Melanism patches up the defective cuticular morphological traits through

promoting the up-regulation of cuticular protein-coding genes in *Bombyx mori*

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Abstract

Melanin and cuticular proteins are important cuticle components in insect. Cuticle

defects caused by mutations in cuticular protein-encoding genes can hinder melanin

deposition. However, the effects of melanin variation on cuticular protein-encoding

genes and the corresponding morphological traits associated with these genes are

remain largely unknown. Using *Bombyx mori* as a model, we showed that the

melanism levels during larval cuticle pigmentation correlated positively with the

expression of cuticular protein-encoding genes. This correlation stemmed from the

simultaneous induction of these genes by the melanin precursors. More importantly,

the effect of the melanism background on the cuticles induced the up-regulation of

other functionally redundant cuticular protein-encoding genes to rescue the

morphological and adaptive defects caused by the dysfunction of some mutated

cuticular proteins, and the restorative ability increased with increasing melanism

levels, which gives a novel evidence that melanism enhances insect adaptability.

These findings deepen our understanding of the interactions among cuticle

components, as well as their importance in the stabilizing of the normal morphology

and function of the cuticle.

Introduction

The prerequisite for the benefits of melanism to insect is not only the integrity of

the melanin biosynthesis and regulatory pathway (Wilson *et al.* 2001; Liu *et al.* 2015;

Mallet and Hoekstra 2016), but also the normal presence of the platform it relied on

(Wittkopp *et al.* 2003; Wittkopp and Beldade 2009; Andersen 2010; Moussian 2010;

Van Belleghem *et al.* 2017). For the insects, the most important fundamental platform

for the color pattern drawing is the cuticle (Hopkins and Kramer 1992; Andersen 2010;

Moussian 2010). During shaping the cuticle, the maintenance and stability of the

cuticle features depends on normal functional cuticular proteins and the interactions

with other components (such as chitin) (Hopkins and Kramer 1992; Guan *et al.* 2006;

Suderman *et al.* 2006; Andersen 2010; Moussian 2010; Chaudhari *et al.* 2011; Noh *et*

al. 2016). Due to the crucial roles of cuticular proteins on cuticle development, when

their coding genes are loss of function, the abnormal or defective cuticle will likely

affect the deposition and attachment of melanin, which is not conducive to the

performance of color pattern (Kanekatsu *et al.* 1988; Oota and Kanekatsu 1993;

Arakane *et al.* 2012; Jasrapuria *et al.* 2012; Wang 2013; Noh *et al.* 2015). Yet little is

known about the corresponding response mode of cuticular protein-encoding genes

via the alteration of the melanin biosynthesis or regulatory pathway.

Recently, several high throughput expression surveys showed that the abundance of

cuticular protein-encoding genes in different colored integuments varied in some

insect species, especially with evidently up-regulated in the melanic regions

(Futahashi *et al.* 2012; He *et al.* 2016; Wu *et al.* 2016; Tajiri 2017), and some of those

shared very similar expression patterns and functions (Nakato *et al.* 1994; Nakato *et*

al. 1997; Shofuda *et al.* 1999; Okamoto *et al.* 2008; Liang *et al.* 2010; Tang *et al.*

2010; Qiao *et al.* 2014).These studies implied that there are probably some

relationships between the promotion of melanism and the expression of cuticular

protein-encoding genes. Prior to this study, further exploring and understanding of the

potential relationships were unclear. Additionally, when melanism-promoting

instructions and defective cuticle proteins occur simultaneously, what are the effects

of their relationships on the presence of the corresponding morphological traits？

In the Lepidoptera model, *Bombyx mori*, an intriguing phenomenon has been

reported that a larval melanic mutant, *Striped* (p^S, 2-0.0) is able to rehabilitate the

malformed body shape, as well as the adaptability defects of the *stony* mutant (*st*,

8-0.0) (Xiang 1995; Banno *et al.* 2005). A recent study clarified that a transcription

factor, *Apontic-like*, which boosts the expression of melanin synthesis genes in

epidermal cells, is responsible for the p^S mutant (Yoda *et al.* 2014). Besides this, there

are also multiple alleles with different melanism degrees at the *p* locus, including p^B,

p^M, *etc* (Xiang 1995; Banno *et al.* 2005; Yoda *et al.* 2014). And the *stony* mutant (*st*,

8-0.0) which precisely caused by the deletion of a RR1-type larval cuticular

protein-encoding gene, *BmLcp17* (or *BmorCPR2*) showed hard and tight touch feeling,

uncoordinated ratios of the length of the internodes and the intersegment folds (I/IF),

bulges at intersegment folds, and severely defective locomotion and behavioral

activities in the larval stage (Qiao *et al.* 2014). In addition, the similarity of the gene

expression patterns and functional characteristics among some members of the

RR1-type larval cuticular protein-encoding gene family, such as *BmLcp18*, *BmLcp22*,

BmLcp30 also suggest that they may play very similar roles as *BmLcp17* in shaping

the larvae cuticle (Nakato *et al.* 1994; Nakato *et al.* 1997; Shofuda *et al.* 1999;

Okamoto *et al.* 2008; Liang *et al.* 2010; Tang *et al.* 2010; Qiao *et al.* 2014). These

dispersed findings are linked through the epistasis of p^S to *stony*, then provide the

breakthrough and the exceptional genetic resources for exploring the interactions

between melanin and cuticular protein-encoding genes.

Here we illustrated that the transcripts levels of four cuticular protein-encoding

genes were positively correlated with the melanism degree of larval cuticle, which

were due to the simultaneous induction these genes by the intracellular melanin

precursors. Moreover, by importing melanism-promoting instruction into the *stony*

mutant, the cuticle deficiency rescued through functionally redundant compensation

by some other up-regulated cuticular protein-encoding genes, which a new evidence

that melanism as a beneficial trait. These findings deepen our understanding of the

interactions among genetic factors which shape morphological features in

lepidopteran, and emphasize the ecological and evolutionary significance of these

mutual interactions.

Materials and Methods

Silkworm strains

Wild-type strains Dazao ($+^p$) and melanic mutant strains p^M, p^S and p^B (Xiang 1995;

Banno *et al.* 2005; Yoda *et al.* 2014) were analyzed in this study. The darkness of

pigment was measured as mean OD value using Image J (https://imagej.nih.gov/ij/).

In terms of melanism degree, the body color of an individual that is homozygous at

one of the aforementioned melanic loci is darker than that of a heterozygous

individual (Xiang 1995; Banno *et al.* 2005). The albinism mutant *albino* (*al*) (Banno

et al. 2005; Fujii *et al.* 2013), non-diapause wild-type strain N4 (used for melanin

inhibition treatment) and *BmLcp17* deletion strain Dazao-*stony* (near isogeneic line of

Dazao, which have been backcrossed with Dazao over 26 generations) were supplied

by the Silkworm Gene Bank in Southwest University. The N4 strain and *al* mutant

were fed with artificial diet at 28℃, and the others were fed fresh mulberry leaves

under a 12 h/12 h light/dark photoperiod at 24 °C.

Chemicals and cell lines

140 L-dopa (D9628), dopamine (H8502), tetrahydrofolic acid (BH_4) (T3125) and

141 2,4-Diamino-6-hydroxypyrimidine (DAHP) (D19206) were purchased from

142 Sigma-Aldrich (St. Louis, MO, USA). *BmNs* cell line was kept in our laboratory.

Mating combinations and progeny phenotypes identification

The p^S and p^M strains were crossed with the Dazao-*stony* strain to generate the F_1

generation, respectively. The F_2 generation were produced by an F_1 self-cross, and

individuals of day 5 of the fifth-instar were collected to further use. The p^B strain was

crossed with the Dazao-*stony* strain to generate F_1 progeny, which mated with the

Dazao-*stony* strain to generate the BC_1 generation, and fed them until at day 5 of the

fifth-instar. Firstly, individuals of the F_2 or BC_1 generations were separated by their

cuticle pigmentation. Subsequently, their phenotypes were further classified by

morphological characteristics, touch feeling, and the ratios between the number of

internodes and intersegmental folds in the second, third, and fourth abdominal

segments based on an earlier method (Qiao *et al.* 2014).

Genotyping

Because the p^S, p^M and p^B mutations are alleles at the p locus, they should be

located in proximity each other on the chromosome 2 (Xiang 1995; Banno *et al.* 2005;

Yoda *et al.* 2014). Based on the reported sequence of the gene corresponding to the p^S

allele, a polymerase chain reaction (PCR)-based molecular marker within of the

genomic region of the *Apontic-like* was designed. PCR screening were performed in

the p^X ($X=M$, S and B) and Dazao-*stony* to obtain the polymorphism molecular marker

for p locus. Similarly, molecular marker was also designed within genomic region of

the *BmLcp17* for polymorphism screening of the *stony* locus. The primers used in this

study are listed in Table S1.

Association analysis of gene expression, phenotype and genotype

Day 5 fifth-instar larvae of the Dazao or Dazao-*stony* strains were selected for

cuticle dissection. The cuticles of the semi-lunar marking region and the non-melanic

portion between the paired semi-lunar marking were finely dissected. Gene

expression levels of *BmLcp17*, *BmLcp18*, *BmLcp22* and *BmLcp30* in these regions

were compared. Gene expression patterns were determined for the dorsal epidermis of

abdominal segments (from semi-lunar marking to star marking) from day 5 of fifth

instar larvae (Dazao, $p^S/+$, $p^M/+$, $p^B/+$). Day 1 of second instar larvae of the *al* and

Dazao strains were also investigated. The same regions of dorsal epidermis regions

were collected from F_2-generation individuals with the p^S/p^S, *st/s*t, and $p^S/+$, *st/st*

genotypes, as well as the p^M/p^M, *st/st* and $p^M/+$, *st/st* genotypes for gene expression

analyses. For all genotyped individuals, the ratios of the intersegment length to the

intersegment fold were also analysed using image J.

Melanin precursors-promoting and inhibition treatments

The preparation and concentration of L-dopa and dopamine solutions were slightly

modified according to the description by Futahashi (Futahashi and Fujiwara 2005),

and filtered with a 0.22 μm membranes before use. Cells were washed three times

with Grace medium without melanin precursors to remove metabolites and other

contaminants on the cell surfaces. Medium (0.8 mL) containing L-dopa or dopamine

was added separately into each well, and the medium without melanin precursors was

used as the control. Culture plates were sealed with tape, wrapped with foil and

incubated at 28℃ for 24 h for a gene expression analysis. For BH_4 feeding assays,

the 30mM working solution was prepared by dissolving tetrahydrofolic acid into

ddH_2O, and spreading it on an artificial diet for *al* mutants. The control was ddH_2O.

Phenotype were observed and recorded from the second instar stage, and expression

of cuticular protein-encoding genes was analysed. For the melanism-inhibition

experiments, the wild-type strain N4 was used. Newly-hatched larvae were divided

into treatment and control groups. Individuals in the treatment group fed with DAHP

dissolved in 0.1M NaOH (15g/L), and individuals in the control group fed 0.1M with

NaOH. Phenotypes observation and gene expressions detection were performed on

day 1 of the second-instar larvae.

Quantitative RT–PCR

Total RNA extraction, reverse transcription and Quantitative reverse

transcription-PCR (qRT-PCR) conducted as described previously (Qiao *et al.* 2014).

Three biological replicates were prepared for each condition, and *BmRPL3* was used

as the internal control. Primers are listed in Table S1.

Results

Entirely distinct expression patterns of cuticular protein-encoding genes and the

cuticle appearance between melanic and non-melanic regions

Expression patterns of *BmLcp17*, *BmLcp18*, *BmLcp22* and *BmLcp30* in melanic or

non-melanic epidermal regions are shown in Figure 1. These results, together with

earlier studies (Futahashi *et al.* 2012; He *et al.* 2016; Wu *et al.* 2016), revealed that the

gene expressions were significantly higher in melanic parts of the cuticle than in

non-melanic parts. Moreover, micro protrusions were more intensive in the melanic

regions than in the non-melanic regions, and accompanied by a higher amounts of

chitin (another important cuticle component (Hopkins and Kramer 1992; Moussian *et*

al. 2006; Andersen 2010; Moussian 2010; Chaudhari *et al.* 2011; Qiao *et al.* 2014),

and the reduction chitin content was reported to impede cuticle melanism (Moussian

et al. 2005)) (Figure S1A). These results showed that, regardless of the different

genetic basis of the melanic mutants or the melanic markings in the non-melanic

strains, excessive accumulation of melanin in the cuticle (accompany by the changes

in the surface structure and the chitin content of the melanic cuticle) was closely

related to the high expression levels of the cuticular protein-encoding genes.

Expression level of larval cuticular protein-encoding genes positively correlated

with the degree of cuticle melanism

To obtain further insights into the relationships between the melanin accumulation

and the expression of cuticular protein-encoding genes, we investigated gene

expression patterns using four p locus alleles (Dazao ($+^p$), p^M, p^S and p^B), which

showed gradually increasing melanin accumulation (Figure 2A). Gene expression

levels were gradually and significantly up-regulated with the increase in the degree of

melanism in cuticle (Figure 2B). These results further showed that the expression

levels correlated positively with the degree of melanism. Thus, the quantities of

cuticular proteins with similar or redundant functions was increased greatly by the

increasing the degree of melanism.

Typical stony phenotyped individuals masked after the introduction of melanic loci

into the BmLcp17 defection strain

We assessed the effects of modulating the melanic background on the phenotypic

defects caused by the deletion of *BmLcp17*. After mating the p^B and *stony* parental,

the percentage of BC_1 individuals with melanism and the normal body shape in the

backcrossed population of the $p^B \times stony$ cross was 52 % (290/553; and theoretically, it

was 25 %), yet individuals with the melanism cuticle and *stony*-type body shape were

not found (theoretically should be almost equivalent to the number of individuals with

melanism cuticle and normal body shape) (Figure 3). In the cross of $p^S \times stony$, ~10.8%

(36/331) of F_2 progeny had an lighter melanism body color and smaller body size

(Figure 3). These individuals exhibited a little hard and tight touched body, but the

body was much softer than that of the *stony* mutant. Their intersegment folds bulged

slightly, and the length were significantly shorter than that of the internodes;

accordingly, their phenotypes slightly resembled the morphological features of the

stony mutant (Figure 3 and 4A). Even so, We did not find individuals with the typical

stony-type body shape and also defective adaptability under the melanism background

(theoretical ratio is 3/16). (Figure 3). Similarly, in the $p^M \times stony$ corss, only

approximately ~11.7% (51/437) of the individuals of the F_2 population were very

lightly melanic, but they exhibited obviously unusual morphological features (Figure

3, Figure 4A). When compared with the ~10.8% F_2 individuals (aforementioned) from

the $p^S \times stony$ cross, the touch feelings of individuals from the $p^M \times stony$ cross were

tighter and firmer, and the intersegment folds bulged more obviously and had a higher

proportion among the segments, meaning that their body features were closer to the

phenotype of the *stony* mutant (Figure 3 and 4A) (Qiao *et al.* 2014). Nevertheless,

melanic individuals showing the typical *stony*-type body features and defective

adaptability still did not appear (theoretical ratio is 3/16) in the progeny from the $p^M \times$

stony cross. Therefore, induction of the melanic mutation into individuals with a

defective cuticular protein-encoding gene could mask the adverse phenotypes, and the

masking effect was more remarkable with the increasing degree of melanism.

Other larval cuticular protein-encoding genes up-regulated evidently in the melanic

and non-stony phenotypic, but with mutated BmLcp17 genotypic offspring

Using the molecular markers (closely linked to the *p* and *st* loci, respectively), we

further genotyped the progenies with melanic color pattern and non-*stony* (including

ambiguous *stony*-like) body shape. The results revealed that approximately 49% of

the individuals showing a melanic color and normal body shape in the BC_1 population

from the $p^B \times stony$ cross was the $p^B/+^{pB}$, *st/st* genotype (Figure 4A). The ratios of the

length of the internodes and the intersegment folds (I/IF) were ~4, which is very

similar to that in $p^B/+^{pB}$, $+^{st}/st$ individuals, and also no significant differences as that in

the wild-type individuals (Figure 4B) (Qiao *et al.* 2014). In the F_2 generation from the

$p^S \times stony$ cross, approximately 9.3% of the individuals with the p^S/p^S, st/st genotype

and very few individuals (~1%) with the genotype $p^S/+^{pS}$, st/st exhibited a melanic

color and the normal body shape (Figure 4A). For p^S/p^S, st/st genotyped individuals,

the I/IF value was approximately 3.3 (Figure 4B). Although the I/IF value was lower

than that in $p^S/\_$, $+^{st}/\_$ individuals (approximately 4, Figure 4B), it was much higher

than that in the *stony* mutant (approximately 1.6 (Qiao *et al.* 2014)). Despite the

slightly smaller body size of the p^S/p^S, st/st individuals, their body shape was

essentially normal (Figure 4A). The genotypes of those lightly melanic individuals,

whose body shape was slightly like that of the *stony* phenotype (mentioned in Result

3), were all the $p^S/+^{pS}$, st/st, and the I/IF value of these individuals was approximately

2.7 (Figures 4A and 4B). In progeny of the $p^M \times stony$ cross, ~10.5% of the individuals

with the p^M/p^M, st/st genotype and ~0.7 % of the individuals with the $p^M/+^{pM}$, st/st

genotype showed a melanic color and subtle *stony* features (just very slight bulges)

(Figure 4A). The body size of the p^M/p^M, st/st individuals were smaller than those of

the $p^M/\_$, $+^{st}/\_$ individuals. They exhibited a certain sense of hardness, and the I/IF

ratio was approximately 2.8, which is in good agreement with their phenotypes

(Figure 4B). Additionally, individuals showing very slight melanism and

morphological features that were more similar to that of the *stony* mutant (mentioned

in Result 3) were all the $p^M/+^{pM}$, st/st genotype, and their I/IF values were

approximately 1.8, which is closer to that of the *stony* mutant (Figures 4A and 4B). In

addition, the expression of cuticular protein-encoding genes in p^S/p^S, *st/st* individuals

were significantly higher than that in $p^S/+^{pS}$, *st/st* individuals (Figure. 4C); a similar

result was also obtained from the p^M/p^M, *st/st* and $p^M/+^{pM}$, *st/st* individuals (Figure 4C).

Taken together, these results revealed that more cuticular proteins with similar

functions were accumulated in the cuticle of melanic homologous individuals at the *p*

locus. Based on the comprehensive and association analysis, we infer that melanic

background effectively drove the expressions of cuticular protein-encoding genes

with similar expression patterns and redundant functions, which compensated for the

morphological and adaptability defects caused by the dysfunctional *BmLcp17* gene;

and the law of compensatory abilities was $p^B/+^{pB}$, *st/st* > p^S/p^S, *st/st* > p^M/p^M, *st/st* ⪆

$p^S/+^{pS}$, *st/st* > $p^M/+^{pM}$, *st/st*, which corresponds well with the gradual weakening of the

degree of melanism (Figures 4 and Figure S3).

Content variations of melanin precursors affect the transcript amount of the

cuticular protein-encoding genes

Due to the crucial material basis for the cuticle melanism (no matter what kind of

genetic basis caused by) is the extensive accumulation of melanin precursors in the

epidermal cells; thus, the essence that melanism tend to increase the expression of

some cuticular protein-encoding genes should be driven by the relations between the

accumulation of melanin precursors and the transcripts amount of the cuticular

protein-encoding genes. The basal expressions of four cuticular protein-encoding

genes were detected in *BmNs* cells (Figure S5) and indicated that regulatory pathways

controlling the expression of cuticular protein-coding genes in this cell line. Thus, this

cell line was used to examine the effect of melanin precursors on gene expressions.

After incubating *BmNs* cells with melanin precursors, the expression of cuticular

protein-encoding genes was significantly higher in cells treated either by L-dopa or

dopamine, compare with that in the control group (Figure 5 top (left)). Second-instar

al mutant are characterized by albinism and a porous cuticle due to a mutation in

sepiapterin reductase, which leads to the insufficient synthesis of the co-factor BH_4

during the synthesis of melanin precursors (Banno *et al.* 2005; Fujii *et al.* 2013).

When treated with BH_4, these mutants had normal melanic body color (Lab

unpublished contribution) (Fujii *et al.* 2013), and gene expressions were obviously

higher than that in the control group (Figure 5 top (right)). These results suggested

that the expression of cuticular protein-encoding genes was induced by increasing

amounts of melanin precursors. We investigated the effects of DAHP, an inhibitor of

guanylate cyclase hydrolase (GTPCHI), an important rate-limiting enzyme in the

synthesis of BH_4 (Hamadate *et al.* 2008). Inhibiting BH_4 synthesis in wild-type

second-instar larvae by blocking the synthesis of melanin precursors in epidermal

cells caused individuals to lose their original melanic body color (Lab unpublished

contribution). The gene expressions were also significantly reduced when compared

with that in the control group (Figure 5 bottom). Thus, the content and variation of the

intracellular melanin precursors were important factors for regulating the expression

of cuticular protein-encoding genes. We concluded that the inducing effect of the

melanin precursors on the expression of cuticular protein-encoding genes is the basis

for melanism promoting genes' transcription.

Discussion

Melanin is mainly deposited in the epicuticle and exocuticle, cross-linked with

proteins in this layer, and involved in the formation of the melanic cuticular

characteristics (Hopkins and Kramer 1992; Suderman *et al.* 2006; Andersen 2010;

Moussian 2010; Hu *et al.* 2013). Some cuticular proteins located in the endocuticle

may not be directly associated with the melanin, but the maintaining of the normal

cuticle need to rely on the normal development and coordination between each cuticle

layer, and these proteins are indispensable element in shaping the endocuticle

(Moussian 2010; Cohen and Moussian 2016). Thus, we suppose that these

endocuticular proteins may influence the transportation or accumulation of melanin

through some indirect ways, to contribute to the unique features of the melanic cuticle.

We indeed clearly observed that extensive star-like protrusions were present on the

cuticle when a large amount of melanin accumulated (Figure S1). Similar correlations

between cuticle structure and body color have been reported multiple times (Futahashi

et al. 2012; Noh *et al.* 2016; Tan *et al.* 2016), imply that there should be some

interactions between cuticular proteins and melanin. Although the exact interaction

pattern between these two cuticular components is unknown, microscopic observation

clearly shows that the deposition of an excessive amount of melanin affected the

cuticle characteristics.

The expression profile of cuticular protein-encoding genes in melanic silkworm

mutants and the black markings of *Papilio* larvae supported the hypothesis that

up-regulated cuticular proteins probably participate in the transport or maintenance of

the corresponding pigments in a specifically colored cuticles (Figures 1, 2 and Figure

S1) (Futahashi *et al.* 2012; He *et al.* 2016; Wu *et al.* 2016). Yet over-expression of

cuticular protein-encoding genes *in vivo* cannot trigger (or induce) the formation of

melanic cuticle (Tajiri *et al.* 2017). Therefore, we hypothesized that if instructions for

melanin accumulation were included in the developmental program for which

melanism is the original factor, other cuticle features and structures would adapt to the

level of melanin accumulation, regardless of genetic background. The up-regulation

of cuticular protein-encoding genes should be a necessary adaptation for the

maintenance and stability of the structural characteristics and physical properties of

melanic cuticles. The relationship of melanin and cuticular proteins at the

ultrastructural level deserves special attention in follow-up studies.

Although the elaborate regulatory mechanism by which melanin precursors affect

the expression of cuticular protein-encoding genes is unclear, our results revealed the

existence of this regulatory phenomenon (Figure 5). Cuticle formation is regulated

stringently by temporal and spatial patterns, the accumulation and oxidization of

melanin precursors, and interactions such as crosslinking among other components

should occur after the initial cuticle formation (Moussian 2010; Sobala and Adler

2016; Tajiri 2017). Therefore, we propose that the cuticular proteins induced by

melanin precursors are used to create a platform for further shaping and perfection of

the melanic cuticle. When melanin-associated protein-encoding genes have similar

expression patterns and functions (Nakato *et al.* 1994; Nakato *et al.* 1997; Shofuda *et*

al. 1999; Okamoto *et al.* 2008; Liang *et al.* 2010; Tang *et al.* 2010; Qiao *et al.* 2014),

the production of large amounts of functionally similar cuticular proteins would be

driven by the melanic background to guarantee construction and stability of the

melanic cuticle. During this process, melanism would unlocks the complementary

features of melanin-associated cuticular proteins (such as *BmLcp18*, *BmLcp22*,

BmLcp30), rescued cuticular malformation caused by the loss of function of some

cuticular proteins (such as defected *BmLcp17* in *stony* mutant). Because a special

cuticle-forming pattern appears to be regulated by melanin accumulation, melanism

may enhance insect adaptability to avoid the impairment of survival caused by

mutations and/or functional loss of some cuticular proteins, and our results also add

new evidence to explain how melanism can be a beneficial trait (Wilson *et al.* 2001;

True 2003; Wittkopp *et al.* 2003; Wittkopp and Beldade 2009; Liu *et al.* 2015; Mallet

and Hoekstra 2016).

As far as we known, there is no evidence to suggest that the four larval cuticular

proteins (as structure proteins) and their orthologous can enter the nucleus and

regulate gene expression by acting as transcription factors. In addition, our findings

and some other studies showed that changes in the expression of one cuticular

protein-encoding genes of R&R family did not affect the expression of other members

(Figure S6, S7) (Arakane *et al.* 2012; Noh *et al.* 2015). Moreover, several studies

reveal that organisms optimize resources allocation at gene or protein expression level

(Liebermeister *et al.* 2004; Dekel and Alon 2005), and there is no report that the

cuticular proteins have long range morphogen effects (such as wingless) to regulate

their encoding genes by feedback regulation from the cuticle to the epidermal cells;

thus, the expression of melanin associated cuticle protein-encoding genes should be

appropriately and simultaneously coordinated with the sufficient accumulation of

intracellular melanin precursors, as a relatively direct, economical, efficient and

convenient strategy. Furthermore, DAHP inhibits GTPCHI activity, but it does not

directly impair the extracellular accumulation of melanin and protein–protein

interactions in the cuticle. Finally, there is some evidence that melanin precursors

regulate gene expressions via the receptors (Konradi *et al.* 1996; Berke *et al.* 1998;

Westin *et al.* 2001). In summary, we hypothesized that the up-regulation of the four

BmLcp genes were induced simultaneously by excessive amounts of melanin

precursors, but should not be the interaction among these four genes on regulation

level. To test our hypothesis, further analyses will be performed to determine the

expression of the remaining *BmLcp* genes in some *BmLcps* mutant (such as defective

BmLcp17) cell line by increasing or decreasing the melanin precursors content.

The markings were lighter in the *stony* mutant than in the wild-type strain, and

were accompanied by the downregulation of melanin synthase genes (Figure S4). If

the dysfunction of some cuticular proteins cannot be effectively supplemented,

abnormalities of the cuticle structure and interactions of various cuticular components

may occur, probably resulting in barriers to melanin deposition and metabolism. This

dysfunction might be the reason why the $p^S/+^{pS}$, *st/st* (or $p^M/+^{pM}$, *st/st*) genotypes was

lighter than the $p^S/+^{pS}$, $+^{st}/\_$ (or $p^M/+^{pM}$, $+^{st}/\_$) genotypes and had a different body

shape. Because the homozygous of p^B mutation is lethal (Xiang 1995; Banno *et al.*

2005), we were unable to obtain F$_2$ progeny with the p^B/p^B, *st/st* genotype. However,

we note that the $p^B/+^{pB}$, $+^{st}/st$ and $p^B/+^{pB}$, *st/st* genotype individuals had almost the

same body shape and melasnism (Figure 4A). Therefore, we propose that during

cuticle formation, if the signal promoting melanism is sufficiently strong, sufficient

melanin precursors should be generated. The contents of functionally redundant

proteins induced by melanin precursors are sufficient to fill the gap generated by the

dysfunction of some cuticular proteins, guaranteeing the normal accumulation of

melanin. Therefore, we hypothesize a threshold for the melanism-promoting effect.

When the accumulation of melanin precursors spans this threshold, the requirements

for cuticular proteins with similar function are not lowered, even when one of them

loses the function. This effect would guarantee the formation of a normal cuticle

structure, ensuring subsequent pigmentation.

We propose the following regulatory model: when large amounts of melanin

precursors induced by endogenous- and/or exogenous melanism-promoting factors, it

may directly or indirectly induce the up-regulation of some cuticular protein-encoding

genes. This upregulation guarantees the formation of normal structural features of the

melanic cuticle. During this process, if some cuticular proteins lose function, other

functionally redundant cuticular proteins induced by melanin precursors compensate

for the functional defects. Compensatory intensity increases with increasing melanin

accumulation. When melanin accumulation spans a certain threshold, compensation

completely masks the defective phenotype caused by the malfunctioning genes. This

model adds to growing evidence that melanism may have pleiotropic effects that

enhance adaptability over and above the effect of melanin accumulation itself (Figure

6). Due to the coexistence of excess melanin and cuticular proteins is common in

other insects, and homologues the four *BmLcp* gene are widely distributed in the

Lepidoptera (Table S2), we presume that the above reciprocal action and its

corresponding biological significance are conserved in other Lepidoptera insects to

that in *Bombyx mori*.

In summary, we used crucial cuticle components to elucidate the mutual effects

among some key genetic factors, and the physiological significance of these mutual

effects during the development of the morphological features. Our findings contribute

a realistic basis for in-depth study on the interaction patterns of melanin and cuticular

proteins, and will also guide relevant studies in other Lepidopteran insects or other

insect species.

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Figure legends

Figure 1. Expression of four larval cuticular protein-encoding genes in melanic and

non-melanic integuments. A and B represent the gene expressions between the

semi-lunar marking (black box) and the non-melanic region (between the semi-lunar

marking, red box) in Dazao or Dazao-*stony*, respectively. Scale bar: 2 mm. C.

Comparative analysis of gene expressions in the dorsal side of abdominal segments

(from the third to the fourth segment, red box) between the p^S and Dazao strains.

Scale bar: 1 cm. D. Comparison of gene expressions between the second-instar *al*

mutant and the Dazao strain (melanic). The red hashtag symbol indicates the Fig. 1 D

we are showing is cited from the previous study of our lab group (Min *et al.* 2016)

with modification. Scale bar: 2 mm. *t*-test, n=3; * $p < 0.05$; ** $p < 0.01$; *** $p <$

0.001.

Figure 2. Expression of cuticular protein-encoding genes in integuments showing

different degree of melanism. A and B display comparisons of degree of melanism

and cuticular protein-encoding gene expression levels among four strains with mutant

alleles at the *p* locus. Scale bar: 1 cm. Ratios represent the ratios of gene expression

levels between two strains. Symbols (–, +, ++, and +++) represent the increment of

the   degree   of   melanism.   Star   represents   the   melanin-associated   cuticular

protein-encoding genes. *t*-test, n=3; * $p < 0.05$; ** $p < 0.01$.

Figure 3. Segregation patterns of the phenotypes of progenies from different crosses

of melanic mutant strains and the *stony* strain. In the segregated progenies, the first

item in the list (B,N), (B,st), (S,N), (S,st), (M,N), (M,st), (N,N) and (N,st) represents

p^B-, p^S- p^M- and Normal color patterns, respectively. The second item indicates body

shape features marked with non-*stony* type (N) and the *stony* type (st). It is

noteworthy that in (S, st-am+) and (M, st-am+), the second item represents the

ambiguous *stony* body shape. The size of "+" symbol represents the corresponding

degree of the ambiguous *stony* body shape. Superscript "T"s represent theoretical

values. Superscript "A"s represent actual values. Backslashes indicates that a value

was not obtained. Chi-squared test, $* p < 0.05$; $** p < 0.01$.

Figure 4. Association analysis of the genotypes, phenotypes and gene expression

levels in segregated progenies from different crosses. A. Correlation analysis between

the genotypes and phenotypes in self-crossed or backcrossed progenies. Scale bar: 1

cm. White and red stars represent polymorphic bands at the $+^p$ and p^X loci ($X = B$, S or

M), respectively. Red and white hash-tag represent polymorphic bands at the st and $+^{st}$

locus, respectively. Solid and dotted red arrows indicate the relative degree of bulging

(solid > dotted), respectively. Slashes show the genotypes within one phenotypic

category. The thickness of the slash represents the proportion of the corresponding

genotypes. Dotted backslashes indicate that the number of individuals with the

corresponding genotype is quite low. B. Ratios of the length of internodes and

intersegmental folds in the second, third, and fourth abdominal segments of

individuals with different genotypes (showing melanic body color) in the self-crossed

or backcrossed progenies. n≥3, t-test, $** p < 0.01$. C. Gene expression analysis of

cuticular protein-encoding genes in homogeneous and heterogeneous individuals at

the p locus from self-crossed progenies of $p^S \times stony$, and $p^M \times stony$ under the

condition that the cuticle was melanic and the genotype was homozygous recessive at

st locus. n = 3, t – test, $* p < 0.05$; $** p \leq 0.01$.

Figure 5. Effect of melanin precursors (top left: in cells) and BH_4 (top right: in *vivo*)

treatments on the expression of cuticular protein-encoding genes (t-test; n = 3, * $p <$

0.05; ** $p < 0.01$), and variations of gene expression levels in larval cuticle treated

with the inhibitor DAHP (bottom). n = 3, t-test, ** $p < 0.01$.

Figure 6. Schematic overview of the effect of melanin precursors on the expressions

of cuticular protein-encoding genes. Black solid circles represent the melanin.

Triangles represent the BmLcps with similar expression patterns and functions. Solid

and hollow triangles represent the normal and defective functions, respectively.

Brown rhombi represent other components (such as chitin) in the cuticle. Solid

double-headed arrow indicates the probable interaction between the endocuticular

proteins and other components. Combination of the single-headed arrow and the letter

'R' indicate the repair of the potential defects through functional complementary.

Purple arrows show the direction in which the melanin precursors or cuticular proteins

flow from the haemolymph to the epidermal cells as well as from the epidermal cells

to the cuticle. Red arrows indicate the increased contents of melanin precursors or the

up-regulation of cuticular protein-encoding genes. Purple polyline arrows indicate

melanism-promoting factors produced by other genetic instructions. Double dovetail

arrows indicate the effect of melanin precursors on cuticular protein-encoding genes.

The question mark indicates that the details of induction (directly or indirectly) are

unclear.

Figure 1

Figure 2

A

B

Figure 3

Figure 4

A

B

Second

$p^B/+,+/st$

$p^B/+,st/st$

$p^S/\_,+/\_$

$p^S/p^S,st/st$

$p^S/+,st/st$

$p^M/\_,+/\_$

$p^M/p^M,st/st$

$p^M/+,st/st$

$p = 0.459$

**

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**

**

Ratios

Third

$p = 0.806$

**

**

**

**

Ratios

Fourth

$p = 0.876$

**

**

**

**

Ratios

C

Figure 5

Figure 6