



# Molecular Mechanisms Underlying Pupal Protective Color Switch in *Papilio polytes* Butterflies

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Mimicry is a survival strategy in which organisms deceive their predators by imitating other organisms or the surrounding environment. One example of this involves pupal color polymorphism, which is widely observed in butterflies and moths. It has been suggested that the pupal colors of *Papilio* butterflies are selected according to the tactile stimulation experienced before pupation (P). Specifically, larvae crawling on smooth leaves become green pupae, but those crawling on rough stems become brown pupae. These protective colors fit with the surrounding environment. However, the detailed molecular mechanisms underlying pupal polymorphism have generally remained unknown. To reveal these mechanisms, we first established control over the green and brown pupal coloration in *Papilio polytes* under laboratory conditions and clarified temporal and spatial changes of pupal pigments under both conditions. We also analyzed the expression of coloration genes during the pre-pupal stage and at P in the epidermis under the green and brown conditions, by RNA sequencing and quantitative RT-PCR. These analyses revealed that the brown pupal color is regulated mainly by melanin synthesis genes, *tyrosine hydroxylase (TH)* and *laccase 2*. In contrast, the green pupal color was suggested to be formed mainly through the expression of both multiple *bilin binding protein (BBP)*-related genes responsible for blue pigmentation and multiple *juvenile hormone binding protein (JHBP)*-related genes responsible for yellow pigmentation. Electroporation-mediated RNAi showed that the knockdown of *TH* or *laccase 2* blocked the brown pupal coloration, and that the knockdown of *BBP*- or *JHBP*-related genes caused yellow or blue coloration in the green-conditioned pupae, respectively, supporting the above hypothesis. We here report how genes involved in the pupal coloration of *P. polytes* are regulated, which sheds light on the evolutionary process of pupal protective colors among Lepidoptera.

**Keywords:** pupal polymorphic color switch, *Papilio polytes*, swallowtail butterfly, electroporation-mediated gene knockdown, mimicry, melanin synthesis genes, bilin binding protein, juvenile hormone binding protein

## INTRODUCTION

More than 150 years ago, pioneering evolutionary biologists identified the phenomenon of mimicry, in which prey deceives its predator by adopting a color, shape, or other characteristic to mimic other objects (Bates, 1862; Wallace, 1865). Among several types of mimicry, concealing mimicry, also called camouflage or crypsis, is one of the most interesting traits involved in prey-predator interactions and has long attracted the interest of many researchers (Prudic et al., 2007;

Futahashi and Fujiwara, 2008; Li et al., 2015; Fujiwara and Nishikawa, 2016; Jin et al., 2019). Pupae of many butterfly species belonging to the families Papilionidae, Pieridae, Nymphalid, and Lycaenid exhibit pupal cryptic colors, which fit with the background color to enable the avoidance of predators (Maisch and Bückmann, 1987; Jones et al., 2007). Pupae of most species in the *Papilio* family show green or brown colors in response to the environment (Figure 1). Hidaka et al. (1959) reported that brown and green pupae of *Papilio xuthus* were preyed upon less in lawns with the same background color, suggesting that pupal color dimorphism is effective for protection against predators. It has also been suggested that some environmental factors such as temperature, relative humidity, wavelength of light, and photoperiod perceived during the larval growth period influence the protective pupal coloration in some Papilionid and Lycaenid species (Ishizaki and Kato, 1956; Bückmann, 1960; Smith, 1978; Honda, 1981; Yamamoto et al., 2011). However, in *P. xuthus* and *P. protenor*, it was shown that the pupal color was not determined by the background color (Ohnishi and Hidaka, 1956).

In *P. xuthus*, determination of the alternative pupal coloration is mainly dependent on the tactile stimuli received in a critical period both before and after girdling (G) (Hiraga, 2006). Under conditions of sufficient light, larvae on smooth surfaces such as leaves or stalks in this period become green pupae (Figure 1). On the other hand, larvae on rough surfaces such as branches or trunks become brown pupae (Figure 1). It is suggested that the stimulus from a rough surface is transmitted to the brain and some unknown neuropeptide for brown color (termed pupal cuticle melanizing hormone: PCMH) is produced and secreted to the larval hemolymph (Awiti and Hidaka, 1982).

Most body colors in the larvae and pupae of butterflies are made by various chemical pigments, such as melanin, bilin, carotenoid, and their related pigments. Our previous studies on *Papilio* larval color revealed the association of the specific expression of several genes with each coloration: black, red, blue, and yellow. The expression of melanin-related genes, *tyrosine hydroxylase (TH)*, *dopa decarboxylase (DDC)*, *yellow*, *ebony*, *tan*, and *laccase 2*, is involved in black pigmentation (Futahashi and Fujiwara, 2005, 2006, 2007, 2008; Futahashi et al., 2010, 2012). Expression of the *ebony* gene, which converts dopamine to *N*-beta alanyl dopamine (NBAD), is associated with the eyespot-specific red pigmentation (Shirataki et al., 2010). In addition, from the comparison of the larval patterns of three *Papilio* species (*xuthus*, *machaon*, and *polytes*), it was suggested that the green color is produced by the co-expression of *bilin-binding protein (BBP)* (blue) and *yellow-related gene (YRG)* or *carotenoid binding protein 1 (CBP1)* (Shirataki et al., 2010; Futahashi et al., 2012). This information on larval pigmentation suggests that the same or a similar set of genes involved in larval pigmentation is used for producing green or brown pupae in *Papilio* species, although the detailed mechanism of pupal coloration and regulation of pigmentation genes has not been clarified.

To clarify how green and brown pupal coloration occurs via the expression of pigmentation-specific genes, we here used *P. polytes* as an experimental model. Using this species, we studied the molecular mechanisms of larval marking formation (Shirataki et al., 2010) and female-specific Batesian mimicry

(Iijima et al., 2018, 2019); we also recently clarified the whole-genome sequence of this species (Nishikawa et al., 2015), which facilitated analysis of the gene regulation involved in pupal coloration. Furthermore, we established a method of electroporation-mediated gene analysis (Ando and Fujiwara, 2013; Nishikawa et al., 2015) that can provide direct evidence of the functional role of each gene.

In this study, we first established control over green and brown pupal coloration in *P. polytes* under laboratory conditions. Using this system, we specified temporal and spatial changes of pupal pigments in brown and green pupae anatomically. We further identified pigmentation genes responsible for producing green and brown pupae, and clarified their functional roles in pigmentation by electroporation-mediated RNAi. We found that some genes exhibit expression patterns for pupal color induced specifically under green or brown conditions. When the expression of these genes is reduced by electroporation-mediated knockdown, the specific coloration is blocked. We here report how genes involved in the pupal green and brown coloration of *P. polytes* are regulated, which sheds light on the evolutionary process of pupal protective colors among Lepidoptera.

## MATERIALS AND METHODS

### Insect Rearing

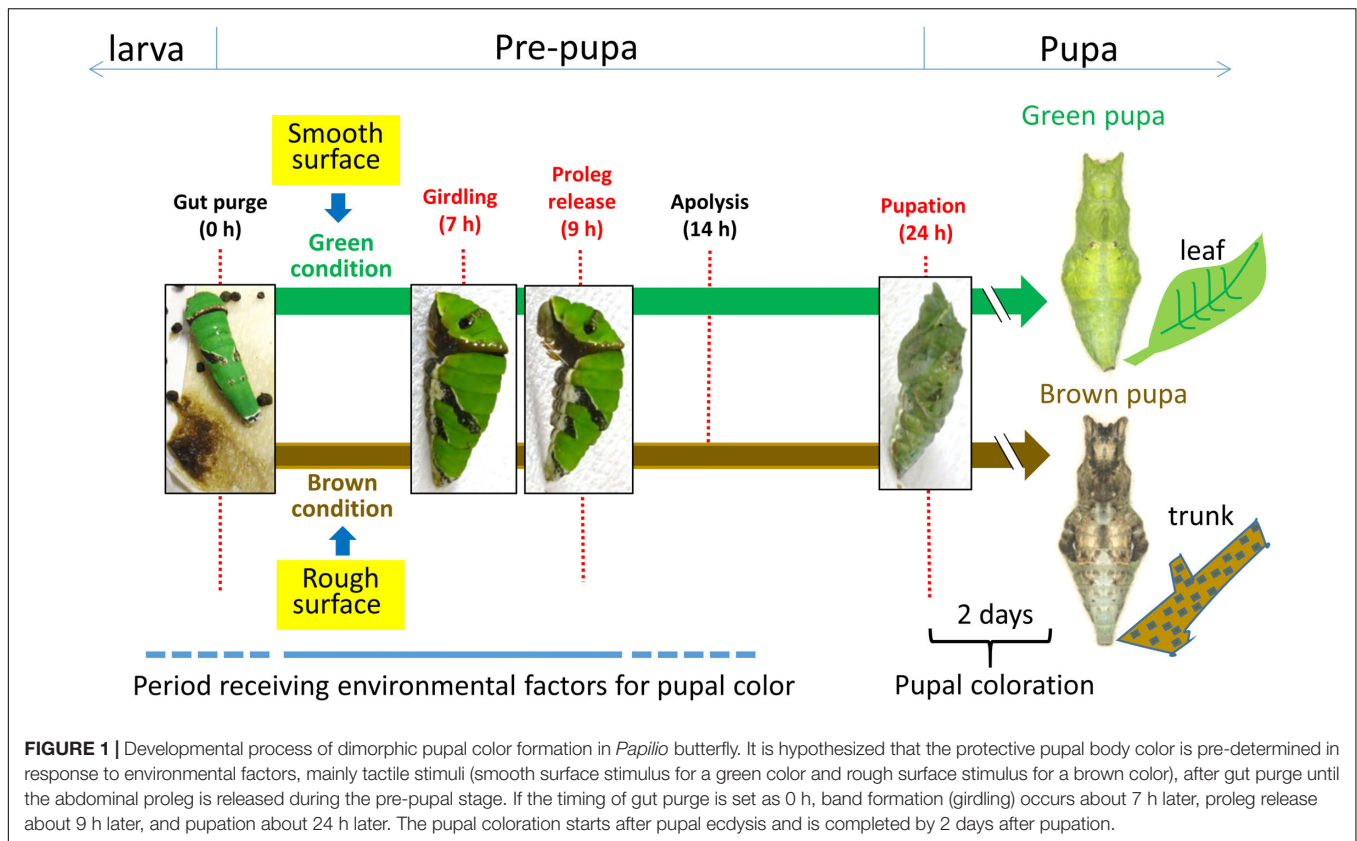
Adults of the swallowtail butterfly, *P. polytes*, were purchased from Chokan-Kabira (Okinawa, Japan). We obtained eggs from mother butterflies and reared the hatched larvae at 25°C under conditions of 16 h light and 8 h darkness (16L/8D). Larvae were fed with *Citrus natsudaidai* (Rutaceae) leaves or an artificial diet including *Citrus* leaf powder. Young larvae (first to fourth instar) and last-instar larvae (fifth instar) were reared in Petri dishes and plastic containers, respectively (Shirataki et al., 2010). Under these conditions, fifth-instar larvae pupated 24 h after gut purge (GP) and emerged 12 days after pupation (P).

### Pupal Coloration System

To obtain green and brown pupae under laboratory condition, we followed the method by Hiraga (2006). The color of pupae was controlled as follows. To obtain green pupae (green pupal conditions), fifth-instar larvae within 1 h after GP were put in a plastic container and placed on the brightest shelf (nearest to the light) in an incubator. To obtain brown pupae (brown pupal conditions), fifth-instar larvae within 1 h after GP were put in a plastic container, the interior of which was covered with the rough side of Kimtowel® (Nippon Paper Crexia Co., Tokyo, Japan) and placed on the darkest shelf in the incubator.

### RNA Isolation and Quantitative Reverse-Transcription Polymerase Chain Reaction

*Papilio polytes* pre-pupae at G (6–7 h after GP), proleg release (PR) (8–10 h after GP), and P (24–30 h after GP) were used for RNA sampling. Epidermal samples from four to six abdominal segments were dissected and washed in cold phosphate-buffered



saline (PBS). Total RNA was isolated using precooled TRI reagent (400  $\mu$ l per sample; Sigma-Aldrich) and treated with 0.2 U of DNase I (Takara) at 37°C for 15 min, purified by phenol–chloroform extraction, and then reverse-transcribed with a random primer (N6) using Verso cDNA synthesis kit (Thermo Fisher Scientific). Reverse transcription was carried out at 42°C for 60 min, followed by inactivation by incubation at 95°C for 2 min. The StepOne™ Real-Time PCR System (ABI) was used to carry out quantitative real-time PCR. Analysis was carried out using StepOne™ Software v 2.1 by the relative standard curve method. PCR reaction was carried out using Power SYBR Green PCR Master Mix (ABI) at 95°C for 15 s and 60°C for 1 min for 40 cycles. **Supplementary Table S1** summarizes the primers used for Quantitative Reverse-Transcription Polymerase Chain Reaction (qRT-PCR).

### Screening of Differentially Expressed Genes in Prepupae/Pupae Under Green and Brown Conditions

Epidermal RNA was extracted and purified by the above method. BGI Japan Corporation was contracted to carry out the RNA-seq analysis. Sequencing was carried out with 100 bp paired-end reads using the Illumina HiSeq 2500 platform. The obtained read data were mapped to the Ppolytes.v1.0.0 transcriptome [with full-length open reading frame of *BBP6* (XM\_013280002.1) and *DDC* (XM\_013278236.1)] (Nishikawa et al., 2015) using the analytical software Bowtie 2 (Langmead and Salzberg, 2012). We

used DESeq R package (Anders and Huber, 2010) for estimating differential gene expression. After calculating the expression level of each gene as the FPKM (fragments per kilobase of transcript per million mapped reads) value, genes with significantly different expression levels ( $p_{adj} < 0.1$ ) in comparisons between green and brown conditions during G, PR, and P stages were identified. Heatmaps were generated in R Bioconductor using the heatmap.2 function of the gplots package<sup>1</sup>.

### Functional Analysis by RNAi Using *in vivo* Electroporation

siRNA to be injected was designed using siRNA design support software siDirect version 2.0<sup>2</sup> (Ui-Tei et al., 2004; Yamaguchi et al., 2011). After obtaining the sequence of the open-reading frame region of the gene of interest from PapilioBase (Nishikawa et al., 2015), candidate sequences were searched for, using siDirect and based on the sequence information. To reduce off-target effects, we used the BLAST search function of PapilioBase to investigate the specificity of the designed sequence to the gene of interest and selected one with high specificity from among the candidate sequences. Synthesis of siRNA was contracted to FASMAC Corporation. **Supplementary Table S2** summarizes the details of the prepared siRNAs. The siRNA was dissolved to 500  $\mu$ M in annealing buffer [100 mM CH<sub>3</sub>COOK, 2 mM Mg (CH<sub>3</sub>COO)<sub>2</sub>, 30 mM HEPES-KOH, pH 7.4]. For the injection,

<sup>1</sup><http://cran.r-project.org/web/packages/gplots/index.html>

<sup>2</sup><http://sirect2.rnai.jp/>

the microinjector FemtoJet (Eppendorf) was used, and the glass needle for injection was fabricated using a puller to process a glass tube with a core. The siRNA was drawn up into the glass needle and placed in a micromanipulator M 401 with a glass needle set under a stereoscopic microscope; the siRNA (250  $\mu$ M; 2  $\mu$ l) was injected from the intersegmental membrane between the fifth and sixth abdominal segments into hemolymph at the prepupal stage within 1 h after GP. Immediately after injection, 0.1% PBS agarose gel was placed on the left dorsal side of the abdominal fourth and fifth segments and siRNA was introduced (five square pulses of 30 V, 280 ms width) by electrostimulation using an electroporator (Ando and Fujiwara, 2013).

## Construction of Phylogenetic Trees

To comprehensively obtain lipocalins from eight lepidopteran species (*P. polytes*, *P. xuthus*, *P. machaon*, *P. glaucus*, *Danaus plexippus*, *Heliconius melpomene*, *Manduca sexta*, and *Bombyx mori*), we downloaded Refseq/predicted proteins with InterProScan annotation files from Lepbase<sup>3</sup> (Challis et al., 2016) and selected genes that were annotated as being members of the lipocalin family (PF00061). Codon alignment was generated from a multiple sequence alignment of predicted amino acid sequences by MUSCLE (Edgar, 2004). The phylogenetic tree was constructed using the neighbor joining method with the MEGA6 program (JTT model) (Tamura et al., 2013). The confidence levels for various phylogenetic lineages were assessed by bootstrap analysis (1,000 replicates).

## Gene Synteny of Lipocalins in Lepidopteran Species

To obtain orthologous scaffolds for the *P. polytes* *BBP* locus from seven lepidopteran species (*P. xuthus*, *P. machaon*, *P. glaucus*, *D. plexippus*, *H. melpomene*, *M. sexta*, and *B. mori*), we used the following four neighboring genes of the *BBP* locus, which were highly conserved in almost all of these species. We performed a Blastp search on Lepbase (Challis et al., 2016) based on two genes upstream of the *BBP* locus, *uncharacterized LOC106101173* (XM\_013280315.1) and *hemicentin-1-like* (XM\_013280340.1), and two genes downstream of it, *uncharacterized LOC106101173* (XM\_013280315.1) and *hemicentin-1-like* (XM\_013280340.1); we then obtained the corresponding scaffolds from each species. We obtained the transcriptional orientation and genomic location of each gene from Lepbase (Challis et al., 2016). To obtain orthologous scaffolds for *P. polytes* *JHBP* (*CBP1*) locus from the seven species, we used *toll-like* gene (XM\_013282841.1) as a landmark which is conserved in most of the species. Since the scaffold length of several species is relatively short and lacks the *toll-gene*, we used a reciprocal blast strategy to look for *P. polytes* *JHBP* orthologs: *P. polytes* sequences were blasted (blastp, *E*-value <  $e^{-10}$ ) against protein database of the seven lepidopteran species of interest as well as the opposite strategy (blastp, *E*-value <  $e^{-10}$ ). Only when both pairs (*P. polytes* vs. the others) of top blast hits were the same, we considered them as true orthologs.

<sup>3</sup><http://lepbase.org/>

## Statistics and Reproducibility

Statistical analysis was performed in three biological replicates, except for analyses on the RNA-Seq data. All data are presented as mean  $\pm$  standard deviation (SD). All statistical tests were two-sided. The statistical significance of differences was analyzed using Student's *t*-test; *p* < 0.05 was considered statistically significant.

## RESULTS

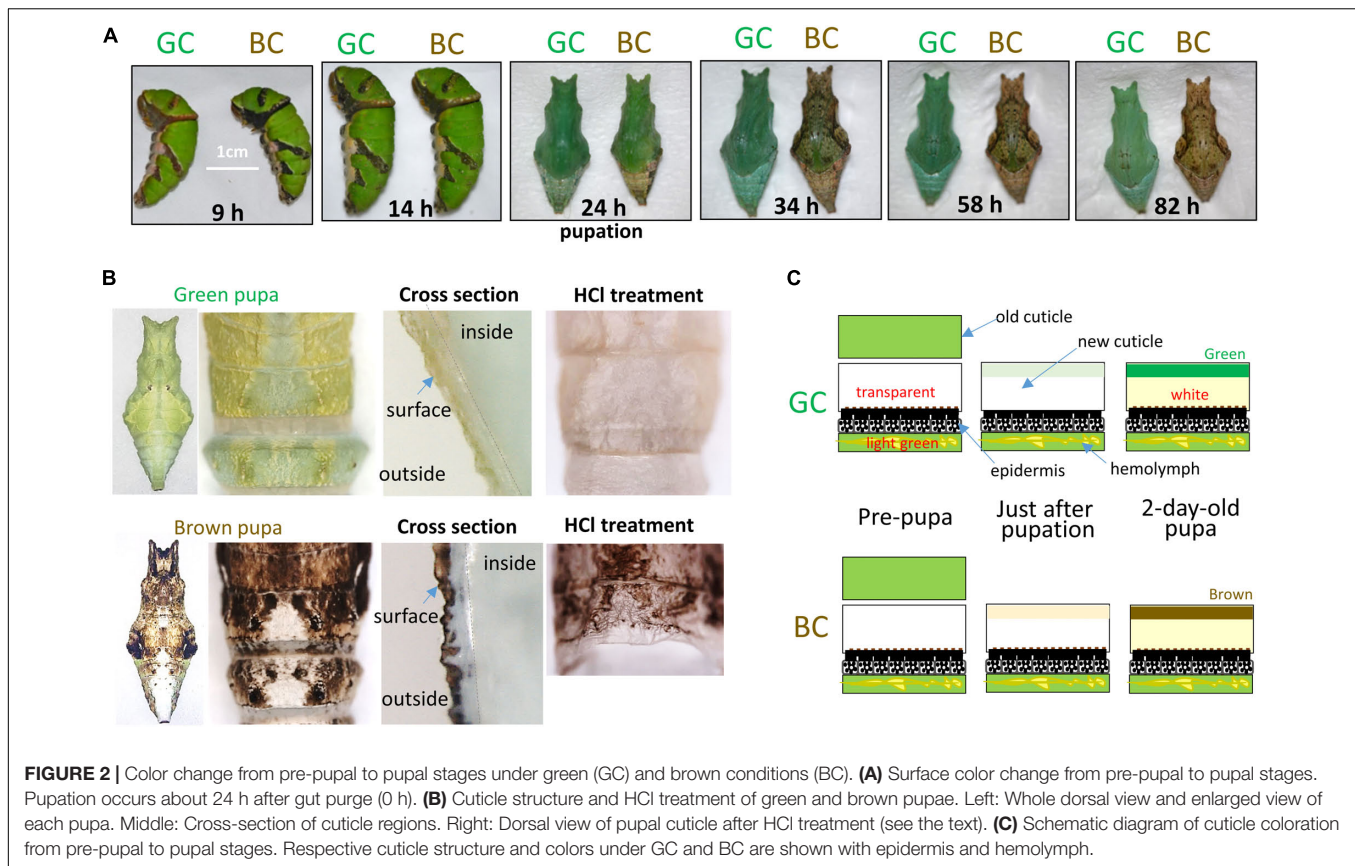
### Temporal and Spatial Change of Color Pigments in Green and Brown Pupae

To the best of our knowledge, the conditions resulting in the production of green and brown pupae in *P. polytes* have not been reported. Thus, with reference to the detailed conditions for selective pupal coloration in *P. xuthus* (Hiraga, 2006), we attempted to establish these conditions in *P. polytes* (see the section "Materials and Methods") to produce green and brown pupae. When last-instar larvae just before GP were placed in a plastic cup on the shelf exposed to the most amount of light in an incubator, 50 of 50 pre-pupae (100%) developed into green pupae. In contrast, when last-instar larvae just before GP were placed on a Kimtowel wiper in a plastic cup and positioned on the shelf exposed to the least amount of light in the incubator, 49 of 50 pre-pupae (98%) developed into brown pupae (**Supplementary Figure S1**).

Almost all of the pupae just after pupal ecdysis (P), about 24 h after GP, appeared green in terms of their external appearance in both conditions. To clarify when and where the green or brown color pigments were expressed, we observed the pigmentation process from pre-pupal to pupal stages under both conditions (**Figure 2A**). During the pre-pupal stage to P, there was little difference in the external appearance. In the pre-pupal stage, the old cuticle layer appeared green and new cuticle appeared transparent in both conditions. Even just after pupal ecdysis, new cuticle appeared transparent, except for only the outer region of cuticle, which exhibited fragmentary green or brown pigment. Since the hemolymph was green in all stages in both conditions, the green color of pre-pupae and pupae at P corresponds to the hemolymph color, which is seen through the transparent cuticle (**Figure 2**).

The green and brown pupal colors were clearly differentiable about 1.5 days after GP (**Figure 2A**, 34 h). When 2-day-old pupae were observed, both the external color and the color of the cuticle surface were completely differentiated into two colors between the green and brown conditions, while the colors of other regions and hemolymph were the same in these two conditions (**Figure 2B**). This suggests that the green or brown appearance of pupae depends mainly on the pigments localized in the cuticle surface. To further characterize the pigments, we treated the cuticles with 6N HCl at 90°C for 120 min (**Figure 2B**, right photos). We found that the green color in green pupae was lost upon this treatment, while the brown color of brown pupae was retained, suggesting that the brown color but not the green color is mainly composed of melanin-related





pigment (Shirataki et al., 2010). The above observation indicates that green and brown pupal coloration (pigment formation) occurs at the exterior of the cuticle (epicuticle) mainly after P (Figure 2C). We also observed the *P. xuthus* samples and found that they exhibited a coloration process similar to that in *P. polytes*.

## Genes Differentially Expressed Between Green and Brown Conditions

It has been suggested that the *P. xuthus* pre-pupae sense environmental factors (mainly in the form of tactile stimulation) before and after G (about 7 h after GP) and that the pupal colors are pre-determined during the pre-pupal stage (Hiraga, 2006). It is assumed that an unidentified neuropeptide that induces the brown coloration is excreted from the brain at least before P because the brown (and green) pigment formation mainly occurred after P, as shown above. Thus, we speculate that gene regulation during the pre-pupal stage to P is essential for dimorphic pupal coloration.

Next, as an attempt to screen the genes involved in green and brown pupal coloration, we performed RNA sequencing (RNA-seq) analyses using RNAs prepared from G (7 h after GP), PR (9 h after GP), and P (24 h after GP), under green and brown conditions (Supplementary Figure S2). We mapped short read sequences on the transcriptome (*Ppolytes* v1.0.0; Nishikawa et al., 2015) and calculated the read count for each gene using Bowtie 2

(Langmead and Salzberg, 2012). Total read numbers in RNA-seq were 112,039,832 (G, under the green condition), 112,716,020 (G, under the brown condition), 101,964,764 (PR, GC), 137,897,900 (PR, BC), 133,278,184 (P, GC), and 108,870,344 (P, BC). Next, we screened the genes differentially expressed (DE) between green and brown conditions using DESeq (see the section “Materials and Methods”), with *padj* (adjusted *p*-value) < 0.1. Under these conditions, we identified DE genes as follows: 48 (G, GC), 72 (G, BC), 62 (PR, GC), 169 (PR, BC), 73 (P, GC), and 53 (P, BC) (Supplementary Figure S2). RNA-seq-based screening in this study mainly has an exploratory value because each comparison consists of one biological replicate.

Notably, seven lipocalin family genes, *BBP1*, 2, 4, 5, two *insecticyanin-A-like* genes (*PpolytesGene0006744*, *PpolytesGene0006745*), and *lopap-like*, were included in the list of green condition-specific genes at P. *BBP* genes are reported to be involved in the blue coloration in the green region of final-instar larvae of *Papilio* species (Shirataki et al., 2010), suggesting that these genes are involved in the green pupal coloration.

In the list of brown-specific genes, some pigmentation-related genes were screened: *opsin-1-like* (at G) is related to red pigmentation of *Drosophila eye*; *kynurenine formamidase* (at PR) is related to brown/red pigment papiliochrome R; and *yellow-c* and *yellow-h3* (at G) are related to melanin synthesis (Supplementary Figure S2) (Futahashi et al., 2012). In addition, some Toll signaling pathway genes, namely, *trypsin-1-like*, *peptidoglycan recognition protein-like*, *serine protease easter-like*,

*trypsin-7-like* (all at G), and serine protease *snake-like* (at PR), were also screened (**Supplementary Figure S2**). It is possible that these genes are involved in the brown coloration because the Toll signaling pathway genes are suggested to be involved in red/black pigmentation in earlier reports on *P. polytes* wings (Nishikawa et al., 2013) and silkworm *Zebra* mutant (KonDo et al., 2017).

## Comparison of Expression Profiles of Genes Putatively Involved in Brown Pupal Coloration – Melanin-Related Genes

Since the brown pupal color is composed of melanin-related pigments as shown above, we next used RNA-seq to analyze the expression profiles of six melanin-synthesis genes at three stages: G, PR, and P (**Figure 3B**). In eyespot marking of the fifth-instar larvae of *P. xuthus*, we previously found that *TH*, *yellow*, *laccase 2*, and *tan* are expressed specifically in the black region, and *ebony* specifically in the red region (Futahashi et al., 2010; Shirataki et al., 2010). Based on the RNA-seq data for the abdominal region at P, *TH*, *ebony*, and *yellow* were expressed more highly under the brown condition than under the green one (**Figure 3A**). In contrast, *tan* exhibited the opposite pattern. Since the purpose of the RNA-seq analysis was to screen notable genes using only one sample, we next performed quantitative RT-PCR for five genes (not for *DDC*) with triplicate samples to confirm the above results. The expression profiles of *tan* and *ebony* were similar between the RNA-seq and qRT-PCR, suggesting the dominant expression of *tan* at P under green conditions, but the dominant expression of *ebony* at P under the brown condition. Comparison of the data obtained by the two methods also showed similar patterns in *TH* and *laccase 2*, but a different one in *yellow*. Judged from the results of qRT-PCR, significant differences in expression were observed in *tan* at P, which was induced specifically in the green condition, and in *laccase 2* at P, which was induced specifically in the brown condition.

## Comparison of Expression Profiles of Genes Putatively Involved in Green Pupal Coloration: *BBP*- and *JHBP*-Related Genes

Our earlier results on larval coloration in *Papilio* species showed that *BBP* and *CBP1* genes are related to blue and yellow color formation, respectively, and that the co-expression of both genes produces a green region (Shirataki et al., 2010; Futahashi et al., 2012). According to phylogenetic analysis, *BBP* is categorized as a kind of lipocalin; here, we found a new *BBP* gene named *BBP6* from RefSeq (XP\_013135456.1) (**Supplementary Figure S3**). Based on the whole-genome sequence of *P. polytes*, we revealed that one single scaffold, NW\_013525180.1, included six *BBP* genes and five lipocalin genes (I–V), suggesting that these genes are clustered at a genetic locus on chromosome (linkage group) 25 (**Figure 4**). Although this gene cluster is conserved among lepidopteran species with a sequenced genome, *P. polytes* has the largest number of *BBP* and lipocalin family genes. *CBP1* (*PpolytesGene0004815*) is categorized as

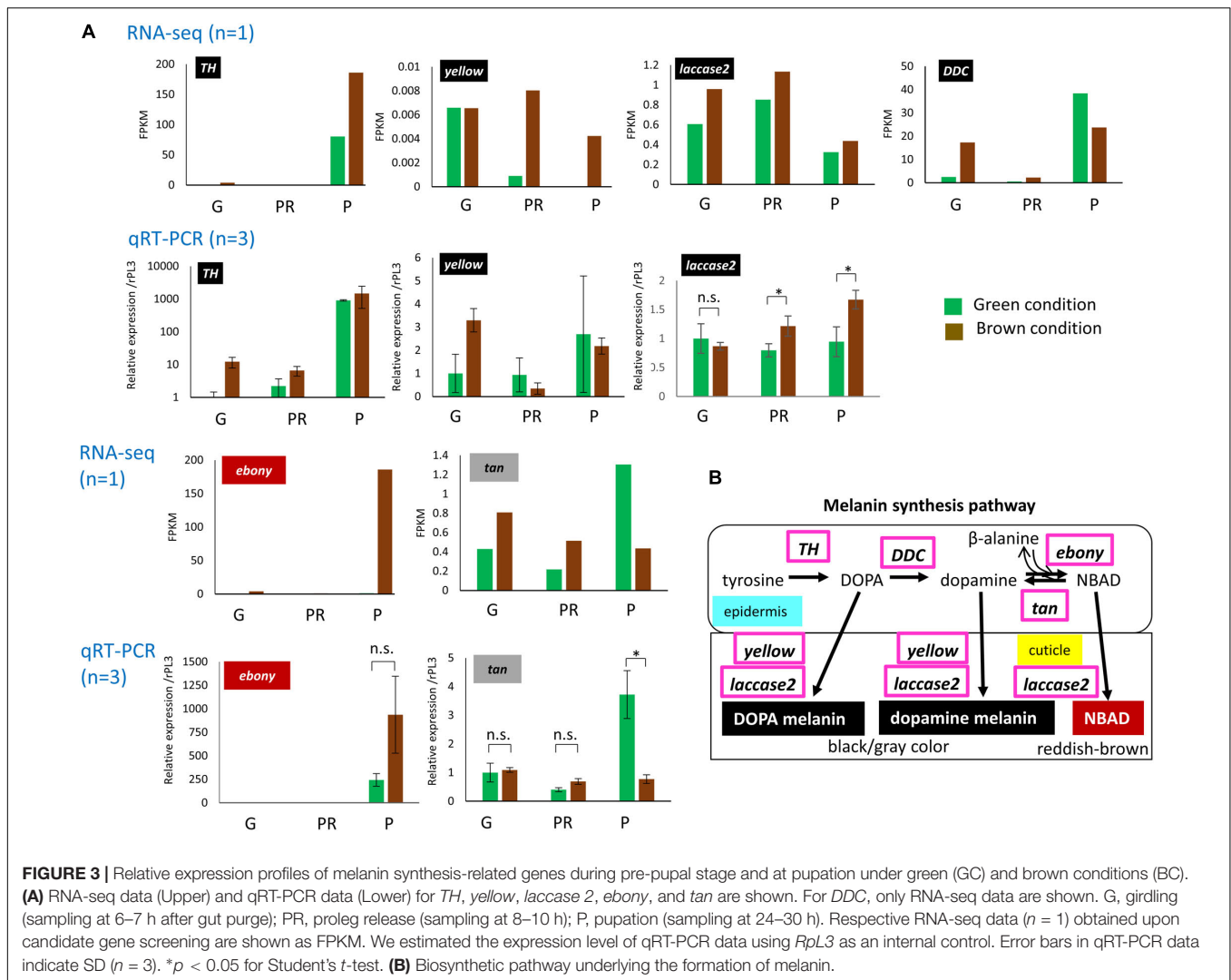
a juvenile hormone binding protein (*JHBP*), and six *JHBP* genes (*PpolytesGene0004811* to *0004816*) were included in a single scaffold (NW\_013525336.1). This suggests that these genes constitute another genomic locus seems to reside in chromosome (linkage group) 23 (**Supplementary Figure S4**). We compared the *JHBP* synteny with other lepidopteran species as for *BBP* genes. The analysis of the *CBP1* locus comparisons revealed that *JHBP* genes including *CBP1* are clustered in all *Papilio* species, indicating that *JHBP* (*CBP1*) cluster is highly conserved between *Papilio* species (**Supplementary Figure S4**). Although we could not detect true *CBP1* orthologs in the rest of lepidopteran species (*D. plexippus*, *H. melpomene*, *M. sexta*, and *B. mori*), we found *JHBP* gene repeats in the corresponding locus (except for *M. sexta*) but the gene numbers are different between species, suggesting that *JHBP* genes have been gained or lost independently in the evolutionary process of Lepidoptera. The variation of *JHBP* gene copies might involve body color formation but future study is needed.

To determine the possibility that these genes are involved in the pupal green color formation, we analyzed their expression profiles between the green and brown pupal conditions (**Figure 5**). In the results of RNA-seq analysis, we found that the expression of five *BBP* genes, *BBP1*, 2, 4, 5, and 6, was induced only at P more highly under the green condition than under the brown condition. However, *BBP3* was expressed in three stages under both color conditions. By qRT-PCR, we confirmed this expression profile of four *BBP* genes (1, 2, 4, and 5) found in RNA-seq. Considering that the pupal coloration occurs mainly after pupal ecdysis, as shown above, it is suggested that the green-specific expression of these *BBP* genes just after P is involved in blue pigmentation.

We further compared the expression patterns of six *JHBP* genes, and found that *PpolytesGene0004811* (*JHBP4811*) and *PpolytesGene0004813* (*JHBP4813*) showed expression profiles similar to those of *BBP* genes, the expression of which is induced at P in the green condition but not in the brown one (**Supplementary Figure S5**). *CBP1* (*PpolytesGene0004815*) and *JHBP4812* showed similar levels of expression between green and brown conditions in all stages. The qRT-PCR analysis of *CBP1* confirmed its expression profile as identified by RNA-seq. The expression of two *JHBP*-related genes [*PpolytesGene0004814* (*JHBP4814*) and *PpolytesGene0004816* (*JHBP4816*)] was induced in the brown condition at the PR stage and at the G stage, respectively.

## Functional Analyses of Genes Involved in Brown and Green Pupal Colors

To determine the functional role of each gene, we next performed electroporation-mediated knockdown experiments. The siRNA for each gene was injected into the dorsal side of the abdominal segment and incorporated into the right side of the abdominal region (the fourth and fifth segments) by touching the plus (+) electrode. We found that the knockdown of *TH* in nine brown-conditioned pupae caused decreased brown coloration in the siRNA-introduced region of eight samples, compared with that on the non-introduced side (**Figure 6**). Similarly,



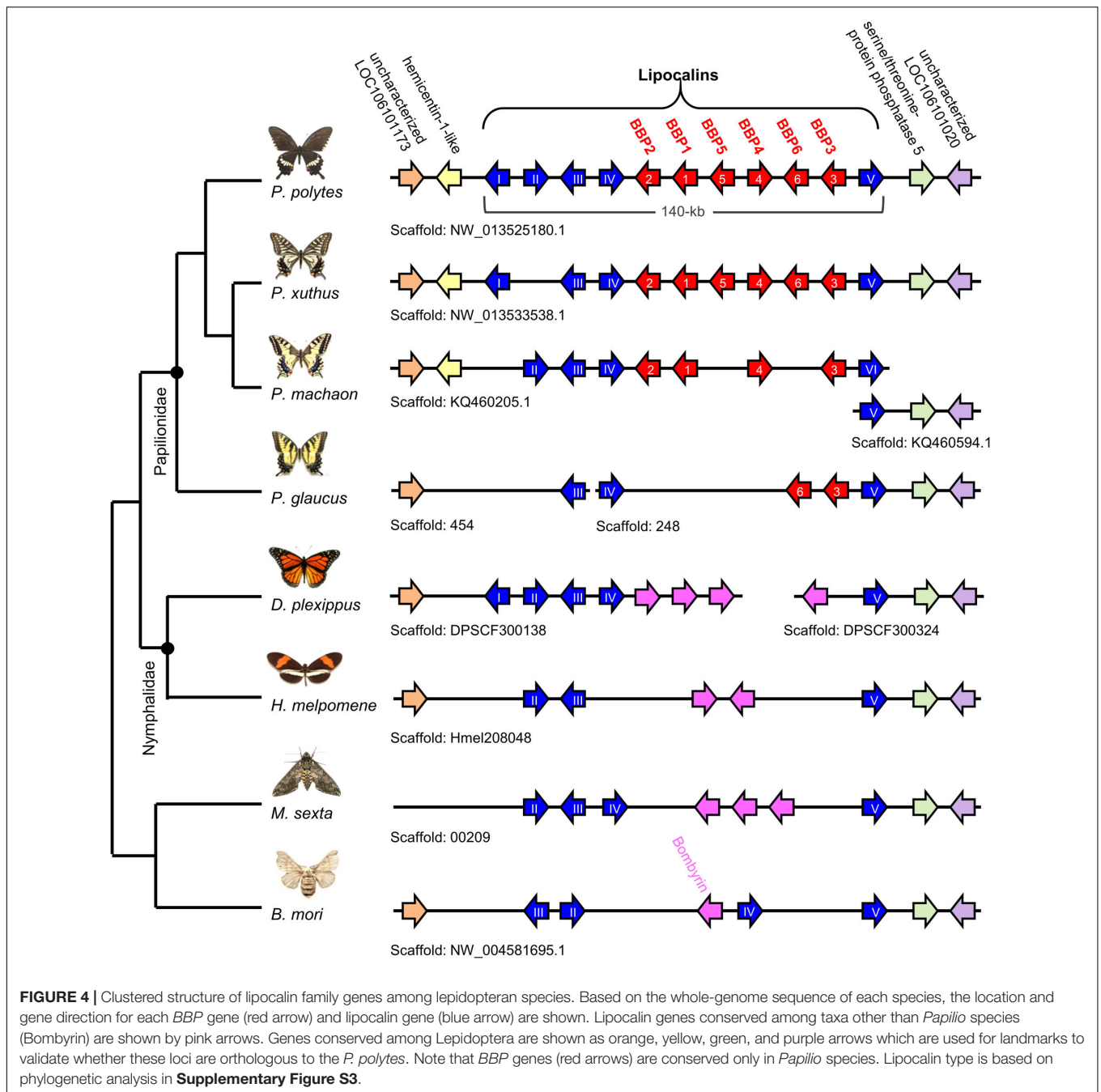
**FIGURE 3 |** Relative expression profiles of melanin synthesis-related genes during pre-pupal stage and at pupation under green (GC) and brown conditions (BC). **(A)** RNA-seq data (Upper) and qRT-PCR data (Lower) for *TH*, *yellow*, *laccase 2*, *ebony*, and *tan* are shown. For *DDC*, only RNA-seq data are shown. G, girdling (sampling at 6–7 h after gut purge); PR, proleg release (sampling at 8–10 h); P, pupation (sampling at 24–30 h). Respective RNA-seq data ( $n = 1$ ) obtained upon candidate gene screening are shown as FPKM. We estimated the expression level of qRT-PCR data using *Rpl3* as an internal control. Error bars in qRT-PCR data indicate SD ( $n = 3$ ). \* $p < 0.05$  for Student's *t*-test. **(B)** Biosynthetic pathway underlying the formation of melanin.

knockdown of *laccase 2* in four brown pupae resulted in the decrease of brown coloration in all cases. Notably, the same siRNA-introduced region showed the complete loss of black pigmentation in the later adult stage (Figure 6). Thus, melanin-synthesis pathway genes, *TH* and *laccase 2*, are involved in the brown coloration. Although we attempted to knock down the expression of other melanin-related genes (*yellow*, *tan*, and *ebony*), we could not detect phenotypic changes in the brown coloration.

To check whether *BBP* and *JHBP* genes are involved in the green pupal coloration, we next knocked down genes whose expression was induced by the green conditions at P (*BBP1*, *BBP2*, *BBP4*, *BBP5*, *JHBP4811*, and *JHBP4813*). Single gene knockdown of *BBP1* or *BBP2* in green-conditioned samples showed a yellowish color change in the introduced region, indicating the loss of blue pigmentation (Figure 7). However, no phenotypic change was observed upon the single knockdown of *BBP4* or *BBP5* (Figure 7). Since the blue coloration could potentially be formed by redundant

functions of *BBP* family genes, we further knocked down several genes simultaneously. Double knockdown of *BBP1/BBP2* or *BBP4/BBP5*, and simultaneous knockdown of four *BBP* genes, made the change from green to yellow in the siRNA-introduced region clearer than the case upon single gene knockdown (Figure 7). This suggests that each of the four *BBP* genes has a redundant function and cooperatively contributes to the pupal blue color formation.

Furthermore, we knocked down two *JHBP* genes in the green pupae, *4811* and *4813*, since their expression was observed specifically under the green conditions at P. While a small change of color from green to blue was observed upon the single knockdown of each gene, double knockdown of both genes showed the blue region more clearly due to the decreased yellow pigmentation (Figure 8). Although *CBP1* (*4815*) was indicated to be involved in the larval green coloration (Shirataki et al., 2010; Futahashi et al., 2012), its knockdown did not show a phenotypic change in the green pupae, suggesting that the expression of *CBP1* is not related to the pupal green coloration.



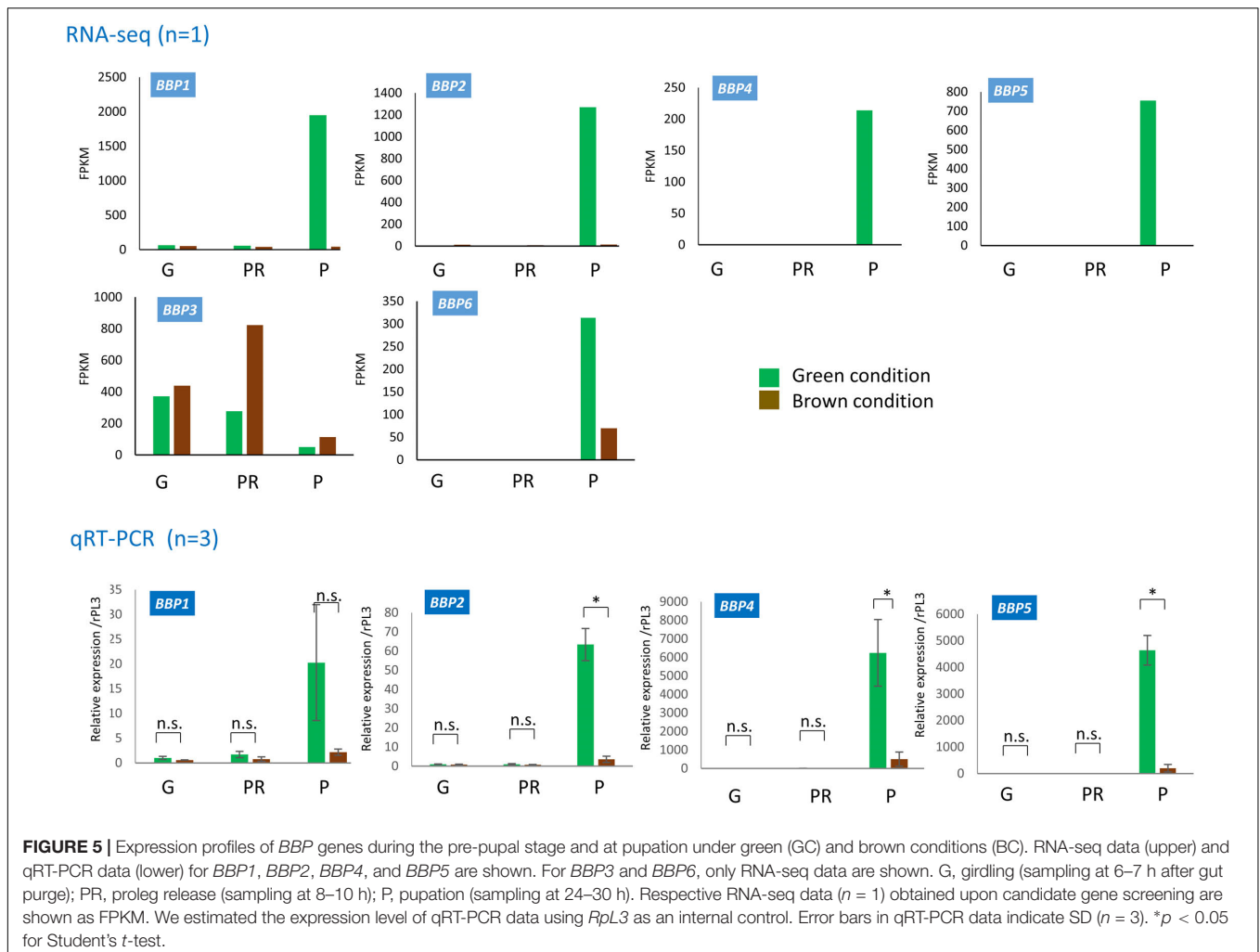
## DISCUSSION

Hiraga (2006) reported that *P. xuthus* pupae become green when the surface of their pupation site is smooth, while they become brown when the surface is rough, under conditions with sufficient light. Although light suppresses the induction of the brown pupal coloration, the main environmental factors influencing pupal coloration are considered to be tactile signals in this *Papilio* species. Hiraga (2005) also found that the protective pupal coloration in another butterfly in Papilioninae, *Graphium sarpedon*, is strongly dependent on the brightness in

the pupation site. Although some environmental factors, such as light, humidity, temperature, and photoperiod, influence the pupal coloration of *Papilio* species, none of these factors appears to be the main determinant (Hazel and West, 1979; Honda, 1981). In *P. polytes*, *P. demoleus*, and *P. polyxenes*, Smith (1978) reported that brown pupae were often found on rough surfaces and green pupae on smooth ones, although the main environmental factor affecting pupal coloration was not identified.

In this study, we observed that the pupal coloration of *P. polytes* occurs via a process similar to that in *P. xuthus*, and established the laboratory conditions causing an almost

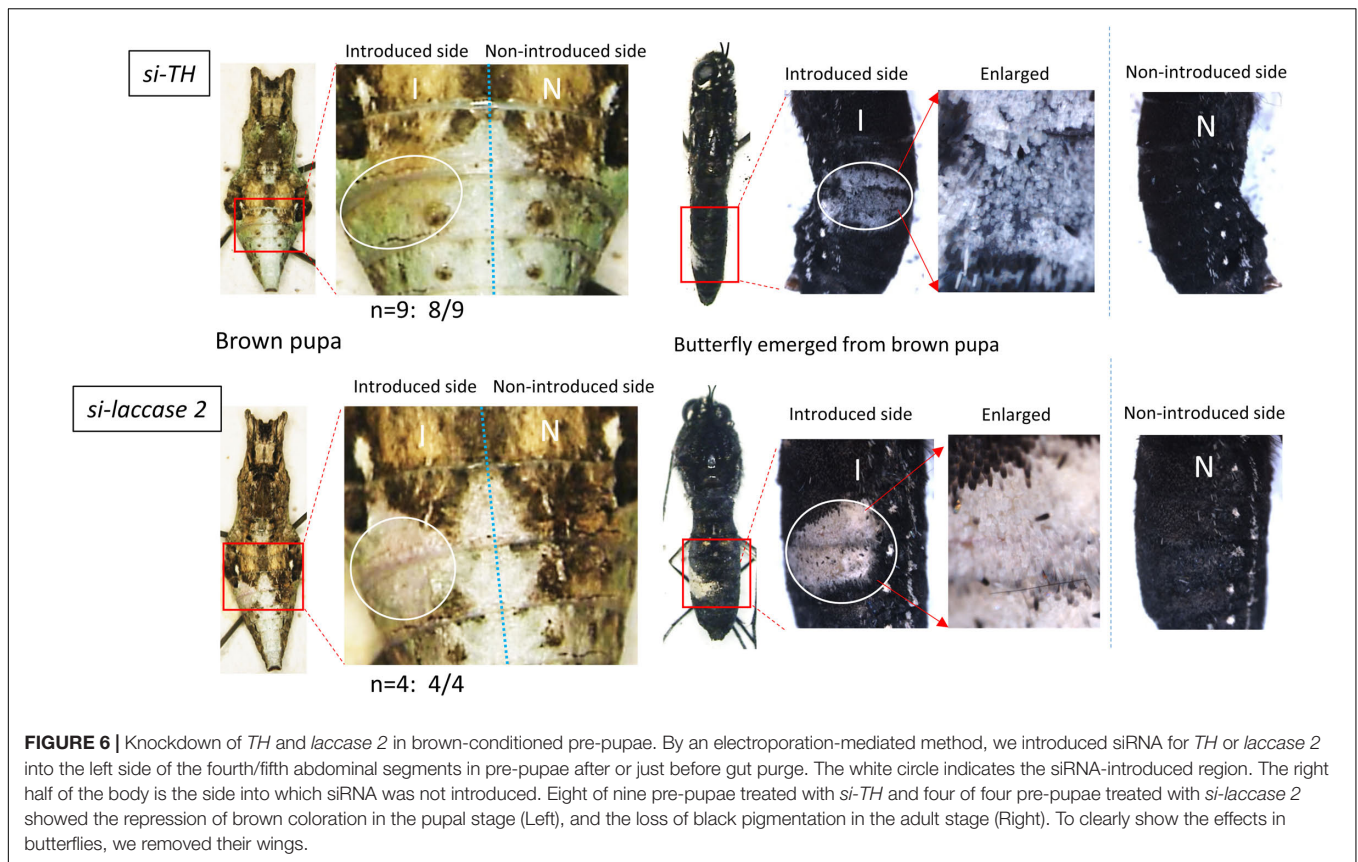




complete switch in the pupal coloration to green or brown (98–100%) (**Supplementary Figure S1**). This enables us to pursue the gene expression profiles leading to each coloration precisely after the pre-determining period before the pigmentation period. Hiraga (2006) suggested that a critical period for exposure to environmental factors pre-determining pupal coloration ranges from before G (about 7 h after GP) to after G. We found that all pre-pupae and pupae just after P (about 24 h after GP), even under brown conditions, appeared almost completely green in appearance, and brown pupae were visible at least 24 h after P (**Figure 2**). Just after P, the green color of hemolymph was seen through the transparent cuticle newly synthesized in pupae under both green and brown conditions. Notably, the green and brown pigments were located mainly in the epicuticle of newly synthesized pupal cuticle. This indicates that there is a time gap, of at least 1 day, between the pre-determination and pigmentation periods.

We thought that the genes controlling the pigmentation processes should be expressed before pupal coloration, which appear to occur mainly after P. Thus, we analyzed

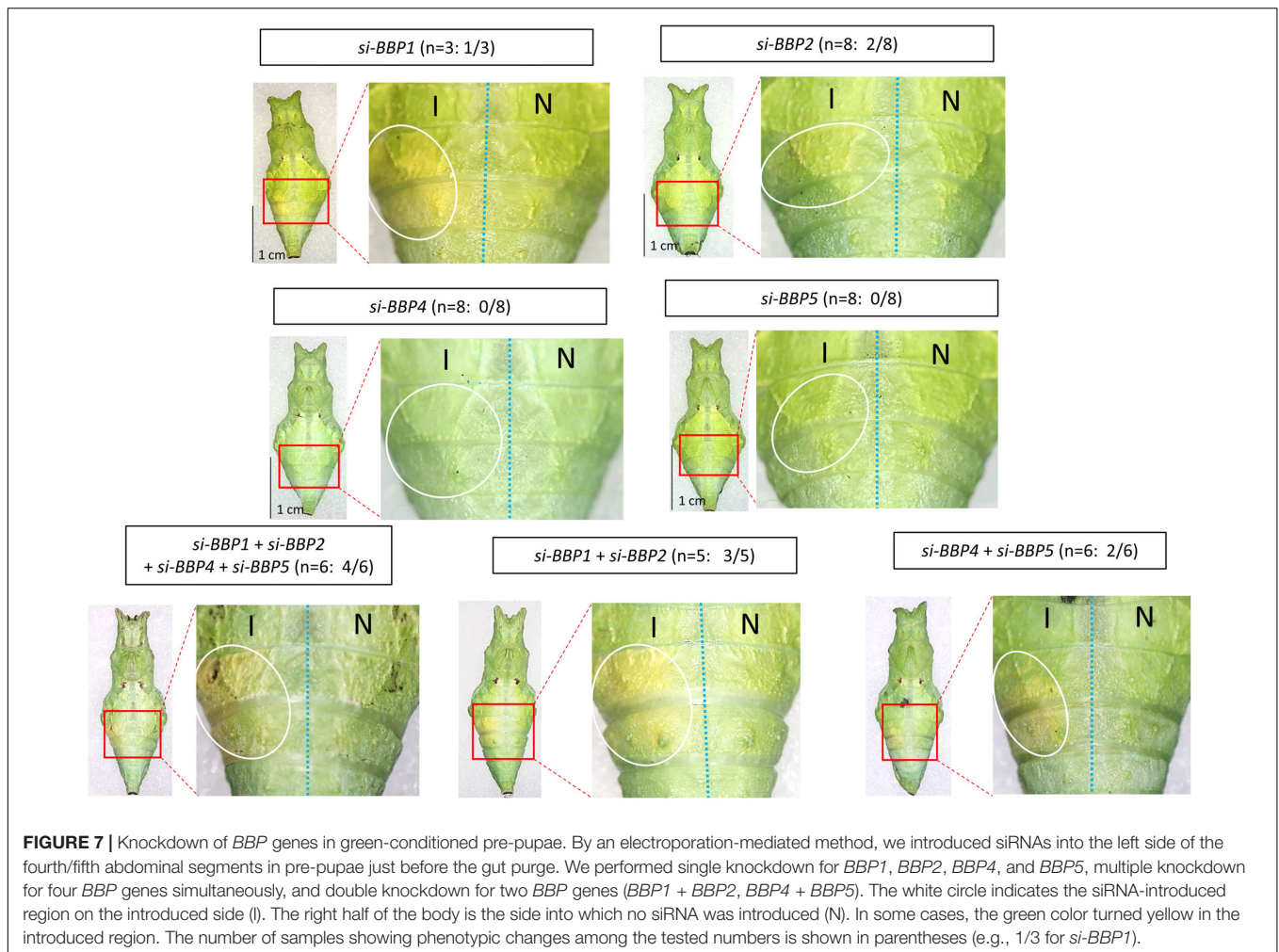
the temporal expression patterns of genes by RNA-seq and qRT-PCR at three developmental stages: G, PR, and P. Among the melanin synthesis pathway genes, *TH*, *laccase 2*, *ebony*, and *tan* showed similar expression patterns between RNA-seq and qRT-PCR analyses (**Figure 3**). However, *yellow* showed different patterns between the two analyses, probably because its expression level was very low and easily fluctuated. The expression levels of *TH* and *laccase 2* were higher under the brown condition in all three stages. Combining this observation with the result that the knockdown of each gene caused the loss of melanin pigments (**Figure 6**), it is supposed that *TH* and *laccase 2* are essential factors for inducing the brown pupal coloration. Interestingly, the expression of *ebony* and *tan* was induced at P specifically under the brown and green conditions, respectively. However, *ebony* knockdown in the brown pupae or *tan* knockdown in the green pupae did not alter the color. Although we do not know the exact reason for this result, the timing of injection of siRNA before the GP may not be appropriate for effective knockdown of the expression of these genes.



*BBP1* and *BBP2* were shown to be expressed specifically in the blue spot region of *P. polytes* larvae (Futahashi et al., 2012), suggesting that these genes are involved in the blue pigmentation. In addition, *BBP1*, *BBP2*, and *CBP1* were also co-expressed in the larval green region of *P. polytes* (Futahashi et al., 2012), suggesting that *CBP1* is involved in the yellow pigmentation, at least during larval development. Notably, five *BBP* genes (1, 2, 4, 5, and 6) were highly expressed in the green-conditioned pupae only at the P stage (Figure 5). However, these *BBP* genes were not expressed in two other pre-pupal stages, nor under the brown condition. Among six *JHBP* genes tested, *4811* and *4813* also showed similar P-specific expression only under the green condition (Supplementary Figure S5), while *CBP1* (4815) was constantly expressed under both green and brown conditions. Therefore, it is suggested that the expression of these genes is regulated to ensure that they work together at P to effectively produce a green color. Each protein encoded by these genes should be produced after P, which is consistent with the observation that green (or brown) coloration occurs mainly after P. Although single knockdown of *BBP4* or *BBP5* could not block the green coloration, double knockdown of both genes repressed it (Figure 7). In addition, simultaneous knockdown of multiple *BBP* or *JHBP* genes generally showed the green coloration more effectively than single-gene knockdown (Figures 7, 8). This indicates the redundant function of *BBP* and *JHBP* genes. It is noteworthy that *CBP1* is involved in the larval green coloration, but

seemingly not in the pupal green (Figure 8). In contrast, *BBP4* and *BBP5* could not block the larval green coloration, even upon their double knockdown, so these genes are involved only in the pupal green coloration (Supplementary Figure S6 and Figure 7). These results suggest different color regulation between larval and pupal stages, even in the same individual of *P. polytes*.

This study also clarified the existence of multiple copies of the *BBP* and *JHBP* gene families, which are each located in a single locus of the genome. In *P. polytes*, six *BBP* and five other lipocalin genes are clustered in a 140 kb genomic region, the structure of which is conserved among *Papilio* species, while the gene numbers differ in each species (Figure 4). *P. glaucus*, the pupae of which are only brown in color, has only two *BBP* genes (*BBP3* and *BBP6*), while *P. machaon*, *P. xuthus*, and *P. polytes*, the pupae of which show dimorphic green and brown colors, have four, six, and six *BBP* genes, respectively (Figure 4). This indicates that the numbers of *BBP* genes have increased (or decreased) as an adaptation to the environment experienced during each life cycle over the course of evolution. In contrast to other *BBP* genes, *BBP3* was expressed under both conditions and was not suggested to be involved in pupal green coloration, which is supported by the above finding in *P. glaucus*. The *BBP* gene cluster is conserved specifically among the *Papilio* species, while it is replaced by lipocalin family *bombyrin* genes in other lepidopteran species (Figure 4 and Supplementary Figure S3).



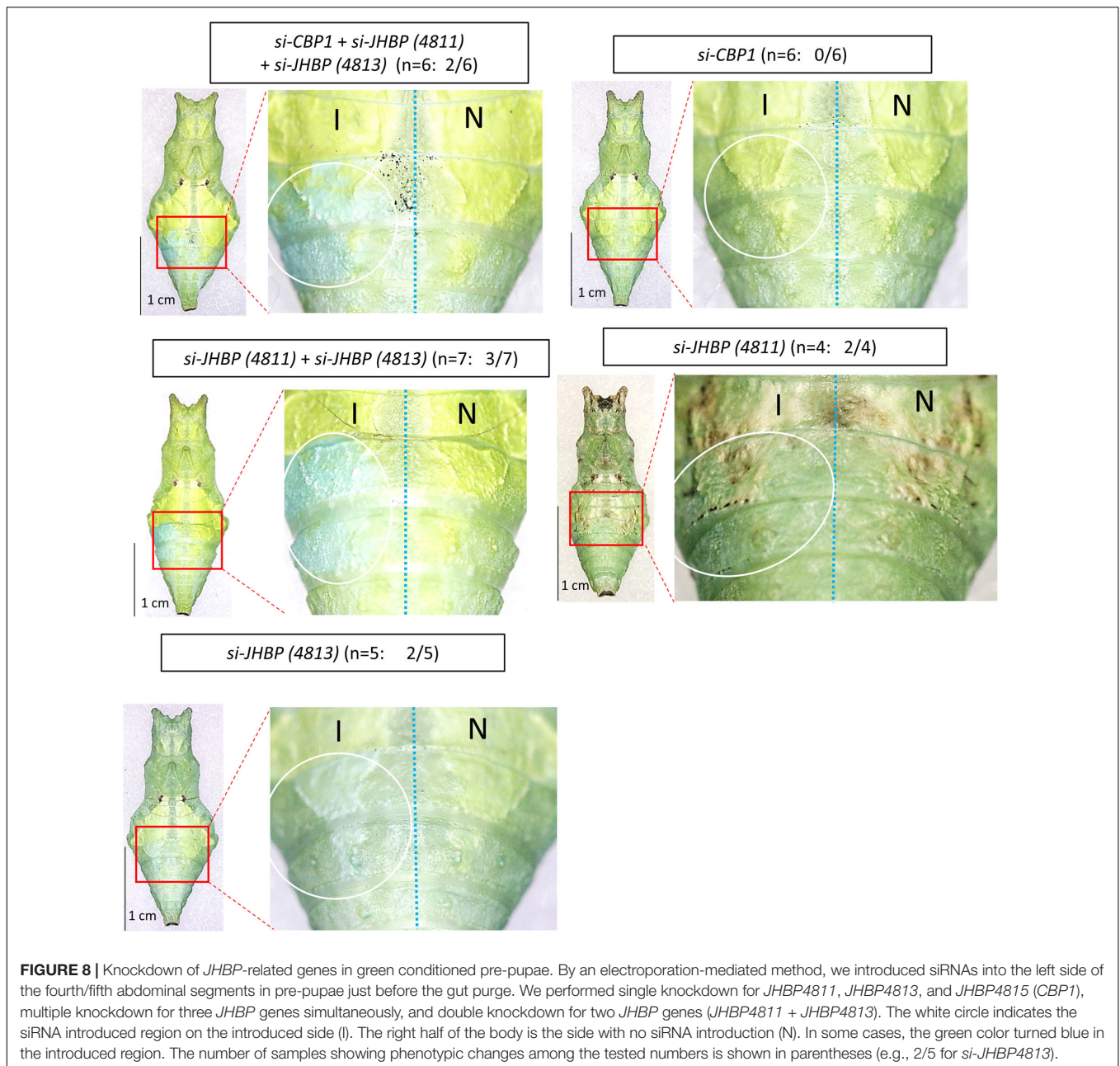
**FIGURE 7 |** Knockdown of *BBP* genes in green-conditioned pre-pupae. By an electroporation-mediated method, we introduced siRNAs into the left side of the fourth/fifth abdominal segments in pre-pupae just before the gut purge. We performed single knockdown for *BBP1*, *BBP2*, *BBP4*, and *BBP5*, multiple knockdown for four *BBP* genes simultaneously, and double knockdown for two *BBP* genes (*BBP1* + *BBP2*, *BBP4* + *BBP5*). The white circle indicates the siRNA-introduced region on the introduced side (I). The right half of the body is the side into which no siRNA was introduced (N). In some cases, the green color turned yellow in the introduced region. The number of samples showing phenotypic changes among the tested numbers is shown in parentheses (e.g., 1/3 for *si-BBP1*).

Bombyrin in *B. mori* is known to be localized in the central nervous system (Sakai et al., 2001), but its function has not yet been clarified. It would be interesting to determine the functional and evolutionary relationships between *BBP* and *bombyrin* family genes.

The clustered structure of *BBP* and *JHBP* multigene families may provide a clue to understanding the evolution and mechanisms underlying the green color formation. Each set of *BBP* genes located side by side in the *P. polytes* genome appears to have different functional roles: the set of *BBP1* and *BBP2* is involved in both larval and pupal green coloration, the set of *BBP4*, *BBP5*, and *BBP6* is involved only in pupal green coloration, while the set of *BBP3* appears to be a prototype because *BBP3* is the only gene that is conserved in *Papilio* species (Figure 4). The main *JHBP* genes involved in pupal green coloration, *4811* and *4813*, are located on the same locus (Supplementary Figure S4), and the distance between them is about 10 kb. These findings indicate that genes with similar functions in green coloration have evolved as sets. A regulatory *cis*-element enabling the similar expression patterns may be shared among each gene set, but this has not yet been clarified. A famous example of multigene families in

insects is chorion (eggshell protein) genes involved in the eggshell formation (choriogenesis). In *Bombyx*, it has been shown that 127 chorion genes are clustered within 750 kb on chromosome 2 (Chen et al., 2015). The vast majority of *Bombyx* chorion genes are organized as coordinately expressed pairs that are transcribed from opposite strands, under the direction of a short shared 5' flanking promoter located on the middle of the pair (Kafotos et al., 1995). Recent study showed that this bidirectional promoter contains *cis*-elements interacting with transcription factors such as HMG A, GATA, and C/EBP to achieve precise timing of chorion genes' expression (Tsatsarounos et al., 2015). In contrast to chorion genes, regulatory mechanisms for the differential expression of *BBP* and *JHBP* genes remained unknown, but it is suggested that the pupal color switch in Papilionid depends on the release of a brown inducing factor, pupal cuticle melanizing hormone (PCMH), which is secreted from brain subesophageal ganglion thoracic ganglion (Br-SG-TG) complexes during the pharate pupal stage (Awiti and Hidaka, 1982; Starnecker and Hazel, 1999; Yamanaka et al., 1999). Interestingly, *Papilio glaucus*, a species that only produces brown pupae, midbody ligation causes the posterior compartment to become green,





and the brown color can be rescued with an injection of PCMH. The observation indicates that environmentally cued pupal color could evolve by facultative inhibition of PCMH release (Jones et al., 2007; Nijhout, 2010). By performing gel filtration and high-performance liquid chromatography (HPLC), Yamanaka et al. (1999) estimated that the molecular weight of the *B. mori* factor showing PCMH activity was 3,000–4,000 Da, although the PCMH molecule has not yet been clarified not only in *B. mori* but also in *Papilio* species. Identifying the PCMH itself should help address the crosstalk between the PCMH-related hormonal pathway and the gene networks involved in the pupal color polymorphism in *Papilio* species.

## DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in NCBI BioProject, NCBI Accession No. PRJDB9103.

## ETHICS STATEMENT

We conducted the study in accordance with the policy of life science research ethics and safety in the University of Tokyo. The ethics committee of the University of Tokyo declares that higher invertebrates are not included in regulated animals.



## AUTHOR CONTRIBUTIONS

EO and MN provided the organisms. EO and SY isolated the RNA and performed qPCR and RNAi, RNA-seq, and phylogenetic analyses. HF and SY wrote the manuscript. HF supervised the project.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fevo.2020.00051/full#supplementary-material>

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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