Laboratory of
Forest Protection

Identification and function of molting fluid chitinases in the Japanese pine sawyer beetle, *Monochamus alternatus*

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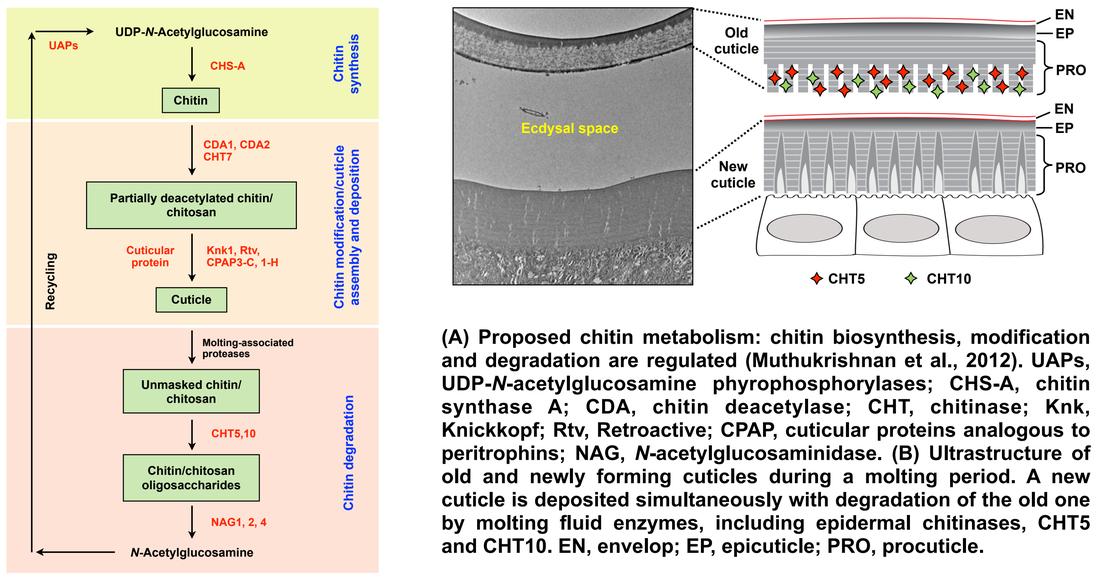
Introduction

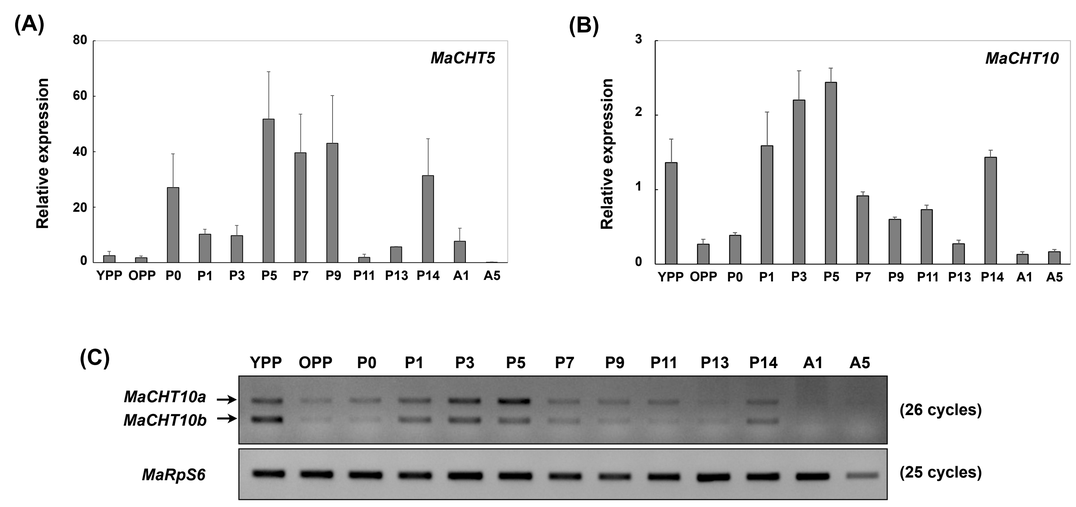
Insect cuticle is an extracellular matrix formed primarily from two different biopolymers, chitin and protein. During each molt cycle, a new cuticle is deposited simultaneously with degradation of the old one by molting fluid cuticle degrading-enzymes, including epidermal group I and II chitinases (CHTs). Insect chitinase belongs to family 18 glycosylhydrolase (GH-18) and have been classified into at least eleven groups based on phylogenetic analysis. In this study, we report a physiological functions of the molting fluid chitinases, MaCHT5 (group I) and MaCHT10 (group II), including two alternatively spliced isoforms of the later, MaCHT10a and MaCHT10b, from the Japanese pine sawyer beetle, *Monochamus alternatus*. RNA interference (RNAi) studies reveal that MaCHT5 is required for both larval-pupal and pupal-adult molts, while depletion of MaCHT10a has little or no effect on those molts. RNAi for both *MaCHT10a* and *MaCHT10b*, however, causes failure of pupation and adult eclosion. All of these results suggest functional specialization of insect molting fluid chitinase genes.

Chitin metabolic pathway in insect cuticle formation and turnover



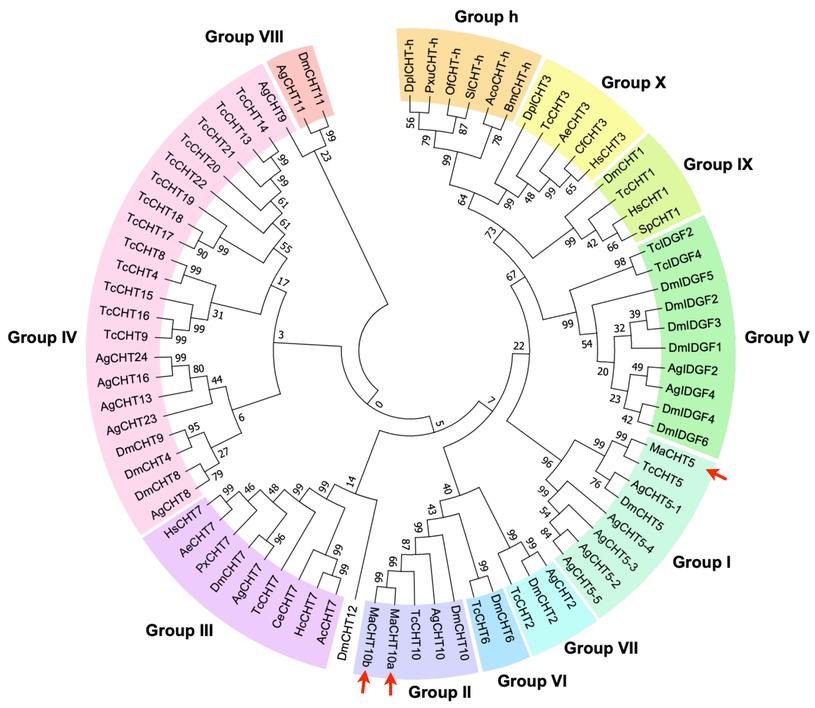
(A) Proposed chitin metabolism: chitin biosynthesis, modification and degradation are regulated (Muthukrishnan et al., 2012). UAPs, UDP-*N*-acetylglucosamine phyrophosphorylases; CHS-A, chitin synthase A; CDA, chitin deacetylase; CHT, chitinase; Knk, Knickkopf; Rtv, Retroactive; CPAP, cuticular proteins analogous to peritrophins; NAG, *N*-acetylglucosaminidase. (B) Ultrastructure of old and newly forming cuticles during a molting period. A new cuticle is deposited simultaneously with degradation of the old one by molting fluid enzymes, including epidermal chitinases, CHT5 and CHT10. EN, envelop; EP, epicuticle; PRO, procuticle.

Expression profiles of *MaCHT5* and *MaCHT10* during late stages of developmental



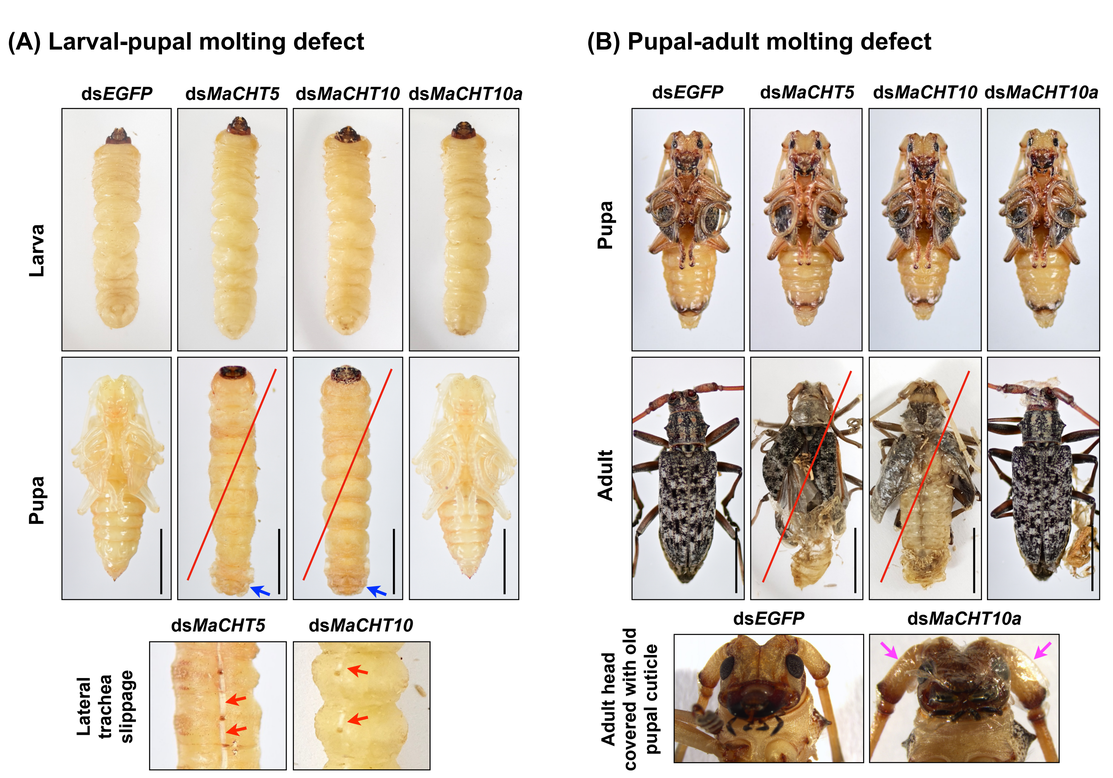
Expression patterns of *MaCHT5* (A) and *MaCHT10* (B) during the late developmental stages ranging from young pharate pupal to mature adult stages were analyzed by real-time PCR. Transcript levels of *M. alternatus* ribosomal protein S6 (*MaRpS6*) were measured to normalize for differences in the concentration of cDNA templates among samples. The expression levels for *MaCHT5* or *MaCHT10* are presented relative to the levels of expression in young pharate pupae (YPP). (C) To analyze expression profiles of two alternatively spliced isoforms of *MaCHT10*, *MaCHT10a* and *MaCHT10b,* we further performed semi-quantitative one-pot two RT-PCR. A primer set that targets *MaCHT10a* and *MaCHT10b* amplified 609 and 401 bp DNA fragments, respectively. Transcripts for *MaRpS6* was used as an internal loading control. YPP, young pharate pupa; OPP, old pharate pupa; P0, day 0 pupa; P1, day 1 pupa; P3, day 3 pupa; P5, day 5 pupa; P7, day 7 pupa; P9, day 9 pupa; P11, day 11 pupa; P13, day 13 pupa; P14, day 14 pupa; A1, day 1 adult; A5, day 5 adult.

Phylogenetic analysis of insect chitinases and chitinase-like proteins



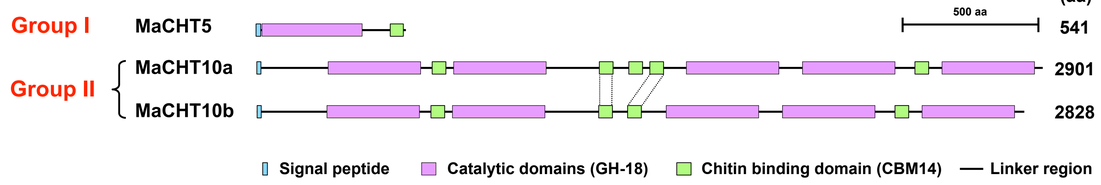
ClustalW software was used to perform multiple sequence alignments prior to phylogenetic analysis. The phylogenetic tree was constructed by MEGA 7.0 software using Neighbor-joining method (Kumar, 2016). CHTs and Chitinase-like proteins are grouped into 11 different groups (Tetreau et al., 2015). The molting fluid chitinases, MaCHT5 (group I) and MaCHT10 (group II) from *M. alternatus,* including two alternatively spliced isoforms of the later, MaCHT10a and MaCHT10b, are indicated by red arrows. Ac, *Ancylostoma ceylanicum*; Aco, *Agrius convolvuli*; Ae, *Acromyrmex echinatior*; Ag, *Anopheles gambiae*; *Agl, Anoplophora glabripennis;* Bm, *Bombyx mori;* Ce, *Caenorhabditis elegans*; Cf, *Camponotus floridanus*; Dm, *Drosophila melanogaster*; *Dpl, Danaus plexippus*; Hc, *Haemonchus contortus;* Hs, *Harpegnathos saltator*; Of, *Ostrinia furnacalis*; Px, *Plutella xylostella*; Pxu, *Papilio xuthus*; Sl, *Spodoptera litura*; Sp, *Strongylocentrotus purpuratus*; Tc, *Tribolium castaneum*.

Loss of function phenotypes produced by RNAi for *MaCHT5* and *MaCHT10*



(A) Injection of dsRNA (2 μg per insect) for neither *MaCHT5* (ds*MaCHT5*) or *MaCHT10* (ds*MaCHT10*; knockdown both *MaCHT10a* and *MaCHT10b* transcripts) into last instar larvae had no effect on larval growth and development (top panels). However, the subsequent larval-pupal molt was adversely affected. Although the resulting pharate pupae exhibited apolysis and slippage of the larval cuticle at the posterior extremity (blue arrows) and lateral trachea (red arrows), they failed completion of molting and died entrapped in their larval exuviae (red lines). (B) Injection of ds*MaCHT5* or ds*MaCHT10* into day 0-1 pupae had no effect on pupal development (top panels). However, the resulting pharate adults failed adult eclosion and died entrapped in their pupal exuviae (red lines). Interestingly, unlike knockdown both *MaCHT10a* and *MaCHT10b* described above, injection of dsRNA specific for *MaCHT10a* (ds*MaCHT10a*) into last instar larvae had no effect on pupation (right panels in A) and adult eclusion (right panels in B). The resulting adults, however, were unable to extricate several their appendages such as antennas (magenta arrows) and leg tarsi. dsRNA for *EGFP* (ds*EGFP*) was injected as a negative control. Scale bar = 1 cm.

Domain organization of MaCHT5 and MaCHT10 proteins



SMART program was used to predict the domain architecture of MaCHT5, MaCHT10a and MaCHT10b proteins. MaCHT5 contains a predicted signal peptide, a catalytic domain (GH18 domain) and a C-terminal chitin binding CBM14 domain (CBD), while MaCHT10a have a signal peptide, five catalytic domains and four or five CBDs (the 3rd CBD in MaCHT10a is alternatively spliced out in MaCHT10b).

Conclusion

- Identified and cloned cDNAs encoding the molting fluid chitinases from *M. alternatus,* MaCHT5 (group I) and two alternatively spliced isoforms of MaCHT10, MaCHT10a and MaCHT10b (group II).
- The high *MaCHT5* transcripts were detected in day 5 and 7 pupa, while both *MaCHT10a* and *MaCHT10b* were highly expressed in young pharate pupae and young pupae with some validation.
- RNAi for either *MaCHT5* or *MaCHT10* causes larval-pupal and pupal-adult molting defects.
- Unlike RNAi for *MaCHT10*, depletion of *MaCHT10a* transcripts had little or no effect on larval-pupal and pupal-adult molts, suggesting that two MaCHT10a and MaCHT10b isoforms compensate for each other in its loss of function situation or the later functions primarily in the pupation and adult eclosion.
- All of these results indicate that MaCHT5 and MaCHT10 have critical roles in degradation and turnover of chitinous cuticle during molting, which is critical for continuous growth, development and survival in *M. alternatus*, and probably in other insect species.

Reference

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Acknowledgement

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