

(要約)

Molecular genetic studies on the oily mutants in the silkworm, *Bombyx mori*

(カイコの油蚕変異体に関する分子遺伝学的研究)

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Chapter 1:

Reduced expression of the *dysbindin*-like gene in the *ov* mutants exhibiting mottled translucency of the larval skin

1.1 Introduction

Uric acid accumulates in the form of urate granules in the epidermis of *Bombyx mori*, making the larval skin color generally white or nontranslucent. Defects in the synthesis and accumulation of uric acid result in transparent larval skin. Because this skin resembles oiled paper, silkworms genetically deficient in urate granules are called oily mutants. More than 35 oily mutants of the silkworm have been reported (Fujii et al., 1998). These mutants show various transparent phenotypes, owing to variation in the capability of synthesis and accumulation of uric acid (Tamura and Sakate, 1983). For instance, the *oal* (oal mottled translucent) mutant of silkworm presents a mottled translucent skin, the *ok* (kinshiryu translucent) mutant has highly transparent skin, whereas the *ov* (mottled translucent of Var) mutant, unlike other oily mutants, shows varying degrees of transparency in the skin among individuals, from high to almost normal (Doira et al., 1981). More than 20 of them have been mapped on the chromosomes (Doira, 1992).

Kômoto and his colleagues have identified the genes for two oily mutants, *oq* (q-translucent) and *og* (Giallo ascoli translucent) as DNA sequences (Kômoto 2002; Kômoto et al. 2003). These two mutants, presenting highly transparent larval skin, occur by mutations in the genes encoding xanthine dehydrogenase (XDH) and molybdenum cofactor (MoCo) sulfurase, respectively, both of which are required for the synthesis of uric acid (Bursell, 1967; Bray et al., 1996). In recent years, with the publication of *Bombyx* complete genome sequence (Mita et al., 2004; Xia et al., 2004; The

International Silkworm Genome Consortium 2008) and a highdensity linkage map (Yamamoto et al., 2006, 2008), molecular genetic studies on the oily mutants have become more active, and the responsible genes for oily mutants have been further identified. The *ow* (waxy translucent) mutant has a 25-bp insertional mutation in the *BmVarp* gene, a *Bombyx* homolog of the *varp* gene encoding a vacuolar protein for sorting (VPS) domain-containing protein in mammals (Ito et al., 2009). Fujii et al. (2008, 2010) reported that the *od* (distinct translucent) mutant results from a molecular defect in a *Bombyx* homolog of the BLOS2 subunit of the human biogenesis of lysosome-related organelles complex-1(BLOC-1). BLOC-1 and VPS are required for the proper biogenesis of lysosome-related organelles (LROs) including the melanosome and delta granules in vertebrates and the pigment granules in *Drosophila* (Huizing et al. 2008), suggesting that the silkworm urate granules are formed by the mechanism similar to LROs. Kômoto et al. (2009) reported that the *w-3* (white egg 3) mutant, characterized by white eggs and eyes and translucent larval skin, results from a single base deletion in an ATP-binding cassette (ABC) transporter gene. In addition, an amino acid transporter is responsible for the *os* (sex-linked translucent) mutant (Kiuchi et al., 2011). These findings indicate that membrane transporters are involved in the transport of uric acid in silkworm epidermal cells. Although the previous studies have elucidated that the oily mutants express their phenotypes through various different steps, I still do not know how the urate granules are formed. Studies of the many oily mutants that have not been analyzed at the DNA level should shed light on the mechanisms of formation of urate granules in silkworm epidermis.

ov, a recessive oily mutant, was found as a spontaneous mutant in a European strain, Var (Doira et al., 1981). It is classified into a group of oily mutants accumulating abnormal levels of uric acid in the epidermis (Tamura and Sakate, 1983). In the *ov* mutant, the degree of transparency of the skin varies among individuals from high to almost normal. The *ov* gene has been mapped at 15.2 cM on chromosome 20. Later, *ov*^p

was found as another spontaneous mutant from the m60 strain and was shown to be allelic to *ov* (Doira et al., 1981).

Here I have described the results of linkage analysis of the *ov* locus and compared the mRNA expression levels of the *ov* mutant and wild type for predicted genes in the responsible region.

1.2 Materials and Methods

1.2.1 Silkworm strains

The *ov* mutant strain o66 and the *ov^p* mutant strain o67 are genetic stocks maintained at Kyushu University, Fukuoka, Japan. The wild type strain m60, from which the o67 strain was derived, was also supplied by Kyushu University. The wild type strain p50T used for the silkworm genome analysis is maintained at the University of Tokyo, Japan. The wild type strain No. 523 was donated by the National Institute of Agrobiological Sciences (NIAS), Tsukuba, Japan. The wild type strain N4 (non-diapause strain) was a kind gift from Dr. Haruhiko Fujiwara (the University of Tokyo) and Dr. Kozo Tsuchida (National Institute of Infectious Diseases, Japan). For the genetic linkage analysis, highly and moderately transparent BC₁ individuals from 11 pairs of backcrosses between the o67 female and the F₁ (p50T female × o67 male) male were used. All silkworm larvae were reared on mulberry leaves at 25 °C.

1.2.2 Genetic map

For mapping the *ov* locus onto the genomic DNA sequence, amplified fragment length polymorphisms in polymerase chain reaction (PCR) and single nucleotide polymorphisms (SNP) were identified at various positions on a sequence scaffold, nscf 2789 on chromosome 20, and markers that showed polymorphism between o67 and p50T were used for the genetic analysis of BC₁ individuals with the *ov* phenotype.

1.2.3 Genomic PCR

Genomic DNAs of parents and F₁ individuals were extracted from the moths using the DNeasy Blood and Tissue Kit (QIAGEN, Valencia, Calif., USA) according to the manufacturer's protocol. Those of BC₁ individuals were isolated from the bodies of fourth or fifth-instar larvae using the Wizard SV 96 genomic DNA purification system (Promega, Madison, Wis., USA) according to the manufacturer's protocol. PCR was performed using Ex *Taq* DNA polymerase (TaKaRa Bio, Otsu, Japan) and the primer sets designed from the *Bombyx* draft genome sequence (Mita et al., 2004; Xia et al., 2004). PCR conditions were as follows: initial denaturation at 94 °C for 2 min, 35–40 cycles of 94 °C for 30 s, 55–57 °C for 30 s, and 72 °C for 1.5 min, followed by 72 °C for 5 min.

1.2.4 Reverse transcription PCR (RT-PCR) analysis

Total RNA were purified using TRIzol (Invitrogen, Carlsbad, Calif., USA) according to the manufacturer's protocol and subjected to cDNA synthesis using the oligo (dT) primer and AMV reverse transcriptase contained in the TaKaRa RNA PCR kit (TaKaRa). RT-PCR was performed using Ex *Taq* and primer sets designed from the *Bombyx* draft genome sequence. RT-PCR conditions were as follows: initial denaturation at 94 °C for 2 min, 25–35 cycles at 94 °C for 30 s, 57–60 °C for 30 s, and 72 °C for 30–90 s, followed by 72 °C for 5 min.

1.2.5 Real-Time RT-PCR

Total RNA was prepared and reverse-transcribed as described above. The real-time RT-PCR was performed with Power SYBR Green PCR master mix (Applied Biosystems, Warrington, UK) using the ABI StepOne Plus Real-Time PCR System (Applied Biosystems) according to the manufacturer's recommended procedure. The

real-time PCR condition was denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The primer sets used in real-time RT-PCR are listed.

1.2.6 Cloning of the candidate gene responsible for *ov*

Total RNAs were isolated from the epidermis of fifth-instar day 3 larvae of strains p50T, m60, o66, and o67 as described above and were used for 5'-and 3'-RACE experiments with the GeneRacer kit (Invitrogen). RT-PCR was performed using Ex *Taq* under the following conditions: initial denaturation at 94 °C for 2 min, 35 cycles at 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 1.5 min, followed by 72 °C for 5 min. The PCR products were subcloned into the pGEM-T Easy vector (Promega) and sequenced using an ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and an ABI Prism 3130 DNA Sequencer (Applied Biosystems).

1.2.7 RNAi experiments

For RNAi experiments, siRNA sequences were designed on the basis of the ORFs of *Bmdysb* and *EGFP* encoding enhanced green fluorescent protein (EGFP). Target positions were selected using siDirect ver. 2.0 (<http://sidirect2.rnai.jp/>). Two and one pair of optimal sequences for *Bmdysb* and *EGFP*, respectively, were selected from a list of potential candidates provided by siDirect as described previously (Yamaguchi et al., 2011). *EGFP* was used as a control. The siRNA sequences are listed. Double-stranded siRNAs were synthesized by FASMAC Corp (Japan) and were dissolved in the injection buffer [100 mM KOAc, 2 mM Mg(OAc)₂, 30 mM HEPES-KOH, pH 7.4] and stored at -20°C until use. For RNAi experiments, *Bmdysb* 1 and 2 solutions were mixed together. The siRNA solutions were then diluted to 25, 50, and 100 μM with the injection buffer and 1–5 nL was injected into embryos of the N4 strain at the preblastoderm stage. The injection was performed according to the method described by Yamaguchi et al (2011) using the injector (IM 300 Microinjector, Narishige Japan). Injected embryos were

incubated at 25°C in a moist Petri dish until hatching. The larval phenotypes were observed on the first instar stage on day 2. For real-time RT-PCR analysis, total extracted RNA from each first instar day 2 larva injected with siRNA was extracted and cDNA was synthesized as described in the 1.2.4 of Materials and Methods. The ribosomal protein 49 gene (*rp49*) was used to normalize transcript levels. Primers used for real-time RT-PCR are listed. Real-time RT-PCR was performed in duplicates. Statistical tests were performed using SPSS 16.0. *P* value of < 0.05 was considered significant.

1.2.8 Quantification of uric acid

Uric acid was extracted individually from first instar day 2 larvae injected with *EGFP* and *Bmdysb* siRNA, and Val and o66 strians, and was performed according to the method described by Kobayashi et al (2010). Each larva was homogenized in 150 µl distilled water. The homogenate was heated for 10 min at 100°C. After centrifugation at 15,000 rpm for 10 min, the supernatant was collected. Uric acid concentration in the supernatant was determined by the Uricase TOOS method using a Uric acid C Test kit according to the manufacturer's instructions (Wake, Japan).

1.2.9 Homology search and phylogenetic analysis

The homologous proteins in metazoans were searched by BLAST at the websites of NCBI (<http://www.ncbi.nlm.nih.gov/mapview/>) and Ensembl (<http://metazoa.ensembl.org/Multi/blastview>). And the amino acid sequences were aligned with CLUSTALW (<http://www.genome.jp/tools/clustalw/>) with the default setting. The gaps in the sequences were removed as described in the Results, and a phylogenetic tree was constructed by the Neighbor-Joining method using MEGA5 (Tamura et al. 2011) with the default settings. The reliability of the tree was tested by bootstrap analysis with 1000 replications.

1.3 Results

1.3.1 Mapping of the *ov* mutation

Genes *Lp* (20-6.2) and *vit* (20-23.0) have been shown to encode 30K proteins in larval hemolymph (Sakai et al., 1988; Kishimoto et al., 1999) and the vitellogenin receptor (Meng et al., 2010), and their sequences occupy 3.54 and 10.6 Mb on chromosome 20, respectively. Gene *ov* has been mapped at 15.2 cM on the same chromosome (Doira et al., 1981). The genetic distances between *Lp*, *ov*, and *vit* suggest that *ov* is located on the genomic sequence scaffold, nscaf 2789, of chromosome 20. To narrow the region responsible for *ov*, I performed genetic linkage analysis using the primer sets designed on the *Bombyx* genome sequence (Mita et al., 2004; Xia et al., 2004). I used seven pairs of primer sets designed from the sequence of nscaf 2789 and performed detailed mapping using 2112 BC1 individuals. I found that a 178582 bp long region between two positions of primers ovmap28 (P28) and ovmap41 (P41) was responsible for the *ov* mutant. There were nine predicted protein-coding genes in this 179-kb region.

1.3.2 Identification of a candidate *ov* gene

The *ov* mutant is deficient in the ability of incorporating uric acid from hemolymph into urate granules of the epidermal cells (Tamura and Sakate, 1983; Tamura and Akai, 1990). This indicates that the process of uptake of uric acid or formation of urate granules is abnormal in the *ov* mutant. These two processes may be controlled by the molecular mechanisms of the epidermal cells. Furthermore, the mottled phenotype of the *ov* mutant suggests the cell-autonomous function of the *ov* gene in epidermal cells. Therefore, I analyzed the expression of the nine candidate genes in the epidermis of day

3 fifth-instar larvae by semiquantitative RT-PCR. Three of the genes were not expressed, and of the other six, the expression level of only one, *BGIBMGA004333*, was severely suppressed in the mutant strains o66 (*ov*) and o67 (*ov^p*) compared with the wild type (p50T) and was moderately suppressed in individuals whose skins were more transparent; the remaining five genes were equally expressed in mutants and the wild type. *BGIBMGA004333* is homologous to *DTNBPI* encoding dysbindin in human as described below in detail, and I named it as *Bmdysb* (*B. mori dysbindin*-like gene) in the present study. I paid special attention to this gene because the defects of the *dysbindin* gene cause the hypopigmentation in human and mouse due to incomplete biogenesis of melanosomes (Li et al., 2003) as discussed in detail below. I further compared the relative expression level of *Bmdysb*'s mRNA in the epidermis between the mutant o67 (*ov^p*) and wild types (p50T and m60 (original strain of *ov^p*)) using real time RT-PCR. The mRNA level in highly translucent individuals was only one-thirtieth that of the wild types. Accordingly, these results suggest that *Bmdysb* is the most probable candidate for the gene responsible for the *ov* mutant.

I also determined the coding sequences (CDS) of *Bmdysb* and the other five predicted genes (*BGIBMGA004280*, *BGIBMGA004332*, *BGIBMGA004334*, *BGIBMGA004335*, and *BGIBMGA004336*) that were equally expressed in the epidermis of the mutant strains o66 (*ov*), o67 (*ov^p*), and the wild type m60. Because the CDS of these six genes were identical in the three strains, I concluded that the formation of translucent skin of the *ov* mutant is because of the reduced expression of *Bmdysb*.

1.3.3 Characterization of *Bmdysb*

As described above, the CDS of *Bmdysb* was identical among o66 (*ov*), o67 (*ov^p*), and m60 (wild type). I also resequenced the *Bmdysb* CDS in the standard strain p50T and found that it was different from those of o66, o67, and m60. These three strains showed nucleotide substitutions at 11 positions in the coding region compared with

p50T. Three were nonsynonymous and eight were synonymous. Next, I performed RACE experiments and determined the sequences of the 5'-and 3'-UTR regions from the four strains. As with the CDS, the UTRs in only the p50T strain were different from those in the other three strains o66, o67, and m60. They differed at 23 nucleotide sites in the 5'-UTR and seven in the 3'-UTR. Although the m60 strain does not carry the *ov^p* gene, the *Bmdysb* CDS was identical to that of o67. Accordingly, I speculated that the low expression of *Bmdysb* in the mutants may be attributed to the variation of transcription regulatory sequences in the upstream region or elsewhere.

The RACE results showed that *Bmdysb* expresses two splice isoforms, both in the wild types and the mutants. One is 1259 bp in length and is designated as the A type and the other is 1158 bp in length and designated as the B type. The A-type isoform has a 101-bp insertion in its 5'-UTR. Semiquantitative RT-PCR showed that the expression level of the B type is markedly lower in the mutant o67 (*ov^p*) than in the wild type p50T. The semiquantitative and qualitative abnormalities observed in the mRNA expression of *Bmdysb* in o67 suggest that it is related to the formation and accumulation of urate granules in the silkworm epidermis.

1.3.4 Knockdown of *Bmdysb* gene by embryonic RNAi

To determine whether a decrease in the amount of *Bmdysb* mRNA causes the depressed content of uric acid in the *ov* mutants compared to that of the normal type (Tamura and Sakate, 1983), siRNA experiments were performed to suppress the expression of the corresponding gene in wild type strain N4 eggs within 4–8 h after oviposition. In the *ov* mutants, the larval skin showed distinctly translucent phenotype on the second day after hatching. The phenotype of each first instar day 2 larvae injected with siRNA was examined, and no clear difference between the larval phenotypes injected with *EGFP* or *Bmdysb* siRNAs was observed. The mRNA expression level analysis of *Bmdysb* was performed for randomly chosen larvae injected

with *EGFP* or *Bmdysb* siRNAs, and the remaining larvae were used for determining the uric acid content. The results showed that the expression level of *Bmdysb* mRNA decreased to 73%, 43%, and 60% after injecting with 25, 50, and 100 μ M, respectively, of *Bmdysb* siRNA compared with that after injecting *EGFP* siRNA. In contrast, the uric acid content after injecting 25, 50, and 100 μ M of *Bmdysb* siRNA decreased to 98%, 78%, and 68%, respectively, compared with that after injecting *EGFP* siRNA. The *Bmdysb* mRNA expression level and uric acid content showed significant decrease after injecting with 50 and 100 μ M *Bmdysb* siRNA compared with that of *EGFP* siRNA injections ($P < 0.05$, t test), whereas there was no significant difference in the *Bmdysb* mRNA expression level and uric acid content after injecting 25 μ M *Bmdysb* siRNA and *EGFP* siRNA. *Bmdysb* mRNA expression level and uric acid content of the *ov^p* mutant larvae were determined at the same stage. These results indicated that repression of *Bmdysb* affected the accumulation of uric acid in the silkworm and that uric acid content was well correlated with *Bmdysb* mRNA expression level. These results also indicated that the low expression of *Bmdysb* was responsible for the translucent phenotype of the *ov* mutants.

1.3.5 Spatial expression profiles of *Bmdysb*

I compared the expression of *Bmdysb* in tissues other than epidermis in p50T and o66 (*ov*) using semiquantitative RT-PCR. The expression level of *Bmdysb* in the testis and ovary was almost identical for p50T and o66, whereas the levels in the brain, fat body, midgut, Malpighian tubules, and hemocytes of o66 were less than those of the wild type p50T, similar to the results for the epidermis.

1.3.6 Phylogenetic analysis of *Bmdysb*

To find the genes homologous to *Bmdysb*, I searched the genomes of eight insects, five vertebrates, one echinoderm, and one cnidarian. My BLASTP search against the

proteins encoded in each genome found a single homolog (E-value < 0.01) for all the 15 species. Conversely, the BLASTP searches against the *B. mori* genome using the 15 homologs as queries hit only one protein, Bmdysb, respectively (E-value < 0.01). The overall amino acid sequence of Bmdysb showed 27% homology and 50% similarity to the *Drosophila* dysbindin and 27% homology and 47% similarity to the human dysbindin (dystrobrevin-binding protein 1). I aligned the amino acid sequences of the 15 dysbindin-like proteins with CLUSTALW. Because the resultant alignment showed that three regions were conserved among them, I deleted nonconserved regions, concatenated these three conservative regions, and constructed a phylogenetic tree by the Neighbor-Joining method. In the tree, the homologs from insects and deuterostomes formed the respective clusters if the cnidarian homolog was put as their outgroup. The branching pattern of the proteins within insects, however, did not coincide well with their phylogenetic relation of the eight species, while some of the intermediate branches were not well supported by the bootstrap test (60%–68%). The results of the bidirectional BLAST and phylogenetic analysis indicate that Bmdysb is homologous to the dysbindins in human and *Drosophila* though it is uncertain whether Bmdysb is orthologous to them.

1.3.7 Sequence comparison of *Bmdysb* and its flanking region among strains

I determined the 14663-bp genomic sequence from nucleotide 927311 to 941974 on nscaf 2789 including the exons and introns of *Bmdysb* and the upstream neighbor gene *BGIBMGA004282* in the mutant strains o66 (*ov*), o67 (*ov^p*), and the wild types m60 and p50T. Comparison of their sequences showed that the nucleotide sequence was identical among strains o66, o67, and m60 in the 14.7-kb region (DDBJ accession no. AB728503), whereas that of the p50T strain differed at many sites (DDBJ accession no. AB728505). I also determined the sequence of *Bmdysb* and the partial sequence of its flanking regions in No. 523 (Var; original strain of o66) maintained at NIAS, and found

that the sequence differed from o66 at multiple sites (DDBJ accession no. AB728504). In all, I found 3 haplotypes, A (p50T), B (o66, o67, and m60), and C (No. 523), in the 14.7-kb region that were not directly associated with the translucent phenotype. This result suggests that the sequence variation responsible for *ov* and *ov^p* is located outside the 14.7-kb region, given that the phenotype of m60 is that of the wild type, so that the sequences must differ between m60 and the mutant strains (o66 and o67) at the responsible site.

1.4 Discussion

In the *ov* and *ov^p* mutants of the silkworm, urate granules do not develop well in epidermal cells, compared with normal larvae (Tamura and Sakate, 1983). In the present study, I found that the expression of *Bmdysb* was severely repressed in the epidermis of the *ov* and *ov^p* mutants, and the uric acid content was decreased after performing the knockdown of *Bmdysb*. I concluded that the transcriptional repression of *Bmdysb* causes incomplete or reduced formation of urate granules in the epidermis of the *ov* mutants. In support of this point, Cheli et al. (2010) found that eye-specific knockdown of *dysbindin* brought about reduced accumulation of red pigment granules containing drosopterin (one of the pteridines) in the *Drosophila* compound eyes. Because both the urate granules in the silkworm epidermis and pigment granules in *Drosophila* compound eyes are small membrane-bordered organelles, it is plausible that these granules are formed by BLOCs-like machineries involving dysbindin (Cheli et al., 2010). In addition, comparison of spatial expression patterns of *Bmdysb* between the wild type p50T and mutant o66 (*ov*) indicated that the expression level of *Bmdysb* was also decreased in other somatic cells, such as fat body, brain, and midgut. This result indicates that the transcriptional regulation of *Bmdysb* is common in somatic cells.

In mammals, the dysbindin protein is a subunit of BLOC-1, which exists as a stable octamer formed by dysbindin, muted, pallidin, snapin, cappuccino, BLOS1, BLOS2, and BLOS3 (Dell'Angelica, 2004; Morgan et al., 2006). BLOC-1 is ubiquitously expressed in various tissues and required for the normal biogenesis of specialized organelles of the endosomal and lysosomal systems in mammalian cells. It is known that abnormalities in dysbindin and BLOS3 of the human cause the Hermansky–Pudlak syndromes (HPS), HPS-7 and HPS-8, respectively (Li et al., 2003; Morgan et al. 2006). HPS patients generally show some degree of hypopigmentation of skin, hair, and iris. In the mouse, mutants of the genes encoding subunits of BLOC-1, *pallid*, *muted*, *cappuccino*, *sandy*, and *reduced pigmentation*, display coat pigmentation dilution (Feng et al., 1997; Moriyama and Bonifacino, 2002; Zhang et al., 2002; Ciciotte et al., 2003; Li et al., 2003). Hypopigmentation is caused by abnormality in the biogenesis of melanosomes in HPS patients and mouse mutants (Huizing et al., 2008). In mice, dysbindin was found to interact at the molecular level with pallidin, muted, and BLOS3 (Li et al., 2003; Starcevic and Dell'Angelica, 2004). In contrast, *Drosophila* homologs of the subunits of BLOC-1 belong to the "granule group" of eye pigmentation. Similar interactions were observed in *Drosophila* BLOC-1 subunits. Pallidin interacts with dysbindin, BLOS3, and cappuccino, and snapin also interacts with dysbindin and BLOS2 (Cheli et al., 2010). These similarities indicate that the molecular machinery of BLOC-1 is conserved between *Drosophila* and mammals. My phylogenetic analysis showed that *Bmdysb* is an ortholog to *Drosophila dysbindin*. As mentioned earlier, *Drosophila dysbindin* functions in the accumulation of red pigment granules in the *Drosophila* compound eyes (Cheli et al., 2010). In addition, the genes encoding other components of BLOC-1 in *Drosophila*, such as *pallidin*, *Blos1*, and *Blos4*, were also associated with the accumulation of red pigment granules (Cheli et al., 2010). They suggest that other components of BLOC-1 are also involved in the accumulation of red pigment granules in *Drosophila*. Recently, Fujii et al. (2008; 2010) reported that the

defect in *BmBLOS2*, orthologous to *BLOS2*, resulted in the *od* mutant of silkworm. *od* is a typical mutant showing larval translucency as a result of the reduced formation of urate granules in epidermal cells. Thus, it is likely that the defects of other subunits of BLOC-1 result in similar translucency through the process of urate granule formation. In this study, I found that the transcriptional repression of *Bmdysb* causes the reduction of the uric acid content. These results strongly support my conclusion that the repression of *Bmdysb* results in the formation of the translucent larval skin in *ov* mutants.

The phenotypes of *ov* and *ov^p* are different from common larval translucent mutants such as *od*, *ow*, and *w-3^{oe}*. The translucency in *ov* and *ov^p* manifests as mottling, indicating that their translucent phenotype is cell autonomously expressed in the epidermis. The inverse correlation between the phenotype and the *Bmdysb* mRNA expression level suggests that *Bmdysb* is differentially expressed among cells. Similar phenotypes have been found in other translucent mutants, *od^m* (Hatamura, 1939; Tamura and Sakate, 1983) and *oal* (Takasaki, 1940; Tamura and Sakate, 1983). Fujii et al. (2010) showed that transgenic silkworms in which BmBLOS2 was driven by the GAL4-UAS system showed the mottled translucency phenotype, indicating that the promoter function affects the heterogeneous expression of the translucency genes.

I compared the 14.7-kb genomic sequences including *Bmdysb* and the upstream neighbor gene *BGIBMGA004282* among 4 strains, o66 (*ov*), o67 (*ov^p*), m60, and p50T. This sequence contained all exons, introns, and 5' and 3' flanking regions of *Bmdysb*. Unexpectedly, the results did not indicate any differences in the 14.7-kb sequence among o66, o67, and m60 although the sequence of p50T (haplotype A) shows many SNPs and insertions/deletions with respect to the other 3 strains (haplotype B). This observation indicates that the 14.7-kb region does not contain the mutation responsible for the transcriptional repression of *Bmdysb* in *ov* and *ov^p*. Therefore, the responsible mutation site may be located in the 27-kb upstream segment or 138-kb downstream

segment at least for *ov*. This situation is similar to another mutant, *C*, in which the transcription of a transmembrane-protein coding gene is upregulated by a mutation in the upstream regulatory region and the yellow cocoon phenotype is manifested (Sakudoh et al., 2010). The mutated sequence of the unknown responsible region may cause the repression of transcriptional activity of *Bmdysb*. Further study is needed to identify the responsible mutation and elucidate the reason for its repression of *Bmdysb* transcription.

Because m60 is the original strain from which the *ov^p* mutant was derived, it is reasonable that they share the same haplotype in the 14.7-kb segment. The *ov* mutant was found in the Var strain by S. Sakate in 1972 (Doira et al., 1981). Although I determined the 8.4-kb (nucleotide 933516 to 941974 on nscaf 2789) sequence of the *Bmdysb* and its flanking regions in the Var strain (No. 523, NIAS), I found that the sequence of Var (haplotype C) was different from that (haplotype B) of o66 (*ov*), o67 (*ov^p*), and m60. This result suggests that the original Var strain contained both haplotypes B and C and that the mutation occurred in haplotype B, which has been lost in the brood of the No. 523 examined.

The spatial expression profiles showed that *Bmdysb* was ubiquitously expressed in the tissues. *BmBLOS2* is also expressed in various tissues other than epidermis (Fujii et al., 2010). These results suggest that a BLOC-1-like complex may be present in various tissues in *B. mori* and have multiple functions besides the formation of urate granule in epidermis. Although the expression of *Bmdysb* was repressed in epidermis as well as other somatic tissues including the brain, I observed no other abnormal phenotype except the oily phenotype in the *ov* and *ov^p* mutants. In *Drosophila*, the deficiency of *dysbindin* affects eye pigmentation as well as the function of the central nervous system (Cheli et al., 2010; Shao et al., 2011). Further studies of the *ov* mutants are needed to elucidate the function of *Bmdysb* in tissues other than epidermis.

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Chapter 2 :

Deficiency of a novel ABC transporter gene in the *ok* mutants lacking the incorporation of uric acid in epidermis

2.1 Introduction

Uric acid is one of the end products of nitrogen metabolism in terrestrial insects (Bursell, 1967; Wright, 1995). It is excreted as a waste product as well as retained and deposited in the body tissues of most insects. Storage of uric acid in the fat body has been reported among several insects, including *Manduca sexta* (Bucker and Caldwell, 1980; Williams-Boyce and Jungreis, 1980), *Hyalophora cecropia* (Junreis and Tojo, 1973; Tojo et al., 1978), and *Periplaneta americana* (Cochran, 1979). Certain insects also store uric acid in outer tissues, such as the adult wing scales and larval integuments. Lafont and Pennetier (1975) demonstrated that the white regions of adult *Pieris brassicae* wings contained high uric acid levels, while the dark regions contained lower levels. In addition, Ninomiya et al. (2006) revealed that cells under the white stripe of larva *Pseudaletia separate* integuments comprised a number of granules including uric acid, while these were absent from cells in the black stripe. It has been suggested that providing coloration is one of the major functions for the storage of uric acid in external tissues of the insects (Cochran, 1985).

The silkworm, *Bombyx mori*, stores uric acid as urate granules in the integument during all larval stages of the silkworm, imparting a white, non-translucent appearance to the larval skin. Uric acid accounts for approximately 11% of the dry weight of the integument of the normal silkworm larva (Tamura and Sakate, 1983). It is well known that the genetic absence or insufficiency of urate granules in the integument leads to striking phenotypes with translucent larval skin, referred to as “oily” mutants. At present, 24 non-allelic mutants that exhibit various degrees of translucency are preserved in the Graduate School of Agriculture, Kyushu University (<http://www.nbrp.jp/report/reportProject.jsp?project=silkworm#top>). The degree of translucency is correlated to the content of uric acid in the integument of the respective mutants (Tamura and Sakate, 1983; Tamura and Akai, 1990). The genes responsible for oily mutants have been identified, including those encoding proteins involved in biosynthesis (Komoto, 2002; Komoto et al., 2003), transport (Komoto et al., 2009; Kiuchi et al., 2011), and accumulation (Fujii et al., 2008, 2010, 2013; Ito et al., 2009; Chapter 1) of uric acid. In spite of these discoveries, the mechanism of urate granule formation in the silkworm larval epidermis, particularly the transport and accumulation of uric acid remains unclear. Further studies on the oily mutants would help us elucidate the molecular mechanism of the urate granule formation.

In this study, I report the isolation and identification of the gene responsible for the *ok* mutant “kinshiryu translucent,” a recessive oily mutant (Tanaka and Matsuno, 1929). The integument of the *ok* mutant larva accumulates very few urate granules, which results in highly transparent skin (Tamura and Akia, 1990). The *ok* locus has been mapped at 4.7 cM on chromosome 5, and its allele is known to be “okusa translucent” (Yokoyama, 1939). I performed positional cloning of the *ok* locus using the information in the assembled *Bombyx* genome sequences (Mita et al., 2004; Xia et al., 2004), and found that the *ok* locus corresponds to *Bm-ok*, encoding an ATP-binding cassette (ABC) transporter homologous to *Drosophila brown*. Here I present two lines of evidence

demonstrating that the *ok* mutants are involved in the structural and functional defects in *Bm-ok*, and that the RNA interference (RNAi) of *Bm-ok* reproduces the larval translucent phenotype.

2.2 Materials and Methods

2.2.1 Silkworm strains

The *ok* mutant strains, o21 (okusa translucent) and o22 (kinshiryu translucent), were obtained from Kyushu University, Fukuoka, Japan through the National BioResource Project. The wild type strain p50T, used for the silkworm genome analysis was maintained at the University of Tokyo, Japan. The wild type strain N4 (non-diapause strain) was a kind gift from Dr. Haruhiko Fujiwara (the University of Tokyo) and Dr. Kozo Tsuchida (National Institute of Infectious Diseases, Japan). All silkworm larvae were reared on mulberry leaves at 25 °C.

2.2.2 Positional cloning of *ok*

The o21 (okusa translucent) and p50T strains were used as the parental strains for linkage analysis. Between the *ok* female and the F₁ male (*ok* female × p50T male), 480 segregants of 4-pair backcrosses (BC₁) were used. To map the *ok* locus to the genomic DNA sequence, the amplified fragment length polymorphisms in polymerase chain reaction (PCR) and single nucleotide polymorphisms (SNPs) were searched at various positions along the nucleotide sequence scaffold, nscaf 2529, on chromosome 5. DNA markers that showed polymorphism between o21 and p50T were used for the genetic analysis of BC₁ individuals.

2.2.3 Isolation of genomic DNA and total RNA

Genomic DNA was extracted from the adult legs of o21, p50T, and the F₁ hybrids as described in Chapter 1. DNA of the BC₁ individuals was isolated from the whole bodies of fourth or fifth-instar larvae using the Wizard SV 96 genomic DNA purification system (Promega) according to the manufacturer's protocol. PCR was performed using Ex *Taq* DNA polymerase (Takara) and the primer sets designed from the *Bombyx* genome sequences (Mita et al., 2004; Xia et al., 2004). The PCR conditions were as follows: initial denaturation at 94 °C for 2 min, 35–40 cycles of denaturation at 94 °C for 30 s and 55–57 °C for 30 s, extension at 72 °C for 1.5 min, followed by final extension at 72 °C for 5 min.

Total RNA isolated from several tissues (brain, fat body, midgut, Malpighian tubules, integument, hemocytes, testis, and ovary) on day 3 of the fifth instar was purified using TRIzol (Invitrogen, Carlsbad, Calif.) according to the manufacturer's protocol. One µg RNA was subjected to reverse transcription using the oligo (dT) primer and AMV reverse transcriptase contained in the TaKaRa RNA PCR kit (Takara). Semiquantitative RT-PCR was performed using Ex *Taq* and primer sets designed from the *Bombyx* draft genome sequence. The conditions of RT-PCR were as follows: initial denaturation at 94 °C for 2 min, 25–35 cycles of denaturation at 94 °C for 30 s and 57–60 °C for 30 s, extension at 72 °C for 30–90 s, followed by final extension at 72 °C for 5 min.

2.2.4 Real-Time RT-PCR

Total RNA was prepared and reverse-transcribed as described above. Real-time RT-PCR was performed using a KAPA SYBR FAST ABI Prism qPCR kit (KAPA Biosystems) and the ABI StepOne Plus Real-Time PCR System (Applied Biosystems) according to manufacturer's recommended procedures. For real-time RT-PCR, the cDNA was subjected to denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. The primer sets used in real-time RT-PCR are listed

in. The mRNA was normalized to the level of the ribosomal protein 49 gene (*rp49*).

2.2.5 Cloning of the full-length cDNA of *Bm-ok*

Total RNA was isolated from the integument of the day 3 fifth-instar larvae of strains p50T, o21, o22, and N4, as described above. The full-length cDNA was obtained by 5'- and 3'-RACE experiments with the GeneRacer kit (Invitrogen). RT-PCR was performed using Ex *Taq* under the following conditions: initial denaturation at 94 °C for 2 min, 35 cycles at 94 °C for 30 s, 63 °C for 30 s, and 72 °C for 1.5 min, followed by 72 °C for 5 min. The PCR products were subcloned into the pGEM-T Easy vector (Promega) and sequenced using an ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and an ABI Prism 3130 DNA Sequencer (Applied Biosystems).

2.2.6 RNA interference (RNAi) experiments

The short interfering RNA (siRNA) sequences listed were designed based on the ORF sequences of *Bm-ok* and *EGFP* (enhanced green fluorescent protein) as described by Yamaguchi et al. (2011). Double-stranded siRNAs were purchased from FASMAC Corp (Japan), dissolved in annealing buffer (100 mM KOAc, 2 mM Mg(OAc)₂, 30 mM Hepes-KOH, pH 7.4), and stored at -20 °C until use. Eggs used for injecting were prepared as described previously (Yamaguchi et al., 2011). Before injection, “*Bm-ok* 1” and “*Bm-ok* 2” siRNA solutions for *Bm-ok* were mixed together. Injection protocol was performed according to the method described by Yamaguchi et al. (2011) using the injector (IM 300 Microinjector, Narishige Japan) 1–5 nl 50 μM siRNA solution was injected into the eggs of the N4 strain within 4–8 h after oviposition. siRNA was used as a control for *EGFP*. Injected embryos were incubated at 25 °C in a moist Petri dish until hatching. To perform real time RT-PCR analysis, total RNA from a first-instar day 3 larva injected with siRNA was extracted, and cDNA was synthesized as described above. The *rp49* was used to normalize transcript levels. Primers used for real time

RT-PCR are listed.

2.2.7 Homology search and phylogenetic analysis

Proteins homologous to Bm-ok were identified using TBLASTN and BLASTP from the websites of NCBI (<http://www.ncbi.nlm.nih.gov/mapview/>) and Ensembl (<http://metazoa.ensembl.org/Multi/blastview>) in 10 insect species (*Bombyx mori*, *Danaus plexippus*, *Drosophila melanogaster*, *Anopheles gambiae*, *Culex quinquefasciatus*, *Apis mellifera*, *Bombus terrestris*, *Tribolium castaneum*, *Acyrtosiphon pisum*, and *Pediculus humanus corporis*) and a crustacean (*Daphnia pulex*). The amino acid sequences were aligned using CLUSTALW (<http://clustalw.ddbj.nig.ac.jp/>) with the default parameters. The Neighbor-Joining phylogenetic tree was constructed based on the Gonnet matrix of pairwise distances, and bootstrapping with 1000 replicates was performed using the MEGA5 program for Bm-ok and its homologs (Tamura et al., 2011).

2.3 Results

2.3.1 Positional cloning of the *ok* locus

To identify the gene responsible for the *ok* mutants, I performed a genetic linkage analysis using BC₁ progenies obtained by crossing females of the o21 strain homozygous for *ok* with males of the F₁ hybrids obtained after a cross between an o21 female and a p50T male. I developed two polymorphic markers, M19 and M13, on nscaf 2529 of chromosome 5, and first mapped the *ok* locus to the intervening 6.7 Mb region. Furthermore, I developed five other polymorphic markers in the 6.7 Mb region and finally mapped the *ok* locus to the 700 kb region between markers M35 and M43. Within this region, 25 protein-coding genes (*P1–P25*) had been predicted from gene

models (The International Silkworm Genome Consortium, 2008).

2.3.2 Identification of the *ok* candidate gene

Previous studies have elucidated that the phenotype of the *ok* mutants is cell autonomously and exclusively expressed in the larval epidermis (See Discussion), indicating that the gene responsible for the *ok* mutants is restricted to the larval epidermis. Therefore, I examined the mRNA expression levels of 25 candidate genes in the epidermis of the wild type p50T and two *ok* mutant strains o21 (okusa translucent) and o22 (kinshiryu translucent) by RT-PCR. Eleven genes were excluded because results of the 35 RT-PCR cycles indicated that their mRNA was not expressed in the larval integument. The other 14 candidate genes were expressed in the integument. Among them, it is noteworthy that the mRNA expression levels of *P11* and *P12* were lower in the *ok* mutants, o21 and o22, compared with that in the wild type p50T, whereas the expression levels of the other 12 candidate genes were identical between the wild type and *ok* mutants. These data suggest that *P11* and *P12* are likely candidate genes responsible for the *ok* mutant.

Subsequently, I performed BLASTP searches of these and the neighbor genes against public protein databases in NCBI, and found that *P10*, *P11*, and *P12* were homologous to ATP-binding cassette (ABC) transporter genes. Using primers, RTokF1 and RTokR1, RT-PCR revealed that *P11* and *P12* expressed a single transcript encoding an ABC transporter, and that *P10* was an independent gene distinct from *P11* and *P12*. The detailed structures of *P11*, *P12*, and *P10* are described in sections 3.3 and 3.4. These data suggest that the ABC transporter gene corresponding to *P11* and *P12* is the gene that is most likely responsible for the *ok* mutants. Therefore, I designated this ABC transporter gene as *Bm-ok*, and analyzed its structure and function.

2.3.3 Structure of *Bm-ok* and its defects in the *ok* mutants

To clarify the structure of *Bm-ok*, I performed RACE experiments, which elucidated the full-length cDNA sequence, and the genomic structure of *Bm-ok* in the wild type p50T, and two *ok* mutant strains, o21 and o22, respectively. In the p50T strain, *Bm-ok* comprise 12 exons encoding a protein of 645 amino acid residues (AB780440). The encoded protein hypothetically possessed a single nucleotide-binding domain (NBD) containing the highly conserved Walker A and B, and Loop 3 regions, and a transmembrane domain (TMD) comprising six transmembrane helices. These results indicate that *Bm-ok* is a half-type ABC transporter (Ewart and Howells, 1998; Jones and George, 2004). A BLASTP search of the amino acid sequence against the public protein database revealed that *Bm-ok* was homologous to the well-known half-type ABC transporters, White, Scarlet, and Brown in *Drosophila*, as well as other ABC transporters of the “ABC-G” family.

In the *ok* mutant strain o21, the exon 3 sequence, which comprised 49 bp, was missing compared with the wild type (AB780441). Deletion of the mRNA sequence of *Bm-ok* mutants in o21 caused a frame-shift, generating a premature stop codon, which is predicted to result in the loss of NBD and TMD. In the *ok* mutant strain o22, two types of transcripts were detected by the RACE experiments and RT-PCR. The first, minor type, was identical to that of the wild type (AB780442), while the other, major type, exon 3 of 49 bp and exon 4 of 184 bp were duplicated in tandem (exon 3–exon 4–exon 3–exon 4; AB780443), which also resulted in a frame-shift and a premature stop codon. Similar to the case of o21, NBD and TMD were predicted to be lost in the amino acid sequence encoded by the major-type transcript in o22.

To investigate the reason for the abnormal *Bm-ok*-splicing in the two *ok* mutants, I compared the genomic sequences of *Bm-ok* in o21 and o22 with that of the wild type and found that the first base of intron 3 was changed from G to C in the o21 strain (AB780444), which violated the GT/AG rule (Breathnach and Chambon, 1981). This disturbance may cause inaccurate intron-3 splicing. In the o22 strain, I found that

approximately 3 kb genomic segment, including exons 3 and 4, was duplicated (AB780445), which may explain the occurrence of two types of *Bm-ok* transcripts in o22.

These predicted structural defects in the *Bm-ok* gene in two independent *ok* mutants led us to propose the hypothesis that *Bm-ok* was responsible for the mutations.

2.3.4 Identification of a second ABC transporter gene (*Bm-brown*) adjoining *Bm-ok*

The neighbor gene, *P10*, was juxtaposed to *Bm-ok* in a "tail-to-tail" manner. Its nucleotide sequence showed homology to *Bm-ok*, and also encoded a novel protein homologous to half-type ABC-transporters such as *Drosophila* White, Scarlet, and Brown. Its amino acid sequence contains a single NBD in the amino terminus followed by six putative transmembrane helices like *Bm-ok*, *Bmwh3*, and *Bm-w-2*. As explained in 3.6, *P10* was orthologous to *brown* in *Drosophila*, and accordingly designated as *Bm-brown*, which implied the *Bombyx mori* gene orthologous to *brown*. I compared the coding sequences (CDS) among p50T (AB780446), N4 (wild types; AB780447), o21 (AB780448), and o22 (AB780449). Seven amino acid sites were dimorphic between strains. The substitution specific to o21 was N309I in a non-conservative region. On the other hand, the o22-specific substitution, V557L, located in the transmembrane domain of *Bm-brown* (The transmembrane regions were predicted at <http://smart.embl-heidelberg.de/>). Because the two residues N309 and V557 were located in non-conserved regions, the possibility seemed to be low that their substitutions in o21 and o22 caused the functional defects of the *Bm-brown* protein.

2.3.5 RNAi of *Bm-ok*

To clarify whether *Bm-ok* is essential for the opacity of the larval integument, I performed an RNAi experiment. I injected *Bm-ok* siRNA into the eggs of N4 4–8 h after oviposition and injected *EGFP* siRNA as a negative control under the same conditions.

The results are shown. Similar to *ok* mutants at the same developmental stage, the integument of the hatched larvae from eggs injected with *Bm-ok* siRNA turned translucent within 2 days after hatching. In contrast, the eggs injected with *EGFP* siRNA after hatching did not display a transparent integument. To confirm that the phenotype induced by RNAi is caused by specific knockdown of *Bm-ok*, I determined the mRNA levels of *Bm-ok* and *P10* in the first-instar day 3 larvae from the eggs injected with *Bm-ok* siRNA. The expression level of *Bm-ok* mRNA decreased to approximately 25% after injecting *Bm-ok* siRNA compared with that after injecting *EGFP* siRNA. The expression level of *P10*, which also encodes an ABC transporter, was not affected by injection of *Bm-ok* or *EGFP* siRNAs. These results strongly support the hypothesis that *Bm-ok* is responsible for the *ok* mutants, and is essential for the formation of urate granules in the epidermis.

2.3.6 Homology search and phylogenetic analysis of Bm-ok among insects

To deduce the function and evolution of *Bm-ok*, I performed TBLASTN and BLASTP searches against the predicted proteins encoded in the genomes of 10 insects (*Bombyx mori*, *Danaus plexippus*, *Drosophila melanogaster*, *Anopheles gambiae*, *Culex quinquefasciatus*, *Apis mellifera*, *Bombus terrestris*, *Tribolium castaneum*, *Acyrtosiphon pisum*, and *Pediculus humanus corporis*) and a crustacean (*Daphnia pulex*), whose whole genomes have been analyzed. Using the amino acid sequence encoded by *Bm-ok* as the query, I searched for homologous sequences with the identity at the amino acid level of $\geq 25\%$ and identified 169 homologs from the 11 species. The overall amino acid sequences of these 169 homologs were used to construct a phylogenetic tree by the Neighbor-Joining method. The phylogenetic tree revealed that *Bm-ok* and *P10* formed a single cluster with other 45 homologs from 11 species, and that all 122 other homologs were placed outside this cluster. Subsequently, I aligned the amino acid sequences of 47 closely homologous proteins with *Bm-ok* using

CLUSTALW, and constructed a phylogenetic tree using two distantly-related “ABC-G” family transporters, *Drosophila* FBpp0077116 and *Bombyx* BGIBMGA005094-PA as the outgroups.

The phylogenetic tree depicts *Bombyx* P10 grouped with *Drosophila* Brown and Brown orthologs in other insects. Therefore, I named this protein Bm-brown. Bm-ok clustered with seven other ABC transporters, whose functions have not yet been established. I defined this group as the Bm-ok subfamily. As a result, the 47 homologs of Bm-ok were classified into four subfamilies, Brown, Bm-ok, Scarlet, and White. The phylogenetic tree also showed that White and Scarlet orthologs were found in all the investigated species, whereas Bm-brown and Bm-ok orthologs were absent in some insects. The *Bm-ok* ortholog was not found in the genomes of dipterans, *Drosophila*, *Culex*, and *Anopheles*, although the *Bm-brown* ortholog was present in these three genomes. In a hemipteran, *Acyrtosiphon*, four *Bm-ok* orthologs were present, whereas the *Bm-brown* ortholog was absent in this insect. In *Tribolium*, *Pediculus*, and *Daphnia*, neither the *Bm-brown* ortholog nor the *Bm-ok* ortholog were found.

Next, I compared the chromosomal positions of the orthologs of *Bm-ok*, *brown*, *scarlet*, and *white* among insect species. In *Bombyx*, *Bm-ok* and *Bm-brown* were juxtaposed in a tail-to-tail orientation on the nscaf 2529 sequence of chromosome 5. This pairwise arrangement of the *Bm-brown* ortholog and the *Bm-ok* ortholog was found not only in *Bombyx* but also in the lepidopteran, *Danaus* (scaffold DPSCF300006), and the hymenopterans, *Bombus* (chromosome 15) and *Apis* (chromosome 15). The juxtapositional localization of the genes is also observed between the orthologs of *white* and *scarlet* in *Bombyx* (Tatematsu et al., 2011), *Danaus*, *Tribolium*, *Apis*, and *Bombus*, although their orientations were not tail-to-tail but head-to-tail as 5'-*white-scarlet*-3'. In all the examined species, the “*Bm-ok-brown*” pair and “*white-scarlet*” pair were located on different chromosomes or different scaffolds.

2.3.7 Spatial expression pattern of *Bm-ok* mRNA

I used semi-quantitative RT-PCR to investigate the spatial expression profiles of the *Bm-ok* and *Bm-brown* mRNAs in various larval tissues of the day 3 fifth instar of p50T. Although the expression of *Bm-ok* and *Bm-brown* was detected in all examined tissues, *Bm-ok* was expressed most highly in the integument, where uric acid is accumulated. In contrast, *Bm-brown* mRNA was expressed most highly in the Malpighian tubules, and was slightly detected in the integument.

2.4 Discussion

In this study, I obtained a lot of evidence to support the hypothesis that a new gene, *Bm-ok*, is responsible for the *ok* mutants. First, the gene responsible for the *ok* mutant was genetically mapped within a 700-kb region. Second, abnormal *Bm-ok* splicing as a result of structural aberrations was detected in the two independent *ok* mutant strains o21 and o22. Both mutants code for incomplete, nonsense proteins. Third, the embryonic RNAi for *Bm-ok* successfully elicited the translucent larval phenotype similar to that of the *ok* mutants. Fourth, *Bm-ok* and its orthologs formed a new subfamily, which was distinct from known ABC transporters. Taken together, I conclude that *Bm-ok* is responsible for the *ok* mutants, and essential for the transport of uric acid in the silkworm epidermis.

Bm-ok encodes a protein belonging to the ABC-G family of ABC transporters, which includes *brown*, *scarlet*, and *white*. ABC transporters comprise a large protein superfamily whose members are transmembrane proteins utilizing energy from ATP, mainly to transport various substrates across membranes, and which contain highly conserved NBD and TMD. Metazoan ABC transporters are classified into six families, ABC-A, -B, -C, -D, -E/F, and G. The ABC-G family proteins are referred to as

“half-type” ABC transporters. The amino acid sequence of the ABC transporters comprises a single NBD in the amino terminus and a single TMD in the carboxyl terminus (Jones and George, 2004). In contrast, amino acid sequence of “full-type” ABC transporters comprises two NBDs and two TMDs, and these proteins function as monomeric transporters. In contrast, half-type ABC transporters often constitute homodimeric or heterodimeric functional transporters (Jones and George, 2004).

In *Drosophila*, Brown forms a heterodimer with White and functions in the transport of drosopterin, one of the pteridines, while White and Scarlet form a heterodimer that mediates transport of the ommochrome precursors in compound eyes (Dreesen et al., 1988; Ewart and Howells, 1998; Mackenzie et al., 2000). In contrast, it has been hypothesized that Bmwh3, the silkworm ortholog of *Drosophila* White and Bm-w-2, the ortholog of Scarlet, form a heterodimer that transports the ommochrome precursors in the eggs and compound eyes of *Bombyx mori* (Tatematsu et al., 2011). Previous studies have also reported that the molecular defect of Bmwh3 in a *w-3* mutant, which leads not only to white eggs and white compound eyes but also translucent larval skin (Guan et al., 2002; Komoto et al., 2009). These data indicate that Bmwh3 is a transporter involved in the transport of two structurally distinct chemicals, ommochrome precursors and uric acid (Komoto et al., 2009; Tatematsu et al., 2011). In addition, the larval skin of *w-2* mutant, which has a defect in the *Bm-w-2* gene, does not exhibit a translucent larval phenotype, indicating that *Bm-w-2* is not responsible for the transport of uric acid. These facts suggest that another partner molecule is required by Bmwh3 to transport uric acid. In the present study, I found that *Bm-ok* was responsible for the translucency of the larval integument. Therefore, it is plausible that Bm-ok and Bmwh3 form a heterodimer for the transport of uric acid in the larval epidermis. The eggs and adult compound eyes of the *ok* mutants have dark brown (wild type) coloration, suggesting that *Bm-ok* is not involved in the accumulation of ommochromes in the eggs and eyes. It should be noted that the pteridines do not contribute to the coloration of the eggs and

eyes in the wild type of *Bombyx mori* (Tazima, 1978). In addition, the previous studies reported that *Bmwh3* was involved in the process of pteridine formation in the silkworm (Tsujita and Sakurai, 1964a). In present study, I found that *Bm-brown* was orthologous to the *Drosophila* brown. So It is possibility that the heterodimer of *Bmwh3* and *Bm-brown* transport pteridine precursors in some tissues of the silkworm though the function of *Bm-brown* is unknown. Additional experimental analyses, such as functional reconstruction of heterodimers in the membrane of cultured cells, or investigation of physical interaction between the respective monomers, will help elucidate the biological properties of the transporter composed by *Bm-ok* and its putative partner protein *Bmwh3*.

In the silkworm, uric acid is mainly synthesized in the fat body, incorporated into epidermal cells via the hemolymph and accumulated as urate granules (Hayashi, 1960; Tamura and Sakate, 1983). The epidermal cells of the *ok* mutants contain very few or no urate granules due to deficiency in incorporation and storage of uric acid in the epidermal cells (Eguchi, 1961; Tamura and Sakate, 1983; Tamura and Akai, 1990). Moreover, it is known that the translucent larval phenotype of *ok* is expressed cell autonomously in the epidermis of the genetic mosaic individuals (Mine et al., 1983). Collectively these observations indicate that the *ok* gene is preferentially expressed in epidermal cells. *Bm-ok* mRNA is more highly expressed in the epidermis than in other larval tissues. Structural defects in the mRNAs of the two *ok* mutants result not only in the appearance of premature stop codons but also in the decrease of the mRNA content of the epidermis. This decrease of mRNAs in the *ok* mutants can be attributed to nonsense-mediated decay (NMD) of mRNA containing a premature stop codon (Kervestin and Jacobson, 2012). In summary, the expression of the *Bm-ok* mRNA correlates well with the function of the *ok* gene.

Phylogenetic analysis revealed that the *Drosophila* genome does not contain a gene orthologous to *Bm-ok*. Although the biological reason for this is unknown, it is worthy

of note that the larval epidermis of *Drosophila* does not accumulate uric acid to a great extent, and appears transparent. The phylogenetic relationship between *Bm-ok* and *brown* orthologs suggests that the ancestral gene of *Bm-ok* existed prior to the split between hemimetabolous and holometabolous insects, and that the *brown* orthologs may have differentiated from an ancestral *Bm-ok* gene. Most likely, orthologs of *Bm-ok* were lost before species diversification in the lineages of Diptera, Coleoptera, and Phthiraptera.

I also found that *Bm-brown* and *Bm-ok* were juxtaposed in a tail-to-tail orientation on chromosome 5 of the silkworm. This tandem arrangement is also seen in the orthologs of another lepidopteran, *Danaus*, and two hymenopteran insects, *Bombus* and *Apis*. Based on this data, and the fact that phylogenetic analysis suggests that the emergence of the *ok* orthologs was earlier than the *brown* orthologs, it can be reasonably speculated that the ancestral *brown* gene was acquired through duplication and subsequent differentiation of the ancestral *Bm-ok* gene. Tatematsu et al. (2011) have proposed that *scarlet* and *brown* originated from the duplication of an ancestral gene. I speculate that *Bm-ok* orthologs functionally differentiated into uric acid transporters after duplication of an ancestral *scarlet* gene, and that *brown* orthologs may have been generated by duplication of an ancestral *Bm-ok* gene. Further analysis of genomic data from more diverse species will improve the understanding of the evolutionary scenario of the closely related ABC transporter genes, i.e., the orthologs of *scarlet*, *white*, *Bm-ok*, and *brown*.

In this study, I reported that the ABC transporter, *Bm-ok*, is essential for transporting uric acid by genetic analyses of *ok*, one of the oily mutants of the silkworm. Uric acid has been considered as one of the basic substances contributing to larval pigmentation in many insects. Furthermore, uric acid contributes to body color as well as to the antioxidant activity in insects. The white decoration of the body surface derived from uric acid is not specific to silkworms but is generally observed in insects. The white

pigmentation in the *Anopheles* mosquito larvae is caused by the accumulation of uric acid and functions in protection against solar radiation (Benedict et al., 1996). Collectively these data suggest the general role of uric acid in insects. Because most intensive studies on the genetics of uric acid accumulation have been performed in the silkworm, further analyses of oily silkworm mutants will help us better understand the mechanisms of uric acid accumulation in insects.

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Chapter 3:

Note: I am planning to publish the content of Chapter 3 as an original article in an academic journal in a few years (at latest by April 9, 2018). To keep the originality of my research results until the submission to the journal, I do not publicize the content of this Chapter for the present.