

A New Arylalkylamine *N*-Acetyltransferase in Silkworm (*Bombyx mori*) Affects Integument Pigmentation

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Abstract Dopamine is a precursor for melanin synthesis. Arylalkylamine *N*-acetyltransferase (AANAT) is involved in the melatonin formation in insects because it could catalyze the transformation from dopamine to dopamine-*N*-acetyldopamine. In this study, we identified a new *AANAT* gene in the silkworm (*Bombyx mori*) and assessed its role in the silkworm. The cDNA of this gene encodes 233 amino acids that shares 57 % amino acid identity with the *Bm-iAANAT* protein. We thus refer to this gene as *Bm-iAANAT2*. To investigate the role of *Bm-iAANAT2*, we constructed a transgenic interference system using a 3xp3 promoter to suppress the expression of *Bm-iAANAT2* in the silkworm. We observed that melanin deposition occurs in the head and integument in transgenic lines. To verify the melanism pattern, dopamine content and the enzyme activity of AANAT were determined by high-performance liquid chromatography (HPLC). We found that an increase in dopamine levels affects melanism patterns on the heads of transgenic *B. mori*. A reduction in the enzyme activity of AANAT leads to changes in dopamine levels. We analyzed the expression of the *Bm-iAANAT2* genes by qPCR and found that the expression of *Bm-iAANAT2* gene is significantly lower in transgenic lines. Our results lead us to conclude that *Bm-iAANAT2* is a new arylalkylamine *N*-acetyltransferase gene in the silkworm and is involved in the metabolism of the dopamine to avoid the generation of melanin.

Keywords Arylalkylamine *N*-acetyltransferase (AANAT) · Dopamine · Melanin · Transgenic silkworm · *Bombyx mori* · Integument color

Introduction

Pigmentation patterning in silkworm (*Bombyx mori*) has long interested biologists, and its study integrates diverse branches of biology, including ecology, developmental biology, genetics, and physiology [1]. Melanism is one of the most important mutations in silkworm. The melanism mutation in insects is strong black phenotype, which formed at their larval and adult stages. During the melanin production process, tyrosine is hydroxylated to DOPA by tyrosine hydroxylase (TH) and DOPA can then be transformed into dopamine by DOPA

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decarboxylase (DDC) [2–4]. Dopamine is a key compound for both sclerotization and melanin formation. Arylalkylamine *N*-acetyltransferase (AANAT) may catalyze the conversion of dopamine to dopamine-*N*-acetyldopamine. It is thought that AANAT is an enzyme that consumes compound for melanin synthesis (dopamine) and indirectly affects melanin synthesis in insects [5, 6].

In vertebrates, AANAT is an important enzyme in the tryptophan and tyrosine metabolic pathways. It not only affected integument pigmentation but also regulated animal's circadian rhythm [7–9]. The silkworm AANAT was first reported by Itoh and found that the activity of AANAT exhibits day/night fluctuations in silkworm's head [10]. Makio cloned and characterized the full-length cDNA of *Bm-iAANAT* [11]. Dai and Zhan found that dopamine levels increased in silkworm loss-of-function mutants (*Bm-iAANAT*), and this led to the development of a silkworm melanism (*mln*) mutant, respectively [12, 13]. That were the first report that demonstrated arylalkylamine-*N*-acetyltransferase played a role in color pattern mutations in Lepidoptera.

In this study, we used the differential display of reverse transcriptional PCR(ddRT-PCR) to study diapause induction in silkworm and identified an *AANAT* gene which highly expressed the multivoltine *N4*. We then investigated its function in the integument pigmentation.

Materials and Methods

Silkworm Strains

The diapause-free *N4* strain of *B. mori* was used for cloning and constructing the interference system. The silkworms were reared on mulberry leaves at 25 °C with natural light. Both strains were provided by the College of Biotechnology, Institute of Sericulture and Systems Biology, Southwest University (Chongqing, China).

Total RNA Isolation and cDNA Synthesis

Total RNA was purified using TRIzol reagent (Invitrogen, Shanghai, China) according to the manufacturer's instructions. cDNA was synthesized using oligo (dT) primers and a GoScript Reverse Transcription System (Promega, Madison, USA).

Cloning

cDNA from the heads of 5th instar *N4* larvae was used as PCR templates to amplify *Bm-iAANAT2*. The primers were 5'-cgcgcatccTGCTCGGCTCAAGGAGCGAGTT-3' and 5'-ccgctcgagGTTTAAACCGTGTTAAATATTTAT-3'. PCR reactions were conducted under the following conditions: 94 °C for 3 min, 30 cycles of 94 °C for 30 s, 55 °C for 1 min, and 72 °C for 1.5 min, with a final extension of 72 °C for 10 min. PCR products were isolated and cloned into a pMD18-T Simple vector (Takara, Dalian, China) and sequenced (Genscript, Nanjing, China).

Recombinant Protein Expression and Purification

Bm-iAANAT2 fragment was amplified from cDNA using the following oligonucleotides: *Bam*HI-forward: 5'-cgcgcatccATGTCCAAAGAAACGACT-3', *Xho*I-reverse: 5'-ccgctcgagTCACGTTTTATTATGAAC-3'. The underlined nucleotides indicate restriction sites

that can be cut by *Bam*HI and *Xho*I (Takara, Dalian, China). DNA-amplified fragments were inserted into a pGEX-4T-1 expression vector and transformed into DH5 α cells following instructions. Positive vectors were sequenced by Genscript (Genscript, Nanjing, China) using T7 primers and transformed into the *Escherichia coli* strain BL21 (DE3). Purified recombinant protein was made according to De Angelis et al. [14]. The protein concentration post-dialysis was 0.5 mg/ml as determined by a Bradford assay referenced to a bovine serum albumin standard. The protein was cleaved with thrombin and then purified according to the manufacturer's instructions (GE, USA) to produce GST-free Bm-iAANAT2 at a protein concentration determined by the Bradford assay. After Centricon ultrafiltration (Millipore, USA), the protein was stored at -80°C to maintain stable enzyme activity.

Plasmid Construction

To generate the effector vector containing cDNA for *Bm-iAANAT2* RNAi, a segment of *Bm-iAANAT2* coding sequence was cloned from *B. mori*. The primer sequences were (up1) 5'-ccggaattcTCACGTTTTATTATGAACA-3', (down1) 5'-cgcggatccTTCCAAGTGACAAA GTT -3', (up2) 5'-cccaagcttTTCCAAGTGACAAAGTT-3', and (down2) 5'-ctagtctagaTCAC GTTTTATTATGAACA-3', which contained *Xba*I, *Hind*III, *Bam*HI, and *Eco*RI sites (underlined). The two PCR fragments (with primer pairs of up1/down1 or up2/down2) were treated with restriction enzymes and ligated tail to tail into multiple cloning sites of the vector PMD-18-T simply to form the vector PMD-18-T-*Bm-iAANAT2*. An intron from the fibroin light chain gene was PCR-amplified from silk gland genomic DNA with primers (up) 5'-TGCTCTAGAGCAGATGTTAAGCTTGGCTGAAACAGACAAG-3' and (down) 5'-CCGGAATTCCCGCAGATAGGATCCCGGTGAGCTCATCGATTC-3' containing *Hind*III and *Bam*HI sites (underlined). The amplified fragment was treated with *Xba*I and *Eco*RI and ligated into the vector PMD-18-T-*Bm-iAANAT2*. The 3xp3 promoter was cloned into vector L4440 (up) 5'-ccgctcgagGTTCCCACAA TGGTTAAT-3' and (down) 5'-ccggaattcATGGTGGCGACCGGTGG-3' containing *Xho*I and *Eco*RI sites. The double-stranded *Bm-iAANAT2* *Bm-iAANAT2*-intron-SV40-polyA fragment from this construct was excised with *Bg*III and *Xho*I and ligated into the vector PL4440-3xp3 to form the vector p3xp3-*Bm-iAANAT2*. Subsequently, the p3xp3-*Bm-iAANAT2*-SV40 polyA fragment was excised and inserted into the vector pSLfa1180fa to obtain the vector pSLfa1180fa-3xp3-*Bm-iAANAT2*. Finally, the 3xp3-*Bm-iAANAT2*-SV40 polyA fragment was inserted into the vector pBac (pBac[3xP3-*DsRedaf*]) to generate the effector vector pBac (3xp3-*DsRedaf*-3xp3-*Bm-iAANAT2*). The sequence of the PCR products and resulting plasmids were confirmed by sequencing performed by Genscript (Genscript, Nanjing, China).

Transgenesis and Screening of Silkworms

The vector pBac3xp3-*DsRedaf*-3xp3-*Bm-iAANAT2* was purified with the QIAGEN Plasmid Midi kit (Qiagen K.K., Japan). A nonautonomous plasmid, pHA3PIG, was used as the helper for the production of transposase [15]. Embryo injection was performed as described by Tomita [16]. After injection, the embryos were allowed to develop at 25°C . G0 moths were mated randomly, and the G1 embryos were screened by detecting DsRed fluorescence under an Olympus SZX12 fluorescent stereomicroscope (Olympus, Tokyo, Japan). G1-positive transgenic individuals were mated within the same family to generate the G2 descendants.

Quantitative RT-PCR

Total RNA was isolated from the head of transgenic and wild-type larva at the 4th instar stage. Quantitative RT-PCR was performed on an ABI Prism 7000 sequence detection system (Applied Biosystems) using SYBR green. The primers used for *Bm-iAANAT2* were (up) 5'-GTCTTTCAAAGCCGTCGAG-3' and (down) 5'-TTTAGGATTGGGACATCGAA-3'. The primers used for *Bm-iAANAT* were (up) 5'-TGAATCTCGCCGTCATCTG-3' and (down) 5'-GAAACTCCATCGCTCAAGGTAG-3'. Internal control primers for the eukaryotic translation initiation factor 4A (silkworm microarray probe ID: sw22934) were (up) 5'-TTCGTA CTGGCTCTTCTCGT-3' and (down) 5'-CAAAGTTGATAGCAATTCCT-3'.

Analysis of Dopamine Content

Dopamine was extracted from the head of larva at the 4th instar stage of each strain (200 mg of tissue from the head). The tissues were homogenized in 0.5 ml of 1.2 M HCl containing 5 mM ascorbic acid in a centrifuge tube and centrifuged at 14,000 rpm at 4 °C for 10 min. Supernatants were incubated at 100 °C for 10 min and then added to 0.5 ml chloroform and shaken before further centrifugation at 12,000 rpm at 4 °C for 10 min. Supernatants were analyzed by HPLC. Chromatography was performed on a Waters e2695 separations module HPLC equipped with a Waters 2475 multi λ fluorescence detector and an xbridge C18 (5 m, 4.6 150 mm) column. The flow velocity of the mobile phases (69.5 mM KH₂PO₄, 5.5 mM K₂HPO₄ (pH 5.8): methanol (Sigma, St. Louis, MO) 98:2) was 1 ml/min of isocratic elution. The column elution was monitored at 280 nm. The standard sample was dopamine hydrochloride (Sigma, St. Louis, MO).

AANAT Activity Assay

The head was homogenized in 1 ml of ice-cold 0.25 M potassium phosphate buffer (pH 6.5) containing 1.4 mM acetyl-CoA (Sigma, St. Louis, MO). The homogenate was centrifuged at 12,000g for 10 min at 4 °C. Aliquots (75 μ l) of the supernatant were mixed with 25 μ l of 8 mM tryptamine (Sigma, St. Louis, MO) in 0.25 M potassium phosphate buffer (pH 6.5) and incubated at 37 °C for 5 min. The reaction was stopped by the addition of 20 μ l of 6 M perchloric acid. After centrifugation at 12,000g for 10 min at 4 °C, 4 μ l of the supernatant was subjected to HPLC using the same column and fluorometric detector as those used for melatonin determination. The detector was set to an excitation wavelength of 285 nm and an emission wavelength of 360 nm. The mobile phase consisted of 50 mM phosphoric acid, 33 % methanol (vol/vol), and 0.65 mM sodium octyl sulfate adjusted to pH 3.5 with sodium hydroxide and pumped at a flow rate of 1.5 ml/min. Peaks were identified by retention time, and *N*-acetyltryptamine was quantified by peak height. *N*-Acetyltryptamine was synthesized from tryptamine and acetic anhydride.

Results

Identification and Biochemical Characterization of *Bm-iAANAT2*

In order to identify new AANAT gene from *B. mori*, we used the fragment obtained from ddRT-PCR to search the *B. mori* EST database (<http://sgp.dna.affrc.go.jp/>). The closely matching EST sequences were jointed into a novel *AANAT* gene, which contains 1119 bp

bases and encodes a protein of 233 amino acid residues. cDNA was generated from extractions of the fat bodies of 5th instar *N4* larvae and used as PCR template to amplify the candidate gene (Fig. 1a). The deduced amino acid sequence was similar to Bm-*i*ANAT with 57 % amino acid identity. We thus named it as *Bm-iANAT2* (GenBank accession number KJ865242). The calculated molecular mass was 26,301 Da, and the isoelectric point of the protein was 5.55. Analysis with ClustalX and MEME revealed that Bm-*i*ANAT2 belonged to the *N*-acetyltransferase (GNAT) superfamily and contains five motifs (Fig. 1b). A BLAST search in NCBI suggested that the motifs 1 and 3 are specific to the insect AANATs. Motifs 4 and 5 appeared to correspond to bind acetyl-CoA and *N*-acetyltransferase domain, respectively.

Tissue Expression of *Bm-iANAT2*

We examined the expression profiles of *Bm-iANAT2* at the transcriptional levels (Fig. 2). The transcripts from nine different tissues were investigated on the third day of the 5th instar larvae. The results showed that *Bm-iANAT2* gene was strongly expressed in the head, fat body, midgut, and integument tubules, and a weak signal was detected in the ovary, testis, and

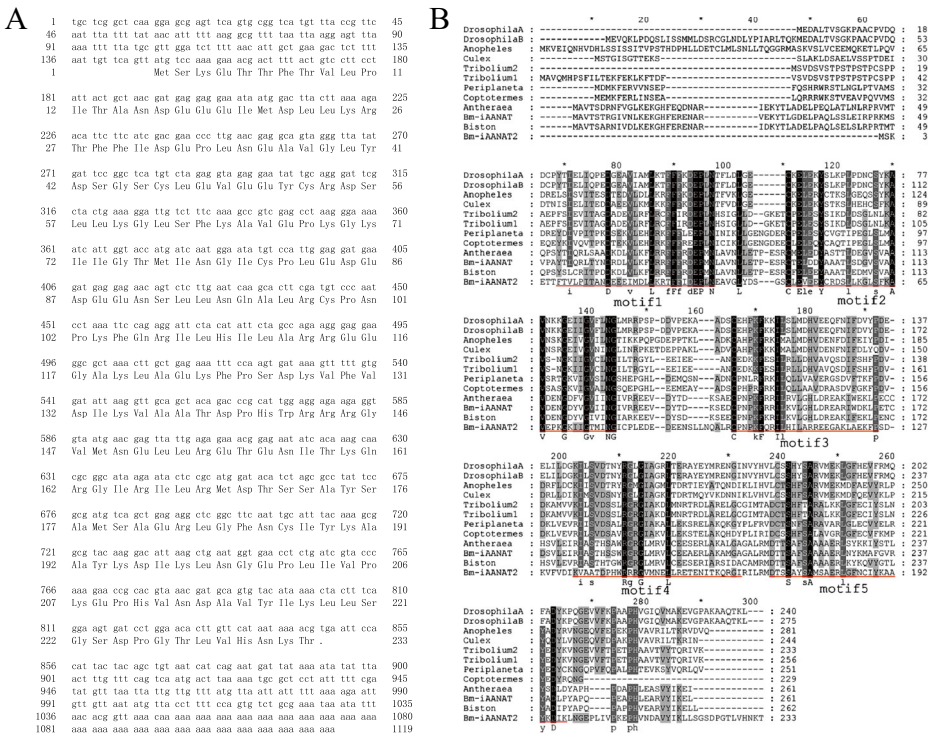


Fig. 1 a Nucleotide and deduced amino acid sequences of *Bm-iANAT2*. b Multiple alignment of *Bm-iANAT2* and AANAT proteins identified from other insects. Residues with black backgrounds are conserved residues. Residues with grey backgrounds are similar residues. *Drosophila melanogaster* dopamine *N*-acetyltransferase, isoform A (GenBank #: NP_523839), *D. melanogaster* dopamine *N*-acetyltransferase, isoform B (GenBank #: NP_995934), *Culex quinquefasciatus* (GenBank #: XP_001865527), *Anopheles darlingi* (GenBank #: ETN67902), *Bombyx mori* (GenBank #: NP_001073122), *Biston betularia* (GenBank #: ADF43200), *Coptotermes formosanus* (GenBank #: AGM32619), *Tribolium castaneum* dopamine *N*-acetyltransferase isoform 1 (GenBank #: NP_001139380), *Tribolium castaneum* dopamine *N*-acetyltransferase isoform 2 (GenBank #: NP_001139379), *Periplaneta Americana* (GenBank #: BAC87874), *Antheraea pernyi* (GenBank #: ABD17803)

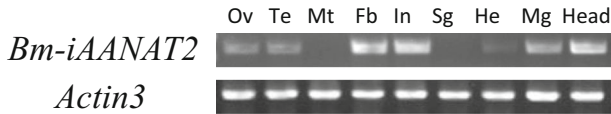


Fig. 2 Tissue expression pattern of *Bm-iAANAT2* at the transcriptional level. Total RNAs of nine different tissues from the third day of the fifth instar larvae were used in the RT-PCR analysis. The silkworm *Actin 3* gene was used as the control. *In* integument, *Mg* midgut, *Sg* silk gland, *He* hemocyte, *Fb* fat body, *Mt* Malpighian tubule, *Te* testis, *Ov* ovary

hemocyte. *Bm-iAANAT2* expressed in the head and integument which tissues needed frequent melanin synthesis and cuticle hardening. It indicates that the gene may take part in the melanin biosynthetic pathway.

Detection of AANAT Activity In Vitro

The *Bm-iAANAT2* gene was expressed in *E. coli*, and recombinant proteins with GST-tags were purified with Glutathione Resin (Genscript, Nanjing, China). Gel electrophoresis of the *Bm-iAANAT2* and GST fusion proteins revealed specific bands corresponding to molecular weights of 25 and 50 kDa, respectively (Fig. 3a).

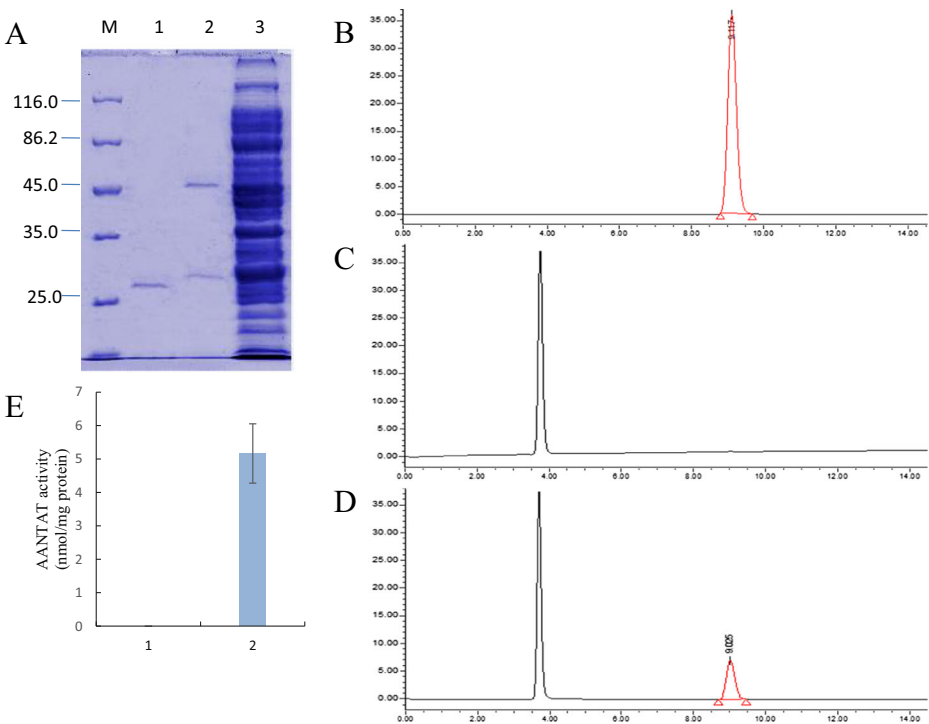


Fig. 3 Heterologous expression of the *Bm-iAANAT2* gene in an *E. coli* expression system and enzymatic activity of recombinant *Bm-iAANAT2*. **a** SDS-PAGE analysis of the heterologous expression for *Bm-iAANAT2*. Lane 1 GST-free *Bm-iAANAT2*, lane 2 GST-*Bm-iAANAT2*, lane 3 soluble protein in the supernatant. **b** *N*-Acetyltryptamine standard (*N*-acetyltryptamine, 9.117 min). **c** Control enzymatic reaction without the recombinant protein. **d** Enzyme assay of the recombinant *Bm-iAANAT2*. **e** Activity of recombinant *Bm-iAANAT2*. 1 Control enzymatic reaction without the recombinant protein. 2 Enzyme assay of the recombinant *Bm-iAANAT2*

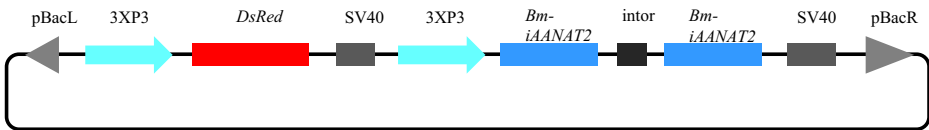


Fig. 4 Physical map of activator and effector transgenic vectors to create a *Bm-iAANAT2* RNAi system. The activator transgenic vector is pBac (3xp3-*DsRedaf*-BmA3-*Bm-iAANAT2*). The transgenic line screening marker is a red fluorescent protein gene (*DsRed*) in conjunction with a 3xp3 expression promoter specific to the eye

AANAT activity in silkworm heads was assayed using the method of Thomas et al. [17] with the modification that excess substrates were added to the purified protein. As shown in the reversed phase HPLC chromatograms, tryptamine from the product of the putative *Bm-iAANAT2* was transformed into *N*-acetyltryptamine, whereas there was no detectable transformation in the control group (Fig. 3b–d). These results suggest that recombinant *Bm-iAANA* T2 protein expressed in vitro exhibits weak AANAT activity (Fig. 3e).

Silkworm *Bm-iAANAT2* Knockdown Mutant

In order to explore the function of the *Bm-iAANAT2* gene in silkworm, we developed a transgenic RNAi-inhibited *Bm-iAANAT2* gene system with a 3xp3 promoter, a system that has been used in many other studies [18, 19]. The 3xp3 promoter is an artificial promoter that drives gene expression in the silkworm nervous system [20]. A physical map of the plasmids in the *Bm-iAANAT2* RNAi system is illustrated in Fig. 4. The *Bm-iAANAT2* sense and antisense cDNA sequences were joined tail to tail and inserted downstream of the 3xp3 promoter in order to transcribe *dsBm-iAANAT2*. Plasmids contained the enhanced red fluorescent protein gene (*DsRed*) to serve as a screening marker for transgenic lines.

The 1078 eggs of the *N4* strain were microinjected, and the outcomes of the transgenesis are summarized in Table 1. We obtained positive transgenic individuals with *DsRed* expression in 16 broods in the G1 embryos of the *N4* strain (Table 1). The reporter gene *DsRed* allowed us to detect positive transgenic silkworm, and its expression is driven by an eye and nervous tissue-specific promoter, 3xp3. In this case, we detected transgenic individuals by their red eyes (Fig. 5a). The insertion of a foreign gene into the genome of the transgenic lines was confirmed by inverse PCR using genomic DNA extracted from their parents which were positive transgenic moths (Table 2). A search in SilkDB (<http://www.silkdb.org>) confirmed that these junction sequences were derived from the silkworm genome.

To avoid analyzing unexpected positional effects, we selected three positive transgenic broods derived from different parents for further study.

Inhibition of *Bm-iAANAT2* Increases Melanin Deposition in Instar Larvae and Adults

We found that inhibition of *Bm-iAANAT2* led to body color changes in the transgenic lines (Fig. 5b). Black coloration mainly developed on the heads of larvae in the G1 transgenic group compared to coloration in the control group of wild-type silkworms. Some of the lateral

Table 1 Summary of microinjection for generating transgenic silkworm lines

| No. of injected embryos | No. of Hatched larvae | No. of red+ pupa | No. of broods with red+ |
|-------------------------|-----------------------|------------------|-------------------------|
| 1078 | 188 | 28 | 16 |

integument streaks in the transgenic group became pronounced, but were weak compared to coloration on the head. This difference was obvious even during the entrance eclosion stages when the scales of transgenic moths were only slightly darker than in the control group.

To confirm that the black coloration in silkworm is caused by the accumulation of dopamine, we measured dopamine content in the heads of silkworms in the 4th larvae stage (Fig. 5c). HPLC analysis indicated that dopamine content was 29.29 $\mu\text{g/g}$ in the control groups and significantly higher (35.96, 35.98, and 37.09 $\mu\text{g/g}$) in the three transgenic groups. Previous report has indicated that dopamine is a precursor for melanin synthesis and is a substrate of AANAT. To investigate whether the accumulation of dopamine is due to the change of AANAT activity, we measured the AANAT activity in both the transgenic silkworms and the control group (Fig. 5d). Three repetitions were done, and each repetition contains ten heads. HPLC analysis indicated that AANAT activity in the three transgenic groups was 290.87, 288.03, and 289.5 pmol/min/head, but AANAT activity in the control group was measured as high as 340.28 pmol/min/head. This correlation is suggestive that the melanin patterning is related to *Bm-iAANAT2* gene activity.

We also analyzed the changes of *Bm-iAANAT* and *Bm-iAANAT2* mRNAs in the transgenic groups (Fig. 5e). The real-time PCR results indicated that our interference system succeeded in knocking down the expression of the *Bm-iAANAT2* gene to 87.7, 87.4, and 87.5 % lower than expression levels in the control group. However, we found that the *Bm-iAANAT* mRNA did not show significant difference between the transgenic and control groups, indicating the phenotype in the transgenic group is due to the knockdown of the *Bm-iAANAT2*.

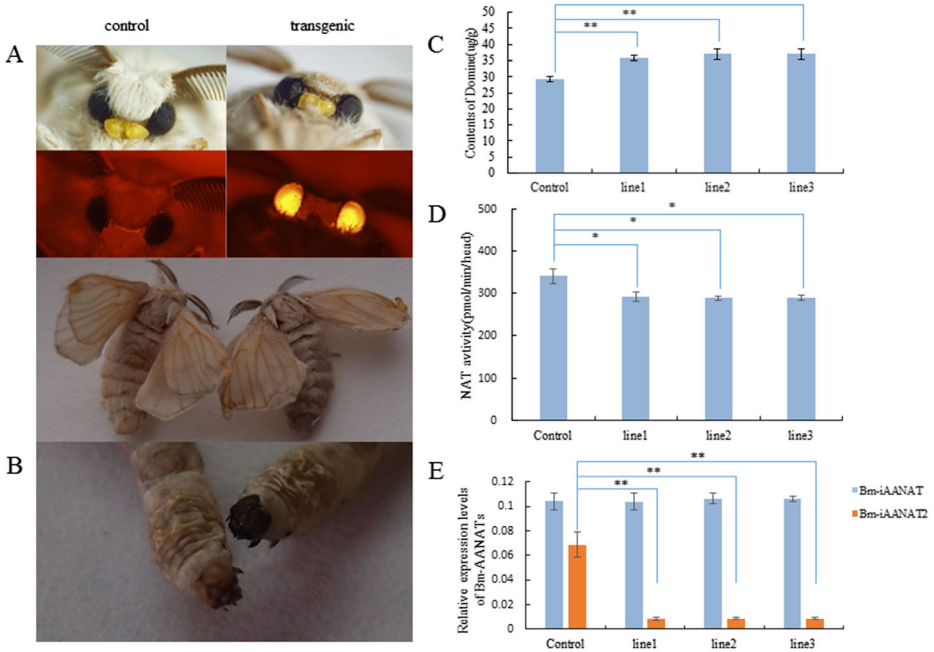


Fig. 5 Effect of knockdown *Bm-iAANAT2* gene using an interference system. **a, b** Phenotype of silkworm with a knockdown *Bm-iAANAT2* gene with interference system in **a** adults and **b** 4th instar larvae. **c** Levels of dopamine ($\mu\text{g/g}$) in silkworm heads in the 4th instar larvae. **d** AANAT activity (pmol/min/head) in silkworm heads in the 4th instar larvae. **e** Relative expression levels of *Bm-iAANAT2* in silkworm heads in the 4th instar larvae. Control wild-type silkworm; lines 1–3 transgenic lines 1, 2, and 3 (Student's *t* test; $n=3$, $*p<0.05$; $**p<0.01$)

Table 2 Inserted loci of the three transgenic lines

| | Scaffold | 5'Genomic sequences | 3'Genomic sequences |
|--------|-----------|-----------------------------------|---------------------|
| Line 1 | nscaf2833 | GGCTAATTTTTGA-TTAA-piggyBac-TTAA- | TCGAGACCCGTTGAG |
| Line 2 | nscaf3031 | AAACAAACATTTA-TTAA-piggyBac-TTAA- | CGTAACTTAGTGATA |
| Line 3 | nscaf3098 | GTTCGCTGTGTC-TTAA-piggyBac-TTAA- | TCGTCGCGGTCTGCG |

Discussion

Melanism is also well studied in other insects [21, 22]. Dopamine is a key compound for both sclerotization and melanin formation. There are four parallel branches in the tyrosine metabolic pathway that can produce substances that influence the color patterns of insects. The downstream products of dopa-melanin or dopamine-melanin pigments combine with cuticular proteins and are ossified in insect integuments to result in a black color. When the precursors dopamine-*N*-acetyldopamine or *N*- β -alanyldopamine are present in concentrated amounts, the integument will be yellow or colorless [23–27]. AANAT could catalyze dopamine to dopamine-*N*-acetyldopamine and indirectly affects melanin synthesis in insects. In this study, we found a new *AANAT* gene from *B. mori*, which contains essential motifs found in other insect *AANAT* genes and shared 57 % identity with the reported *AANAT* gene, suggesting that it is a member of the *AANAT* gene family and thus named it as *Bm-*iAANAT2**. Many members of this gene family have been found in bacteria, fungi, vertebrates, and insects. In some insects, such as *Drosophila* [28, 29], there also are two *AANAT* genes. In order to investigate the function of the *Bm-*iAANAT2** gene, recombinant proteins were expressed in the *E. coli*. Recombinant proteins showed weak AANAT activity to catalyze the conversion of tryptamine to *N*-acetyltryptamine. We think that the weak AANAT activity is due to the *E. coli* expression system that does not allow the post-translational modification. However, weak AANAT activity was still enough to show the function of the *Bm-*iAANAT2** gene.

In previous reports, dopamine existed in the silkworm heads especially in the nervous system [30], so we construct a specific interference system using eyes and nervous-specific promoter to analyze the function of the *Bm-*iAANAT2** gene. We speculated that knocking down of the *Bm-*iAANAT2** gene would change the dopamine levels in the silkworm heads. To avoid any unexpected positional effects, we selected three positive transgenic broods derived from different parents for further study. Our experimental results found that black coloration presented mainly on the head, and dopamine also accumulated in the heads of the three transgenic lines. Moreover, our real-time PCR results indicate that there is no significant difference in the expression levels of *Bm-*iAANAT** mRNA in the transgenic and control groups. In silkworm, AANAT is mainly encoded by *Bm-*iAANAT** and *Bm-*iAANAT2** genes. The protein level of these two proteins will influence the total AANAT activity. Because the amino acid sequence of *Bm-*iAANAT2** is similar to *Bm-*iAANAT**, the antibody may cross-reactive between *Bm-*iAANAT2** and *Bm-*iAANAT**. It is hard for us to quantitatively determine the change of the *Bm-*iAANAT2** protein in transgenic groups. However, according to the result of qPCR and AANAT activity assay, it is obvious that with the inhibition of *Bm-*iAANAT2** gene, the *Bm-*iAANAT2** protein is decreased in transgenic groups and finally decreased the total AANAT activity. According to the above results, we convinced that the melanin synthesis in transgenic silkworm head is due to the knockdown expression of the *Bm-*iAANAT2** gene. This result is inconsistent with the C12 gene in the study of Zhan et al. [13]. We think that the observed inconsistency may be caused by different major genes in different silkworm varieties.

In summary, we identified a new arylalkylamine *N*-acetyltransferase gene (*Bm-iAANAT2*) in silkworm and provide evidence that the *Bm-iAANAT2* gene plays an important role in melanin synthesis. We hope that our research will extend our understanding of melanin synthesis in the silkworm and other insects.

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