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

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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 homoygous 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),
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), 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 ommims (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 color defects, suggesting that the Bm-re gene homologs are conserved and widespread contributors to ommochrome biosynthesis in insects.

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°) 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°) 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° mutant male were each back-crossed to a re° 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), and 2 mutant strains (re° and re°), 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
Novel Ommochrome Pathway Gene in Insects

**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-080104-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. To perform PCR analysis, total RNA was amplified by PCR with gene-specific primers containing T7/H11032 polymerase site at the 5′ end. Products were amplified by vector-specific primers containing a T7/H11032 polymerase site at the 5′ end. DNA fragments were subcloned into pGem-T Easy vector (Promega) according to the manufacturer’s instructions. The dsRNA solutions were diluted to 500 ng/µl according to the manufacturer's instructions. The diluted samples were used for 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. 2A, 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. 2A) (45). From a blastx search, we found that BGIBMGA003497 and BGIBMGA003694 had homology to retinol dehydratase (CGG6704 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-
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. 2B). Among the 6 candidate genes, BGIBMGA003497-1 did not give rise to any bands after 40 cycles of PCR. Moreover, the expression level of BGIBMGA003498 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, re911, the strain used for linkage analysis, and re28, 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. 3A). In both re911 and re28 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 (911 and 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. 3C, striped boxes, and supplemental Fig. S2). Comparison with the published genomic sequence indicated that the splice donor site in 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.
predicted type 1 and 2 proteins were located in the 11th and 10th transmembrane domains (TMDs), respectively, yielding only 11 and 10 TMDs (Fig. 3C).

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\textsuperscript{28} had a 541-bp insertion, which had high sequence similarity to bm\textsubscript{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 retroelement. Notably, the insertion sequence was a nonautonomous transposable element such as a SINE (short interspersed nuclear element) or a retroelement. The 2-bp target site duplication in re\textsuperscript{28} was also conserved in the genomic sequence of both wild-type and re mutant strains (47), which was also conserved in the predicted gene sequence TcasGA2\_TC013631 except lacking a 12-bp (993–1004) portion and a 57-bp (1114–1170) portion. To investigate if the re\textsubscript{11} function 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 functions 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 multicompartment 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 eggs and re\textsuperscript{11} eggs, indicating both strains had the same origin. By contrast, eggs injected with BGlMGA003497-1 dsRNA became a normal egg pigmentation, which was absent in both re\textsuperscript{11} and WT strains (36). This is consistent with our TLC results indicating that re\textsuperscript{11} 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...
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_{5}O_{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.
**Novel Ommochrome Pathway Gene in Insects**

**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 oxidase (vermillion), formylkynurenine is converted to kynurenine by kynurenine formamidase, kynurenine is converted into 3-hydroxykynurenine by kynurenine 3-hydroxylase (cinnaabar, 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.

**References**


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Novel Ommochrome Pathway Gene in Insects


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