flour containing 5% brewer's yeast at 30°C under standard conditions.

dsRNA synthesis

Double-stranded RNAs (dsRNAs) were prepared by using Ampliscribe T7-Flash Kit (Epicentre Technologies, Madison, WI) according to the manufacturer's protocol. Two unique regions were chosen as templates for synthesis of each dsRNA of CPAP1-C, 1-H, 1-J, and 3-C (Jasrapuria, 2010).

dsRNA injection

dsRNAs were mixed in injection buffer (0.1 mM potassium phosphate buffer containing 5 mM potassium chloride, pH, 7.4), and injected into young larvae and pharate pupae (200 ng /insect; n=20). Treated insects were kept under standard conditions as described previously (Arakane et al., 2003).

Measurement of transcripts for CPAP after RNAi

RT-PCR was carried out to measure the transcript levels after dsRNA injections. Insects were injected with dsRNA at young larval stage and total RNA for each treatment was isolated from four pupae (4-day old) by using RNeasy Min Kit (Qiagen, Valencia, CA). cDNA was prepared from total RNA by using SuperScript III First-Strand Synthesis Kit (Invitrogen). RT-PCR was

performed to determine the transcript level using gene-specific primers after dsRNA injection. *T. castaneum* ribosomal protein S6 (RpS6) RT-PCR amplification products were used as an internal loading control.

Western blot analysis

Insects (n = 20) were injected as young larvae with dsRNAs of *TcCPAP1-C*, *TcCPAP1-H*, *TcCPAP1-J* and *TcCPAP3-C*. dsRNA Vermilion-treated group (n =20) was used as the control group. Crude extracts of proteins were obtained from 20 pairs of elytra from the pharate adult stage for each group. The homogenate containing proteins in phosphate buffered saline (PBS) was centrifuged to obtain the supernatant and pellet fractions. The antigens, TcCPAP1-C and TcCPAP1-H, were detected using antibodies raised in rabbits using the corresponding proteins expressed in the Hi-5 insect cell line, (Jasrapuria, Ph. D. thesis, Kansas State University 2011). TcCPAP3-C was detected using 2A12 antibody (monoclonal, Developmental Studies Hybridoma Bank, IA). The western blots were developed using secondary antibodies to the primary hosts' antisera conjugated with HRP. To check the specificity of antibody detection, SDS-PAGE and western blot were performed on extracts or media from Hi-5 cells expressing these proteins according to the manufacturer's protocol (Bio-Rad).

Immunohistochemistry and confocal analysis

Insects were treated with dsRNA of *CPAP1-C*, *1-H* and *3-C* at the young larval stage and collected as pharate adults (n=5). They were dissected to collect the middle part of the body with the whole elytron attached, fixed in 4% para-formaldehyde solution and then subjected to a series of sucrose gradients (12, 15, 18 and 20%). Cryosectioning was performed on a Leica CM

1800 microtome. The sections were rehydrated with 0.2% PBST buffer followed by 1 h incubation in 2% BSA (in PBST) blocking buffer at room temperature. Primary antibodies to TcCPAP1-C (1:100, in rabbit), 1-H (1:100, in chicken), 3-C (1:10, in mouse) and Knk (1:100, in rabbit) were applied to the cryosections (thickness = 20 μm) to detect the corresponding proteins. Alexa 488-conjugated goat anti-rabbit, chicken or mouse IgG's? (1:1000) were applied as secondary antibodies for primary antibody detection, respectively. After incubation at room temperature for 3 h, Rhodamine- or Cy3 conjugated chitin-binding probe (1:100) was used for chitin staining at 4°C overnight. The sections were rinsed with 0.2% PBST the next day followed by nuclei staining with 4, 6-diamidino-2-phenylindole-dihydrochloride (DAPI, 10 ng/ μl , 1:30) for 8 min. The sections were rinsed quickly. Fluoromount (Sigma) was added and sealed properly. The sections were stored at 4°C until confocal analysis. Both ventral body wall and elytron cuticles were the major foci for our experiments.

The Zeiss Leica 510 confocal microscope was used for scanning images using an objective (oil, 40 X/1.3) with zoom of 8X (body wall) and 4X (elytron), and with lasers capable of detecting DAPI (405 nm), Alexa (488nm) and Rhodamine- or Cy3- conjugated chitin-binding probe (543 nm). Finally, images were analyzed using ImageJ and LSM 510 software (NIH).

Transmission electron microscopy

dsRNA-injected insects were collected as pharate adults (pupal day-5) (n=5). They were dissected to obtain middle abdominal segments of the body and whole elytron. Samples were fixed in a solution containing 4% para-formaldehyde and 0.1% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2-7.4), and were kept with constant rotation overnight at room temperature. The samples were washed with 0.1 M sodium cacodylate buffer 3 times for 5 min.

Dehydration was carried out using an ethanol gradient series (50%, 60%, 70%, 80%, 90%, 95% and 100%). Resin filtration with LR white resin (EMS, CAT# 14383-CA) was applied following the protocol (provided by Kwang-ho Lee), and samples were vacuum infiltrated for 2 h. Samples were embedded in gelatin capsules (EMS, cat# 70104) filled with resin, capped and polymerized at 60° for 24 h in an oven.

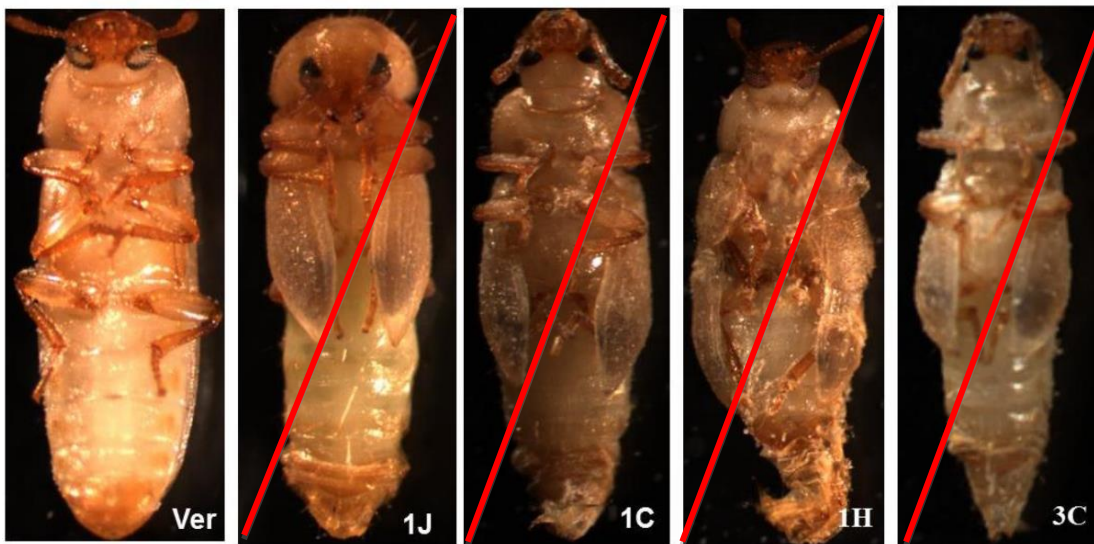
Time course analysis on cuticle development

Twelve stages were considered for the cuticle development time-course study, consisting of time points one day apart from each other and starting with young larva through adult day 3 (n=5 for each stage). Larvae that had a fairly long body size (~3* mg) and were actively moving were chosen as 6th instar larvae. Larvae that had a curled body (“C” shape) with very little movement were collected as pre-pupae (before the larval-pupal molt). Freshly emerged pupae were collected as pupal day 0. For developmental stages beyond adult day 0, insects were collected at 24-h intervals based on its previous stage. For instance, adult day 1 insects were collected precisely 24 h right after pupal emergence (pupal day 0). All samples were dissected and fixed for immunohistochemistry and confocal analysis according the standard protocol as described previously (Chaudhari et al., 2011).

Results and discussion

To functionally characterize selected *CPAP* genes, RNA interference (RNAi) experiments were used to down regulate the corresponding mRNAs. To observe the phenotype after each knockdown, 20 young larvae were selected and injected with dsRNA, and dsRNA *Vermilion* treated insects (lack of eye pigment) were used as the control group (Lorenzen et al., 2002). *CPAP1-C*, *CPAP1-H*, *CPAP1-J* and *CPAP3-C* dsRNA injected insects were all arrested at the pharate adult stage (Fig. 1A). In addition, *dsCPAP1-H* treated insects showed improper folding of appendages (Fig. 1bB) abnormal development of eyes, including pink eye pigment instead of black and convex-shaped eyes (Fig. 1B, IC). As they grew to pharate adult stage, eye pigment stayed pink and eyes became flat while some liquid leaked out. *dsCPAP1-H* treated insects had not completed abdominal contraction and/or elytral expansion, and had a longer in body length as comparing to control insects and possessed disorganized appendages.

A



B

dsVer

dsCPAP1-H



C

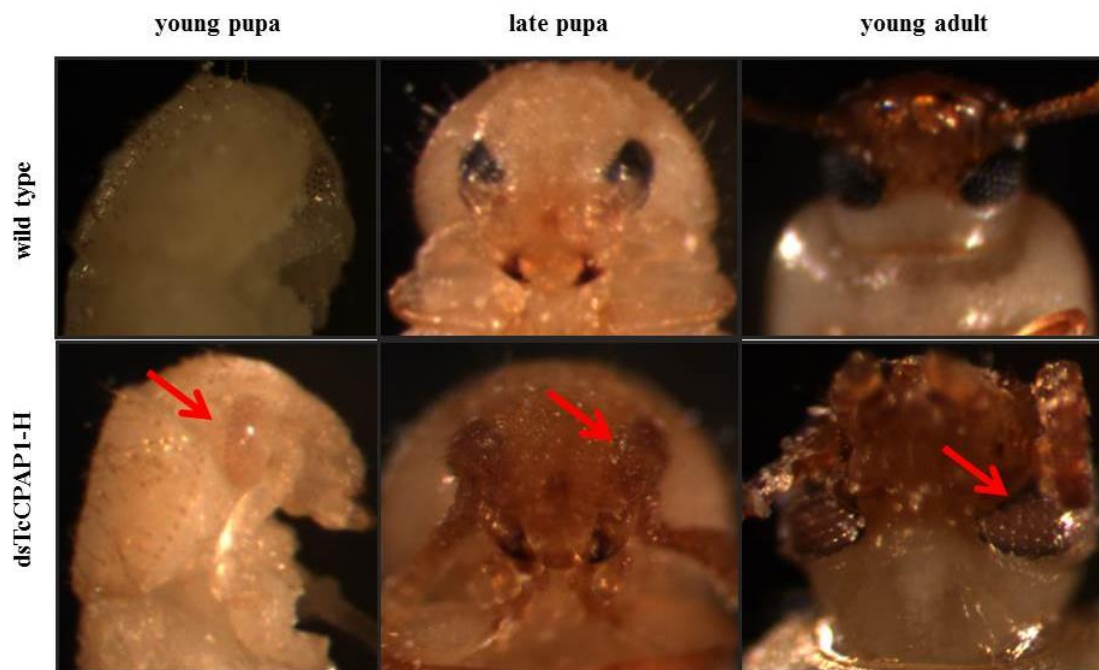


Figure 2.1 Phenotypes after ds RNA treatments.

Young larvae (n=20 for each group) were injected with dsRNA (800 ng) of *TcCPAP1-C*, *TcCPAP1-H*, *TcCPAP1-J*, and *TcCPAP3-C*, and photographs were taken at the pharate adult stage. *dsVer* injected insects (n=20) were used as the control group, which shows white eye pigmentation without any other visible effects on development of morphology. Images (A) represent the terminal stage and phenotypes after each dsRNA injection (except control). The

insects were arrested at the pharate adult stage and had 100 percent mortality dsRNA *TcCPAP1-H* treated insects (B) display improperly folded elytra and appendages after the larval to pupal molt. dsRNA *TcCPAP1-H* treatment also affected eye development (Fig. 2C).

Measurement of transcripts for CPAP after RNAi

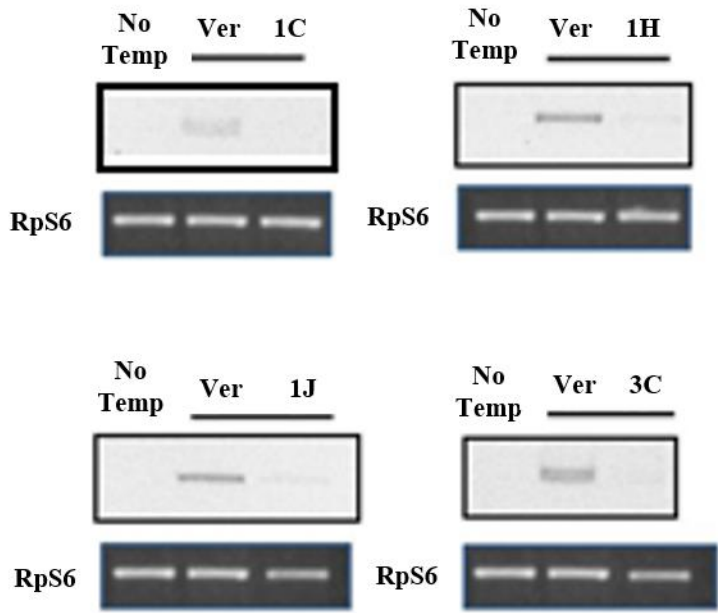


Figure 2.0.2 RT-PCR to determine the knockdown ability of dsRNAs .

Total RNAs were extracted from 4-d old pupae (n=4). cDNAs were prepared from total RNAs according to kit manufacture’s protocol. RT-PCR (28 cycles) was performed using gene-specific primers designed by Jasrapuria (Ph. D, thesis, KSU, 2011). RpS6 (24 cycles) was used as an internal loading control.

Western blot analysis for antibody detection

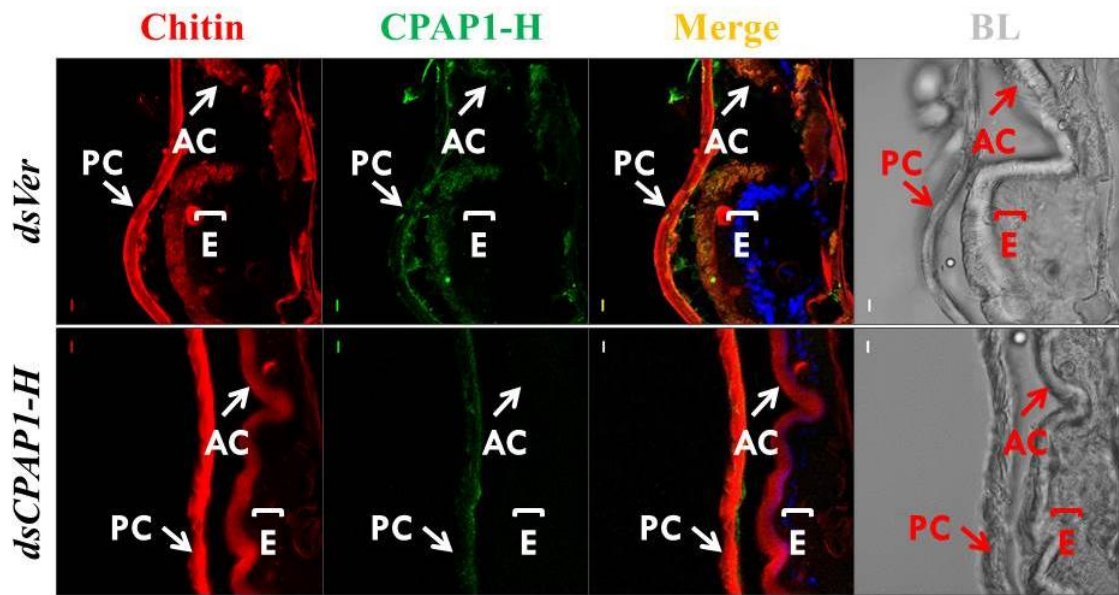
To verify the specificity of the antisera available in the lab, western blot experiments were performed using extracts of proteins from insects subjected to RNAi for *Ver*, *CPAP1-H*, *CPAP1-C* and *CPAP1-J*. Western blot for each antibody was performed using 10 µl of Hi-5 cell culture medium 3 days after infection with the recombinant baculovirus containing either the full length protein or a fragment of the corresponding protein to check antibody specificity. TcCPAP1-C antibody (raised in rabbit) has low specificity. Antibody detected proteins in all pellet fractions, even after *TcCPAP1-C* knockdown. TcCPAP1-H antibody (raised in chicken) detected pure TcCPAP1-H protein and dsRNA treated TcCPAP1-J pellet fraction. CPAP3-C antibody (raised in mouse) is commercially available from Developmental Studies Hybridoma Bank, IA.

Immunohistochemistry and confocal analysis

TcCPAP1-H protein is co-localized with chitin throughout the procuticle

TcCPAP1-H was detected with polyclonal antibody raised in chicken (Jasrapuria, Ph. D. thesis, KSU, 2011). TcCPAP1-H protein was detected throughout the procuticle (green) and co-localized (yellow in merge) with chitin (red) in both elytral and ventral body wall cuticle at the pharate adult stage. Down-regulation of *TcCPAP1-H* transcripts was expected to result in loss of corresponding protein. As expected, no CPAP1-H was detected in dsRNA *TcCPAP1-H* treated samples. Newly formed adult body wall cuticle shows a reduced thickness. This result is consistent with the results of biochemical analysis of chitin content, which revealed approximately 45% reduction of chitin (Jasrapuria, 2011). However, there was little or no evidence of reduction of the thickness of the elytral cuticle.

A



B

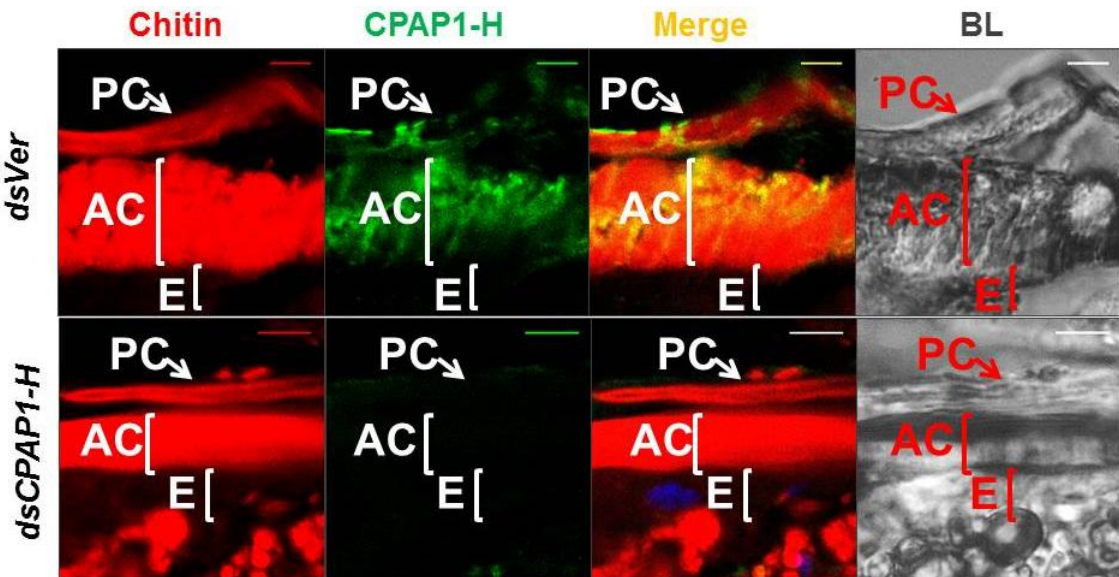


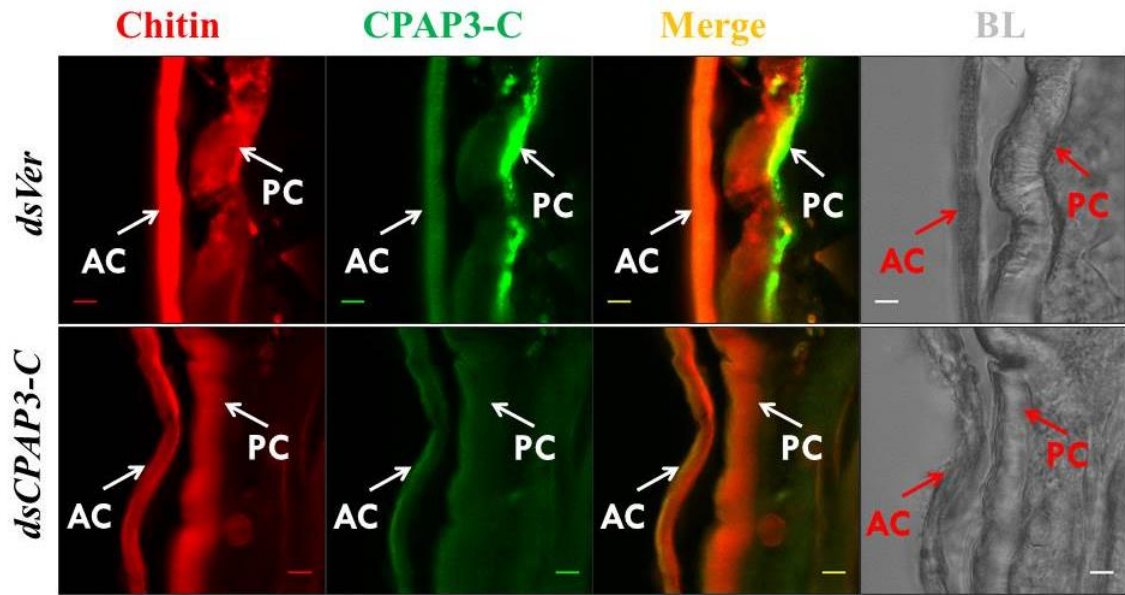
Figure 2.3 TcCPAP1-H protein is co-localized with chitin throughout the procuticle.

Immunohistochemistry followed by confocal analysis shows that TcCPAP1-H is present in the procuticle and co-localizes with chitin in elytra cuticle (A) and body wall (B). PC, pupal cuticle; AC, adult cuticle; E, epidermis. Immunostaining color coding; chitin (red), TcCPAP1-H protein (green), nuclei (blue) and co-localization of chitin and protein (yellow). BL, bright field. Scale bar = 5 μm .

TcCPAP3-C protein is localized in the assembly zone

TcCPAP3-C protein was detected with monoclonal antibody 2A12 (1:10 dilution), which was raised against *Drosophila melanogaster* tracheal protein, later identified to be obstructor C protein (Samakovlis, 1996). The antibody was raised in mouse, and is commercially available from the Developmental Studies Hybridoma Bank, IA. TcCPAP3-C protein was detected in the assembly zone (lower part of procuticle) (green) in control group. However, it does not completely overlap (yellow in merge) with chitin (red) in both elytral and ventral body wall cuticle at the pharate adult stage. Down-regulation of TcCPAP3-C transcripts substantial reduction of CPAP3-C protein in dsRNA *TcCPAP3-C* treated samples.

A



B

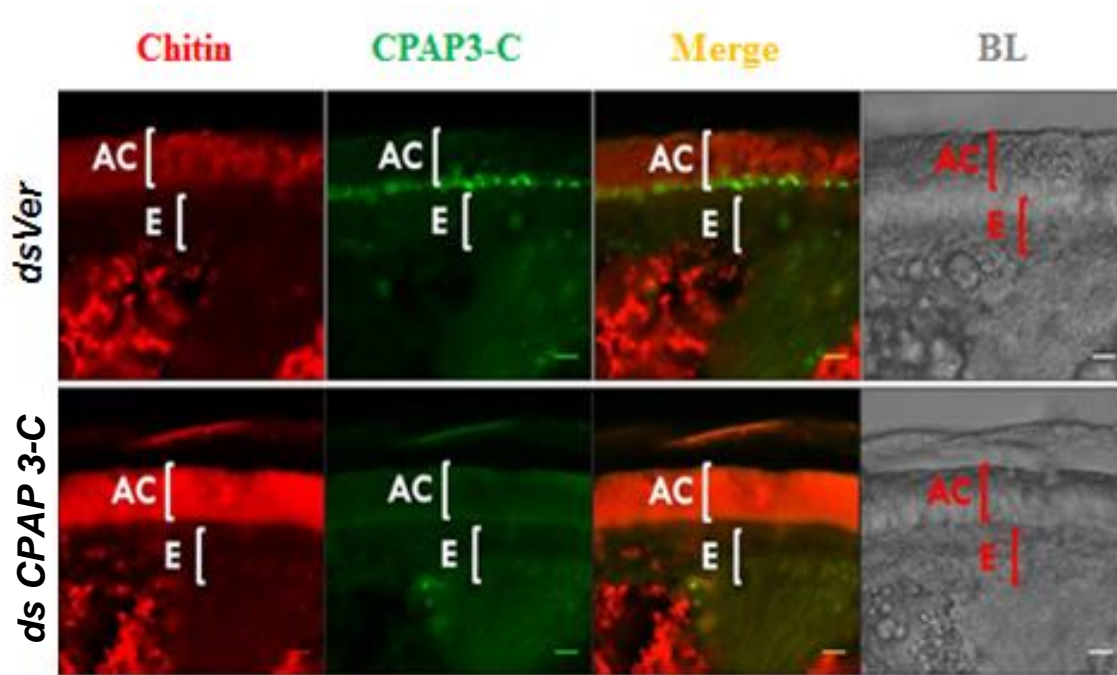


Figure 2.4 TcCPAP3-C protein is localized in the assembly zone

Immunohistochemistry followed by confocal analysis indicates that TcCPAP3-C is present in the assembly zone (lower part of procuticle) of both elytral (A) and ventral body wall cuticle (B), and overlaps with chitin only in the basal part of the procuticle. PC, pupal cuticle; AC, adult cuticle; E, epidermis. Immunostaining color coding: chitin (red), TcCPAP3-C protein (green), nuclei (blue); merge of chitin and protein staining (yellow). BL, bright field. Scale bar = 5 μ m.

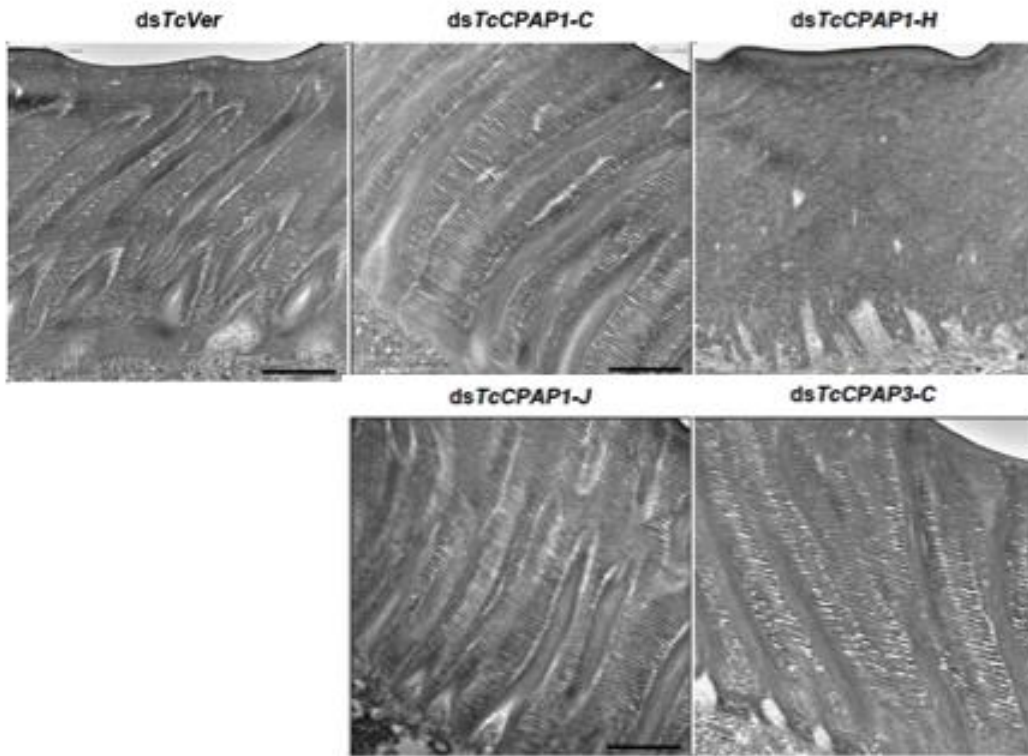
Transmission electron microscopic analysis on cuticle after RNAi for selected CPAP genes

Transmission electron microscopic analysis (TEM) of adult elytral cuticle revealed the role for CPAP1-H in organization of chitin laminae. Following RNAi of CPAP1-H a complete loss of normal laminar architecture in the procuticle is seen (Fig. 2.5 panels A). Further the plasma apical membrane protrusions into the procuticle, which seem to be the originating points of the pore

canals are quite irregular relative to *dsVer*-treated controls (Fig. 2, panel C). Clearly the epidermal cell- cuticle boundary is not clear and smooth as in the control. *CPAP3-C* knockdown also results an abnormal architecture of assembly zone, the region just above the epidermal cell apical plasma membrane where chitin is presumably secreted and arranged into laminae. While the parallel chitin laminae can be discerned, the pore canals seem to be surrounded by numerous fibrous structures, which do not form normal compact vertical pore canals. However, the arrangement of chitin laminae at other parts of the procuticle is apparently not affected following RNAi for CPAP3C. Further the epidermal cell procuticle boundary is not regular and flat. These results suggest CPAP1-H is essential for chitin laminar organization as new cuticle is synthesized, and CPAP3-C is important for organization of chitin fibers into the pore canals at the cell boundary.

In contrast to the results of RNAi for these two genes, down regulation of transcripts for CPAP1-C and CPAP1-J did not result in any obvious cuticular abnormalities in the elytra or body wall. (Fig. 2A)

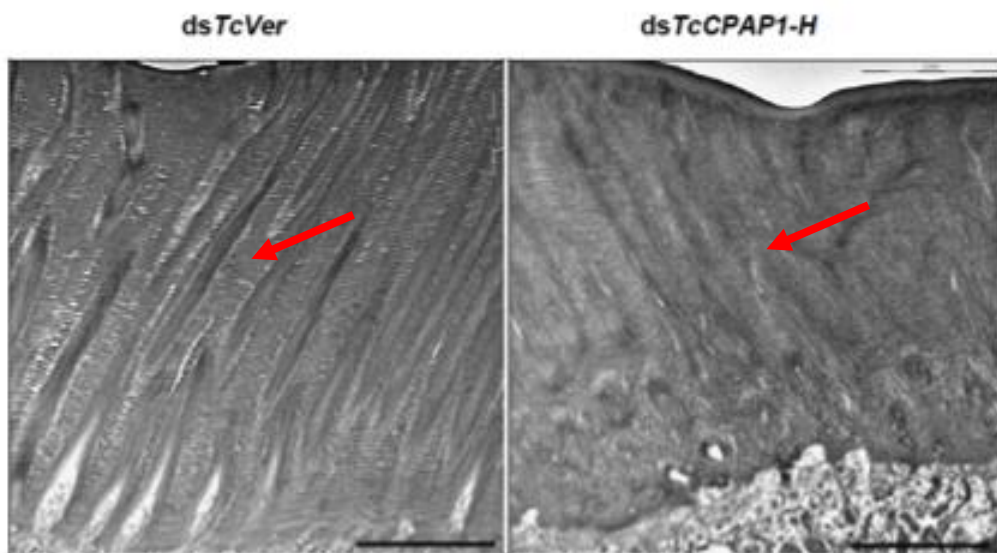
A



Scale bar=1 μ m

B

Elytral cuticle



Scale bar=2 μ m

C

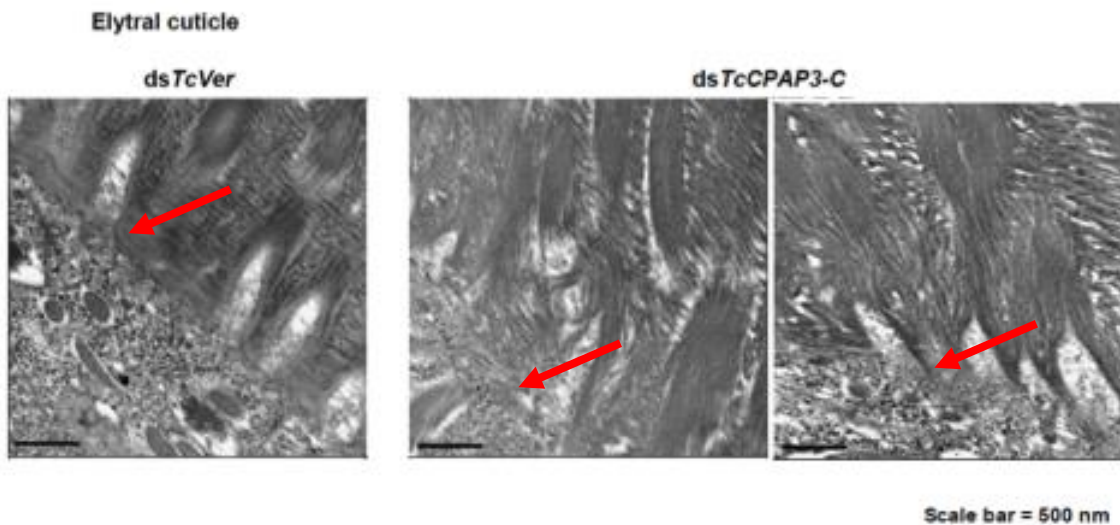


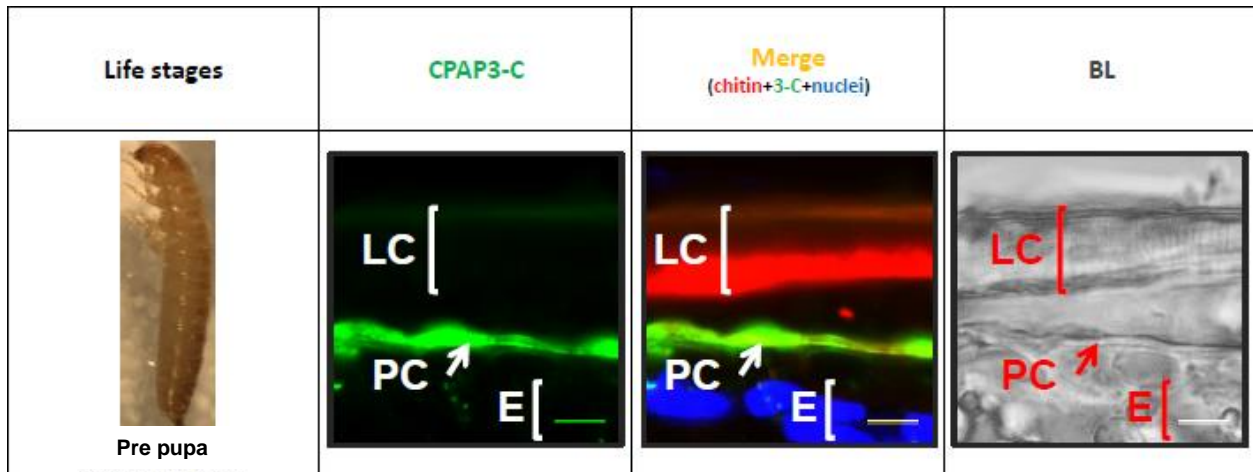
Figure 2.5 Transmission electron microscopic analysis of elytral cuticle of pharate adults.

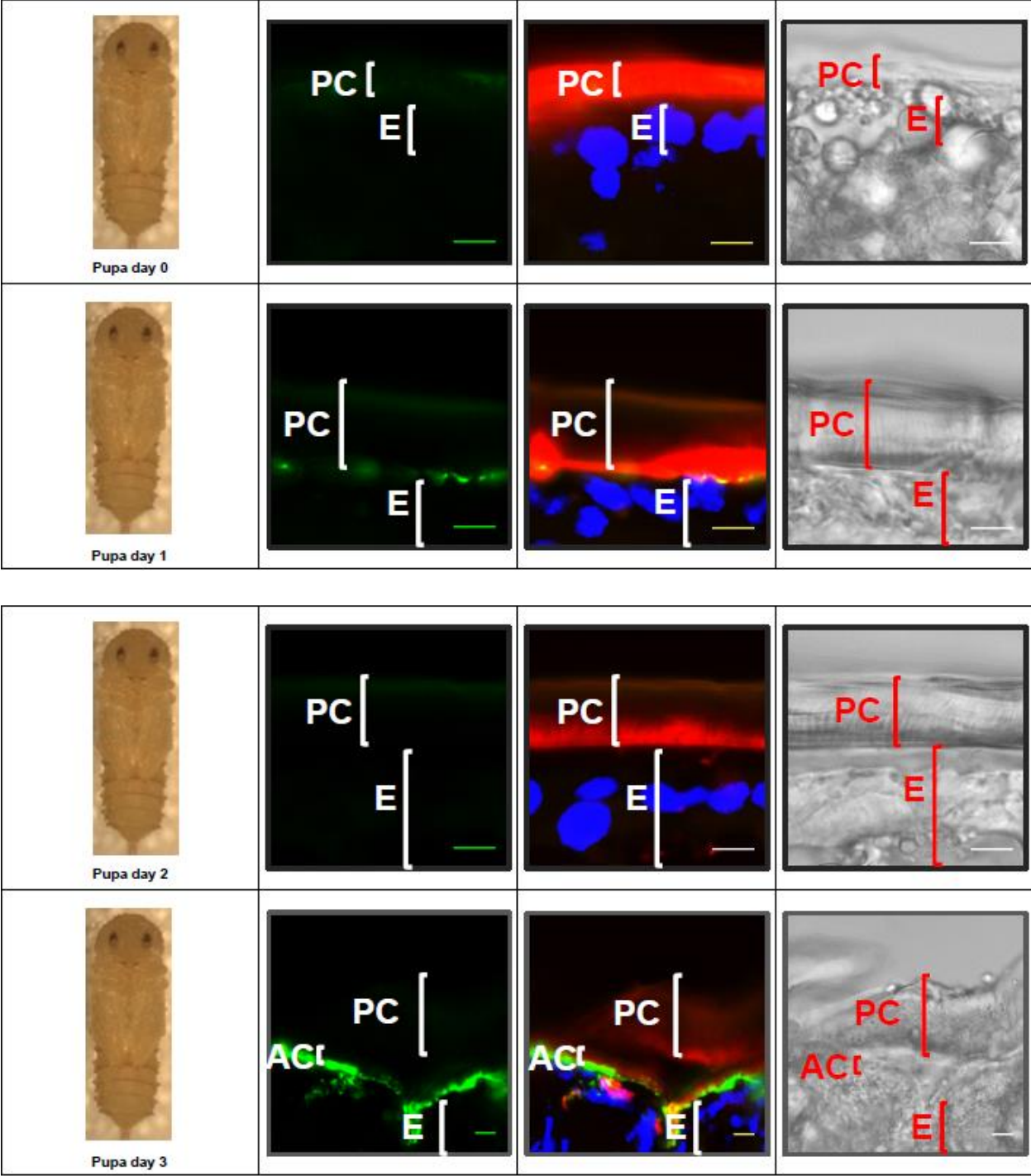
dsRNA *Vermilion* (*dsTcVer*)-treated insects were used as control group for all experiments. Cuticle defects after RNAi for each gene were observed, respectively (A). *CPAP1-H* knockdown showed loss of the horizontal chitin laminae (red arrow) (B). *CPAP3-C* knockdown caused an abnormal organization of the assembly zone (red stars) versus the control (yellow star). No visible defects were observed following RNAi for *CPAP1-C* or *CPAP1-J*.

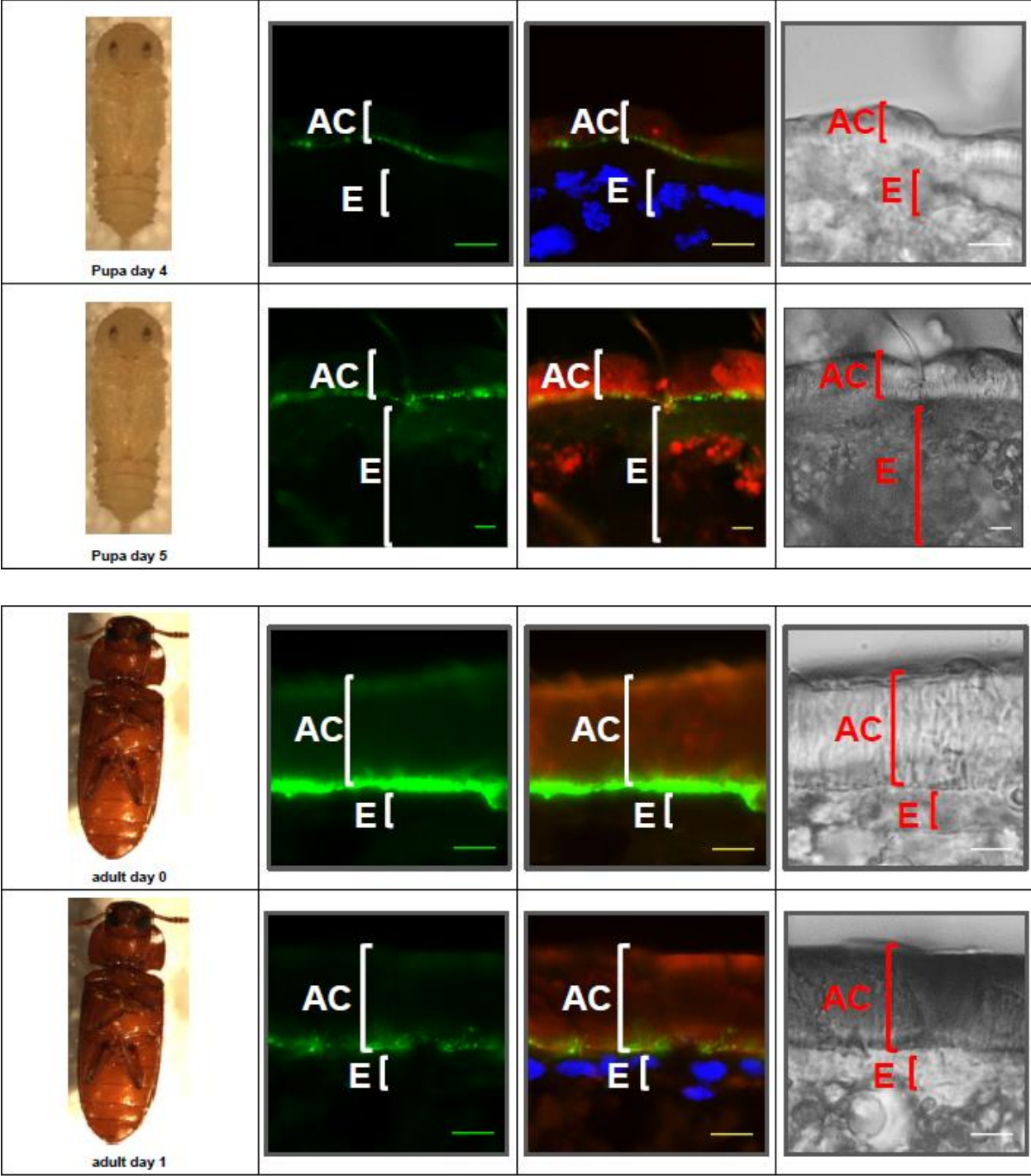
Dynamics of turnover of CPAP3-C during pupal-adult metamorphosis.

Twelve stages were considered for expression of CPAP proteins during pupa-adult development including prepupa through adult day 3 (n=5 for each stage). Immunohistochemistry followed by confocal analysis reveal that TcCPAP3-C protein is localized in the assembly zone (lower part of procuticle, next to the apical plasma membrane) of the newly formed cuticle, and is never detected in the apical parts of the procuticle at any stage of development analyzed here (Fig. 2.6; pupal d 1). This protein (indicated in green color) disappears from the old cuticle during molt

and appears early during cuticle deposition. For example, at the prepupal stage CPAP3-C is missing in the larval cuticle but is found in the newly forming pupal cuticle. CPAP3C-protein disappears from the pupal cuticle on pupal day 2. The timing of appearance of CPAP3-C, its location and the effects of RNAi of the transcripts of this gene which leads to loss of laminar architecture without any apparent changes in chitin content are consistent with the notion that TcCPAP3-C may be responsible for organization of chitin either directly or in association with other with proteins before procuticle deposition. Its absence in the old cuticle as molting occurs, combined with the molt arrest following RNAi of this gene at the pharate adult stage may suggest that turn-over of this protein is correlated with molting. Alternatively TcCPAP3-C may be reabsorbed and recycled for new cuticle synthesis at this stage.







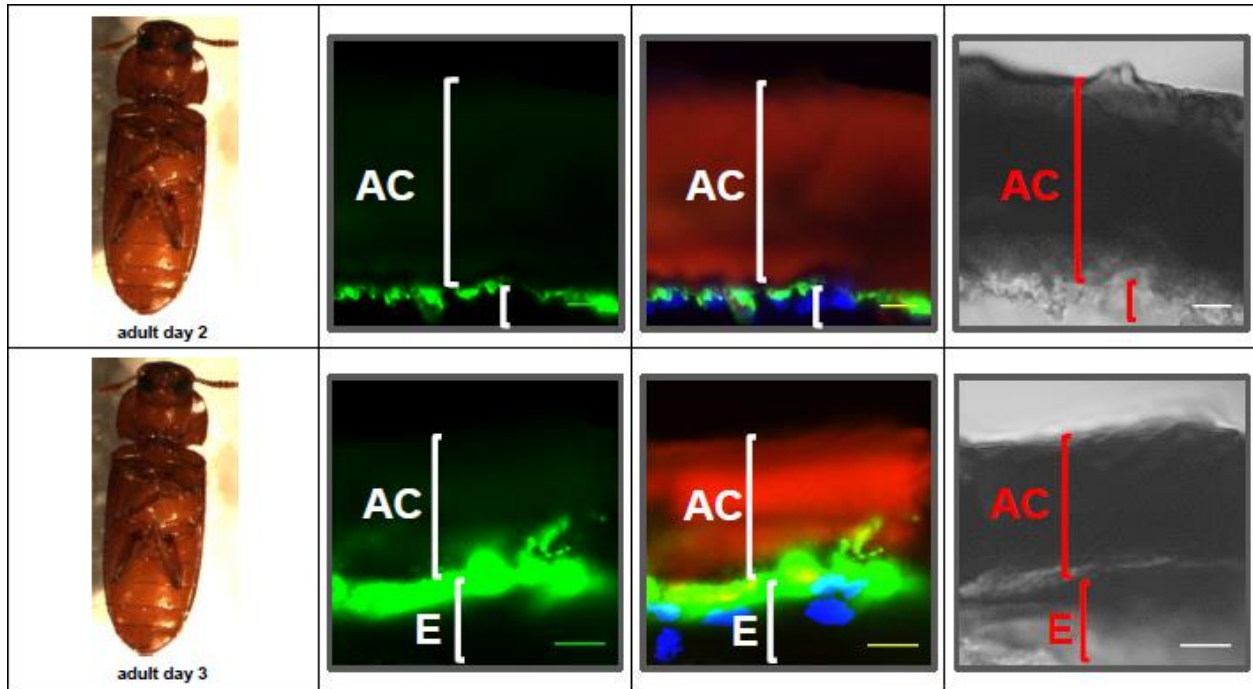


Figure 2.6 TcCPAP3-C protein expression time course determined by confocal analysis.

A composite of ventral body wall cuticle immunostained with CPAP3-C antibody shows that TcCPAP3-C is present in the lower part of the procuticle, the so-called assembly zone region, and it turns over on pupal day 2 when molting fluid is secreted to actively degrade the endocuticle. PC, pupal cuticle; AC, adult cuticle; E, epidermis. Immunostaining color coding: chitin (red), TcCPAP3-C protein (3-C, green), nuclei (blue) and co-localization of chitin and protein (yellow). BL, bright field. Scale bar = 5 μ m.

Discussion

Previous studies have shown that *T. castaneum* *CPAP* family genes have a very broad expression pattern over the entire period of insect growth and development (Jasrapuria et al., 2011, 2014). Transcripts for these genes are found in all cuticle-forming tissues (e.g. elytron, hindwing, and carcass) rather than in the midgut, which suggests that *CPAP* genes are involved in deposition and maintenance of insect cuticle. Many of them are essential for molting or cuticle integrity. For instance, down regulation of *CPAP3-B* transcripts causes a severe appendage problem that results in adult walking defects. Knocking down transcripts of *CPAP3-D1* and *CPAP3-D2* causes rough and shortened adult elytra. In addition, down-regulating *CPAPI-C*, *CPAPI-H*, *CPAPI-J* and *CPAP3-C* results in 100% lethality as the pupa attempts to molt to the adult stage. The insects showed evidence that ecdysis had started; however, there was failure to shed the old pupal cuticle and the insects were arrested at the pharate adult stage. Biochemical analysis for chitin content indicated that insects treated with dsRNA and collected as pharate adults (whole insects) did not have any change in chitin content except for those injected with dsRNA for *CPAPI-H*. Down-regulation of *CPAPI-H* transcripts reduced chitin content by 45%, suggesting that the protein product of this gene may promote chitin synthesis (or protect chitin) during development. When dsRNA *CPAPI-H* treated elytra were boiled at 95°C and stained with fluorescein isothiocyanate conjugated chitin-binding protein (FITC-CBD), the elytra were completely dissolved, whereas control elytra remained intact after identical treatment, suggesting that *CPAPI-H* gene encoded protein must function to maintain the integrity of the cuticle.

All *CPAP* gene family-encoded proteins are predicted to have cleavable signal peptides. They may be either transported into the procuticle or remain in the assembly zone as revealed by the

different locations of CPAP1-H and CPAP-3C. The CPAP proteins contain one or three copies of the peritrophin-A domain and are predicted to possess the ability to bind to chitin. The peritrophin-A domain has the consensus sequence, CX₁₃₋₂₀CX₅₋₆CX₉₋₁₉CX₁₀₋₁₄CX₄₋₁₄, containing six cysteines (C = cysteine; other amino acids denoted by the letter X) (Tellam et al., 1999). Jasarapura et al. (2010) aligned the highly conserved amino acid residues of peritrophin-A domains in CPAP1 and CPAP3 proteins by Weblogo analysis and found that in addition to the cysteines other residues flanking the cysteines are also conserved (Jasarapura Ph. D. Dissertation, 2011, Kansas State University). However, the precise functions of these proteins are not well known yet. In this study, I have focused on the functions of the *CPAP* genes encoding four proteins, CPAP1-C, CPAP-1 H, CPAP1-J and CPAP3-C because these are the only *CPAP* genes that appear to be essential for insect survival or molting.

***CPAP1-H* encoded protein may help to keep the structural integrity of the cuticle**

When transcripts for the *CPAP1-H* gene were down regulated, the insect was arrested as a pupa prior to adult molting. Besides the molting arrest, additional phenotypes have been also observed and may imply secondary functions of this gene product. First of all, dsRNA *CPAP1-H* treatment of insects resulted in abnormal compound eye development (Fig. 2.1C). In wild type *T. confusum*, each compound eye contains several ommatidia and is covered by a layer of cuticle. At an early stage, the ommatidium grows convex towards to the outer margin while the cuticular lenses are forming (Marshall, 1927). The cuticle provides strength to keep the eyes in proper shape. However, dsRNA *CPAP1-H* treated insects showed convex out growth in the eyes at the early pupal stage, no black eye pigment granules were observed, and light pink colored compound eyes formed at the pharate adult stage. Second, dsRNA treated newly emerged pupa

revealed an apparently longer body size when compared to control insects (Fig. 2.1B). Third, elytra were extremely fragile during dissection and they were tightly stuck to the forewings. Fourth, dehydration may have occurred in the dsRNA-treated insects as they exhibited a concaved abdomen. All of the above phenotypes imply that lack of CPAP1-H protein causes severe physical changes involving cuticle containing tissues. Reduction of cuticular chitin content may result in reduced mechanical strength of the cuticle, which prevents the insects from contracting their bodies or developing eyes in the proper shape. In addition, loss of chitin content may increase the cuticle permeability, thus causing dehydration. Immunohistochemistry and confocal analysis revealed that CPAP1-H protein is present throughout the procuticle and is co-localized with chitin. dsRNA *CPAP1-H* treated insects showed significant thickness reduction in body wall cuticle by approximately 50% (Fig 2-3), which explains why dsRNA *CPAP1-H* treated insects had a 45% loss of chitin content by chemical analysis (Jasrapuria et al., 2013). However, no evidence so far showed reduction in thickness of the elytral cuticle (Fig. 2.5 B). TEM analysis revealed down regulation of *CPAP1-H* transcripts caused a complete loss of horizontal laminae in the cuticle observed on pupa day 5 (Fig. 2.3B). This result suggests that the *CPAP1-H* gene may play an important role in organizing cuticle chitin into laminae during insect development. The fragility of the elytron of CPAP1-H dsRNA treated insects suggests that this protein may interact with chitin during cuticle deposition. It may be responsible for holding adjacent chitin microfibrils directly as the single cuticle layer is assembled. Alternatively, CPAP1-H protein may be present in between cuticle layers while binding to other protein(s) that wrap around the chitin bundles to keep the cuticle layers attached and organized properly.

***CPAP3-C* encoded protein may be responsible for cuticle turnover.**

CPAP3-C gene is expressed in different developmental stages and in various cuticle forming tissues. Immunohistochemistry and confocal analysis revealed that the *CPAP3-C* protein is localized in the assembly zone region and not the entire procuticle. Down-regulation of *CPAP3-C* mRNA causes insect death during pupa to adult molting. Biochemical and TEM analysis of these insects revealed that there was no obvious difference in chitin content, or thickness of the cuticle or the laminar architecture of the procuticle as compared to control insects versus dsRNA-treated insects. However there were subtle differences in the structure of the epidermal cell procuticle boundary, which lead to an abnormal looking assembly zone where the plasma membrane protrusions are irregular. The densely packed pore canal fibers seen in the *dsVer* control insects are missing, but they are replaced by less organized, loosely packed fibers. It suggests that the *CPAP3-C* protein may be responsible for packing chitin and some cuticular proteins into proper combinations inside the pore canals. Although cuticle is composed of multilayers, it can be as thick as 200 μm in elytra and as thin as 1 μm in the hind gut (Vincent et al., 2004). It is possible that some internal cuticle-lined tissues did not form properly after dsRNA treatment, such as the taenidia in the tracheal system. It has been shown that two isoforms of Knickkopf, named *KNK2* and *KNK3* are important for the normal structure of bodywall denticles and taenidia (Chaudhari et al., 2014). At each molt, the cuticular lining of the tracheae is shed and a new intima replaces it. RNA interference was carried out at the 6th instar larval stage, and the insects showed mortality at the pharate adult stage. This might be because the adult tracheal system did not form properly to either provide optimal oxygen exchange and/or to power muscle contraction during ecdysis, or both. The time course of *CPAP3-C* protein expression revealed that this protein is localized in the assembly zone of newly formed cuticle

and is never transported out to the upper regions of the procuticle. This protein turns over on pupal day 2 and reappears on pupa day 3 when adult cuticle starts to form. Therefore, it is concluded that CPAP3-C protein is responsible for new cuticle assembly; it stays in the assembly zone constantly to pack chitin with proteins, and later to transport chitin laminae out to the procuticle. While CPAP3-C protein has not been detected inside of the epithelial cells at any stage, it is possible that this protein is transported from Golgi to assembly zone very quickly. Another possibility is that the formation of antigenic epitopes occurs after the signal peptide is cleaved off and that the protein is later recognized by antibody in the assembly zone.

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Chapter 3 - Time course of expression and localization of Knickkopf protein

Abstract

Unlike vertebrates' internal skeleton, which grows along with their body size, insects have to form the new cuticle and discard the old cuticle through periodical molting process in order to develop. Paradoxically, the insect must degrade chitin in the old cuticle while making new cuticular chitin when chitinolytic enzymes are secreted into the molting fluid. Previous studies have suggested that chitin in the newly synthesized cuticle receives protection a cuticular protein, Knickkopf (Knk), which not only prevents molting fluid enzymes from attacking chitin, but also helps with chitin laminar organization. In this study, we analyzed cuticle at multiple developmental stages of the red flour beetle, *T. castaneum*, to determine the distribution of Knk protein in cuticle before, during and after the molting process as well as during the formation of the new cuticle. We report that the Knk protein is expressed in the cuticle secreting epidermal cells from the 6th larval instar to adult day 3. Knk is detected in the newly synthesized cuticle, co-localized with chitin in the procuticle near the epidermal cell surface, as well as inside the epithelial cells. Just prior to apolysis, KNK disappears from the old cuticle even as it accumulates in the newly forming cuticle. These results indicate that Knk turnover is correlated with the digestion of the new cuticle.

Introduction

Insects have to periodically undergo the process of molting that consists of casting off their old outer exoskeletons in order to allow the organism to develop. Molting includes digestion of the inner procuticle (endocuticle), sloughing off the outer procuticle (exocuticle) and waxy layer, recycling of the digested products (amino acids and sugar), and construction of the new cuticle (Chang, 1993). It takes place in response to hormonal changes, such as 20-hydroxyecdysone and juvenile hormone. As the molting process begins, the inner procuticle (endocuticle) detaches from the epidermis, exuvial space appears and is later filled with molting fluid (Jenkin and Hinton, 1966). The epidermal cells secrete the first layer of the new cuticle (the envelope or the outer epicuticle) to cover the epidermis. The newly synthesized cuticle continues to grow while the molting fluid degrades the old cuticle. Cuticle biogenesis is a process during which chitin chains form coaxial bundles, assemble into laminae and complex with proteins, especially chitin-binding proteins and cuticular proteins with the Rebers & Riddiford chitin-binding consensus sequence, which are predicted to have the ability to bind to chitin and become arranged in the form of a laminar cuticle, which can be arranged in pseudoorthogonal or helicoidal bundles.

Chaudhari et al. (2011) have provided experimental evidence that the protein Knickkopf (Knk) protects new cuticle from degradation by molting fluid while digestion of old cuticle occurs. In addition, Knk helps with chitin laminae organization in the procuticle. In this study, the localization and dynamics of turnover of the protein Knk in insect cuticle from the young larva to adult day 3 are presented. We demonstrate that Knk appears early during cuticle deposition and is co-localized with chitin in the newly forming pupal procuticle, but the protein turns over as the pupa metamorphoses into the adult. This protein is highly expressed in the newly synthesized

adult cuticle, which is consistent with the suggestion that Knk protects the new cuticle from degradation by chitinases even as molting is progressing

Materials and Methods

Insect culture Results

Red flour beetle, *T. castaneum* GA-1 strain was used for all experiments. Insects were reared in wheat flour containing 5% brewer's yeast at 30 °C under standard conditions.

Time course analysis of cuticle development

Twelve stages were considered for the cuticle development time-course study consisting of time points one day apart from each other, starting with young larva through adult day 3 (n=5). Larvae that had fairly long body size and were actively moving were chosen as 6th instar larvae. Larvae that had a curled body ("C" shape) with very little movement were collected as pre-pupae (before larval-pupal molt). Freshly emerged pupae were collected and labeled as pupal day 0 sample. For developmental stages beyond adult day 0, insects were collected at 24-h intervals based on its previous stage. For instance, adult day 1 insects were collected precisely 24 h after pupal-adult molt. All samples were dissected and fixed for immunohistochemistry and confocal analysis according to the standard protocol described before.

Immunohistochemistry and confocal analysis


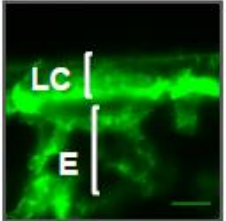
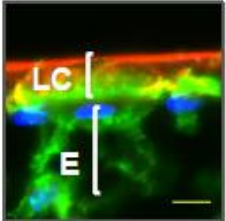
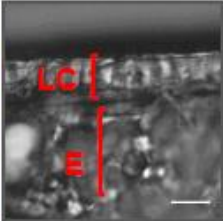

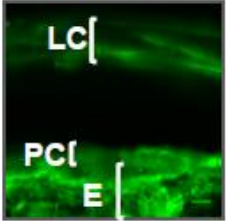
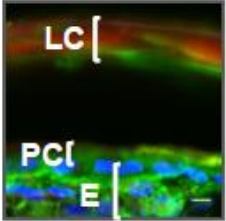
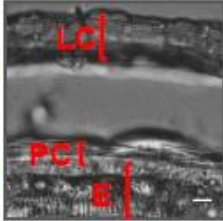

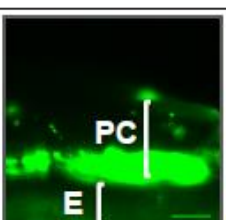
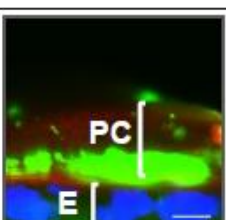
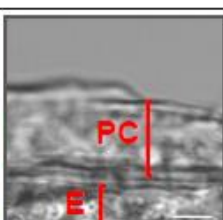

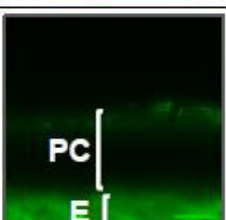
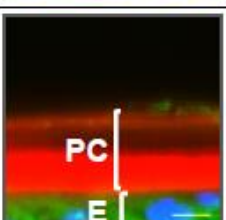
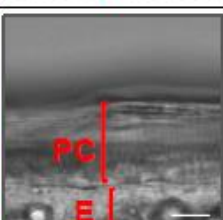
For each developmental stage, insects (n=5) were dissected to collect middle part of the body with elytron attached (abdominal segments), and fixed in 4% para-formaldehyde solution and then subjected to a series of sucrose gradients (12, 15, 18 and 20%). Cryosectioning was performed on Leica CM 1800 microtome. The sections were rehydrated with 0.2% PBST buffer (1X Phosphate Buffered Saline solution with Tween-20) followed by 1 h incubation in 0.2% BSA (in PBST) blocking buffer at room temperature. Primary antibodies of TcKnk (1:100, in rabbit) were applied to the cryosections (thickness = 20 μm) to detect the corresponding proteins. Alexa 488-conjugated goat anti-rabbit (1:1000) was applied as secondary antibodies for primary antibody detection. After incubation at room temperature for 3 h, Cy3 conjugated chitin-binding probe (1:100) was used for chitin staining at 4°C overnight. The sections were rinsed with 0.2% PBST next day followed by nuclei staining with 4, 6-diamidino-2-phenylindole-dihydrochloride (DAPI, 10 ng/ μl , 1:30) for 8 min. The sections were rinsed quickly, Fluoromount (Sigma) was added, and sealed properly. The sections were store at 4°C until confocal analysis. Both ventral body and elytral cuticles were the major foci for our experiments.

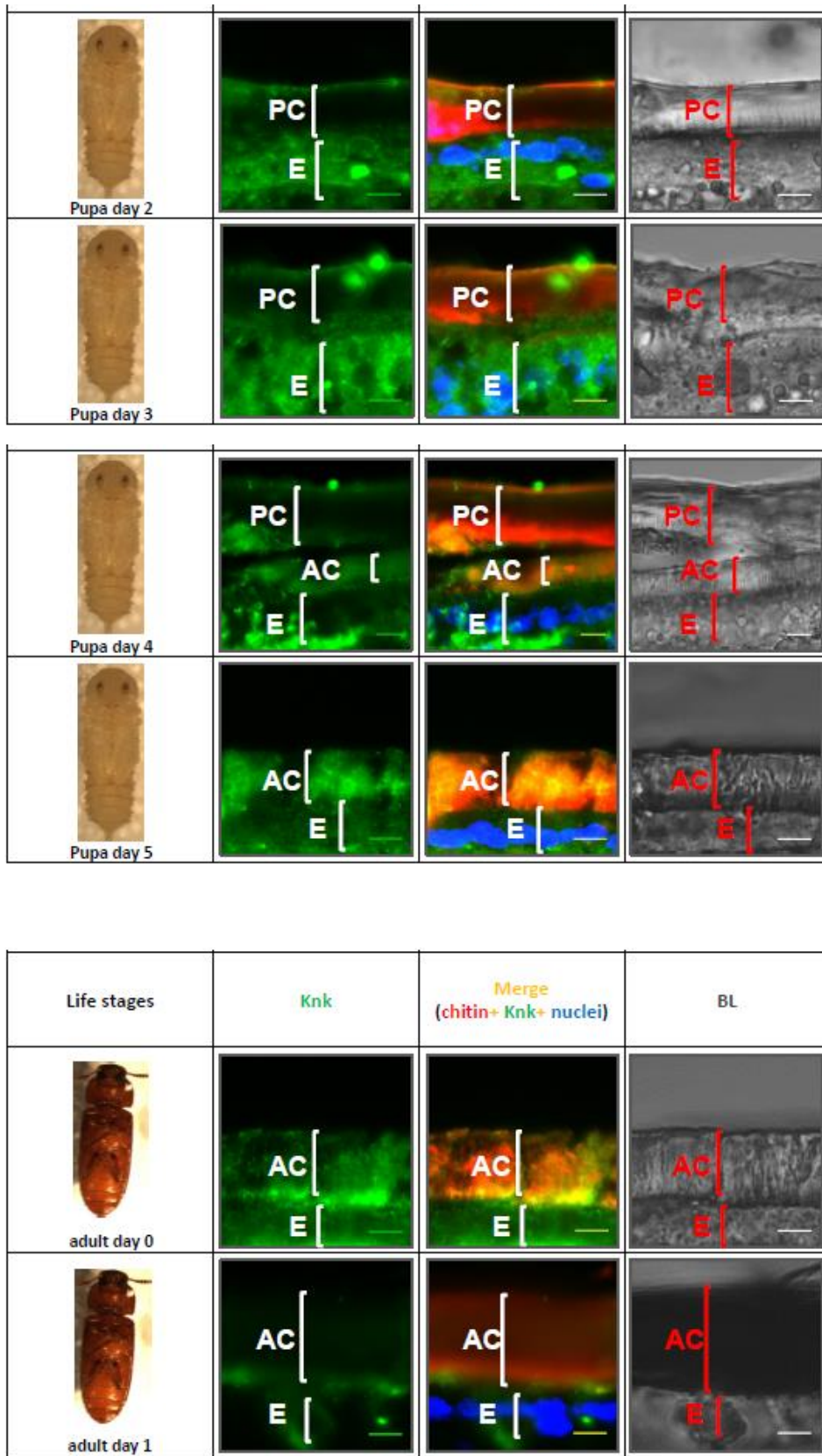
Zeiss Leica 510 confocal microscope was used for scanning images using an objective (oil, 40 X/1.3) with zoom of 8 X (body wall) and 4 X (elytron), and with lasers capable of detecting DAPI (405 nm), Alexa (488 nm) and Cy3-conjugated chitin-binding probe (543 nm). Finally, images were analyzed using ImageJ and LSM 510 software (NIH).

Results

Time course study on cuticle development determined by confocal analysis

Twelve stages were considered for cuticle development study, 6th instar larva (young larva) through adult day 3 (n=5). Immunocytochemistry followed by confocal analysis revealed that TcKnk protein is localized throughout the procuticle as well as inside epithelial cells. In young larvae, Knk can be observed at the basal region of the epithelial cells as well in the apical plasma membrane and in the lower parts of the procuticle just above the epidermal cell apical plasma membrane (Chaudhari et al., 2011). Significant staining of KNK could also be seen inside the cell including the basal part of the epidermal cell. This is probably because Knk is a GPI-anchored protein synthesized in the endoplasmic reticulum and is transported via a vesicular transport pathway to the apical plasma membrane, where it is cleaved by a phosphoinositide-specific phospholipase-C and incorporated into the procuticle (Chaudhari et al., 2011).

Life stages	Knk	Merge (chitin+ Knk+ nuclei)	BL
 <p data-bbox="414 659 514 680">Young larva</p>	 <p data-bbox="613 512 782 548">LC [</p> <p data-bbox="646 604 669 640">E [</p>	 <p data-bbox="847 512 1016 548">LC [</p> <p data-bbox="880 604 902 640">E [</p>	 <p data-bbox="1081 512 1120 548">LC [</p> <p data-bbox="1114 604 1136 640">E [</p>
 <p data-bbox="423 894 505 915">Pre pupa</p>	 <p data-bbox="630 705 685 741">LC [</p> <p data-bbox="613 831 669 867">PC [</p> <p data-bbox="662 873 685 909">E [</p>	 <p data-bbox="863 705 919 741">LC [</p> <p data-bbox="847 831 902 867">PC [</p> <p data-bbox="896 873 919 909">E [</p>	 <p data-bbox="1097 705 1136 741">LC [</p> <p data-bbox="1081 831 1120 867">PC [</p> <p data-bbox="1146 873 1169 909">E [</p>
 <p data-bbox="414 1142 514 1163">Pupa day 0</p>	 <p data-bbox="678 1045 717 1081">PC [</p> <p data-bbox="662 1115 685 1150">E [</p>	 <p data-bbox="896 1045 935 1081">PC [</p> <p data-bbox="880 1115 902 1150">E [</p>	 <p data-bbox="1146 1045 1185 1081">PC [</p> <p data-bbox="1162 1115 1185 1150">E [</p>
 <p data-bbox="414 1377 514 1398">Pupa day 1</p>	 <p data-bbox="630 1281 669 1316">PC [</p> <p data-bbox="662 1350 685 1386">E [</p>	 <p data-bbox="880 1281 919 1316">PC [</p> <p data-bbox="896 1350 919 1386">E [</p>	 <p data-bbox="1097 1281 1136 1316">PC [</p> <p data-bbox="1114 1350 1136 1386">E [</p>



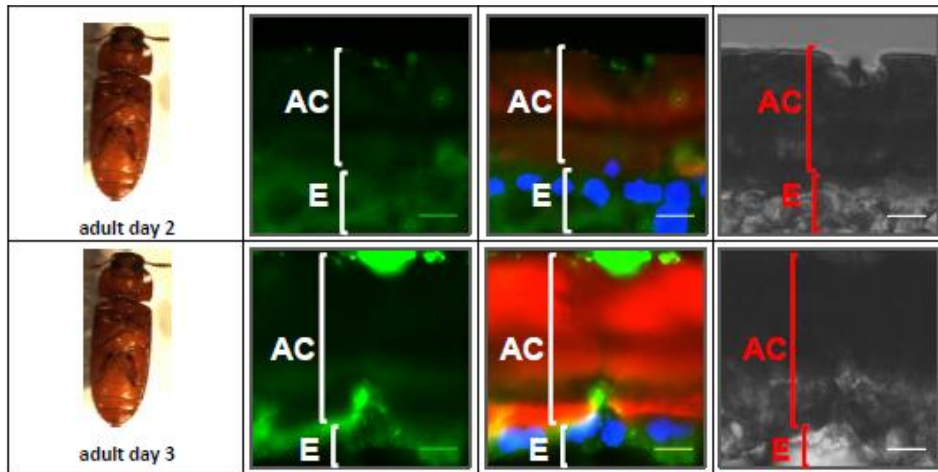


Figure 3.1 Time course analysis of TcKnk protein expression as determined by confocal analysis.

Immunohistochemistry followed by confocal analysis indicates that TcKnk protein is localized throughout the procuticle as well as inside the epithelial cells. PC, pupal cuticle; AC, adult cuticle; E, epidermis. Immunostaining color coding: chitin (red), TcKnk protein (green), nuclei (blue) and co-localization of chitin and protein (yellow). BL, bright field. Scale bar = 5 μm .

Discussions

The protein encoded by the gene *Knickkopf* (*Knk*) has been reported to have an unexpected mechanism of action during insect molting (Chaudhari et al., 2011) in the red flour beetle, *T. castaneum* and *D. melanogaster* (Moussian et al., 2006). This finding disproved the classic dogma, which postulated that the epicuticle protected the new cuticle from enzymatic degradation. *Knk* is responsible for protection of newly synthesized cuticle from chitinases and assists with laminar organization of chitin microfibrils. The gene *Knk* is expressed from the embryonic stage up to the pharate adult stage, and in various tissues including hindgut and carcass. Knock-down of the mRNA of *Knk* revealed lethality at all stages of development (larval-larval, larval-pupal, pupal-adult molts), strongly suggesting that *Knk* is needed for each molting process as well as new cuticle deposition. RT-PCR showed that *Knk* is not expressed in the adult stage (Chaudhari et al. 2011), suggesting that *Knk* protein may turn over once synthesis of new cuticle as well as molting are completed. Additionally, biochemical analysis showed that *Knk* dsRNA treatment affects the total chitin content.

Prior study of *Knk* was focused on the pharate adult stage (prior to adult molting) and on the adult cuticle. Whereas in this study, we studied the expression of *Knk* protein in multiple developmental stages and in different cuticles. The finding that while abundant amounts of *Knk* protein can be found in the pupal cuticle during early pupal stages, the levels of this protein are substantially diminished in later pupal periods. This is consistent with the suggestion that turnover of *Knk* is a prerequisite for degradation of chitin in the old cuticle (Chaudhari et al., 2011). Likewise, *Knk* is abundant in the larval cuticle during feeding stages, but close to the time of the larval-pupal molt, this protein is greatly diminished in the larval cuticle. Even after adult molt, the protein is undetectable in the adult cuticle. However, we are unable to conclude

whether this was due to turnover of the Knk protein or due to a technical failure to detect this protein as the cuticle becomes heavily tanned after day 1 of the adult stage. However, we can still find this protein inside the cell at least until day3 of the adult stage indicating continued expression of this gene and/or the persistence of the protein product inside the cell.

Immunohistochemistry followed by confocal analysis revealed that from the 6th instar larval stage to adult day 3, Knk protein is distributed throughout the procuticle and co-localized with chitin. It is detectable at the basal as well as the apical regions of the epithelial cells presumably due to its transport in intracellular vesicles. Knk, a GPI-anchored protein is presumed to be transported from the ER to the plasma membrane via the vesicular transport pathway to the final destination, namely the apical plasma membrane, which explains why a significant amount of Knk is present inside of the cell. GPI-anchored proteins are thought to be facing the outside (in this case the procuticle). Knk has been shown to be released to the medium from Hi-5 cells expressing a recombinant form of this protein by a phosphoinositide-specific phospholipase C (Chaudhari et al., 2011) Another protein, Retroactive (Rtv), is also needed for Knk transportation and for cuticle differentiation during new cuticle formation (Moussian et al., 2006; Chaudhari et al., 2014). There is a low level of Knk observed in the partially digested old pupa cuticle (exocuticle), which might be due to cross-linking of this protein in newly synthesized cuticle.

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Chapter 4 - Summary and Discussions

General Conclusions

Chitin, β -1, 4-linked N-acetylglucosamine, is a major component of the cell walls of fungi as well as the exoskeletons of most arthropods and nematodes (Merzendorfer, 2006). Chitin in the cuticle is secreted by a membrane bound protein chitin synthase-A. Adjacent chitin chains are linked via hydrogen bonds between adjacent antiparallel chains and tend to form chitin microfibrils. Many such microfibrils running parallel to each other form thin layers, called laminae which can then become stacked up on top of one another to form orthogonal or helicoidal bundles. In insects chitin has been found in the cuticle-forming tissues, such as elytra, body wall and trachea as well as in the peritrophic matrix (Kramer and Muthukrishnan, 2005). Chitin together with lipids, proteins and catecholamines undergo certain modifications to assemble into cuticle in order to function as the physical barrier. For instance, it protects insect from mechanical and biological damages and dehydration. The mechanism of proteins and chitin cross-linking, however, remains poorly understood.

In previous studies from our laboratory, cuticular proteins analogous to peritrophins (CPAP) and Knickkopf (Knk) were identified and characterized mainly at the pharate adult stage during development of *T. castaneum*. However, the dynamic assembly of both CPAP and Knk into the cuticle, their turnover and functional characterizations of CPAP are still not fully understood.

In this study we investigated how these cuticular proteins are assembled into the cuticle during different developmental stages and carry out their functions in *T. castaneum*. RNA interference (RNAi) resulted in down-regulation of transcripts for *CPAP 1-C*, *CPAP 1-H*, *CPAP 1-J*, *CPAP 3-C* or *Knk* genes and also to molting defects and mortality. Confocal and transmission electron

microscopic analysis revealed protein expression over twelve stages, spanning young larva through adult day 3 stages. CPAP3-C and Knickkopf showed different localizations in the insect cuticle, suggesting that these two proteins have different functions during molting and new cuticle synthesis. Both CPAP3-C and KNK turn over during insect growth. Turnover of CPAP3-C on pupal day 2 may be because of the degradative action by molting fluid proteases that appear in the apolytic space during this time. CPAP3-C stays in the lower part of the endocuticle in the so-called assembly zone and it does not distribute throughout the procuticle with chitin. We predict that CPAP3-C assists in the assembly of chitin bundles before their assembly into horizontal laminae. This protein appears in the newly forming procuticle and is substantially depleted just prior to the next molt. Down-regulation of CPAP 3-C transcripts resulted in a disorganized assembly zone. However, no loss of chitin content or the laminar architecture of the procuticle was observed above the assembly zone.

While Knk protein was present throughout the procuticle, some protein was inside the epithelial cells. Knk turns over in the old cuticle during the molting process when the old cuticle needs to be digested and no longer needs protection from chitinases. During formation of new cuticle, Knk accumulated initially at the apical region of the epithelial cells and in the plasma membrane. Later it is carried out to the procuticle to protect chitin from molting fluid associated chitinases.

Future Directions

There are several ways to continue with the study of CPAPs and Knk. CPAP proteins contain one or three repeats of the peritrophin-A domain, suggesting the ability for chitin binding. Thus the chitin-binding assay is needed for testing chitin-binding ability of these proteins. We are currently making progress on raising antibodies to CPAP1-C and CPAP1-J for immunolocalizing these two proteins in the cuticle. CPAP-3C is present only in the assembly zone region and predicted to function as the mediator of chitin-protein assembly. To determine its exact localization in the cuticle, we are currently localizing CPAP3-C protein with a secondary antibody conjugated with gold. The turnover of CPAP3-C on pupal day 2 suggests that this protein is digested prior to molting by some proteases, such as chymotrypsin-like proteins found in the molting fluid (references). It will be interesting to immunostain CPAP3-C in the assembly zone after eliminating these proteases. The reason why low levels of Knk protein persist in the old cuticle is unknown. It might be due to sclerorization that occurs in the exocuticle. Knockdown of some genes, which are responsible for cuticle sclerorization may be carried out to see if Knk can be completely eliminated from the old cuticle during molting.