Insect science

Insect Science (2019) 26, 35–43, DOI 10.1111/1744-7917.12501

ORIGINAL ARTICLE

karmoisin and *cardinal* ortholog genes participate in the ommochrome synthesis of *Nilaparvata lugens* (Hemiptera: Delphacidae)

Shu-Hua Liu*, Ju Luo*, Bao-Jun Yang, Ai-Ying Wang and Jian Tang

State Key Laboratory of Rice Biology, China National Rice Research Institute, Hangzhou, China

Abstract Ommochrome is the major source for eye coloration of all insect species so far examined. Phenoxazinone synthetase (PHS) has always been regarded as the terminal step enzyme for ommochrome formation, which is encoded by *cardinal* or *karmoisin* genes. Our previous study indicated that the *karmoisin* ortholog gene (*Nl-karmoisin*) product in the brown planthopper (BPH) was a monocarboxylate transporter, while not a PHS. Here, based on full-length complementary DNA, the *cardinal* ortholog gene in BPH (*Nl-cardinal*) product was predicted to be a haem peroxidase rather than a PHS. We suggest for the first time that neither *karmoisin* nor *cardinal* encodes the PHS, but whether PHS participates in BPH eye pigmentation needs further research. Nymphal RNA interference (RNAi) experiments showed that knockdown *Nl-cardinal* transcript led the BPH ocelli and compound eye to color change from brown to red, while knockdown *Nl-karmoisin* only made the ocelli present the red phenotype. Notably, not only the *Nl-cardinal* transcript, dscd injection (*Nl-cardinal* targeting double-stranded DNA (dsRNA)) also significantly reduced the *Nl-karmoisin* transcript by 33.7%, while dska (*Nl-karmoisin* targeting dsRNA) injection did not significantly change the *Nl-cardinal* transcript. Considering the above RNAi and quantitative real-time polymerase chain reaction results, we propose that *Nl-cardinal* plays a more important role in ommochrome synthesis than *Nl-karmoisin*, and it may be an upstream gene of *Nl-karmoisin*. The present study suggested that both *karmoisin* and *cardinal* ortholog genes play a role in ommochrome synthesis in a hemimetabolous insect.

Key words *cardinal*; eye color; *karmoisin*; *Nilaparvata lugens*; ommochrome; phenoxazinone synthetase

Introduction

Ommochrome is the major source for eye coloration of all insect species so far examined, such as the fruit fly, silkworm, moths, bees, beetles, bugs, grasshopper and mosquitoes (reviewed by Grubbs *et al.*, 2015). Genetic analyses of *Drosophila* eye color mutants have revealed that ommochrome is derived from tryptophan through several enzyme-catalyzed reactions. In brief, tryptophan is oxidized by tryptophan dioxygenase (TDO) to *N*-formyl-*L*-kynurenine (NFK), followed by the hydrolysis of NFK to kynurenine by kynurenine formamidase (KFA); kynurenine may further be hydroxylated to 3-hydroxykynurenine (3-HK) by kynurenine 3-monooxygenase (KMO) (Rasgon & Scott, 2004; Han *et al.*, 2012). In *Drosophila*, TDO and KMO are encoded by genes of *vermilion* and *cinnabar*, respectively, which are named by the mutation eye-color of their corresponding enzyme (Searles & Voelker, 1986; Walker *et al.*, 1986; Warren *et al.*, 1996).

Correspondence: Jian Tang, State Key Laboratory of Rice Biology, China National Rice Research Institute, Hangzhou 311401, China. Tel: +86 571 63370331; fax: +86 571 63370399; email: tangjian@mail.hz.zj.cn

*These two authors contributed equally to this work.

3-HK is the precursor of ommochrome and is synthesized in cytoplasm, then is transported into pigment granules for final processing by heterodimeric ABC (adenosine triphosphate-binding cassette) transmembrane transporters, encoded by *white* and *scarlet* genes (Tearle *et al*., 1989; Pepling & Mount, 1990; Mackenzie *et al*., 2000). The early steps of ommochrome synthesis pathway are conservative in different insects and are clearly understood (Quan *et al*., 2002; Quan *et al*., 2007; Kômoto *et al*., 2009; Tatematsu *et al*., 2011). However, the molecular mechanisms involved in the conversion of 3-HK into ommochrome pigment are largely unknown, even in *Drosophila*.

Because ommochrome is a phenoxazinone chromophore compound, Phillips' team have indicated that it is formed from bimolecular oxidative condensation of 3-HK, and phenoxazinone synthetase (PHS) is just the catalyzing enzyme of this step (Phillips *et al*., 1970; Phillips & Forrest, 1970; Phillips *et al*., 1973; Wiley & Forrest, 1981). In some studies, the *Drosophila cardinal* gene product has been regarded as a PHS (Howells *et al*., 1977; Harris *et al*., 2011), because the *cardinal* mutant of *D. melanogaster* showed delayed eye pigmentation resulting from slow conversion of 3-HK to ommochrome (Howells *et al*., 1977; Mackenzie *et al*., 2000; Harris *et al*., 2011). Like *cardinal* mutant, *Drosophila karmoisin* mutant also accumulates excess 3-HK during larval life (Howells *et al*., 1977). So, in some other papers, *karmoisin* gene product was deemed as the PHS (Lloyd *et al*., 1998; Grubbs *et al*., 2015). Unfortunately, the relationships among *cardinal*, *karmoisin* and PHS have remained unclear until to now.

Osanai-Futahashi *et al*. (2016) indicated that *Drosophila cardinal* ortholog gene plays a major role in ommochrome synthesis of *Bombyx mori* and *Tribolium castaneum*, which are both holometabolous insects. This is the first evidence at the molecular level for the presence of *cardinal* ortholog gene functions in ommochrome synthesis. Whether *cardinal* ortholog gene plays a conserved role in hemimetabolous insects or not is still unknown. The *cardinal* ortholog gene product in *B. mori* has in fact been proven to be a haem peroxidase, not a PHS (Osanai-Futahashi *et al*., 2016). The *Drosophila karmoisin* ortholog gene product in *Nilaparvata lugens* (Stål) (Hemiptera: Delphacidae) is not a PHS either, which is predicted to be a member of MCTs (monocarboxylate transporters) (Liu *et al*., 2016). However, whether the *karmoisin* gene functions in eye pigmentation or not has not been further analyzed at the molecular level.

The brown planthopper (BPH), *N. lugens*, is a hemimetabolism insect and a notorious rice pest in East Asian countries (Cheng, 2009). The wild-type compound eye color of BPH is brown, while red-eye color mutation phenotype can also be observed (Seo *et al*., 2011; Liu *et al*., 2014). In order to clarify the ommochrome synthesis pathway of this insect pest, we cloned and characterized the *cardinal* ortholog gene from BPH. RNA interference (RNAi) study showed that BPH *cardinal* ortholog gene also plays a major role in ommochrome synthesis. Moreover, we also analyzed the function of *karmoisin* gene and its relationship with *cardinal* gene. This study is expected to contribute to the understanding of the ommochrome synthesis pathway of BPH and other similar insects. It also can help to reveal the red-eye color mutation mechanism of this insect pest.

Materials and methods

Insects

The BPH was a laboratory strain originally collected from China National Rice Research Institute in 2000. Insects were kept in a constant temperature incubator with rice plants at 27 ± 1°C, 80% ± 10% relative humidity, and a 16 h : 8 h L : D photoperiod. Synchronized insects were collected with the same method as Liu *et al*. (2015).

Total RNA isolation and cDNA synthesis

The total RNA was extracted using Trizol® reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. First-strand complementary DNA (cDNA) for RT-PCR (reverse transcription-polymerase chain reaction) was synthesized from 2 μg of total RNA using the reverse transcriptase (Moloney murine leukemia virus) with oligo dT18 (Promega, Madison, WI, USA). cDNAs for RACE (rapid-amplification of cDNA ends) were synthesized according to the Smart Race kit protocol (Clontech, Mountain View, CA, USA). cDNAs for quantitative RT-PCR (qRT-PCR) were synthesized from 1 μg of total RNA using the Primescript$^{\mathrm{TM}}$ RT reagent Kit with genomic DNA eraser (perfect real time) (TaKaRa, Dalian, China), according to the manufacturer's instructions.

Gene cloning and sequence analysis

For cloning the *cardinal* ortholog gene from BPH (*Nl-cardinal*), a translated Basic Local Alignment Search Tool nucleotide (tBLASTn) search of the *N. lugens* egg transcriptome database (unpublished) using the *D. melanogaster* Cardinal protein (Genbank number: NP_651081.1) as query, revealed one *cardinal-like*

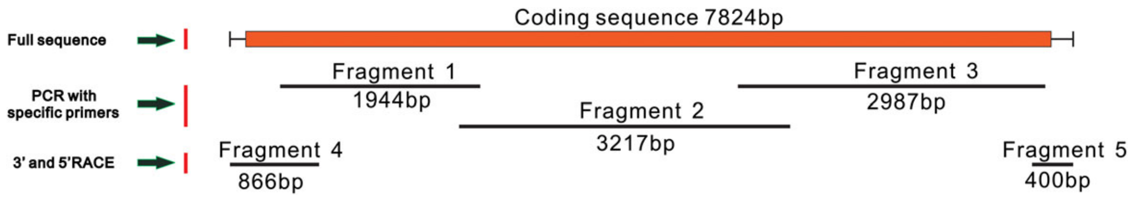

Fig. 1 The strategy used to amplify the full-length complementary DNA (cDNA) sequence of the *Nl-cardinal* gene. Lines below the full-length cDNA sequence represent each of the polymerase chain reaction fragments mentioned in Table 1. Fragment 2 was obtained from our transcriptome database. Fragments 1 and 3 were amplified with primers located in the predicted exons.

unigene with 3 501 bp in length (BGI_novel_G002008). This unigene was first used as the query to blast the BPH genome database (Genbank number: AOSB00000000.1), a scaffold was obtained (scaffold1 196). Then the genes in this scaffold were predicted with AUGUSTUS (http://augustus.gobics.de/). The predict gene including the sequence of BGI_novel_G002008 was collected for further research. Combining bioinformatics analysis, RT-PCR and RACE, the full-length cDNA sequence of *Nl-cardinal* was obtained with the strategy in Figure 1. Gene-specific primers (GSPs) designed to obtain overlapping PCR products are shown in Table 1.

The isoelectric point (pI) and molecular weight (MW) were analyzed by ExPASY Proteomic Server (http://ca.expasy.org/tools/pi_tool.html). The TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) was used to predict the transmembrane helices. The putative motifs and domains were analyzed with

Table 1 Oligonucleotide primers used for reverse transcription polymerase chain reaction (PCR), rapid-amplification of complementary DNA ends (RACE), quantitative real-time PCR and double-stranded RNA synthesis.

Purpose	Primer name[†]	Primer sequence (5′ to 3′)[‡]	PCR product (bp)
Gene clone	Cd-F1	CAGAGCCTCCAAGCCAAGGATG	1944
	Cd-R1	CAGACTGTCACAGGTCACTCTG	
	Cd-F2	GACCAGTTCAAGAGACTGAAAGTTGG	3217
	Cd-R2	TCTCTTCATTGTCAGGCTTGGGTG	
	Cd-F3	CTCAAGGCAGTATGCAGTTTGGAG	2987
	Cd-R3	GTCAGGCGAGTAATGACCTAGAAG	
5′RACE	Cd-T1 (outer)	GATCATCTCTGTATTCGGGTCTGTGG	>680
	Cd-T2 (inner)	TGGAGCTAGAACCCTTCTGAAC	>571
3′RACE	Cd-S1 (outer)	CTAGCTTATGCTCCATCTCAGGATG	>642
	Cd-S2 (inner)	TTGACGCATTCATGACAGCCGGTCAG	>146
RNA interference	dsCd-F	ggatcctaatacgactcactataggACGATTTCCTGTTGCGATGT	509
	dsCd-R	ggatcctaatacgactcactataggGCCACTATTTTCCTGCTTTC	
	dsKa-F	ggatcctaatacgactcactataggATTTGCTGCGTCTTTCTCC	314
	dsKa- R	ggatcctaatacgactcactataggGCGTATCATCAGCCGTAAT	
	dsGFP-F	ggatcctaatacgactcactataggATGCCACCTACGGCAAGCT	360
	dsGFP-R	ggatcctaatacgactcactataggTCGGCCATGATATAGACGTT	
Quantitative real-time PCR	Cd RT-F	CGACTATGCTGATGGTGT	100
	Cd RT-R	GTATTCGGGTCTGTGGAT	
	Ka RT-F	GGTCCGATTGCGTTCGACTTGTG	284
	Ka RT-R	TGGATGAGTTGCGAGGTGGCTGT	
	RPS15-F	CCGATCGTGTGGCGTTGAAGGG	150
	RPS15-R	ATGGCCGACATTCTTCCAGGTCC	
	TUB-F	ACTCGTTCGGAGGAGGCACC	174
	TUB-R	GTTCCAGGGTGGTGTGGGTGGT	

[†] Cd and Ka represent the *Nl-cardinal* and *Nl-karmoisin* gene of *N. lugens*, respectively.

[‡] The lower case letters in primers are the T7 promoter sequence.

InterPro (http://www.ebi.ac.uk/interpro/). The Molecular Evolutionary Genetic Analysis software version 6.0 (MEGA 6.0) (http://www.megasoftware.net/) was used to construct the phylogenetic tree using the neighbor-joining (NJ) method and the bootstrap values were calculated on 1000 replications.

RNAi experiments

In order to explore the function of *Nl-cardinal*, a 509 bp *Nl-cardinal* cDNA fragment was synthesized by PCR (exons 7–9), using GSPs incorporating the T7 RNA polymerase promoter sequence (Table 1). A previous verified plasmid was used as template. PCR product was purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) and used for double-stranded RNA (dsRNA) synthesis using the T7 Ribomax Express RNAi System (Promega, Madison, WI, USA). The synthesized dsRNA was respectively isopropanol-precipitated, resuspended in nuclease-free water, quantified by a spectrophotometry (NanoDrop 1 000, Thermo Fisher Scientific, USA) at 260 nm, and kept at –80°C until use.

Furthermore, to understand whether the *Drosophila karmoisin* ortholog gene participated in the ommochrome synthesis in BPH, we also synthesized the dsRNA targeting the *Nl-karmoisin* gene (Genbank number: KT304312), which is 314 bp in length (exons 2–4). As a control, a 400 bp enhanced green fluorescent protein (GFP) gene (Genbank number: GQ404376.1) dsRNA was also produced as described above.

Following carbon dioxide anesthesia, early third-instar nymphs were immobilized on the 1% agarose plate and 50 nL of purified dsRNA (5 μg/μL) was injected with the same method as that of Liu *et al.* (2010). The eye color phenotype was observed every day after injection, and the messenger RNA (mRNA) levels of *Nl-cadinal* and *Nl-karmoisin* were determined in 4 days after injection.

Quantitative real-time PCR

N. lugens were collected at 4 days after injection of dsRNA targeting *Nl-cardinal*, *Nl-karmoisin* and GFP to perform a digital gene expression (DGE) experiment. Total RNAs were extracted from the whole body using a Trizol kit. Quantitative real-time PCR reactions were performed on an ABI 9600 Real-time PCR system (Applied Biosystems, Foster City, CA, USA) using Power SYBR® Green PCR Master Mix (Applied Biosystems, Warrington, UK). Quantitative real-time PCR was performed in a 20 μL total reaction volume containing 4 μL diluted cDNA, 0.4 μL of each primer (10 μmol/L), 10 μL Master Mix (2×) and 5.2 μL ddH$_2$O. Ribosomal protein S15e (RPS15) (Genbank number: ACN79501.1) and α-tubulin (Genbank number: ACN79512.1) were used as internal controls, and the primers are the same as in Yuan *et al.* (2014). Thermocycling conditions were set as a standard quantitative PCR protocol according to the manufacturer's instruction. Data were analyzed by the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). Each quantitative real-time PCR experiment was performed in three independent biological replicates and analyzed in three technical replications. The specific primer pairs for each gene are provided in Table 1.

Results

Cloning and sequence analysis of the Nl-cardinal gene

Based on the initial cDNA fragment/unigene from the egg transcriptome database and its genome database (Xue *et al.*, 2014), the full-length cDNA sequence of the *cardinal* ortholog gene of *N. lugens* (hereafter referred to as *Nl-cardinal*) was obtained from multiple PCR amplifications, and the GenBank number is KY769575 (Fig. 1). The full-length *Nl-cardinal* cDNA (8 167 bp) contains an ORF (open reading frame) of 7824 nucleotides that encodes 2607 amino acid residues and it predicted a protein with molecular mass of 288.26 kDa and pI of 7.06 (Fig. S1). At the 3´- end of the *Nl-cardinal* cDNA sequence, a polyadenylation signal sequence AATAAA was apparent upstream of the poly A tail.

The genomic DNA (gDNA) sequence of the *Nl-cardinal* gene was found by BLASTn searching against the brown planthopper Genome Database with the full-length cDNA sequence of *Nl-cardinal* as a query. Subsequent genomic structure analysis showed that the *Nl-cardinal* gene contains 14 exons and a large last exon, following the GT-AG splicing rule (Fig. 2A). The first exon of *Nl-cardinal* is predicted not to encode amino acids but the untranslated region, which is the same as the *cardinal* gene of *B. mori* (Osanai-Futahashi *et al.*, 2016).

As shown in Figure S1 and Figure 2B, the predict protein of *Nl-cardinal* (hereafter referred to as Nl-Cardinal) has a hydrophobic transmembrane (TM) domain and a haem peroxidase domain at the positions of 37–59 and 211–747, respectively. In *B. mori* and *D. melanogaster*, *cardinal* homolog genes also encode proteins with a TM domain at the amino terminus and a haem peroxidase domain, respectively (Harris *et al.*, 2011; Osanai-Futahashi *et al.*, 2016). With the *D. melanogaster* chorion peroxidase gene product as an outgroup protein, phylogenetic

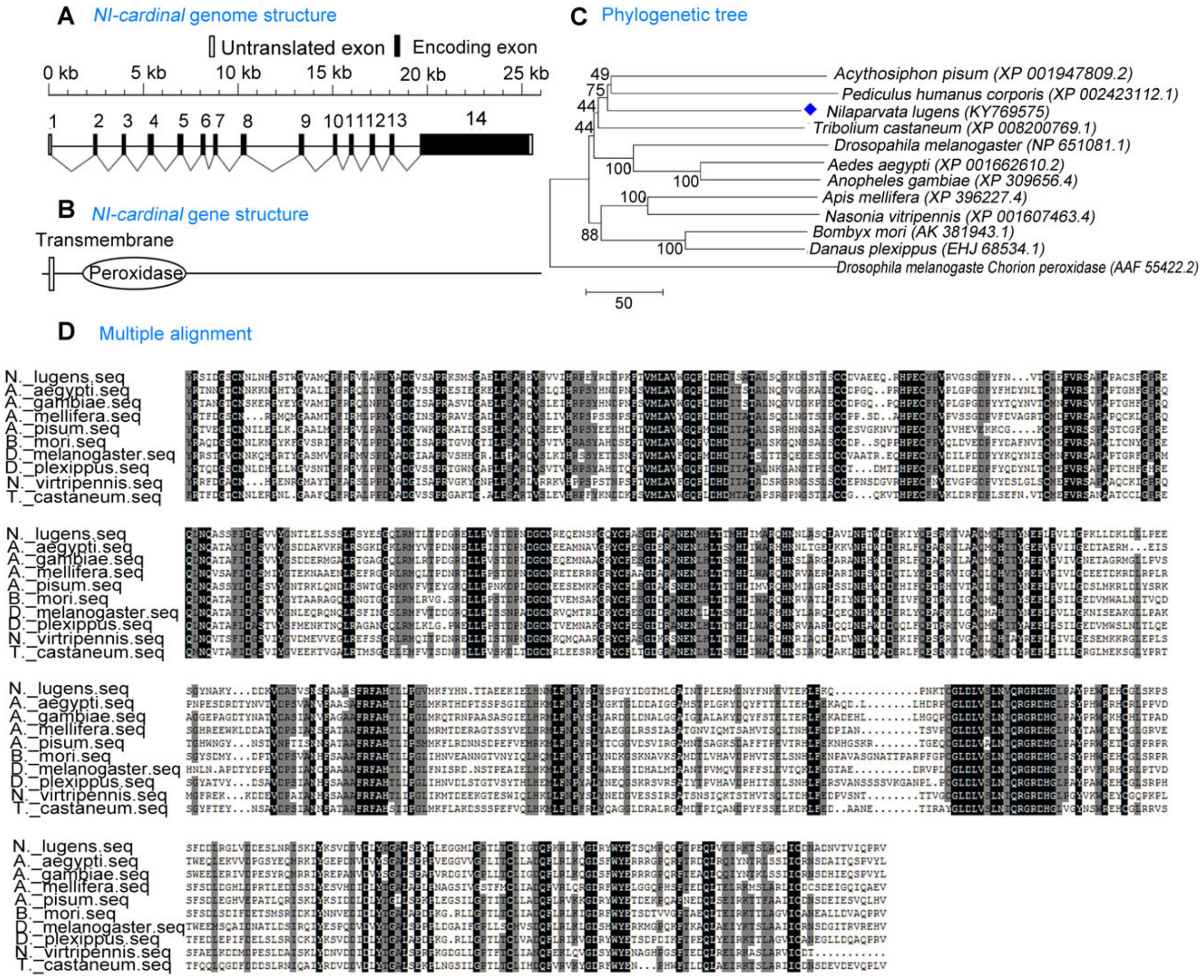

Fig. 2 Structure and phylogenetic analyses of the *Nl-cardinal* gene. (A) Genomic structure of the *Nl-cardinal* gene. The exons encoding amino acids are indicated by solid boxes, the untranslated exons are indicated by open boxes and the spaces between two boxes indicate the introns. The figure is drawn to scale, and the corresponding scale bar is shown. (B) The predicted protein structure of the *Nl-cardinal* gene product. (C) Phylogenetic relationship of the *cardinal* homolog gene products in insects. The *Drosophila melanogaster* chorion peroxidase gene product was included as an outgroup protein. Accession numbers are in brackets. (D) Alignment of peroxidase domains of the insect *cardinal* product homologs.

analysis revealed that proteins deduced from insect *cardinal* genes formed a monophyletic cluster (Fig. 2C) and amino acid sequences of the peroxidase domain were highly conserved among insects (Fig. 2D). These results indicated that the gene *Nl-cardinal* we cloned is the *cardinal* ortholog gene of *N. lugens*.

RNAi experiments

To investigate whether the *Nl-cardinal* gene is essential for BPH compound eye pigmentation, we performed nymphal RNAi experiments. The *Nl-cardinal* transcript in individuals injected with dscd (dsRNA targeting *Nl-cardinal*) decreased to 19.8% of that in the control ones at the 4th day after injection (Fig. 3A). Meantime, the compound eye color changed from brown to partially bright red, and this phenotype persisted throughout the rest of the life of this insect (Fig. 4A). The ocelli color also presented red since the 3rd day after injection (Fig. 4A). These results suggested that *Nl-cardinal* plays a major role in ommochrome synthesis affecting the pigmentation of not only the compound eye but also ocelli.

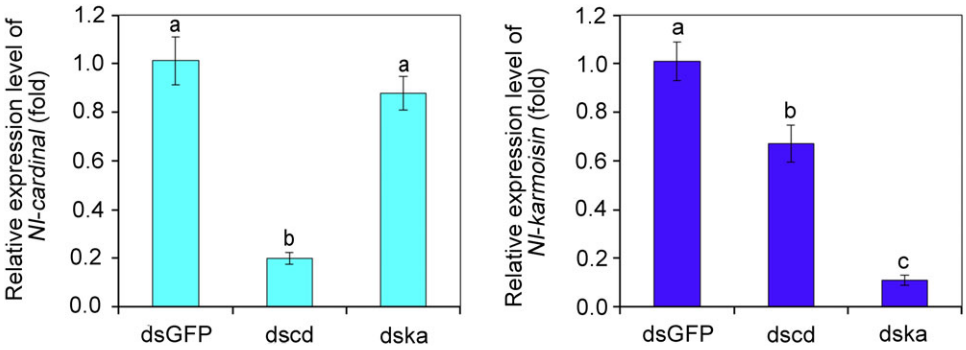

Fig. 3 Knockdown of *Nl-cardinal* and *Nl-karmoisin* transcripts by RNA interference (RNAi). (A) The relative expression level of *Nl-cardinal* at 4 days post-RNAi with dscd and dska. (B) The relative expression level of *Nl-karmoisin* at 4 days post-RNAi with dscd and dska. dsGFP, double-stranded RNA (dsRNA) targeting green fluorescent protein; dscd, dsRNA targeting *Nl-cardinal*; dska, dsRNA targeting *Nl-karmoisin*. Different lowercase letters above bars indicate significant differences between treatments ($P < 0.05$; Tukey's test; $n = 4$).

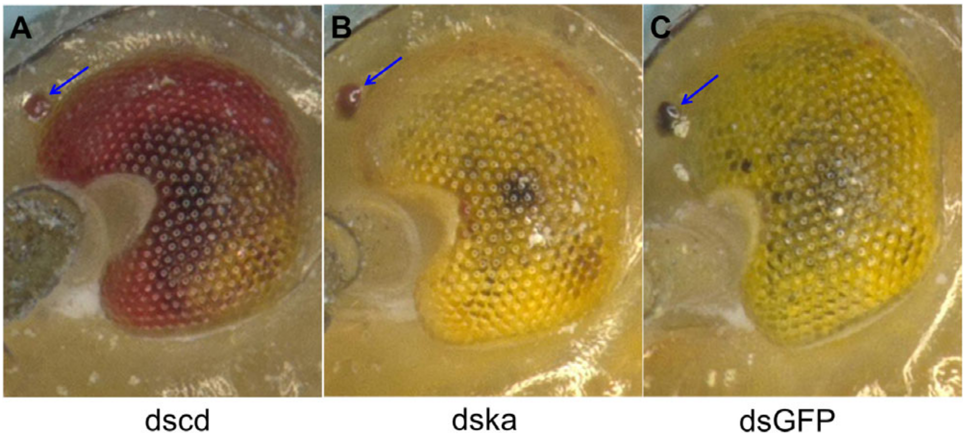

Fig. 4 Effects of RNA interference on compound eye and ocelli pigmentation at 2 days after eclosion. (A) Individuals injected with dscd (double-stranded RNA (dsRNA) targeting *Nl-cardinal*); (B) Individuals injected with dska (dsRNA targeting *Nl-karmoisin*); (C) Individuals injected with dsRNA targeting green fluorescent protein (dsGFP). Blue arrows indicate the ocellus.

Apart from *cardinal* gene, *karmoisin* gene product has also been deemed as the PHS, which catalyzes the terminal step of 3-HK to ommochrome (Phillips & Forrest, 1970; Phillips *et al*., 1973). So, in this study, we also evaluated the function of *karmoisin* ortholog gene in BPH, which is reported in our previous study (Liu *et al*., 2016). Injection of dska (dsRNA targeting *Nl-karmoisin* gene) reduced the *Nl-karmoisin* transcript by 89.1%, compared with dsRNA targeting GFP (dsGFP) injected individuals after 4 days (Fig. 3B). Different from dscd, the compound eye color of dska injected individuals had no change. However, the ocelli color changed from brown to red (Fig. 4B), which is the same as the dscd injected individuals. Furthermore, dscd injected individuals also had significantly lower *Nl-karmoisin* transcript compared with dsGFP injected individuals, which was only 66.3% of the control individuals (Fig. 3B). Different from dscd, dska injection did not significantly change the *Nl-cardinal* transcript level (Fig. 3A).

Discussion

Here, we cloned the full-length *cardinal* ortholog gene from BPH, which is named *Nl-cardinal*. Knockdown *Nl-cardinal* transcript can lead the compound eye color change from brown to partially red, and the ocelli also presented red phenotype throughout the whole adult lifetime, similar to individuals in which ommochrome-related gene *Nl-scarlet* was knocked down (Liu *et al*., 2017). So, it is reasonable to speculate that *Nl-cardinal* participated in the ommochrome synthesis of BPH, which is a hemimetabolous insect. *cardinal* ortholog gene is also proven to play a major role in ommochrome synthesis of holometabolous insects, such as *B. mori*, *T. castaneum* and *D. melanogaster* (Osanai-Futahashi *et al.*, 2016). The conservation of *cardinal* homolog function in *T. castaneum*, *B. mori*, *D. melanogaster* and *N. lugens*, and the widespread distribution of *cardinal* homologues in insects (Fig. 2C), suggest that *cardinal* ortholog genes play a conserved role in the ommochrome synthesis pathway of insects.

As mentioned in the Introduction section, PHS has always been deemed to catalyze the conversion of 3-HK to ommochrome of *D. melanogaster* (Ryall *et al.*, 1976; Yamamoto *et al.*, 1976; Wiley & Forrest, 1981). The original *Drosophila cardinal* gene product was regarded as a PHS (Howells *et al.*, 1977; Harris *et al.*, 2011). However, the *Nl-cardinal* product is predicted to be a haem peroxidase with a transmembrane domain and the peroxidase domain, which is the same as *Bm-cardinal* product (Osanai-Futahashi *et al.*, 2016). So it is safe to say that *cardinal* is not the PHS encoding gene. However, apart from *cardinal*, PHS has also been regarded as the *karmoisin* product in some other papers (Lloyd *et al.*, 1998; Grubbs *et al.*, 2015), because *Drosophila karmoisin* mutant has been shown to accumulate 3-HK just like *cardinal* mutant (Howells *et al.*, 1977; Tearle, 1991). The full-length cDNA has already been cloned in our previous study (Liu *et al.*, 2016). Homologous analysis showed that BPH *karmoisin* product is a member of MCTs (monocarboxylate transporters) with 11 predicted TMDs (transmembrane domains), while also not being a PHS (Liu *et al.*, 2016). The present study firmly suggested that neither *cardinal* nor *karmoisin* genes encode PHS, and PHS may be the product of a mysterious unknown gene. However, no reports have described the sequence of PHS in insects. Nie *et al.* (2014) indicated that *Streptomyces antibioticus* PHS like gene (BGIBMGA006740) may only function in melanin pigmentation rather than ommochrome pigmentation in *B. mori*. So, whether PHS participated in the eye pigmentation of insects needs further study.

Different from *Nl-cardinal*, knockdown *Nl-karmoisin* transcript did not change the compound eye color, but made the ocelli color turn into red (Fig. 4B). These results indicate that *Nl-karmoisin* plays a key role in ocelli pigmentation, while not the compound eye coloration. Note that *Nl-karmoisin* is not a tissue-specific gene, which can be detected in body wall, ovary, fat body, midgut and Malpighian tubule (Liu *et al.*, 2016). So *karmoisin* homolog genes may also have other than ocelli pigmentation functions in insects, which needs further study. Furthermore, dscd injected individuals also had significantly lower *Nl-karmoisin* transcript (Fig. 3B), while dska (dsRNA targeting *Nl-cardinal*) injection did not significantly change the *Nl-cardinal* transcript level (Fig. 3A). So, it is hard to conclude whether the red ocelli phenotype of dscd injected individuals is caused by the lower level of *Nl-cardinal* or *Nl-karmoisin*, which needs further research. Considering above RNAi and quantitative real-time PCR results, we propose that *Nl-cardinal* plays a more important role in ommchrome synthesis than *Nl-karmoisin*, and *Nl-cardinal* may be an upstream gene of *Nl-karmoisin*. Although they indeed affect the eye pigmentation, the specific roles in ommochrome synthesis are still unclear.

BPH is an important insect pest of rice (Cheng, 2009). Except the wild-type brown eye, red-eye color mutation phenotype can also be observed (Seo *et al.*, 2011; Liu *et al.*, 2014). Moreover, red eye mutation phenotype is also present in other Hemipterans, such as *Sogatella furcifera*, *Laodelphax striatellus* (Wang *et al.*, 2013) and several bugs (Shimizu & Kawasaki, 2001; Pires *et al.*, 2002; Snodgrass, 2002; Moraes *et al.*, 2005; Allen, 2013; Hull *et al.*, 2014). However, the mutation mechanisms of these insects are still unknown, limited by the lack of clarity regarding the eye pigment synthesis pathway. This is the first report suggests that *cardinal* and *karmoisin* genes participated in the ommochrome synthesis of a hemimetabolous insect, and *cardinal* may be an upstream regulation gene of *karmoisin*. These results will accelerate the research on the eye color mutation mechanisms of the above-mentioned Hemipteran mutants.

Acknowledgments

This work was supported by Zhejiang Provincial Natural Science Foundation of China (LY17C140004), the Special Fund for Basic Expenditure for Scientific and Research of Central Non-Profit Scientific Research Institutions (2014RG005-3) and the National High Technology Research and Development Program of China (2013AA102402). We would like to thank the Rice Pest Management Research Group of the Agricultural Science and Technology Innovation Program of China Academy of Agricultural Science.

Disclosure

The authors declare no conflict of interest.

References

Allen, M.L. (2013) Genetics of a sex-linked recessive red eye color mutant of the tarnished plant bug, *Lygus lineolaris*. *Open Journal of Animal Sciences*, 3, 1–9.

Cheng, J.A. (2009) Rice planthopper problems and relevant causes in China, in Planthoppers: new threats to the sustainability of intensive rice production systems in Asia. (eds. K. Heong & B. Hardy), pp. 157–178. International Rice Research Institute, Los Baños, the Philippines.

Grubbs, N., Haas, S., Beeman, R.W. and Lorenzen, M.D. (2015) The ABCs of eye color in *Tribolium castaneum*: orthologs of the *Drosophila white*, *scarlet*, and *brown* genes. *Genetics*, 199, 749–759.

Han, Q., Robinson, H. and Li, J. (2012) Biochemical identification and crystal structure of kynurenine formamidase from *Drosophila melanogaster*. *Biochemical Journal*, 446, 253–260.

Harris, D.A., Kim, K., Nakahara, K., Vásquez-Doorman, C. and Carthew, R.W. (2011) Cargo sorting to lysosome-related organelles regulates siRNA-mediated gene silencing. *The Journal of Cell Biology*, 194, 77–87.

Howells, A.J., Summers, K.M. and Ryall, R.L. (1977) Developmental patterns of 3-hydroxykynurenine accumulation in *white* and various other eye color mutants of *Drosophila melanogaster*. *Biochemical Genetics*, 15, 1049–1059.

Hull, J.J., Chaney, K., Geib, S.M., Fabrick, J.A., Brent, C.S. and Walsh, D. *et al*. (2014) Transcriptome-based identification of ABC transporters in the western tarnished plant bug *Lygus hesperus*. *PLoS ONE*, 9, e113046.

Kômoto, N., Quan, G.X., Sezutsu, H. and Tamura, T. (2009) A single-base deletion in an ABC transporter gene causes white eyes, white eggs, and translucent larval skin in the silkworm *w-3*^{oe} mutant. *Insect Biochemistry and Molecular Biology*, 39, 152–156.

Liu, S.H., Wang, A.Y., Yang, B.J., Luo, J. and Tang, J. (2017) Knockdown of an ABC transporter leads to bright red eyes in the brown planthopper, *Nilaparvata lugens* (Stål) (Hemiptera: Delphacidae). *Journal of Asia-Pacific Entomology*, 20, 421–428.

Liu, S.H., Yao, J., Yao, H.W., Jiang, P.L., Yang, B.J. and Tang, J. (2014) Biological and biochemical characterization of a red-eye mutant in *Nilaparvata lugens* (Hemiptera: Delphacidae). *Insect Science*, 21, 469–476.

Liu, S.H., Ding, Z.P., Zhang, C.W., Yang, B.J. and Liu, Z.W. (2010) Gene knockdown by intro-thoracic injection of double-stranded RNA in the brown planthopper, *Nilaparvata lugens*. *Insect Biochemistry and Molecular Biology*, 40, 666–671.

Liu, S.H., Tang, J., Luo, J., Yang, B.J., Wang, A.Y. and Wu, J.C. (2016) Cloning and characterization of *karmoisin* homologue gene (*Nlka*) in two brown planthopper strains with different eye colors. *Rice Science*, 23, 104–110.

Liu, S.H., Yang, B.J., Luo, J., Tang, J. and Wu, J.C. (2015) A comparative study on the population fitness of three strains of *Nilaparvata lugens* (Hemiptera: Delphacidae) differ in eye color related genes. *Journal of Economic Entomology*, 108, 1675–1682.

Livak, K.J. and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods*, 25, 402–408.

Lloyd, V., Ramaswami, M. and Krämer, H. (1998) Not just pretty eyes: *Drosophila* eye-color mutations and lysosomal delivery. *Trends in Cell Biology*, 8, 257–259.

Mackenzie, S.M., Howells, A.J., Cox, G.B. and Ewart, G.D. (2000) Sub-cellular localisation of the White/Scarlet ABC transporter to pigment granule membranes within the compound eye of *Drosophila melanogaster*. *Genetica*, 108, 239–252.

Moraes, A.S., Pimentel, E.R., Rodrigues, V.L. and Mello, M.L. (2005) Eye pigments of the blood-sucking insect, *Triatoma infestans* Klug (Hemiptera, Reduviidae). *Brazilian Journal of Biology*, 65, 477–481.

Nie, H., Liu, C., Cheng, T., Li, Q., Wu, Y. and Zhou, M. *et al*. (2014) Transcriptome analysis of integument differentially expressed genes in the pigment mutant (*quail*) during molting of silkworm, *Bombyx mori*. *PLoS ONE*, 9, e94185.

Osanai-Futahashi, M., Tatematsu, K., Futahashi, R., Narukawa, J., Takasu Y. and Kayukawa, T. *et al*. (2016) Positional cloning of a *Bombyx pink-eyed white egg* locus reveals the major role of *cardinal* in ommochrome synthesis. *Heredity*, 116, 135–145.

Pepling, M. and Mount, S.M. (1990) Sequence of a cDNA from the *Drosophila melanogaster white* gene. *Nucleic Acids Research*, 18, 1633.

Phillips, J.P., Forrest, H.S. and Kulkarni, A.D. (1973) Terminal synthesis of xanthommatin in *Drosophila melanogaster*. III. Mutational pleiotropy and pigment granule association of phenoxazinone synthetase. *Genetics*, 73, 45–56.

Phillips, J.P. and Forrest, H.S. (1970) Terminal synthesis of xanthommatin in *Drosophila melanogaster*. II. Enzymatic formation of the phenoxazinone nucleus. *Biochemical Genetics*, 4, 489–498.

Phillips, J.P., Simmons, J.R. and Bowman, J.T. (1970) Terminal synthesis of xanthommatin in *Drosophila melanogaster*. I. Roles of phenol oxidase and substrate availability. *Biochemical Genetics*, 4, 481–487.

Pires, H.H.R., Abrão, D.O., Machado, E.M.d.M., Scho-field, C.J. and Diotaiuti, L. (2002) Eye color as a genetic marker for fertility and fecundity of *Triatoma infestans* (Klug, 1834) Hemiptera, Reduviidae, Triatominae. *Memórias do Instituto Oswaldo Cruz*, 97, 675–678.

Quan, G.X., Kim, I., Kômoto, N., Sezutsu, H., Ote, M. and Shimada, T. *et al*. (2002) Characterization of the kynurenine 3-monooxygenase gene corresponding to the *white egg 1* mutant in the silkworm *Bombyx mori*. *Molecular Genetics and Genomics*, 267, 1–9.

Quan, G.X., Kobayashi, I., Kojima, K.A., Uchino, K., Kanda, T. and Sezutsu, H. *et al*. (2007) Rescue of *white egg 1* mutant by introduction of the wildtype *Bombyx* kynurenine 3-monooxygenase gene. *Insect Science*, 14, 85–92.

Rasgon, J.L. and Scott, T.W. (2004) *Crimson*: A novel sex-linked eye color mutant of *Culex pipiens* L. (Diptera: Culicidae). *Journal of Medical Entomology*, 41, 385–391.

Ryall, R.L, Ryall, R.G. and Howells, A.J. (1976) The ommochrome biosynthetic pathway of *Drosophila melanogaster*: The Mn^{2+}-dependent soluble phenoxazinone synthase activity. *Insect Biochemistry*, 6, 135–142.

Searles, L.L. and Voelker, R.A. (1986) Molecular characterization of the *Drosophila vermilion* locus and its suppressible alleles. *Proceedings of the National Academy of Sciences USA*, 83, 404–408.

Seo, B.Y., Jung, J.K. and Kim, Y. (2011) An orange-eye mutant of the brown planthopper, *Nilaparvata lugens* (Hemiptera: Delphacidae). *Journal Asia-Pacific Entomology*, 14, 469–472.

Shimizu, T. and Kawasaki, K. (2001) Red-eye mutants in Orius bugs (Heteroptera: Anthocoridae). *Applied Entomology and Zoology*, 36, 185–187.

Snodgrass, G.L. (2002) Characteristics of a red-eye mutant of the tarnished plant bug (Heteroptera: Miridae). *Annals of the Entomological Society of America*, 95, 366–369.

Tatematsu, K., Yamamoto, K., Uchino, K., Narukawa, J., Iizuka, T. and Banno, Y. *et al*. (2011) Positional cloning of silkworm *white egg 2* (*w-2*) locus shows functional conservation and diversification of ABC transporters for pigmentation in insects. *Genes Cells*, 16, 331–342.

Tearle, R.G., Belote, J.M., McKeown, M., Baker, B.S. and Howells, A.J. (1989) Cloning and characterization of the *scarlet* gene of *Drosophila melanogaster*. *Genetics*, 122, 595–606.

Tearle R.G. (1991) Tissue specific effects of ommochrome pathway mutations in *Drosophila melanogaster*. *Genetics Research*, 57, 257–266.

Walker, A.R., Howells, A.J. and Tearle, R.G. (1986) Cloning and characterization of the *vermilion* gene of *Drosophila melanogaster*. *Molecular and General Genetics*, 202, 102–107.

Wang, L., Zhuang, Z., Li, Y. and Fang, J. (2013) Biological characteristics and mating advantage of the red-eye mutant of the small brown planthopper, *Laodelphax striatellus* (Hemiptera: Delphacidae). *Acta Entomologica Sinica*, 56, 878–883.

Warren, W.D., Palmer, S. and Howells, A.J. (1996) Molecular characterization of the cinnabar region of *Drosophila melanogaster*: identification of the *cinnabar* transcription unit. *Genetica*, 98, 249–262.

Wiley, K. and Forrest, H.S. (1981) Terminal synthesis of xanthommatin in *Drosophila melanogaster*. IV. Enzymatic and nonenzymatic catalysis. *Biochemical Genetics*, 19, 1211–1221.

Xue, J., Zhou, X., Zhang, C.X., Yu, L.L., Fan, H.W. and Wang, Z. *et al*. (2014) Genomes of the rice pest brown planthopper and its endosymbionts reveal complex complementary contributions for host adaptation. *Genome Biology*, 15, 521.

Yamamoto, M., Howells, A.J. and Ryall, R.L. (1976) The ommochrome biosynthetic pathway in *Drosophila melanogaster*: the head particulate phenoxazinone synthase and the developmental onset of xanthommatin synthesis. *Biochemical Genetics*, 14, 1077–1090.

Yuan, M., Lu, Y., Zhu, X., Wan, H., Shakeel, M. and Zhan, S. *et al*. (2014) Selection and evaluation of potential reference genes for gene expression analysis in the brown planthopper, *Nilaparvata lugens* (Hemiptera: Delphacidae) using reverse-transcription quantitative PCR. *PLoS ONE*, 9, e86503.

Manuscript received March 27, 2017
Final version received June 2, 2017
Accepted June 7, 2017

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1 Nucleotide and deduced amino acid sequence of the *Nl-cardinal* gene. Numbers on the left indicate the nucleotide (upper) and amino acid (lower) position of the *Nl-cardinal* gene. The start codon (ATG), stop codon (TAA), and putative polyadenylation signal (AATAAA) of the complementary DNA sequence are highlighted in green with bold characters. The predicted transmembrane domain is indicated in brown with red characters. The signature motif of haem peroxidase gene is shaded in yellow with black characters.