Function of *yellow-y* in adult cuticle melanization of *Monochamus alternatus*

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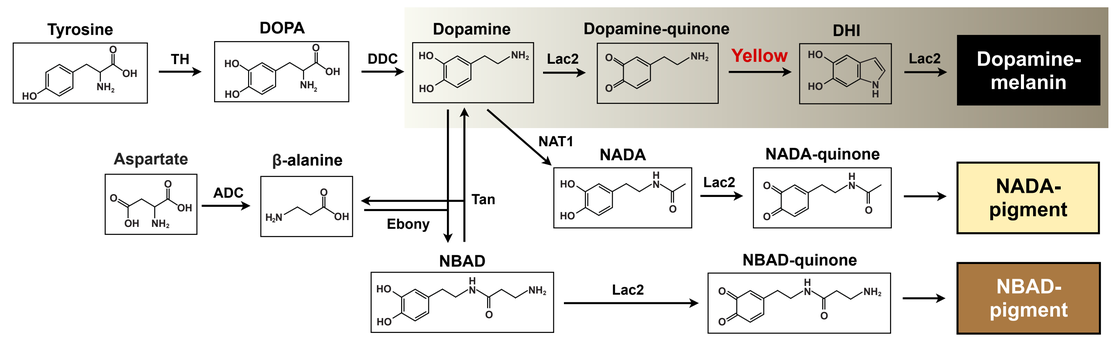
Introduction

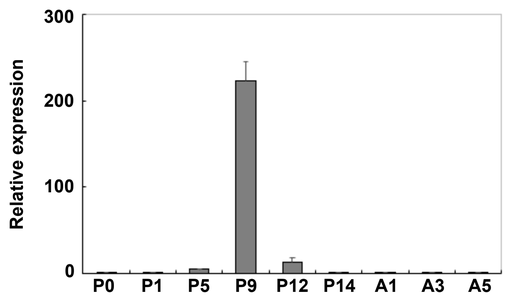
Cuticle tanning (pigmentation and sclerotization) is an important physiological event in insect development. In this vital metabolism initiated with tyrosine, dopachrome conversion enzymes (DCEs) encoded by the *yellow* genes significantly accelerate melanization reaction. Insect *yellow* is a rapidly evolving gene family generating functionally diverse paralogs. In this study we identified, cloned cDNA and investigated the function of *yellow-y* (*MaY-y*) in adult cuticle melanin-type pigmentation of the Japanese pine sawyer beetle, *Monochamus alternatus*, which is a major vector of the pinewood nematode, *Bursaphelnchus xylophilus* that causes Pine wilt disease. Real-time qPCR revealed that *MaY-y* was sharply induced in day 9 pupae and declined thereafter during late developmental stages. Loss of function of *MaY-y* caused by RNAi had no effect on larval and pupal development. However, the resulting adults exhibited a reddish-brown body wall and elytra as well as bristles instead of a black coloration in the control animals. These results indicate that MaY-y has a critical role in normal black pigmentation of *M. alternatus* adult.

Tyrosine-mediated cuticle pigmentation metabolic pathway in *M. alternatus*



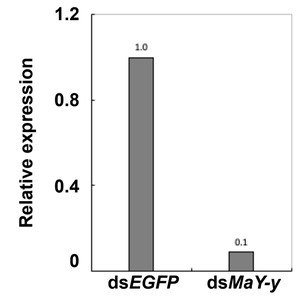
Dopamine is major precursor for synthesis of both melanin and *N*-acylquinoid-derived pigments. Yellow protein, which accelerates melanin production, is highlighted in red. DOPA, 3,4-dihydroxyphenylalanine; Dopamine, 3,4-dihydroxyphenethylamine; NADA, *N*-acetyldopanime; NBAD, *N*-β-alanyldopamine; TH, 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; Noh et al., 2021).

Expression profiles of *MaY-y* during late stages of development



Transcript levels of *MaY-y* 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 *MaY-y* 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).

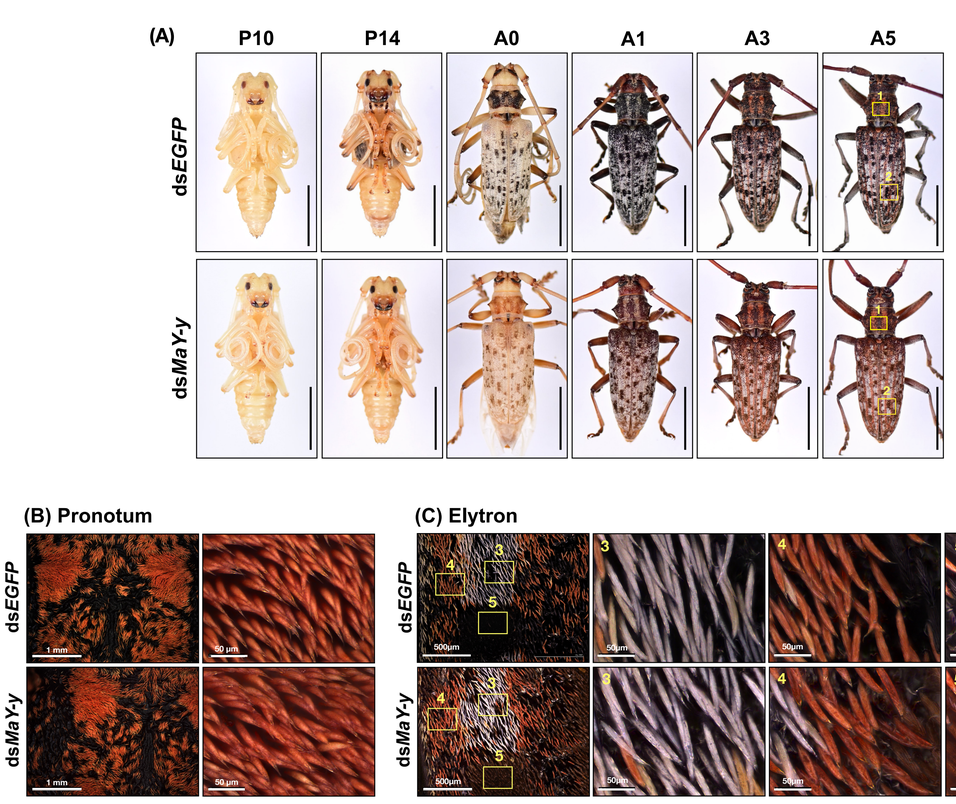
Knockdown transcript levels of *MaY-y*

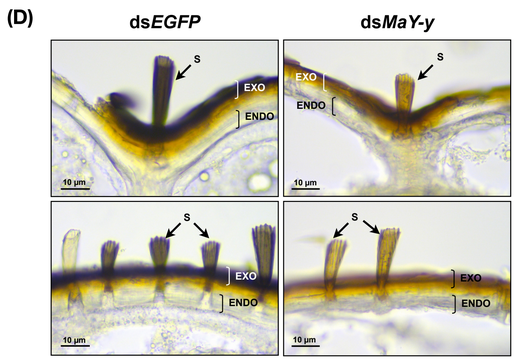


Knockdown level of *MaY-y* transcripts was analyzed by real-time PCR. Total RNA was isolated from day 9 pupae that had been injected with double-stranded RNA (dsRNA) for *MaY-y* (ds*MaY-y*) or *EGFP* (ds*EGFP*) (2 μg per insect) into day 2 pupae. Transcript level of *MaY-y* is presented relative to the level in ds*EGFP*-treated controls.

Amino acid alignment of insect Yellow-y sequences

Effect of RNAi for *MaY-y* on the adult cuticle pigmentation

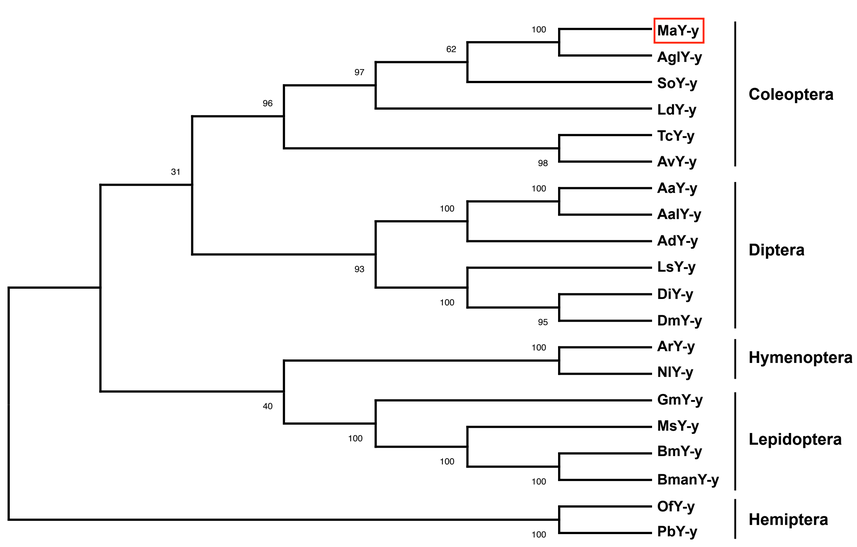




ds*MaY-y* or ds*EGFP* (2 μg per insect) were injected into day 1-5 pupae. (A) Injection of ds*MaY-y* had no effect on pupal development or pupal-adult molting. However, unlike ds*EGFP*-control adults that developed black, reddish-brown and gray pigments in the body wall and elytral cuticles by day 5, the whole body color of ds*MaY-y* adults was obviously lighter than that of ds*EGFP* controls, exhibiting light reddish hue. P10, day 10 pupa; P14, day 14 pupa; A0, day 0 adult; A1, day 1 adult; A3, day 3 adult; A5, day 5 adult. Scale bar = 1 cm. (B and C) The coloration and morphology in equivalent regions of cuticles such as the pronotum and elytron (boxes in 1 and 2 in A, respectively) from the day 5 adults were further analyzed by digital microscopy (Zeiss, smartzoom 5). In the elytron, grayish (box 3), reddish-brown (box 4) and black (box 5) pigmented regions are enlarged. (D) Cryosections (~14 μm) of the elytral cuticle dissected from day 5 adults were observed under an optical microscope. RNAi of *MaY-y* reduced pigmentation of the black exocuticle and scales (arrows). EXO, exocuticle; ENDO, endocuticle; S, scale. (E) Hindwings from the day 5 adults. There was no effect of RNAi of *MaY-y* on pigmentation of the veins. However, injection of ds*MaY-y* caused significant defect in the dark/black pigmented membranous region (arrow heads). Scale bar = 1 cm.

Multiple sequence alignment of insect Yellow-y proteins was made using ClustalW software. The predicted signal peptide and catalytic (MRJP) domain are bolded and boxed, respectively. The putative *N*-glycosylation sites are highlighted in gray. Ma, *Monochamus alternatus*; Agl, *Anoplophora glabripennis*; So, *Sitophilus oryzae*; Ld, *Leptinotarsa decemlineata*; Tc, *Tribolium castaneum*; Av, *Asbolus verrucosus*; Aa, *Aedes aegypti*; Aal, *Aedes albopictus*; Ad, *Anopheles darlingi*; Ls, *Lucilia sericata*; Di, *Drosophila immigrans*; Dm, *Drosophila melanogaster*; Ar, *Athalia rosae*; Nl, *Neodiprion lecontei*; Gm, *Galleria mellonella*; Ms, *Manduca sexta*; Bm, *Bombyx mori*; Bman, *Bombyx mandarina*; Of, *Oncopeltus fasciatus*; Pb, *Platymeris biguttatus*

Phylogenetic analysis of insect Yellow-y proteins



MEGAX 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. Ma, *Monochamus alternatus*; Agl, *Anoplophora glabripennis*; So, *Sitophilus oryzae*; Ld, *Leptinotarsa decemlineata*; Tc, *Tribolium castaneum*; Av, *Asbolus verrucosus*; Aa, *Aedes aegypti*; Aal, *Aedes albopictus*; Ad, *Anopheles darlingi*; Ls, *Lucilia sericata*; Di, *Drosophila immigrans*; Dm, *Drosophila. melanogaster*; Ar, *Athalia rosae*; Nl, *Neodiprion lecontei*; Gm, *Galleria mellonella*; Ms, *Manduca sexta*; Bm, *Bombyx mori*; Bman, *Bombyx mandarina*; Of, *Oncopeltus fasciatus*; Pb, *Platymeris biguttatus*

Conclusion

- **High transcript levels of *MaY-y* were detected by real-time PCR at middle stages of pupal development (Day 9 pupae).**
- **RNAi of *MaY-y* caused significant defect black melanin production, resulting in lighter adult body color, exhibiting abnormal reddish hue.**
- **These results indicate that *MaY-y* is required for normal cuticle coloration, in particular, black melanin-type pigment of *M. alternatus* adult.**

References

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