ORIGINAL ARTICLE

Positional cloning of a *Bombyx pink-eyed white* egg locus reveals the major role of *cardinal* in ommochrome synthesis

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Ommochromes are major insect pigments involved in coloration of compound eyes, eggs, epidermis and wings. In the silkworm *Bombyx mori*, adult compound eyes and eggs contain a mixture of the ommochrome pigments such as ommin and xanthommatin. Here, we identified the gene involved in ommochrome biosynthesis by positional cloning of *B. mori* egg and eye color mutant *pink-eyed white egg* (*pe*). The recessive homozygote of *pe* has bright red eyes and white or pale pink eggs instead of a normal dark coloration due to the decrease of dark ommochrome pigments. By genetic linkage analysis, we narrowed down the *pe*-linked region to ~258 kb, containing 17 predicted genes. RNA sequencing analyses showed that the expression of one candidate gene, the ortholog of *Drosophila* haem peroxidase *cardinal*, coincided with egg pigmentation timing, similar to other ommochrome-related genes such as *Bm-scarlet* and *Bm-re*. In two *pe* strains, a common missense mutation was found within a conserved motif of *B. mori cardinal* homolog (*Bm-cardinal*). RNA interference-mediated knockdown and transcription activator-like effector nuclease (TALEN)-mediated knockout of the *Bm-cardinal* gene produced the same phenotype as *pe* in terms of egg, adult eye and larval epidermis coloration. A complementation test of the *pe* mutant with the TALEN-mediated *Bm-cardinal*-deficient strain showed that the mutant phenotype could not be rescued, indicating that *Bm-cardinal* is responsible for *pe*. Moreover, knockdown of the *cardinal* homolog in *Tribolium castaneum* also induced red compound eyes. Our results indicate that *cardinal* plays a major role in ommochrome synthesis of holometabolous insects. *Heredity* (2016) 116, 135–145; doi:10.1038/hdy.2015.74; published online 2 September 2015

INTRODUCTION

Ommochromes represent a major group of insect pigments and are divided into two subgroups, ommins and ommatins, depending on their liability to alkaline conditions (Butenandt *et al.*, 1958; Linzen, 1974). Ommins are stable under alkali conditions, a compound with a high degree of polymerization, and function as screening pigments in the eyes of most insects except for higher dipterans. Ommins are also found in the eyes of many arthropod and cephalopod species, and in the epidermis of the cricket *Gryllus bimaculatus*, the stick insect *Carausius morosus* and the mantis *Mantis religiosa* (Linzen, 1974). Ommatins are unstable under alkali conditions, are of rather low molecular weight and are found in epidermis, wings, excreta and eyes (Linzen, 1974; Nijhout, 1997). Xanthommatin is a common ommatin pigment found in the eyes of most insects. Xanthommatin has two forms, an oxidized form and a reduced form, that are associated with brown/red color differences of *Heliconius* butterfly wings (Gilbert *et al.*, 1988) and yellow/red body color change of red dragonflies (Futahashi *et al.*, 2012).

The genes involved in ommochrome biosynthesis have been identified mostly by genetic analyses of *Drosophila* eye color mutants. The primary ommochrome precursor is tryptophan that is

converted into 3-hydroxykynurenine by three enzymes encoded by *vermillion*, *kynurenine formamidase* and *cinnabar* (Searles and Voelker, 1986; Searles *et al.*, 1990; Warren *et al.*, 1996). Subsequently, 3-hydroxykynurenine is incorporated into pigment granules by the heterodimeric ABC (ATP-binding cassette) transporters encoded by *scarlet* and *white* (Tearle *et al.*, 1989; Pepling and Mount, 1990). However, the molecular mechanisms involved in the conversion of 3-hydroxykynurenine into ommochrome pigments are largely unknown, and this may be because of the lack of ommin pigments in *Drosophila melanogaster* (Linzen, 1974).

It has been reported that the eggs and adult compound eyes of the silkworm *Bombyx mori* contain a combination of the ommochrome pigments, ommin and xanthommatin (Kawase and Aruga, 1954; Koga and Osanai, 1967; Linzen, 1974; Sawada *et al.*, 2000). Many *Bombyx* egg and eye color mutants have been preserved and used to study insect genetics such as homologous recombination (Ariga and Murakami, 1975; Ariga and Murakami, 1976) and parthenogenetic induction (Hirokawa, 1993; Hirokawa, 1996). *B. mori* egg and eye color mutants *w-1*, *w-2* and *w-3* have been reported to correspond to *Drosophila* eye color mutants *cinnabar*, *scarlet* and *white*, respectively, indicating that genes involved in the early steps of the ommochrome

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synthesis pathway are conserved between the two species (Quan *et al.*, 2007; Komoto *et al.*, 2009; Tatematsu *et al.*, 2011). In contrast, some *B. mori* egg color mutants could not be explained by simple adaptation of *Drosophila* eye color mutants. We have reported that the gene responsible for *B. mori* red egg (*re*) mutant is a novel member of a major facilitator superfamily transporter that is conserved in most insects but is lost in *Drosophila* (Osanai-Futahashi *et al.*, 2012). The *re* mutant is deficient in ommin synthesis, suggesting that the loss of the *re* gene ortholog in higher Diptera coincides with the lack of ommins in their eyes (Linzen, 1974). Owing to them having a more complex ommochrome composition than *Drosophila* and still unrevealed ommochrome mutants, *B. mori* is suitable for investigating the final steps of ommochrome biosynthesis.

Pink-eyed white egg (*pe*) is a recessive egg and eye color mutant of *B. mori*. The recessive homozygote of *pe* generally has white eggs and pink eyes, a brighter shade than those of the *re* mutant (Figure 1). Notably, the non-diapausing eggs of the *pe* mutant turn pink after several weeks of preservation at low temperature. The *pe* locus has been mapped to linkage group 5, the same as the *re* locus. In the *pe* mutants, the level of 3-hydroxykynurenine, the ommochrome precursor, accumulates compared with wild type, suggesting that *pe* mutants have defects in the conversion of 3-hydroxykynurenine to final ommochromes (Inagami, 1954). It has been suggested that *pe* functions downstream of *w-2* (*scarlet*) and upstream of *re* from genetic studies (Uda, 1932).

Here, we identify the gene corresponding to the *pe* locus by positional cloning. We report a missense mutation in a gene encoding peroxidase orthologous to the *cardinal* gene of *D. melanogaster* (Harris *et al.*, 2011), and reproduce the *pe* phenotype by RNA interference (RNAi) gene knockdown and transcription activator-like effector nuclease (TALEN)-mediated gene knockout. We also conducted a

complementation test by crossing gene knockout individuals with *pe* mutants, and found that these were unable to rescue the *pe* phenotype, demonstrating that the *B. mori* *cardinal* homolog is the gene responsible for the *pe* phenotype. We also confirmed the decrease of red/purple pigments (supposed ommin), decarboxylated xanthommatin and xanthommatin in *pe* mutants by thin-layer chromatography (TLC) and high-pressure liquid chromatography–mass spectrometry (HPLC-MS). Instead, the ommochrome precursor 3-hydroxykynurenine and uncharacterized pigments seem to accumulate in *pe* mutants. Moreover, in the red flour beetle *Tribolium castaneum*, knockdown of the *cardinal* homolog also induced the red eye phenotype, suggesting that the *cardinal* gene has a major role in biosynthesis of normal ommochrome pigments, at least in holometabolous insects.

MATERIALS AND METHODS

Silkworm and *Tribolium* strains

We used two *pe* strains, *pe* ^{re⁹¹¹} and *pe* ^{re²⁰}, for this study. The *pe* ^{re²⁰} mutant strain was provided from the silkworm stock center of Kyushu University (Fukuoka, Japan) supported by the National BioResource Project (Banno *et al.*, 2010). The wild-type silkworm strain p50T was maintained in the Transgenic Silkworm Research Unit, National Institute of Agrobiological Sciences (Tsukuba, Japan), and other silkworm strains C108, *pnd*, *p^S* and *pe* ^{re⁹¹¹} were maintained in the Genetic Resource Center, National Institute of Agrobiological Sciences. Silkworms were reared on mulberry leaves or on an 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* is derived from a strain that was provided from the National Food Research Institute (Tsukuba, Japan) and was raised on whole wheat flour at 30 °C.

Positional cloning of *pe*

For recombination mapping, six F₁ heterozygous males, which were obtained from a single-pair cross between a C108 wild-type female and a *pe* ^{re⁹¹¹} mutant male, were each backcrossed to a *pe* ^{re⁹¹¹} mutant female. A total of 656 BC₁ eggs (282 normally pigmented eggs, 260 white eggs and 114 red eggs) were used for analyses. Genomic DNA was extracted from parent moths, F₁ moths and from each BC₁ neonate larva using DNAzol solution (Invitrogen, Carlsbad, CA, USA). For genetic analysis, 14 single-nucleotide polymorphism (SNP) markers previously reported on chromosome 5 (Yamamoto *et al.*, 2008) and 16 new SNP markers that were constructed for this study were used. The primers for each SNP marker are listed in Supplementary Table S1.

Phylogenetic analysis

Deduced amino acid sequences of *cardinal* gene homologs were aligned using the Muscle program implemented in MEGA6 (Tamura *et al.*, 2013). Molecular phylogenetic analysis was conducted by neighbor-joining and maximum-likelihood methods using MEGA6. The evolutionary distances were computed using the JTT or Poisson model for neighbor-joining or maximum-likelihood analysis, respectively. The confidence of the phylogenetic lineages was assessed by bootstrap analysis of 1000 resamplings.

RNA sequencing (RNAseq) experiment

Total RNA was isolated from at 0, 24, 48 and 72 h after eggs were laid from two wild-type *B. mori* strains (p50T and C108) and a *pe* mutant strain *pe* ^{re²⁰} that is different from the *pe* ^{re⁹¹¹} strain used for linkage analysis. ISOGEN (Nippon Gene, Tokyo, Japan) and RNeasy mini kit (Qiagen, Dusseldorf, Germany) were used for the RNA extraction from egg samples. RNAseq libraries were prepared using TruSeq Sample Preparation Kit v2 (Illumina Inc., San Diego, CA, USA), and sequenced using 100 bp paired-end strategy on a HiSeq 2500 sequencer (Illumina). Mapping to the most recent *B. mori* gene list (Suetsugu *et al.*, 2013) was performed with BWA-mem software using MASER pipeline provided by the Cell Innovation Project team at the National Institute of Genetics (http://cell-innovation.nig.ac.jp/public/contents/service_en.html#pf_maser). The relative expression of each gene was estimated to calculate fragments per kilobase of exon per million (FPKM) values. The SNPs of the

Figure 1 Wild-type and *pe* mutant of the silkworm *B. mori*. Eggs (top), adult compound eyes (middle) and eyespot markings of final-instar larva (bottom) of wild-type (+*pe*/*pe*, left) and *pe* ^{re²⁰} mutant (*pe/pe*, right) are shown. Around the eyespot markings, red markings are found in the *Striped* (*p^S*) strain (+*pe*/*pe*; *p^S/p^S*, bottom left) but not in double mutants of *p^S* and *pe* (*pe/pe*; *p^S/p^S*, bottom right).

genes were analyzed by Integrative Genomics Viewer. The RNAseq data set is deposited in DDBJ (DNA Data Bank of Japan) under accession number DRA003068.

RNAi experiments

A small interfering RNA (siRNA) targeted to *Bm-cardinal* was designed using siDirect program version 2.0 (<http://sidirect2.rnai.jp/>) (Naito *et al.*, 2009), following the guidelines reported in Yamaguchi *et al.* (2011). The sequences of the siRNA are as follows: *pe*-1 sense strand, 5'-GCGAUUACAGCACUUUC AAUG-3'; antisense strand, 5'-UUGAAAGUGCUGUAAUCGCUU-3'. We used the following siRNA for *Bm-white* as reported in Ando and Fujiwara, 2013; sense strand, 5'-CAUUUAUGGCCCAAAACGUUA-3'; antisense strand 5'-ACGUUUUUGGGCCAUAAAUGAA-3'. To produce templates for double stranded RNA (dsRNA) synthesis the following primers were used for *T. castaneum cardinal*: Tccd-F3 5'-GACGATGTCAGGGGGTGAAC-3' and Tccd-R2 5'-GTTATAGCCAACTAATCCATGGTC-3'.

For embryonic RNAi of *B. mori*, 2–3 nl of 100 μ M siRNA was injected into eggs 3–5 h after laying as reported previously (Osanai-Futahashi *et al.*, 2012). For larval and pupal RNAi of *B. mori*, an electroporation-mediated method was performed as reported by Ando and Fujiwara, 2013. The larval epidermis of the third- or fourth-instar *p^S* strain and the compound eye region of day 1–3 pupae were injected with 0.25–0.5 μ l of 100 μ M siRNA solution. Immediately after injection, platinum electrodes and droplets of gel for Ultrasound GELH-250 (Nepagene, Ichikawa, Japan) were placed nearby and five pulses of 45 V (280 ms s⁻¹) were applied by the electroporator Cure-Gene (CellProduce Co., Ltd, Tokyo, Japan). To perform RNAi using the beetle *T. castaneum*, double-stranded RNAs were prepared and 0.5 μ g μ l⁻¹ of dsRNA solution was injected into the abdomens of last- or penultimate-instar larvae as described previously (Osanai-Futahashi *et al.*, 2012).

Knockout experiment using TALEN

TALEN knockout experiments were conducted as reported previously (Takasu *et al.*, 2013). A manual search was performed for a TALEN target site for *Bm-cardinal* in a conserved region among *p50T*, *C108*, and *pe^{e20}* strains. 5'-GCTGAGGGTCGGGAG-3' and 5'-TTGGATCAGATGGAAGGA-3' in exon 8 of *Bm-cardinal*, which is a part of the peroxidase domain, were selected as the recognition sequences. The TAL portions were prepared by Golden Gate TALEN and TAL Effector Kit (Addgene, Cambridge, MA, USA), following the method described by Cermak *et al.* (2011), using an *in-vitro* expression vector pBlue-TAL. The constructed plasmids were purified by HiSpeed Plasmid Midi kit (Qiagen), linearized by *Xba*I, treated with proteinase K, extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform, precipitated with ethanol and washed with 70% ethanol three times. TALEN mRNAs were transcribed by mMESSAGE mMACHINE kit (Applied Biosystems, Foster City, CA, USA), followed by lithium chloride precipitation and were then washed with 70% ethanol three times. The resulting TALEN mRNAs were dissolved into injection buffer (0.5 mM phosphate buffer (pH 7.0), 5 mM KCl) to achieve a final concentration of 0.5 mg ml⁻¹ each, and were microinjected into non-diapausing *B. mori pnd* (wild-type egg color) strain 3–5 h after laying. Of the 192 injected eggs, 105 hatched and 47 became adults. Sixteen G0 males were mated with sibling G0 females to obtain G1 generation eggs, and 10 of the G0 males were re-mated with diapausing *pe^{e20}* females for complementation test. The eggs for complementation test were subjected to acid treatment 24 h after laying to prevent entering diapause. For genotyping mutations, genomic DNA was isolated from adult heads of moths emerged from pink eggs from the G0 and *pe^{e20}* crosses, and G1 moths, using Maxwell 16 Tissue DNA purification Kit (Promega, Fitchburg, WI, USA) following the manufacturer's instructions. To identify the indels introduced by TALENs, the targeted region was amplified by PCR with primers 5'-AACCAAGCCACAGCCTTTATC-3' and 5'-TGATTC AAAACAATAGCGTCCTT-3', and directly sequenced by ABI prism 3130 Genetic Analyzer (Applied Biosystems).

Quantitative reverse transcriptase-PCR

Total RNA from *B. mori* eggs was extracted using ISOGEN (Nippon Gene) and used as a template for synthesis of cDNA with a first-strand cDNA synthesis kit (GE Healthcare, Buckinghamshire, UK). The expression level of *Bm-cardinal*

mRNA was quantified using a Light-Cycler 1.5 (Roche, Basel, Switzerland). The *B. mori* rpL3 gene was analyzed to normalize transcript levels. Quantitative PCR was performed in duplicate. The *P*-values were obtained using Student's *t*-test. Primers used for quantitative PCR are listed in Supplementary Table S2.

Pigment extraction

Ommochrome pigment was directly extracted from four pairs of *B. mori* compound eyes by 100 μ l of 1% hydrochloric acid in methanol (v/v). Because we found that decarboxylated xanthommatin was removed by methanol wash, we did not conduct washes before pigment extraction. After centrifugation, supernatants were collected and used for analyses.

Thin-layer chromatography

TLC was performed by spotting 20 μ l of the compound eye extracts on to pre-coated silica gel plates with concentrating zone (60 F254, Merck, Billerica, MA, USA). They were developed using the upper layer of collidine/lutidine/water (1:1:2, v/v) as solvent (Ajami and Riddiford, 1971; Sawada *et al.*, 2000). The autofluorescence of the separated pigments was observed by 302 nm irradiation using a minitransilluminator (Funakoshi, Tokyo, Japan). 3-hydroxykynurenine was purchased from Sigma (St Louis, MO, USA), and synthesized xanthommatin and decarboxylated xanthommatin were kindly provided by Dr Hiroaki Mano (National Institute for Basic Biology, Okazaki, Japan).

High-pressure liquid chromatography–mass spectrometry

HPLC-MS analysis was conducted following the method used for analysis of *Junonia coenia* wing ommochrome pigments (Daniels and Reed, 2012). The experiment was conducted by using a Hewlett Packard HP 1100 MSD system fitted with a CAPCELL PAK C18 TYPE UG80 reversed-phase column (3.0 mm ϕ $\times$ 150 mm, 5 μ m particle size, Shiseido, Tokyo, Japan), running on a linear gradient protocol. The primary solvent was 0.5% acetonitrile with 0.2% acetic acid and increased linearly to 95% acetonitrile in 0.2% acetic acid in 15 min at a flow rate of 0.5 ml min⁻¹. Ultraviolet/visible spectra were monitored with a photodiode array detector. Electrospray ionization in the positive mode was used for the MS detection.

RESULTS

Positional cloning of the *pe* locus

To identify the candidate region for the *pe* gene, we conducted a genetic linkage analysis using a high-resolution SNP linkage map (Yamamoto *et al.*, 2008) and silkworm genome sequence information (The International Silkworm Genome Consortium, 2008). First, we obtained F₁ heterozygous males from a cross between a single male of *pe re* double-mutant strain *pe re⁹¹¹* and a single female of a wild-type egg and eye colored strain C108. We backcrossed the F₁ heterozygous males with *pe re⁹¹¹* females, and obtained 282 normally pigmented BC₁ eggs (*+pe^e+re^e/pe re*), 114 red BC₁ eggs (*+pe^ere/pe re*) and 260 white BC₁ eggs (*pe re/pe re* or *pe +re^e/pe re*). It should be noted that red BC₁ eggs are homozygous for the *re* allele but heterozygous for the *pe* allele, as *pe* gene functions above *re*. By analyzing the SNPs of 656 BC₁ eggs, we narrowed down the *pe*-linked region to within 258 kb on chromosome 5, from position 1 156 521 to 1 414 291 (Supplementary Figure S1 and Supplementary Table S1). Within this region, 17 and 12 genes were predicted by the most recent *B. mori* gene list (Suetsugu *et al.*, 2013) and canonical gene model (The International Silkworm Genome Consortium, 2008), respectively (Figure 2a).

Comprehensive transcriptome analysis of wild-type and *pe* eggs

As the morphological differences between *pe* and wild-type eggs become distinct 48–72 h after the eggs are laid, the responsible gene is likely to be expressed during or just before this period. We therefore compared the expression pattern and deduced amino acid sequences

of all 17 candidate genes among two wild-type strains p50T and C108, and *pe*^{e20} mutant strain, which is a different *pe* strain from *pe* re⁹¹¹, by RNAseq at 0, 24, 48 and 72 h after eggs were laid. In both wild-type

and *pe* strains, seven genes (*Gene2414*, 17856, 2416, 2417, 17857, 2424 and 2425) and two genes (*Gene2415* and 2426) were hardly (FPKM < 5) or only weakly expressed (FPKM < 20), respectively (Figure 2b).

As *pe* egg color phenotype is not subject to maternal inheritance, we excluded Genes 2418, 17858, 17859, 2420 and 2423 from the candidate genes, because their mRNA detected immediately post laying were very likely to be maternally derived and should cause maternal effect if they were the responsible gene. Expression patterns of two genes (*Gene2421* and *2422*) were very similar between wild-type and *pe* strains and no nucleotide changes were found in the *pe* strain. In the remaining gene (*Gene2419*), the level of expression was elevated at ~24 h after laying, just before egg pigmentation occurred, similar with other ommochrome genes such as *Bm-re*, *Bm-scarlet* and *Bm-white* (Figure 2b) (Tatematsu et al., 2011; Osanai-Futahashi et al., 2012). For *Bm-white*, we summed the FPKMs of two predicted genes that were both alleles of *Bm-white* derived from different strains. Because maternally synthesized 3-hydroxykynurenine is mainly used for egg pigmentation, the low expression level of *vermilion* and *cinnabar* at this period was consistent. We found two paralogous genes for kynurenine formamidase in *B. mori* (*Gene8740* and *8743*), and only *Gene8743* was expressed around this stage. We found two

missense mutations in *Gene2419* of the *pe*^{e20} strain (Figures 3a and d, Supplementary Figure S2). We therefore determined the open reading frame sequence of *Gene2419* in another *pe* mutant strain, *pe* re⁹¹¹ (hereafter referred to simply as *pe*⁹¹¹), which was originally used for linkage analysis, and detected one shared missense mutation (arrowhead in Figure 3d, Supplementary Figure S2). This suggested that the missense mutation in *Gene 2419* is a strong candidate for the cause of *pe* phenotype. Although in the RNAseq experiment *pe*^{e20} had twice the higher expression at 24 h post oviposition, no significant differences were found between wild-type p50T and *pe*^{e20} or *pe*⁹¹¹ strains by quantitative reverse transcriptase-PCR (*t*-test; *P*=0.25 and 0.62, respectively) (Supplementary Figure S3).

Gene 2419 encodes a peroxidase gene orthologous to *cardinal*

Gene2419 encoded a haem peroxidase, the ortholog of the *cardinal* gene in *D. melanogaster* (hereafter referred to as *Bm-cardinal*) (Figure 3c). Notably, the *cardinal* mutant of *D. melanogaster* shows delayed eye pigmentation because of slow conversion of 3-hydro-

Figure 3 Structure and phylogenetic relationship of the *Bm-cardinal* gene. (a) Genomic structure of the *Bm-cardinal* gene. The open reading frame is indicated by solid boxes, the untranslated region is indicated by open boxes and introns are indicated by diagonal lines. The tryptophan to arginine mutation that is specific for the two *pe* strains is located in exon 9. Exon 13 is mapped into an unmapped scaffold Bm_scaf1910; thus, the exact size of adjoining introns is unknown. (b) The predicted gene structure of the *Bm-cardinal* gene. The transmembrane domain was predicted by SOSUI (Hirokawa et al., 1998). (c) Phylogenetic relationship of the *cardinal* gene homolog in insects. *Homo sapiens* myeloperoxidase, eosinophil peroxidase and peroxidase gene from *B. mori* and *D. plexippus* with the second highest *e*-value by blastp search using *Bm-cardinal* protein were included as outgroups. A neighbor-joining phylogeny inferred from 1735 aligned amino acid sites is shown, and maximum-likelihood phylogeny was found to exhibit substantially similar topologies. On each node, bootstrap values are indicated in the order of neighbor-joining method/maximum-likelihood method. Bootstrap values over 50% are indicated. Accession numbers or annotation IDs are in brackets. (d) Alignment of insect *cardinal* homologs. The tryptophan to arginine mutation shared by the two *pe* strains is indicated by arrowheads, and the aspartic acid to alanine mutation that is specific to *pe*^{e20} strain is indicated by asterisk.

Figure 2 Positional cloning of the *pe* locus. (a) A physical map of the *pe*-linked region. The *pe*-linked region was narrowed within the 258 kb region between SNP markers pe-080514-L-R7_F1 and pe-080514-R-R18_F20 (Supplementary Table S1). In this region, there are 17 and 12 predicted genes of recently revised gene model (Suetsugu et al., 2013) and canonical gene model (numbers in parenthesis, (The International Silkworm Genome Consortium, 2008)). (b) Expression analysis of 17 predicted genes within the *pe*-linked region and 7 ommochrome-related genes by RNAseq. The numbers on the x axis indicate hours after oviposition. The numbers on the y axis indicate the level of relative expression (FPKM values). The dark gray, light gray and diagonally lined column indicate the p50T (wild type), C108 (wild type) and *pe*^{e20} strain, respectively.

xykynurenine to xanthommatin (Harris *et al.*, 2011), thus indicating that *Cardinal* mediates this process. The predicted *cardinal* gene product has a transmembrane region and a peroxidase homology domain (Harris *et al.*, 2011), and the two mutations in the *Bm-cardinal* gene of *pe*²⁰ were located within the peroxidase domain (Figures 3a and b). Molecular phylogenetic analysis revealed that insect *cardinal* genes formed monophyletic cluster (Figure 3c) and amino acid sequences of the peroxidase domain were highly conserved among insects (Figure 3d). The tryptophan residue, which was converted to arginine in the two *pe* strains, was widely conserved in holometabolous insects (arrowhead in Figure 3d), whereas the amino acid at position of the *pe*²⁰-specific mutation was not conserved (asterisk in Figure 3d). These results support the hypothesis that the shared mutation in the *Bm-cardinal* gene is a strong candidate for the cause of the *pe* phenotype.

Functional analysis of *Bm-cardinal* gene by RNAi

To investigate whether the *Bm-cardinal* gene is essential for normal egg pigmentation, we performed embryonic RNAi experiments. Most eggs injected with *Bm-cardinal* siRNA failed to turn into the normal purplish-brown color, but became white like the eggs of the *pe* mutant (Figure 4a). In contrast, all eggs injected with the injection buffer

developed into the normal dark color (Figure 4a). We next analyzed the effect of RNAi on adult compound eyes. Because simple systemic RNAi by injection of dsRNA does not work in the larval and pupal period in Lepidopteran species, including *B. mori*, in general (Terenius *et al.*, 2011), we conducted recently developed electroporation-mediated RNAi as reported by Ando and Fujiwara (2013). We found that one side of compound eyes became reddish by electroporation of *Bm-cardinal* siRNA, like the *pe* mutant, whereas it became white by electroporation of *Bm-white* siRNA like *w-3* (*Bm-white*) mutant (Figure 4b). All individuals injected with the injection buffer had the normal dark compound eyes (Figure 4b).

Ommochromes are also involved in red and brown pigmentation of Lepidopteran larval markings. In the *p^s* strain, conspicuous red markings are recognized around eyespot markings of final-instar larva. We found that this red marking disappears with individuals on a *pe* background (Figure 1). We therefore examined the effect of *Bm-cardinal* RNAi on larval pigmentation. By using electroporation-mediated RNAi, we confirmed that red pigmentation could be suppressed by introduction of either *Bm-cardinal* siRNA or *Bm-white* siRNA, but not with injection buffer only (Figure 4c), suggesting that these genes are essential for the formation of red markings on larval epidermis. Overall, the *pe* phenotype was

Figure 4 Knockdown of the *Bm-cardinal* gene by RNAi. (a) The effect of RNAi on egg pigmentation. *Bm-cardinal* siRNA or injection buffer was injected into eggs at 3–6 h post oviposition, and eggs were then incubated at 25°C. The injected eggs were photographed after 3 days. (b) The effect of RNAi on the pigmentation of adult compound eyes. One side of the eyes (arrowheads) was subjected to electroporation-mediated RNAi at day 0 pupa. (c) The effect of RNAi on pigmentation around eyespot markings of the fifth-instar larva. One side of eyespot markings (arrowheads) was subjected to electroporation-mediated RNAi at the fourth or third instar.

Figure 5 Disruption of the *Bm-cardinal* gene using designed TALENs. **(a)** The genomic structure of the *Bm-cardinal* gene and the TALEN binding sites. The TALEN target sites are located in exon 8. **(b)** The mosaic coloration phenotype of G0 eggs and adult compound eyes injected with TALEN mRNA targeting *Bm-cardinal*. TALEN mRNA was injected into eggs 3–6 h post oviposition, and eggs were then incubated at 25°C. The eggs and compound eyes showed various ranges of mosaic coloration. Injected eggs were photographed after 3 days. **(c)** Summary of the G0 phenotype resulting from TALEN injection. **(d)** Adult compound eyes of the G1 generation. Eggs obtained from sibling mating of G0 moths derived from eggs injected with TALEN mRNA targeting *Bm-cardinal*, and an adult derived from a pink G1 egg is shown. **(e)** Wild-type neonatal larva (left), G1 generation of *Bm-cardinal* targeting TALEN experiment, derived from a pink egg (middle), and a *pe^{e20}* mutant strain. Both *pe*- and *Bm-cardinal*-disrupted neonatal larva lack brown pigmentation in the abdominal legs. **(f)** Compound eyes of an adult derived from pink egg produced by crossing *pe^{e20}* females with G0 male moths targeted for *Bm-cardinal*. The specimen in this picture corresponds to CT-E-F-1 of **(g)**. **(g)** Sequences of small, induced insertions and deletions in *Bm-cardinal* for 13 G1 mutants, and 3 adults from the complementation test. The inserted sequences are underlined. Only the alleles with TALEN-mediated indels are shown for the complementation test (the *pe^{e20}*-derived allele has the same sequence as wild type (WT) in this region).

reproduced in coloration of eggs, adult eyes and larval epidermis by *Bm-cardinal* RNAi.

Functional analysis of *Bm-cardinal* gene using TALEN

We next performed genetic knockout experiments for *Bm-cardinal* by TALEN. TALEN target sites were designed in exon 8 (Figure 5a), 132 nucleotides upstream of the *pe*-specific mutation (Figure 2b). A large portion of the eggs injected with TALEN mRNA showed a mosaic color phenotype between white and normal purple brown to varying degrees (Figures 5b and c). In the G0 generation, we observed two adults with red eyes like *pe*, 20 adults with mosaic colored eyes and 25 adults with black eyes (Figures 5b and c). We obtained 16 G1 egg batches from G0 sibling pairing, among which 15 batches contained pink eggs (Supplementary Figure S4). Notably, pink egg coloration is because of prolonged preservation of non-diapausing eggs at low temperature, and was not observed in non-diapausing eggs left to develop at room temperature, as well as in diapaused eggs. All adults derived from pink G1 eggs had pink-red eyes like the *pe* mutant (Figure 5d). We also found that neonatal larvae from pink G1 eggs, as well as *pe* mutants, have lighter coloration in their abdominal prolegs (Figure 5e), suggesting that the brownish color around the prolegs in the wild type are caused by ommochrome pigments.

To confirm TALEN-mediated gene disruption, we extracted genomic DNA from 16 G1 adults with pink eyes from batches 1 and 2 (Supplementary Figure S3), and sequenced the 200 bp region containing the TALEN target sites. Direct sequencing of the PCR product showed that three individuals had overlapping sequences just after the TALEN target sites, possibly heterozygous for indels. All of the remaining 13 individuals had non-overlapping sequences with a small deletion and/or an insertion (Figure 5g), indicating that the *Bm-cardinal* gene is disrupted in these individuals.

Complementation test of *pe* mutant female with *Bm-cardinal*-deficient male

Results from the expression analyses, RNAi and TALEN experiments strongly suggest that the *Bm-cardinal* gene corresponds to the *pe* locus. To confirm this, we performed a complementation test by crossing *pe* mutant females with *Bm-cardinal*-deficient G0 males. We obtained 10 batches of eggs, all of which contained pink eggs (Supplementary Figure S5). We found that all adults derived from pink eggs had red eyes like *pe* (Figure 5f), indicating that the *pe* phenotype could not be rescued by a *Bm-cardinal*-deficient male. Furthermore, we extracted genomic DNA from the adult heads and amplified the 200 bp region containing the TALEN target sites; we found that these individuals were heterozygotes of a *pe*²⁰-derived sequence that is the same as that of the wild-type individuals for this exon, and a sequence with insertions or deletions in the region between the two TALEN target sites (Figure 5g). The above results demonstrate that the *Bm-cardinal* gene is responsible for the *pe* phenotype.

Pigment analyses of *B. mori* adult eyes

To analyze the changes of eye pigment composition between wild type and *pe*, we extracted ommochrome pigments from adult compound eyes with acidic methanol for pigment analysis (Supplementary Figure S6a). We omitted washes before the pigment extraction to detect decarboxylated xanthommatin (Futahashi *et al.*, 2012). The extracted pigments from *pe* or *Bm-cardinal* knockout individuals showed lighter coloration than that from wild-type p50T strain (Supplementary Figure S6a).

In TLC analysis, we analyzed the extracted pigments from p50T and *pe* eyes, along with 3-hydroxykynurenine, synthesized xanthommatin

and synthesized decarboxylated xanthommatin in collidine/lutidine/water (1:1:2) solvent system on silica gel plate. A purple-red band of RF=0.46 was observed in p50T eye extract that was faintly found in *pe* eye extract and was absent in the standard pigments (Supplementary Figure S6b). This band was in good correspondence with the one of the ommin spots (RF=0.46) reported from saturniid moth eye (Ajami and Riddiford, 1971). We observed autofluorescence in *pe* eye extract at the similar mobility with 3-hydroxykynurenine (Supplementary Figure S6b), suggesting the accumulation of 3-hydroxykynurenine in the *pe* eye as reported previously (Inagami, 1954).

We also performed HPLC-MS analyses for acidic methanol extract of eyes from *Bm-cardinal* knockout individuals and p50T. In comparison with chromatogram and the mass data of the synthesized xanthommatin and decarboxylated xanthommatin, we found that both xanthommatin and decarboxylated xanthommatin were obviously present in p50T eye extracts (Supplementary Figures S7–S10). Although xanthommatin was also detected in *Bm-cardinal* knockout individuals, it was not a major component based on electrospray ionization mass spectra of the mass peak of corresponding retention time and the extracted ion chromatogram (Supplementary Figures S11 and S12). Decarboxylated xanthommatin was hardly detected in *Bm-cardinal* knockout eye extract (Supplementary Figures S11 and S12). Notably, several uncharacterized substances seem to be major components of *Bm-cardinal* knockout extract around the xanthommatin and decarboxylated xanthommatin retention time (Supplementary Figures S11 and S12). These results suggest that both ommin and ommatin composition of *Bm-cardinal* deficient insect differs largely from that of wild type.

Knockdown of the *cardinal* ortholog gene in the beetle *T. castaneum*

Like *B. mori*, most insects have blackish eyes because of ommin pigments (Linzen, 1974; Osanai-Futahashi *et al.*, 2012). To investigate the function of the *cardinal* gene ortholog in other insects, we performed RNAi of *cardinal* ortholog in *T. castaneum* (*Tc-cardinal*). All pupae treated with *Tc-cardinal* dsRNA (*n* = 16) did not develop eye pigmentation throughout the pupal stage and newly emerged adults had white compound eyes (Figure 6), similar to individuals in which ommochrome-related genes *Tc-vermilion*, *Tc-white* and *Tc-scarlet* were knocked down (Broehan *et al.*, 2013). All pupae treated with EGFP dsRNA (*n* = 17) had normal eye pigmentation (Figure 6). Notably, the adult compound eyes of individuals treated with *Tc-cardinal* dsRNA became reddish several days after eclosion (Figures 6b–d), similar to the *cardinal* mutant of *D. melanogaster*. These results indicate that the *Tc-cardinal* gene functions in eye pigmentation and ommochrome biosynthesis in *T. castaneum* as it does in *B. mori*.

DISCUSSION

In this study, we found that a missense mutation in the *Bm-cardinal* gene is responsible for the silkworm *pe* locus. The original *Drosophila cardinal* gene product has been regarded as a phenoxazinone synthase for converting 3-hydroxykynurenine into xanthommatin (Howells *et al.*, 1977; Harris *et al.*, 2011). Although both the *Bombyx pe* mutant and the *Drosophila cardinal* mutant seem to accumulate the ommochrome precursor 3-hydroxykynurenine (Supplementary Figure S6a, Inagami, 1954; Howells *et al.*, 1977), it should be noted that they have different eye color phenotypes. *Drosophila cardinal* has reduced ommochrome levels at eclosion and becomes close to the wild-type compound eye color during aging, but the pink eyes of the *Bombyx pe* mutant do not become black like those of the wild type. Notably, eye pigments of most insects, including *Bombyx*, have been reported to be

Figure 6 Knockdown of a cardinal homolog by RNAi in *T. castaneum*. The last- or penultimate-instar larvae were injected with 170 nl of dsRNA (500 ng μ l⁻¹) for the cardinal homolog in *T. castaneum* (*Tccd*) or *EGFP*. (a) Ventral view of pupae at day 4. Arrowheads indicate compound eyes. (b) Ventral view of adult at day 0, or 5 days after eclosion. The specimen of day 0 *Tc-cardinal* RNAi (left) was photographed just after eclosion. (c) Magnified view of adult compound eyes of (b). (d) Summary of the pattern of eye pigmentation during developmental stages of the RNAi experiments.

a mixture of purple ommin pigments and reddish xanthommatin, which gives eyes black coloration in many insects, whereas ommin is absent in eyes of higher Dipteran species including *Drosophila* (Linzen, 1974). In the present study, we found that decarboxylated xanthommatin is also a major pigment of wild-type *B. mori* eyes (Supplementary Figure S10). Because decarboxylated xanthommatin can be removed by methanol wash, it has been missed in the previous study. By TLC and HPLC-MS results, we confirmed obvious decrease of not only red-purple pigment that is a good candidate for ommin, but also xanthommatin and decarboxylated xanthommatin in *Bm-cardinal*-deficient insect eyes. Instead, we found that substances with different molecular weights are the majority of the mass peak of the same retention time with xanthommatin and decarboxylated xanthommatin, suggesting that other substances, possibly pigments, are produced in these insects. We could not identify these substances as well as ommins by HPLC-MS, and this deserves future studies.

In addition to eye and egg coloration, we also found that *Bm-cardinal* is essential for ommochrome pigmentation in the larval epidermis. A recent study on *Bombyx* ommochrome and melanin larval mutant *quail* hypothesized that *BGIBMGA006740* (*Gene5459* in Suetsugu *et al.*, 2013) is the phenoxazinone synthetase gene (Nie *et al.*, 2014). Because *Gene5459* was hardly expressed around the egg pigmentation period (Figure 2b), and has sequence similarity with the *laccase2* gene, it may function in melanin pigmentation rather than ommochrome pigmentation. The observed phenotype of the *pe* mutant and the results from RNAi experiments suggests that *Bm-cardinal* is involved in ommochrome biosynthesis in larval epidermis, at least for the red markings in the *p^S* strain (Figures 1 and 4c). Although ommochrome pigments are absent in white *Bombyx* wings, they are present in wings of Nymphalidae butterflies

such as *Heliconius* species and *Precis coenia* (Butenandt and Schafer, 1962; Gilbert *et al.*, 1988; Nijhout, 1997), and phenoxazinone synthetase activity has been detected in pupal *P. coenia* wings (Nijhout, 1997). We confirmed the presence of *cardinal* gene homolog in the developing wing expressed sequence tag data set of *Heliconius erato* (IC33431AfEcon6544) (Hines *et al.*, 2012) by tBlastn search (<http://butterflygenome.org/>), and the *cardinal* gene homolog was also reported in the transcriptomes of Hawaiian happy-face spiders (Croucher *et al.*, 2013). It may be of interest to determine whether the *cardinal* gene ortholog is involved in coloration of butterfly wings and spider bodies.

Notably, the effects of *Bm-cardinal* suppression on pigmentation varied among tissues. As mentioned above, red ommochrome coloration was always observed in compound eyes of *pe* mutants and the individuals that had *Bm-cardinal* gene experimentally knocked out/down with TALEN and RNAi, respectively, whereas embryonic RNAi produced white eggs in general and both *pe* mutants and individuals treated with *Bm-cardinal* siRNA suppressed red pigmentation of larval epidermis. This suggests that the absence of *Bm-cardinal* activity results in complete or strong repression of conversion of 3-hydroxykynurenine into ommochrome pigments in eggs and larval epidermis, whereas it results in altered ommochrome synthesis in adult compound eyes. Difference of the coloration phenotype between tissues in *cardinal* mutants is also reported in *D. melanogaster*. Compound eyes of *cardinal* mutants produce xanthommatin during aging, but their ocelli are colorless. One explanation to account for variation of the pigmentation phenotype between tissues is the difference in the efficiency of 3-hydroxykynurenine auto-oxidation into xanthommatin that is known to occur spontaneously *in vitro* (Wiley and Forrest, 1981). Considering the coloration phenotype and the pigment analysis results, we propose that the loss of *cardinal* activity basically represses the ommochrome synthesis and possibly substances with reddish coloration similar with xanthommatin are produced by auto-oxidation in the mutant eyes in a delayed manner. Notably, when the non-diapausing destined eggs were preserved for several weeks at low temperature, *pe* and *Bm-cardinal* TALEN knockout eggs turned white to pink (Supplementary Figure S4), and this was not observed in diapaused eggs. One contributing factor that prevents *cardinal* independent red pigment formation may be the recently reported *Bombyx* homolog of human oxidation resistance 1 (OXR1) protein that has higher mRNA expression in diapaused eggs than in non-diapausing eggs (Kobayashi *et al.*, 2014). From biochemical experiments with *Drosophila* wild-type and eye color mutants, it has been proposed that xanthommatin itself also acts as an oxidizing agent for its own precursor 3-hydroxykynurenine, and is concomitantly converted into its reduced form, dihydroxanthommatin, which is the major form in insect tissues (Wiley and Forrest, 1981). As the catalytic activity by xanthommatin is absent at the onset of xanthommatin synthesis, it is most likely that peroxidase, which is encoded by the *cardinal* gene, triggers xanthommatin synthesis in the pupal ommatidia of both in *B. mori* and *D. melanogaster* (Harris *et al.*, 2011).

In addition to *B. mori*, we found that the *cardinal* ortholog in *T. castaneum* was also involved in normal eye pigmentation (Figure 6). Notably, the newly emerged *Tc-cardinal* RNAi beetle displayed white eyes, suggesting that xanthommatin synthesis occurred after eclosion as it occurred in the *cardinal* mutant of *D. melanogaster* (Figure 6). The differences in pigmentation timing in the *cardinal* mutant among species coincides with their pupal periods; *D. melanogaster* has a short pupal period of 4 days and pigment synthesis starts after 49 h, *T. castaneum* has a short pupal period of 6 days and the ommatidia starts to color at day 2 and *B. mori* has a pupal period of 12–14 days

and compound eye coloration can be detected at 5 days before eclosion. Because of a short pupal period, *cardinal*-independent red pigment synthesis may be incomplete at the time of eclosion in the *cardinal*-deficient *Drosophila* and *Tribolium* individuals.

The conservation of *cardinal* homolog function in *Tribolium*, *Bombyx* and *Drosophila*, and the widespread distribution of *cardinal* homologues in insects (Figure 3c), suggest that *cardinal* functions in black eye coloration in many insect species. Notably, some mutants are quite similar to *pe* mutants. In *T. castaneum*, the *cardinal* homolog is located in the same linkage group X that harbors several red- or pink-eyed mutants such as *red-1*, *peach*, *pink Ndg* and *pink Tiw* (Lorenzen *et al.*, 2002). The *Culex pipiens* mutant *crimson* is also a recessive mutant with colorless ocelli at first-instar larva that gradually turn red during larval development (Rasgon and Scott, 2004). Eye pigments detected by the head squash method of teneral *crimson* adults are yellow and turn to red-brown with age, whereas teneral and aged wild-type eye pigments are black, and the teneral adult eyes have excess 3-hydroxykynurenine. Both the *tangerine* mutant of blowfly *Lucilia cuprina* and the *chartreuse* mutant of honeybee *Apis mellifera* have excess 3-hydroxykynurenine and impaired ommochrome pigment synthesis (Dustmann, 1968; Linzen, 1974; Summers and Howells, 1980). Comparative studies on these mutants should increase our understanding of ommochrome biosynthesis.

DATA ARCHIVING

Data available from the DDBJ repository: accession number DRA003068.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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