Laboratory of
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Tyrosine hydroxylase and DOPA decarboxylase are required for adult cuticle morphology and pigmentation of *Monochamus alternatus*

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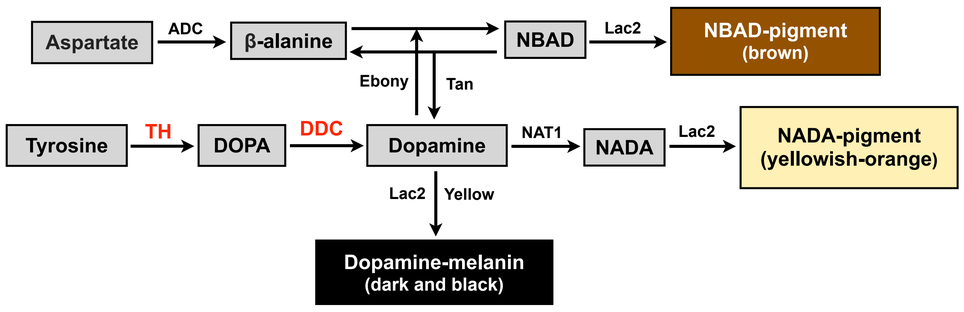
Introduction

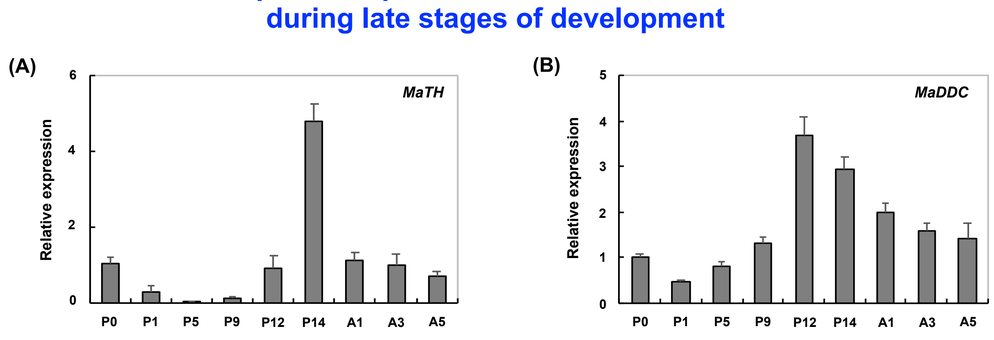
In insects, tanning is a complex and vital physiological process in cuticle coloration (pigmentation) and hardening (sclerotization). With tyrosine as the initial substrate, the early steps in the tanning pathway are the hydroxylation of tyrosine to produced 3,4-dihydroxyphenylalanine (DOPA) by tyrosine hydroxylase (TH), followed by the decarboxylation of DOPA to dopamine by DOPA decarboxylase (DDC). In this study, we report the physiological functions of MaTH and MaDDC from the Japanese pine sawyer beetle, *Monochamus alternatus*, which is a major vector of the pinewood nematode, *Bursaphelnchus xylophilus* that causes Pine wilt disease. We identify, clone and characterize the *MaTH* and *MaDDC* cDNAs. Loss of function(s) of *MaTH* and *MaDDC* by RNA interference (RNAi) causes abnormally pale/white and light yellow-brown cuticle of *M. alternatus* adults, respectively. These results indicate that both MaTH and MaDDC play critical roles in normal cuticle coloration of *M. alternatus* adult.

Proposed tyrosine-induced cuticle pigmentation metabolic pathway



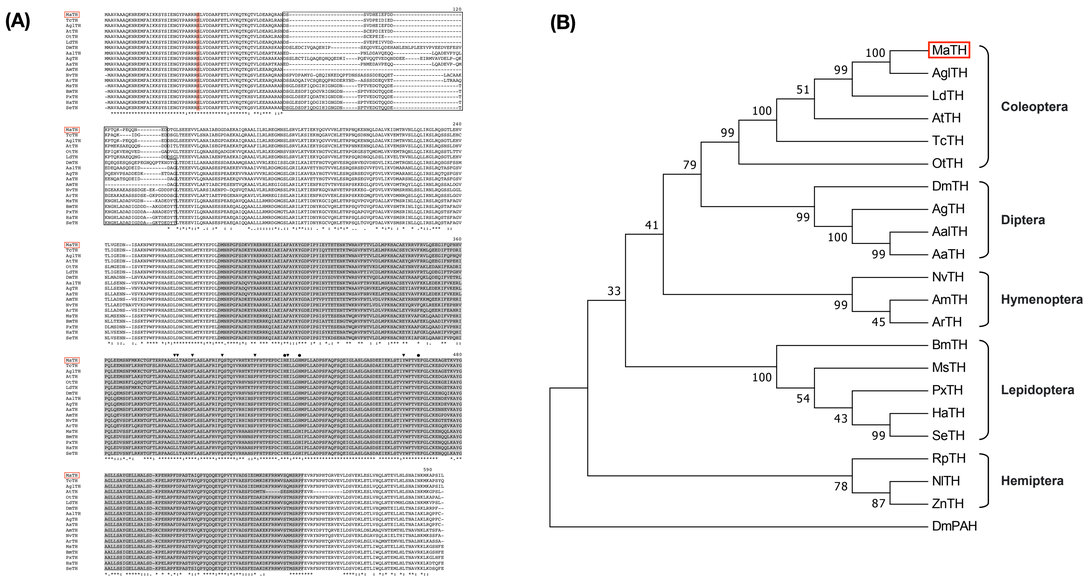
TH and DDC related with insect cuticle pigmentation are highlighted in red. DOPA, 3,4-dihydroxyphenylalanine; Dopamine, 3,4-dihydroxyphenethylamine; NADA, *N*-acetyldopanime; NBAD, *N*-β-alanyldopamine; TH, tyrosine hydroxylase; DDC, dopa decarboxylase; Yellow, dopachrome conversion enzyme (DCE); NAT1, *N*-acetyltransferase 1; Lac2, laccase 2; ADC, aspartate 1-decarboxylase; Ebony, NBAD synthase; Tan, NBAD hydrolase (Arakane et al., 2016; Mun et al., 2020).

Expression profiles of *MaTH* and *MaDDC* during late stages of development



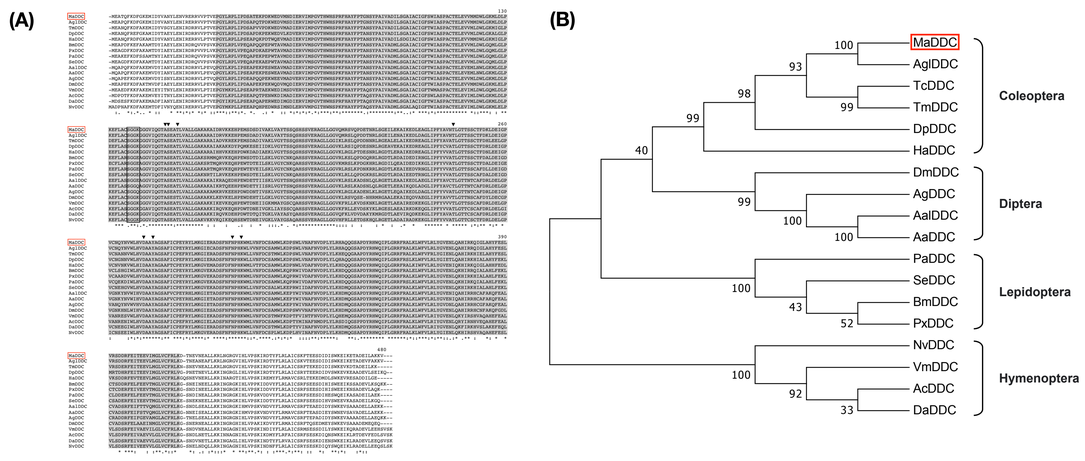
Expression profiles of *MaTH* (A) and *MaDDC* (B) during late developmental stages. Transcript levels of the genes relative to that of *M. alternatus* ribosomal protein S6 (*MaRpS6*) were determined by real-time PCR. P0, day 0 pupae; P1, day 1 pupae; P5, day 5 pupae; P9, day 9 pupae; P12, day 12 pupae; P14, day 14 pupae; A1, day 1 adults; A3, day 3 adults; A5, day 5 adults. Expression levels of *MaTH* and *MaDDC* are presented relative to the levels of expression in the day 0 pupae (P0). All data are shown as the mean value ± SE (n = 3).

Amino acid sequence alignment and phylogenetic analysis of insect TH proteins



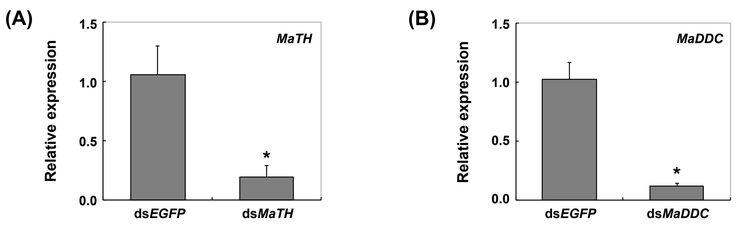
(A) Amino acid sequence alignment of insect TH proteins. Multiple sequence alignment of insect TH proteins was made using ClustalW software. DmTH from *Drosophila melanogaster* is regulated by phosphorylation of Ser32 by cAMP-dependent protein kinase and is conserved among insect THs analyzed (highlighted in red). An acidic region with low sequence conservation in the N-terminal regulatory domain is boxed. The putative catalytic domain is highlighted in gray. The amino acid residues that are critical to form the binding sites for an iron atom and for a cofactor tetrahydrobiopterin (BH_4) are indicated by closed circles and triangles, respectively. (B) Phylogenetic analysis of insect TH proteins. MEGA 7.0 software was used to construct the phylogenetic tree using the Neighbor-Joining method. Numbers by each branch indicate results of bootstrap analysis of 5000 replications. Phenylalanine hydroxylase (DmPAH) from *D. melanogaster* was used as an outgroup. Ma, *Monochamus alternatus*; Agl, *Anoplophora glabripennis*; Ld, *Leptinotarsa decemlineata*; At, *Aethina tumida*; Tc, *Tribolium castaneum*; Ot, *Onthophagus taurus*; Dm, *D. melagaster*; Ag, *Anopheles gambia*; Aal, *Aedes albopictus*; Aa, *Aedes aegypti*; Nv, *Nasonia vitripennis*; Am, *Apis mellifera*; Ar, *Athalia rosae*; Bm, *Bombyx mori*; Ms, *Manduca sexta*; Px, *Papilio xuthus*; Ha, *Helicoverpa armigera*; Se, *Spodoptera exigua*; Rp, *Rhodnius prolixus*; Nl, *Nilaparvata lugens*; Zn, *Zootermopsis nevadensis*.

Amino acid sequence alignment and phylogenetic analysis of insect DDC proteins



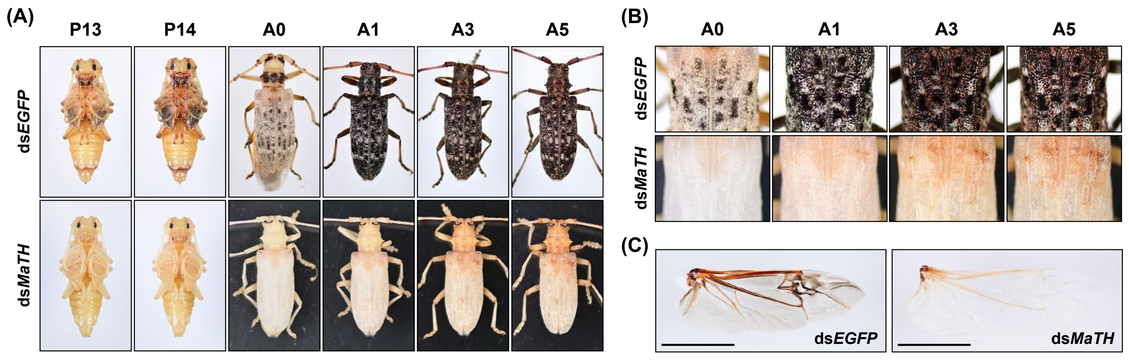
(A) Amino acid sequence alignment of insect DDC proteins. Multiple sequence alignment of insect DDC proteins was made using ClustalW software. The pyridoxal-5-phosphate (PLP)-dependent decarboxylase domain is highlighted in gray. A PLP-binding motif is boxed. Closed triangles indicate the amino acid residues that are critical to form the binding sites for the cofactor PLP. (B) Phylogenetic analysis of insect DDC proteins. MEGA 7.0 software was used to construct the phylogenetic tree with the UPGMA method. Numbers by each branch indicate results of bootstrap analysis of 5000 replications. Tm, *Tenebrio molitor*; Dp, *Dendroctonus ponderosae*; Ha, *Harmonia axyridis*; Pa, *Pararge aegeria*; Vm, *Vespa mandarinia*; Ac, *Apis cerana*; Da, *Diachasma alloeum.*

Knockdown transcript levels of *MaTH* and *MaDDC*



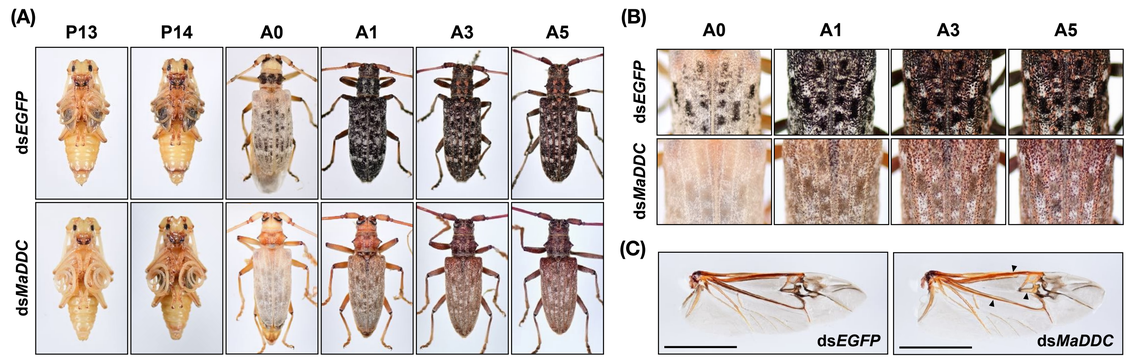
Knockdown levels of *MaTH* (A) and *MaDDC* (B) transcripts were analyzed by real-time PCR. Total RNA was isolated from day 14 pupae that had been injected with double-stranded RNA (dsRNA) for *MaTH* (ds*MaTH*), *MaDDC* (ds*MaDDC*) or *EGFP* (ds*EGFP*) (2 μg per insect) into day 0-2 pupae. Transcript levels of *MaTH* and *MaDDC* are presented relative to their levels in ds*EGFP*-treated controls. An asterisk indicates a significant difference in transcript levels of *MaTH* (p = 0.03, t-test) and *MaDDC* (p = 0.004, t-test) between control and test animals. All data are shown as the mean value ± SE (n = 3).

Pale/white adult cuticle phenotype produced by RNAi of *MaTH*



ds*MaTH* or ds*EGFP* (2 μg per insect) were injected into day 0-2 pupae. (A) ds*EGFP*-control pupae exhibited a normal pigmentation of pharate adult cuticles such as mandibles, legs and wings, which was visible through their translucent pupal cuticle on pupal day 14 (P14). The resulting adults developed rapidly typical black, reddish-brown and gray pigments in the head, antennae, legs and elytra by day 5 (A5). In contrast, ds*MaTH*-treated pupae exhibited little or no pigmentation of the pharate adult cuticles. Approximately 20% of MaTH-deficient pupae failed to molt to adults. The resulting adults (~80%), in addition, showed nearly complete inhibition of pigmentation by the end of the observation period. P13, day 13 pupa; P14, day 14 pupa; A0, day 0 adult; A1, day 1 adult; A3, day 3 adult; A5, day 5 adult. (B) The elytra from the resulting day 0-5 day adults are enlarged. (C) The hindwing dissected from the resulting day 5 adults. RNAi for *MaTH* caused obvious defects in the pigmentation of the pterostigma and veins of the hindwings. Scale bar = 1 cm.

Lighter cuticle pigmentation phenotype produced by RNAi of *MaDDC*



(A) Injection of dsRNA for *MaDDC* (ds*MaDDC*) into day 0-2 pupae had no effect on pupal development or pupal-adult molting. However, the body color of the resulting adults was obviously lighter than that of ds*EGFP*-control animals, in particular, black pigments of the elytra were drastically reduced. P13, day 13 pupa; P14, day 14 pupa; A0, day 0 adult; A1, day 1 adult; A3, day 3 adult; A5, day 5 adult. (B) The elytra from the resulting day 0-5 adults are enlarged. (C) Hindwing dissected from the resulting day 5 adults. RNAi of *MaDDC* reduced dark brown pigmentation in the hindwings (arrows). Scale bar = 1 cm.

References

- Arakane, Y., Noh, M.Y., Asano, T., Kramer, K.J., 2016. Tyrosine metabolism for insect cuticle pigmentation and sclerotization, Extracellular Composite Matrices in Arthropods. Springer, pp. 165-220.
- Mun, S., Noh, M.Y., Kramer, K.J., Muthukrishnan, S., Arakane, Y., 2020. Gene functions in adult cuticle pigmentation of the yellow mealworm, *Tenebrio molitor*. Insect Biochem. Mol. Biol. 117, 103291.

Acknowledgement

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Conclusion

- High transcript levels of *MaTH* and *MaDDC* were detected by real-time PCR at pharate adult stages right before eclosion.
- RNAi of *MaTH* caused little or no pigmentation of the adult cuticles, including rigid body wall and elytra as well as the membranous hindwings.
- MaDDC-deficient adults exhibited lighter cuticle pigmentation, in particular, black pigments were substantially decreased.
- These results suggest that MaTH and MaDDC are required for normal cuticle coloration, including black, reddish-brown and gray pigments of *M. alternatus* adult.