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The silkworm (*Bombyx mori*) neuropeptide orcokinin is involved in the regulation of pigmentation

Pingyang Wang[a,c,1], Qiaoling Zhao[a,b,*,1], Zhiyong Qiu[a,b,1], Simin Bi[a], Wenbo Wang[a], Meina Wu[a], Anli Chen[d], Dingguo Xia[a,b], Xiaobai He[a,b], Shunming Tang[a,b], Muwang Li[a,b], Guozheng Zhang[a,b], Xingjia Shen[a,b,**]

[a] *Jiangsu Key Laboratory of Sericultural Biology and Biotechnology, School of Biotechnology, Jiangsu University of Science and Technology, Zhenjiang, Jiangsu, 212018, China*

[b] *Key Laboratory of Silkworm and Mulberry Genetic Improvement, Ministry of Agriculture and Rural Affairs, Sericulture Research Institute, Chinese Academy of Agricultural Sciences, Zhenjiang, Jiangsu, 212018, China*

[c] *Guangxi Zhuang Autonomous Region Research Academy of Sericultural Science, Guangxi, Nanning, 530007, China*

[d] *The Sericultural and Apicultural Research Institute, Yunnan Academy of Agricultural Sciences, Mengzi, Yunnan, 661101, China*

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ABSTRACT

The natural colorful cuticles of insects play important roles in many physiological processes. Pigmentation is a physiological process with a complex regulatory network whose regulatory mechanism remains unclear. *Bombyx mori* pigmentation mutants are ideal materials for research on pigmentation mechanisms. The purple quail-like ($q\text{-}l^{p}$) and brown quail-like ($q\text{-}l^{b}$) mutants originated from plain silkworm breeds 932VR and 0223JH respectively exhibit similar cuticle pigmentation to that of the quail mutant. The $q\text{-}l^{p}$ mutant also presents a developmental abnormality. In this study, genes controlling $q\text{-}l^{p}$ and $q\text{-}l^{b}$ mutants were located on chromosome 8 by positional cloning. Then the neuropeptide gene *orcokinin* (*OK*) was identified to be the major gene responsible for two quail-like mutants. The *B. mori orcokinin* gene (*BommoOK*) produces two transcripts, *BommoOKA* and *BommoOKB,* by alternative splicing. The CRISPR/Cas9 system and orcokinin peptides injection were used for further functional verification. We show a novel function of *BommoOKA* in inhibiting pigmentation, and one mature peptide of orcokinin A, OKA_type2, is the key factor in pigmentation inhibition. These results provide a reference for studying the function of *orcokinin* and are of theoretical importance for studying the regulatory mechanism of pigmentation.

1. Introduction

Orcokinin (OK), a unique neuropeptide of Arthropoda, was first discovered in *Orconectes limosus* in 1992. Its mature peptide sequence is NFDEIDRSGFGFN, and it is referred to as orcokinin because of its myotropic character (Stangier et al., 1992). Many OKs were subsequently discovered in other crustacean species (Christie et al., 2011; Li et al., 2002; Skiebe et al., 2002; Yasuda-Kamatani and Yasuda, 2000). An OK was found in insects (*Blattella germanica*) for the first time in 2004 (Pascual et al., 2004). Thereafter, OKs were found in other insects including *Schistocerca gregaria* (Hofer et al., 2005), *Drosophila melanogaster* (Liu et al., 2006), *Leucophaea maderae* (Hofer and Homberg, 2006), *Locusta migratoria* (Clynen and Schoofs, 2009), *Rhodnius prolixus* (Ons et al., 2009, 2011) and *Bombyx mori* (Yamanaka et al., 2011).

OKs are multifunctional neuropeptides. They enhance motility in the hindgut in *O. limosus* (Stangier et al., 1992), and they regulate the extension of stomatogastric nervous systems and pericardial organs in adult crustaceans (Li et al., 2002), the phase-dependent shift in circadian locomotor activity in *L. maderae* (Hofer and Homberg, 2006), awakening behavior in *Tribolium castaneum* (Jiang et al., 2015) and insect ecdysis, heartbeat contraction and midgut peristaltic contraction

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[*] Corresponding author. School of Biotechnology, Jiangsu University of Science and Technology, Nanxu Road, Runzhou Ditrict, Zhenjiang City, Jiangsu Province, 212018, China.

[**] Corresponding author. School of Biotechnology, Jiangsu University of Science and Technology, Nanxu Road, Runzhou Ditrict, Zhenjiang City, Jiangsu Province, 212018, China.

E-mail addresses: qlzhao302@126.com (Q. Zhao), shenxjsri@163.com (X. Shen).

[1] These authors contributed equally to this work.

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in *R. prolixus* (Wulff et al., 2017, 2018). OKs are also involved in the transcriptional regulation of vitellogenin in the fat body in *B. germanica* (Ons et al., 2015). In *B. mori*, BRFa (Bommo-FMRFamide) regulates the cAMP content, and ultimately regulates the synthesis of ecdysone. Orcokinin exhibits prothoracicotropic activity and may weaken the facilitation of the effect of BRFa on ecdysone synthesis after binding to an unknown receptor (Tanaka, 2011; Yamanaka et al., 2011).

Orcokinins produce three types of neuropeptides (orcokinin A, orcokinin B and orcomyotropins), but two of them present only minor differences (Chipman et al., 2014; Veenstra, 2016). *Orcokinin* genes always exhibit two types of transcripts encoding OKA and OKB respectively in Insecta (Sterkel et al., 2012) while in other groups of Arthropoda, *orcokinin* genes encode both OKA and OKB peptides in the same precursor (Sterkel et al., 2012; Veenstra, 2016). In *R. prolixus*, the third transcript *RhoprOKC* is identified, but it is highly similar to *RhoprOKB* and all the peptides with conserved core sequence encoded in the *RhoprOKC* are OKA or OKB peptides (Wulff et al., 2017). *Orcokinin* transcripts are transcribed from two separate genes in *Stegodyphus*, *Mesobuthus,* etc. and from one gene by alternative splicing in *Strigamia*, *Zootermopsis*, *Drosophila,* etc. (Veenstra, 2016). In *D. melanogaster*, *OKA* is expressed mainly in the central nervous system and ventral nerve cord, while *OKB* is expressed in intestinal enteroendocrine cells (Chen et al., 2015). In *T. castaneum*, *OKA* and *OKB* are co-expressed in 5–7 pairs of brain cells and in midgut enteroendocrine cells (Jiang et al., 2015). The pattern is similar in *B. germanica*, where *BlageOKA* is expressed in brains and *BlageOKB* is detected in brains and midgut (Ons et al., 2015). In *R. prolixus* both *RhoprOKA* and *RhoprOKB* peptides were detected in male and female gonads, besides the conserved expression of *OKA* in nervous system and *OKB* in nervous system and midgut (Wulff et al., 2017). In *B. mori*, *BommoOK* gene is mainly expressed in the ventral nerve cord, central nervous system and intestinal endocrine cells (Yamanaka et al., 2011). Research has also shown that *BommoOKA* is mainly expressed in the ventral nerve cord, with the lowest expression at 18 h after molting of the 4th instar, while *BommoOKB* is expressed at higher levels in the head and midgut than in other body parts and is expressed at higher levels during molting than in other periods in *B. mori* (Wang et al., 2018b).

The silkworm mutants purple quail-like ($q\text{-}l^p$) and brown quail-like ($q\text{-}l^b$) were arose from the plain silkworm breeds 932VR and 0223JH, respectively. The larval epidermis of quail-like mutants exhibits similar pigmentation to that of quail mutants (Kato et al., 2006; Zhao et al., 2014). The body colors of $q\text{-}l^p$ and $q\text{-}l^b$ larvae are light purple and brown, respectively. During feeding process, the $q\text{-}l^p$ mutant delays 2–3 days in development and emerges developmental abnormality resulting in high mortality, low appetite, a small larval size, and a small cocoon size compared to the wildtype 932VR, while the $q\text{-}l^b$ mutant shows synchronous compared to the wildtype 0223JH (Wang et al., 2018a; Zhao et al., 2014) (Fig. S1).

Genetic analysis reveals that both the $q\text{-}l^b$ and $q\text{-}l^p$ mutant are controlled by a recessive gene and that $q\text{-}l^b$ and $q\text{-}l^p$ are allelic variants of the same gene at genetic level, where $q\text{-}l^b$ is dominant to $q\text{-}l^p$ (Zhao et al., 2014). Expression analysis between two quail-like mutants and their wildtypes showed significant differences in pigment synthesis-related genes, cuticular protein genes, chitin synthesis-related genes, nuclear receptor genes, etc. In particular, the expression of genes on the melanin synthesis pathway was shown to be upregulated, implying an increase in pigment in the quail-like mutants; for example, the *black* gene is greatly upregulated in the two quail-like mutants, implying that the *black* gene is associated with the major causal gene of the mutants (Qiu et al., 2018; Wang et al., 2017). In this study, we identified the major gene responsible for the $q\text{-}l^b$ and $q\text{-}l^p$ mutants as the neuropeptide gene *orcokinin* (*BommoOK*), and we determined the locus of *BommoOK* by positional cloning. We further verified *BommoOK*'s new function in pigmentation regulation by CRISPR/cas9 gene editing and mature peptide injection. CRISPR/cas9 is an efficient genome editing technology (Hsu et al., 2014) that is maturely and extensively applied in the silkworm (Wei et al., 2014). This is the first report of the pigmentation regulation function of the *BommoOK* gene and its mature peptide OKA_type2, which is of theoretical importance for the study of the mechanism of insect pigmentation and provides another pathway of insect pigmentation.

2. Materials and methods

2.1. The silkworm strains

The $q\text{-}l^p$ mutant, $q\text{-}l^b$ mutant, wildtype 932VR, 0223JH, Dazao and Qiufeng silkworm strains were supplied by the Sericulture Research Institute, Chinese Academy of Agricultural Sciences (Zhenjiang, China). The larvae were fed fresh mulberry leaves under standard conditions: 25 ± 2 °C, 12-h light/12-h dark photoperiod and 65 ± 5% relative humidity. When the silkworms had developed to the appropriate stage, tissues were dissected and subsequently stored at −80 °C.

2.2. Hybridized combined materials for positional cloning

The Dazao and $q\text{-}l^p$ ($q\text{-}l^p/q\text{-}l^p$) mutant silkworm strains were hybridized to acquire F_1 offspring. Dazao, F_1 offspring and the $q\text{-}l^p$ mutant were used to screen molecular markers with polymorphisms. Then, BC_1F (♀F_1 × ♂$q\text{-}l^p$) and BC_1M (♀$q\text{-}l^p$ × ♂F_1) were obtained by backcrossing F_1 offspring and the $q\text{-}l^p$ mutant, from which the $+/q\text{-}l^p$ and $q\text{-}l^p/q\text{-}l^p$ offspring of BC_1F were used for linkage analysis, and the $q\text{-}l^p/q\text{-}l^p$ offspring of BC_1M were used for fine mapping. The hybridized combination materials for $q\text{-}l^b$ ($q\text{-}l^b/q\text{-}l^b$) and Dazao ($+/+$) were the same as the $q\text{-}l^p$ ($q\text{-}l^p/q\text{-}l^p$) mutant.

2.3. Positional cloning of the two quail-like mutants

Genome sequences were downloaded from the Silkworm Genome Database (SilkDB, http://www.silkdb.org/silkdb/). Then, simple sequence repeat (SSR) markers were screened by the software SSR Hunter, and primers (Supplemental Table S1) were designed with the software Oligo 7. Molecular markers showing polymorphism were screened with the genomic DNA of Dazao, the $q\text{-}l^p$ mutant and the F1 offspring (Dazao × $q\text{-}l^p$) as a template. These molecular markers with polymorphisms were used to verify BC_1F offspring (10 individuals with a wildtype phenotype and 10 individuals with a mutant phenotype). The mutant gene and the molecular marker could be considered to be linked on the same chromosome if the DNA classification of 10 individuals with a wildtype phenotype was the same as that of the F1 offspring and the DNA classification of 10 individuals with a mutant phenotype was the same as that of the $q\text{-}l^p$ mutant. Additional molecular markers exhibiting polymorphism were screened on the linked chromosome and verified with BC_1M individuals. Finally, the mutant gene was located between two molecular markers according to the positional cloning principle. Every attempt was made to limit the physical distance of these two markers was limited to less than 300 kb. The methods of positional cloning used for the brown quail-like mutant $q\text{-}l^b$ were the same as those for the purple quail-like mutant $q\text{-}l^p$.

2.4. Screening of candidate genes

Since the allelic variants of the same gene controlling the characteristics of the $q\text{-}l^p$ and $q\text{-}l^b$ mutants, we focused on the overlapping portion of their positionally cloned regions when screening the candidate genes. Analysis of the structure and expression levels of predicted genes in the overlapping region was performed based on the Silkworm Genome Database SilkDB and Silkbase (http://silkbase.ab.a.u-tokyo.ac.jp/cgi-bin/index.cgi) to identify the differences in candidate genes between the wildtype and mutant strains for confirmation of the mutant gene.

2.5. Extraction of genomic DNAs

The posterior silk glands of 5th-instar larvae were used for genomic DNA extraction, and DNA extraction buffer (0.01 mol/L Tris, 0.1 mol/L EDTA, 0.5% SDS, pH 8.0) was then added after sufficient grinding when solid particles are invisible to the naked eye. Followed by a phenolic extraction process, and the precipitate was washed with 75% ethyl alcohol. Then, the precipitate was dissolved in deionized water. Finally, the DNA solution was diluted to 100 ng/μL after determination of the concentration by a spectrophotometer.

2.6. Total RNA extraction and cDNA synthesis

Approximately 0.1 g of silkworm tissue was transferred to a precooled mortar in liquid nitrogen and sufficiently ground. Then, 1 mL of RNAiso Plus (TaKaRa, China) was added. When the lysate appeared clear after sufficient grinding, the lysate was transferred to a new 1.5 mL centrifuge tube. Then, we extracted the total RNA referring to the RNAiso Plus protocol and measured the concentration by a spectrophotometer. Subsequently, cDNA was synthesized according to the PrimeScript™ RT Master Mix (TaKaRa, China) protocol after treatment with DNase (TaKaRa, China). Finally, the cDNA solution was diluted to 100 ng/μL after determination of the concentration by a spectrophotometer.

2.7. Quantitative reverse transcription PCR

Quantitative RT-PCR is an expression analysis method based on the primary template of PCR. The cDNA samples were diluted to 100 ng/μL and used as the template for qRT-PCR. Each 20-μL reaction included 1 μL of primers (10 μmol/L, primers are listed in Supplemental Tables S1) and 1 μL of cDNA, 10 μL of 2 × SYBR Premix Ex Taq™ (Tli RNaseH Plus) (TaKaRa, China) and 8 μL ddH$_2$O. After a rapid centrifugation step, qRT-PCR was performed using a LightCycler 96 real-time PCR system (Roche, Switzerland) with the following three-step reaction program: 10 min of predegeneration at 95 °C, followed by 40 cycles of 95 °C for 10 s, 58 °C for 10 s and 72 °C for 10 s, and then a final melting step. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), employing *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) and *BmActin3* as the reference genes (Primers are listed in Supplemental Table S1). The final relative expression level was presented like this: the relative expression levels of wildtype and mutant calculated using the $2^{-\Delta\Delta Ct}$ method were all divided by that of wildtype for every group. Thus, the relative expression levels of wildtype were assumed as “1”, while the relative expression levels of mutant represented as the fold change. Melting analysis was performed to verify the quality of the qRT-PCR results.

2.8. CDS and genomic DNA cloning

CDS cloning: Newly hatched larvae were used as materials for CDS cloning. First, total RNA was extracted and cDNA synthesis was performed. Cloning primers were designed from the initiation codon to the termination codon of CDS for every gene based on reference sequence in SilkDB. RT-PCR was performed following by electrophoresis, and the PCR products were cloned to pMD-18T (TaKaRa, China) clone vector, and sequenced (entrusting Sangon Biotech, https://www.sangon.com/). Then CDS structures between wildtype and mutant were compared to check out the structure difference.

Genomic DNA cloning: The genome DNA extracted from $q\text{-}l^e$ was used as the template and that of Qiufeng serving as the reference. Primers were designed on two flanks of target sites. Then, PCR, cloning and sequencing were consecutively performed.

2.9. Functional verification of BommoOK by the CRISPR/Cas9 system

In this study, cas9 protein and guide RNA was used for genome editing. First, guide RNA with the target site was synthesized *in vitro*: the specific primer (sequence: TTCTAATACGACTCACTATAG(N$_{20}$)GTTTTAGAGCTAGA, N20 represented the target site) with the T7 promoter (sequence with underline) were used for sgRNA synthesis referring to the protocol of EnGen® sgRNA Synthesis Kit (New England Biolabs, USA) (https://international.neb.com/). After purification and determination of concentration, these sgRNAs were used for the silkworm eggs injection, together with commercial cas9 protein (New England Biolabs, USA).

A digital micromanipulator workstation (Eppendorf, Germany) was used for microinjection. A pair of sgRNA (1000 ng/μL (about 31 μmol/L) each) and cas9 protein (20 μmol/L) were mixed by the ratio of 1:1. Then the cas9-sgRNA mixture was injected into eggs of Qiufeng silkworm strain within 2 h after oviposition with an average dosage of 8 nL per egg. After sealing the wound, these eggs were incubated under 25 °C with a humidity of 85%. After hatching, larvae were fed with fresh mulberry leaves in normal feeding conditions. Finally, positive individuals were screened out by phenotypic identification and genomic verification of the target sites cloning and sequencing (primers are listed in Supplemental Table S1). In this study, we designed two target sites in exon 3 (OK_sgRNA_1: GGCATCGTAGCGTTCCCCAGAGG) and exon 2 (OK_sgRNA_2: AACTGCTGGAGTGCTTGCCGTGG) of the *BommoOK* gene (the underlined sequence represents PAM (Protospacer Adjacent Motif)) using an online software CRISPRdirect (http://crispr.dbcls.jp/).

2.10. Peptide synthesis and injection

Six kinds of mature peptides, as predicted by a report in the literature (Yamanaka et al., 2011), were synthesized by Genscript company (https://www.genscript.com, USA). The purity was up to 95% tested by HPLC (high performance liquid chromatography) and verified by MS (Mass Spectrum) (Fig. S2). Peptide solubility test was also performed, of which 3% ammonium hydroxide and dimethylsulfoxide were deadly to silkworm (Table 1). Then the remaining four peptides OKA-type2, OKA-type4, OKA-type5 and OKB-type1 were used for experiments. Since the phosphate buffer was similar to deionized water that had no effect on development of silkworms, the deionized water was used as the only control. These mature peptides were dissolved in a suitable solvent at a concentration of 5 mmol/L (Table 1). A microsyringe was used for subcutaneous injection. After sterilizing, the peptide solution was inhaled into microsyringe, and injected into silkworm close to the cuticula. Newly exuviated 4th-instar larvae of the $q\text{-}l^p$

Table 1
Injection of mature orcokinin peptides.

Name	Sequence	Solvent	$q\text{-}l^b$ (rescue rate)	$q\text{-}l^p$ (rescue rate)	Attentions
OKA_type1	NFDEIDRSSLNTFV	3% Ammonium hydroxide	–	–	Ammonium hydroxide has a strong damage to the silkworm
OKA_type2	NFDEIDRSSMPFPYAI	Deionized water	100%	81.5%	–
OKA_type3	FYHLYGQNFLDIDSPVSSFD	Dimethylsulfoxide	–	–	Dimethylsulfoxide has a strong damage to the silkworm
OKA_type4	EARHSGYLPYQMF	Deionized water	–	–	–
OKA_type5	YDYISPYG	Deionized water	–	–	–
OKB_type1	NLDSLGGANF	0.1 mol/L Phosphate buffer	–	–	Phosphate buffer has rarely effects to the silkworm

and q-l^{b} mutants were subjected to subcutaneous injection with a volume of 2 μL for each silkworm of each peptide, and deionized water served as the control. Then, the phenotypic changes of these larvae were carefully observed including changes of skin color, markings and development. Besides, the average weight of individuals (n = 30) was determined.

2.11. Homologous comparison

The protein precursor sequences of orcokinin were downloaded from NCBI (National Center of Biotechnology Information, https://www.ncbi.nlm.nih.gov/). Then mature peptide predicting was performed based on the rule of mono- and dibasic proteolytic cleavage sites (Veenstra, 2000). Alignment was performed using the software MEGA, and finally established the atlases with the online software WebLogo 3 (http://weblogo.threeplusone.com/).

3. Results

3.1. Positional cloning of the mutant genes of two quail-like mutants

Because the two quail-like mutants q-l^{p} and q-l^{b} are controlled by a recessive gene (Zhao et al., 2014), the positional cloning method was used to independently map the mutant genes of the two quail-like mutants. First, simple sequence repeat (SSR) markers with polymorphisms (primers are listed in Supplemental Table S1) that were screened from the genome were verified in BC$_1$Fs (the first offspring of backcross employing F$_1$ progeny as the female parent), and the mutant genes of the q-l^{p} and q-l^{b} mutants were all found to be located on the 8th chromosome of the silkworm, *B. mori* (Fig. 1A and E). Then, BC$_1$Ms (the first offspring of backcross employing F$_1$ progeny as the male parent) were used for fine mapping, and q-l^{b} was finally localized to an interval of approximately 230 kb with 18 predicted genes (Fig. 1B and C, Fig. S3A), and q-l^{p} was localized to a region of 250 kb with 14 predicted genes (Fig. 1C and D, Fig. S3B).

Comparison between the mapping intervals of the two quail-like mutants revealed an approximately 150 kb overlap region, in which there were 6 predicted genes (BGIBMGA00**5417**, BGIBMGA00**5418**, BGIBMGA00**5419**, BGIBMGA00**5420**, BGIBMGA00**5421**, BGIBMGA00**5422**; the last four digits are used as the abbreviation of the gene accession number in the following description, Fig. 1B–D) based on SilkDB and Silkbase. Structural analysis of these 6 genes indicated that *5421* and *5422* are two different transcripts of *orcokinin* (*BommoOKA* (GenBank No. MH321199 - MH321202) and *BommoOKB* (GenBank No. MH321203)) formed by alternative splicing. The *BommoOKB* transcript is the *5421* gene, while the other transcript *BommoOKA*, consists of exon 1 to exon 3 of the *5421* gene as well as all four exons of the *5422* gene (Wang et al., 2018b) (Fig. 2A, Fig. S4).

3.2. The variation in its structure and expression level of BommoOK in two quail-like mutants

CDS cloning of the above 5 genes (primers listed in Supplemental Table S1) showed that there were no structural variations in *5417*, *5418*, *5419* and 5420 that were detectable in the two quail-like mutants compared with their wildtypes. However, a one-base deletion was detected in exon 3 of *BommoOK* in the q-l^{p} mutant (Fig. 2A), which led to a nonsense mutation in *BommoOKA* and *BommoOKB* and resulted in the complete loss of *BommoOK* function. In the q-l^{b} mutant, we cloned two types of *BommoOKA*, one of which had lost exon 6 (q-l^{b}-type1), while another one had lost both exon 5 and exon 6 (q-l^{b}-type2) (Fig. 2B). The 10 times of random sequencing results showed that q-l^{b}-type1 was the major type (80%) of *BommoOKA*. Besides, two types of mutant *BommoOKA* presented simultaneously in one individual as heterozygosis.

To further validate the mutation in the CDS of *BommoOK* in the q-l^{p} mutant, the corresponding region of the genome was cloned (primers are listed in Supplemental Table S1) and sequenced. The results showed that a single-base deletion occurred in the same position of the genome in the q-l^{p} mutant, which was completely consistent with the mutation in the CDS. In the q-l^{b} mutant, a much larger fragment (approximately 7 kb) was obtained compared with that from the wildtype (approximately 2.5 kb) (Fig. S5). The sequencing results for the amplified fragment from q-l^{b} indicated that there was a pair of positive AGATG GCTTCC in exon 6 of *BommoOK*, and a 4318 bp unknown sequence was inserted between these two repeats (Figs. S5 and S6). A poly (A) structure was found at the 3′ end of the inserted sequence, and the inserted sequence exhibited multiple copies in the silkworm genome. After a structural analysis of the inserted sequence, a coding region of reverse transcriptase (GenBank No. AAA17752) and a partial coding region of gag-like protein (GenBank No. BAA76303) were also found inside the inserted sequence (Fig. S6). In a non-LTR (non-long terminal repeat) retrotransposon L1Bm, it also contained two ORFs encoding a gag-like protein and reverse transcriptase, respectively (Abe et al., 1998; Ichimura et al., 1997). Meanwhile non-LTR retrotransposons are characterized by frequent truncation at the 5′-end and always have 3′-tails of either poly (A) trace or a simple A-rich repeat (Eickbush, 1992; Ichimura et al., 1997; Ohshima et al., 1996). Thus, we speculated that the inserted fragment might be a retrotransposon, and the 11 bp positive repeats might be the recognition sequence for transposons in the genome (Abe et al., 1998; Ichimura et al., 1997).

Epidermis of larvae was used for qRT-PCR analysis (primers are listed in Supplemental Table S1). The results showed no significant differences (fold change $<$ 2) in the expression levels of *5417*, *5418*, *5419* and *5420* between the mutant and its wildtype (n = 5 for epidermis, Fig. 3A and B), of which *5417* and *5420* were neither expressed in wildtype 932VR nor in mutant q-l^{p} (C_T value $>$ 35). Expression analysis of *BommoOKA* in the ventral nerve cord and *BommoOKB* in the epidermis on the 3rd day of the 5th instar larvae was performed by qRT-PCR (n = 5 for epidermis, n = 10 for ventral nerve cord), and the results indicated that *BommoOKA* and *BommoOKB* were significantly downregulated (fold $<$ 0.5) in both the q-l^{p} and q-l^{b} mutants compared with their wildtypes (Fig. 3C and D).

On the basis of the above structural and expression analyses, the *orcokinin* gene was thought to be the major gene that was primarily responsible for the two quail-like mutants.

3.3. Quail-like pigmentation appeared in normal silkworm larvae after BommoOK gene knockout via the CRISPR/cas9 method

After co-injection of sgRNA (Fig. 4A) and the cas9 protein into eggs in the early embryonic stage from Qiufeng individuals with normal markings, one individual (*BommoOK*-edited quail-like, defining as q-l^{e}) was obtained in the G$_0$ generation, which showed a similar phenotype to the quail-like mutant. Compared with the wildtype larvae, in the body markings of q-l^{e}, the quail-like markings were superimposed on the normal markings (Fig. S7). Orcokinins play as neuronal prothoracicotropic factors in *B. mori* (Yamanaka et al., 2011). Ecdysone plays important roles in development regulation in insect (Niwa and Niwa, 2014). As ecdysteroid biosynthesis regulatory factors, mutation of orcokinin should present certain abnormal development. Actually, the phenotype of q-l^{e} was also similar to that of the q-l^{p} mutant in terms of the character of the observed developmental abnormalities. The q-l^{p} mutant developed irregularly (Fig. S1) and exhibited 10%–30% smaller than the wildtype 932VR, especially in 5th instar (Wang et al., 2018a). Besides, compared to wildtype Qiufeng, the silkworms developed slowly delaying about two days, ate little mulberry leaf and eventually died (Figs. S1 and S7).

We have cloned the genomic DNA of flanks of two target site (primers are listed in Supplemental Table S1), and the results indicated that a single base was deleted in exon 3 of the *BommoOK* gene, within the OK_sgRNA_1 region in q-l^{e}, whereas there were no changes at another site, OK_sgRNA_2, in q-l^{e}. After more than 10 times repetition of

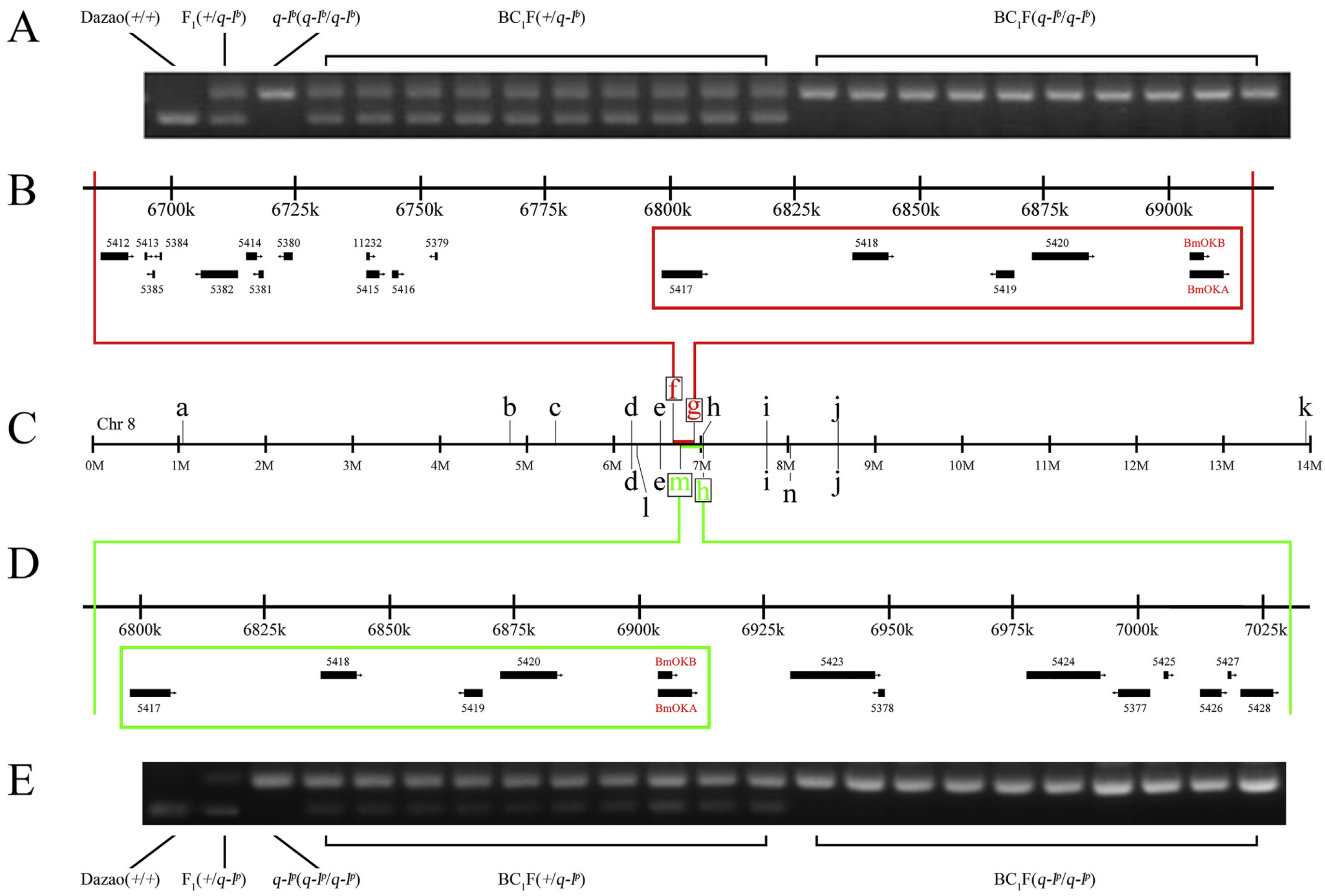

Fig. 1. Positional cloning of *q-l*^{p} and *q-l*^{b}. **(A)** Linkage analysis of *q-l*^{b}. The mutant gene was located on chromosome 8. **(B)** Positional region of *q-l*^{b}. There were 18 predicted genes in the positional region. **(C)** Molecular markers showing polymorphism on chromosome 8 (Supplemental Table S1). Markers above the line belong to the *q-l*^{b} mutant, while those below the line belong to the *q-l*^{p} mutant. *Q-l*^{b} was positioned between molecular markers f and g, while *q-l*^{p} was between m and h. The letters a-n represent the molecular markers S0805, S0816, S2827–22, S2828–234, S2828–076, S2828–085, S2828–092, S2828–063, S2828–226, S2828–013, S2970–7, S2828–238, S2828–109 and S2828-011, respectively. **(D)** Positional region of *q-l*^{p}. There were 18 predicted genes in the positional region. Genes in the green box and genes in the red box in pale (B) represent the overlapping portion of the positional regions of the two quail-like mutants. **(E)** Linkage analysis of *q-l*^{p}. The mutant gene was located on chromosome 8. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

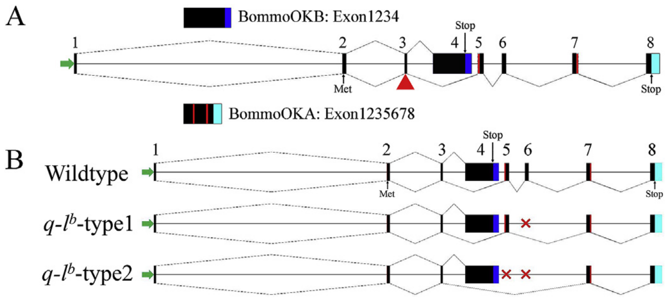

Fig. 2. Structural analysis of the *BommoOK* gene in quail-like mutants. **(A)** Structure pattern diagram of the *BommoOK* gene. The green arrow represents the promoter, boxes represent exons, black lines represent introns, and blue and cyan boxes represent 3′ untranslated regions of *BommoOKB* and *BommoOKA,* respectively. The red boxes represent polymorphic splicing of BommoOKA. The imaginary line indicates the alternative splicing of *BommoOKA* and *BommoOKB* (Wang et al., 2018b). The red triangle indicates the one-base-deletion site of the *BommoOK* gene in the *q-l*^{p} mutant. In addition, met represents the initiation codon, while stop represents the termination codon. **(B)** Structural variation of the *BommoOK* gene in the *q-l*^{b} mutant. Exon 6 was absent in *q-l*^{b}-type1, and both exons 5 and 6 were absent in *q-l*^{b}-type2.

sequencing, the *q-l*^{e} mutant was identified as homozygote in mutant site. Interestingly, both *q-l*^{p} and *q-l*^{e} exhibited a one-base deletion in exon 3 of *BommoOK*, and the deletion sites of *q-l*^{e} and *q-l*^{p} were separated by only a 24 bp interval (Figs. S7A–C). Besides, expression analysis of *BommoOKA* and *BommoOKB* revealed that both *BommoOKA* and *BommoOKB* were downregulated in *q-l*^{e} mutant (Fig. 4B), which were also similar to *q-l*^{p} and *q-l*^{b} mutants. Thus, the *q-l*^{e} mutant almost reproduced the *q-l*^{p} mutant. The results above demonstrated that the *BommoOK* gene plays a crucial role in the mutant characteristics of quail-like mutants, and is directly responsible for the two quail-like mutants and that the *BommoOK* gene inhibits pigmentation.

3.4. *Rescue of mutant characteristics by injecting peptides of orcokinin*

Newly exuviated 4th-instar larvae of the two quail-like mutants, *q-l*^{p} and *q-l*^{b,} were subcutaneously injected with 4 types of mature orcokinin peptide solution separately, and their phenotypes were observed in the 5th instar. The results showed that only one group that injecting the OKA-type2 peptide had phenotypic modulation in pigmentation namely that more than 80% of the mutants were rescued in both *q-l*^{p} mutant and *q-l*^{b} mutant (Fig. 5A, Table 1). The phenotype of the rescued *q-l*^{b} silkworms looked similar to that of the wildtype, 0223JH, but the phenotype of rescued *q-l*^{p} silkworms seemed different from that of the

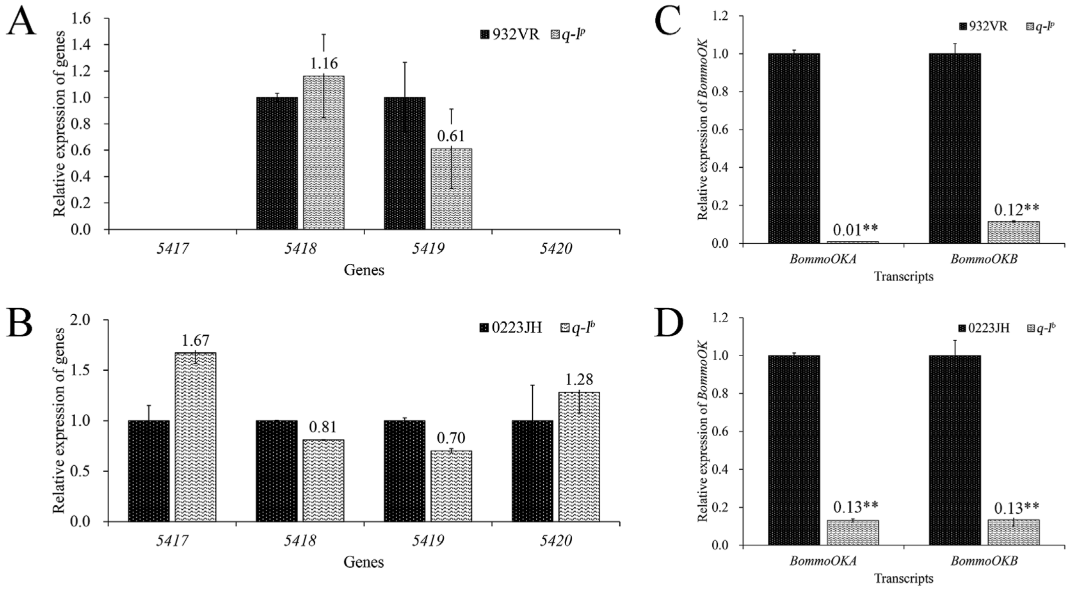

Fig. 3. Expression analysis of candidate genes of quail-like mutants. **(A)–(B)** Quantitative analysis of 4 genes (except 2 transcripts of *BommoOK*) in the positional region. All genes showed no significant difference (fold change < 2) between the wildtypes and mutants. **(C)–(D)** *BommoOKA* and *BommoOKB* were both significantly downregulated (fold < 0.5) in $q\text{-}l^p$ and $q\text{-}l^b$ mutants tested by qRT-PCR. ** represented a *t*-test result of $P < 0.01$.

wildtype, 932VR. Although the markings of the rescued silkworm larvae of $q\text{-}l^p$ disappeared or faded, their body color was still light purple (Fig. 5A). The peptide was found to be most effective when injected into newly hatched larvae of the 4th instar, whereas no effect was observed when injection was performed in gluttonous stage, premolting stage and molting stage in 4th instar, which implied that signal transduction related to pigmentation might have been initiated earlier. These results fully confirmed that the *BommoOK* gene was the key gene of the two quail-like mutants. Additionally, this study is the first to confirm that the *BommoOK* gene inhibits pigmentation and that the mature peptide OKA_type2 is the key factor in the regulation of pigmentation.

Because the development of the $q\text{-}l^p$ mutant is abnormal, its average body weight was measured every 24 h. The results showed that the average body weight of larvae injected with OKA_type2 and OKA_type4 increased slightly, although not significantly so, while the average body weight of the larvae injected with OKA_type5 and OKB_type1 increased significantly on the 7th and 8th days of the 5th instar, which indicated that OKA_type5 and OKB_type1 had effects on silkworm development (Fig. 5B–E).

3.5. Homologous comparison of orcokinin A and orcokinin B

Homologous comparison of orcokinin A and orcokinin B was performed. We used 10 species for each type of orcokinin peptides, and the results showed highly conservative in phylogenesis especially in N-terminal (Fig. S8), which is accordant to previous studies (Jiang et al., 2015). The first 5 to 6 amino acids in N-terminal of orcokinin A was extremely conservative, with a sequence of NFDEID-. There were also several amino acids conservative in middle. These rules also applied to orcokinin B. These results implied that N-terminal might related to binding domain, and C-terminal might relate to functional domain.

4. Discussion

In this study, we showed that the cause of the mutant characteristics of $q\text{-}l^b$ and $q\text{-}l^p$ mutants is changes in the gene structure and expression level of *BommoOK*. Moreover, we verified that *BommoOK* is closely

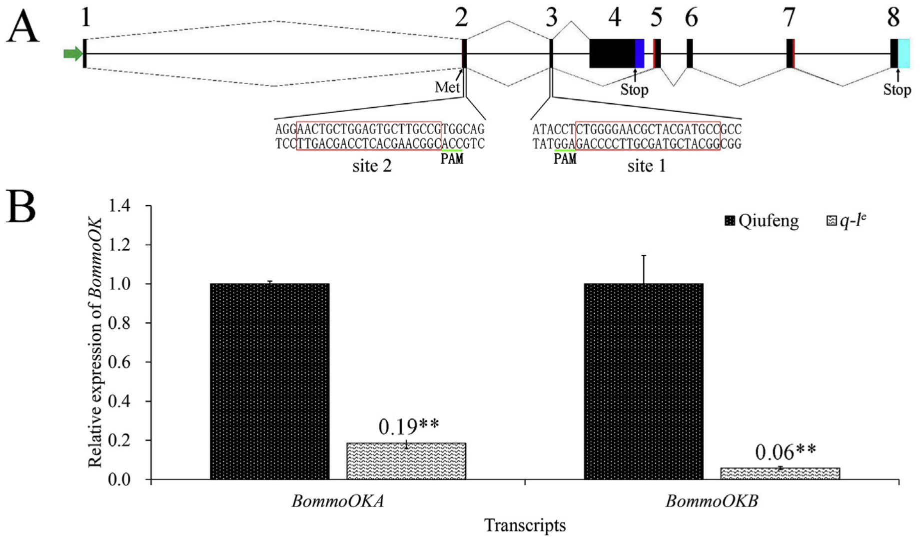

Fig. 4. Verification of the function of the *BommoOK* gene with the CRISPR/cas9 system. **(A)** The target sites OK_sgRNA_1 and OK_sgRNA_2 in the *BommoOK* gene. The green underline represents the PAM. **(B)** Relative expression of *BommoOKA* and *BommoOKB* between the wildtype Qiufeng and $q\text{-}l^e$ mutant. ** represented a *t*-test result of $P < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

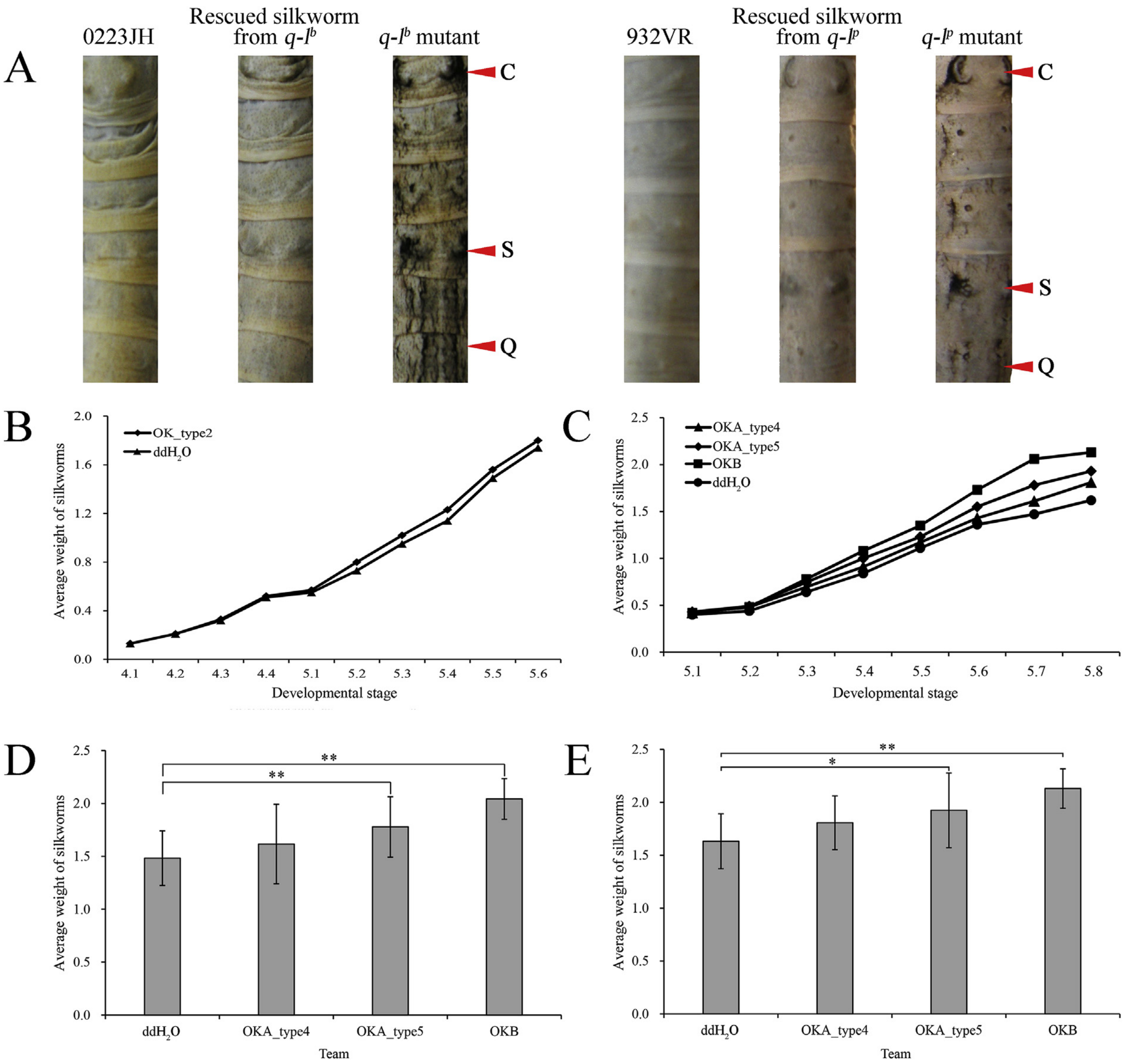

Fig. 5. Rescue of the mutant by peptide injection. **(A)** Rescue of the mutant by injecting the peptide OKA_type2. The six types of epidermis from left to right correspond to newly hatched larva in the 5th instar of wildtype 0223JH, the rescued silkworm from *q-l^b*, the mutant *q-l^b*, wildtype 932VR, the rescued silkworm from *q-l^p* and the mutant *q-l^p*. The red triangles represent different markings. E: eye spot; C: crescent spot; S: star spot; Q: dots and lines with longitudinal wave markings of quail-like mutant. **(B)** OKA_type2 had no effects of the silkworm weight. **(C)** OKA_type4, OKA_type5 and OKB_type1 affected the weight of silkworms. **(D)–(E)** Analysis of the significance of the difference in silkworm weight on the 7th **(D)**and 8th **(E)** days of the 5th instar between the experimental group that injected with the peptides OKA_type4, OKA_type5 and OKB_type1 and the control group injected with ddH$_2$O. * represented a *t*-test result of $0.01 < P < 0.05$, and ** represented a *t*-test result of $P < 0.01$.

related to larval body pigmentation, which is another new function of orcokinin identified in *B. mori* (and in insects overall), following the discovery of the function of *BommoOK* in ecdysone biosynthesis (Yamanaka et al., 2011). The *q-l^b* and *q-l^p* mutants provided a favorable model for studying insect pigmentation mechanisms.

Genetically, the genes controlling the mutations of *q-l^p* and *q-l^b* are allelic variants of the same gene, and *q-l^b* is dominant to *q-l^p* (Zhao et al., 2014). In fact, we had verified it at the molecular level that the neuropeptide gene *BommoOK*, encoding orcokinin, was the key causative gene of the two quail-like mutants. The structure of *BommoOKA* was altered in *q-l^b*, but there were no changes in *BommoOKB*. In addition, the nonexistent 99-base exon of *BommoOKA* in *q-l^b* implied that only one amino acid fragment was deleted in the encoded product, and some mature peptides might still be spliced from the defective protein precursor. Thus, the products of both transcripts of *BommoOK* in *q-l^b* might have retained functions. In contrast, nonsense mutations occurred in both transcripts of *BommoOK* that led to complete loss of gene functions in *q-l^p* mutant. Thus, *q-l^b* was dominant to *q-l^p* at the molecular level.

Peptides are always composed of two or more amino acids and play important roles in vivo. Neuropeptides are a group of peptides that are synthesized and secreted by nerve cells. Some neuropeptides are transported to target tissues by hemolymph, after which they bind to cell surface receptors. Then, intracellular signaling pathways are activated to induce a cell response and achieve the objective of regulating physiological processes (Zupanc, 1996). Some neuropeptides, such as neuropeptide F, are involved in neuroregulation as neurotransmitters (Roller et al., 2008).

The neuropeptide gene *BommoOK* was confirmed to be the causative gene of the two quail-like mutants. Meanwhile, the expression analysis of pigmentation-related genes reveals different expression level between the quail-like mutant and wildtype, among which the *black* gene is greatly upregulated in both *q-l*^{p} and *q-l*^{b} mutant (Qiu et al., 2018; Wang et al., 2017). Pigmentation is an important and complex biological characteristic in insects. There are three main types of pigments melanin, ommochromes and pteridines (Insausti and Casas, 2008; Meng et al., 2009). They were regulated by many transcription factors. The pigmentation mutant *Multilunar* (*L*) was resulted from the ectopic expression of *Wnt1* gene in the epidermis (Yamaguchi et al., 2013). Another silkworm markings, the normal silkworm pattern, are primarily controlled by one allele ($+^p$) of the *p* locus, and a transcription factor apontic-like accounts for the silkworm p locus (Yoda et al., 2014). *BommoOK* gene ought to be another regulation factor for pigments synthesis and degradation. As the major gene, the mature peptide OKA_type2 of *BommoOK* gene should play roles in melanin synthesis pathway.

The previous studies of orcokinin in *B. mori* are neuronal regulation of ecdysteroidogenesis (Tanaka, 2011; Yamanaka et al., 2011). Neuropeptides prothoracicotropic hormone (PTTH) plays roles in synthesis and secretion of ecdysone in the prothoracic gland (PG) (McBrayer et al., 2007). Prothoracicostatic peptide (PTSP) and Bommo-myosuppressin (BMS) are identified to be new prothoracicostatic factors in *B. mori* (Yamanaka et al., 2005, 2010). Bommo-FMRF related peptide (BRFa) and orcokinin are also neuropeptides in regulation of ecdysteroidogenesis (Tanaka, 2011; Yamanaka et al., 2011). Thus, some mature peptides processed from *BommoOKA* and *BommoOKB* might eventually act on ecdysone-mediated signal pathway to regulate the growth and development of silkworms. In our previous study, we have identified a development-regulated gene *BmAR* (aldose reductase) that is significantly downregulated in *q-l*^{p} mutant compared with the wild-type 932VR, and it is closely related to the mutant phenotype of developmental abnormalities and decreased vitality (Wang et al., 2018a). AR is the rate-limiting enzyme in the polyol pathway (Anil Kumar and Bhanuprakash Reddy, 2007). We speculate that *BmAR* gene might be one of the downstream genes regulated by *BommoOK*.

Author contributions

Q.Z. and P.W. designed the research, P.W., Z.Q., S.B., W.W., M.W., and A.C. performed experiments, P.W., D.X., X.H., S.T., M.L., G.Z. and X.S. analyzed data, P.W., Q.Z. and X.S. wrote the article.

Conflicts of interest

The authors declare that they have no conflicts of interest with the contents of this article.

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Appendix ASupplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2019.103229.

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