The BTB-ZF gene *Bm-mamo* regulate pigmentation in caterpillars

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Abstract:

Color pattern of insects is one of the most dazzling adaptive evolutionary phenotypes. However, the molecular regulation of this color pattern is not clear. In this paper, we found a transcription factor, *Bm-mamo*, is responsible for *bd* (*black dilute*) allele mutants in silkworm. It belongs to BTB zinc finger family, and is ortholog to *mamo* of *Drosophila melanogaster*, which It is found that this gene has conservative function in gamete production, and evolved a pleiotropic function in regulation of color patterns in caterpillar. We found that the *Bm-mamo* can comprehensively regulate the expression of related pigment synthesis and cuticular protein genes to form color patterns. This suggests that the deposition of pigment particles in caterpillars' epidermis requires not only the spatiotemporal expression of pigment synthesis genes, but also the correct expression of related cuticular protein genes. This study provides a new data for the setting of color patterns.

Background:

The insects, living under natural light, often have ingenious color patterns that provide great aid to them, such as in behavior[1], immunity[2], thermoregulation[3], UV protection[4], and especially in antagonist to visual predators by either aposematic[5], mimicry[6], cryptic color patterns or combination among them[7]. Impressively, the color patterns of insects, such as cryptic color, can deceive visual animals, based on their clever designed which highly similar the surrounding environment. In addition, the color patterns are divergent and convergent among populations[8]. Due to this gorgeous visual feature and highly active adaptive evolutionary phenotype, lead to the genetic basis and evolution mechanism of color patterns have been highly concern for a long

time.

The body color of insects is actually main formed by the absorption, reflection or/and diffraction of natural light by the body surface, or/and some appendage (such as bristles and scales)[9]. Thus, the difference in epidermal structure and the difference in pigment deposits can form divergent color patterns. Cuticular proteins (CPs) are the main structural proteins that form the epidermis. There are large number genes encoding cuticular proteins in arthropods genome. For example, in *Bombyx mori* over 220 genes belong to the family of CPs[10]. These cuticular proteins belong to more than 10 subfamilies, including CPR, CPF, CPFL, CPH, Tweedle, CPT, CPG and et al[11]. However, the fine localization molecular mechanism of cuticular proteins in epidermis is still unclear. Moreover, the biochemical metabolic pathways of pigments, which used for color patten of insect, such as melanins, pterins, ommochromes, have been reported [12]. The deficient of enzymes in pigments metabolism pathway can lead to the change of color pattern[13]. However, some pleiotropic factors, such as *wnt1*[14], *Apontic-like*[15], *clawless*, *abdominal-A*, and *Abdominal-B*[16], *bric* à *brac* (*bab*)[17], *Distal-less* (*Dll*)[18], play an important role in the regulation of color pattern. Nevertheless, the molecular mechanism by which these factors participate in the regulation of body color patterns is unclear. In addition, there are many undiscovered factors involved in the gene regulatory network (GRN) involved in the regulation of insect color patterns. Hence, the identification of new color pattern regulatory genes and the study of molecular mechanisms are helpful to further understand the color patterns plan.

Silkworm, *Bombyx mori* (*B. mori*), is a completely domesticated Lepidoptera, for more than 5000 years, is famous in silk fiber production [19]. Because of long-term domestication, artificial selection and genetic research, its genetic background is clear, and about 200 strains of mutants involved in color pattern have been preserved[20], which provides a good resource for color pattern research. The *black dilute* (*bd*) mutant, complied with the recessive Mendelian inheritance law, showed a dark gray larvae body color and its female is sterile. The bd^f (*black dilute fertile*) is one of its alleles, the body color is lighter than that of *bd* and the female is fertile (fig.1). The two mutants were mapped to a single locus on 22.9 centimorgan (cM) of linkage group 9 [20,21]. Because there are no pigmentation related genes reported at this locus. For it, research may discover a new color pattern related gene, which have aroused our concern.

Result:

Candidate gene of *bd* allele of silkworm

To identify the genomic region responsible for the *bd* alleles, the positional cloning of *bd* locus was performed. Due to the female infertility of the *bd* mutant and the female of its allele bd^f is fertile. So, we used bd^f and wild type Dazao strain as the parents for mapping analysis.

The 1162 back-crossed filial 1 (BC1M) generation individuals, between bd^f and Dazao, were used for fine mapping by using molecular markers (fig 2). It found that the genome region of about 390 kb (kilo base) was responsible for *bd* phenotype (table s1). This region included five predicted genes (*BGIBMGA012516*, *BGIBMGA012517*, *BGIBMGA012518*, *BGIBMGA012519* and *BGIBMGA014089*). And then, qRT-PCR (Fluorescence quantitative PCR) was performed, which found that *BGIBMGA012517* and *BGIBMGA012518* were significant down-regulation expressed at day 3 of five instar of larvae in the *bd* phenotype individuals (fig s1). In addition, the two genes are consistent expression trend in those strain, respectively. To determine the gene structure of the *BIGBMGA012517* and *BIGBMGA012518*, we using forward primers of the *BIGBMGA012517* gene and reverse primers of the *BIGBMGA012518* gene to amplify the cDNA from wild type strain Dazao. By gene cloning, the predicted genes *BGIBMGA012517* and *BGIBMGA012518* were proved to be one gene. For the convenience of description, we temporarily call it *12517/8* gene.

The *12517/8* gene produce two transcripts, the long transcripts contain an open reading frame (ORF) with 2397 bp, and the ORF of short one was 1824 bp, which share the same 5'-terminal in wild type strain Dazao (fig s2). The *12517/8* is significant low expression in bd^f mutant, and were found multiple variations in nearby region of this gene by analysis of comparative genome between bd^f and Dazao (table s2). In addition, the *12517/8* is silence completely due to the deletion of DNA fragments (about 168 Kb) from first intron to upstream, and insert 3560 bp in *bd* mutant (fig s3).

To estimate the function of the gene *12517/8*, we performed a BLAST search using its full-length sequence and found a transcription factor, *mamo* (*maternal gene required for meiosis*), in *D. melanogaster*, which had a highly sequence similarity to that of the *12517/8* gene (fig s4). So, we named the *12517/18* gene *Bm-mamo*.

The gene *mamo*, belong to Broad-complex, Tramtrack and bric à brac/poxvirus

zinc finger protein (BTB-ZF) family. The BTB-ZF family, which use the zinc finger as recognition motif of DNA specific sequences, and the BTB domain promotes oligomerization and the recruitment of other regulator factors[23]. Most of these factors are typically repressors of transcription, such as N-CoR (nuclear receptor corepressor) and SMRT (silencing mediator for retinoid and thyroid hormone receptor)[24], but also some are activators, such as p300[25]. Therefore, these displaying features of commonly serve as regulator of gene expression. The *mamo* is enriched in embryonic primordial germ cells (PGCs) in *D.melanogaster*. Deficiency of *mamo* are able to undergo oogenesis, but fail to execute meiosis properly and leading to female infertility in *D.melanogaster*[26]. However, the lack of the *Bm-mamo* gene not only leads to sterility, but also causes significant melanism, in caterpillar.

Expression pattern analysis of *Bm-mamo*

To analyze the expression profiles of *Bm-mamo*, we examined the quantitative RT–PCR. The different developmental stage expression level of *Bm-mamo* gene was investigated in the whole body from embryonic stage to adult stage in Dazao strain. The gene is highly expressed at molting stage of caterpillar and the expression was up-regulated in the later pupa and adult stage (fig 3A). This suggests that the *Bm-mamo* gene may respond to ecdysone titer and participate in the process of molting and metamorphosis of silkworm. In the investigation of tissue expression levels in the 5th instar 3rd day larvae of Dazao strain, the midgut, head, epidermis, have highly expression level, and the trachea, nerve, silk glands, testis, ovary, muscle, wing disc and malpighian tubules have moderate expression level, in blood and fat body were low expression level (fig 3B). This result suggests that the *Bm-mamo* may be involved in the development regulation of multiple tissues of silkworm. Due to the melanism of the epidermis of the *bd* mutant and the high expression level of *Bm-mamo* gene in the epidermis, we detected the expression level of this gene in the epidermis of the 4th to 5th instar of Dazao. In the temporal expression of epidermis, the *Bm-mamo* gene was up regulated in the molting period, and the highest expression was at the beginning of molting (fig 3C).

Functional analyses of *Bm-mamo* for pigmentation

In order to study the function of the *Bm-mamo* gene, we carried out RNAi experiment. Five microliter of siRNA were injected into haemolymph form stigma of day 3 of fourth instar, before the expression peak of *Bm-mamo* in larval stage, and

conducted the electroporation experimental[27], immediately. We found that there was significant melanin pigmentation in the epidermis of the newly molted 5th instar larvae. This indicate that deficient of *Bm-mamo* can cause melanin pigmentation (fig 4).

In addition, the gene knockout was performed. Two sgRNAs were designed to target exon 4 of *Bm-mamo* by CRISPRdirect (http://crispr.dbcls.jp) online program. The ribonucleoproteins (RNPs) formed by gRNA and recombinant Cas9 protein were injected into 450 silkworm embryos. Then, the phenotype identification and DNA cloning of the individuals of third generation were performed. In the G0 generation, individuals with mosaic melanization phenotype were found. These melanistic individuals were raised to moth and then crossed. The homozygous line of gene knockout was obtained through generations of screening. It is revealed that the gene edited individuals with significantly melanism body color and the female moth is sterile (fig 5).

It is indicated that the *Bm-mamo* gene has negative regulation on melanin pigmentation in caterpillar and participated in the reproductive regulation of female moths.

Downstream target gene analysis

Because *Bm-mamo* belongs to zinc finger proteins family, which specifically recognize downstream DNA sequences based on their zinc fingers. We have identified homologous genes of mamo in multiple species and conducted phylogenetic analysis (fig s5). In addition, by sequence alignment, found that the amino acid residues of the zinc finger motif of mamo-S protein are much conserved among 57 species, and the mamo-L protein are conserved among 30 species (fig s6). Then, we used a software (http://zf.princeton.edu)[28] to predict the DNA recognition sequence of *Bm-mamo*, especially the core sequence, GCGTG, which highly consistent with the binding sequence of mamo in those species (fig s7). Using the predicted position weight matrices (PWMs) of recognized sequences of Bm-mamo and FIMO (Find Individual Motif Occurences) software of MEME[29] suit to perform whole genome scanning for possible downstream target gene. The 2 Kb genomic regions, which locate in upstream and downstream of the total 14623 predicted genes, were investigated. It is found that there are 10622 genes contain the recognition sites of Bm-mamo protein in silkworm genome (fig 6). Moreover, we used a comparative transcriptome data

between homozygotes and heterozygotes of *bd* mutant in 4th instar beginning molting, which is high expression level stage of *Bm-mamo*[30]. In the integument tissue of this period, there were 182 genes with significant expression differences between homozygotes (*bd*/*bd*) and heterozygotes (+/*bd*) (table s3). Further, we found 147 genes that are differentially expressed in the transcriptome data and have binding sites of Bm-mamo protein near the upstream and/or downstream.

The protein function annotation was performed, and found that a group of cuticular protein genes were significantly up-regulation expressed in homozygotes mutant. We detected the expression levels of 20 cuticular protein genes and eight key genes of melanin synthesis were investigated in heterozygous and homozygous gene knockout line of *Bm-mamo*. Among them, *yellow*, *tan*, *DDC* (*DOPA decarboxylase*) (fig 7) and 19 cuticular protein genes were significantly up-regulated, and cuticular protein gene *CPR11* were significantly down-regulated, in the homozygous *Bm-mamo* knockout individuals (fig 8).

The up-regulation of gene expression level of *yellow*, *DDC* and *tan* can promote the generation of melanin. Moreover, some cuticular protein gene, for example *CPH24*, when it is defective can lead to the remarkable decreased of pigmentation [31].

Therefore, the *Bm-mamo* gene can affect the color pattern of caterpillar by regulating the key genes of melanin metabolism pathway and cuticular protein genes.

Discussion

Insects are known as one of the most successful populations in evolution, which evolved many important phenotypes in the process of adapting to the environment. Among these, color pattern is one of the most magnetic traits. The biochemical metabolic pathways of pigment, such as melanin, ommochrome, pteridine have been identified in insects[12]. However, the regulation of pigment metabolism related genes, and the process of transport and deposition of pigment substances is not clear. In this paper, we discover the *Bm-mamo* gene negative regulate melanin pigmentation in caterpillar. When this gene is deficient, the body color of silkworm is signification melanism, changes in the expression of some melanin synthesis genes and some cuticular protein genes are also significant change.

Deficient of some genes in the melanin synthesis pathway can lead to variations in the color pattern, such as *TH*, *yellow*, *ebony*, *tan*, *aaNAT* [32-34]. However, those often only lead to localized pigmentation changes in later instar caterpillar. For example, the

yellow mutant of silkworm, which eye spot, lunar spot, star spot, spiracle plate, head, foot hook and some sclerotization area appears reddish brown, and the other epidermis is consistent with the wild-type[35]. Why not exhibit a yellow phenotype in other parts of the larval epidermis? The possible reason is that, the expression of *yellow* is limited to a certain region by transcription regulator, or\and other factors, such as transporter, cuticular protein in the epidermis limit the pigmentation of melanin. Because, the biochemistry synthesized pigment substances need to be transported from epidermis cells and embedded into the cuticle to form coloring. Therefore, the correct location of cuticular protein in epidermis may be one of the important factors of pigmentation. Previous studies have shown that the lack of cuticular protein, CPH24, can lead to defects in pigmentation which supports this argument[31].

How does cuticular protein affect pigmentation? Previous research finding that some cuticular proteins can form "pore canals" to transport some macromolecules[36]. In addition, some cuticular proteins can be crosslinked with catecholamines which synthesized by melanin metabolism pathway[37]. Because of no live cell in cuticle, melanin precursor substances may be transported by pore canals, formed by some cuticular proteins, and fixed to specific positions through cross-linking with some cuticular proteins. The cuticular protein, CPR4, high expression in black individuals of *Bm-mamo* knockout line, is required for formation of pore canals in *Tribolium castaneum* [38]. In addition, the vertical pore canal is lacking in less pigmented cuticles of *T. castaneum* [39]. This suggestion that the pore canals constructed by CPR4 may transport the pigments and contribute in epidermis pigmentation. Consequently, to maintain the accuracy of the color pattern, the localization of cuticular proteins in the epidermis may be very important. In one study of microarray analysis, it was found that there are different kinds of cuticular proteins in different colored areas of epidermis, in *papilio xuthus* [40]. These ten cuticular protein genes were conserved in different insects (table s4). In addition, ten cuticular protein genes, which are highly expressed in the black stripes of *papilio xuthus* larvae, are also significantly up-regulated in *Bm-mamo* knockout lines of silkworm (fig s8). Therefore, we believe that the location of cuticular proteins and architecture of epidermis, the transport and cross-linking of pigments in cuticle, is the important bases for the formation of color patterns. Hence, the cuticular proteins and pigments metabolism are close cooperated by the uniformly regulated of transcription factors, such as *Bm-mamo*, thus forming

color patterns in caterpillars.

In *D.melanogaster*, the *mamo* is required for functional gamete production. In silkworm, the *Bm-mamo* has conservative function in the female reproduction. Furthermore, the *Bm-mamo* play an important role in regulation of color pattern of caterpillar. It is generally believed that the main reason for a conserved transcription factor to gain new functions is the evolution of *cis*- regulatory elements (CREs) of target genes. Because CREs have important function in spatiotemporal expression pattern regulation of genes, and large number of them are located in the non-coding region, which are prone to sequence changes compared with the sequences of coding genes[41]. In addition, transcription factors (TFs), because of them recognize relatively short sequences, generally between 4 and 20 bp in length, can have a large number of binding sites on the genome[42]. Therefore, one member of TFs has the potential to regulate almost all genes in one genome. For example, according to the PWM sequence of mamo protein recognize sequence, 10622 genes (~73% of the total number of genes) contain the binding sites in 2 kb of their up/down stream region, in silkworm. If the core recognition sequence (GCGTG) of mamo protein is used to compare these genome regions, there were 13567 genes (~93% of the total number of genes) contain the binding sites in 2 kb of their up/down stream region (table s5). And, such binding sites also exist in intron or/and beyond the 2 kb of their up/down stream region of the remaining 1056 genes. The shorter the recognition sequence, the more widespread it can be in the genome. However, in fact, there are not as many genes whose expression is significantly affected by the change of one TF as expected. Therefore, there are some transcription factors binding sites in a gene, but this gene not necessarily regulated by these factors. The possible reason is gene regulation network restricts the target gene set of regulatory factors by such as, whether the target chromatin is open or not[43], interplay of TFs[44], and the participation of cofactors[45].

We have noticed that a lepidopteran caterpillar has multiple visual factors in its skin, and even when its compound eyes are covered, it can rely on the skin to feel the color of the environment, so as to change its body color to adapt to the environment. This visual gene is common in many *Lepidoptera*[46]. In the industrial melanism event, in a short period of several decades, the body colors of multiple of insects have changed significantly[47]. If we only rely on random mutations in the genome, it cannot be so swift.

We speculate that under the stimulation of the natural environment, caterpillars can distinguish the light wave frequency of the background, so that this signal can guide the epigenetic factors and transcription factors, turn on or off the expression of corresponding gene, such as pigment synthesis gene and cuticular protein genes, thus forming a body color pattern similar to the background. In addition, the BTB-ZF family member can participate in chromatin remodeling, and their zinc finger motifs can recognize DNA sequences[48]. During the opening of chromatin, the probability of mutation of exposed DNA sequence will increase[49]. The transposon insertion occurs in a timely manner upstream of the *cortex* gene in the melanism *Biston betularia*[50], may be caused by the similar binding of transcription factors and opening of chromatin.

Therefore, we speculate that evolution of color pattern may be programmed events of the system, rather than relying on random mutations. Based on this assumption, animals can rapidly undergo phenotypic changes under the influence of the environment, and thus the speed of species evolution may be much faster than previous cognition.

Method

Silkworm strains.

The *bd* and bd^f mutant strains, wild-type strain Dazao and N4 were from the bank of genetic resources of Southwest University. Silkworms were reared on mulberry leaves or artificial diets at 25°C and 73% relative humidity, dark 12 hours and light 12 hours.

Positional cloning of the *bd* locus.

For mapping of the *bd* locus, the F1 heterozygous individuals were obtained from a cross between a bd^f strain and a Dazao strain. Then the female of F1 was crossed with bd^f male (BC1F), and the male of F1 was backcrossed with a bd^f female (BC1M). A total of 1,162 BC1M individuals were used for recombination analysis. Genomic DNA was extracted from the parents, Dazao, bd^f and F1, and each BC1 individuals using phenol chloroform extracting method. Search for available DNA molecular markers through polymorphism screening and linkage analysis. The primers used for mapping is listed in supplementary Table s6.

Phylogenetic analysis.

To determine whether *Bm-mamo* orthologues existed in other species, we

performed the Blastp program on the NCBI nr database (http://www.ncbi.nlm.nih.gov/BLAST/). The sequences of multiple species were executed a multiple sequence alignment of predicted amino-acid sequences by MUSCLE. And then the phylogenetic tree was constructed using the neighbor-joining method with the MEGA7 program (person model). The confidence levels for various phylogenetic lineages were estimated by bootstrap analysis (2,000 replicates).

Quantitative RT–PCR.

Total integument RNA was isolated from the silkworm *bd* mutant, bd^f, *Bm-mamo* knockout line and strain Dazao using TRIzol reagent (life), purified by phenol–chloroform extraction and then reverse transcribed with a random primer (N6) using a PrimeScript™ RT reagent Kit (TAKARA), according to manufacturers' protocol.

qRT-PCR was performed using a CFX96™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA) with a Real-Time PCR System with iTaq Univer SYBR Green Supermix System (Bio-Rad). The cycling parameters were as follows: 95 °C for 3 min followed by 40 cycles of 95 °C for 10 s and annealing for 30 s. The primers used for target genes are listed in supplementary Table s6. Expression levels for each sample were determined with biological replicates, and each sample was analyzed in triplicate. The gene expression levels were normalized against the expression levels of the ribosomal protein L3 (RpL3). The relative expression levels were analyzed using the classical $R=2^{-\Delta\Delta Ct}$ method.

siRNA for gene knockdown.

siRNAs for *Bm-mamo* were designed by siDirect program (http://sidirect2.rnai.jp). The target siRNAs and negative control were synthetized by Tsingke Biotechnology Co., Ltd. The siRNA (5 μl, 1μl/μg) was injected from the abdominal spiracle into haemolymph at the fourth instar day 3 larval stage. Immediately after injection, phosphate buffered saline (PH 7.3) droplets were placed nearby and the 20-voltage pulse was applied 3 times. The phenotype was observed for fifth instar larvae. The left and the right of epidermis were separately dissected from the injected larvae, and the RNA were extract, respectively. Then the cDNA was synthetized and the expression level of gene were detected by quantitative RT–PCR.

sgRNA synthesis and RNP complex assembly

We used CRISPRdirect (http://crispr.dbcls.jp/doc/), an online software originally, to screen appropriate guide RNA (sgRNA) target sequence. The gRNAs were

synthetized by Beijing Genomics Institute. sgRNA templates were transcribed using a T7 polymerase with the RiboMAX™ Large-Scale RNA Production Systems (Promega, Beijing, China) according to the manufacturer's instructions. RNA transcripts were purified using 3M Sodium Acetate (pH 5.2) and anhydrous ethanol (1:30) precipitation, were washed using 75% ethanol and were eluted in Nuclease-Free Water. All injection mixes contained 300 ng/μL Cas9 Nuclease (TAKARA Bio, Dalian, China) and 300 ng/μL purified sgRNA. Before injection, the injection mixtures were incubated for 15 min at 37 °C to reconstitute active ribonucleoproteins (RNPs) directly before use.

Microinjection of embryos

For embryo microinjection, microcapillary needles were manufactured using a PC-10 dual stage glass micropipette puller (Narishige, Tokyo, Japan). Microinjection was performed using Eppendorf's electronic microinjector TransferMan NK 2 (Eppendorf, Hamburg, Germany) with suitable needles. The female moth of wild type silkworm was mated with male moth for 4 hours and laid eggs. The newly laid eggs were adhered onto a clean glass slide. CRISPR/Cas9-messenger RNP mixtures with volumes of approximately 1 nL were injected into the middle of the eggs, and glue the wound. All injected embryos were allowed to develop in the artificial environment of 25 °C and humidity 80%.

Comparative genomics

The reference genome of Dazao was extract from Silkbase (https://silkbase.ab.a.u-tokyo.ac.jp/cgi-bin/index.cgi). The *bd*^{f} genome from silkworm pan-genome project[22].

DNA recognition sequence analysis and Downstream target gene screening

The software (http://zf.princeton.edu) was used to predict DNA binding site for Cys2His2 zinc finger proteins. Chose the confident ZF domains, which scores higher than 17.7. The prediction model chooses RF regression on B1H. And then the sequence logo and position weight matrices (PWM) of DNA binding site was Obtained. The FIMO (Find Individual Motif Occurences) package of MEME suite program was used to search the binding site in silkworm genome.

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Competing interests

The authors declare that they have no competing interests.

Author contributions

FY Dai conceived and designed the experiments. SY Wu and CX Peng performed the study. SY Wu, XL Tong, KP Lu, JW Luo, CL Li, X Ding, YR Lu, XH Duan, CH Zhang, D Tan and H Hu analyzed the data. SY Wu wrote the paper. XL Tong and FY Dai edited and revised the manuscript.

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Figure 1. Larval phenotype of the wild type Dazao, *bd* and bd^f.

The epidermis of *bd* is dark black gray and the epidermis of bd^f is light black gray at 5th instar day 3 of silkworm. The bar indicates 1 centimeter.

Figure 2. Positional cloning of the bd locus

(A) Using 532 BC1 individuals to map the *bd* locus between the PCR marker C and I. The numbers above DNA markers indicate recombination events.

(B) Using 1162 BC1individulas to narrow the bd locus to ~400 kb region.

(C) The partial enlarged view of responsible region of bd. In this region contains 4 predicted genes, *BGIBMGA012517*, *BGIBMGA012518*, *BGIBMGA012519* and *BGIBMGA014589*.

(D) The analysis of nucleotide differences of *bd* responsible region. The green block indicated the deletion of the genome in bd mutants. The grey lines indicated the SNP and the red lines indicated the Indel of bd^f mutants.

Figure 3. Spatiotemporal expression pattern of *Bm-mamo*.

(A) Temporal expression of *Bm-mamo*. The molting stage and adult stage this gene is

(B) M: moulting, W: wandering stage, P: Pupal stage, A: adult stage. The 1th to 5 th denote the first instar of larvae to fifth instar of larvae, respectively.

(B) Tissues expression of the 4th instar molting larvae. The Bm-mamo have relatively high expression level in midgut, head and epidermis.

(C) Detailed analysis of *Bm-mamo* at the 4th larval stage in epidermis of Dazao strain. The *Bm-mamo* is up-regulation expression during molting stage. HCS indicates head capsule stage.

Figure 4. Effects of *Bm-mamo* siRNA injection on pigmentation formation.

(A)The siRNA was introduced by microinjection followed by electroporation. The “+” and “-” indicate the positive and negative poles of the electrical current. Scale bars, 1 cm

(B) Partial magnification of siRNA experimental group and negative control group. Scale bars, 1 cm

(C) and (D) Relative expression levels of *Bm-mamo* in a negative control and RNAi group as determined by quantitative RT–PCR analysis. The means ± s.d. Numbers of samples are shown at the upper right in each graph. **P<0.01, paired Student’s t-test. (NS, not significant).

Figure 5. Disruption of *Bm-mamo* using CRISPR/CAS9.

(A) Larval phenotype in G3 forth instar larva of Dazao targeted for *Bm-mamo*. Scale bars, 1 cm

(B) The after 36 hours of laying eggs, the pigmentation of the eggs indicates that the eggs produced by knockout homozygous females cannot undergo normal pigmentation and development.

(C) Genomic structure of *Bm-mamo*. Open reading frame (blue), untranslated region (black). The gRNA 1 and gRNA 2 sequences are shown.

(D) Sequences of the *Bm-mamo* knock out individuals. Line 1 and line 2 deletion 15 and 71 bp, respectively.

Figure 6. The DNA recognize sequence logo of Bm-mamo protein and the analysis of downstream target genes.

(A) The DNA recognize sequence logo of Bm-mamo-s protein.

(B) The DNA recognize sequence logo of Bm-mamo-L protein.

(C) According to MEME's FIMO program package, potential downstream target genes of Bm-mamo protein in the silkworm genome were identified.

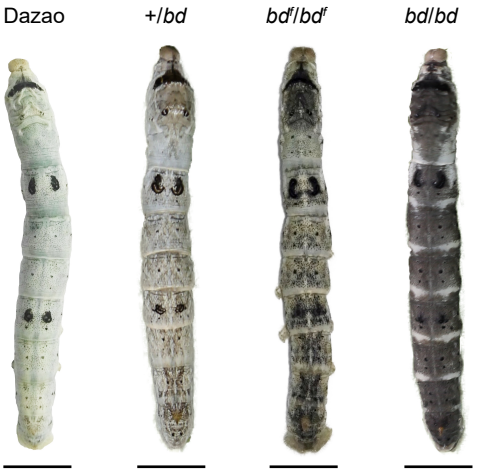
Figure 7. The melanin metabolism pathway and Fluorescence quantitative PCR of relative genes.

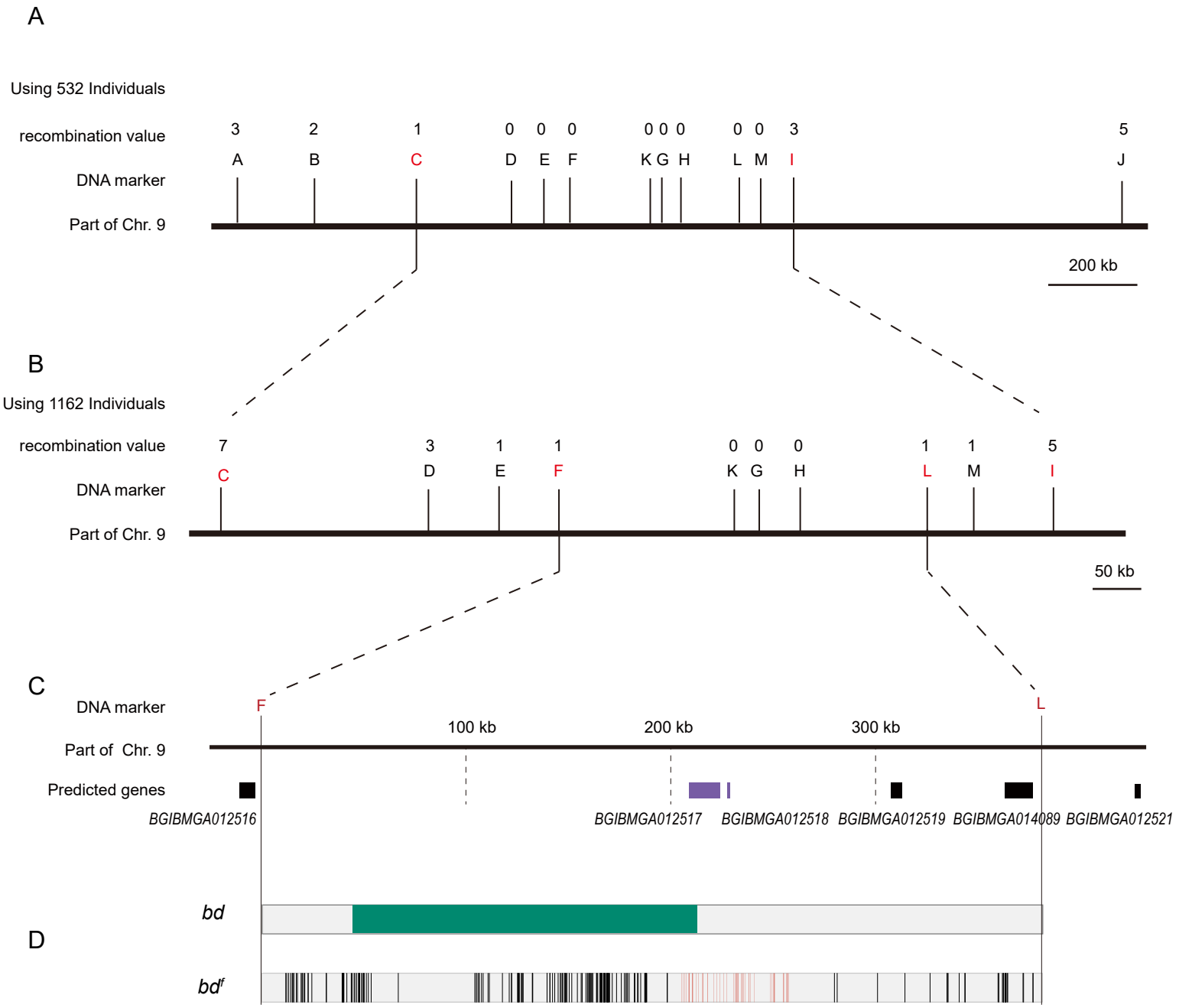
(A)The melanin metabolism pathway. The blue indicates amino acid and catecholamine, the red indicates the name of genes, the black indicates the name of enzymes.

(B) Relative expression levels of eight genes in heterozygous type of *Bm-mamo* knockout group (blue) and homozygous *Bm-mamo* knockout group (red) as determined by quantitative RT–PCR analysis. The means ± s.d. **P<0.01, paired Student’s t-test.

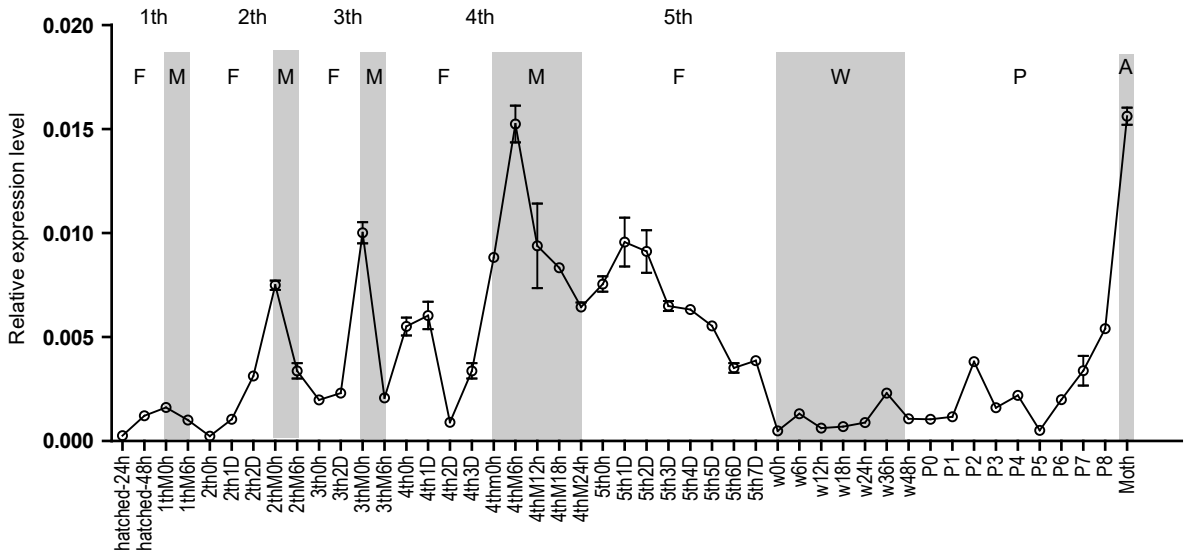
Figure 8. The expression level of cuticular protein genes were detected by qRT-PCR.

The means ± s.d. **P<0.01, paired Student’s t-test.

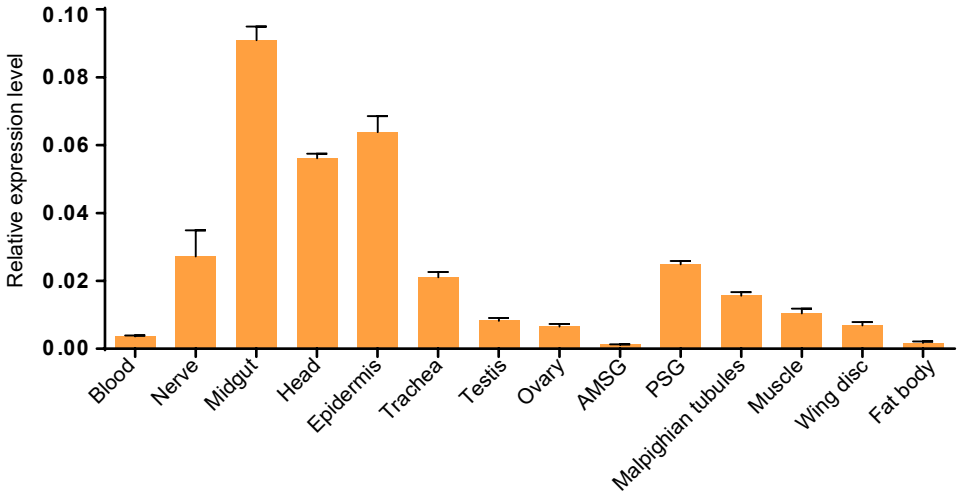

Dazao
+/bd
bd^f/bd^f
bd/bd


A
Using 532 Individuals
recombination value
3 2 1 0 0 0 0 0 0 0 0 3 5
A B C D E F K G H L M I J
DNA marker
Part of Chr. 9
200 kb
B
Using 1162 Individuals
recombination value
7 3 1 1 0 0 0 1 1 5
C D E F K G H L M I
DNA marker
Part of Chr. 9
50 kb
C
DNA marker
F L
100 kb
200 kb
300 kb
Part of Chr. 9
Predicted genes
BGIBMGA012516
BGIBMGA012517
BGIBMGA012518
BGIBMGA012519
BGIBMGA014089
BGIBMGA012521
D
bd
bd^f

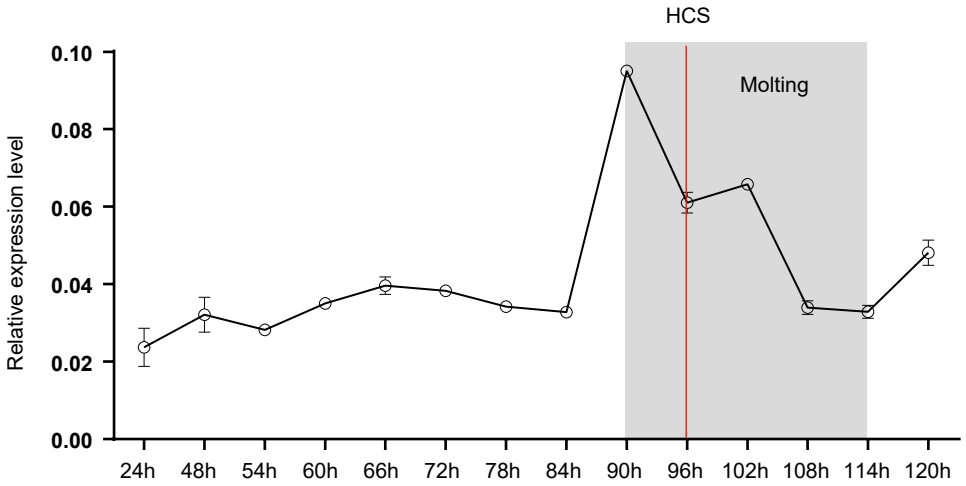
A


1th
2th
3th
4th
5th
F
M
F
M
F
M
F
M
F
W
P
A
Relative expression level
0.020
0.015
0.010
0.005
0.000
hatched-24h
hatched-48h
1thM0h
1thM6h
2th0h
2th1D
2th2D
2thM0h
2thM6h
3th0h
3th2D
3thM0h
3thM6h
4th0h
4th1D
4th2D
4th3D
4thm0h
4thM6h
4thM12h
4thM18h
4thM24h
5th0h
5th1D
5th2D
5th3D
5th4D
5th5D
5th6D
5th7D
w0h
w6h
w12h
w18h
w24h
w36h
w48h
P0
P1
P2
P3
P4
P5
P6
P7
P8
Moth

B


Relative expression level
0.10
0.08
0.06
0.04
0.02
0.00
Blood
Nerve
Midgut
Head
Epidermis
Trachea
Testis
Ovary
AMSG
PSG
Malpighian tubules
Muscle
Wing disc
Fat body

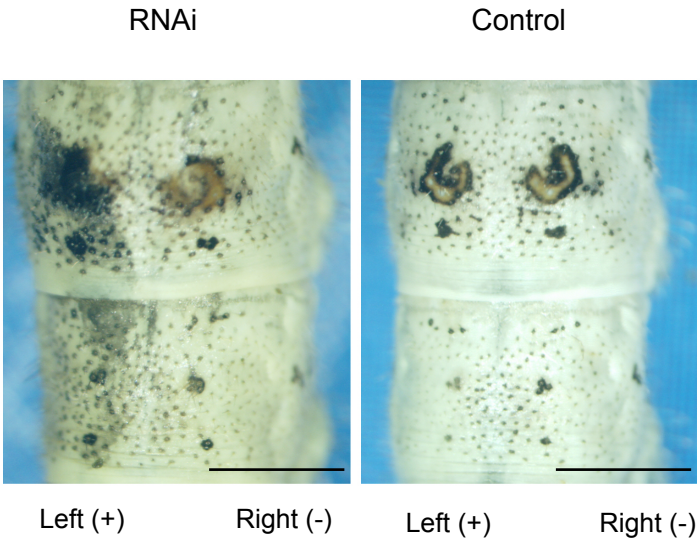
C


HCS
Molting
Relative expression level
0.10
0.08
0.06
0.04
0.02
0.00
24h
48h
54h
60h
66h
72h
78h
84h
90h
96h
102h
108h
114h
120h

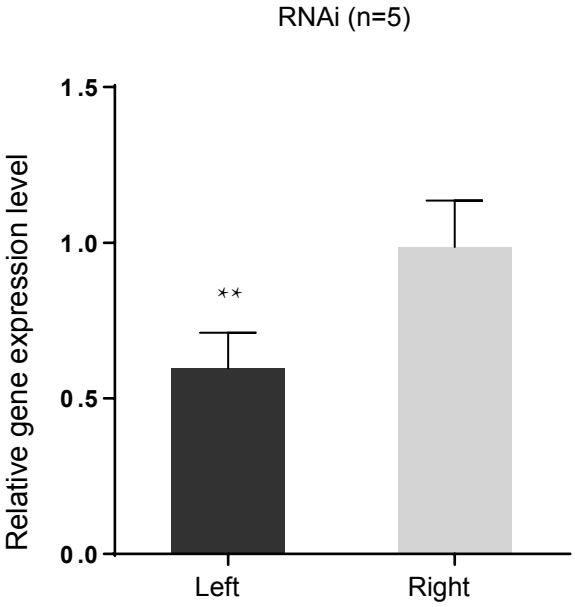
A

RNAi
Control
Left (+)
Right (-)
Left (+)
Right (-)

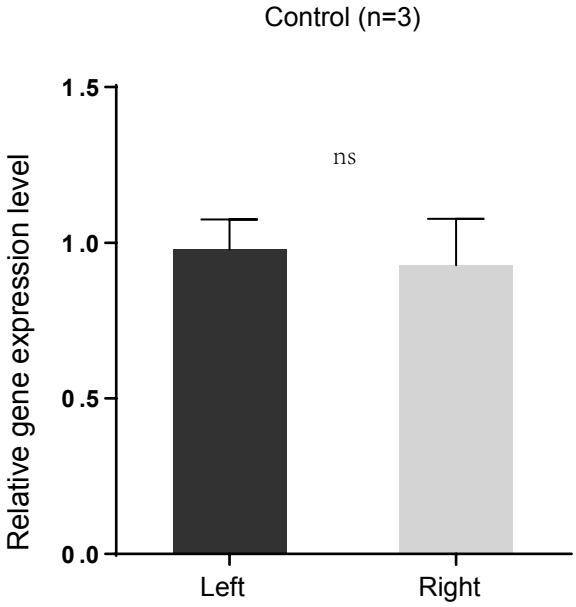
B


RNAi
Control
Left (+)
Right (-)
Left (+)
Right (-)

C


RNAi (n=5)
Relative gene expression level
1.5
1.0
0.5
0.0
**
Left
Right

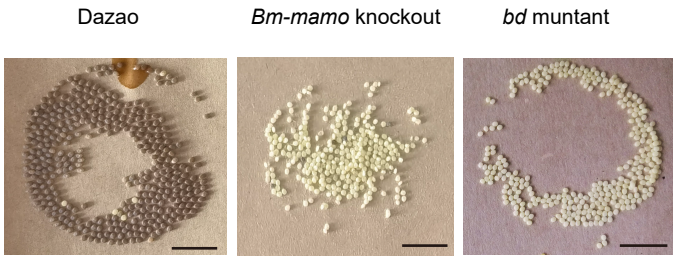
D


Control (n=3)
Relative gene expression level
1.5
1.0
0.5
0.0
ns
Left
Right

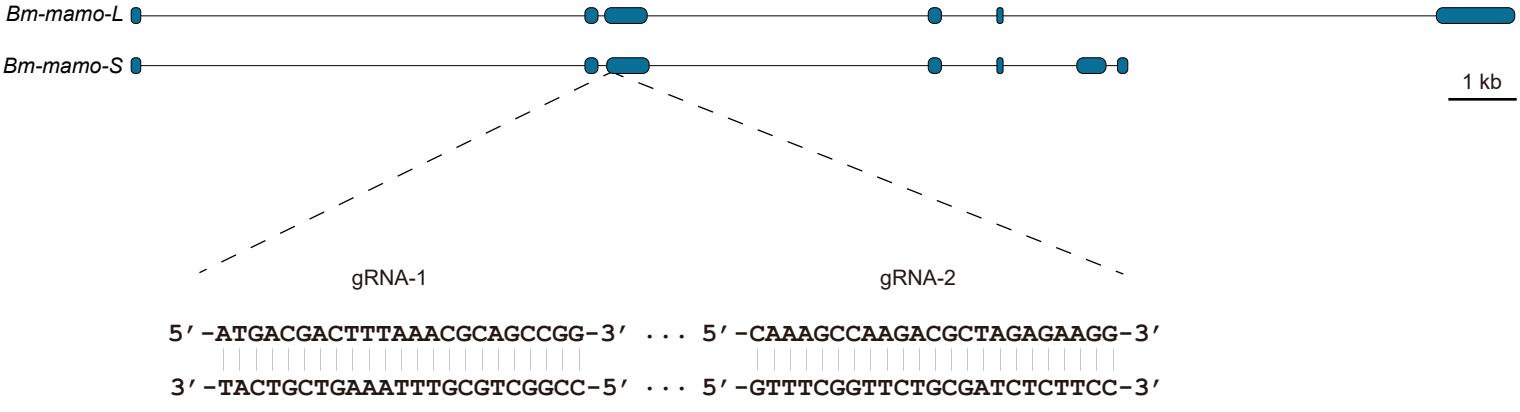
A

Dazao
Bm-mamo knockout

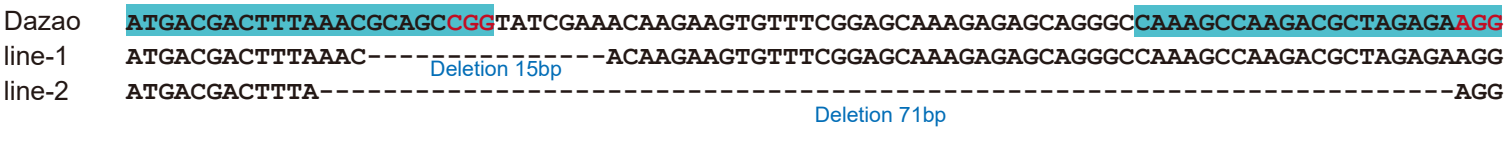
B


Dazao
Bm-mamo knockout
bd muntant

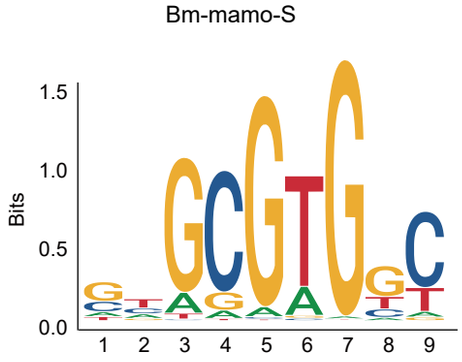
C


Bm-mamo-L
Bm-mamo-S
1 kb
gRNA-1
gRNA-2
5′-ATGACGACTTTAAACGCAGCCGG-3′ ... 5′-CAAAGCCAAGACGCTAGAGAAGG-3′
3′-TACTGCTGAAATTTGCGTCGGCC-5′ ... 5′-GTTTCGGTTCTGCGATCTCTTCC-3′

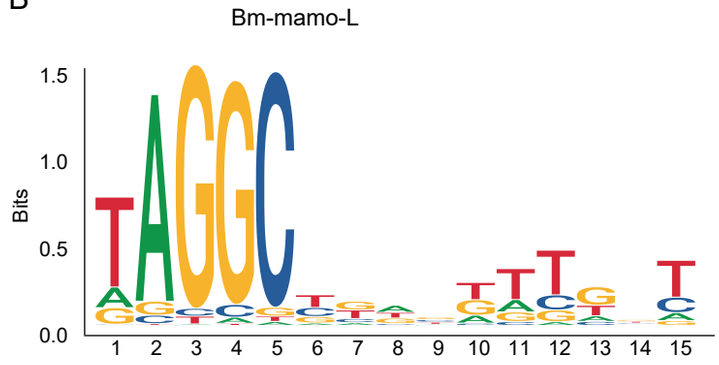
D


Dazao ATGACGACTTTAAACGCAGCCGGTATCGAAACAAGAAGTGTTTCGGAGCAAAGAGAGCAGGGCCAAAGCCAAGACGCTAGAGAAGG
line-1 ATGACGACTTTAAAC----------ACAAGAAGTGTTTCGGAGCAAAGAGAGCAGGGCCAAAGCCAAGACGCTAGAGAAGG
Deletion 15bp
line-2 ATGACGACTTTA----------AGG
Deletion 71bp

A


Bm-mamo-S
Bits
1.5
1.0
0.5
0.0
1 2 3 4 5 6 7 8 9

B


Bm-mamo-L
Bits
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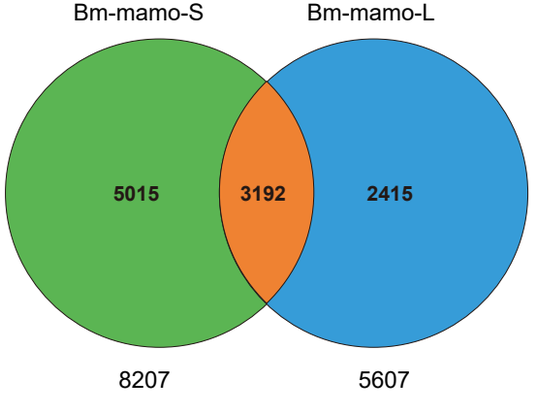
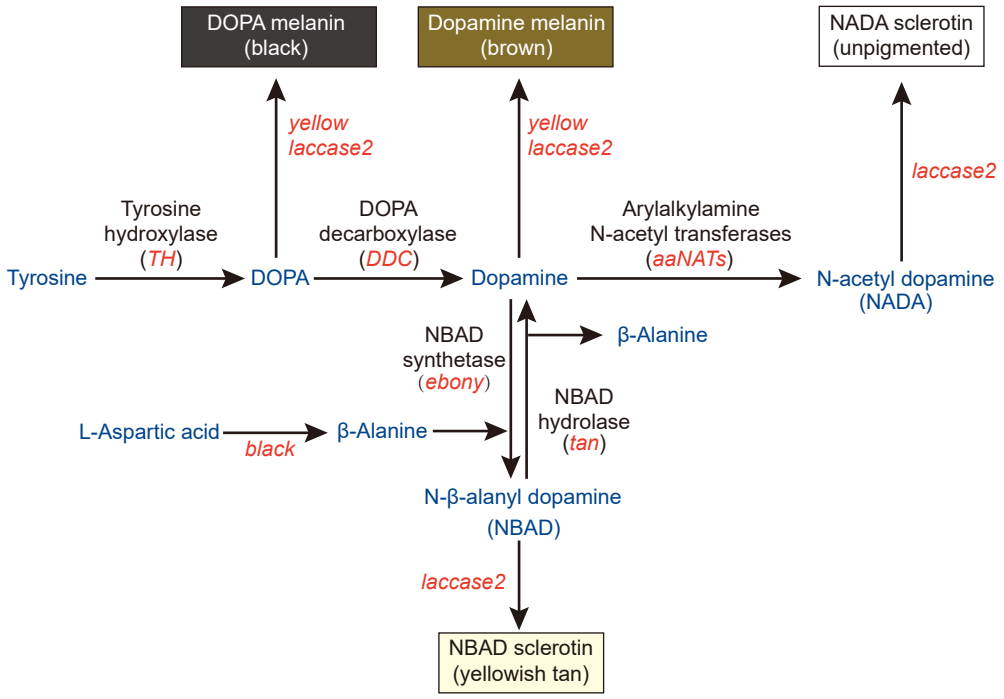
1.0
0.5
0.0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

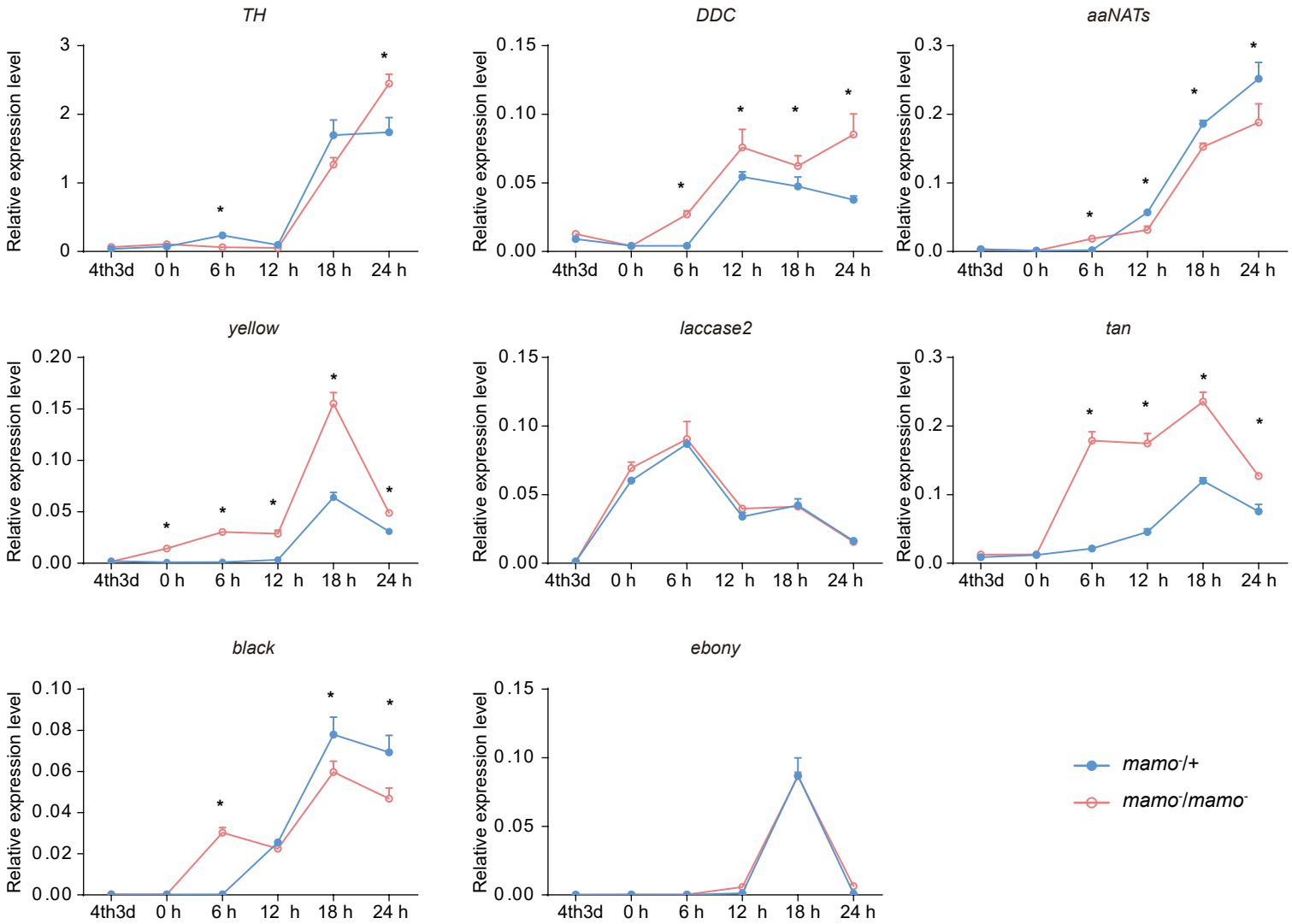
C

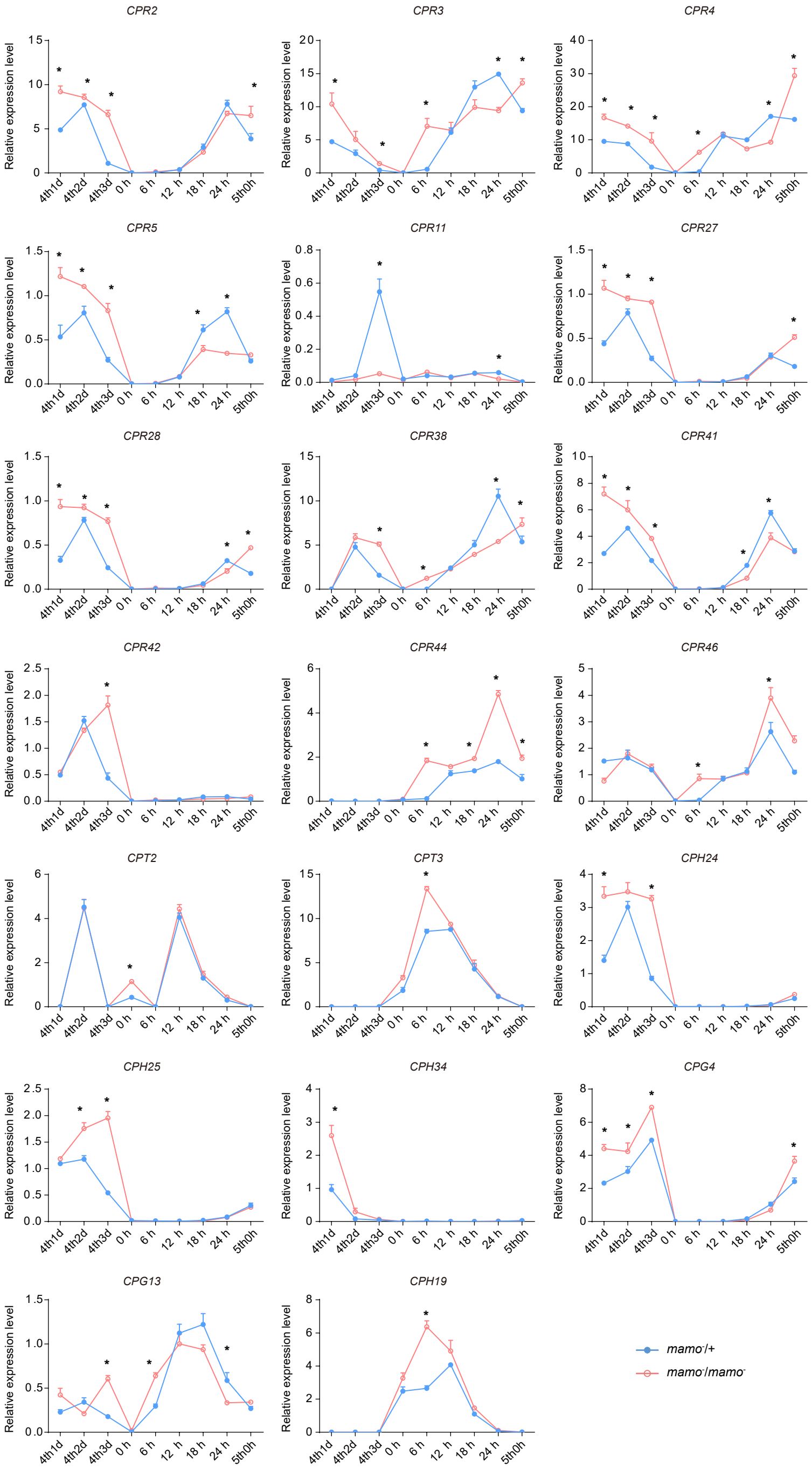

Bm-mamo-S
Bm-mamo-L
5015
3192
2415
8207
5607

A


DOPA melanin
(black)
Dopamine melanin
(brown)
NADA sclerotin
(unpigmented)
yellow
laccase2
yellow
laccase2
laccase2
Tyrosine
hydroxylase
(TH)
DOPA
decarboxylase
(DDC)
Arylalkylamine
N-acetyl transferases
(aaNATs)
Tyrosine
DOPA
Dopamine
N-acetyl dopamine
(NADA)
NBAD
synthetase
(ebony)
β-Alanine
NBAD
hydrolase
(tan)
L-Aspartic acid
black
β-Alanine
N-β-alanyl dopamine
(NBAD)
laccase2
NBAD sclerotin
(yellowish tan)

B


TH
DDC
aaNATs
yellow
laccase2
tan
black
ebony
Relative expression level
4th3d
0 h
6 h
12 h
18 h
24 h
mamo-/+
mamo-/mamo-


CPR2
CPR3
CPR4
CPR5
CPR11
CPR27
CPR28
CPR38
CPR41
CPR42
CPR44
CPR46
CPT2
CPT3
CPH24
CPH25
CPH34
CPG4
CPG13
CPH19
Relative expression level
4th1d
4th2d
4th3d
0 h
6 h
12 h
18 h
24 h
5th0h
mamo-/+
mamo-/mamo-