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Identification of the *Bombyx Red Egg* Gene Reveals Involvement of a Novel Transporter Family Gene in Late Steps of the Insect Ommochrome Biosynthesis Pathway*[S]◆

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Mizuko Osanai-Futahashi[‡1,2], Ken-ichiro Tatematsu[‡1], Kimiko Yamamoto[§], Junko Narukawa[§], Keiro Uchino[‡], Takumi Kayukawa[¶], Tetsuro Shinoda[¶], Yutaka Banno[‖], Toshiki Tamura[‡], and Hideki Sezutsu[‡]

From the [‡]Transgenic Silkworm Research Unit, the [§]Insect Genome Research Unit, and the [¶]Insect Growth Regulation Research Unit, National Institute of Agrobiological Sciences, Tsukuba, Ibaraki 305-8634, Japan and the [‖]Laboratory of Insect Genetic Resources, Faculty of Agriculture, Kyushu University, Fukuoka 812-8581, Japan

Background: Ommochromes are major pigments in insect eyes and eggs.
Results: Based on mutant analysis, a novel transporter family gene involved in egg/eye pigmentation was identified and characterized in *Bombyx* and *Tribolium*.
Conclusion: A novel transporter family gene was associated with insect ommochrome biosynthesis.
Significance: This study sheds light on the molecular mechanisms of the final ommochrome pigment biosynthesis.

Ommochromes are one of the major pigments involved in coloration of eggs, eyes, and body surface of insects. However, the molecular mechanisms of the final steps of ommochrome pigment synthesis have been largely unknown. The eggs of the silkworm *Bombyx mori* contain a mixture of ommochrome pigments, and exhibit a brownish lilac color. The recessive homozygous of egg and eye color mutant, *red egg* (*re*), whose eggs display a pale orange color instead of normal dark coloration, has been long suggested to have a defect in the biosynthesis of the final ommochrome pigments. Here, we identify the gene responsible for the *re* locus by positional cloning, mutant analysis, and RNAi experiments. In the *re* mutants, we found that a 541-bp transposable element is inserted into the ORF of *BGIBMGA003497-1* (*Bm-re*) encoding a novel member of a major facilitator superfamily transporter, causing disruption of the splicing of exon 9, resulting in two aberrant transcripts with frameshifts yielding nonfunctional proteins lacking the C-terminal transmembrane domains. *Bm-re* function in pigmentation was confirmed by embryonic RNAi experiments. Homologs of the *Bm-re* gene were found in all insect genomes sequenced at present, except for 12 sequenced *Drosophila* genomes, which seemed to correlate with the previous studies that have demonstrated that eye ommochrome composition is different from other insects in several Dipterans. Knockdown of the *Bm-re* homolog by RNAi in the red flour beetle *Tribolium castaneum* caused adult compound eye coloration defects, indicating a conserved role in ommochrome pigment biosynthesis at least among holometabolous insects.

Ommochromes are a major group of pigments that are widely distributed in eyes, eggs, and body surface in insects and several molluscs (1). Ommochrome pigments generally correspond to the black or brownish eye coloration and reddish coloration of epidermal and wing tissues of numerous insects. Ommochromes are divided into two groups, ommatins and ommins, according to their stability in alkali (2). Ommatins (*e.g.* xanthommatin) are labile in alkali, whereas ommins (*e.g.* ommin A) are stable in alkali. Ommatins are found in insect excreta, eyes, epidermis, and wings (1, 3), whereas ommins are found nearly ubiquitously in the insect eyes and also in epidermis of several insects such as cricket *Gryllus bimaculatus* (1, 4). In ommochrome biogenesis, tryptophan is converted to 3-hydroxykynurenine, which is then incorporated into pigment granules, where ommatins and ommins are hypothesized to be synthesized through oxidative condensation (5–7).

Several genes in the ommochrome synthesis pathway have been identified through eye color mutants of *Drosophila melanogaster*. Because of their easily recognized coloration, some of these genes have been used as molecular markers in *Drosophila* (8, 9). However, the findings are mainly restricted to the synthesis pathway from tryptophan to 3-hydroxykynurenine (*e.g. vermilion* and *cinnabar*) (10–12), incorporation of 3-hydroxykynurenine into pigment granules (*e.g. white* and *scarlet*) (13, 14), and pigment granule formation (*e.g. deep orange, garnet, light, carmine, carnation, lightoid, claret,* and *pink*) (15–24). Molecular mechanisms in the pathway after 3-hydroxykynurenine is incorporated into pigment granules are largely unknown.

In the silkworm *Bombyx mori*, eggs and adult compound eyes contain a combination of ommochrome pigments (1). The newly laid eggs are yellowish white, and start to color after around 40 h, turning into a brownish lilac color by 72 h (Fig. 1),

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[S] This article contains supplemental Figs. S1–S7 and Tables S1–S3.
The nucleotide sequences reported in this study has been submitted to DDBJ with accession numbers AB663084 and AB663085.
[1] Both authors contributed equally to this work.
[2] To whom correspondence should be addressed: Transgenic Silkworm Research Unit, National Institute of Agrobiological Sciences, 1-2 Owashi, Tsukuba, Ibaraki 305-8634, Japan. Tel.: 81-29-838-6091; Fax: 81-29-838-6091; E-mail: mfutahashi@nias.affrc.go.jp.

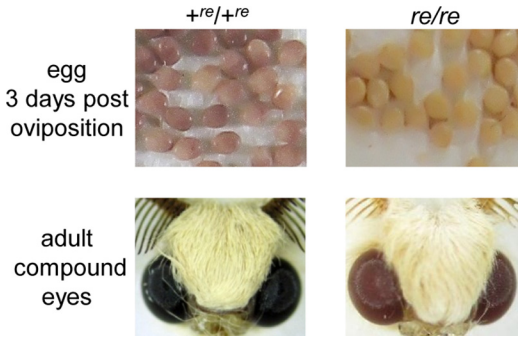

FIGURE 1. **Eggs (*top*) and compound eyes (*bottom*) of the silkworm, *B. mori*.** Wild-type ($+^{re}/+^{re}$), *left*, and *red egg* mutant (*re*/*re*, *bottom*) are shown. By 3 days post-oviposition, the egg serosa color of wild-type ($+^{re}/+^{re}$) becomes a brownish lilac color, whereas that of *re* mutants becomes a pale orange color. The adult compound eye color of wild-type is black, whereas that of the *re* mutant is dark red.

then growing darker with time (25). Several egg and eye color mutants have been found in *B. mori* (26). The gene responsible for three white egg and eye color mutants *w-1*, *w-2*, and *w-3* correspond to the orthologs of *cinnabar*, *scarlet*, and *white* of *Drosophila*, respectively (27–30), suggesting that genes involved in the early steps of the ommochrome synthesis pathway are conserved between *Bombyx* and *Drosophila*. Several other *Bombyx* egg and eye color mutants have been hypothesized to be involved in late steps of ommochrome synthesis pathway. One of them is the *red egg* (*re*),[3] a mutant characterized by crimson red eggs and dark red eyes found as a spontaneous mutant a century ago (31, 32). The *re* locus is mapped to position 31.7 of silkworm genetic linkage group 5 (33). The eggs of the recessive homozygote of the *re* mutant displays a pale orange color at 72 h post-laying, which darkens into crimson red with time (Fig. 1) (25, 32, 34). Similarly, the adult compound eyes of the *re* mutant are dark red instead of normal black (Fig. 1) (25, 32, 34). Whereas the egg/eye pigments of the wild-type silkworm contain both ommatin and ommins (1, 35, 36), those of the *re* silkworm contain only ommatin (xanthommatin or a related pigment) (36). In addition, the *re* locus acts downstream of all known silkworm egg/eye coloration loci according to genetical studies (31), which suggest that the *re* gene may be the missing link between 3-hydroxykynurenine and the final ommochrome pigments.

Here, we identify the gene corresponding to the *re* locus by positional cloning. We found an insertion of a transposable element in a novel gene (*Bm-re*) encoding a member of a major facilitator superfamily transporter in *re* mutants, and reproduced the *re* phenotype using RNAi with embryos. Furthermore, *Bm-re* gene homologs were widely found in insects and other organisms excepting *Drosophila*. In the red flour beetle *Tribolium castaneum*, gene knockdown of the *Bm-re* homolog also showed eye coloration defects, suggesting that the *Bm-re* gene homologs are conserved and widespread contributors to ommochrome biosynthesis in insects.

[3] The abbreviations used are: *re*, red egg; SNP, single nucleotide polymorphism; EGFP, enhanced green fluorescent protein; RNAi, RNA interference; dsRNA, double-stranded RNA; TMD, transmembrane domain; MFS, major facilitator superfamily.

EXPERIMENTAL PROCEDURES

Silkworm and Tribolium Strains—The *re* mutant strain e28 (hereafter referred to as re^{e28}) was provided from the silkworm stock center of Kyushu University supported by the National BioResource Project. The *re* mutant strain 911 (hereafter referred to as re^{911}) was provided from the National Institute of Agrobiological Sciences (NIAS, Kobuchizawa, Japan). The wild-type silkworm strain p50T was maintained in the Transgenic Silkworm Research Unit, NIAS, and wild-type silkworm strains C108 and pnd^{+} were maintained in the Genetic Resource Center, NIAS. Silkworms were reared with mulberry leaves or artificial diet (Nihon Nosan Kogyo, Yokohama, Japan) under a 16-h light, 8-h dark photoperiod at 25 °C. The wild-type strain of *T. castaneum* was provided by the National Food Research Institute, Tsukuba, and raised on whole wheat flour at 30 °C.

Positional Cloning of re—For recombination mapping, six F1 heterozygous males obtained from a single-pair cross between a C108 wild-type female and a re^{911} mutant male were each backcrossed to a re^{911} mutant female. 396 BC1 eggs (282 normally pigmented eggs, and 114 red eggs) were used for analysis. Genomic DNA was extracted from parent moths, F1 moths, and each BC1 neonate larva using DNAzol solution (Invitrogen). For genetic analysis, 18 SNP markers previously reported on chromosome 5 (37), and 21 new SNP markers were used. The primers for the SNP markers used in the linkage analysis are listed in supplemental Table S1.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Rapid Amplification of cDNA Ends (RACE)—Total RNA was isolated from the silkworm *B. mori* eggs at 0, 24, 48, and 72 h after laying from wild-type strains (p50T, C108), two mutant strains (re^{911} and re^{e28}), and the pupae of the red flour beetle *T. castaneum* 1 day before adult eclosion using ISOGEN (Nippon Gene), and reverse transcribed with a random primer (N6) using the first-strand cDNA synthesis kit (GE Healthcare) as previously described (38). PCR conditions were as follows: 35–40 cycles of 94 for 30 s, 58 for 30 s, and 72 for 30 s. The identity of each RT-PCR product was confirmed by DNA sequencing. Primers used for PCR are listed in supplemental Table S2. The gene for *ribosomal protein L3* (*rpL3*) of *B. mori* was used as an internal control for normalization of equal sample loading. The full-length cDNA was obtained by the RACE technique using a Marathon cDNA amplification kit (Clontech).

Genomic PCR—Genomic DNA was extracted from silkworm adult legs using the DNeasy Blood & Tissue Kit (Qiagen). PCR was conducted using primers 3497N-F10 and 3497-intron-R1 (supplemental Table S2).

Phylogenetic Analysis—To investigate whether *re* orthologs were found in species other than *B. mori*, we performed phylogenetic analysis using genes included in the Blastp hits ($p < e^{-20}$). We aligned the sequences using Clustal W, and constructed phylogenetic trees by the neighbor-joining method with the MEGA5 program (39). The confidence of the various phylogenetic lineages was assessed by bootstrap analysis.

RNAi Experiments—We prepared template DNA fragments to synthesize double-stranded RNAs (dsRNAs) as follows. For

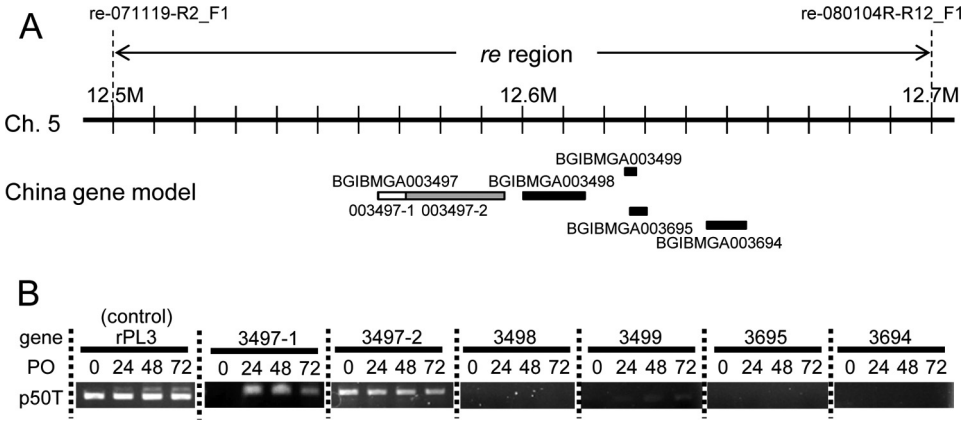

FIGURE 2. **Mapping of the *re* gene.** *A*, physical map of the *re* region. The *re* region was narrowed within the ~202-kb region between SNP markers re-071119-R2_F1 and re-080104R-R12_F1 of chromosome 5. Five genes were predicted inside the *re* region by the gene model. *BGIBMGA003497* was misannotated and was actually two genes, which we designated as *BGIBMGA003497-1* and *BGIBMGA003497-2*. *BGIBMGA003497-1* is indicated by an *open box*, *BGIBMGA003497-2* is indicated by a *gray box*. *B*, expression analysis of genes in the *re* locus. *p50T*, wild-type strain. *PO*, hours post-oviposition. *Ribosomal protein L3* (*rPL3*) was amplified as a positive control.

Bm-re and *Tc-re*, first, 916- and 713-bp fragments were amplified with primer sets 3497N-F5/3497N-R1 and Tc-re-F1/Tc-re-R2, respectively, using cDNA synthesized from total RNA extracted from *B. mori* or *T. castaneum* pupae. The resulting DNA fragments were subcloned into pGem-T Easy vector (Promega), and confirmed by sequencing. Next, the subcloned fragments were amplified by vector-specific primers containing a T7 polymerase site at the 5′ end. For *EGFP*, a 520-bp fragment was amplified by PCR with gene-specific primers containing T7 polymerase sites using the plasmid pBac-UAS 3xP3GFP (40) as a template. The resulting PCR products were purified and submitted to dsRNA synthesis using a MEGAscript RNAi Kit (Ambion) according to the manufacturer's instructions. The dsRNA solutions were diluted to 500 ng/μl in injection buffer (0.5 mM phosphate buffer (pH 7.0), 5 mM KCl). For performing RNAi with *B. mori* embryos, 2–3 nl was injected into eggs of the wild-type strain (*pnd*+) at the preblastoderm stage as described previously (28), and followed by incubation at 25 °C in a moist Petri dish. To perform RNAi using *T. castaneum*, ~170 nl of dsRNA was injected into the abdomens of last instar larvae as described previously (41). After injection, animals were raised individually in 24-well microtiter plates with whole wheat flour at 30 °C. To perform quantitative RT-PCR analysis, total RNA from a single *B. mori* egg injected with dsRNA was extracted at 60 h after laying using ISOGEN (Nippon Gene), or total RNA from a single *T. castaneum* day 4 pupa injected with dsRNA at larval stage was extracted, and used as a template for the synthesis of cDNA with a first-strand cDNA synthesis kit (GE healthcare). The expression level of *Bm-re* mRNA and *Tc-re* mRNA was quantified using a Light-Cycler 1.5 (Roche Applied Science). The *B. mori rpL3* gene and *T. castaneum Rp49* gene were analyzed to normalize transcript levels. Quantitative PCR was performed in duplicate, and melting curve analysis was performed on all reactions to ensure homogeneity of the amplified PCR products. The *p* values were obtained using a Student's *t* test ($n = 4$ and 3 for *B. mori* and *T. castaneum*). Primers used for dsRNA template synthesis and quantitative PCR are listed in supplemental Table S2.

Thin Layer Chromatography of Silkworm Egg Pigments—Egg pigment was extracted by a modified protocol described previously (36). About 30 eggs 72 h post-oviposition were collected, crushed in acetone, and washed in methanol twice, and extracted with 60 μl of 0.5% hydrochloric acid in methanol. After centrifugation, the supernatant was collected, reduced by adding 5 μl of 1% ascorbic acid (Sigma). Thin layer chromatography was performed by spotting 30-μl aliquots of the reduced egg pigment extract onto pre-coated silica gel plates (60 F254, Merck). They were developed using the upper layer of collidine/lutidine/water (1:1:2, v/v) as solvent (42, 43). The TLC images were scanned with EPSON LP-M5000A and analyzed by Adobe Photoshop elements software.

RESULTS

Positional Cloning of re Locus—To identify a candidate region for the *re* gene, we performed a genetic linkage analysis based on a high resolution SNP linkage map (37, 44) and information from the silkworm genome sequence (supplemental Table S1). For linkage analysis, we obtained F1 heterozygous males between a re^{911} (an *re* strain) male and a C108 (a wild-type egg colored strain) female that we backcrossed 6 individuals with re^{911} mutant females to obtain BC1 eggs. By analyzing the SNPs of 396 BC1 eggs (282 normally pigmented eggs and 114 red eggs), we narrowed the region responsible for the *re* phenotype to within ~202 kb on chromosome 5 (Fig. 2*A*, supplemental Fig. S1, and supplemental Table S1). Within this region, five genes, *BGIBMGA003497*, *BGIBMGA003498*, *BGIBMGA003499*, *BGIBMGA003694*, and *BGIBMGA003695* were predicted by the gene prediction model (Fig. 2*A*) (45). From a blastx search, we found that *BGIBMGA003694* and *BGIBMGA003695* had homology to retinol dehydratase (CG6704 in *D. melanogaster*), *BGIBMGA003498* to agrin (CG32354 in *D. melanogaster*), and *BGIBMGA003499* had no significant similarity with known proteins. For the gene annotated as *BGIBMGA003497*, we found that the 3′ region of this annotated gene had sequence similarity with a chromodomain helicase-DNA-binding protein, but its predicted 5′ region encoded a major facilitator superfamily (MFS) transporter. This suggested that the gene originally annotated as *BGIBMGA003497* actually consisted of two different genes. Thus, we designated the 5′ portion of *BGIBMGA003497* as *BGIB-*

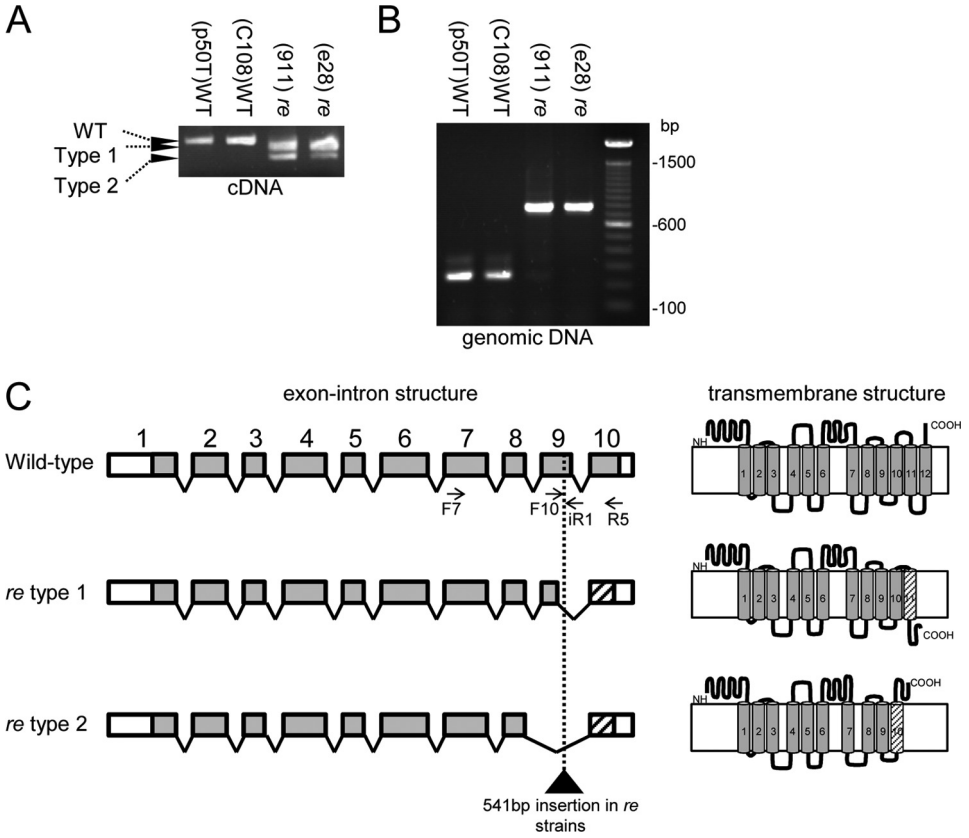

FIGURE 3. **Comparison of *BGIBMGA003497-1* among wild-type (*WT*) and *re* mutants.** *A*, RT-PCR of *BGIBMGA003497-1* by primers F7 and R5 (*C*). Two smaller sized products (*type 1* and *type 2*) were amplified for *re* strains (911 and e28). *B*, genomic PCR of *BGIBMGA003497-1* with F10 and iR1 primers (*C*). Larger sized products were amplified for *re* strains (911 and e28). DNA size markers are shown on the *right*. *C*, comparison of gene structure among wild-type and *re* mutants. *Left*, exon-intron structure of *BGIBMGA003497-1*. In *re* type 1, the splice donor site of exon 9 was located 5′ to the original site. In *re* type 2, exon 9 was completely skipped. The missing sequences caused frameshifts in both *re* types 1 and 2, thus the coding frame of exon 10 were both different from wild-type. *Solid boxes*, open reading frames; *open boxes*, untranslated regions; *striped boxes*, aberrant open reading frames; *diagonal lines*, introns. e28 and 911 both had identical 541-bp insertions (*solid triangle*) in exon 9. Primers used in *A* and *B* are depicted by *arrows*. *Right*, transmembrane domain predictions of *BGIBMGA003497-1* for wild-type and *re* mutants. In the *re* mutants, the amino acid sequences differed from the wild-type in the 10th or 11th transmembrane domain. Transmembrane domains with different amino acid sequences from wild-type are *striped*.

MGA003497-1 and the 3′ portion as *BGIBMGA003497-2*, which brought the number of genes within the *re* candidate region to six.

Expression Analysis of Six Candidate Genes in re Locus—Because none of the six genes in the candidate region were homologs of previously characterized ommochrome-related genes, we next examined their expression patterns in early embryos. Because the eggs start to color from around 40 h after oviposition, we performed RT-PCR with total RNA extracted from eggs of a wild-type strain (p50T) at 0, 24, 48, and 72 h after laying (Fig. 2*B*). Among the 6 candidate genes, *BGIBMGA003498*, *BGIBMGA003694*, and *BGIBMGA003695* did not give rise to any bands after 40 cycles of PCR. Moreover, the expression level of *BGIBMGA003499* was very low, and *BGIBMGA003497-2* was expressed in all samples. Interestingly, the expression level of *BGIBMGA003497-1* increased at 24 h, which preceded the timing of pigmentation of the egg, and persisted at 48 and 72 h. We next conducted RT-PCR with cDNAs of two *re* strains, re^{911}, the strain used for linkage analysis, and re^{e28}, a strain preserved in Kyushu University. The expression patterns for all six candidate genes were nearly identical between wild-type and *re* strains. Importantly, the amplified size of *BGIBMGA003497-1* was different between wild-type strains and *re* strains (Fig. 3*A*). In both re^{911} and re^{e28} strains, no products of the same size as wild-type were amplified, but two smaller sized products were observed. This suggested that the abnormality of the *BGIBMGA003497-1* transcript was a strong candidate for the cause of the *re* phenotype.

Analysis of BGIBMGA003497-1 Transcripts and Genomic Structure in re Mutants—We determined the complete cDNA sequences of the *BGIBMGA003497-1* genes amplified by RT-PCR and RACE from the two wild-type strains (p50T and C108) and two *re* strains (re^{911} and re^{e28}). In both wild-type strains, the *BGIBMGA003497-1* cDNA encoded a 494-amino acid protein (accession number AB663084). In both *re* strains, two types of cDNA were detected (supplemental Fig. S2). In both types of transcripts, 76 (type 1) and 151 bp (type 2) were missing from exon 9, both causing frameshifts in exon 10 (Fig. 3*C*, *striped boxes*, and supplemental Fig. S2). Comparison with the published genomic sequence indicated that the splice donor site in exon 9 was located 5′ of the original splice donor site in the type 1 transcript, and that exon 9 was entirely skipped in the type 2 transcript (Fig. 3*C*). Type 1 encoded a 459-amino acid protein, and type 2 encoded a 434-amino acid protein. The wild-type protein was predicted to have a 12-pass transmembrane protein by the SOSUI (57) program (Fig. 3*C*). The frameshifts in the

TABLE 1
Summary of embryonic RNAi experiment for *Bm-re* (*BGIBMGA003497-1*)

	Bm-re dsRNA	*EGFP* dsRNA
	500 ng/μl	
Number of injected eggs	134	66
Number of eggs with *re* color	110	0
Number of eggs with WT color	24	66

predicted type 1 and 2 proteins were located in the 11th and 10th transmembrane domains (TMDs), respectively, yielding only 11 and 10 TMDs (Fig. 3*C*).

To investigate the cause of missplicing in *BGIBMGA003497-1* transcripts of the *re* mutants, we analyzed the genomic sequence of both wild-type and *re* mutant strains. In exon 9, re^{e28} had a 541-bp insertion, which had high sequence similarity to bm_1218, a repetitive sequence in the *Bombyx* genome (46) (Fig. 3, *B* and *C*, and supplemental Fig. S3). Although it did not have sequence similarity to known proteins encoded by mobile elements, we found a 2-bp target site duplication (supplemental Fig. S3, *yellow boxes*, *TA*), suggesting that the insertion sequence was a nonautonomous transposable element such as a SINE (short interspersed nuclear element) or a MITE (miniature inverted-repeat transposable element). re^{911} had an identical insertion as re^{e28} (supplemental Fig. S3), thus indicating both strains had the same origin.

Knockdown of BGIBMGA003497-1 Gene by Embryonic RNAi—To test whether the *BGIBMGA003497-1* gene was essential for normal egg pigmentation, we performed RNAi experiments using embryos. We injected double strand RNA corresponding to a 916-bp portion of the *BGIBMGA003497-1* gene (supplemental Fig. S4) into wild-type eggs 5 to 8 h after laying. Details of the *BGIBMGA003497-1* RNAi results are shown in Table 1. As shown in Fig. 4, eggs injected with *BGIBMGA003497-1* dsRNA failed to take on the normal brownish lilac color, but became a pale orange color like the eggs of the *re* mutant (see Fig. 1*A*). In contrast, eggs injected with *EGFP* dsRNA became a normal brownish lilac color (Fig. 4). This result clearly showed that the defect of the *BGIBMGA003497-1* gene was responsible for the *re* phenotype, and that it functioned in the ommochrome biosynthesis pathway of *B. mori*. Furthermore, thin layer chromatography of the egg pigment extracts separated a purple-red pigment presumably representing an ommin from wild-type egg pigments, which was absent in both *re* eggs and *BGIBMGA003497-1* dsRNA-injected eggs (supplemental Fig. S5, *red arrow*). Thus, we designated the *BGIBMGA003497-1* gene as *Bm-re*.

Homologs of Bm-re Gene Among Other Organisms—As described above, the *Bm-re* gene has sequence similarity with a MFS transporter. They contain 12 or 14 TMDs, and a well conserved MFS-specific motif (G*X*3(D/E)(R/K)*X*G)*X*((R/K)(R/K)) located between TMD2 and TMD3 (47), which was also conserved in the *Bm-re* gene (supplemental Fig. S6, *red asterisks*). From a search among other insects and organisms, we found the *Bm-re* homologs ($p < e^{-20}$) from vertebrates to plants (supplemental Table S3 and Fig. S7), but none of these genes were previously characterized. Genes with significant sequence similarity to the *Bm-re* gene were found in *Pediculus*, *Acrythosiphon*, *Camponotus*, *Solenopsis*, *Harpegnathos*, *Apis*, *Nasonia*, *Tribolium*, *Anopheles*, *Culex*, and *Aedes* (supplemental Table S3 and Fig. S7), but notably, could not be found in any of the 12 sequenced *Drosophilidae* species. Phylogenetic analyses indicated that many insects had two homologs and one gene from each insect species clustered with the *Bm-re* gene (Fig. 5, supplemental Fig. S7).

Knockdown of Tribolium re Ortholog by RNAi—As with many other insects, the pigments of the adult compound eye of the red flour beetle *T. castaneum* contains ommochromes (48). We cloned the *Bm-re* homolog of *T. castaneum* (*Tc-re*, AB663085, supplemental Fig. S4) using the cDNA of *T. castaneum* pupae and the predicted gene sequence TcasGA2_TC013631 (EFA11449.1) as reference. The cloned sequence encoded a 457-amino acid protein and was identical to TcasGA2_TC013631 except lacking a 12-bp (993–1004) portion and a 57-bp (1114–1170) portion. To investigate if the *Tc-re* functions in eye pigmentation as observed in the *re* mutant of *B. mori*, we injected dsRNA corresponding to a 713-bp portion of the *Tc-re* ORF (supplemental Fig. S4) into final instar larvae. As shown in Fig. 6, all of the compound eyes of the newly molted adults injected with *Tc-re* dsRNA became light brown ($n = 15$), whereas those of newly molted adults injected with control *EGFP* dsRNA became black like wild-type insects ($n = 10$). These results indicated that the *Tc-re* gene also functioned in eye pigmentation and ommochrome biosynthesis in *T. castaneum* like in *B. mori*.

DISCUSSION

In this article, we found that the defect in a novel MFS transporter family gene *Bm-re* is responsible for the *red egg* mutation of the silkworm, *B. mori*. This is the first report of a gene involved in ommochrome biosynthesis after the incorporation of 3-hydroxykynurenine into the pigment granules. Similar results from a RNAi experiment in *T. castaneum* highlighted its conserved biological function across diverse insect taxa.

Putative Role of re Gene in Ommochrome Biosynthesis in Insects—MFS transporters have been identified in all organisms from bacteria to mammals. Whereas ABC transporters are in general multicomponent transporters capable of transporting both small molecules and macromolecules, MFS transporters are single-polypeptide transporters capable of transporting only small solutes, such as amino acids, sugars, drugs, nucleosides, vitamins, and organic acids (47).

How exactly does the *Bm-re* gene contribute to ommochrome synthesis? The pigments of the *B. mori* eggs and eyes are reported to be a mixture of several ommochrome pigments. Kawase and Aruga (36) reported that three pigments, named chrome-I, -II, and -III, were isolated by paper chromatography from extracts of wild-type silkworm eggs and eyes. In contrast to wild-type, only chrome-I was isolated from *re* strains (36). This is consistent with our TLC results indicating that *re* eggs and *Bm-re* RNAi eggs have at least one missing pigment. The pigment present in *re* (chrome-I) has been assumed to be xanthommatin (1, 36), or a substance whose degradation products include xanthommatin but not organic sulfate (49). Chrome-II and -III are assumed to be ommins (1). From the similarity of the UV spectra, it is likely that chrome-III is ommin A, which is

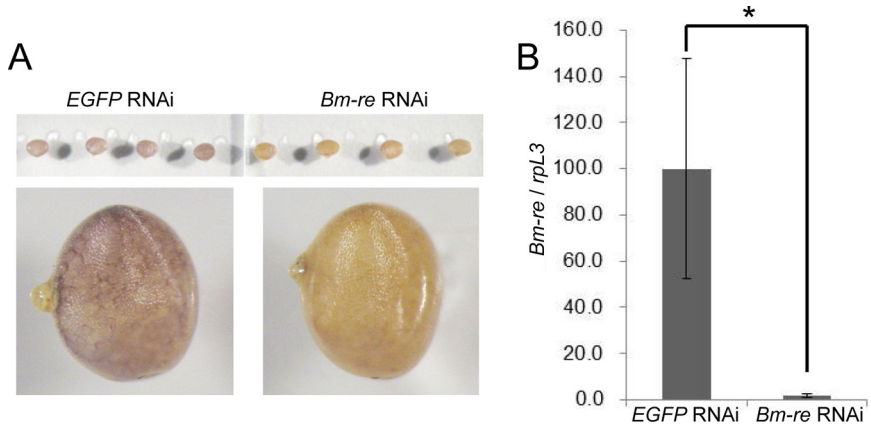

FIGURE 4. **Knockdown of the *B. mori re* gene (*Bm-re, BGIBMGA003497-1*) in silkworm eggs by RNAi causes *re* phenotype.** *A*, dsRNA for *BGIBMGA003497-1* (*right*) or *EGFP* (*left*) was injected into eggs at 5 h post-oviposition, and incubated at 25 °C. The injected eggs were photographed after 6 days. Magnified images are shown at the *bottom*. *B*, RT-PCR analysis of *Bm-re* RNAi eggs. Total RNA was extracted from the eggs at 60 h post-laying and used as template. The expression levels of *Bm-re* were normalized to *rpL3*, and the value for *EGFP* RNAi was designated as 100. The *bars* indicate mean ± S.D. of mRNA expression ($n = 4$). *, $p < 0.05$ (*t* test).

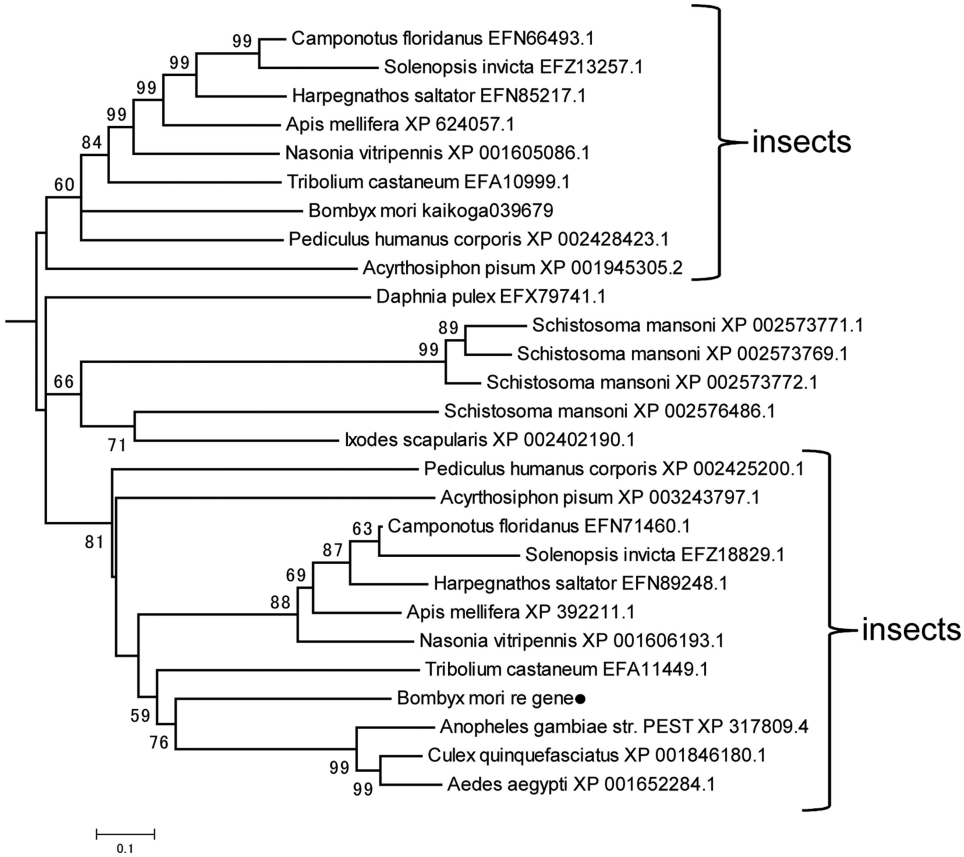

FIGURE 5. **Neighbor-joining tree of the *Bm-re* gene and related insect genes based on their amino acid sequences.** The *Bm-re* gene is indicated by a *black circle* to the *right*. A phylogenetic tree including *Bm-re*-related genes of plants and vertebrates is shown in supplemental Fig. S7.

reported to be detected in the wild-type eggs and eyes of the silkworm (50). Ommin A has a chemical formula of $C_{30}H_{27}N_5O_{10}S$, and from incorporation experiments of ^{35}S-labeled precursors, it is indicated that its sulfur is derived from cysteine or methionine, but not from sulfate and sulfide (4). These studies suggest that a key difference between the pigments of wild-type and *re* is the presence of sulfur derived from cysteine or methionine, and the *Bm-re* gene may function in transporting these amino acids into the pigment granules (Fig. 7).

MFS family genes related to *Bm-re* were found in a wide range of species including plants and vertebrates (supplemental Table S3 and Fig. S7). Some species including insects had more than one gene. The defect in eye pigmentation caused by *Tc-re* RNAi suggested that insect genes monophyletic to *Bm-re* function in ommochrome biosynthesis. Ommochromes are distributed mainly among arthropods and cephalopoda, but not reported in Chordata and plants at present. Thus, genes paraphyletic to *Bm-re* may function in processes other than ommochrome biosynthesis.

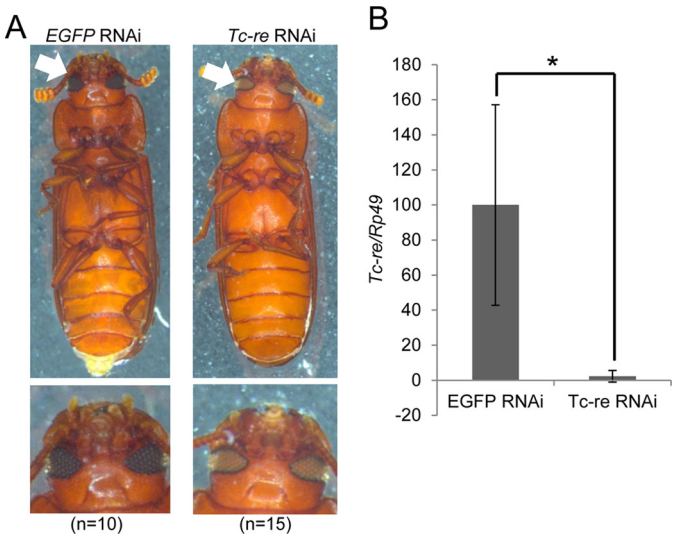

FIGURE 6. **Knockdown of a *re* homolog by RNAi in *T. castaneum* causes eye pigmentation defects.** *A,* last instar larvae were injected with ~170 nl of dsRNA (500 ng/μl) for the *re* homolog in *T. castaneum* (*Tc-re*) (*right*) or *EGFP* (*left*). *Top panel*, whole body of the dsRNA-treated individuals at the adult stage. Compound eyes are indicated by *white arrows*. *Bottom panel*, magnified images of the heads. *B,* RT-PCR analysis of *Tc-re* RNAi pupae. Total RNA was extracted at 4th day of pupal stage and used as template. The expression levels of *Tc-re* were normalized to *TcRp49*, and the value for *EGFP* RNAi was designated as 100. The *bars* indicate mean ± S.D. of mRNA expression ($n = 3$). *, $p < 0.05$ (*t* test).

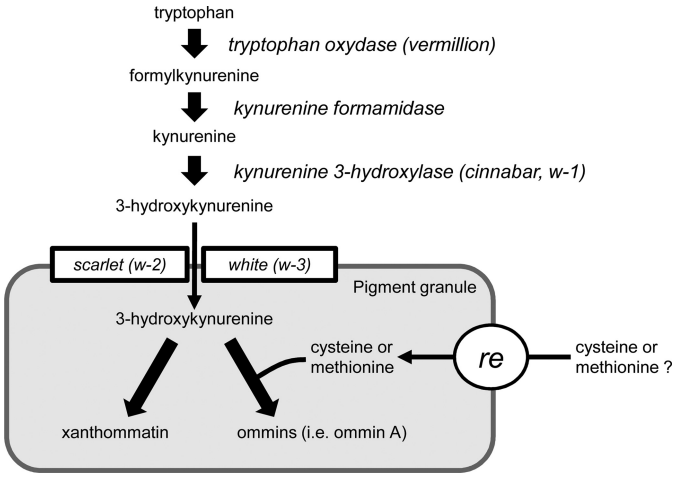

FIGURE 7. **Model of the ommochrome biosynthesis pathway in *Bombyx* egg pigmentation.** *Drosophila* and *Bombyx* mutant gene names for the enzymes are in *brackets*. Tryptophan is converted to formylkynurenine by *tryptophan oxydase* (*vermillion*), formylkynurenine is converted to kynurenine by *kynurenine formamidase*, kynurenine is converted into 3-hydroxykynurenine by *kynurenine 3-hydroxylase* (*cinnabar*, *w-1*). 3-Hydroxykynurenine is incorporated into pigment granules of the egg by the heterodimer of ABC transporters *scarlet* (*w-2*) and *white* (*w-3*). Ommochrome pigments (*e.g.* ommins and xanthommatin) are synthesized from 3-hydroxykynurenine in the pigment granules by oxidative condensation. The ommochrome pigments of *Bombyx* eggs are reported to be a mixture of several pigments, including ommin A (36). The *re* gene may function in incorporating other ommin precursors such as cysteine or methionine.

Relationships between re Gene and Ommochrome Pigments in Insects—In this study, we found *Bm-re* gene homologs in all insect species with sequenced genomes except for *Drosophila*. Because a *Bm-re* homolog is found in mosquito species, the *re* gene seems to have been lost in the course of speciation in dipterans. From the results of RNAi experiments, we found that the *Tc-re* gene also functioned in pigment synthesis in adult compound eyes of *Tribolium*, which is estimated to have diverged from *B. mori* more than 270 million years ago (51), suggesting that the *Bm-re* homolog may function in ommochrome synthesis in many insect species. These findings are consistent with previous studies indicating that Ommin A is present ubiquitously in insect eyes except for some Diptera and Orthoptera (1). It has been reported that all investigated species of Cyclorrhapha (including *Drosophila*) lack ommin in the compound eyes and xanthommatin appears to be the only ommochrome pigment present. The loss of the *re* gene may be the cause of loss of ommins in the eyes of the Cyclorrhapha species.

Possible Usage of re Gene as Transgenic Marker—The distinct egg color of *re* and independence from the maternal genotype suggest the potential of a *Bm-re* gene as a visible transgenic marker. Currently, a 3xP3-*EGFP* marker, which drives expression of green florescence protein by a 3xP3 promoter in the eyes (52, 53), and a *KMO* (*kynurenine 3-hydroxylase*) transgenic marker, which turns the integument of first instar larvae brown (54), are used as transgenic markers in silkworms. However, *EGFP* screening under the fluorescence microscope is a laborious procedure for large-scale screening, and the *KMO* marker requires the use of the *w-1* (*kynurenine 3-hydroxylase* mutant) white egg strain, and is subject to maternal inheritance. Thus, markers, which could be used for normal egg color strains, are not subject to maternal inheritance and do not need fluorescence microscopy have been desired for the analysis of numerous mutant strains and recombinant protein production. One strategy for changing the normal egg color is knockdown of related genes by transgenic RNAi (55, 56). All egg color (ommochrome related) mutants with genes identified thus far exhibit a white egg phenotype, which is very similar to the color of non-diapausing or unfertilized eggs. However, the *re* phenotype in the eggs and eyes is distinct, so it avoids misjudging false-positive eggs. In addition, the *Bm-re* gene has the advantage that the mutant phenotype can be detected from an early stage (3 days after laying), compared with the *EGFP* marker (7 days after laying). Thus, transgenic RNAi using the *Bm-re* gene has several potential advantages for a transgenic marker that can be used for most strains with a wild-type ommochrome pathway. Because *Bm-re* homologs were found to be widespread in insects, and shown to function in eye pigmentation in *T. castaneum*, a transgenic marker utilizing the *re* gene may be applicable in diverse insect taxa.

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