Expression of one isoform of GTP cyclohydrolase I coincides with the larval black markings of the swallowtail butterfly, *Papilio xuthus*

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**Abstract**

The larva of the swallowtail butterfly *Papilio xuthus* changes its body markings during the fourth ecdysis. We found that stage-specific cuticular black markings are mainly regulated by co-localization of two melanin synthesis enzymes; tyrosine hydroxylase (TH) and dopa decarboxylase (DDC). TH converts tyrosine to dihydroxyphenylalanine (dopa), and tyrosine itself is converted from phenylalanine by phenylalanine hydroxylase (PAH). Guanosine triphosphate cyclohydrolase I (GTPCHI) is essential for the synthesis of tetrahydrobiopterin (BH4) that is a cofactor of TH and PAH. In this report, we found that a GTPCHI inhibitor prevents pigmentation in cultured integuments, suggesting that the GTPCHI activity is also involved in cuticle pigmentation. We have cloned *GTPCHI* and *PAH* cDNAs from *P. xuthus* and investigated their spatial expression patterns in epidermis by whole-mount in situ hybridization. There are two isoforms of *GTPCHI* in larval epidermis (*GTPCHIa* and *GTPCHIb*). *GTPCHIa* is expressed at the black markings of the subsequent instar, similar to *TH*, whereas *GTPCHIb* is expressed uniformly, similar to *PAH*. This suggests that the region-specific expression of *GTPCHIa* supplies sufficient BH4 reinforcing the TH activity in black marking area. Our results imply that larval markings are regulated by not only melanin synthesis enzymes but also the cofactor supplying enzyme.

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**1. Introduction**

The larva of swallowtail butterfly *Papilio xuthus* changes its body markings markedly during the fourth ecdysis. The young-instar larva is brownish-black in color with white markings, which is believed to mimic a bird dropping in order to avoid predation (Fig. 1Aa). On the other hand, the final-instar larva has green camouflage color (Fig. 1Ab). The final instar has a pair of large false eyespots on the thoracic segment 3 (Fig. 1Ab, arrowhead) and a V-shaped marking on the abdominal segments 4–5 (Fig. 1Ab, arrow). We found that the stage-specific black and reddish-brown markings on the cuticle are mainly regulated by co-localization of three melanin synthesis enzymes; tyrosine hydroxylase (TH; EC 1.14.16.2), dopa decarboxylase (DDC; EC 4.1.1.28) and *ebony* (*Futahashi and Fujiwara, 2005*). TH and DDC convert tyrosine to dopa and dopa to dopamine, respectively (Fig. 1B). Expressions of *TH* and *DDC* are strongly correlated with the black markings of subsequent instar, and addition of dopamine to integuments caused overall darkening without specific markings. In the larval body cuticle of *Manduca sexta*, dopamine is the primary precursor of melanin (*Hiruma et al.*, 1985), and *P. xuthus* larval melanin is likely dopamine melanin although it has not been determined.

Tyrosine is a normal component of the diet, and it also can be obtained from phenylalanine by phenylalanine hydroxylase (PAH; EC 1.14.16.1), a rather ubiquitous enzyme in insect tissues (*Kramer and Hopkins*, 1987) (Fig. 1B). Both TH and PAH require tetrahydrobiopterin (BH4) (Fig. 1B). BH4 serves as an electron donor for the hydroxylation of the aromatic amino acids phenylalanine, tyrosine, and tryptophan (*Fitzpatrick*, 2003). In...
Drosophila, BH$_4$ is essential to the enzyme which catalyzes tyrosine to dihydroxyphenylalanine (dopa) as a cofactor during the cuticle sclerotization and melanization (O’Donnell et al., 1989; Neckameyer and White, 1993; Piedrafita et al., 1994), although in the case of the biosynthesis of melanin in the mammalian melanocytes, the formation of dopa from tyrosine, that is catalyzed by tyrosinase, does not require BH$_4$ as a cofactor (Riley, 1997; Hearing, 2000).

Guanosine triphosphate-cyclohydrolase I (GTPCHI; EC 3.5.4.16) which converts GTP to dihydroneopterin triphosphate is the first enzyme in the biosynthesis of pteridines, including pteridine pigments and BH$_4$ (Nichol et al., 1985). It has been suggested that the GTPCHI activity also correlates with the black pigment synthesis in several insects. In the wing of buckeye butterfly, *Precis coenia*, the temporal expression level of GTPCHI gene and the GTPCHI activity is highest around the black and gray pigmentation stage (Sawada et al., 2002). In *Drosophila melanogaster*, a mutant of GTPCHI affects both melanization and sclerotization. The unpigmented phenotype of embryo in the GTPCHI mutant is very similar to the embryonic lethal phenotype of TH mutant (Wright, 1987). It was reported that the dopa, dopamine and N-acetyldopamine contents are reduced in the both PAH and GTPCHI mutant strains (Piedrafita et al., 1994). However, the correlation between black markings and GTPCHI spatial expression is still unclear. In addition, we do not know whether PAH is involved in the black pigment distribution.

We first examined the effect of GTPCHI inhibitor on pigmentation of the cultured integument. The addition of GTPCHI inhibitor blocked the region-specific pigmentation completely, which is similar to the effect of TH inhibitor. To elucidate the correlation between larval black markings and GTPCHI, we cloned the GTPCHI gene from *P. xuthus*, and found that two isoforms, GTPCHI$_{a}$ and GTPCHI$_{b}$, which have different N-terminal sequences, were expressed in epidermis. Interestingly, one isoform (GTPCHI$_{a}$) was expressed at the black markings in a stage-specific manner, while the other (GTPCHI$_{b}$) uniformly in the whole area. These results suggest that not only TH but also GTPCHI are associated with black pigment distribution patterns. Sufficient level of a cofactor BH$_4$ supplied by the region-specific expression of GTPCHI$_{a}$ may reinforce the strictness of region specificity regulated mainly by a melanin synthesis enzyme TH.

2. Materials and methods

2.1. Experimental animals and developmental staging

*P. xuthus* was purchased from Eiko-kagaku (Osaka, Japan), or kindly provided by Dr. A. Yamanaka (Yamaguchi University, Japan). Larvae were reared with leaves of *Zanthoxylum ailanthoides* (Rutaceae) at 25°C under long-day conditions (16 h light: 8 h dark). The staging of molting period was based on the time when head capsule slippage (HCS) occurred.

2.2. Epidermis culture

Epidermis culture was carried out as described in a separate paper (Futahashi and Fujiwara, 2005). The epidermis of the thoracic segment 3 was dissected from the...
fourth instar at 12 h after HCS and was cut into about 3 x 3 mm^3 pieces, which were cultured in 0.5 ml Grace’s Medium (GIBCO BRL) during 24 h at 25 °C. Incubation was performed in 1.5 ml centrifuge tube rotating in order that the cuticle was exposed to the air. l-dopa (Sigma) was applied to 5 mM. A 3-iodo tyrosine saturated solution and a 2,4-Diamino-6-hydroxypyrimidine saturated solution were made by dissolving to a final concentration of 5 mM gradually during rotating condition with cultured epidermis. Around 10–55 pieces of integument were used in each condition.

2.3. Cloning and sequencing

Total RNA was isolated from the whole larval epidermis at 16 h after HCS during the fourth molt using the TRI reagent (Sigma) according to the manufacturer’s instructions. RNA (2 μg) was reverse transcribed with random primer (N6) using the First-Strand cDNA Synthesis kit (Amersham).

Degenerate primers were: 5’-AYGARGAYCAYGARYGARATGG-3’ and 5’-ACCNCGATNACCATRCAC-3’ for P. xuthus GTPCHI (Sawada et al., 2002), and 5’-TCYACGTNTAYTGGTTYAC-3’ and 5’-AGATGCAANATYTCNGTRTT-3’ for P. xuthus PAH. The cycling conditions of polymerase chain reaction (PCR) were 35 cycles of 94 °C for 0.5 min, 52 °C for 1 min and 72 °C for 1.5 min. PCR products were isolated and subcloned into the TA cloning vector (pGEM-T Easy vector, Promega) and sequenced by a 310 DNA sequencer (ABI, USA). Full-length cDNA was isolated by the rapid amplification of cDNA ends (RACE) technique using the Marathon cDNA Amplification kit (Clontech, USA).

2.4. Phylogenetic analysis

Sequences were aligned using Clustal_X (Thompson et al., 1997). Phylogenetic tree was constructed by the Neighbor joining method with the MEGA2 program (Kumar et al., 2001). The confidence of the various phylogenetic lineages was assessed by the bootstrap analysis. We also compared several related genes with the phylogenetic analysis. The following sequences were used to create the diagram (see Fig. 2): PxPAH, P. xuthus PAH (AB220983), DmPAH, D. melanogaster PAH (CAA66798); HsPAH, Homo sapiens PAH (NP000268); MmPAH, Mus musculus PAH (AAH13458); PxTH, P. xuthus TH (AB178006); DmTH, D. melanogaster TH (AAF50648); HsTH, H. sapiens TH (NP000351); MmTH, M. musculus TH (NP033403).

2.5. Northern analysis

Total RNA (10 μg) was separated on a formaldehyde-agarose (1%) gel and transferred to a Hybond-N nylon membrane (Amersham). Hybridization was performed at 42 °C for 18 h in 50% formamide, 5 x SSC (1 x SSC is 0.15 M sodium chloride and 0.15 M sodium citrate, pH 7.4), 10 x Denhardt’s solution (0.2% each of bovine serum albumin, Ficol, and polyvinylpyrrolidone), 25 μg/ml sonicated salmon sperm DNA, 50 mM sodium phosphate (pH 7.0) and 32P-labeled DNA. Each DNA probe was labeled with [γ-32P] dCTP using BcaBEST Labeling Kit (TaKaRa). DNA probes were synthesized by PCR using the following primers: 5’-ATATCGGTATTACCAGT GTGAATC-3’ and 5’-AGATGCAANATYTCNGTRTT-3’ for PAH; 5’-TGCTAAATCTCGCACTATGTC-3’ and
5'-GAATGCTGATCCTGCCTAGG-3' for GTPCHIa; 5'-GGACTCAACCAGGTGTAACC-3' and 5'-ATCAC CAACAAATGGTACG-3' for GTPCHIb. The membranes were washed twice at room temperature for 20 min in 2 × SSC with 0.1% sodium dodecyl sulfate (SDS). The further washes were followed by 30 min at 65 °C successively in 2 × SSC with 0.1% SDS and in 0.2 × SSC with 0.1% SDS.

2.6. In situ hybridization

Larval epidermis were dissected and then fixed immediately in 4% paraformaldehyde in phosphate-buffered saline. In situ hybridization was carried out as described in a separate paper (Futahashi and Fujiwara, 2005). Hybridization was performed at 60 °C for 16 h in 50% formamide, 5 × SSC, 5 × Denhardt’s solution, 25 μg/ml sonicated salmon sperm DNA, and 0.1% Tween20 with digoxigenin-labeled antisense RNA probe. RNA probes for PAH, GTPCHIa, GTPCHIb and GTPCHI (a and b) were prepared using the Roche Biochemicals kit. The primers for PAH, GTPCHIa and GTPCHIb were used as described above in the section of Northern analysis. The primers for common region of GTPCHIa and GTPCHIb were shown as follows: 5’-ATCCAGAGACAGGGTCTG-3’ and 5’-CAATAA CAACAGGACACCA-3’. Digoxigenin-labeled sense strand probes were used as negative controls. The color reaction was performed at room temperature in 100 mM Tris-HCl, 100 mM NaCl, 50 mM MgCl2, pH 9.5 containing 3.5 μl/ml 5-bromo-4-chloro-3-indolyl-phosphate, 4-toluidine salt (Roche) and 4.5 μl/ml nitroblue tetrazolium chloride (NBT; Roche). Around 12–72 pieces of integument were used in each probe.

2.7. Quantitative reverse transcription-PCR

Larval epidermis was divided into the black and non-black (presumptive green) regions during the fourth molt, and total RNA was reverse transcribed into cDNA using the First-Strand cDNA Synthesis Kit (Amersham). Quantitative reverse transcription-PCR (RT-PCR) was performed using the Smart Cycler (TaKaRa) with the SYBR Green premix Ex Taq (Perfect Real Time) (TaKaRa). The gene of interest and total RNA was reverse transcribed into cDNA using the Reverse Transcription SuperScript III Kit (Invitrogen). The gene of interest and total RNA were reverse transcribed into cDNA using the Reverse Transcription (RT) Kit (Futahashi and Fujiwara, 2005). The primers for common region of GTPCHIa and GTPCHIb were prepared using the Roche Biochemicals kit. The primers for PAH, GTPCHIa and GTPCHIb were used as described above in the section of Northern analysis.
3.3. Temporal expression profiles of Gtpchi, and Pah mRNAs during molting period

In P. xuthus larva, three melanin synthesis enzymes; TH, DDC, and eboni, are specifically expressed just at the pigmentation stage (Futahashi and Fujiwara, 2005). We investigated the precise developmental profile of GTPCHI and PAH expression in the epidermis during the fourth molt by Northern hybridization. Both two isoforms of GTPCHI were uniformly expressed during the fourth molt (Fig. 3A left and center). PAH was also uniformly expressed during molt (Fig. 3A right). We found that a weak TH expression was detected 4 h after HCS (Fig. 3B dotted arrow) and a strong TH expression was detected around the time of pigment synthesis (Futahashi and Fujiwara, 2005). Unlike TH, expressions of GTPCHI and PAH mRNAs were not correlated with the time of pigment synthesis (Fig. 3B).

3.4. Correlation between the spatial expression pattern of GTPCHIA and larval cuticular markings

In P. xuthus larva, the TH expression on epidermis during molt (Fig. 4Ab,g,l) is strongly correlated with the subsequent black markings (Fig. 4Aa,f,k) (Futahashi and Fujiwara, 2005). We used whole-mount in situ hybridization of the larval epidermis to investigate the relationships between the mRNA distribution of GTPCHI and PAH, and larval cuticular markings. Expression pattern of GTPCHI was different between two isoforms. The GTPCHIA expression was correlated with black markings in eyespots and abdominal segments at 15 h after HCS during the fourth molt and 14 h after HCS during the third molt (Fig. 4Ac,h,m). During both the third and fourth molts, the GTPCHIA expression patterns coincided with the black melanin markings of the subsequent instar (Fig. 4Ac,h,m). On the other hand, GTPCHIB was expressed uniformly in the whole area of integuments (Fig. 4Ad,i,n). PAH was also expressed irrespective of the subsequent black marking patterns (Fig. 4Ae,j,o). The results of in situ hybridization using probe for common region of GTPCHI were similar to that of GTPCHIA-specific probe (Fig. 4B), suggesting that GTPCHIB expression was weak. Both GTPCHI and PAH were less expressed at the white false reflection area in the center of the eyespot. The cross-section of epidermis showed that the white false reflection area (Fig. 4C, brace) was attached to the muscular tissues (Fig. 4C, bracket) and lack of epidermal cells, suggesting that this special physical structure is associated to the white coloration. From 12 to 16 h after HCS, GTPCHIA expression correlated with the black markings (data not shown). Before 12 h after HCS, it was difficult to detect the positive signal by in situ hybridization, because the old cuticle was not taken off. To investigate whether GTPCHIA expression always occurs at the place of the black region, we compared the relative expression between the presumptive black and non-black region by quantitative RT-PCR. At 4 and 8 h after HCS during the fourth molt, the expression level of GTPCHIA in the black region was similar to that in the non-black region (Fig. 4D left). At 12 and 16 h after HCS when the pigment synthesis occurs, the expression level of GTPCHIA in the black region was stronger than that in the non-black region (Fig. 4D left) as expected by the results of in situ hybridization. The expression level of GTPCHIB in the black region was similar to that in the non-black region all the time during the fourth molt (Fig. 4D right). These results suggest that GTPCHI expressions of both isoforms are not localized at the early phase of molt (4–8 h after HCS), but GTPCHIA expression becomes predominant in the black region during the pigment synthesis (12–16 h after HCS), but GTPCHIB does not. The above results showed that during the pigment synthesis, the expression of GTPCHIA as well as TH was correlated with the black marking patterns in the subsequent instar, suggesting that the GTPCHIA activity is involved in the cuticular pigment patterns, whereas GTPCHIB and PAH are not (Fig. 4E).

4. Discussion

4.1. Functional role of GTPCHI during pigment distribution in P. xuthus

We found that GTPCHIA expression is correlated with the black markings. In P. xuthus, TH is highly expressed in the presumptive black region (Futahashi and Fujiwara, 2005).
2005), which suggests that the marking-specific GTPCHIa expression enhances the TH activity in the black region. In contrast, PAH and GTPCHIb was uniformly expressed irrespective of the black marking. Both TH and PAH require BH4 as a cofactor, and we expected that the overall expression of GTPCHI and its downstream pathways in epidermis could be appropriate both for the uniformly expressed PAH and the marking-specifically expressed TH to receive BH4. GTPCHI activity is necessary for not only the synthesis of catecholamine, a precursor of melanin but also pteridines-related pigments (Takahashi and Nakagoshi, 1994; Ziegler et al., 2000). BH4 is also an essential cofactor for nitric oxide synthase (Marletta, 1993; Imamura et al., 2002). Therefore, the uniform expression of GTPCHIb should be essential for all other enzymes than TH. It seems not indispensable that GTPCHI should be expressed in a region-specific manner. However, the active reaction of TH in the very limited developmental stage...
needs plenty of substrates and cofactor and thus we speculate that the concurrent expression of *GTPCHI* with TH is necessary to make the explicit melanin markings.

It is reported that *GTPCHI* and TH are co-localized in the central nervous system and co-immunoprecipitated in *D. melanogaster* (Krishnakumar et al., 2000). Mutations of *GTPCHI* gene reduce the TH activity, but the addition of BH$_4$ could not fully rescue the TH activity (Krishnakumar et al., 2000), suggesting that regulation of the TH activity depends not only on the BH$_4$ concentration but also on its association with *GTPCHI*. The putative association with TH may be different between the *GTPCHI* isoforms in the *P. xuthus* isoforms.

In *P. coenia*, it is suggested that *GTPCHI* expression is associated with the white pigments (Sawada et al., 2002). Pteridines are contained in the larva of lepidopteran species (Tsusue et al., 1990; Sawada et al., 1998). Pteridines are considered to be contained in the white region of young larva of *P. xuthus*; however, the correlation between *GTPCHI* expression and the white region was not found in our study.

### 4.2. Structural comparison of *GTPCHI* isoforms among insects

There are at least two isoforms of the *GTPCHI* gene in *P. xuthus*. Both of the isoforms retain the core region necessary for the correct enzyme activity and the protein folding (Maier et al., 1995). By the analysis of crystal structure, human and rat *GTPCHI* have flexible N-terminal regions, and it has been suggested that this region has little effect on the activity of this enzyme (Auerbach et al., 2000; Maita et al., 2004). The N-terminal domain of *GTPCHI* is not conserved even between *Papilio* and *Drosophila*, suggesting that these domains are highly diversified among species.

In *P. coenia*, there are also two isoforms of *GTPCHI* in wing during pupation, and these isoforms are differentially regulated by 20-hydroxyecdysone, while the structures of two isoforms were not characterized (Sawada et al., 2002). In *P. xuthus* larva, however, the expression of *GTPCHI* isoform mRNAs during fourth molt was basically at the same level (Fig. 3) and thus seemed uncorrelated with the 20E concentration. In *D. melanogaster*, more than 60 mutant alleles of *GTPCHI* gene were generated, and the ensuing genetic characterization demonstrated that *GTPCHI* gene is a complex locus (Mackay et al., 1985; Reynolds and O’Donnell, 1988). It is noted that there are two isoforms even in the same tissue and same stage in *P. xuthus*. It is possible that there are other isoforms of *GTPCHI* gene among the different tissues and stages in *P. xuthus*.

### 4.3. Multi-step regulation of pigment localization

We reported that the cuticular melanic pattern is regulated by co-localization of melanin synthesis enzymes. Both TH and DDC are correlated with cuticular pigment patterning by regulation dopamine distribution, and the addition of dopamine into integuments causes overall darkening without specific markings (Futahashi and Fujikawa, 2005). In this study, we found that expression of *GTPCHIa* is also correlated with the black marking during the pigment synthesis. Our results suggest that the larval body markings are regulated by not only melanin synthesis enzymes itself but also another enzyme which supplies the cofactor. The expression of *GTPCHIa* and *GTPCHIb* seems to be controlled by different kinds of transcription factors, and *GTPCHIa* and *TH*, as well as *DDC*, may be regulated by the same transcription factor. Identification of the marking-specific transcription factor will clarify how several enzymes are expressed coordinately in region-specific manner.

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### References


