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A visible dominant marker for insect transgenesis

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Transgenesis of most insects currently relies on fluorescence markers. Here we establish a transformation marker system causing phenotypes visible to the naked eye due to changes in the color of melanin pigments, which are widespread in animals. Ubiquitous overexpression of arylalkylamine-*N*-acetyl transferase in the silkworm, *Bombyx mori*, changes the color of newly hatched first-instar larvae from black to a distinctive light brown color, and can be used as a molecular marker by directly connecting to baculovirus *immediate early 1* gene promoter. Suppression of black pigmentation by *Bm*-arylalkylamine-*N*-acetyl transferase can be observed throughout the larval stages and in adult animals. Alternatively, overexpression in another gene, *B. mori* β-alanyl-dopamine synthetase (*Bm-ebony*), changes the larval body color of older instars, although first-instar larvae had normal dark coloration. We further show that ectopic *Bm*-arylalkylamine-*N*-acetyl transferase expression lightens coloration in ladybird beetle *Harmonia axyridis* and fruit fly *Drosophila melanogaster*, highlighting the potential usefulness of this marker for transgenesis in diverse insect taxa.

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Genetic modification of insects is an essential and basic technique for studying developmental biology, as well as for applications such as pest control and recombinant protein production[1]. Transgenic techniques have been developed for many dipterans and lepidopteran insects[2,3], and also for insects in other orders such as the red flour beetle *Tribolium castaneum*[4], the ladybird beetle *Harmonia axyridis*[5], the sawfly *Athalia rosae*[6] and the cricket *Gryllus bimaculatus*[7].

In *Drosophila melanogaster*, transgenic flies are generally identified by mutant-based genetic markers with visible phenotypes (for example, the *white* gene for eye pigmentation)[8]. However, most insect species do not have a set of suitable recipient mutant strains and the functional transgene to rescue the phenotype. Instead, fluorescence markers such as 3xP3-enhanced green fluorescent protein (EGFP), which drives the eye-specific expression of green fluorescent protein, are used[9–12]. As this transgene is dominant, it is especially useful for nontraditional model insects. However, screening under a fluorescence microscope is a laborious procedure for large-scale projects, and insect eyes normally have dark pigmentation, which makes detecting fluorescence difficult. Thus, the development of a dominant visible marker applicable to various strains would be beneficial for studies of non-Drosophila insects.

For transgenic screening, it would be convenient if the altered phenotype could be easily identified externally at an early developmental stage. Suitable characteristics include unique pigmentation of eggs or neonatal larvae. One of the major pigments in insects is melanin, which is usually black or brown in color and is widespread in the exoskeletal cuticle[13–19]. Melanin-related pigments are mainly derived from dopamine[18,20,21], which is synthesized from tyrosine by the enzymes tyrosine hydroxylase and dopa decarboxylase.

Besides black and brown melanin pigments, dopamine is involved in the synthesis of light-coloured pigments and also in sclerotization. *N*-β-alanyl-dopamine (NBAD), a derivative of dopamine, is a precursor for the yellow/red cuticular pigments of *Drosophila*[18] and the larva of swallowtail butterfly, *Papilio xuthus*[15], and for NBAD-*o*-quinone, which crosslinks cuticle proteins in sclerotization[22,23]. Another derivative of dopamine, *N*-acetyl-dopamine (NADA), is a precursor for NADA-*o*-quinone, which is also involved in sclerotization of transparent or very light-coloured cuticles[22–24]. Dopamine is converted to NBAD by the β-alanyl-dopamine synthetase encoded by *ebony* gene, whereas NADA is synthesized from dopamine by arylalkylamine-*N*-acetyl transferase (aaNAT) activity.

The *ebony* mutant of *Drosophila* has darker overall coloration than wild type, and the ectopic expression of *Dm-ebony* changes the dark coloration of the adult abdomen to yellow[18]. In the silkworm *Bombyx mori*, the mutant allele of *Bm-ebony* is called *sooty*, which displays a distinct black pupal coloration instead of the normal light brown color[16]. The larvae and adults of *sooty* have a smoky color, which is less conspicuous than in pupae.

The identity of the gene responsible for aaNAT activity in cuticle sclerotization is less clear. In insects, aaNAT activity is not only involved in sclerotization, but also in neurotransmitter catabolism and melatonin precursor formation[25–32]. Two *aaNAT* genes have been identified in *Drosophila*[25,26], one of which is expressed in the brain. However, their involvement in sclerotization has not been reported, leaving open the possibility that an unidentified aaNAT enzyme may be involved in sclerotization in this insect. From the recent results of *in vitro* aaNAT activity analysis, *aaNAT*1 and *aaNAT*2 are assumed to be involved in sclerotization, and neurotransmitter inactivation in *Aedes aegypti*[33]. In *B. mori*, only one *aaNAT* gene, *Bm-aaNAT* has been characterized[28]. Notably, the mutant allele *melanism* (*mln*) does not display serious defects in sclerotization, but the adult shows darker overall pigmentation, and larvae show dark pigmentation in the head, forelegs and tail spot[34,35]. However, no reports exist for phenotypic effects of the *mln* mutant in the pupa and most parts of the larval epidermis[34,35]. Several *aaNAT*-like genes have been predicted in the *B. mori* genome based on sequence similarity[35], which suggests that some of them may function in sclerotization in different regions and stages. As the substrates of aaNAT include dopamine[27,31], it may be able to interfere with the synthesis of the dark dopamine-melanin. However, ectopic expression of *aaNAT* has never been tested *in vivo* in insects for converting dopamine to NADA.

In silkworms, the neonatal larvae are black in color due to melanin, and are traditionally called 'ants' in Japanese. The development of a transgene that could change the color of the cuticle in insects of this stage in a dominant manner would be beneficial for the production and maintenance of transgenic silkworm strains.

In this study, we tested whether ectopic expression of *Bm-aaNAT* and *Bm-ebony* interferes with the black melanin pigmentation of silkworms. Using the GAL4/UAS binary system, we found that ectopic expression of the *Bm-aaNAT* gene alters the coloration of neonatal larva from a dark ant-like color to light brown. In addition, overexpression of the *Bm-aaNAT* and *Bm-ebony* genes changed the pigmentation in the cuticle of second- to final-instar larva and the adult antennae from black/brown to a lighter color. We also demonstrate that expressing the *Bm-aaNAT* gene under the direct control of a constitutive baculovirus *immediate early 1* (*IE1*) gene promoter produced a color transformation in neonatal larvae. Furthermore, the *Bm-aaNAT* gene had ability to lighten dark melanin coloration in ladybird beetle *H. axyridis* and fruit fly *D. melanogaster*, which strongly indicates the utility of *Bm-aaNAT* gene as a dominant molecular marker in insects.

Results

***Bm-aaNAT* lightens body color of neonatal larvae in silkworm.** To express *Bm-aaNAT* and *Bm-ebony* in *B. mori*, we first used the binary GAL4/ upstream activating sequence (UAS) system. Effector vectors that carried the *Bm-aaNAT* gene or *Bm-ebony* gene linked to an UAS and an EGFP reporter gene under the control of the eye-specific 3xP3 promoter were constructed (Fig. 1a). The effector lines were generated by germline transformation. After sib mating and selection of G1 progeny based on the presence of EGFP fluorescence, G1 male moths were crossed with female moths of the *actin A3*-GAL4 driver line 193-2, which drives ubiquitous expression of GAL4, and can be identified by the 3xP3-DsRed2 marker (Fig. 1a)[36]. The overexpression of *Bm-aaNAT* and *Bm-ebony* was first tested in the w1-pnd strain, which is generally used to produce transgenic silkworms because the white-egg phenotype is convenient for fluorescence screening of the embryonic eye. In contrast to the blackish coloration of control *w1* neonatal larvae, the head and the body of the newly hatched larvae were light brown in color in all three *actin A3*-GAL4; UAS-*NAT* lines (Fig. 1b, Supplementary Fig. S1 online). This suggests that *Bm-aaNAT* interfered with the dopamine-melanin synthesis cascade in the cuticle of neonatal larvae, and produced NADA, leading to lighter melanin pigmentation. However, only a subtle change in coloration was observed in *actin A3*-GAL4; UAS-*ebony* neonatal larvae (Fig. 1c, Supplementary Fig. S1), although we checked several lines.

***Bm-aaNAT* and *Bm-ebony* suppress larval black pigmentation.** In wild-type silkworm strains, after the second instar, the epidermis is mainly white and opaque due to the accumulation of uric acid granules, and has several black markings consisting of

melanin and ommochrome pigmentation. To observe the effects of ubiquitous overexpression of *Bm-aaNAT* and *Bm-ebony* in older instar larvae, we chose to use the black striped strain *striped* (p^S; Fig. 2). This strain has a wide black stripe in each segment induced by the dominant *striped* gene p^S, in addition to the melanin pigmentation in eyespot markings of the second thoracic segment, crescent markings in the second abdominal segment, and star spot markings in the fifth abdominal segment, which are present in wild-type strains. We crossed the *striped* gene p^S homozygotes with *actin A3*-GAL4; UAS-*NAT* and *actin A3*-GAL4; UAS-*ebony* lines (Supplementary Fig. S2). As expected, the ectopic expression of *aaNAT* caused marked changes in pigmentation although the coloration of each instar differed. In the both second- and the third-instar larvae of *actin A3*-GAL4; UAS-*NAT* (p^S) lines, the black body stripes, and the crescent and star spot markings on the second and fifth abdominal segments were a very light tan color (Supplementary Fig. S3). In the fourth-instar larvae of these lines, the black body stripes were a light brownish grey color, and crescent markings and star spot markings were a lighter brown color. In addition, the brown-coloured area was larger compared with wild type, especially in the crescent markings (Supplementary Fig. S4). In the fifth instar, the stripes were light grey instead of black (Fig. 2). Notably, the pigmented areas of eyespot markings in the second thoracic segment, crescent markings, and star spot markings were markedly smaller and the shapes of the crescent and star spot markings were altered (Fig. 2). These observations indicate that the ectopic expression of *Bm-aaNAT* not only affects the synthesis of melanin pigments in neonatal larvae, but throughout the larval stages.

Although the *actin A3*-GAL4; UAS-*ebony* showed no marked changes in neonatal larval coloration, a change in pigmentation was observed from the second instar in p^S-crossed individuals. Like *actin A3*-GAL4; UAS-*NAT* (p^S) lines, larval pigmentation differed between stages, but was also different from *aaNAT*-overexpressing lines. The color of the markings in the second-instar larvae of *actin A3*-GAL4; UAS-*ebony* (p^S) lines changed from black to pale yellow. In the third-instar larvae, most of the black regions changed to a darker orange-yellow color, which was

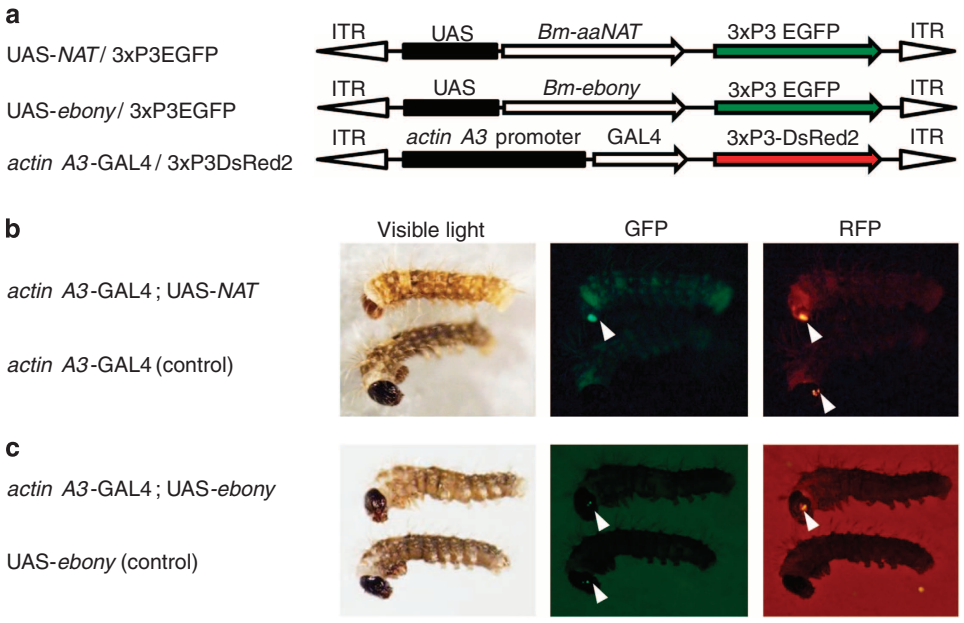

Figure 1 | *Bm-aaNAT* overexpression alters cuticle coloration in neonatal silkworm larvae. (**a**) Structures of UAS-*NAT*/3xP3EGFP, UAS-*ebony*/3xP3EGFP and *actin A3*-GAL4/3xP3DsRed2 (not to scale). 3xP3, eye-specific promoter; ITR, inverted terminal repeat of the *piggyBac* transposon. Effect of ectopic expression of *Bm-aaNAT* (**b**) and *Bm-ebony* (**c**) using the GAL4/UAS binary system (w1-pnd background). Left, bright field; middle, GFP; and right, red fluorescent protein (RFP). White arrowheads indicate fluorescence in the ommatidia.

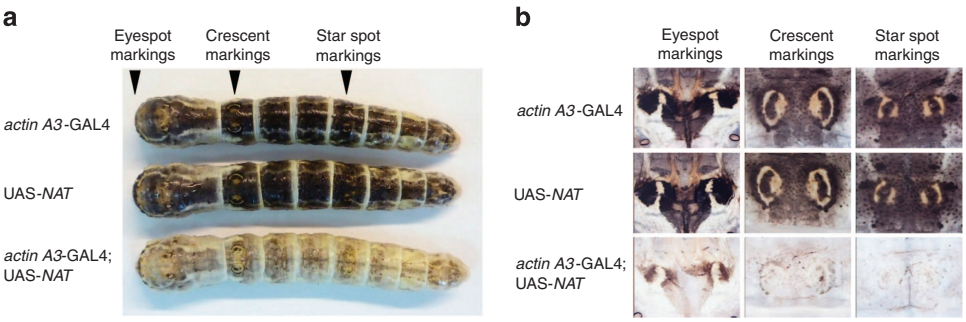

Figure 2 | *Bm-aaNAT* overexpression lightens pigmentation in the black striped silkworm strain. The fifth-instar larvae shown here have the dominant *striped* (p^S) genetic background. (**a**) Dorsal view of the larvae. The positions of the markings magnified in **b** are indicated by the arrowheads. (**b**) Magnified images of the larval markings.

different from that in *actin A3*-GAL4; UAS-*NAT* (p^S) larvae of the same instar (Fig. 3). Although black pigmentation was not observed in the third-instar larvae of *actin A3*-GAL4; UAS-*NAT* (p^S) lines, it remained in *actin A3*-GAL4; UAS-*ebony* (p^S) lines in several areas such as the margins of the crescent marking. In the fifth-instar larvae, ectopic *ebony* overexpression did not induce yellowish coloration, and the change of pigmentation was modest compared with that in younger larvae. However, they could be distinguished from non-overexpressing lines by the loss of pigmentation in the lateral area especially in areas surrounding the spiracles, and in the dorsal region of the second and third thoracic segments (Supplementary Fig. S5).

As the silkworm tracheae are also pigmented with black melanin, we inspected the tracheae of 3-day-old fifth-instar larvae of *actin A3*-GAL4; UAS-*NAT* and *actin A3*-GAL4; UAS-*ebony* lines, and found them to be pale brown in color, indicating that dopamine was converted into NADA or NBAD instead of dopamine-melanin (Supplementary Fig. S6 online). Taken together, our data show that ectopic expression of *Bm-aaNAT* affected melanization of the epidermal cuticle in larvae of all stages and the tracheae, and that ectopic expression of *Bm-ebony* affected the melanization of the second- to fifth-instar larvae and the tracheae.

***Bm-aaNAT* and *Bm-ebony* lighten adult antenna coloration.** No clear differences were observed between control insects and *actin A3*-GAL4; UAS-*NAT*- and *actin A3*-GAL4; UAS-*ebony*-carrying insects in the wing scales and body hair of adult silk moths, which are normally white. However, a distinct change in the color of the antennae was observed (Fig. 4). In contrast to the normal brown pigmentation, the antennae of *Bm-aaNAT*- and *Bm-ebony*-overexpressing insects were light brown in color. This suggests that the pigment of the antennae in adult silk moths is derived from dopamine-melanin, which was converted into NADA and NBAD by ectopically expressed *Bm-aaNAT* and *Bm-ebony*, respectively.

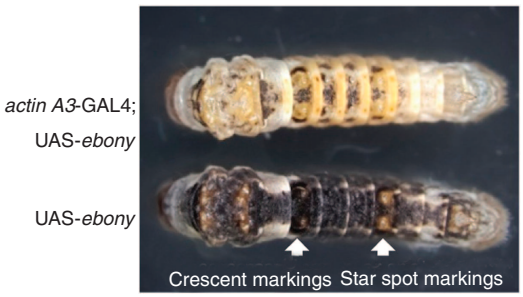

Figure 3 | *Bm-ebony* overexpression alters silkworm larval pigmentation in older instars. The black striped silkworm (p^S) strain, which the effect on melanin pigmentation is easy to observe is used. The dorsal view of third-instar larvae is shown. Ubiquitous overexpression of *Bm-ebony* changed most areas of black pigmentation.

Development of a dominant marker with the *Bm-aaNAT* gene. As the phenotype produced by ectopic *Bm-aaNAT* expression seemed to be ideal for a transformation marker, we next tested whether *Bm-aaNAT* directly linked to a promoter alters the black melanin coloration. We tested the *B. mori actin A3* promoter and baculovirus *IE1* gene promoter to drive expression of the transgene (Fig. 5a). Although no large difference in coloration was observed using the *actin A3* promoter, the body color of neonatal larvae carrying the *IE1-NAT* transgene was light brown (Fig. 5b, left panel, white arrowheads, Supplementary Fig. S7) in all eight G1 strains expressing EGFP fluorescence in the eye, and the difference in color was easily distinguishable without the aid of a microscope. The transformation efficiency for *IE1-NAT* transgene was 19% (8/43), which compared favourably with that achieved with the *actin A3*-EGFP transgene (14%) and 3xP3EGFP transgene (12%) using the same system[37]. In addition, no significant difference was detected in the survival rate, and developmental time was only delayed for average of 1 day in *IE1-NAT* G1 silkworms (average 81.3%, s.d. = 15.1%, $n = 4$) compared with their non-transgenic siblings (average 87.4%, s.d. = 3.9%, $n = 4$; $P = 0.47$, Student's *t*-test), and the subesequent generation also grew normally, and had no notable difference with non-transgenic siblings in fecundity (Supplementary Fig. S8, Supplementary Table S1). Furthermore, the light brown coloration was observed for larvae with 50% segregation obtained from an F1 backcross, which suggests that insertion of a single copy is enough to alter pigmentation. Moreover, when we used C146 (a commercial silkworm strain homozygous for the wild-type *w1* (*kynurenine 3-mono oxygenase*) gene, which has brown ommochrome pigments in the epidermis in addition to melanin pigments; and 1.9-fold cocoon yield of w1-pnd strain, Supplementary Fig. S8), as the host for germline transformation, G1 transformants could easily be distinguished without microscopy by their orange-brown color (Fig. 5b, right panel, white arrowheads), with only small decrease in cocoon weight (Supplementary Fig. S8), verifying that the *IE1-NAT* transgene can function as a dominant visible transgenic marker in practical wild-type silkworm strains.

***Bm-aaNAT* suppresses pigmentation also in other insect taxa.** Finally, to check the potential usefulness of *Bm-aaNAT* in diverse insect taxa, we tested heterologous expression of *Bm-aaNAT* in fruit fly *D. melanogaster* and ladybird beetle *H. axyridis*. First, we generated UAS-*NAT* effector fly lines with the same construct that used for silkworm, and crossed with *heat shock protein 70 (hsp)*-GAL4, and *pannier*-GAL4 (ref. 38) (which expresses GAL4 in a broad stripe along the dorsal midline of developing flies) lines. As a result, *hsp*-GAL4; UAS-*NAT* individuals had overall clearly lighter pigmentation and white bristles in the thoracic segment (Fig. 6a), and black pigmentation of *pannier*-GAL4; UAS-*NAT* individuals was diminished in the *pannier* expression domain (Fig. 6b, bracket), in a similar manner to the *pannier*-GAL4; UAS-*ebony* phenotype reported by (ref. 12). The light coloration phenotype was observed for both of the two UAS-*NAT*

Figure 4 | Ectopic expression of *Bm-aaNAT* and *Bm-ebony* lightens adult antenna coloration. Moths carrying only *actin A3*-GAL4/3xP3DsRed2 were used as controls.

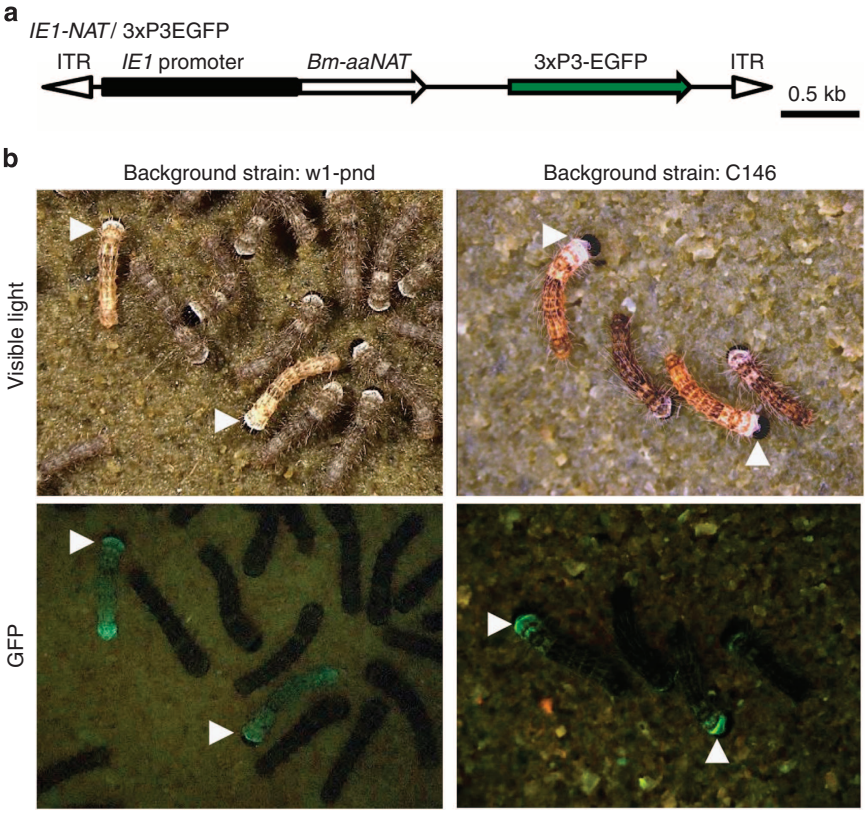

Figure 5 | Development of a dominant marker *IE1-NAT* that alters neonatal larval coloration. (**a**) Structure of the *IE1-NAT*/3xP3EGFP transgene. *Bm-aaNAT* expression was under the direct control of the constitutive baculovirus *IE1* promoter. (**b**) Neonate larvae carrying *IE1-NAT* transgene can be easily distinguished by its light coloration (white arrowheads), whether w1-pnd (*kynurenine 3-mono oxygenase* (*KMO*)-deficient strain, left panels) or commercial strain C146 (with intact *KMO* gene, right panels) is used as the host strain for transgenesis. Top panels, bright field; bottom panels, GFP filter.

effector fly lines we obtained. These results indicate that the ectopic expression of *Bm-aaNAT* suppress black coloration also in *Drosophila*. For the ladybird beetle *H. axyridis*, we used the *IE1* promoter and the *Drosophila hsp* promoter (Supplementary Fig. S9A) to drive *Bm-aaNAT* expression (Fig. 6c). Although the *IE1-NAT* transgene did not show any coloration effects, one of the three transgenic strains with *hsp-NAT* transgene produced markedly light-coloured larvae (Fig. 6c, Supplementary Fig. S9B), possibly due to the influence of a nearby enhancer. The light coloration phenotype of this strain was enhanced by heat shock treatment (Fig. 6c), and *Bm-aaNAT* expression was confirmed by reverse transcription–polymerase chain reaction (Supplementary Fig. S9C), supporting that the light coloration is caused by the effect of *Bm-aaNAT*. Overall, these results indicate that *Bm-aaNAT* has the ability to lighten melanin coloration also in several insect taxa other than Lepidoptera.

Discussion

We have shown that ectopic expression of *Bm-aaNAT* or *Bm-ebony* is sufficient to alter the black melanin pigmentation, which demonstrates their potential usefulness as dominant transformation markers. Although ubiquitous expression of *Bm-aaNAT* or *Bm-ebony* may affect the dopamine amount in the nervous system, there were no serious effects on fitness (Supplementary Fig. S8) and behaviour such as eating and mating in the present experiments. In addition to facilitating the screening procedure, it is also beneficial regarding the choice of host strains for germline transformation. For instance, in silkworms, *w1* mutant strain with white egg and eyes have been the feasible host for germline transformation because the eye-specific fluorescent markers are difficult to detect in wild-type strains with dark eggs and eyes. This has been a drawback for the analysis of most silkworm strains other than *w1*, and it is preferable to use commercial strains for production of biomaterials such as spider silk[39] because of their high productivity of silk. The use of *aaNAT* marker would be of benefit on such cases, and moreover, the widespread distribution of melanin-related pigments, conservation of the melanin synthesis pathway and ability of *aaNAT* to suppress black coloration even in flies and ladybird beetles (Fig. 6, Supplementary Fig. S9) suggest its potential as a transformation marker in diverse insect taxa. In the ladybird beetle, suppression of black pigmentation occurred only in the larval stage of a single strain out of three transgenic strains and was not observed in the adult stage, suggesting that the *Bm-aaNAT* expression in this strain is under the influence of a nearby enhancer. However, as the *IE1* promoter is derived from *Autographa californica* polyhedrosis virus, it is likely that *IE1-NAT* transgene could be directly applied to lepidopteran insects, which many species are known to be agricultural pests, and practical use of *Bm-aaNAT* gene should be possible for other taxa in combination with suitable promoters. Furthermore, *aaNAT* marker can be used in parallel with fluorescent markers because it does not interfere with the fluorescent signals, which enables the addition of an extra transgene.

The data presented in this study also have significant impact for the understanding of molecular basis of melanin/cuticle synthesis. Although NADA is the most common precursor for colourless cuticle in the melanin/cuticle synthesis pathway of insects[22,23], the gene involved in its synthesis had long remained

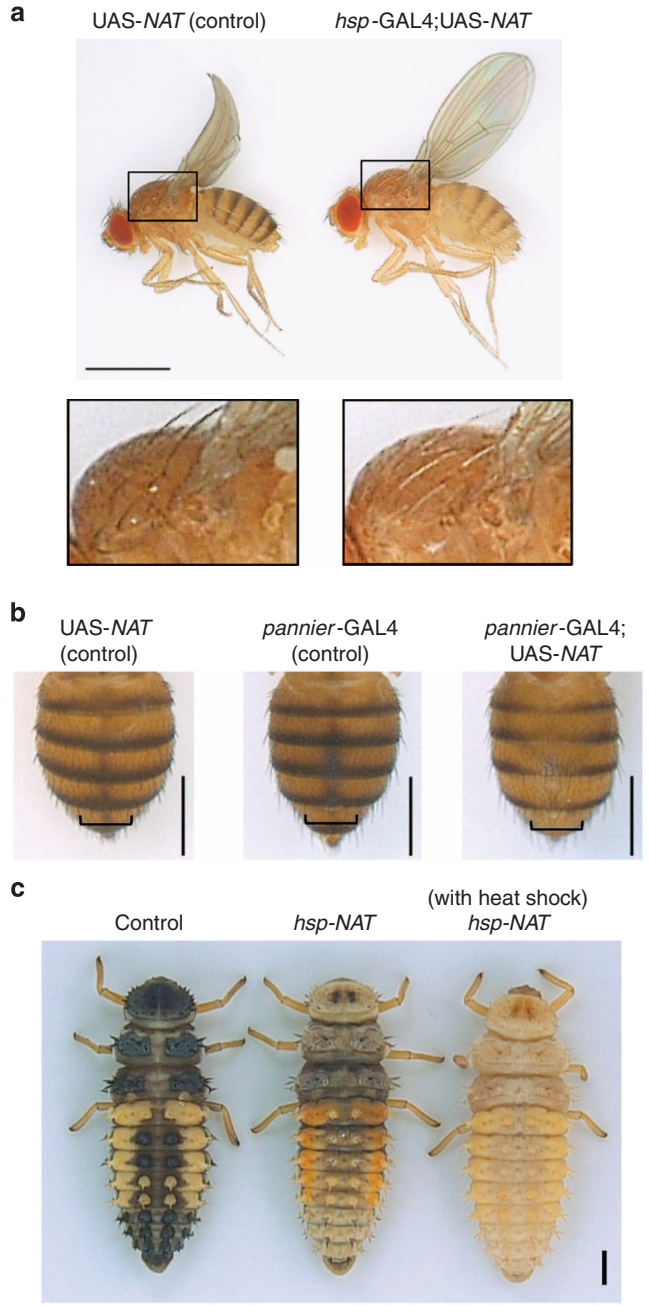

Figure 6 | *Bm-aaNAT* lightens coloration in other insect orders. (**a**,**b**) Effect of ectopic *Bm-aaNAT* expression in *D. melanogaster*. The flies are at least 4 days (100 h) old. Scale bar, 1 mm (**a**), 0.5 mm (**b**). (**a**) Shows the effect of *Bm-aaNAT* driven by *hsp*-GAL4. The fly shows overall lighter pigmentation, and as shown in the bottom panels (the magnified view of the square in the top panel) bristle coloration are also suppressed by *Bm-aaNAT* overexpression. (**b**) Shows the effect of *Bm-aaNAT* driven by *pannier*-GAL4, which drives expression in a broad stripe along the dorsal midline (brackets). Abdomen of adult flies is shown. (**c**) Effect of *hsp-NAT* transgene on *H. axyridis* fourth-instar larvae. Left, insect with no transgene; middle, insect with *hsp-NAT* transgene; right, insect with *hsp-NAT* transgene with heat shock treatment. Scale bar, 1 mm.

unclear, until the recent discovery that *Bm-aaNAT* gene was disrupted in the *B. mori* mutant *melanism*[34,35]. Our results provide a direct evidence that Bm-aaNAT has the ability to lighten coloration in adults as well as the whole epidermal cuticle in silkworm larvae. Although *Bm-aaNAT* mutation in the mutant *melanism* has little effect on larval coloration, overexpression of *Bm-aaNAT* in this study induced marked changes throughout the larval stages (Figs 1, 2, 4–6, Supplementary Figs S1, S3, S4 and S7 online). The *aaNAT* gene was conserved in other lepidopterans such as monarch butterfly *Danaus plexippus* (GenBank accession code EHJ67891.1), and also was detected in 23 species out of 25 arthropods (excepting tick *Ixodes scapularis* and jewel wasp *Nasonia vitripennis*) when searched by OrthoDB (http://cegg.unige.ch/orthodb4). Recently, upregulated *aaNAT* expression linked with yellow pigmentation was reported in *Heliconius* butterfly hindwing[40], which suggests that *aaNAT* functions as a modifier in melanin pigmentation of other insects.

As for *ebony*, it has been described as a suppressor of black coloration in *Drosophila* that delimits the black pigmentation on the wing and abdomen[18,41], and reported to change black melanin pigmentation to a tan color by ectopic expression (Fig. 7)[18]. Ebony is also reported to be associated with the yellow/red pigments in the larval eyespot markings and adult wing of the swallowtail and *Heliconius* butterflies[40,42–44]. Our results showing that ectopic *ebony* expression alters the color of melanin pigments in larvae and adults are consistent with these reports, and the conservation of the *ebony* gene in arthropods (24 out of 25 species in OrthoDB, not detected only in *I. scapularis*) suggest that it may contribute to light-coloured pigmentation in other lepidopterans.

Notably, ectopic expression of *Bm-aaNAT* and *Bm-ebony* had different effects in silkworm larvae. In general, *Bm-ebony* overexpression induced a more yellowish coloration than *Bm-aaNAT* in the black striped p^S strain (Fig. 7). This is consistent with the previous observations that cuticles sclerotized exclusively by NADA (product of *aaNAT*) are colourless or very lightly straw-coloured, and grow darker brown the more NBAD (product of *ebony*) dominates in the process (Fig. 7)[23]. Furthermore, in contrast to the control silkworms whose markings were black throughout the larval stage, coloration differed between stages in *Bm-aaNAT*- and *Bm-ebony*-overexpressing silkworms (Fig. 7). For example, p^S larvae with ectopic *Bm-aaNAT* expression showed gold-brownish coloration in the third and fourth instars (Supplementary Figs S3 and 4 online), and greyish coloration in the fifth instar (Fig. 2). As both aaNAT and Ebony use dopamine as a substrate and do not affect the production of dopa-melanin, the differences in the coloration between larval stages may reflect the increase of grey dopa-melanin in the elder instars (Fig. 7). In the control larvae, grey dopa-melanin may be concealed by black dopamine-melanin, leaving its presence undetectable.

In the *Bm-ebony*-overexpressing larvae, more significant differences in coloration between larval stages and areas were observed. It is noteworthy that only a subtle color change was observed in neonatal larvae and some areas remained black in the third-instar larvae with *Bm-ebony* overexpression (Figs 1, 3 and 7). As β-alanine is required for the conversion of dopamine to NBAD by Ebony (Fig. 7), a possible cause of these observations may be a difference in the uptake or accumulation of β-alanine. β-Alanine is produced by the action of the product of *black* gene (Fig. 7) in *D. melanogaster* and *T. castaneum*, whose adult *black* mutants are dark and black, respectively[45]. In the silkworm, a previous microarray analysis shows that the *black* gene is not expressed in the first instar[46], suggesting that ectopic *Bm-ebony* expression could not alter neonatal larval coloration because of lack of *β*-alanine. The ubiquitous effect of *aaNAT* may be because the widely abundant acetyl-coA is its cofactor for the conversion of dopamine to NADA.

Until now, the 'visible markers' used for insect transgenesis were based on *D. melanogaster* mutants and mostly restricted to the same species, due to the lack of mutants to use as host strains

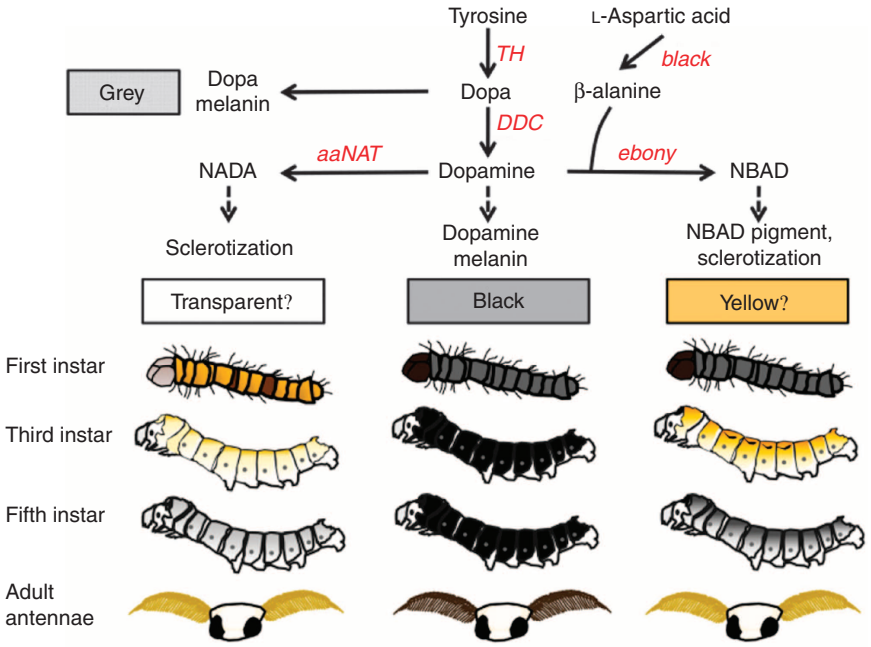

Figure 7 | Effects of overexpression of *Bm-aaNAT* and *Bm-ebony* on silkworm pigmentation. The *striped* strain p^S is used in the model for larval coloration. The melanin synthesis pathway is adapted from Futahashi *et al.*[16] The melanic pigmentation of silkworm larvae and adult antennae is affected by *Bm-aaNAT* and *Bm-ebony*, which use dopamine as substrate and produce transparent and yellowish cuticle, respectively. Notably, *Bm-aaNAT* effect on melanic pigmentation can be easily detected at the first instar, whereas *Bm-ebony* does not have significant effect in this stage. TH, tyrosine hydoxylase; DDC, dopa decarboxylase.

in other insects. As pigmentation is realized by the action of many genes, a dominant molecular marker that produces pigments where they did not originally exist seemed to be challenging. Our experiments indicate that a 'visible marker' based on melanin pigments can be developed using the opposite approach, that is, by reducing pigmentation. As this strategy led to visible changes in larvae of all stages and even in adults, the *aaNAT* transgene should enable the screening of transgenic insects during most of their lifetime (Fig. 7). The application of the *aaNAT* transformation marker should facilitate the use of transgenic techniques and promote molecular studies in insects.

Methods

Insects. The silkworm strains w1-pnd, *actin A3*-GAL4/3xP3-DsRed2 (*w1*)[47] and C146 are maintained at the Transgenic Silkworm Research Unit of NIAS, and the p^S strain at the Genetic Resource Center of NIAS. Silkworms were reared at 25 °C with mulberry leaves or artificial diets (Nihon Nosan Kogyo, Yokohama, Japan) under a 16-h light: 8-h dark photoperiod. *D. melanogaster* was reared on a standard medium at 27 °C. The embryos used as recipients for DNA injection to generate transgenic lines were Oregon R. The GAL4 lines used in this study is as follows; *hsp*-GAL4 (*w[*]; P{w[+mC]=GAL4-Hsp70.PB}2/CyO*) and *pannier*-GAL4 (ref. 38) (*y1 w1118; P{w+mW.hs=GawB}pnrMD237/TM3, P{w+mC=UAS-y.C}MC2,Ser1*). *H. axyridis* was purchased from Agrisect Inc. (Japan) and maintained as described previously[5,48]. The insects were reared at 30 °C, and heat shock cycles were applied from second instar. One cycle consisted of heat shock at 40 °C 1 h followed by recovery at 30 °C for 5 h.

Donor plasmid construction. To construct the pBacUAS-*NAT*/3xP3EGFP plasmid, the *Bm-aaNAT* ORF was amplified from p50T pupal wing cDNA with the primers pBacMCS-UAS-BlnI-Kozak-BmNAT1-ORF-s (5′-GCCTAGTAGACCTAGAAAAATCAAAATGGCTGTTACAAGTACAAGAGGC-3′) and pBacMCS-UAS-BlnI-BmNAT1-ORF-a (5′-GTATGGCTGACCTAGTTACAGCTCCTTAATGTAGACCCTG-3′), using Phusion Hot Start II High-Fidelity DNA Polymerase (New England Biolabs). The resulting PCR product was gel purified using a QIAquick Gel Extraction Kit (Qiagen) and subsequently cloned into the *Bln*I site of pBacUASMCS-3xP3EGFP using In-Fusion HD Cloning Kit (Clontech). The sequence was confirmed by the Sanger method using an ABI3130xl Genetic Analyser (Applied Biosystems). The *Bm-aaNAT* ORF sequence is the same as that with GenBank accession no. DQ256382.

To construct the pBacUAS-*ebony*/3xP3EGFP plasmid, the *Bm-ebony* ORF was amplified from p50T pupal wing cDNA with the primers pBacMCS-UAS-BlnI-Kozak-Bm-ebony-ORF-s (5′-GCCTAGTAGACCTAGAAAAATCAAAATGGGCTCCTTGCCTCGCGT-3′) and pBacMCS-UAS-BlnI-Bm-ebony-ORF-a (5′-GTATGGCTGACCTAGTTAGTCCAAGGGTTTCCAGCAC-3′). Subsequent procedures are the same as described above. The sequence of the *Bm-ebony* ORF was the same as that for GenBank accession no. AB455231.

To construct the pBac*IE1-NAT*/3xP3EGFP plasmid, the *IE1* portion of pIEx-2 (Novagen) was amplified with the primers hr5-IE1-BglII-s (5′-CATTGCGTAGATCTCGCGTAAAAC-3′) and hr5-IE1-BlnI-a (5′-AAAAAAAACCTAGGTCACTTGGTTGTTCACGATC-3′) and ligated into the 6-kb portion of *Bgl*II- and *Bln*I-digested pBacUASMCS-3xP3EGFP. The resulting plasmid was named pBac-*IE1*-3xP3EGFP. Next, the *Bm-aaNAT* ORF was amplified from pBacUAS-*NAT*/3xP3EGFP with the primers pBacIE1BlnIKozakBmNAT1ORF-s-2 (5′-AACCAAGTGACCTAGGAAAAATCAAAATGGCTGTTACAAGTACAAGAGGC-3′) and pBacMCS-UAS-BlnI-BmNAT1-ORF-a (5′-GTATGGCTGACCTAGTTACAGCTCCTTAATGTAGACCCTG-3′), and was subcloned into the *Bln*I site of pBac-*IE1*-3xP3EGFP using an In-Fusion HD Cloning Kit.

To construct the pBac-*hsp-NAT*/3xP3EGFP plasmid, the *hsp* promoter portion was amplified with the primers Dmhsp70p-BglII-s (5′-AAAAAAAAAGATCTAAGCTTCTAGAATCCCAAAACAAACTGG-3′) and Dmhsp70p-BlnI-a (5′-AAAAAAAACCTAGGGAATTCCCAATTCCCTATTCAGAG-3′), and *p10* gene 3′ UTR (to enhance protein production[49]) portion was amplified with primers p10-5′-BlnI-s (5′-AAAAAAAACCTAGGATGAATCGTTTTTAAAATAACAAATC-3′) and p10-3′-PstI-a (5′-AAAAAACTGCAGGTTAACTCGAATCGCTATCCAAG-3′), ligated into the 5.5-kb portion of *Bgl*II- and *Pst*I-digested pBacUASMCS-3xP3EGFP. The resulting plasmid was named pBac-*hsp*-3xP3EGFP. Next, the *Bm-aaNAT* ORF was amplified from pBacUAS-*NAT*/3xP3EGFP with the primers Syn21-NAT-s (5′-TTGGGAATTCCCTAGGAACTTAAAAAAAAAAATCAAAATGGCTGTTACAAGTACAAGAGGC-3′) and P10-NAT-a (5′-AACGATTCATCCTAGGTTACAGCTCCTTAATGTAGACCCTG-3′), and was subcloned into the *Bln*I site of pBac-*hsp*-3xP3EGFP using an In-Fusion HD Cloning Kit.

Generation of transgenic insects. Germline transformation of silkworms using the w1-pnd strain was conducted as described previously[50]. Transgenic silkworm generation using the C146 strain was conducted according to a previously described method[51], with modifications. To prevent entering into diapause, eggs were treated with 20% HCl for 30 min at 25 °C 4 h after oviposition[51]. Donor and helper plasmid DNAs were injected into 476 eggs 30 min to 3 h after acid treatment. After injection, the embryos were incubated at 25 °C to allow them to hatch. The hatch rate for HCl-treated C146 eggs was 26% (125 out of 476) for injected eggs and ~90% without injection. G1 screening was conducted by visual inspection on day 1 of first instar and was confirmed by fluorescence microscopy.

Germline transformation of *D. melanogaster* was conducted as described previously[52,53], using Oregon R as the host strain. *PiggyBac*-mediated germline transformation of ladybird beetle *H. axyridis* was conducted as described by Kuwayama *et al.*[5] Transgene screening was conducted by fluorescence microscopy at first instar.

Fitness assessment of *IE1-NAT* silkworms. For each experiment, non-transgenic siblings were used as control. Survival rate, percentage of eclosed adults compared with first-instar larvae; developmental time, amount of days it took from hatching to eclosion; fecundity, number of eggs laid per female within 24 h of separation from male; coccon weight, weight of coccon after removal of pupa.

Expression analysis of *Bm-aaNAT* in *H. axyridis*. Total RNA was isolated from *H. axyridis* larva just before third moult using ISOGEN (Nippon gene), and reverse transcribed with a random primer (N6) using the first-strand cDNA synthesis kit (GE Healthcare) as previously described[16]. PCR conditions for *Bm-aaNAT* and *Ha-rp49* (*H. axyridis ribosomal protein 49* gene) were as follows: 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s. Primers used for PCR are as follows; Ha-rp49 no. 1 (5′-GCGATCGCTATGGAAAACTC-3′), Ha-rp49 no. 2 (5′-TACGATTTTGCATCAACAGT-3′), BmNAT1-F1 (5′-CTGCCGGAGACTT GTGACTC-3′) and BmNAT1-R2 (5′-CATCTTGTAATTGAGCCTCTC-3′).

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Author contributions

M.O., R.F., T.T. and H.S. designed research; M.O., K.U., J. H., T. O. and T.N., performed research; M.O. and T. N. analysed the data; M.O. wrote the paper with the help of R.F., T.T., T.N. and H.S.

Additional information

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