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Long-wave opsin involved in body color plastic development in *Nilaparvata lugens*

Jia-Bao Lu^{1†}, Ze-Dong Li^{2†}, Zhuang-Xin Ye¹, Hai-Jian Huang¹, Jian-Ping Chen¹, Jun-Min Li¹ and Chuan-Xi Zhang^{1,2*}

Abstract

Background As one of the components of visual photopigments in photoreceptor cells, opsin exhibits different spectral peaks and plays crucial roles in visual function. Besides, it is discovered to evolve other functions despite color vision. However, research on its unconventional function is limited nowadays. With the increase in genome database numbers, various numbers and types of opsins have been identified in insects due to gene duplications or losses. The *Nilaparvata lugens* (Hemiptera) is a rice pest known for its long-distance migration capability. In this study, opsins were identified in *N. lugens* and characterized by genome and transcriptome analyses. Meanwhile, RNA interference (RNAi) was carried out to investigate the functions of opsins, and then the Illumina Novaseq 6000 platform-based transcriptome sequencing was performed to reveal gene expression patterns.

Results Four opsins belonging to G protein-coupled receptors were identified in the *N. lugens* genome, including one *long-sensitive opsin* (*Nllw*) together with two *ultraviolet-sensitive opsins* (*NIUV1/2*) and an additional new *opsin* with hypothesized UV peak sensitivity (*NIUV3-like*). A tandem array of *NIUV1/2* on the chromosome suggested the presence of a gene duplication event, with similar exons distribution. Moreover, as revealed by spatiotemporal expression, the four *opsins* were highly expressed in eyes with age-different expression levels. Besides, RNAi targeting each of the four *opsins* did not significantly affect the survival of *N. lugens* in phytotron, but the silencing of *Nllw* resulted in the melanization of body color. Further transcriptome analysis revealed that silencing of *Nllw* resulted in up-regulation of a *tyrosine hydroxylase* gene (*NITH*) and down-regulation of an *arylalkylamine-N-acetyltransferases* gene (*Nlaa*NAT) in *N. lugens*, demonstrating that *Nllw* is involved in body color plastic development via the tyrosine-mediated melanism pathway.

Conclusions This study provides the first evidence in a Hemipteran insect that an opsin (*Nllw*) takes part in the regulation of cuticle melanization, confirming a cross-talk between the gene pathways underlying the visual system and the morphological differentiation in insects.

Keywords Opsin, Melanization, Body color, RNAi, Transcriptome, Genome, *Nilaparvata lugens*

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Background

The visual system is essential for insects to collect abundant information from their surroundings and respond appropriately, including making choices among alternative behaviors, foraging, and mating [1–4]. Generally, the spectral sensitivity of photoreceptor cells is determined by the photopigment, a complex of chromophore and opsin [5]. The chromophore, usually the 11-*cis*-retinal derived from vitamin A, forms a covalent bond with a lysine residue (K) in the opsin, a light-activated G-protein coupled receptor, via a Schiff base [6–8]. Opsins can be usually categorized into three groups based on their spectral peaks, namely, long-wavelength-sensitive (LW), blue-sensitive (B), and ultraviolet (UV)-sensitive proteins [9]. The majority of insects possess this ancestral trichromatic visual system, such as *Drosophila melanogaster* (Diptera) [10, 11], *Apis mellifera* (Hymenoptera) [12], *Spodoptera exigua* (Lepidoptera) [13], and *Nephotettix cincticeps* (Hemiptera) [14]. As more and more genome databases have been constructed, insects are found to possess various numbers or types of opsins due to gene duplications or losses [15]. For instance, one UV-sensitive, five B-sensitive, and up to ten LW-sensitive opsins are present in *Sympetrum frequens* (Odonata) [16]; meanwhile, the ancestral B-sensitive opsin is lost in Coleoptera insects, but the trichromacy at least twelve times is regained by duplicating the UV-sensitive and LW-sensitive opsin genes [17, 18].

In *Drosophila*, Rh1-6 opsins are expressed in the compound eyes, among which, Rh1 long-sensitive opsin is expressed in photoreceptor cells 1–6, Rh3/4 UV-sensitive opsins in photoreceptor 7 cells, whereas photoreceptor 8 cells express the Rh5 opsin for blue light detection and the Rh6 opsin for green light detection [11, 19, 20]. Another opsin, Rh7, found in the central brain, helps regulate circadian light entrainment by circadian pacemaker neurons [21]. In addition to the above ancestral visual functions, insect opsins have also evolved additional functions that are crucial for insect survival and fitness [22, 23]. For instance, in *Drosophila*, Rh1 plays a crucial role in the initiation of thermosensory signaling cascades for thermotaxis [24]; additionally, two other opsins, Rh5 and Rh6, function in the brain and body wall to support thermal preference during the late-3rd instar larvae [25]; furthermore, Rh6 is also responsible for the cooling-induced activation of bitter gustatory receptor neurons [26]; and opsins are also suggested to be linked to morphological differentiation, like the evolution of distinct yellow wing pigment along with a second UV opsin in *Heliconius* butterflies [27]. Despite the extensive research regarding the impact of opsins on the visual function across various insect species [23], the unconventional roles of these opsins in insects remain largely mysterious.

The *Nilaparvata lugens* (Hemiptera), belonging to the Delphacidae family, is the most destructive rice pest in Asia. It is particularly notable for its wing dimorphism and the capability of long-distance migration, allowing it to exploit its exclusive rice host in temperate regions and inflict significant damage on rice crops [28]. A previous study has identified three opsin genes in *N. lugens* genome, including one LW- and two UV-absorbing opsins (*Nllw*, and *NIUV1/2*, respectively) [14]. However, it is still unknown whether there are other functions except for visual sensing. In this study, opsin genes in the *N. lugens* were systematically investigated based on transcriptomic and genomic data, and an additional new opsin with hypothesized UV peak sensitivity (*NIUV3-like*) was identified. Thereafter, the functions of these four opsin genes were investigated, respectively. According to our results, *Nllw* took part in the melanization of body color and might be involved in the tyrosine-mediated cuticle melanism pathway. As far as we know, this study is the first to reveal the unconventional function of opsins in Hemiptera and suggests a cross-talk between gene pathways underlying the visual system and the morphological differentiation in insects.

Results

Identification of opsin genes from the genome and transcriptome databases

To identify the potential opsin genes in *N. lugens*, the *Drosophila ninaE* protein sequence (NP_524407.1) was used as a query to search the corresponding genome and transcriptome databases of *N. lugens* [29, 30]. As a result, altogether four opsin genes were identified, including the previously reported *Nllw* (GenBank accession number: AB761147.1), *NIUV1* (GenBank accession number: AB761148.1), *NIUV2* (GenBank accession number: AB761149.1), and an additional paralog. Blast analysis of the paralog revealed a 99% identity with a UV-sensitive opsin predicted from an *N. lugens* genome sequence (GenBank accession number: NC_052505.1), indicating that a UV-sensitive-like opsin (*NIUV3-like*) (GenBank accession number: XP_022205432) was detected in *N. lugens*.

Sequence and phylogenetic analysis

The four opsins, *Nllw*, *NIUV1/2*, and *NIUV3-like* were predicted to be G protein-coupled receptors with seven transmembrane α -helices (TMH1-7) [6] and contained a conserved K in TMH7, which covalently binds to the chromophore [7, 8]. A total of 386, 380, 380, and 499 amino acids (aa) peptides were predicted in *Nllw*, *NIUV1/2*, and *NIUV3-like*, respectively. The conserved motif histidine-glutamine-lysine (HEK) involved in G-protein binding [31] was present at the C-terminal side of TMH5 of *Nllw* and *NIUV1/2*, but not in *NIUV3-like*.

(Fig. 1A). Moreover, these opsins were located on different chromosomes in the *N. lugens* genome, with *Nllw* and *NIUV3-like* on chromosomes (Chr) 13 and 01, separately; whereas *NIUV1/2* with tandem distribution on Chr 04 (Fig. 1B). Structural analysis revealed differences in the number of exons spanning TMH1-7, namely, 2nd – 8th (E2-8) in *Nllw*, 2nd – 7th (E2-7) in *NIUV1/2*, and all the four exons (E1-4) in *NIUV3-like* (Fig. 1C). Furthermore, a phylogenetic tree analysis suggested that the *Nllw*, *NIUV1*, and *NIUV2* clustered well with the long-wave opsins, *UVopsin1*, and *UVopsin2* in *Sogatella furcifera* (Hemiptera) and *Laodelphax striatellus* (Hemiptera), respectively, which was consistent with a previous study [14]. However, the *NIUV3-like* was distinct from *NIUV1/2*, which demonstrated strong clustering with the Rh7 (GenBank accession number: NP_524035.2) in *D. melanogaster*, with Rh4 (GenBank accession number: XP_021702394.1) in *Aedes aegypti* (Diptera), as well as with Rhodopsin7 (GenBank accession number: QDQ16904.1) and a UV-like opsin (GenBank accession number: XP_032513103.1) in *Danaus plexippus* (Lepidoptera) (Fig. 2). Overall, the sequence characteristics, Chr location, gene structure, and phylogenetic analysis highlights that *Nllw*, *NIUV1/2*, and *NIUV3-like* are divided into three distinct subfamilies. And the emergence of *NIUV1/2* is likely attributed to the duplication of a common ancestor opsin. Meanwhile, *NIUV3-like* shares homology with other opsin variants, such as Rh7 in *D. melanogaster*, which helps regulate circadian light entrainment in circadian pacemaker neurons [21].

Spatiotemporal expression patterns

Temporal expression patterns were analyzed with the fragments per kilobase per million mapped fragment (FPKM) values based on the transcriptome sequencing data. According to our results, *Nllw* was expressed continuously after the hatching of the 1st instar nymph, with relatively stable expression levels in the nymph and female adult stages, but it reached the highest expression level in the male adult stage. For *NIUV1/2* and *NIUV3-like*, the expression patterns varied obviously. The expression levels of *NIUV1/2* peaked in the male adult stage, similar to *Nllw*, but *NIUV1* was barely expressed from the egg stage to the 4th instar nymph stage and showed low expression in the female adult stage. *NIUV2*, on the other hand, exhibited continuous expression from late embryogenesis, a stage when the cuticle of the 1st instar nymph began to form [32]. Besides, *NIUV2* was periodically expressed with molting, with high levels being detected at the beginning and the end of each instar. Whereas *NIUV3-like* showed a similar periodic expression pattern to *NIUV2*, the expression level of *NIUV3-like* peaked in the hatching stage rather than in the male adult period (Fig. 3A). Overall, *Nllw*, *NIUV1/2*, and *NIUV3-like*

exhibited exceptionally high expression levels in eyes (Fig. 3B), indicating that all the four opsins are involved in visual processing, albeit with the possibility of divergent functions depending on age-related expression patterns.

Nllw is associated with melanin-type pigment

The expression levels of *Nllw*, *NIUV1/2*, and *NIUV3-like* were detected in all the RNAi-treated groups (Fig. 4A). The target genes were all effectively silenced by RNAi with the early 4th instar nymph *N. lugens*, compared with the dsGFP control group. Moreover, the expression levels of the other three opsins were also detected with each RNAi treatment. When *Nllw* was knocked-down, the expression levels of *NIUV1/2* were both significantly up-regulated. Conversely, silencing of either *NIUV1/2* resulted in a significant up-regulation of *Nllw*, suggesting a possible functional complementation between *Nllw* and *NIUV1/2*. However, the down-regulation of either *NIUV1/2* led to the silencing of the other one, probably attributed to their 77.97% sequence similarity. It is noteworthy that the silencing of *NIUV3-like* did not impact the expression of *Nllw* and *NIUV1/2*. Similarly, the silencing of each of *Nllw* and *NIUV1/2* did not affect the expression of *NIUV3-like*, indicating that the expression of *NIUV3-like* is independent of *Nllw* and *NIUV1/2*. Among the four opsins in *N. lugens*, RNAi against each target gene did not lead to an apparent lethal phenotype, and there was no significant difference in survival rate compared with the dsGFP control group (Fig. 4B). However, an unconventional function was observed in the ds*Nllw*-treated group (Fig. 4C). To be specific, gradual accumulation of melanin occurred when *Nllw* was knocked-down with the 4th instar nymph. When *N. lugens* developed into the 5th instar, the rigid cuticles of the mesothorax, abdominal dorsal cuticle, leg, wing bud, and head became darker; while the other soft and flexible cuticles, including the abdominal ventral plate as well as the segmacoria, were only slightly affected. When *N. lugens* further developed into adults, melanin-type pigments occurred on almost the whole body surface, except for soft parts of leg bases of the mesothorax. In addition, the short-hind wings also became darker. While in dsGFP-treated control group, *N. lugens* usually remained brown, with only slight darkening in male adult, but the darkening degree was far less than the ds*Nllw*-treated group *N. lugens*, and the hind-wing of adult was light color in general. To avoid the off-target effect of RNAi, the other non-overlapping RNAi region of ds*Nllw* was designed (Fig 1A). RNAi in two different regions featured similar phenotypes, demonstrating that off-targeting did not occur in this experiment.

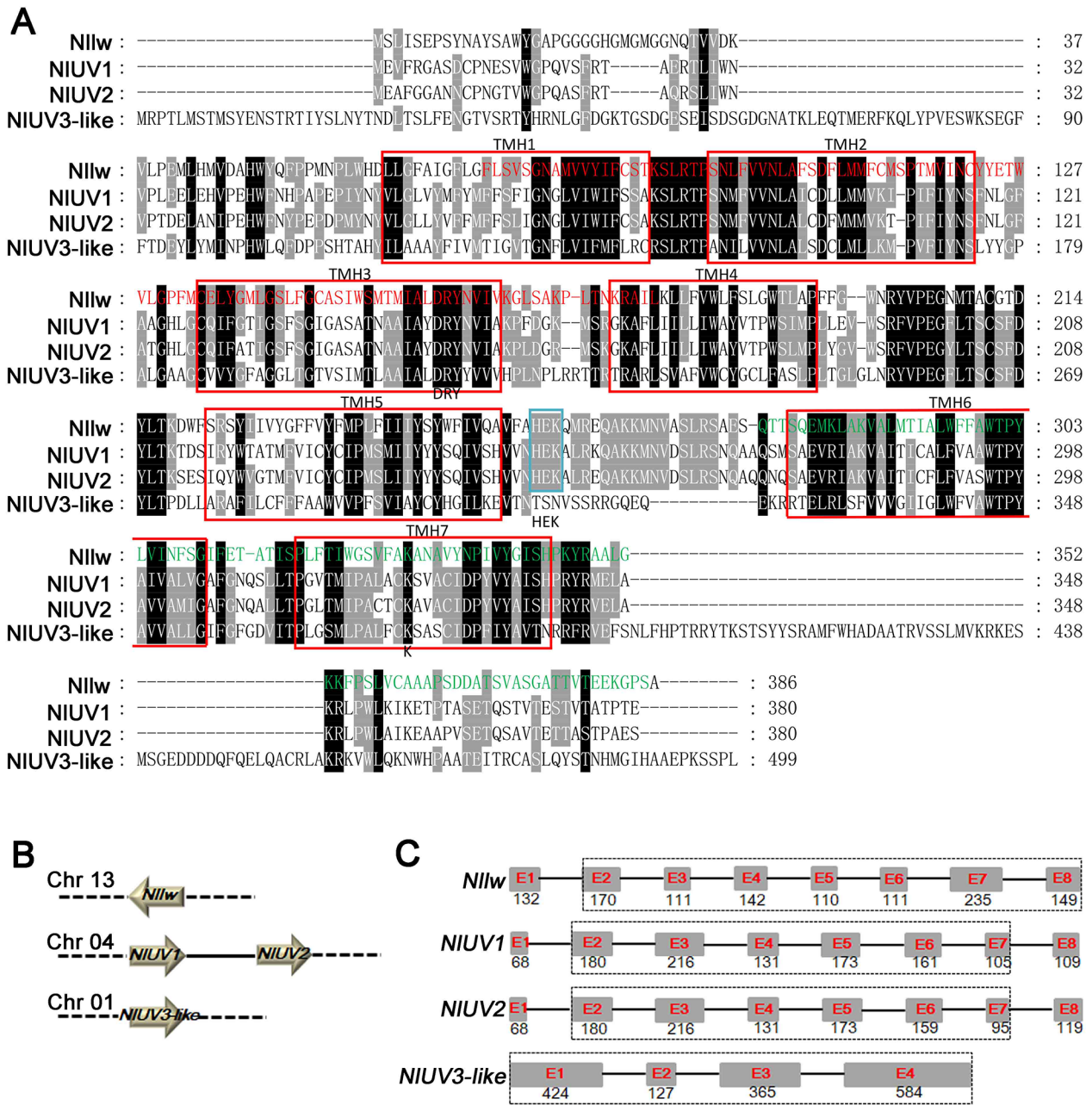


Fig. 1 Sequence characteristics analysis. **A.** Alignment of the amino acid sequences of *Nilw*, *NIUV1/2*, and *NIUV3-like*. Seven transmembrane α -helices (TMH1-7) are indicated with red frames; The conserved motif histidine-glutamine-lysine (HEK) is denoted by a blue frame; The conserved lysine (K) in TMH7 is marked below; Black background: 100% conserved amino acid sites; Gray background: 80% conserved amino acid sites. Red and green words: the 1st and 2nd regions for RNAi against *Nilw*, respectively. **B.** An ideogram of *Nilw*, *NIUV1/2*, and *NIUV3-like* on three chromosomes. Chr 13/04/01: chromosome 13/04/01; The arrow representing *Nilw* pointing to the left: means the *Nilw* transcript is in the opposite direction to Chr 13; The arrow representing *NIUV1/2* and *NIUV3-like* pointing to the right means the *NIUV1/2* and *NIUV3-like* transcripts are in the same direction to Chr 04 and 01; Solid line means tandem replicates of *NIUV1/2*. **C.** The distribution of *Nilw*, *NIUV1/2*, and *NIUV3-like* coding sequences within the genomic sequences. Gray boxes indicate exons (E), and the width of each box is proportional to the size of the corresponding exon. Figures below each exon are the sizes of corresponding exons. Black lines represent introns, which are not proportional to the size of the corresponding introns. The location of seven transmembrane α -helices on exons is marked with black dotted frames

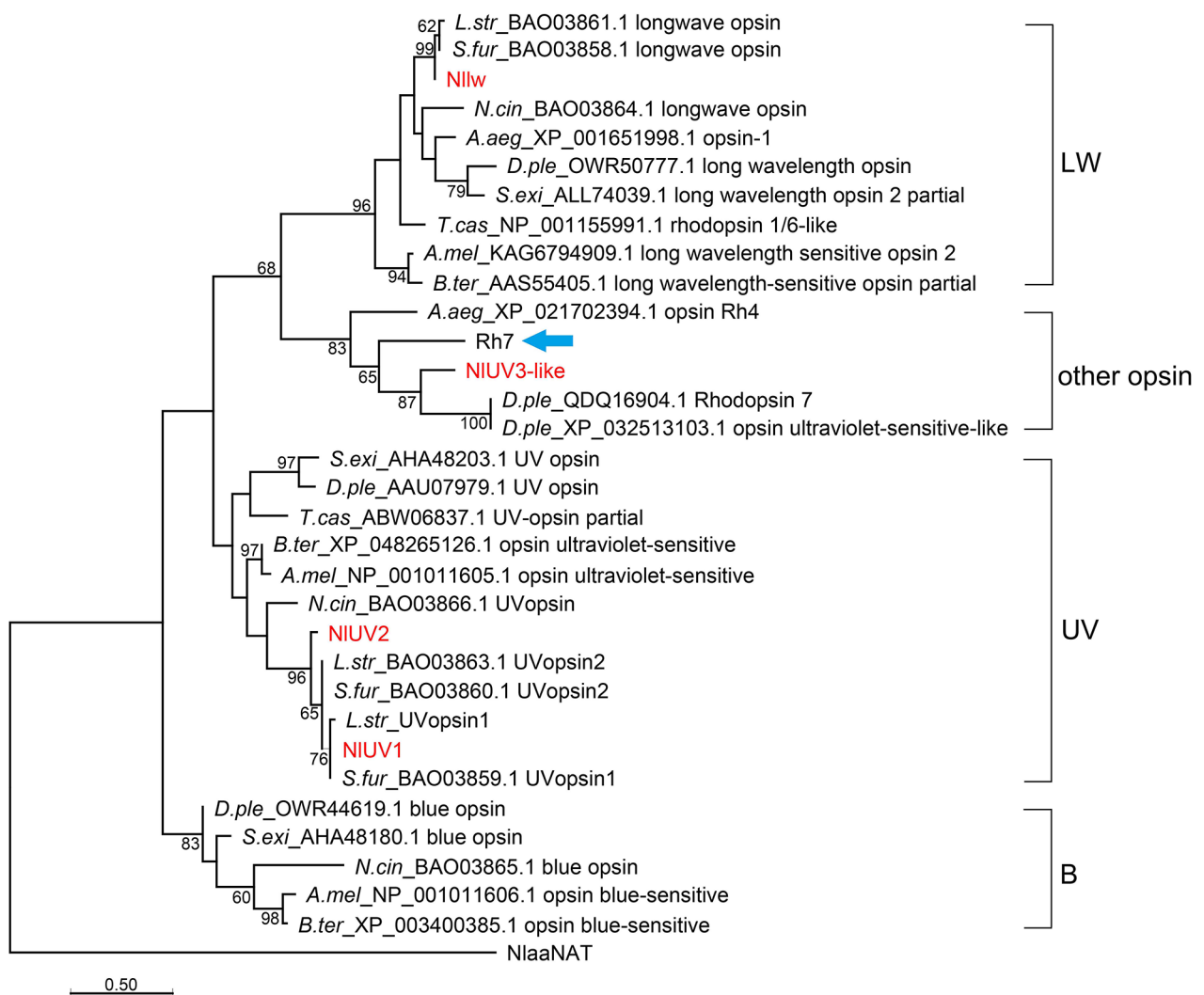


Fig. 2 Phylogenetic analysis. A phylogenetic tree with the amino acid sequences of *Nllw*, *NIUV1/2*, *NIUV3-like*, and 28 homologs from other insects. The names represent the abbreviated Latin name – GenBank accession number as well as functional annotation. LW, UV, B: long-wave-, ultraviolet-, and blue-sensitive opsin; Blue arrow: Rh7 in *Drosophila*. Detailed information regarding the 28 homologs is listed in Supplementary Table 3. Only > 50 bootstrap values are shown

RNA-seq of the transcriptomes revealed potential downstream genes of *Nllw* in the melanism pathway

Among the various types of pigmentation in insects, melanin-type pigment is prevalent [33, 34]. RNA-seq analysis after RNAi targeting *Nllw* revealed a total of 184 differentially expressed genes (DEGs) in 0 h-female adults, comprising 35 down-regulated and 149 up-regulated genes, compared with the *dsGFP* control group (Supplementary Table 1 A, B). Moreover, Gene Ontology (GO) analysis indicated that 3 genes related to the regulation of the circadian sleep/wake cycle (GO:0042749), which was consistent with the classical function of opsin [21], 3 genes associated with positive regulation of morphogenesis of an epithelium (GO:1,905,332), and 2 genes involved in negative regulation of glucose transmembrane transport

(GO:0010829) were down-regulated. In comparison, the up-regulated GO terms were primarily linked to various catabolic processes of organic compounds (Fig. 5A, Supplementary Table 2 A, B).

Particularly, a *tyrosine hydroxylase* gene (*NlTH*) and an *arylalkylamine-N-acetyltransferases* gene (*NlaaNAT*) in *N. lugens* were up- and down-regulated after knockdown of *Nllw*, respectively. The *TH* and *aaNAT* were key genes involved in the tyrosine-mediated melanism pathway [35]. Consistent expression levels of *NlTH* and *NlaaNAT* were verified by qRT-PCR with the newly emerging insects after *Nllw* knockdown (Fig. 5B).

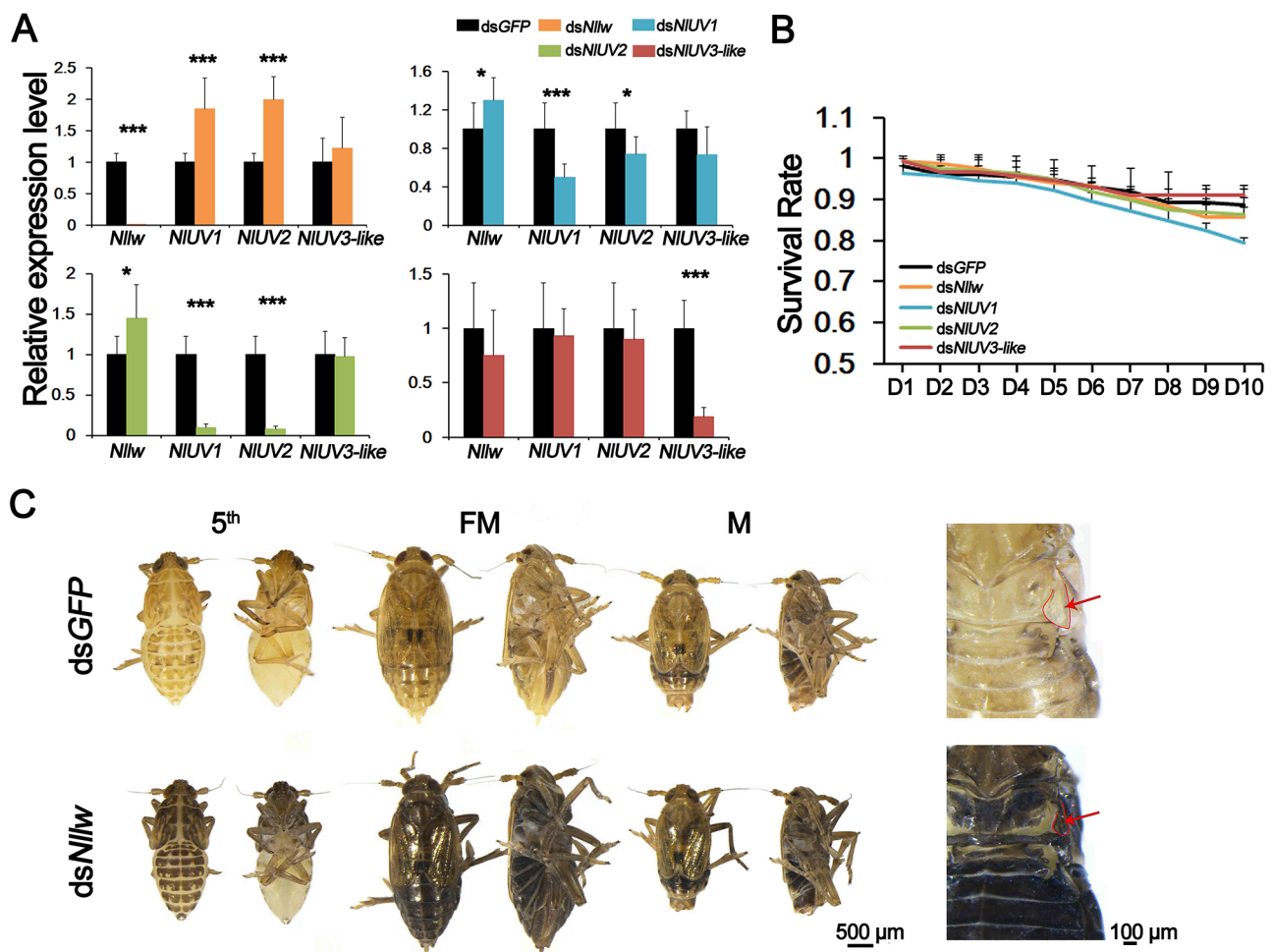


Fig. 4 RNAi against each of *Nilw*, *NIUV1/2*, and *NIUV3-like*. **A**. Relative expression levels of *Nilw*, *NIUV1/2*, and *NIUV3-like* after RNAi against each target gene, dsGFP was used as the control group. **B**. The survival rates of *N. lugens*. For **A** and **B**, data represent the means of three biological replicates (means + SD). A two-tailed unpaired t-test was applied. * $P < 0.05$ and *** $P < 0.001$ indicate significant differences compared with the dsGFP control group. **C**. Melanism of *N. lugens* after knock-down of *Nilw*. 5th: the 5th instar nymph; FM: female adult; M: male adult. RNAi was performed with the 4th instar nymph. In the ds*Nilw*-treated group, when *N. lugens* developed into the 5th instar, the rigid cuticle of the mesothorax and abdominal dorsal cuticle, leg, wing bud, and head became darker; when *N. lugens* eclosed into adult, melanin-type pigments occurred in almost the whole body surface, except for soft parts of the leg bases of the mesothorax. The short-hind wing which was marked with red line and arrow became darker. In the dsGFP-treated control group, *N. lugens* usually remained brown, with a little darker in male adult, and the hind-wing of adult was light color

purpose of ecological adaptation to climatic conditions under low temperatures [47]. In the meantime, melanization takes part in the innate immune system to resist pathogens and non-self-substances, that are involved in a phenoloxidase [48]. However, previous studies have indicated that distinct photoperiod does not significantly affect the changes in body color composition of *N. lugens*, and less than half of the black 5th -instar nymphs retain their black coloration after molting [44]. Typically, the phenomenon of melanization in *Biston betularia* (Lepidoptera) is a well-known example that highlights the crucial role of visual perception in body color change [49]. While in the case of *N. lugens*, the regulatory mechanism underlying body color may be much more intricate than our current understanding, just like in *Locusta*

migratoria (Orthoptera), the pigment of body color is complex and can be modified by various factors, such as endogenous hormone and population density [50]. Moreover, previous research has demonstrated that both body color and wing morphology of *N. lugens* contribute to the regulation of development [51]. *N. lugens* is widely distributed among rice-growing regions in the tropical and temperate Asian Pacific region [52], and there are variations in migration and reproductive preferences among different regional populations of *N. lugens* [53, 54]. Consequently, it is important to conduct extensive investigations into the relationship between body color and the environment in various regions, to provide further insights into the polyphenism of body color in *N. lugens*. In a word, it is speculated that *Nilw* is not only

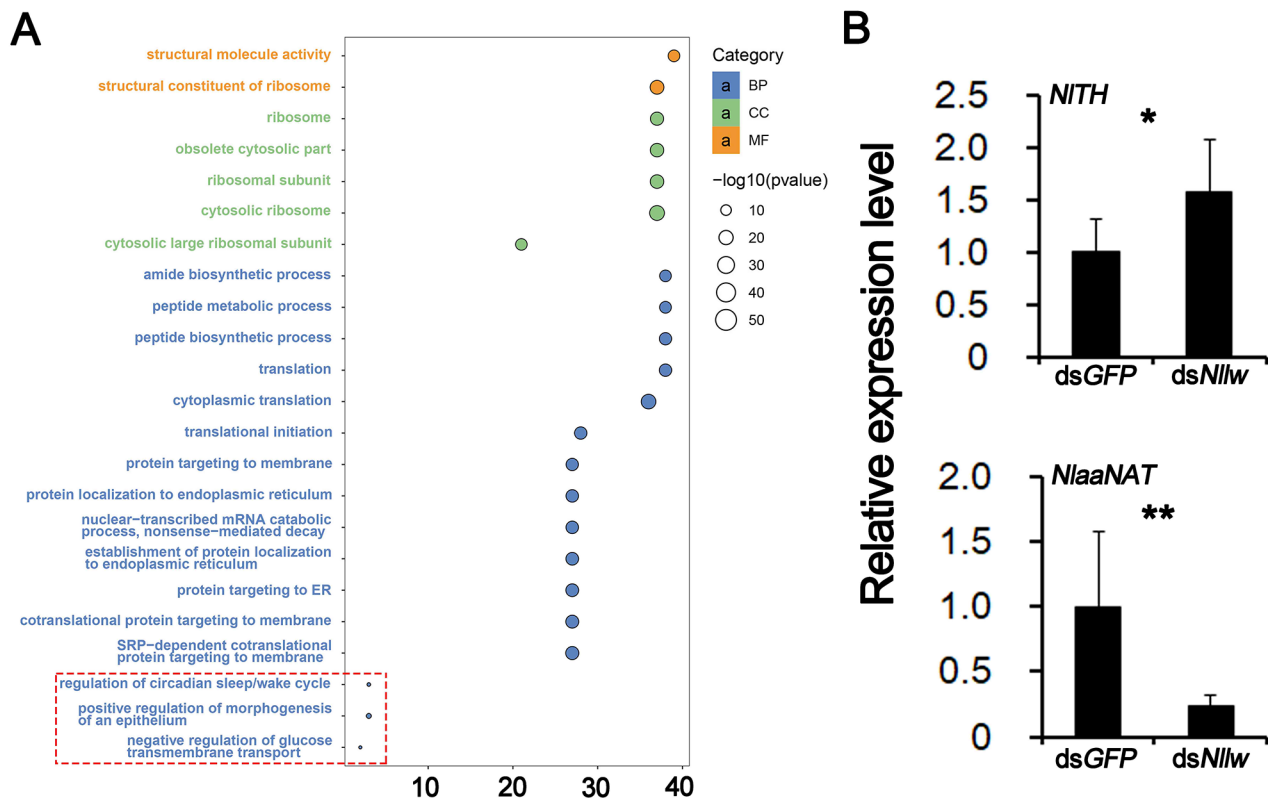


Fig. 5 Analysis of potential downstream genes of *Nllw*. **A**. Gene Ontology (GO) analysis after RNAi against *Nllw*. All of the 3 down-regulated GO terms are marked with a red dotted frame; The others are the top 20 up-regulated GO terms. The result is analyzed with three biological replicates. dsGFP is used as the control group. **B**. qRT-PCR verification of *NITH* and *NlaaNAT* after RNAi against *Nllw*. Data represent the means of three biological replicates (means + SD). A two-tailed unpaired *t*-test was applied. * $P < 0.05$ and ** $P < 0.01$ indicate significant differences compared with the dsGFP control group

responsible for long-wave spectrum absorption but also plays a crucial role in the complex regulatory network between external signals and body color via the tyrosine signal pathway, with the regulation of key enzymes *NITH* and *NlaaNAT*.

In addition, a predicted *NIUV3-like* was identified in *N. lugens*, but the *B-sensitive opsin* was lacking, which was different from *Nephotettix cincticeps*, another rice pest in Hemiptera, that possesses classic trichromacy [14]. Notably, the down-regulation of *Nllw* led to the up-regulation of *NIUV1/2*, and vice versa, suggesting partial functional compensation between *Nllw* and *NIUV1/2*. However, the expression of *NIUV3-like* was independent of the other three opsins, aligning with the phylogenetic analysis result. Additionally, as revealed by the phylogenetic tree analysis, *NIUV1/2* exhibited higher conservation within the Delphacidae family, whereas *NIUV3-like* diverged from *NIUV1/2*. Until now, the mechanisms of vision in *N. lugens* remain unknown, including whether the replicated UV-sensitive opsins can compensate for the missing B-sensitive opsin, like that in beetles [18]. Besides, although the silencing of any one of the *Nllw*, *NIUV1/2*, and *NIUV3-like* did not lead to significant mortality, it

did not necessarily imply that it has no impact on the fitness of *N. lugens*. As crucial components of visual pigments, the changes in preference for spectral sensitivity in *N. lugens* after the silencing of the four opsins are still unknown. Given that the *N. lugens* is a kind of long-distance migratory insect with a specialized feeding on rice, the influence of the spectral preference deserves further investigation. For example, a study in monarch has demonstrated that the specific opsin expansions are involved in sensing skylight cues [55]. Additionally, there is also another opsin reported in *Ciona intestinalis* (Phlebobranchia), which plays an important role in photoreponse during swimming behavior [56].

Conclusions

In summary, this study has highlighted an unconventional role of one opsin (*Nllw*) in the regulation of cuticle melanization through tyrosine signal pathway, implying a cross-talk between the gene pathways underlying the visual system and the morphological differentiation in insects and a complex mechanism existed in them. Besides, another UV-like opsin (*NIUV3-like*), which was homolog to the Rh7 in *Drosophila* was described, and

the detailed study of *Nllw*, *NIUV1/2*, and *NIUV3-like* revealed the conservation and evolution of opsins in *N. lugens*.

Methods

Insect rearing

N. lugens used in this study is reared on the Xiushui rice plant in a phytotron under 26.5°C, 65–75% relative humidity, and a 14/10 h light/dark photoperiod at Ningbo University, Ningbo, China.

Sequence analysis

DNAMAN software was used to predict gene CDS. NCBI blast (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) and NCBI CD search (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) websites were used to reveal potential gene function and conserved domains. Protein sequences were aligned using Clustal X software. Furthermore, through the NCBI Spleign website (<https://www.ncbi.nlm.nih.gov/sutils/spleign/spleign.cgi?textpage=online&level=form>), the distribution of gene CDS on *N. lugens* genomic sequence was displayed.

Phylogenetic analysis

28 amino acid sequences from ten insect species of five orders were downloaded from the NCBI database (Supplementary Table 3). A phylogenetic tree was thereby constructed based on *Nllw*, *NIUV1/2*, *NIUV3-like*, and the above 28 proteins with the software MEGA7, following the maximum likelihood method with 1000 bootstrap replications [57]. Furthermore, the *Nlaa*NAT amino acid sequence was selected as an outgroup member.

Real-time quantitative PCR (qRT-PCR) analysis

The total RNA of the target sample was extracted using the Trizol RNA isolater Total RNA Extraction Reagent (Vazyme Biotech, Nanjing, China) following the manufacturer's protocol. The quality and concentration of the isolated RNAs were assessed using a NanoDrop One Spectrometer (Thermo Fisher Scientific, USA). Next, 400 ng of each RNA was reverse transcribed into cDNA using the HiScript II Q RT SuperMix (+gDNA wiper) kit (Vazyme Biotech, Nanjing, China) in line with the protocol. Finally, qRT-PCR was carried out with an ABI QuantStudio 5 equipment (Thermo Fisher Scientific, USA) in a 10- μ L reaction system, containing 5 μ L of ChamQ SYBR Color qPCR Master Mix (Vazyme Biotech, Nanjing, China), 2.4 μ L of ddH₂O, 2 μ L of 20-fold diluted cDNA, and 0.3 μ L of each 10 μ mol primer. The procedure of qPCR comprised an initial denaturation step at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 10 s, and primer annealing together with amplification at 60 °C for 30 s. With $2^{-\Delta\Delta C_t}$ (Ct: cycle threshold) method [58], the relative expression level of the target

gene was calculated, with *N. lugens* 18 S ribosomal RNA (*NIU8S*, GenBank accession number: JN662398.1) serving as the housekeeping gene. Primers used for qRT-PCR are listed in (Supplementary Table 4 A).

Spatiotemporal expression analysis

Temporal expression patterns of *Nllw*, *NIUV1/2*, and *NIUV3-like* were analyzed with the FPKM values. Data from three biological replicates of each sample were used (Supplementary Table 5 A). By dissecting different tissues containing integument, digestive tract, ovary, testis, fat body, and eye from the 5th instar nymphs and adults, the *Nllw*, *NIUV1/2*, and *NIUV3-like* expression patterns in tissues were analyzed by qRT-PCR. Data from three biological replicates are listed in (Supplementary Table 5B).

RNA interference (RNAi)

One specific sequence of the target gene was cloned to synthesize the double-stranded RNA (dsRNA) with a MEGAscript T7 Transcription Kit (Ambion, Austin, TX), following the manufacturer's protocol. The *green fluorescent protein (GFP)* gene from *Aequorea victoria* (Leptothecata) was used as a negative control. Primers containing the T7 promoter sequences used for cloning are listed in (Supplementary Table 4B). With a FemtoJet Microinjection system (Eppendorf, North America), around 250 ng of dsRNA was injected into each of the 4th instar nymphs. The gene silencing efficiency was detected at 72 h post-injection by randomly selecting five insects from each sample. The insect survival rate after RNAi was counted for 10 days and the insect phenotypes were recorded using a stereomicroscope (Leica 151 S8AP0, Germany).

Transcriptome sequencing (RNA-seq)

Firstly, dsRNA of *Nllw* was injected into the 3rd instar *N. lugens* nymphs, and female adult *N. lugens* was collected at 0 h after emergence since the body color difference was more pronounced than male adults after RNAi. Afterward, ten individuals were homogenized for total RNA extraction, and dsGFP-treated sample was taken as the negative control. Each treatment was replicated thrice for cDNA library preparation and sequencing on the Illumina Novaseq 6000 platform (Novogene, Tianjin, China). Clean reads were aligned to the *N. lugens* reference genome using HISAT2. Furthermore, transcripts per million (TPM) expression values were calculated based on feature counts for genes by Cufflink. DEGs were identified using the DESeq2 package upon the threshold of P-value < 0.05 and log₂ ratio > 1. To explore the biological progress of DEGs, GO analysis was conducted with eggNOG, and GO terms were classified as biological process (BP), cellular component (CC), and molecular function (MF) categories.

Statistical analysis

The data are presented as means + SD in graphic instructions, and three biological repetitions were performed for each experiment. Statistical analysis was performed using GraphPad Prism 7.00 software and Microsoft Excel. The two-tailed Student's *t*-test was conducted to compare the difference. The significance level was set at * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12864-023-09470-7>.

Supplementary Material 1

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Not applicable.

Authors' contributions

Jia-Bao Lu and Ze-Dong Li: experimental operation, data analyzing and original draft writing; Zhuang-Xin Ye: bioinformatic analysis; Hai-Jian Huang: data analyzing and text editing; Jian-Ping Chen, Jun-Min Li and Chuan-Xi Zhang: experimental design; Chuan-Xi Zhang: A guide to the whole experiment, and final text editing. All authors read and approved the final manuscript.

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Data availability

The transcriptome raw sequence data are available in the Genome Sequence Archive in National Genomics Data Center, China National Center for Bioinformation / Beijing Institute of Genomics, Chinese Academy of Sciences (<https://ngdc.cncb.ac.cn/gsub/>), with the GSA CRA010945 under Bioproject PRJCA016793.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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