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Cloning and Characterization of *karmoisin* Homologue Gene (*Nlka*) in Two Brown Planthopper Strains with Different Eye Colors

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Abstract: The brown planthopper (BPH), *Nilaparvata lugens*, is a destructive insect pest of rice throughout Asia. Different from brown-eye color wild type, BPH also has red-eye color mutation phenotype. As a visible genetic marker, the red-eye mutant in BPH is a valuable material. To reveal the eye color mutation mechanism, a *karmoisin* homologue gene (named as *Nlka*) was cloned from BPH. And *karmoisin* is always deemd as a xanthommatin-related gene in other insects, encoding phenoxazinone synthetase (PHS). *Nlka* is consisted of 7 exons and encodes a protein with 502 amino acids (NlKA). NlKA showed high amino acid identities with its insect homologues (48.8%–51.8%). *Nlka* transcripts can be detected at all the developmental stages and in all tissues tested, including egg, nymph, adult, body wall, ovary, fat body, midgut and Malpighian tubule. However, no constant In/Del or non-synonymous mutation was observed between the mutant and the wild type strains. Quantitative real-time PCR experiment also showed that *Nlka* transcript level had no significant differences between them. These results indicated that *Nlka* is not the target gene causing the red-eye color mutation phenotype of BPH. Through the second structure and motif analysis, the present study also showed that all the proteins deduced from the *karmoisin* genes in insects may be members of monocarboxylate transporters (MCTs) rather than PHSs.

Key words: *Nilaparvata lugens*; red-eye mutant; *karmoisin*; monocarboxylate transporter; phenoxazinone synthetase; rice; gene clone

Compound eye color is an important biological characteristic of insects, which is largely determined by the nature of pigments (Ichiki et al, 2007). Xanthommatin (brown pigment) and pteridine (red pigment) are two key eye pigments. In Dipterans, eye color is determined by the presence of xanthommatin and pteridine (Beard et al, 1995; Challoner and Gooding, 1997; Ooi et al, 1997). However, the level and redox status of xanthommatin are the only biochemical basis of eye color in *Culex pipiens* (Rasgon and Scott, 2004), *Bombyx mori* (Kômoto et al, 2009) and *Tribolium castaneum* (Lorenzen et al, 2002). Compared with pteridine, the xanthommatin biosynthesis pathway is relatively well understood. It is started with the oxidation of tryptophan to N-formyl-*L*-kynurenine by tryptophan 2,3-dioxygenase, followed by the hydrolysis of N-formyl-*L*-kynurenine to kynurenine by kynurenine formamidase. Kynurenine is hydroxylated to 3-hydroxykynurenine by kynurenine 3-monooxygenase. Then 3-hydroxykynurenine is transformed to xanthommatin by enzymatic or non-enzymatic reaction (Rasgon and Scott, 2004; Han et al, 2012).

karmoisin is a xanthommatin-related gene (Lloyd et al, 1998; Grubbs et al, 2015). Lloyd et al (1998) have deemed that *karmoisin* encodes phenoxazinone synthetase (PHS), which catalyzes the reaction of

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3-hydroxykynurenine to xanthommatin.

The brown planthopper (BPH), *Nilaparvata lugens* (Stål) (Hemiptera: Delphacidae), is a notorious rice phloem sap-sucker and plant virus vector in East Asian countries. It causes huge economic loss to rice production by direct feeding and virus transmission during its long-distance migration (Cheng, 2009). In 2009, a red-eye BPH mutant colony was established in our laboratory. Our previous research indicated that this mutant has dramatically declined xanthommatin content, which is only 36.3% of those in normal ones (Liu et al, 2014). This preliminary study of mutation mechanism reports the cloning of a *karmoisin* homologue gene (*Nlka*) from BPH, as well as the comparative analysis of its sequence variation and expression level between mutant and wild type strains. In addition, its expression characteristics in different developmental stages and various tissues were also studied. The present study will contribute to the revealing of eye color mutation mechanism of this insect pest.

MATERIALS AND METHODS

Insects and tissues

BPH were collected from the wild type brown-eye colony (NIL-BB) and the red-eye mutant colony (NIL-rr), which are near-isogenic lines (NILs) (Liu et al, 2015). They were reared in light growth chamber under the conditions of 27 ºC ± 1 ºC, 80% ± 10% relative humidity and a photoperiod of 16 h light and 8 h dark. To obtain synchronized insects, newly hatched nymphs were collected every 24 h and placed into glasses containing fresh rice seedlings, which were fixed and moisturized by 1% water agar. The developmental stages were synchronized at each larval molt. The 1st, 3rd, 5th instar nymphs, newly emerged females and males were collected and frozen in liquid nitrogen for RNA extraction.

Body wall, ovary, midgut, Malpighian tubule, head, thorax and abdomen were dissected from female individuals, and fat body was from the 5th instar larvae which were all with brown eyes. They were dissected in cold phosphate buffer solution (1×, pH 7.0), and immediately frozen in liquid nitrogen for RNA extraction.

Total RNA isolation and reverse transcription

Total RNA was extracted using Trizol kit (Life, UK). First-strand cDNA for reverse transcription polymerase chain reaction (RT-PCR) was synthesized from 2 μg of total RNA using the reverse transcriptase (M-MLV) and oligo dT_{18} (Promega, USA). The cDNA for rapid amplification of cDNA ends (RACE) was synthesized according to the Smart Race kit protocol (Clontech, Mountain View, CA). The cDNA for quantitative real-time PCR (qRT-PCR) was synthesized from 1 μg DNA Eraser (perfect real time) (TaKaRa, China) treated total RNA using the Primescript™ RT reagent kit in accordance with the manufacturer's instructions.

Gene cloning and sequence variation detection

Nlka was identified using bioinformatics and RT-PCR. A *karmoisin*-like unigene was found from *N. lugens* transcriptome database. This unigene was firstly verified using primer pairs (Table 1). Full-length cDNA was obtained with gene specific primers (Table 1) and SMARTer™ RACE cDNA Amplification kit (Clontech, Mountain View, CA) following the manufacturer's instructions. RT-PCR conditions, cloning and sequencing methods agree with that of Liu et al (2010). After large-scale cloning and sequencing (10 clones from two independent cDNA batches), a comparison of the full-length *Nlka* cDNA from NIL-BB and NIL-rr strains was performed on the acquired data for detecting sequence variations.

In addition, a BLASTn search of the *N. lugens* genome database (GenBank number: AOSB00000000) using the full-length *Nlka* cDNA (including the 5′-untranslated region and 3′-untranslated region) revealed its alternative splicing forms.

Quantitative real-time PCR (qRT-PCR)

qRT-PCR reactions were performed on an ABI 9600 real-time PCR system (Applied Biosystems, USA) using Power SYBR® Green PCR Master Mix (Applied Biosystems, UK) and gene specific primers (Table 1). *β-actin* (GenBank number: EU179846) was used as an internal control. PCR was performed in 20 μL reaction

Table 1. Oligonucleotide primers used for reverse transcription polymerase chain reaction (RT-PCR), rapid amplification of cDNA ends (RACE) and quantitative real-time PCR (qRT-PCR).

Primer	Sequence (5′–3′)	Description
ka-F1	ATGTCGGAAACCGAGAAAGTGC	RT-PCR
ka-R1	GAACAAACAGAGGCAGGGATGC	
ka-F2	GTCCGATTGCGTTCGACTTGTG	3′-RACE
ka-R2	CTCCGCTGCAACTCCACATAGACC	5′-RACE
ka-F3	GGTCCGATTGCGTTCGACTTGTG	qRT-PCR
ka-R3	TGGATGAGTTGCGAGGTGGCTGT	
β-actin F	ACGCCATCCTCCGTCTGGACTT	
β-actin R	CAAAGTCCAGGGCAACGTAGCA	