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Tribolium castaneum RR-1 cuticular protein TcCPR4 is required for formation of pore canals in rigid cuticle

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Insect cuticle is composed mainly of structural proteins and the polysaccharide chitin. Despite a rather limited composition, insects produce diverse cuticles with the proper combination of mechanical properties (strength, hardness and flexibility). Adult beetles are covered mostly in a hard cuticle, but they can fly because the cuticle is lightweight. The rigid cuticle is comprised of three major functional layers, namely the outermost envelope, the protein-rich epicuticle and the innermost chitin-protein rich procuticle. In addition, there is a large number of vertically oriented columnar structures denoted as pore canals that contain chitin fibers (pore canal fibers, PCFs) that are absent in soft and flexible cuticles.

The CPR family is the largest family of cuticle proteins (CPs), which can be further divided into three subgroups based on the presence of one of the three presumptive chitin-binding sequence motifs denoted as Rebers-Riddiford (R&R) consensus sequence motifs RR-1, RR-2 and RR-3. The TcCPR27 protein containing the RR-2 motif is one of the most abundant CPs present both in the horizontal laminae and in vertical pore canals in the procuticle of rigid cuticle found in the elytron of the red flour beetle, *Tribolium castaneum*. Depletion of TcCPR27 by RNA interference (RNAi) causes both unorganized laminae and pore canals, resulting in malformation and weakening of the elytron. In this study, we investigated the function(s) of another CP, TcCPR4, which contains the RR-1 motif and is easily extractable from elytra after RNAi to deplete the level of TcCPR27. Transcript levels of the *TcCPR4* gene are dramatically increased in 3 d-old pupae when adult cuticle synthesis begins. Immunohistochemical studies revealed that TcCPR4 protein is present in the rigid cuticles of the dorsal elytron, ventral abdomen and leg but not in the flexible cuticles of the hindwing and dorsal abdomen of adult *T. castaneum*. Immunogold labeling and transmission electron microscopic analyses revealed that TcCPR4 is predominantly localized in pore canals and regions around the apical plasma membrane protrusions into the procuticle of rigid adult cuticles. RNAi for *TcCPR4* resulted in an abnormal shape of the pore canals with amorphous

PCFs in their lumen. These results support the hypothesis that TcCPR4 is required for achieving proper morphology of the vertical pore canals and PCFs that contribute to the assembly of a cuticle that is both lightweight and rigid.

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Key words: *Tribolium castaneum*, Cuticle protein, Cuticle/Exoskeleton, Pore canal fiber

Anticancer activities of synthetic analogs of coprisin, an antimicrobial peptide from the dung beetle *Copris tripartitus*

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An insect defensin, named coprisin, was identified from the dung beetle, *Copris tripartitus* immunized against *E. coli*. The peptide was composed of forty-three amino acid residues containing conserved six cysteines with three intramolecular disulphide bridges. Then, we generated coprisin analogs based on structural analysis and selected α-helical region of coprisin. Among the several synthetic analogs, dimeric form of CopA3 has been previously confirmed to have antimicrobial activity. In the present study, we have assessed the anticancer activity of CopA3 dimer peptide against human gastric cancer cell lines. As a result, we determined that the cell viability and cytotoxicity of gastric cancer cells. We also conducted an investigation into CopA3's mechanism against SNU668 cancer cell line. CopA3 was shown to induce necrotic and apoptotic cell death of gastric cancer cells by acridine orange/ethidium bromide staining and flow cytometry analysis. In addition, CopA3 bound to the surface of cancer cells via a specific interaction with phosphatidylserine, which is one of cancer cell membrane components. Intratumoral inoculation of D-CopA3 resulted in a significant decrease in the SNU668 gastric cancer tumor volume in a xenograft mice model. Moreover, histologic analysis revealed that D-CopA3 caused tumor suppressive effect in tumor tissues after peptide treatment compared with the untreated tumors. Collectively, the results suggest potential utility of CopA3 as a cancer therapeutic agent.

Key words: Antimicrobial peptide, Anticancer activity, Necrosis, Phosphatidylserine, CopA3

Chitinase 7 (TcCHT7) is required for chitin deposition and cuticle morphology of *Tribolium castaneum*

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Insect chitinases (CHTs), which belong to family 18 glycosylhydrolases (GH-18), have been detected in molting fluid and gut tissues and are predicted to mediate the digestion of chitin present in the cuticle/exoskeleton and peritrophic matrix (PM) in the gut. Based on amino acid sequence similarity and phylogenetic analysis, insect CHT family proteins have been classified into eight groups (group I to VIII).

CHT7s belong to Group III chitinase contain two catalytic domains and one chitin binding domain (CBD) at the C-terminus. The catalytic domain 1 of this group of chitinases exhibits greater sequence similarity to one another than to the catalytic domain 2 in the same protein(s), suggesting distinct functions and/or evolutionary origins for each of these two catalytic domains. This group of chitinases, unlike most insect CHTs, possesses a predicted transmembrane segment at the N-terminal region. The recombinant *Tribolium castaneum* CHT7 (TcCHT7) protein expressed in Hi-5 insect cells was bound to the cell membrane. Apparently, the catalytic domains of this CHT face the extracellular space as revealed by its ability to hydrolyze an artificial chitin substrate added to the medium.

DsRNA-based functional studies (RNAi) for several *CHT* genes in *T. castaneum* indicated that CHTs belong to groups I (TcCHT5) and II (TcCHT10) are critical for molting and turnover of chitin in the old cuticle during molting and/or metamorphosis. In other hand, RNAi for *TcCHT7* did not affect any types of molting such as larval-larval, larval-pupal and pupal-adult. The resulting pupae or adults, however, failed to wing-expansion and abdominal contraction. Immunohistochemical analysis revealed that TcCHT7 protein is localized in newly synthesized procuticle, suggesting that TcCHT7 could be released from the plasma membrane of epidermal cells by proteolysis. Cuticular chitin appears to accumulate inner region of the procuticle in TcCHT7-deficient animals. Transmission electron microscopy revealed that RNAi for *TcCHT7* resulted in disorganization of horizontal chitin laminar in both rigid cuticle (e.g. elytron) and soft cuticle (e.g. hindwing). In former cuticle, TcCHT7 is

also critical for formation of the vertical oriented pore canals. These results suggest that TcCHT7 have critical roles in the laminar assembly and synthesis and/or deposition of cuticular chitin.

This work was supported by NRF (NRF-2012R1A2A1A01006467).

Key words: *Tribolium castaneum*, Chitinase, Chitin, RNAi, Cuticle/Exoskeleton

Identification and functional characterization of autophagy-related genes in response to *Listeria monocytogenes* infection in the coleopteran model insect, *Tenebrio molitor*

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Autophagy is a lysosomal self-eating process against unused or damaged macromolecules, cellular components and whole organelles. Currently 36 autophagy-related gene (ATG) homologues have been characterized in yeast and higher eukaryotes including insects. Autophagy signals can be induced by extra- or intracellular stressors and signals such as starvation, growth factor deprivation, ER stress, and pathogen infection. In this study, we have screened and identified a comprehensive set of *Tenebrio molitor* autophagy-related genes using transcriptome sequencing and EST analysis that may operate at different levels in the autophagic process. To study autophagy in response to microbial infection, anti-TmAtg8 polyclonal antibody, the marker for autophagy signaling was generated. We investigated expression patterns of autophagy-related genes in different developmental stages and different tissues, and induction patterns of autophagy-related genes against pathogenic infection. Depletion of autophagy-related genes (*TmAtg3*, *TmAtg5* or *TmAtg8*) led to a significant reduction in survival ability of *T. molitor* larvae against an intracellular pathogen, *Listeria monocytogenes*. These data suggested that *Tenebrio* autophagy-related genes may play putative role in mediating autophagy-based clearance of *L. monocytogenes* in *T. molitor* model. We are currently working on potential function of *microtubule-associated protein 1a/1b light chain 3* (*TmLC3*) which is another member of ATG8 gene family.

Key words: RNA-seq, *Tenebrio molitor*, Autophagy-related genes, RNA interference, *Listeria monocytogenes*

PI10

Developing long-term preservation technique for *Tenebrio molitor* larvae

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The mealworm beetle, *Tenebrio molitor* (Coleoptera: Tenebrionidae), is one of the strong candidates for the high protein resource. The fecundity of this beetle is quite high, and the larval period is short (ca. 15~20 weeks). Moreover, this beetle is rapidly gaining weight during the larval period. Based on these advantages, the mealworm larvae have been highly focused as a protein supplement for animal feed and human diet. However, there is no criterion for preserving this beetle. In addition, the beetle colony is frequently collapsed during winter. Therefore, this beetle needs to be preserved in good condition for continuous production. In this study, we tested different conditions for preserving larvae. To test different preserving conditions, 12^{th} and 15^{th} larval instars were preserved at 5 and 10℃. Every 30 days after low temperature preservation, 300 larvae were placed at 25℃. After recovery from low temperature preservation, we checked the each larval period, pupation rate, pupa weight, adult emerging rate, and adult weight. The larvae were no significant different for developing after preserved 15^{th} larvae at 10℃. However, the larval period was reduced as the low temperature preservation period was extended with 15^{th} larvae at 10℃. The larvae died because of developmental arrest or damage from mite when the low temperature preservation period was more than 250 days. Therefore, we concluded that the maximum low temperature preservation period is 250 days for 15^{th} larvae. In addition, the 12^{th} larvae also can be preserved for 250 days at 10℃. At 5℃, 15^{th} larvae could be preserved only 30 days for normal development. However, 12^{th} larvae could not be preserved at 5℃. Based on these result, we suggest that 12^{th} and 15^{th} mealworm larvae can be preserved for 250 days at 10 ℃.

Key words: *Tenebrio molitor*, Larva, Low temperature preservation

PI11

Developmental characteristics of *Zophobas atratus* larvae (Coleoptera: Tenebrionidae) in different instars

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The giant mealworm beetle, *Zophobas atratus*, is a promising food resource for animals including humans. It was officially introduced to Korea in 2011, and has been commercially maintained. This beetle contains balanced nutriment with high protein, so it is a good food resource as animal feed. However, the characteristics of each life stage are not clearly known especially for the larval stage that can be used as commercial products. Therefore, we counted the number of *Z. atratus* instars, and described its characteristics at each larval stage. *Z. atratus* larvae required eight to nine days of incubation periods before hatching. The 1st instar period took three to four days. Except the 1st instar, there were relatively large variations for the each instar period. Before emerging adults, most of the individuals had 15 to 18 instars. The highest pupation rate, 25.71%, was observed in both 16th instars and the 17th instars. The body length was gradually increased with each successive instar and it was reached its maximum at the 18th instar. The color of larvae was white in the first instar, and gradually turned brown after the 2nd instar.

Key words: *Zophobas atratus*, Larva, Body length, Instar

PI16

Identification and characterization of *Pseudomonas poae* in *Aromia bungii*

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The presence of *Aromia bungii* (Coleoptera: Cerambycidae) was recorded for the first time in one location in Germany, and *A. bungii* is a fruit tree pest originating from Asia recently. On the other hand, this insect was investigated as a hobby and therapy animal because of the musk aroma from the *A. bungii*, therefore insect rearing farms of *A. bungii* have been increasing. We detected *Pseudomonas poae* from the diseased *A. bungii* using PCR and sequencing 16SrRNA of *P. poae* and identified 99% similarity from BLAST alignment. Pseudomonadaceae insect infections are generally known that invasive power if this bacteria pathogen is infected within hemocoel. Insect pathogens of members of the family Pseudomonadaceae are investigated *P. aeruginosa, P. aureofaciens, P. chlororaphis, P. fliorescens, P. noctuarum, P. putida, P. savastanoi, and P. septica.*

Key words: *Aromia bungii* (Coleoptera: Cerambycidae), *Pseudomonas poae*, 16SrRNA, PCR

PI23

Pre-treatment conditions on the powder of *Tenebrio molitor* for using as a novel food ingredient

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Although the mealworm larva (*Tenebrio molitor*) is high protein source, aversion feature of the larva made it difficult for consuming as a food. In this study, we established optimal powder manufacturing process for *T. molitor* larva for using as a novel food. For this purpose, it should be feed with the bran sterilized before freeze-drying. The sterilized *T. molitor* was lyophilized and grinded by a blender. A safety of the powder as a food was validated by evaluation of Raw 264.7 macrophage cytotoxicity using MTS assay. As above results, we propose that optimal powder manufacturing process established in this study can be used in industrial production of *T. molitor* as a novel food.

Key Words: *Tenebrio molitor*, Food, Sterilization, Cytotoxicity

PI24

Molecular phylogenetic diversity of *Monochamus saltuarius* (Gebler) based on mitochondrial COI sequence analysis

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Monochamus saltuarius (Gebler), like *Monochamus alternatus*, is a vectors of *Bursaphelenchus xylophilus* Niclke which causes pine wilt disease and lead to huge damage to pine needle trees. *M. saltuarius* is reported that it has a morphological polymorphism, but there is no standard phenotype to distinguish the differences within species. To investigate a molecular phylogenetic analysis, we collect samples of *M. saltuarius* in Chungbuk, Gyeonggi and Gangwon region of Korean Peninsula. Analysis of mitochondrial COI 5' region showed that two distinctive phylogenetic groups with a geographical separation. The first group includes specimens collected in Chungbuk, Kyungwon and Kangwon region while the specimens collected only in Gangwon region makes another group. Two group shows 2.4~2,6 K2P distances but there is no significant difference in morphological character. But a distinctive difference was found in genital organ that the specimens collected only in Ganwon region (the second group) showed a little shorter and wider paramere. More in depth studies will be necessary to clearly distinguish these two groups with more intensive collection of specimens in wider region and try to find out a phenotypic variations.

Key words: *Monochamus saltuarius* (Gebler), Pine wilt disease, Mitochondria, COI, DNA barcode

PI54

Molecular cloning and effects of TmCactin gene silencing on *Tenebrio* larval mortality

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Innate immune system is very important to protect host itself from pathogenic microorganism infection in insect. Cactin, cactus-interacting protein was for the first time identified in Drosophila and was discovered to be involved in dorsal-ventral patterning and intracellular toll signaling cascade. In the present study, we have identified and functionally characterized *Tenebrio Cactin* (*TmCactin*) in the beetle, *Tenebrio molitor* by RNASeq/EST. Analysis of RNA interference indicates that TmCactin plays an important role in Gram-negative and -positive bacteria infection, not fungal infection in *T. molitor* larvae.

Key words: Toll signaling, Cactin, *Tenebrio molitor*, Microbial infection, RNA interference, AMP

PI55

Identification and characterization of TmToll-like receptor 7 (TmTLR7) from the mealworm, *Tenebrio molitor*

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Toll signaling cascade has been well studied especially in *Drosophila* and beetle model. Recently, the role of extracellular spätzle-Toll signaling cascade was identified using *Tenebrio molitor* model. However, there is no information for *Tenebrio* toll-like receptor (TLR) genes. Here, we have screened and so far identified seven TLR genes from RNAseq and expressed sequence taq (EST) generated from *T. molitor*. *Tenebrio Toll-like receptor 7* (*TmTLR7*) gene was cloned and partially characterized. The results show that TmTLR7 contains 3,939 bp of ORF encoding 1,311 amino acid residues, 847 bp of 5'-UTR and 231 bp of 3'-UTR except poly-A tail. Domain analysis shows that the TmTLR7 includes one signal peptide region, seven leucine rich repeat region, one transmembrane domain and one TIR domain. Developmental expression patterns shows that *TmTLR7* mRNA was highly expressed on late instar larvae. Tissue specific expression patterns indicates that *TmTLR7* transcripts were highly expressed in Malpighian tubules at late instar larvae and integument at 5-day old adult. *TmTLR7* was strongly induced at 6 hrs post infection of *Escherichia coli* and *Staphylococcus aureus*, and at 9hrs post infection of *Candida albicans* and *Listeria monocytogenes*.

Key words: Toll-like receptor, TLR7, *Tenebrio molitor*, Microbial infection, Induction patterns

PI56

Functional characterization of Tm14-3-3ζ on autophagy signaling in *Tenebrio molitor*

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14-3-3 is a family whose members are highly conserved eukaryotic proteins that play pivotal roles in the regulation of cell survival, apoptosis, and signal transduction. In this study, two isoforms of the *Tenebrio* 14-3-3 proteins, Tm14-3-3ε and Tm14-3-3ζ, were identified and their functions in countering pathogenic infections were investigated. A peptide-based polyclonal antibody was generated for determination of subcellular localization of Tm14-3-3ζ. Tm14-3-3ζ is localized in the membranes of midgut epithelial cells, nuclei of the fat body and cytosol of hemocytes but little or no in Malpighian tubules. A confocal microscopic analysis, furthermore, revealed that Tm14-3-3ζ protein and the signals for LysoTracker as an autolysosome signal were not merged. During a critical window of larval to pupal transition, expression levels of *Tm14-3-3ζ* were inversely correlated to the acidification levels of lysosomes. Injection of C-2 Ceramide revealed a time-dependent increase in the transcripts of *TmATG8* whereas it decreases in the expression level of *Tm14-3-3ζ* transcripts in the first hour. Depletion of Tm14-3-3ζ triggers the conversion of TmAtg8-I to TmAtg8-II (active form) as determined by Western blot analysis with TmAtg8 polyclonal antibody. Our results suggest that Tm14-3-3ζ protein has negative regulatory roles in autophagy.

Key words: *Tenebrio molitor*, 14-3-3ζ, Autophagy, Atg8