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Identification of the silkworm *quail* gene reveals a crucial role of a receptor guanylyl cyclase in larval pigmentation

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Diverse color patterns on the integument of lepidopteran larvae play important roles in their survival through camouflage, mimicry, sexual signaling, and aposematism. In the silkworm *Bombyx mori*, many color pattern variations have been preserved in inbred strains making them a good model for elucidating the molecular mechanisms that underlie color pattern formation. In this study, we focused on the silkworm *quail* (*q*) mutant, which exhibits abnormalities in multiple pigment biosynthesis pathways. Positional cloning of the *q* gene revealed that disruption of a guanylyl cyclase gene, *BmGC-I*, is responsible for its abnormal pigmentation. In *q* mutants, we identified a 16-bp deletion in the *BmGC-I* transcript, resulting in the production of a premature stop codon. Knockout of the *BmGC-I* gene resulted in the *q*-like abnormal pigmentation, thereby demonstrating that the *BmGC-I* gene is involved in the pigment biosynthesis pathway in the integument. Moreover, quantitative reverse transcription polymerase chain reaction showed that *BmGC-I* was strongly expressed in the fourth instar on day 2. Our results suggest that *BmGC-I* deficiency affects the pigment biosynthesis pathway, which supports the involvement of guanylyl cyclase in larval coloration.

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1. Introduction

Lepidopteran insects exhibit a wide variety of color patterns in the adult wings and larval skin, which confer survival advantages through camouflage, mimicry, sexual signaling, and warning coloration (aposematism; Romoser and Stoffolano, 1994). In the silkworm *Bombyx mori*, dorsal skin color patterns are established by the coordinated allocation of multiple pigments, including melanins, ommochromes, and pteridines (Mazda et al., 1980; Ohashi et al., 1983). More than 500 silkworm mutants exist, including color pattern mutants, and biochemical and genetic studies of several color pattern mutants have identified the genes responsible for controlling the biosynthesis, transport and signaling pathways of the pigments (Dai et al., 2010; Ito et al., 2010, 2012; Yamaguchi et al., 2013). For example, a mutation in the tyrosine hydroxylase gene leads to weaker melanization in the cuticle of neonatal larvae (Liu et al., 2010). In the *lemon* and *lemon lethal* mutants, defects in the sepiapterin reductase gene cause yellow pigmentation in the larval body due to the accumulation of sepiapterin and sepialumazine (Meng et al., 2009). These silkworm mutants are useful experimental tools for investigating the mechanism that underlie larval color pattern formation.

The *quail* (*q*) is a color pattern mutant, which displays unique coloration as a consequence of abnormalities in multiple pigment pathways that cause the increased synthesis of melanin, ommochrome, and pteridine (Kato et al., 2006; Kawase, 1955; Kiguchi, 1973; Ohashi et al., 1983). Therefore, the larval skin is light pink in *q* mutants, with black markings in additional to those of the wild-type larvae (Fig. 1). In dorsal region in addition to shred-like lines, irregular lines to posterior segment from crescent and star spot markings emerge.

The *q* locus has been mapped to 0.0 cM on chromosome 7 (Doira, 1972; Hasimoto, 1934). Based on the analyses of the larval integument transcriptome, Nie et al. (2014) shows that larvae with the *q* gene exhibit changes in the expression levels of several genes, including those involved in juvenile hormone (JH) metabolism,

Abbreviations: BC1, backcross 1; *CREB*, cAMP-response element binding protein; cGMP, cyclic guanosine monophosphate; GTP-CHI, GTP cyclohydrolase I; KO, knockout; MAP, mitogen-activated protein; PCR, polymerase chain reaction; *q*, *quail*; RT-PCR, reverse-transcription polymerase chain reaction; sgRNA, single synthetic guide RNA.

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Fig. 1. Phenotypes of the wild-type and *quail* mutant larvae. (A) The larval integument of the wild-type larva is white and has crescent-shaped markings on the second segments. (B) *quail* mutant larva has additional dark spots and light-pink colored skin. Scale bar, 1 cm.

cuticular protein production, and chitin metabolism, and nuclear receptor-related genes. Thus, the *q* gene influences the expression of different genes. However, the gene responsible for *q* locus remains elusive.

In this study, we discovered by positional cloning that a mutation in the guanylyl cyclase gene, *BmGC-I*, is responsible for the *q* phenotype. Also, a gene knockout experiment confirmed that disruption of this gene caused a *q*-like phenotype. Our results suggest that *BmGC-I* is required for normal larval coloration in the silkworm.

2. Materials and methods

2.1. Silkworm strains

All silkworms were raised at 25 °C on mulberry leaves with a 12-h light/12-h dark cycle. The wild-type silkworm strain p50T and its derived non-diapause egg strain p50TN were maintained in our laboratory. Strains c55, b32, k01, k02, m42, and d33 were donated by the silkworm stock center of Kyushu University (Fukuoka, Japan), which is supported by the National BioResource Project, and strain no. 120, 128, 144, 145, 146, 940, and 941 were provided by the National Institute of Agrobiological Sciences (Kobuchizawa, Japan).

2.2. Positional cloning

For linkage mapping, we obtained 531 backcross 1 (BC1) individuals (220 *q* skin and 311 normal larvae) from the cross between a c55 female and a F1 male (p50T female × c55 male). Genomic DNA was extracted using the HotSHOT method (Truett et al., 2000). The BC1 silkworms were genotyped using polymerase chain reaction (PCR)-based markers designed for chromosome 7. The primer sets used in this study were designed based on the silkworm genome (http://sgp.dna.affrc.go.jp/index.html) and are listed in Table S1 (Supplementary file).

2.3. Reverse transcription polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE)

Total RNA was isolated from whole integuments at various stages and different tissues using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. First-strand cDNA was prepared using an oligo(dT) primer and avian myeloblastosis virus reverse transcriptase from a TaKaRa RNA PCR kit (Takara Bio Inc., Otsu, Japan). RT-PCR was conducted using TaKaRa Ex Taq (Takara Bio Inc.) under the following conditions: 94 °C for 2 min, 35–40 cycles at 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 1–2 min, followed by 72 °C for 5 min. The full-length sequence of *BmGC-I* cDNA was obtained by a rapid amplification of cDNA ends technique (RACE) using the GeneRacer™ Kit (Invitrogen). RT-PCR for RACE was conducted using KOD FX Neo (Toyobo Inc., Osaka, Japan) under the following conditions: 94 °C for 2 min, 40 cycles at 98 °C for 10 s, 58 °C for 30 s, and 68 °C for 0.5–1 min. The primers used for RT-PCR are listed in Table S2 (Supplementary file). The obtained cDNA sequence of *BmGC-I* was deposited in the DDBJ database under accession number LC081227.

2.4. Quantitative reverse transcription polymerase chain reaction

To quantify the amount of mRNA, we performed quantitative RT-PCR with a KAPA SYBR® FAST qPCR kit (Kapa Biosystems, Boston, MA, USA) and an ABI StepOne™ Plus Real-Time PCR System (Applied Biosystems).

The primers used for quantitative RT-PCR are listed in Table S2 (Supplementary file). The transcript level of *rp49* was used for normalization.

2.5. CRISPR/Cas9-mediated gene knockout (KO)

We performed CRISPR/Cas9-mediated gene KO according to the method reported by Bassett et al. (2013). A unique oligonucleotide containing the T7 polymerase binding site and a single synthetic guide RNA (sgRNA) target sequence for sgRNA production were designed for *BmGSTe4* (5′-GAAATTAATACGACTCACTATAGGAGCTCGGAATTTATGACGGTTTTAGAGCTAGAAATAGC-3′) and for *BmGC-I* (5′-GAAATTAATACGACTCACTATAGGAGTCTCTTAACGATAAGGGTTTTAGAGCTAGAAATAGC-3′). The plasmid MLM3613 (Addgene plasmid #42251) was used for *Cas9* mRNA production. We mixed 0.5 μg of sgRNA and 10 μg of *Cas9* mRNA in a 30 μl volume with 3 μl of 3 M sodium acetate (pH 5.2), which was followed by precipitation with 90 μl of ethanol for purification. After centrifugation, the pellet was washed twice with 70% ethanol and resuspended in 11 μl of the injection buffer (100 mM potassium acetate, 2 mM magnesium acetate, 30 mM HEPES-potassium hydroxide, pH 7.4). The mixture of *Cas9* mRNA and sgRNA was injected into p50TN strain eggs within 3 h after the eggs were laid. The injected individuals were crossed with non-injected p50T strain individuals to establish the KO lines of *BmGC-I* or *BmGSTe4*. Generation 1 (G1) individuals carrying a heterozygous KO mutation were intercrossed and homozygous KO animals were produced. Cas9-induced mutations were detected by T7 endonuclease I (NEB Inc., Beverly, MA, USA) and were checked by sequencing.

3. Results

3.1. Positional cloning of the gene responsible for the q locus

To identify and characterize the gene responsible for the *q* phenotype, we performed genetic linkage analysis using PCR-based markers (Fig. 2A). Based on the silkworm genome, primer sets that yielded PCR products of different lengths from the wild-type (p50T) and *q* strain (c55) larvae were designed for chromosome 7. The F1 male offspring of the p50T female and c55 male were backcrossed with c55 females to produce the BC1 population. Initially, we performed rough mapping using 48 BC1 individuals and narrowed down the candidate region for the *q* locus to a 2.0-Mb region

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