Overexpression of Tyrosine hydroxylase and Dopa decarboxylase associated with pupal melanization in Spodoptera exigua

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Melanism has been found in a wide range of species, but the molecular mechanisms involved remain largely elusive. In this study, we studied the molecular mechanisms of the pupal melanism in Spodoptera exigua. The full length cDNA sequences of tyrosine hydroxylase (TH) and dopa decarboxylase (DDC), two key enzymes in the biosynthesis pathway of melanin, were cloned, and their temporal expression patterns in the integument were compared during the larval-pupal metamorphosis process of the S. exigua wild type (SEW) and melanic mutant (SEM) strains. No amino acid change in the protein sequence of TH and DDC was found between the two strains. Both DDC and TH were significantly over-expressed in the integument of the SEM strain at late-prepupa and 0 h pupa, respectively, compared with those of the SEW strain. Feeding 5th instar larvae of SEM with diets incorporated with 1 mg/g of the DDC inhibitor L-α-Methyl-DOPA and 0.75 mg/g of the TH inhibitor 3-iodo-tyrosine (3-IT) resulted in 20% pupae with partially-rescued phenotype and 68.2% of pupae with partially- or fully-rescued phenotype, respectively. These results indicate that overexpressions of TH and DDC are involved in the pupal melanization of S. exigua.

Melanism, occurrence of dark (melanic) forms caused by over-deposition of the pigment melanin, is one of the most conspicuous phenotypic variations in external morphology. It has been documented in many different animal groups, including various insects1–10. In these documented insect species, the spatiotemporal expression profiles of melanization and the resultant advantages and fitness costs are usually different. For example, melanization is expressed locally in the two dipterans Drosophila polymorpha4 and Drosophila immigrans6, but globally in almost all the documented lepidopterans2,3,9–18. In terms of temporal expression pattern, melanic phenotype may express in larva9–11, pupa14, adult2,3,16,19, larva and adult (Bombyx mori mln mutant strain16), larva and pupa (B. mori sooty mutant strain21), or pupa and adult (Helicoverpa armigera15) stages. Further, melanism in these diverse taxa may confer one or more adaptive advantages22 that are associated with a fitness cost (e.g. in Manduca sexta11 and H. armigera15) or a fitness gain (e.g. in Mythimna separate16, Spodoptera littoralis13 and D. immigrans6) in life-history
traits. Such variable expression profiles and adaptive impacts of melanization imply a great diversity of genetic/molecular mechanisms controlling melanism.

The genetic/molecular mechanisms underlying the great diversity of melanism in lepidopteran insects still remain largely a mystery. There are only three melanin mutant species (B. mori, Papilio glaucus and M. sexta) whose genetic/molecular bases of melanization have been elucidated by looking for alternations in the hormone cascades and/or the melanin and other pigment biosynthesis pathway genes. Among the two studied melanic mutants of B. mori, the sooty (so) mutant, which produces smoky larvae and black pupae, is caused by large deletions of the ORF (open reading frame) of the N-β-alamyl dopamine synthase (also known as N-β-alamyl dopamine synthase) gene ebony9, whereas the melanism (mln) mutant, in which melanization is expressed locally in larvae but globally in adults, is resulted from partial deletion of the 4th exon of the arotylalanine-N-acetyltransferase (AANAT) gene and its reduced expression22,23. Studies of the black larval mutant of M. sexta establish a causal link between JH (juvenile hormone) deficiency, higher DDC (dopa decarboxylase) expression, and the melanin phenotype21,22,24. However, such a link is not detected in the dark larval mutant of the butterfly, Bicyclus anynana6. In the melanic female adults of P. glaucus, local deposition of the melanin pigment on the wing is resulted from early suppression of N-β-alamyltransferase encoded by ebony6; in concert with reduced DDC activity25–27. By contrast, the global industry melanization of the adults of the peppered moth (Biston betularia) is not linked to any of the sixteen melanin pathway genes identified so far22. As far as we know, there is no single case of pupal melanism, in which the underlying molecular mechanism has been elucidated.

To advance understanding of the molecular genetic bases of melanism, we choose to study the melanic mechanisms in a pupal melanic mutation strain (SEM) of the beet armyworm [Spodoptera exigua (Hübner)], which was established with 40 black pupae spontaneously occurred within a typical laboratory population (SEW brown-pupa) of S. exigua. The melanic phenotype is globally expressed only in the pupal stage. Relative to its parental wild-type strain SEW, the melanin SEM strain actually develops faster and has higher fecundity. The fact that melanin, rather than brown pigment as in the SEW strain, is synthesized/deposited in the newly-pupated SEM pupae (personal observation) suggest that alternations in one or more melanin-(e.g. tyrosine hydroxylase (TH or pale) and DDC) or other pigments-promoting (e.g. ebony and AANAT) genes are probably responsible for the pupal melanin phenotype in the SEM strain. To test the melanin-promoting gene alternation hypothesis, here we compared the cDNA/protein sequences and temporal expression profiles of TH and DDC, two enzymes responsible for production of the melanin precursors, dopa and dopamine respectively, between the two strains. We also examined the in vivo effects of inhibiting TH and DDC on the melanin phenotype of the melanin SEM strain. We not only detected overexpression of TH and DDC in the integuments of SEM prepupae and pupae, but also found that feeding larvae of the mutant SEM strain with inhibitors of TH and DDC led to conversion of the melanin pupae to wild type (brown) or intermediate (dark brown) color pupae. These results suggest that overexpression of the two melanin precursor-producing genes are responsible for the pupal melanin color morph in the SEM strain.

**Results**

**TH cDNA in the wild type and melanic strains of S. exigua.** The full-length cDNA sequences of TH and DDC from S. exigua were obtained by RT-PCR cloning of a partial fragment, followed by cloning of their 5’ and 3’ ends by 5’ and 3’ RACE (random amplification of cDNA ends). The NCBI accession numbers of the cDNA sequences of TH and DDC from SEW and SEM were JF795467, JF795468, JF795469 and JF795470, respectively. The S. exigua TH (SeTH) cDNA (2259 bp in total) contains a 185-bp 5’ UTR (untranslated region), an open reading frame (ORF) of 1686 bp encoding 561 amino acids, and a 386-bp 3’ UTR (Fig. 1). Sequence analysis shows that SeTH shares 97% amino acid identity with TH from Mythimna separata, 94% from Papilio xuthus, M. sexta and B. mori, 91% from Heliconius melpomene malleti and Samia cynthia ricini. Phylogenetic analysis also shows that SeTH is more closely related to TH from M. separata, which, like S. exigua, belongs to Noctuidae (Fig. 2A). Similar to the structure of other TH proteins, SeTH contains the conserved metal binding site for iron atom (the circled histidine 389, histidine 394 and glutamate 432 in Fig. 1) and the cofactor tetrahydrobiopterin (BH₄) binding site (the boxed Leu251, leu252, phe257, gln267, pro385, glu390 and try429 in Fig. 1).

CDNA sequence alignment uncovers that the SeTH alleles from the wild type and melanin mutant strains only have 1 SNP (single nucleotide polymorphism) in the ORF, 1 SNP and 1 1-bp indel in the 3’ UTR (underlined in Fig. 1). The only SNP in the ORF does not cause amino acid change.

**DDC cDNA in the wild type and melanic strains of S. exigua.** The S. exigua DDC (SeDDC) full-length cDNA is 1688 bp long, containing a 175-bp 5’UTR, an ORF of 1431 bp (from 176 to 1606) encoding 476 amino acids, and a 82-bp 3’UTR (Fig. 3). The predicted SeDDC proteins shows 95% amino acid sequence identity with Mamestra brassicae DDC, 94% with M. separata DDC, 91% with Antheraea pernyi and Papilio machaon DDC. SeDDC is grouped into the same clan with M. separata and M. brassicae DDC, which share a common ancestor DDC with another lineage formed by the Papilionoidea DDC clades (Fig. 2B). Like other DDC, SeDDC has a cofactor binding site for pyridoxal 5’-phosphate (PLP). The PLP binding site is composed of ala148, ser149, thr152, thr242, asp271, try274, asn303 and lys306 (underlined in Fig. 3).
Only one nucleotide difference was found between the DDC cDNA sequences from SEW and SEM. This substitution occurs in the 3'UTR and thus causes no amino acid change between the two DDC alleles (Fig. 3).

**Overexpression of TH and DDC in the melanic SEM strain of *S. exigua***. Two-way ANOVA revealed significant differences in TH expression between strains and among the five developmental stages (Table 1A). There were also significant strain × stage interactions in TH expression. The normalized expression of TH in the integument of the wild type SEW strain was in a descending order of 0-h pupae (22.134 folds) >2-h pupae (4.587 folds) >late prepupa (0.936 folds) >24-h pupae (0.517 folds) >early prepupa (0.136 folds) (Fig. 4A). The temporal expression profile of TH slightly differed in the melanic mutant strain SEM, which had a descending order of 0-h pupae (27.820 folds) >late prepupa (14.219 folds) >2-h pupae (3.703 folds) >24-h pupae (1.190 folds) >early prepupa (0.272 folds). Relative to the wild type SEW strain, the expression of TH was significantly higher in the late prepupae (15.191 folds) of the SEM strain (Fig. 4A).

Two-way ANOVA also revealed significant differences in DDC expression between strains and among the five developmental stages as well as significant strain × stage interactions in DDC expression (Table 1B). Comparison of Fig. 4A,B revealed that DDC transcripts were much less abundant than TH transcripts in both the wild and mutant strains. The normalized expression of DDC in the integument of the wild type strain SEW was ranked as 24-h pupae (0.145 folds) >0-h pupae (0.110 folds) >2-h pupae (0.070 folds) >late prepupa (0.046 folds) >early prepupa (0.044 folds) (Fig. 4B). The temporal expression profile of DDC in the integument of the mutant SEM strain was ranked as late prepupa (1.492 folds) >2-h pupae (3.703 folds) >24-h pupae (1.190 folds) >early prepupa (0.272 folds). Relative to the wild type SEW strain, the expression of DDC was significantly higher in the late prepupa (15.191 folds) of the SEM strain (Fig. 4A).

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**Rescue of the mutant melanic phenotype by inhibiting TH and DDC***. To test if overexpression of TH and/or DDC is responsible for the mutant melanic phenotype in SEM, we fed 5^th^ instar larvae of SEM with control diets, diets incorporated with 0.25, 0.50, or 0.75 mg/g of 3-Iodo-L-tyrosine (3-IT,
Figure 2. Phylogenetic trees of insect TH (A) and DDC (B) based on their amino acid sequences. Phylogenetic trees were constructed using the neighbor-joining method with 1000 bootstrap replicates. The numbers at each tree node are the bootstrap values.
a TH inhibitor), or diets with 0.1, 0.5, and 1.0 mg/g of L-α-Methyl-DOPA (a DDC inhibitor). All the pupae obtained from larvae fed on control diets and diets with 0.1 or 0.5 mg/g of L-α-Methyl-DOPA were as black as the H2O control group (Fig. 5B1; Table 2), which were the same color as the SEM strain (Fig. 5A1), whereas 6 out of the 30 pupae pupated from larvae fed on diets with 1.0 mg/g of L-α-Methyl-DOPA were dark-brown (Fig. 5C; Table 2), i.e. partially rescued. The TH inhibitor 3-IT displayed even stronger rescue of the mutant phenotype. About half of the pupae from larvae fed on diet with 0.25 mg/g of 3-IT were partially recued and had a dark-brown color (Fig. 5D and Table 2), while all the pupae from the PBS control diets remained black (Fig. 5B2; Table 2). As the 3-IT dose increased, both the level of phenotype rescue (compare pupae in Fig. 5D-E) and the percentage of pupae with partially- (Fig. 5D,E) and fully-rescued (brown color pupae in Fig. 5F) simultaneously increased (Table 2), and the

**Figure 3.** Alignment of DDC cDNA sequences from the wild type (SEW) and melanic (SEM) strains of S. exigua. — = cofactor binding site of DDC.

| Source of variation | df | SS   | MS   | F    | P    |
|---------------------|----|------|------|------|------|
| Strain              | 1  | 133.883 | 133.883 | 7.857 | 0.009 |
| Stage               | 4  | 3218.326 | 804.582 | 47.219 | <0.0001 |
| Strain × Stage Interaction | 4  | 272.158 | 68.039 | 3.993 | 0.011 |
| Error               | 28 | 477.097 | 17.039 |      |      |
| Total               | 38 | 6489.238 |      |      |      |

**Table 1.** Two-way ANOVA on the expressions of TH and DDC at different development stages between SEW and SEM strains of S. exigua
rescued phenotype is very close (Fig. 5E) and even lighter (Fig. 5F) than the wild-type morph (Fig. 5A2). The highest rescue rate was caused by 0.75 mg/g 3-IT (68.2%), which was not significantly higher than that elicited by 0.5 mg/g 3-IT (52.4%, \( P = 0.228 \)) and 0.25 mg/g 3-IT (47.1%, \( P = 0.500 \)). There was no significant difference between the rescue rates of 0.5 mg/g 3-IT and 0.25 mg/g 3-IT (\( P = 0.261 \)).

**Discussion**

The great diversity of insect melanism provides rich models for studying the molecular and developmental regulation of phenotypic variations. All the melanic mutant phenotypes, whether occurred locally or globally in space/time and associated with or without a fitness cost, are concentration of one or both of the two insect melanins—Dopa melanin and Dopamine melanin—on a particular body surface at a particular developmental time. This can only happen if the precursors of one or both of the two melanins, Dopa and Dopamine respectively, are overproduced, or less of them are channeled to synthesize the yellow and/or colorless pigments in the melanic insect strains.

To test if one or both of the two melanin precursors are overproduced in the pupal melanic SEM strain of *S. exigua*, we contrasted the cDNA sequences and expression levels of TH and DDC, the two enzymes responsible for production of the Dopa and Dopamine precursors respectively, between the wild type SEW and melanic SEM strains. While we did not found any differences in the amino acid sequences of the two enzymes (Figs 1 and 3), we detected about 15-fold more TH transcripts in the early prepupae of SEM and more DDC transcripts in the late prepupae (32.435 folds) and 0-h pupae (3.082 folds) of SEM (Fig. 4). The fact that inhibiting the overproduced TH and DDC in SEM resulted in fully- and partially-rescued pupae respectively (Table 2 and Fig. 5) demonstrates that overproduction of TH and DDC is responsible for the pupal melanism in the SEM strain of *S. exigua*. Whether less
consumption of the Dopa and Dopamine precursors for synthesis of the yellow and colorless pigments also contributes to SEM’s melanic phenotype has yet to be determined.

As far as we know, our study is the first report of molecular mechanisms underlying melanism occurred only in the pupal stage of lepidoperan insects. Among the limited cases studying the mechanisms of lepidoperan melanism, 2 involved melanization in the larval stage, 1 in the adult stage, 1 in the larval and adult stage, and 1 in the larval and pupal stage. Overproduction of the Dopamine precursor due to enhanced DDC expression is linked to the larval melanism of M. sexta. By contrast, less consumption of the Dopa precursor for synthesis of the yellow pigment by simultaneously-reduced expression of ebony and DDC causes partial melanization on the female adult wing of P. glaucus. Similarly, less consumption of the Dopamine precursor for synthesis of the yellow or colorless pigment by deletion of the ebony ORF or the 4th exon of AANAT is linked to the so (larval and pupal melanization) and mln (larval and adult melanization) mutants of B. mori, respectively. Thus, our finding of TH overexpression as the major contributor to the pupal melanism of S. exigua also represents the first report of the involvement of TH in insect melanism mutation. This finding is consistent with an earlier

Figure 5. Phenotypes of the melanic SEM strain pupae pupated from larvae fed with diets containing the DDC inhibitor L-α-Methyl-DOPA or the TH inhibitor 3-IT. A1. Untreated SEM strain, 24 h old pupa; A2. Untreated SEW strain, 24 h old pupae; B1. SEM strain, H2O control, 5-day old pupae; B2. SEM strain, PBS control, 24 h old pupae; C. SEM strain, 1.0 mg L-α-Methyl-DOPA/g artificial diet, 5-day old pupae; D. SEM strain, 0.25 mg 3-IT/g artificial diet, 24 h old pupae; E. SEM strain, 0.50 mg 3-IT/g artificial diet, 24 h old pupae; F. SEM strain, 0.75 mg 3-IT/g artificial diet, 24 h old pupae.
report that reduced expression of TH is linked to the light neonate color in the sex-linked chocolate (sch) mutant of B. mori.

While we have discovered that overexpression of TH and DDC is responsible for the pupal melanization in the SEM strain of S. exigua, we have not identified the actual alterations that lead to increased expression of the two genes. One possibility would be an alternation in a transcription factor or small RNA that regulates expression of both TH and DDC. Another possibility would be some indel or SNP mutations in the 5' promoter regions of both genes that separately enhance expression of the two genes. The third possibility would be an alternation in the promoter region of TH or its transcription factor/small RNA that only increases TH expression, but the resultant extra Dopa precursor induces the expression of the downstream DDC gene. Clearly, molecular cloning of the promoter regions of the two genes is the first step needed to resolve these speculations.

Methods

Experimental animals. The wild-type strain (SEW, brown color pupae) of S. exigua was established with a field collection from Jingzhou, Hubei, China in 2003 and has been maintained on artificial diet (a solid mixture of soybean flour, barley flour, yeast powder, Vitamin C, benzoic acid, sodium benzoate, acetic acid and agar) at 27 ± 1 °C, 65% relative humidity and a photoperiod of 14:10 (L: D). At the 6th generation, forty black pupae spontaneously occurred within the SEW strain. The adults emerged from the 40 black pupae were crossed to establish the pupal melanic strain (named SEM). The SEM strain has then been maintained on the same artificial diet at the same conditions with the SEW strain since then.

TH and DDC cDNA Cloning. For each strain, three prepupa integuments were dissected out on a filter paper placed on ice, washed with cold 0.1% DEPC-treated water, briefly dried on a clean filter paper, transferred to a ceramic mortar with liquid nitrogen, and ground to powder in liquid nitrogen. The resultant prepupa integument power of each strain was immediately transferred into a 1.5-mL centrifuge tube with about 500 μL of Trizol™ reagent (Invitrogen, USA). Total RNA was isolated from the power according to the manufacturer's instructions. RNA purity was evaluated by 260/280 and 260/230 ratios measured using NanoDrop 1000 (NanoDrop, USA). DNase I (Fermentas, EU) treated RNA (1 μg) was reverse transcribed into cDNA in a 20 μL reaction with random primer (N9) and Oligo dT(18) primer using First-Strand cDNA Synthesis Kit (Fermentas, EU).

The primers for cloning of TH partial cDNA (Table S1) were designed based on the conserved regions of tyrosine hydroxylases of insect. The PCR conditions were 3 min denaturation at 95°C, followed by 40 cycles of 30 sec denaturation at 94°C, 30 sec annealing at 50°C and 1 min extension at 72°C, and a final 9 min extension at 72°C. The primers for cloning of DDC partial cDNA (Table S1) were designed on the basis of S. exigua dopa decarboxylase (DDC) gene (NCBI accession no.: SEU71404). The PCR conditions were 3 min denaturation at 95°C, followed by 40 cycles of 30 sec denaturation at 94°C, 30 sec annealing at 56°C and 30 sec extension at 72°C, and a final 9 min extension at 72°C. The full-length cDNAs of the two genes were obtained by rapid amplification of cDNA ends (RACE) technique using the 3'-Full RACE Core Set Ver. 2.0 (TaKaRa, Japan) and 5'-Full RACE Kit (TaKaRa, Japan) with the corresponding specific primers (Table S1) designed based on their partial cDNA sequences. The open reading frame (ORF) of TH and DDC were amplified from SEW and SEM strains with the corresponding flanking PCR primers (Table S1), respectively. The flanking PCR conditions for TH gene was 3 min denaturation

### Table 2. Statistics of pupae color changes resulted from feeding larvae of the melanic SEM strain of S. exigua with TH and DDC inhibitors.

| Inhibitor            | Concentration (mg/g) | Total number of pupae | Number of pupae with the following color | Rescue rate (%) |
|----------------------|----------------------|-----------------------|------------------------------------------|----------------|
|                      |                      |                       | Black | dark-brown | brown |                      |
| L-α-Methyl-DOPA      | 1                    | 30                    | 24    | 6          | 0     | 20.0                  |
|                      | 0.5                  | 25                    | 25    | 0          | 0     | 0.0                   |
|                      | 0.1                  | 29                    | 29    | 0          | 0     | 0.0                   |
| CK (H2O)             |                      | 28                    | 28    | 0          | 0     | 0.0                   |
| 3-IT                 | 0.75                 | 22                    | 7     | 6          | 9     | 68.2                  |
|                      | 0.50                 | 21                    | 10    | 6          | 5     | 52.4                  |
|                      | 0.25                 | 17                    | 9     | 8          | 0     | 47.1                  |
| CK (PBS)             |                      | 16                    | 16    | 0          | 0     | 0.0                   |

1Rescue rate = (the number of dark-brown and brown pupae/the total number of pupae) × 100%.
at 95 °C, followed by 40 cycles of 30 sec denaturation at 94 °C, 30 sec annealing at 54 °C and 2 min extension at 72 °C, and a final 9 min extension at 72 °C. The flanking PCR conditions for DDC gene was 3 min denaturation at 95 °C, followed by 40 cycles of 30 sec denaturation at 94 °C, 30 sec annealing at 50 °C and 2 min extension at 72 °C, and a final 9 min extension at 72 °C. All the fragments and full ORF were cloned into pMD18-T Vector (TaKaRa, Japan) and at least three clones were sequenced for each fragment or ORF from the two strains.

**Sequence alignment and phylogenetic analysis.** The nucleotide and deduced amino acid sequences of TH and DDC were aligned to identify potential changes between the wild type (SEW) and mutant (SEM) strains. The conserved domains of the deduced protein sequences were localized by blast against the conversed domain database (CDD) (http://blast.ncbi.nlm.nih.gov/). The protein sequences retrieved from NCBI database and used for phylogenetic analysis were TH from the two strains of *S. exigua* (NCBI accession no.: AFG25778.1, AFG25779.1), *Drosophila melanogaster* (AAA62877 and AAA62876), *Musca domestica* (XP_005180802.1), *M. sexta* (ABQ95973.1), *Tribolium castaneum* (ABQ95974.1), *Tenebrio molitor* (ACU77882.2), *H. mellomene malleti* (ADU32895.1), *Bombbyx mandarinia* (ADV56709.1), *M. separata* (BAF32573.1 and BAF32574.1), *B. mori* (BAH11148.1), *Apis mellifera* (NP_001011633.1), *Ernolatia moorei* (ADV32917.1), *Harpeganthes saltator* (XP_011137576.1), *Fopius arisanus* (XP_011299399.1), *Cerapachys biroi* (XP_011336847.1), *Solenopsis invicta* (XP_011157658.1), *Megachile rotundata* (XP_003704650.1), *Bombus terrestris* (XP_003396906.1), *Bombus impatiens* (XP_003486444.1), *Bicyclus anynana* (AGT62463.1), *Anopheles gambiae* (AAA44132), *Acromyrmex echinatior* (EIGI65971.1), *Nasonia vitripennis* (XP_008203627.1), *Acyrthosiphon pisum* (XP_008182999.1), *Camponotus floridanus* (EFN71001.1), *Polyrachhis vicina* (AEC14314.1), *Danaus plexippus* (EHJ75630.1), *Plutella xylostella* (ADK12633.1), *Biston betularia* (ADF43213.1), *Papilio polytes* (Bajar07593.1), *P. xuthus* (BAJ07589.1), and as well as DDC from the two strains of *S. exigua* (AFG25780.1, AFG25781.1), *M. brassicae* (BAC68545.1), *M. separata* (BAC68549.1), *A. pernyi* (AAR23825.1), *Antheraea yamamai* (AAR23824.1), *P. machaon* (BAJ07588.1), *Danaus plexippus* (EHJ3554.1), *M. sexta* (AAC46604.1), *P. xuthus* (BAE43852.2, BAM18274.1), *Papilio polytes* (BAJ07594.1), *B. mori* (NP_001037174.1), *D. melanogaster* (P05031.4), *D. viridis* (EDW57710.1), *Armerges subbalbatus* (AAT75222.1), *A. gambiae* (AAC16249.1 and AAC16247.1), *Aedes aegypti* (AAC31639.1), *Armerges subbalbatus* (AAT75222), *Ceratitis capitata* (CA69968.1), *Polyrachhis vicina* (AFI80897.1), *T. castaneum* (ABU25222.1), *T. molitor* (BAA95568.1) and *H. mellomene malleti* (ADU32894.1). Phylogenetic trees were constructed with the MEGA 4 program, using the neighbor-joining method. The confidence of the various phylogenetic lineages was assessed by the bootstrap analysis (bootstrap = 1000). The phylogenetic tree of TH was rooted by using the sequence of *Harpeganthes saltator* from Hymenoptera, and the phylogenetic tree of DDC was rooted by using the sequence of *Polyrachhis vicina* from Hymenoptera.

**Real-time RT-PCR analysis of TH and DDC expression.** Total RNA was isolated from the dissected integuments from both strains at five time points (early prepupa, late prepupa, 0 h pupa, 2 h pupa, 24 h pupa) and reverse transcribed into cDNA as described above. The cDNAs were used as the templates for quantitative RT-PCR (qRT-PCR) analysis of TH and DDC expression using SYBR® Premix Ex Taq™ II kit (TaKaRa, Japan). Real-time PCR of TH, DDC, β-actin and GAPDH (β-actin and GAPDH were internal reference genes) were performed individually in a 20 μL reaction containing 2×SYBR® Premix Ex Taq™ II 10 μL, 10 μM forward primer and reverse primer (0.8 μM each), 1 μL template cDNA, and 7.4 μL nuclease-free water. Specific primers (Table S1) were designed for real-time PCR of TH, DDC, β-actin and GAPDH to generate the corresponding amplicons of 142 bp, 151 bp, 107 bp and 174 bp, respectively. All real-time PCR reactions were performed in iQ™ 96-well PCR plates (Bio-Rad, USA). A CFX96 real-time PCR detection system (Bio-Rad, USA) was used as the fluorescence detector with the following common PCR conditions for the three genes: an initial denaturing cycle of 95 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 5 s, annealing at 60 °C for 10 s and extension at 72 °C for 30 s, and data collection and real-time analysis enabled at 72 °C. Melting curve analysis from 65 °C to 95 °C was run for each target and reference gene to ensure free of junk products. For the time points of early prepupae, late prepupae, 0 h pupa and 2 h pupa, there were 4 biological replicates of 3 integuments each for each strain for each gene; for the time point of 24 h pupa, 3 biological replicates of 3 integuments each for each gene were tested; each biological replicate was qRT-PCR-analyzed 3 times.

The expression level of all the genes were calculated with their mean Ct and amplification efficiency (E) (Equation 1), and the expression level of the two target genes (TH and DDC) at each time point of each strain were normalized with the geometric mean of the expression of the two reference genes (β-actin and GAPDH) (Equation 2)\(^{32,33}\).

\[
\text{Expression level} = (1 + E)^{-Ct}.
\]

Normalised expression level of target gene
Enzyme inhibitor treatment. The TH inhibitor 3-iodo-tyrosine (3-IT) (Sigma) PBS solution was incorporated into artificial diets at the final concentrations of 0.75, 0.50 and 0.25 mg/g diets according to previous studies\textsuperscript{30,34}. The DDC inhibitor L-\textalpha-Methyl-DOPA (Sigma) water solution was mixed with artificial diets at 1.0, 0.5, and 0.1 mg/g diets according to a previous study\textsuperscript{35}. The control diets for 3-IT and L-\textalpha-Methyl-DOPA were diets containing equal volume of PBS solution and water, respectively. For each inhibitor or control diets, 40 newly molted 5\textsuperscript{th} instar larvae of the melanic SEM strain were fed with the corresponding diets till pupation. The body colors of the SEM pupae were observed and photos were taken with a digital camera (OLYMPUS C5060) under white light. The numbers of pupae and adults obtained were recorded at the end of this experiment. The black pupae resulted from this inhibitor feeding experiment were considered as mutant melanic pupae, whereas the dark-brown and brown pupae obtained were considered as partially- and fully-rescued wild type pupae, respectively. For each concentration of inhibitor, the numbers of melanic, partially-rescued, and fully-rescued pupae were recorded and the rescue rate was calculated using the formula of the number of partially- and fully-rescued pupae/ the total number of pupae × 100%.

Statistical analysis. Two-way ANOVA was performed to examine the impacts of strain, stage and strain \times stage interaction on the expressions of TH and DDC. Paired t-tests were conducted to test the significance of differences in the expression levels of TH and DDC at each of the five developmental stages between the two strains. Tukey HSD tests were performed to test the significance of differences in the expression levels of TH and DDC among the five developmental stages of each strain. The differences in the rescue rate among different concentrations of 3-IT were analyzed with one-tailed Fisher’s exact test. All of the statistical tests were performed by SPSS version 16.0 software for Windows.

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Author Contributions
X.L. and S.L. conceived and designed the experiments. S.L. performed the experiments. X.L. and S.L. analyzed the data. X.L., S.L. and M.W. wrote the manuscript. All authors have read and approved the manuscript for publication.

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