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ORIGINAL PAPER

# A reexamination on the deficiency of riboflavin accumulation in Malpighian tubules in larval translucent mutants of the silkworm, *Bombyx mori*

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## Abstract

A variety of insects accumulate high contents of riboflavin (vitamin $B_2$) in their Malpighian tubules (MTs). Although this process is known to be genetically controlled, the mechanism is not known. In the 1940s and the 1950s, several studies showed that riboflavin contents were low in the MTs of some *Bombyx mori* (silkworm) mutants with translucent larval skin mutations (e.g., *w-3, od, oa*, and *otm*) and that genes responsible for these translucent mutations also affected riboflavin accumulation in the MTs. Since the 2000s, it has been shown that the *w-3* gene encodes an ABC transporter, whereas genes responsible for *od, oa*, and *otm* mutations encode for the biogenesis of lysosome-related organelles. These findings suggest that some genes of ABC transporters and biogenesis of lysosome-related organelles may control the accumulation of riboflavin in MTs. Therefore, we reexamined the effects that translucent mutations have on the accumulation of riboflavin in MTs by using the translucent and wild-type segregants in mutant strains to measure the specific effect that each gene has on riboflavin accumulation (independent of genomic background). We used nine translucent mutations (*w-3*$^{oe}$, *oa, od, otm, Obs, oy, or, oh*, and *obt*) even though the genes responsible for some of these mutations (*Obs, oy, or, oh*, and *obt*) have not yet been isolated. Through observation of larval MTs and measurements of riboflavin content using high-performance liquid chromatography, we found that the *oa, od, otm*, and *or* mutations were responsible for low contents of riboflavin in MTs, whereas the *Obs* and *oy* mutations did not affect riboflavin accumulation. This indicates that the molecular mechanism for riboflavin accumulation is similar but somewhat different than the mechanism responsible for uric acid accumulation in epidermal cells. We found that the genes responsible for *oa, od*, and *otm* mutations were consistent with those already established for uric acid accumulation in larval epidermis. This suggests that these three genes control riboflavin accumulation in MTs through a mechanism similar to that of uric acid accumulation, although we do not yet know why the *or* mutation also controls riboflavin accumulation.

**Keywords** *Bombyx mori* · Translucent mutation · Riboflavin accumulation · Malpighian tubules · ABC transporter · Lysosome-related organelle

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## Introduction

A variety of insects accumulate high contents of riboflavin (vitamin $B_2$) in their Malpighian tubules (MTs) (Ishihara 1956; Nickla 1972). The accumulated riboflavin makes MTs exhibit yellow colors (Ishihara 1958a, b). When MTs lack riboflavin, they exhibit white or light-yellow colors (Ishihara 1958c; Van Breugel 1987). The accumulated riboflavin in MTs is thought to contribute to the homeostasis of flavin adenine dinucleotide and flavin mononucleotide (Sang 1956; Nakamura et al. 1992), which play essential roles in a variety of physiological processes. Because riboflavin cannot be synthesized in metazoans (Bacher et al. 2000; Ladenstein et al. 2013; Tuan et al. 2014), it is mainly obtained from their food (Dadd 1985).

In the silkworm *Bombyx mori*, Koyanagi and Hatamura (1944) first reported that *od* translucent mutants contained little riboflavin in their MTs. In general, translucent mutations such as *od* cause a urate granule deficiency in the epidermal cells and subsequently lead to a striking color change in the larval epidermis, from an opaque white to translucent (Tamura and Akai 1990). Kikkawa (1948) reported that the MTs of the wild-type (WT) silkworms stored riboflavin as needle-shaped crystals, whereas MTs of some translucent mutants did not accumulate riboflavin. Thereafter, Aruga et al. (1952a, b) and Eguchi (1955, 1956) showed that the riboflavin contents in MTs and eggs of WT silkworms were greater than those of *od* translucent mutants. Later, based on his microscopic observations, Ishihara (1958a, b) reported that the needle-shaped granules (crystals) contained high concentrations of riboflavin in their WT MT cells, whereas such granules were absent from MTs of *od* mutants, suggesting that riboflavin accumulated as cytoplasmic "riboflavin granules" in them.

Ishihara (1958c) compared riboflavin accumulation in MTs among lepidopteran species and speculated that this process must be under genetic control. In addition, he found that several silkworm translucent mutants such as *w-3, w-3*$^{106}$, *w-3*$^{ol}$ (both *w-3*$^{106}$ and *w-3*$^{ol}$ are allelic to *w-3*), *oa, od*, and *odk* had very low contents of riboflavin in their MTs. However, his results also indicated that the quantity of uric acid in epidermal cells did not simply correlate with the accumulation of riboflavin in MTs. For example, the mutants *oc, ok*, and *os* contained larger contents of riboflavin than normal silkworms, although they accumulated few urate granules (Ishihara 1958c). Recently, Zhang et al. (2017) reported that another translucent mutant, *otm*, also possesses riboflavin-deficient MTs. Together, these results suggest that the mechanism for uric acid accumulation in epidermal cells is partly coupled with riboflavin accumulation in MTs. However, due to a lack of molecular information, researchers in the 1940s and the 1950s could not elucidate a mechanism for the accumulation of uric acid and riboflavin in MTs.

In this study, we reexamined the effects of translucent mutations on the accumulation of riboflavin in MTs of silkworms by measuring their riboflavin contents using high-performance liquid chromatography (HPLC). We used translucent and WT segregants of mutant strains to determine the independent effect of each translucent mutant gene (regardless of their genomic backgrounds).

## Materials and methods

### Silkworm strains

To examine the effects of translucent mutations on riboflavin accumulation in the MTs, we searched SilkwormBase (http://silkworm.nbrp.jp/), Kyushu University, for silkworm strains in which the translucent mutation genotypes segregated as WT (heterozygous) and translucent segregants (homozygous). Nine independent mutations were selected for study (Fig. 1; Fig. S1; Table S1). All silkworm larvae were raised with fresh mulberry leaves under a continuous 12/12 h light/dark cycle at 25 °C.

As shown in Fig. 1, in seven mutant strains, k32, w05, o90, t52, d50, w07, and r50, non-translucent (WT) and translucent larvae (*oa, otm, Obs, or, oh, obt*, and *oy*) are segregated, and the genetic backgrounds of both segregants are similar between the respective strains, except for the chromosome carrying the mutant gene. The sex-limited black-egg mutant strain (r01) has a fragment of chromosome 10 containing the +$^{w\text{-}3}$ gene translocated onto the W chromosome in the *w-3*$^{oe}$ mutant [T(W;10)+$^{w\text{-}3}$ *w-3*$^{oe}$]. Because female silkworms are heterogametic (ZW) and males are homogametic (ZZ) (Goldsmith et al. 2005), females exhibit WT egg color and larval translucency phenotypes, whereas males exhibit white egg serosa and translucent larval skin due to the *w-3*$^{oe}$ mutation. To obtain *od* segregants with a similar genetic background, we crossed the WT strain p50 (female) with the *od* mutant strain o06 (male). Because the *od* gene is located on the Z chromosome, the $F_1$ of p50×o06 exhibits two different larval skin phenotypes: WT (*od*/+, male) and translucent (*od*/W, female).

### HPLC analysis of riboflavin contents

We collected the MTs on the day just before spinning of the 5th instar because that is when normal silkworms usually store riboflavin at a high content in their MTs (Nakamura et al. 1992; Zhang et al. 2018). We then performed an HPLC analysis in MTs using the same methods described in Zhang et al. (2018).

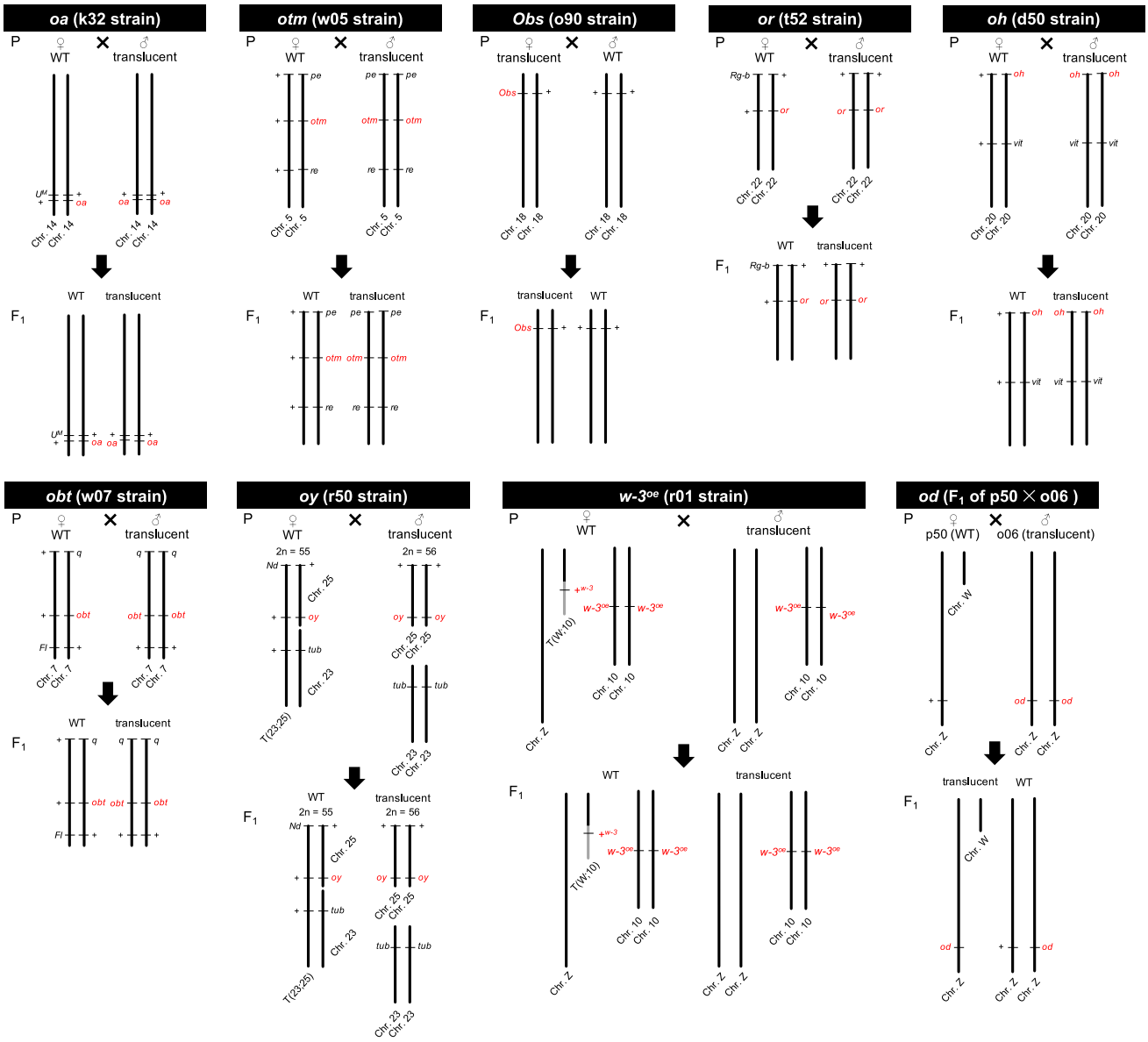


**Fig. 1** Genetic background and cross-schemes for the nine mutations used in this study. Detailed information on the respective genes is available at http://silkworm.nbrp.jp/ and Table S1

## Results

### Observation of MTs in various mutant strains

We investigated nine mutations (*oa, otm, Obs, or, oh, obt, oy, w-3$^{oe}$*, and *od*), using WT and translucent segregants of the k32, w05, o90, t52, d50, w07, r50, r01, and $F_1$ of p50×o06 (Fig. 1) strains, respectively, to show their effects on the accumulation of riboflavin in the MTs.

We were able to classify the nine mutations into three groups according to the phenotypic patterns of the MT color (Fig. 2). Group 1 included four mutations: *oa, od, otm*, and *or*. The translucent larval segregants (homozygotes) exhibited white MTs, suggesting a lack of riboflavin accumulation, whereas the non-translucent segregants (heterozygotes) exhibited normal yellow MTs. In group 1, larval skin translucency was highly correlated with MT whiteness.

Group 2 included two mutations: *Obs* and *oy*. The MTs of larvae with these mutations were yellow regardless of genotype and skin translucency. This suggests that *Obs* and *oy* do not affect riboflavin accumulation in the MTs.

Group 3 included three mutations: *w-3$^{oe}$*, *oh*, and *obt*. In strains with these mutations, MTs were white regardless of genotype and larval skin translucency. In homozygotic segregants, *oh*/*oh*, as well as heterozygotic ones, *oh*/+, MTs appeared white. Similarly, both homozygotic (*obt*/*obt*) and

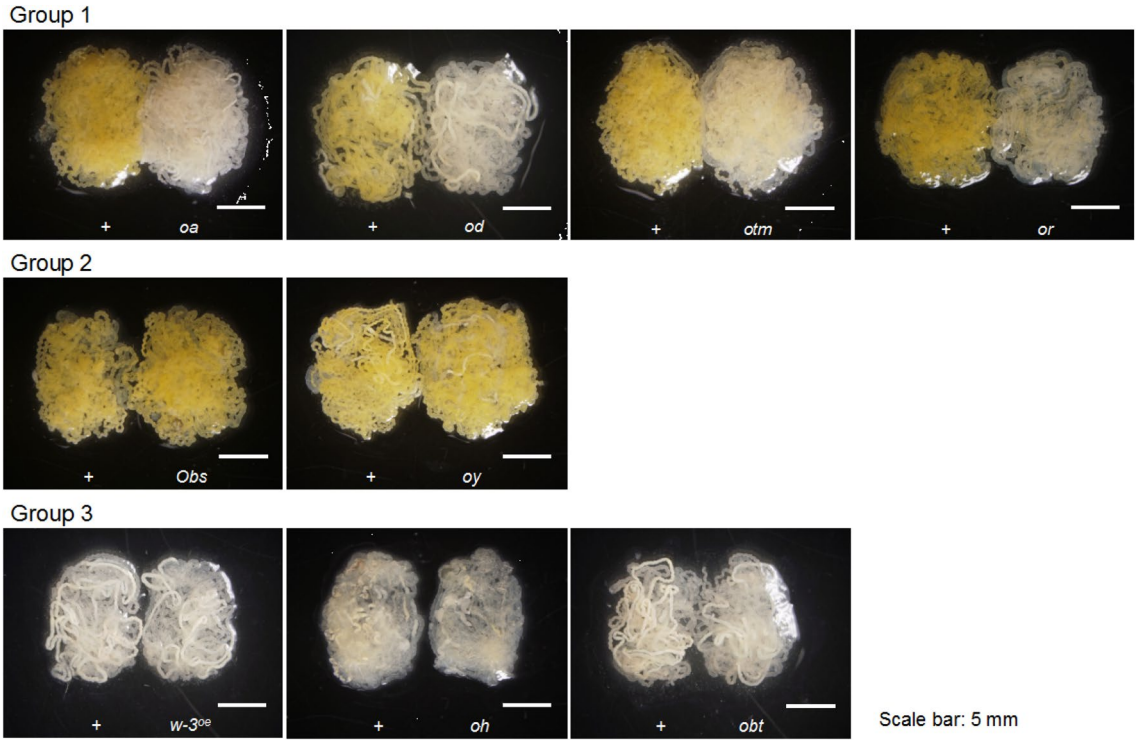


**Fig. 2** Malpighian tubules (MTs) from the WT and translucent segregants in nine mutant strains of silkworm. MTs were collected on the day just before the spinning of the 5th instar. + represents WT

heterozygotic (*obt*/+) segregants had white MTs. One possible explanation is that *oh* and *obt* have a dominant effect on MT whitening. Another explanation is that the genetic backgrounds of the d50 and w07 strains contain unknown mutations that affect riboflavin accumulation in MTs. Although T(W;10)+$^{w\text{-}3}$ *w-3*$^{oe}$/*w-3*$^{oe}$ females exhibit non-translucent larval skin, their MTs appeared white. The MTs of the *w-3*$^{oe}$/*w-3*$^{oe}$ males, whose larval skin is translucent, were also white. This indicates that the +$^{w\text{-}3}$ gene on the W chromosome does not complement the MT color phenotype in *w-3*$^{oe}$ though it is unclear whether the W-linked +$^{w\text{-}3}$ gene is insufficient for riboflavin accumulation, or another hidden mutation in this strain affects the phenotype.

## Riboflavin contents in larval MTs

The riboflavin contents of fresh MTs from each of the nine strains measured using HPLC are shown in Table 1 and Table S2. As with the MT color patterns described above, we were also able to classify the nine mutations into three groups (Table 1 and Table S2), according to the MT riboflavin contents.

**Table 1** Riboflavin contents of larval MTs. The average riboflavin content per fresh MTs (μg/g) is presented for WT and translucent segregants, respectively

| Group | Mutation | Strain | WT | Translucent |
|---|---|---|---|---|
| Group 1 (high vs. low) | *oa* | k32 | 1539.63 ± 144.59 | 89.63 ± 114.41 |
| | *od* | $F_1$ of p50 × o06 | 1292.13 ± 396.69 | 23.45 ± 3.89 |
| | *otm* | w05 | 972.01 ± 128.61 | 53.31 ± 20.92 |
| | *or* | t52 | 786.00 ± 272.14 | 40.04 ± 22.90 |
| Group 2 (high vs. high) | *Obs* | o90 | 1112.38 ± 433.87 | 1421.55 ± 439.26 |
| | *oy* | r50 | 675.51 ± 229.87 | 955.17 ± 328.06 |
| Group 3 (low vs. low) | *w-3* $^{oe}$ | r01 | 27.91 ± 3.49 | 27.44 ± 2.38 |
| | *oh* | d50 | 46.75 ± 9.33 | 40.00 ± 11.30 |
| | *obt* | w07 | 89.31 ± 20.48 | 51.22 ± 11.07 |

Values are means ± SD (n = 4). Detailed information is available in Table S2

In group 1 (*oa, od, otm*, and *or*), the MTs in WT (heterozygous) segregants had high riboflavin contents (ca. 800–1,500 µg/g), whereas the riboflavin contents of the translucent (homozygous) segregant MTs were ca. 20-fold lower. In group 1, the translucency of the larval skin was correlated with the riboflavin accumulation deficiency, suggesting that these mutations affect riboflavin accumulation in the MTs via a mechanism similar to that of uric acid accumulation in the epidermis.

In group 2 (*Obs* and *oy*), translucent as well as non-translucent (WT) segregants contained riboflavin in their MTs at the same relatively high level (ca. 700–1,400 µg/g). This indicates that *Obs* and *oy* have no effect on riboflavin accumulation as expected, based on visual observations.

In group 3 ($w\text{-}3^{oe}$, *oh*, and *obt*), the MTs of both non-translucent and translucent segregants had low riboflavin contents (30–90 µg/g) regardless of larval skin translucency. These results indicate that the $+^{oh}$ and $+^{obt}$ genes cannot rescue the MTs from riboflavin deficiency in strains d50 and w07, respectively. Also, the results indicate that the W-translocated $+^{w\text{-}3}$ gene cannot rescue it in $w\text{-}3^{oe}/w\text{-}3^{oe}$ in strain r01.

## Discussion

Previous studies have shown that epidermal translucency is partly coupled with riboflavin accumulation deficiency in the MTs (Koyanagi and Hatamura 1944; Kikkawa 1948; Aruga et al. 1952a, a, b; Eguchi 1955, 1956; Ishihara 1958a, b, c). Therefore, identification of the genes responsible for translucent mutations contributes to our understanding of the riboflavin accumulation mechanism. To date, 11 genes have been reported to be responsible for translucent mutations, and these are categorized according to three major processes resulting in translucency (Zhang et al. 2017): failure of uric acid biosynthesis (*oq*, xanthine dehydrogenase; *oya*, molybdenum cofactor (MoCo) synthesis-step 1 enzyme; and *og*, MoCo sulfurase) (Kômoto 2002; Fujii et al. 2016; Kômoto et al. 2003); failure of uric acid transport (*w-3*, ATP-binding cassette (ABC) transporter Bmwh3; *os*, solute carrier family OS; and *ok*, ABC transporter Bm-ok) (Kômoto et al. 2009; Kiuchi et al. 2011; Wang et al. 2013a); and failure of membrane trafficking and/or intracellular accumulation of uric acid in epidermal cells (*ow, od, oa, ov*, and *otm*, subunits of biogenesis of lysosome-related organelle complexes (BLOCs) and the adaptor protein-3 (AP-3) interactive proteins) (Ito et al. 2009; Fujii et al. 2010, 2012; Wang et al. 2013b; Zhang et al. 2017).

In the present study, the nine translucent mutations linked to riboflavin accumulation were categorized into three groups according to patterns of MT riboflavin phenotypes (Fig. 2; Table 1). In the first group, four genes (*oa, od, otm*, and *or*) clearly affect riboflavin accumulation, indicating a positive link between uric acid and riboflavin accumulation. The second group (*Obs* and *oy*) appears to have no effect on riboflavin accumulation. The third group ($w\text{-}3^{oe}$, *oh*, and *obt*) seems to affect riboflavin accumulation in both WT and translucent segregants.

In the first group, the genes for the *oa, od*, and *otm* mutations have been identified as *BmHPS5*, which encodes a subunit necessary for BLOC-2; *BmBLOS2*, which encodes a subunit of BLOC-1; and *Bm-muted*, which encodes another subunit of BLOC-1, respectively (Fujii et al. 2010, 2012; Zhang et al. 2017), whereas the gene for *or* is unknown. The genes for *oa, od*, and *otm* are known to play essential roles in uric acid accumulation at the final site, especially in the formation of urate granules in epidermal cells (Fujii et al. 2010, 2012; Zhang et al. 2017). The results of this study indicate that they also probably participate in the final riboflavin accumulation in MT cells. Lastly, although the gene responsible for the *or* mutation is unknown, its MT phenotype is identical to those of *oa, od*, and *otm*, suggesting that the causative *or* gene also plays a role in riboflavin accumulation in the MTs.

In the second group, the *Obs* and *oy* mutations had no significant effect on riboflavin accumulation (Table 1). Coincidentally, Ishihara (1958c) reported that *os, oc*, and *ok* mutations hardly affected riboflavin accumulation in the MTs. Because the genes for *os* and *ok* mutations have been reported to be involved in uric acid transport in larval epidermal cells (Kiuchi et al. 2011; Wang et al. 2013a), the results of this study indicate that *os* and *ok* specifically control uric acid accumulation in the epidermis. If the genes responsible for *Obs, oy*, and *oc* are identified, it will be possible to explain why they affect only uric acid accumulation.

In the third group, the *oh* and *obt* mutations appeared to affect riboflavin accumulation in both heterozygotes and homozygotes. A probable explanation for this is that *oh* and *obt* are likely to behave dominantly for riboflavin accumulation but recessively for epidermal translucency (Fig. 1; Table 1). Another possibility is the effects of their genetic backgrounds. Although the d50 strain carries the *vit* (*scanty of vitellin*) mutation linked to *oh*, the *vit* gene codes for the vitellogenin receptor (Lin et al. 2013) and may not affect riboflavin accumulation in the MTs (Fig. 1). Similarly, the w07 strain carries the *q* (*quail*) mutation linked to *obt*. However, the *q* gene may not affect the MT phenotype because it encodes a guanylyl cyclase gene, *BmGC-I*, which controls the pigmentation pattern of larval skin (Yuasa et al. 2016). Nevertheless, there is another possibility that the genetic backgrounds of d50 and w07 carry unknown genes affecting riboflavin accumulation in MTs, respectively. To clarify the effects of *oh* and *obt*, further genetic experiments are needed.

Furthermore, the effect of $w\text{-}3^{oe}$ should be evaluated thoroughly because we used a special strain, r01, which carries a

**Table 2** The translucent mutations examined by Ishihara (1958c) and in this paper

| Translucent mutation | Examined by Ishihara (1958c) | Examined in this paper | Riboflavin accumulation deficient | Responsible gene | Annotation |
|---|---|---|---|---|---|
| *os* | Y | | | *os* | Solute carrier protein (Kiuchi et al. 2011) |
| *ok* | Y | | | *Bm-ok* | ABC transporter (Wang et al. 2013a, b) |
| *og* | Y | | | *MoCoS* | Uric acid biosynthesis (Kômoto et al. 2003) |
| *oc* | Y | | | Unknown | |
| *odk* | Y | | Y | Unknown | |
| *w-3* | Y (*w-3, w-3*$^{106}$, and *w-3*$^{ol}$) | Y (*w-3*$^{oe}$) | Y | *Bmwh3* | ABC transporter (Abraham et al. 2000; Kômoto et al. 2009; Kobayashi et al. 2010) |
| *oa* | Y | Y | Y | *BmHPS5* | Urate granule formation (Fujii et al. 2012) |
| *od* | Y | Y | Y | *BmBLOS2* | Urate granule formation (Fujii et al. 2010) |
| *otm* | | Y | Y | *Bm-muted* | Urate granule formation (Zhang et al. 2017) |
| *or* | | Y | Y | Unknown | |
| *Obs* | | Y | | Unknown | |
| *oy* | | Y | | Unknown | |
| *oh* | | Y | Y | Unknown | |
| *obt* | | Y | Y | Unknown | |

Y: yes

translocated +$^{w-3}$ gene on the W chromosome (Fig. 1) with a *w-3*$^{oe}$/*w-3*$^{oe}$ background. One possible reason why the +$^{w-3}$ gene cannot rescue the riboflavin accumulation is that the translocated +$^{w-3}$ gene does not encode enough gene products to remedy the riboflavin deficiency even though it is sufficient to remedy epidermal translucency and ommochrome deficiency in the serosa in *w-3*$^{oe}$/*w-3*$^{oe}$. The gene for *w-3* has been identified as *Bmwh3* (Kômoto et al. 2009), which encodes a membrane-spanning ABC transporter involved in uric acid and ommochrome precursor transport (Tatematsu et al. 2011; Wang et al. 2013a). To date, three *w-3* alleles (*w-3, w-3*$^{106}$, *w-3*$^{ol}$) have been reported to affect riboflavin accumulation (Ishihara 1958c; Abraham et al. 2000; Kobayashi et al. 2010) in a genetically recessive manner. These observations suggest that *Bmwh3* controls riboflavin transport in the MT cells via a mechanism similar to that of uric acid and ommochrome precursor transport. Nevertheless, we cannot exclude another possibility that the genetic background of r01 carries a hidden gene affecting riboflavin accumulation in MTs.

Based on the results of both the present study and Ishihara's (1958c) study, we conclude that *odk, w-3, oa, od, otm, or, oh*, and *obt* mutations are involved in the accumulation of both uric acid and riboflavin in the silkworm, whereas *os, ok, og, oc, Obs*, and *oy* mutations function only in uric acid accumulation and do not affect riboflavin accumulation in the MTs (Table 2). This finding supports our hypothesis that the molecular mechanism of riboflavin accumulation bears some similarity to that of uric acid accumulation. Currently, the genes responsible for the *oc, odk, Obs, oy, or, oh*, and *obt* mutations are unknown. Uncovering the genes responsible for these mutations can be achieved by positional cloning. Further molecular biological studies of *odk, or, oh*, and *obt* mutations will provide a better understanding of the common mechanism in riboflavin accumulation and uric acid accumulation.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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