A *Bombyx mandarina* mutant exhibiting translucent larval skin is controlled by the molybdenum cofactor sulfurase gene

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During the maintenance of the wild silkworm, *Bombyx mandarina*, a mutant phenotype exhibiting translucent skin was identified. Based on the crossing experiments with the domesticated silkworm, *Bombyx mori*, we found that the mutant was controlled by molybdenum cofactor sulfurase (MoCoS) gene. We designated the mutant "Ozaki’s translucent" (*ogZ*). We found a 2.1-kb deletion containing the transcription initiation site, exons 1 and 2, and the 5' end of exon 3 of the MoCoS gene. The transcript of the MoCoS gene was not detected in the *ogZ* homozygote. We concluded that *ogZ* is a complete loss-of-function allele generated by a disruption of the MoCoS gene.

**Key words:** uric acid, nitrogen metabolism, *Bombyx mori*, deletion

**INTRODUCTION**

In the domesticated silkworm, *Bombyx mori*, one of the end products of nitrogen metabolism is uric acid. Uric acid is synthesized in the larva and transported to the epidermis; it subsequently accumulates as urate granules, which renders the larval skin opaque, or non-translucent (Tamura and Akai, 1990). Until date, more than 35 translucent mutants have been reported (Fujii et al., 1998). These mutants are caused by defects in the process of synthesis, transport, and accumulation of uric acid (Tamura and Sakate, 1983; Tamura and Akai, 1990). Translucent mutants have easily distinguishable phenotypical characteristics. Therefore, they are considered potential candidate marker genes in the construction of transgenic silkworms (Yasukochi et al., 1998). Thus far, responsible genes for the three translucent mutant, *og* (MoCoS) (Komoto et al., 2003), *oq* (*BmXDH1*) (Komoto, 2002), and *od* (*BmBLOS2*) (Fujii et al., 2008) were clarified.

The wild silkworm, *Bombyx mandarina*, is believed to be the ancestor of *Bombyx mori*. These two species can be crossed reciprocally and produce hybrid progeny (Kawaguchi, 1928; Murakami and Imai, 1974). Some genes such as wild melanism (*Wm*; Hirobe, 1947) and wild wing spot (*Ws*; Hirobe, 1947) which are derived from the *Bombyx mandarina* were introduced into *Bombyx mori* strain through the hybridization. In 1982, many individuals of *Bombyx mandarina* specimens were collected from Hangzhou, China, and reared in our laboratory. During the maintenance of their descendants, several larvae with translucent skin were found in a batch, whose female individuals deposited only unfertilized eggs, while the males were fertile. Therefore, males of the mutant were crossed with *Bombyx mori*. All the *F₁* individuals had non-translucent skin. On the other hand, the *F₂* obtained from sib-mating of the *F₁* resulted in non-translucent and translucent larvae at a ratio of about 3:1 in both female and male offsprings. These results indicate that the characteristic of translucency was inherited as an autosomal recessive mutation. The mutant was tentatively called “Ozaki’s translucent” and the gene was assigned the symbol *oz*; it is the first example of the translucent mutation originated from *Bombyx mandarina*. We have maintained the *oz* allele by repeated crossing of the *+/oz* females with either the *oz/oz* or *+/oz* males.

The *oz/oz* phenotype is characterized by highly translucent larval skin, female sterility, high mortality during pupal period and low viability of moths. In the translu-
cent mutants, op, oq, and og locus cause sterility in Bombyx mori (Fujii et al., 1998). In the op strain, only the male moths of mutant homozygotes are sterile. In the og strains, both the female and male moths of mutant homozygotes are sterile. On the other hand, og causes sterility in females, whereas og/og males are fertile. Additionally, many og individuals perish during pupal period similar to the oz homozygotes. Based on its resemblance to the og phenotype, we speculate that oz is the og gene of Bombyx mandarina.

Here we report genetic analysis of the oz mutant. Based on the crossing experiments, we confirmed that oz is the og gene of Bombyx mandarina. Moreover, sequence analysis revealed that exons 1, 2, and 3 are disrupted in the molybdenum cofactor sulfurase gene.

MATERIALS AND METHODS

Silkworm strain C108 is a standard strain maintained in our laboratory (Promboon et al., 1995). og t homozygotes exhibit translucent larval skin (Fig. 1). The o3t strain bears og t. Homozygotes of oz (og z) exhibit translucent larval skin (Fig. 1). The OZ strain having oz (og z) gene is maintained in our laboratory.

DNA extraction Genomic DNA was extracted from the legs of the moths using InstaGene™ Whole Blood Kit (Bio-Rad) as described previously (Fujii et al., 2006). Genomic DNA was also extracted from larval posterior silk glands as described by Abe et al. (1998).

Genomic PCR Genomic PCR was performed with ExTaQ (TaKaRa) under the following conditions: 94°C for 3 min followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 3 min. When the primer set (gandy1a/gandy1b) was used, the cycle number was increased to 40 and elongation time decreased to 1 min. PCR products were analyzed by electrophoresis on 1% or 2% agarose gels and stained with ethidium bromide. The PCR primer sequences used in this study are shown in Table 1. The sequence of a primer, MSR, has been described by Komoto et al. (2003).

Cloning and DNA sequencing PCR fragments were purified from gels and cloned into a pGEM-T easy vector (Promega). Cloned DNA was sequenced using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit and analyzed by the ABI PRISM 3100 Genetic Analyzer.

RT-PCR Total RNA was extracted from the larval fat body using TRIzol reagent (Invitrogen) and treated with deoxyribonuclease (Wako) according to the manufacturer’s instructions. The total RNA isolated was reverse-transcribed, diluted, and used for PCR. First-strand cDNA reaction was performed using the TaKaRa RNA PCR Kit (AMV) Ver. 3.0 (TaKaRa) using an Oligo dT-adaptor primer. PCR was performed using ExTaQ (TaKaRa) under the following conditions: 40 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min. The cycle number was decreased to 30 for the primer set (EFRTF/EFRTR). The elongation time was increased to 3 min for the primer set (EFRTF/EFRTR). The elongation time was increased to 3 min for the primer set (M13 primer M4/oz4a), and (M13 primer M4/MSF). PCR products were analyzed by electrophoresis on 1% agarose gels and stained with ethidium bromide. Sequences of primers for the MoCoS gene are shown in Table 1. Sequences of the primer set (EFRTF/EFRTF) for the silkworm elongation factor 1α (EF1α) gene have been described by Komoto et al. (2003). The M13 primer M4 is a primer complement to the adaptor region of the Oligo dT-Adaptor primer used in the reverse transcription. It is also provided by the TaKaRa RNA PCR Kit (AMV) Ver. 3.0 (TaKaRa).

GenBank accession number Nucleotide sequences flanked by a primer set (oz0/MSR) derived from the og z allele were deposited in the DDBJ, EMBL, and GenBank.

![Fig. 1. Larval characteristics of the og'/og', og/og (og2), and og/+ (og2+). og' and og (og2) homozygotes exhibit translucent skin. Larval skin of og/+ (og2+) larva is non-translucent (normal).](image-url)
nucleotide databases under the accession number AB426481.

RESULTS AND DISCUSSION

The allelism of oz and the og locus can be tested by crossing the og/+ females and ox/+ males. The og strain is maintained by crossing the og/+ male and og/+ female because both the males and females of the og homogametes are sterile. Therefore, we cannot distinguish +/+ and +/og individuals in the og strain. In the og allele, a 551-bp miniature inverted-repeat transposable element (MITE) named Organdy is inserted in exon 5 of MoCoS (Komoto et al., 2003). To discriminate the +/+ individuals from the +/og ones in the og strain, we constructed a primer set (gandy1a/gandy1b) which amplified the 758-bp fragment containing Organdy from the og allele. DNA was extracted from six wild type moths of the og strain and the amplification pattern was analyzed using a primer set (gandy1a/gandy1b). The 758-bp band was amplified in the Nos. 1, 3, 4, and 5 moths (Fig. 2), which suggests that (1) the genotypes of Nos. 1, 3, 4, and 5 moths were +/og and (2) those of Nos. 2 and 6 were +/+.

To confirm the allelism of oz and og, three og/+ females, and two +/+ females were crossed with oz/+ males obtained by crossing an oz/+ female and an oz/oz male. When oz/+ males were crossed with +/+ females, all larvae exhibited normal skin translucency (Table 2). In contrast, normal and translucent larvae occurred at the ratio of 3:1 in the crosses between oz/+ males and +/og females (χ² = 0.09, P > 0.7) (Table 2). These results indicate that oz is the og gene of Bombyx mandarina. Hence, we revised the genetic symbol of “Ozaki’s translucent” from oz to og.

A 15-kb genomic region containing the MoCoS gene has been sequenced in the standard Bombyx mori strain, C108 (Komoto et al., 2003) (GenBank accession number AB090243). The gene consists of six or seven exons and there are four types of splicing around intron 1 (Komoto et al., 2003). A variant called type A (Komoto et al., 2003) was shown in Fig. 3. To analyze the genomic
structure of the MoCoS gene, we performed genomic PCR analysis. In genomic PCR, two primer sets (oz2a/oz3b and oz5a/MSR) produced amplification products in the normal silkworm larva (C108 strain) (Figs. 4a and 4b), while in the og^z homozygote, the primer set (oz2a/oz3b) did not produce an amplification product (Fig. 4a). These results suggest that there is deletion in the MoCoS gene. To reveal the structural abnormality of the MoCoS gene, we constructed a PCR primer set (oz0/oz5b) based on the sequence data of the C108 strain (AB090243) (Fig. 3). The oz0 primer is designed from 541 bp upstream of exon 1, and the oz5b primer from exon 5 of the MoCoS gene. We performed genomic PCR using the primer set (oz0/oz5b) in the C108 larvae and the ogZ homozygote. In the C108 larva, a 3.3-kb band was obtained, while a 1.2-kb band was amplified in the ogZ homozygote (Fig. 5). To determine the genomic structure of the MoCoS gene, the PCR products amplified by the two primer sets (oz0/oz5b and oz5a/MSR) were sequenced (AB426481). Subsequent sequence analysis of the fragment disclosed the 2.1-kb deletion containing exons 1 and 2, and the 5’ end of exon 3 of the MoCoS gene (Figs. 3 and 5). The 5’ breakpoint was assigned at 4,500 bp in AB020934, and the 3’ breakpoint was mapped to 6,641 bp. An orphan sequence of 10 nucleotides (5’-AAAATTAATA-3’) was present between the 5’ and 3’ breakpoints. The 5’ flanking region of the breakpoint (1-469 bp in AB426481) had perfect homology with 4,032-4,500 bp in AB020943 except for a T-A substitution. The 3’ flanking region of the breakpoint (480-2,426 bp in AB426481) was 98% homologous to the 6,641-8,584 bp region in AB020943. The 2,982-3,332 bp region of AB426481 had perfect homology with 8,907-9,257 bp in AB020943 except for a C-T polymorphism. Additionally, we found a 231-bp insertion sequence (2,535-2,767 bp in accession number AB426481) in intron 6 of the og^z allele. The insertion makes the difference in the sizes of amplification products in the genomic PCR using a primer set (oz5a/MSR) between +/+ and og^z/og^z (Fig. 4b).

We presumed that MoCoS gene is not expressed in the og^z homozygotes because the deletion identified in the ogZ allele contains transcript start site of the MoCoS gene identified by Komoto et al. (2003). To detect the transcripts of the MoCoS gene, we performed RT-PCR analysis. However, clear band was not obtained in the RT-PCR using primer sets (oz2a/oz3b and oz5a/MSR) in the C108 larva and the og^z homozygote (data not shown). Therefore, we performed nested RT-PCR. In one experiment, first PCR was conducted using a primer set (MSF/M13 Primer M4). Second PCR is conducted using a primer set (oz2a/oz3b) (Fig. 6b). In the other experiments, first PCR was conducted using a primer set (oz4a/M13 primer M4). Second PCR is conducted using a primer set (oz5a/MSR) (Fig. 6c). The M13 primer M4 is a primer complement to the adaptor region of the Oligo dT-Adaptor primer used in the reverse transcription. The silkworm elongation factor 1α (EF1α) gene was used as the control for RT-PCR. Expression of the EF1α was detected in the C108 larva and the og^z homozygote (Fig. 6a). However, expression of the MoCoS gene was not
Bombyx mandarina translucent mutant

Table 1

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Fig. 6. Nested RT-PCR analysis of the MoCoS in the C108 larva (+/+ and the oq oq homozygote. The Bombyx mori elongation factor 1α (EF1α) gene was used as the control. RNA was extracted from the larval fat body. Numbers at the left indicate base pairs (bp). a, A primer set (EFRTF/EFRTR) was used. b, In the first PCR, a primer set (MSF/M13 Primer M4) was used. c, In the second PCR, a primer set (oz2a/oz3b) was used. In the second PCR, a primer set (oz5a/MSR) was used. In the first PCR, a primer set (oz4a/M13 primer M4) was used. M13 primer M4 is a primer complement to the adaptor region of the Oligo dT-Adaptor primer used in the reverse transcription. See Fig. 3 for the primer positions.

detected in the oq oq homozygote (Figs. 6b and 6c). These results indicate that MoCoS gene was not expressed in the oq oq homozygote due to the deletion containing exons 1 and 2, and the 5’ part of exon 3 of the MoCoS. On the basis of these results, we concluded that oq oq is a null mutant of the MoCoS gene.

XDH catalyzes the oxidation of hypoxanthine to xanthine and xanthine to uric acid (Bursell, 1967). XDH requires the sulfide form of molybdenum cofactor (MoCo) for its enzymatic activity (Hill, 1996). MoCoS is responsible for converting the oxo form of Moco to its sulfide form (Amrani et al., 2000; Watanabe et al., 2000). In the oq oq homozygotes, we presumed that the (1) lack of MoCoS results in defective XDH activity leading to the translucent phenotype and (2) accumulation of hypoxanthine and xanthine, the substrates of XDH, lead to high mortality during pupal period and low viability of moths.

According to us, the oq oq homozygotes are fertile although they occasionally need assistance for copulation. Besides oq oq, there are two alleles in the oq locus. Males of oq homoygotes are fertile while that of oq oq or oq oq homoygotes are sterile. In the oq homoygotes, XDHβ activity was absent while faint XDHα activity was present. In the oq oq or oq oq homoygotes, both XDHα and XDHβ activity were absent (Komoto et al., 2003). The oq locus encodes the XDH1 gene (Komoto, 2002). oq homoygotes lack XDHα activity and are unable to synthesize uric acid (Tamura, 1977, 1983). Similar to the oq oq or oq oq homozygotes, males of oq oq homoygotes is sterile. Male sterility of the oq oq homoygotes and the oq oq homoygotes can be rescued by the injection of the xanthin oxidase which is an equivalent enzyme of xanthin dehydrogenase. It is concluded that male sterility of them are caused by the defect in the XDH activity (Tamura et al., 1999). Therefore, it is unclear why oq oq/og oq males are fertile although MoCoS gene is not expressed. For the better understanding of the effect of the MoCoS and XDH on the male fertility, XDH activity should be analyzed in the oq oq homoygotes.

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