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Specific transcription factors regulate the expression of *Rh6* in *Bactrocera minax* and *Bactrocera dorsalis* (Diptera: Tephritidae)

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Abstract

Color vision is widely used by herbivorous insects to make host location. We have previously demonstrated that the long-wavelength-sensitive (LWS) opsin gene *Rhodopsin 6* (*Rh6*) confers green preference in *Bactrocera minax* (a specialist) but yellow in *B. dorsalis* (a generalist). However, the transcriptional regulation underlying *Rh6* expression and its association with color preference between these two sister species remains unclear. Here, we cloned the core promoter regions of *BmRh6* and *BdRh6*, and identified the transcription factors (TFs) BmHmx in *B. minax* and BdPtx1 in *B. dorsalis* through bioinformatics and transcriptomic analysis. The functional impact of the two TFs on *Rh6* transcription was validated using the dual luciferase reporter assays and yeast one-hybrid (Y1H) assays. RNA interference (RNAi)-mediated knockdown of the TFs resulted in significant downregulation of *Rh6* expression. Furthermore, silencing of *BmHmx* eliminated the preference for green in *B. minax*, while knockdown of *BdPtx1* in *B. dorsalis* led to

the loss of yellow preference. Our results elucidate the mechanism underlying transcriptional regulation of *Rh6* towards color preferences in tephritids, which also provide new insights into the links between host location and visual ecology in insects.

Keywords: color vision; *Rh6* gene; transcription factor, tephritid fruit flies

Introduction

Insects within complex ecological contexts utilize vision to reduce uncertainty, with color perception serving as a critical visual cue [1-3]. Color vision plays a crucial role in phytophagous insects finding host plants [4-6]. Moreover, the vast majority of insect species are trichromatic, possessing UVS (ultraviolet-sensitive), SWS (short-wavelength-sensitive), and LWS (long-wavelength-sensitive) photoreceptors [7, 8]. Insect color vision depends notably on the number and patterns of opsin expression in photoreceptor cells within compound eyes, with the spectral sensitivity of these cells primarily associated with the expressed opsins, and possible spectral modulation of the opsin sensitivity range conferred by lateral filters [9-11].

Opsin expression in photoreceptor cells is regulated by transcription factors (TFs), enabling their expression at varying developmental stages or within specific regions of the compound eyes [12-15]. This process entails a complex and overlapping series of transcriptional regulatory events that function synergistically to ensure the accurate and stable expression of specific opsins in each cell. Specifically, in *Drosophila* eyes, interdependent regulatory loops have been shown to pattern the retina and dictate opsin expression [16]. Notable examples include a bistable loop involving mutually inhibitory TFs Melted and Warts, a feed-forward loop involving TFs Orthodenticle (Otd/Crx) and defective proventriculus (Dve), and complex feedback loops between stereotyped

and stochastic regulators [17-19]. These interdependent regulatory loops dictate the expression patterns of opsins Rhodopsin 1 (Rh1), Rh3, Rh4, Rh5, and Rh6. However, some insects do not possess the SWS opsin Rh5. For instance, in the flies *B. minax*, *B. dorsalis* and *Ceratitis capitata*, the woodwasp *Sirex noctilio*, the hemipteran bug *Halyomorpha halys*, as well as several beetle lineages, the SWS opsin gene is absent from the genome [20-24]. Furthermore, the diversity of opsin types and their quantities, along with their expression in photoreceptor cells, varies significantly across insect species. Although some TFs involved in photoreceptor cell differentiation and opsin expression have been identified in Lepidoptera and Coleoptera, the transcriptional regulation of opsins in insects remains underexplored [25-27]. Detailed investigations into how these TFs are involved in gene regulatory networks will further contribute to uncovering the mechanisms underlying the development of complex visual gene regulation, which promotes the rich diversity of color vision systems in insects.

Throughout evolution, color perception has undergone adaptations in response to complex environments, enabling insects to adjust to ecological niches [28, 29]. The demonstration of color vision in various insect species can also provide a foundation for developing novel pest control strategies. For example, behavioral and field studies have demonstrated that *Rhagoletis pomonella* prefers red, *Neoceratitis cyanescens* shows a strong preference for orange, and *Rhagoletis indifferens* exhibits a strong attraction to yellow [30-32]. The findings of color preference have promoted the wide application of sticky color traps to attract and kill insect pests [33-36], although the related regulation mechanism remains unclear.

In this study, we focused on two closely related tephritid fruit fly species within the *Bactrocera* genus, the Chinese citrus fly (*B. minax*) and the oriental fruit fly (*B. dorsalis*), which

represent the classic specialist and generalist tephritids, respectively. The hosts of *B. dorsalis* includes hundreds of fruits and vegetables whereas *B. minax* has evolved exclusively towards unripe green citrus fruits. Previous studies have robustly shown that tephritid flies rely on visual stimuli for orientation to suitable oviposition sites, and in particular, that *B. minax* prefers green color while *B. dorsalis* tends to prefer yellow [23, 35, 37-39]. Molecular biology experiments and field assays have provided robust evidence that these preferences are determined by the same opsin gene, *Rh6* [23]. As such, we suggest that the transcriptional regulation of *Rh6* expression may contribute to altering photoreceptor cell spectral sensitivity, and ultimately, color preference in *B. minax* and *B. dorsalis*.

In this study, we set out to investigate candidate molecular mechanisms leading to differential expression of *Rh6* in these closely related tephritids. Using transcriptomic predictions and JASPAR analyses, we identified two key TFs, BmHmx and BdPtx1, which bind to the promoters of *BmRh6* and *BdRh6*, respectively. Additionally, the positive regulatory mechanism involving these TFs and promoters was demonstrated through dual-luciferase activity assays and RNAi. The specific binding of TFs to promoter fragments was further confirmed by Y1H assays. Collectively, our results illustrate an example of molecular mechanisms underlying opsin gene regulatory patterns and provide novel insights into the evolution of insect visual ecology.

Materials and methods

Insect collection and rearing

Pupae of *B. minax* were collected from citrus orchards in Yichang (30°49′ N, 111°04′ E), Hubei Province, China. Only adults that emerged from these wild pupae were used in this study.

The emerged *B. minax* adults were kept in the laboratory on an artificial diet of sucrose and brewer's yeast at 27°C, 14 L:10 D, and 75% relative humidity (RH), following the rearing protocol of Wang et al. [35]. *B. dorsalis* adults were held in the insectary after being sampled from a wild population at Huazhong Agricultural University, Wuhan, China. Larvae and adults were fed at 27°C, with 75% RH and a photoperiod of 14 L:10 D, and provided artificial diets as described by Ren et al. [40].

RNA extraction, cDNA synthesis, and RT-qPCR analysis

Total RNA was isolated following the protocol of RNAiso Plus (TaKaRa, Dalian, China). cDNAs were synthesized from 1 μg of total RNA using Hifair® III 1st Strand cDNA Synthesis SuperMix for qPCR (Yeasen, Shanghai, China) according to the manufacturer's instructions. The mRNA levels were assessed by RT-qPCR with the Hieff UNICON® Universal Blue qPCR SYBR Green Master Mix (YEASEN, Shanghai, China) in 20-μL reactions containing 10-fold dilution of cDNA and 0.2 μM primers using the Roche LightCycler 96 system (Roche, Basel, Switzerland). All RT-qPCRs were conducted with three biological and three technical replications. *GAPDH*, *α-Tubulin*, and *18S* were used as reference genes, and relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method. The RT-qPCR primers were validated by generating melting and standard curves as shown in Table S1.

DNA extraction, PCR amplification, cloning, sequencing and sequence analysis

Genomic DNA (gDNA) was extracted from adult *B. minax* and *B. dorsalis* using the Insect DNA Kit D0926 (Omega Bio-Tek, Norcross, GA, USA). PCR was performed with PrimeSTAR®

Max DNA Polymerase (TaKaRa, Dalian, China). The cycling conditions were: 98°C for 3 min; 35 cycles of 98°C for 10 s, 55°C for 5 s, 72°C for 20 s; and a final extension at 72°C for 5 min. The PCR products were purified using the TSP602-200 Trelief® DNA Gel Extraction Kit (Tsingke, Beijing, China). The PCR-purified products of the full-length promoter and transcription factor open reading frame (ORF) sequences were cloned into a universal vector using the 5 min TA/Blunt-Zero Cloning Kit (Vazyme, Nanjing, China). The PCR products were ligated into vectors for the Dual-luciferase reporter assays and Y1H assay via homologous recombination using the ClonExpress II One Step Cloning Kit (Vazyme, Nanjing, China). All cloned sequences were verified by sequencing (Tsingke, Wuhan, China). The ORF sequences of the cloned genes were identified using the ORF Finder tool (https://www.ncbi.nlm.nih.gov/orffinder/) and subsequently analyzed for predicted domains using SMART (https://smart.embl.de/). The physicochemical properties of the proteins were computed using the ProtParam tool (http://web.expasy.org/protparam/).

Dual-luciferase reporter assays in *Drosophila* S2 cell

This part of the method refers to the description of previous studies [41, 42]. Promoter regions of *BmRh6* and *BdRh6* were screened from the genomes of *B. minax* and *B. dorsalis*. JASPAR (http://jaspar.genereg.net/) and ALGGEN (http://alggen.lsi.upc.edu/) were used to analyze TF binding sites, with a threshold relative profile score of 99 %. Sequentially truncated promoters were amplified and then ligated into the pGL3-basic firefly luciferase reporter vector (Promega, Beijing, China) to construct pGL3-promoter plasmids. TF expression plasmids were generated by cloning the ORF sequences of *BmHmx* and *BdPtx1* into pAc5.1/V5-His A vector

(Invitrogen, Carlsbad, USA). The pRL-AC vector was used as an internal control. All cloned sequences were confirmed by sequencing (Tsingke, Wuhan, China). The primers used for amplifying promoter fragments and TFs are listed in Table S2.

Transfection was performed using Polyethylenimine Linear (PEI) MW40000 (Yeasen, Shanghai, China). PEI was prepared at a concentration of 1 μg/μL in Milli-Q® water, pH adjusted to 6.8–6.9 using 1 mol/L NaOH, and sterilized by filtration through a 0.2 μm membrane filter. A volume of 2 μL of PEI was used per 1 μg of plasmid. For promoter activity analysis, 400 ng promoter constructs and 4 ng pRL-AC plasmid were co-transfected into S2 cells, with the pGL3-basic vector serving as a control. To detect the effects of TFs, 200 ng TF expression plasmids, 200 ng promoter constructs and 4 ng pRL-AC vector were co-transfected into S2 cells, with the empty pAc5.1 vector used as a control. Firefly and Renilla luciferase activities were measured using 20 μL of cell lysate after 48 h of transfection on the SPARK 10M Multimode Reader Platform (Tecan, Männedorf, Switzerland). Relative luciferase activity was normalized by Renilla luciferase activity.

Y1H assay

The Y1H assays were performed using the Y1HGold-pAbAi Yeast One-Hybrid interaction proving kit (Coolaber, Beijing, China) according to the manufacturer's protocol. The sequences of promoter regions were ligated into the pAbAi vector as bait plasmids, and TF sequences were inserted into the pGADT7 vector to generate prey plasmids. The recombinant bait plasmids were linearized using the Bsp119I (*Bst*BI) (Thermo Scientific, Waltham, USA) and finally integrated into Y1HGold yeast cells. Bait strains were isolated with SD/-Ura medium and confirmed by PCR.

Then, the pGADT7-TF vectors were transformed into bait yeast strains, and the transformants were selected on SD/-Leu medium with Aureobasidin A (AbA). The Y1HGold strain containing the pGADT7-p53 and pAbAi-p53 plasmids was used as positive control, while the negative control consisted of Y1HGold strain transformed with the empty pGADT7 vector and pAbAi recombinant vectors. Specific forward and reverse primers are listed in Table S3.

dsRNA preparation and RNAi

RNAi was used to knock down the expression of transcription factor genes *in vivo*. The synthesis and microinjection of dsRNAs used the previously described method [23]. In brief, dsRNA was synthesized and purified using the Transcript Aid T7 High Yield Transcription Kit (Thermo Scientific, Waltham, USA) according to the product manual. *B. minax* adults were treated with 2.5 μg ds*BmHmx*, while *B. dorsalis* adults were injected with 1.5 μg ds*BdPtx1*, and meantime control groups were injected with ds*egfp*. RT-qPCR was used to evaluate target gene expression in the heads of the adult flies 48 hours after injection. All primers are listed in Table S1.

Color preference assay

The color preference tests of *B. minax* and *B. dorsalis* were conducted using the green-yellow two-color maze, as reported by Wang et al. [23]. Green and yellow coated paper, printed according to the Commission Internationale de L'Eclairage color standard, was alternately placed in each cell of the octagonal maze. Approximately 15 flies (6 days old) from each group were anesthetized with CO_2 and restrained at the center of the maze for 10 min to acclimate, after which the number of flies in each cell was counted after 30 min. The attraction rate for each color was calculated as

the number of individuals choosing the focal color compared to the total number of individuals making any choices. Each test was repeated seven times.

Transcriptome and genome data sources

The transcriptome and genomic data used in this study were derived from previous sequencing efforts [23]. Specifically, all sequencing data and assembled genomes utilized in this study were sourced from GenBank under accession codes PRJNA793623 (*B. dorsalis*) and PRJNA793518 (*B. minax*).

Statistical analysis

The gene expression level and color preference assay data were analyzed using an independent samples *t*-test. One-way analysis of variance (ANOVA) was used to determine the significant difference, followed by Tukey's HSD tests for multiple comparisons. Values were expressed as the mean ± SEM from at least triplicate experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ were considered statistically significant.

Results

Transcriptional activity of *BmRh6* and *BdRh6* promoters

To identify the critical regulatory regions for *Rh6* transcription, we generated a series of stepwise deletions of *Rh6* promoters and ligated into the pGL3 vector. These constructs were co-transfected with pRL-AC plasmid in S2 cells. The firefly luciferase activities of the pGL3 vector driven by the deletion fragments were measured and normalized to Renilla luciferase

activity. The relative luciferase activities of deleted fragments indicated that all the constructs had promoter activities compared with the pGL3-basic vector (Fig. 1).

Among the six constructs including BmP1 (−1710/+61), BmP2 (−1253/+61), BmP3 (−980/+61), BmP4 (−682/+61), BmP5 (−456/+61), and BmP6 (−102/+61) in *B. minax*, BmP5 was the most significantly induced, followed by BmP6 and BmP4, indicating that critical responsive elements might be located between −456 and −102 bp of the *BmRh6* promoter (Fig. 1A).

Five pGL3-basic recombinant plasmids that contained the *BdRh6* promoter, including BdP1 (-1792/+69), BdP2 (-1021/+69), BdP3 (-725/+69), BdP4 (-337/+69), BdP5 (-148/+69), were constructed. Among these, BdP4 exhibited the most notable increase among the five constructs, followed by BdP5, indicating that essential TF binding sites may be situated within the -337 to -148 bp region of the *BdRh6* promoter (Fig. 1B).

Screening of transcription factors for *BmRh6* and *BdRh6* promoters

RNA sequencing was performed on *B. minax* samples from newly emerged and sexually mature individuals to explore potential TF binding sites involved in regulating *BmRh6* expression. Using the JASPAR and ALGGEN databases, we identified candidate TF binding sites within the *BmRh6* promoter region (-456 ~ -102 bp, BmRh6Pr). Afterwards, the highly expressed TF BmHmx in sexually mature flies was screened out, which may suggest a crucial involvement in the transcriptional regulation of *BmRh6* (Fig. 2A). RT-qPCR analysis confirmed that both *BmHmx* and *BmRh6* expression levels were significantly higher in sexually mature flies compared to newly emerged ones (Fig. 2C). Similarly, to identify TFs responsible for regulating *BdRh6* expression, JASPAR and ALGGEN databases were used to predict potential binding sites within the −337 to

−148 bp region of the *BdRh6* promoter (BdRh6Pr). Combining these predictions with transcriptomic data from the heads of sexually mature *B. dorsalis*, we identified BdPtx1 as a likely regulatory TF (Fig. 2B). As shown in Fig. 2D, both *BdPtx1* and *BdRh6* showed higher levels of expression in the sexually matured individuals compared with newly emerged *B. dorsalis*. Taken together, these findings suggest the presence of a BmHmx binding site in the *BmRh6* promoter and a BdPtx1 binding site in the *BdRh6* promoter (Fig. S1). *BmHmx* gene encodes a 559-amino acid protein with a predicted molecular weight of 58.6 kDa and a theoretical isoelectric point (pI) of 6.99 (Fig. S2A). *BdPtx1* encodes a 612-amino acid protein with an estimated molecular weight of 64.5 kDa and a theoretical pI of 6.65 (Fig. S2B).

BmHmx and BdPtx1 regulate *Rh6* overexpression

To confirm whether BmHmx and BdPtx1 could bind to the *Rh6* promoter regions, we performed dual-luciferase reporter assays and Y1H assays. The dual-luciferase assay revealed that overexpression of BmHmx significantly increased the promoter activity of *BmRh6* ($P < 0.05$) (Fig. 3A), while BdPtx1 also induced a significant increase in *BdRh6* promoter activity ($P < 0.05$) (Fig. 3B). Subsequently, pAbAi-BmRh6Pr was used as bait plasmid in the Y1H assay and the interaction with BmHmx predicted according to the schematic tested (Fig. S1A). The results showed that the bait yeast containing pGADT7-*BmHmx* can grow normally on SD/-Leu media supplemented with 100 ng/mL AbA after co-transformation, the same as the positive control pAbAi-53 and pGADT7-53 (Fig. 3C). Similarly, Y1H assay was performed to confirm the interaction between BdPtx1 and the *BdRh6* promoter. The yeast cells containing prey pGADT7-*BdPtx1* and bait pAbAi-BdRh6Pr could grow in the medium lacking Leu with AbA (100

ng/mL) (Fig. 3D). Collectively, these results demonstrate that BmHmx and BdPtx1 were able to directly bind to the promoter sequences of *BmRh6* and *BdRh6*, respectively.

RNAi knockdown of *BmHmx* and *BdPtx1* alters color preference in *Bactrocera*

We next examined whether RNAi silencing of *BmHmx* affected the color preference of *B. minax* for green and yellow. The expression level of *BmHmx* decreased significantly after ds*BmHmx* injection (Fig. 4A, D). Compared with the injection of ds*egfp*, RNAi of *BmHmx* significantly decreased the expression of *BmRh6* by 45.76% in female and 43.32% in male (Fig. 4B, E). By releasing the flies into the green-yellow two-color maze, we found that RNAi of *BmHmx* no longer showed green preference in *B. minax* (Fig. 4C, F).

Likewise, to assess the role of *BdPtx1* in color preference and *Rh6* expression regulation, we used RNAi to knock down *BdPtx1* in *B. dorsalis*. RT-qPCR analysis confirmed efficient silencing of *BdPtx1* 48 hours post-injection (Fig. 4G, J). Knockdown of *BdPtx1* significantly decreased mRNA levels of *BdRh6* by 38.39% in female (Fig. 4H), as well as a decrease by 40.16% in male (Fig. 4K). According to the color preference assays, the silencing of *BdPtx1* significantly impacted the *B. dorsalis* preference for the green-yellow two-color maze, as yellow was no longer favored (Fig. 4I, L).

Discussion

The spectral tuning of color vision in insects is primarily determined by the number, function and expression patterns of opsin genes in ommatidial photoreceptor cells, and is further shaped by rapid regulatory evolutionary changes in opsin gene expression [8, 14, 15, 43, 44]. We previously

found that *Bactrocera minax* prefers green while *Bactrocera dorsalis* prefers yellow which is mediated by the same LWS opsin gene *Rh6* [23, 35]. In this study, using Y1H and dual-luciferase assays, our results show that BmHmx directly interacts with the *BmRh6* promoter region, while BdPtx1 directly interacts with the *BdRh6* promoter region (Fig. 3). Furthermore, through RNAi experiments and color preference assays, we further elucidated the regulatory roles of the TFs BmHmx and BdPtx1 in *Rh6* expression (Fig. 4). Collectively, these results suggest that different TFs regulate *Rh6* expression in *B. minax* and *B. dorsalis*, which may contribute to species-specific differences in transcription and expression levels, and, at least partly, explain the observed species-specific color preferences.

As opsins play pivotal roles in cellular physiology and behavior, they serve as key molecular targets for investigating genotype-phenotype relationships in animals [43]. Opsins are crucial components of visual pigments, forming the primary molecular basis of insect color vision. Specifically, in several insect species, opsins exhibit heightened expression after sexual maturity, particularly in the adult head and compound eyes [23, 45, 46]. The diversity of opsin genes in insects is influenced by multiple factors, including light environment, ecological niche, and host organism coloration. For example, selective pressures on opsin genes vary across lepidopteran species and have been shown in several cases to be driven by ecological transitions from dim to bright habitats [47, 48]. In dipterans, the mosquito *Aedes aegypti* exhibits preferences for orange and red hues, likely due to their prevalence in the human skin reflectance spectrum [1]. Similarly, our previous studies showed that the enhanced upregulation of phototransduction genes in the tephritid fly specialist, *B. minax*, confers a preference for green in behavioral assays [23], although the molecular mechanisms remain to be elucidated. The findings that transcriptional regulation of

insect opsins plays a more important role than previously thought in modulating spectral sensitivity [14, 27], support the idea that a detailed understanding of the molecular mechanisms underlying opsin expression diversity may contribute to revealing more spectral adaptations in insect photoreceptor cells.

In the current study, we establish a regulatory connection between the TF BmHmx and the opsin BmRh6 in *B. minax*, as well as TF BdPtx1 regulating opsin BdRh6 in *B. dorsalis*. Notably, *BmHmx* and *BdPtx1* showed high expression levels in the heads of sexually mature adults (Fig. 2C, D), aligned with the elevated expression of the *Rh6* opsin gene. While research on insect opsin TFs has predominantly focused on *Drosophila* in recent decades, the regulatory mechanisms governing opsin expression in other insect species have remained underexplored [25, 49]. Here, we convincingly show, using both *in vivo* and *in vitro* experiments, that BmHmx regulates *BmRh6* at the transcriptional level. In *Drosophila*, knockdown *Hmx* during eye development results in reduced or absent adult eyes, or failure of head development leading to pupal death [50]. Furthermore, the homeobox TF Hmx is integral to the development of vertebrate sensory ganglia [51].

On the other hand, in the case of *B. dorsalis*, our results indicated that the TF BdPtx1 is crucial for the regulation of *BdRh6* expression in the compound eyes. *Drosophila* Ptx1 is homologous to the human PITX1, PITX2, and PITX3 TFs which is recognized as a key regulator in various developmental processes in both mammals and flies [52]. For instance, the loss of PITX TFs is linked to developmental defects in humans, with the absence of PITX2 or PITX3 specifically leading to eye malformations and abnormal optic nerve development [53].

Despite remarkable variations across animal visual systems, distinct subtypes of

photoreceptors depend on the regulation by transcription factors [54, 55]. We found the divergence in key TFs regulating *Rh6* between *B. minax* and *B. dorsalis*, which differs from those identified in *D. melanogaster*. Additionally, the visual systems of *B. minax* and *B. dorsalis* have evolved differently in terms of food habits and host location [23]. These differences may help explain the variations in the predicted TFs within the core promoter region of the *Rh6* opsin gene, even when screened under identical conditions. In addition to transcriptional regulation mechanisms likely to be contributed by Hmx and Ptx1, visual differences in color preference behavior between these two sister species, *B. minax* and *B. dorsalis*, may further rely on additional mechanisms. Hence, recent studies have shown that DNA methylation and histone acetylation directly regulate mouse opsin expression, both *in vitro* and *ex vivo* [56]. Similarly, chromatin reorganization has downregulated an otherwise intact *UVRh2* gene in *Heliconius melpomene*, which affects the ultraviolet receptor spectral sensitivity compared to other sister *Heliconius* butterfly species [27]. Under this context, the potential influence of epigenetic modifications, driven by ecological pressures and host-location behaviors, warrants further investigation.

Future studies could explore whether DNA methylation, histone modifications, chromatin remodeling, and regulation by long noncoding RNAs (lncRNAs) and microRNAs (miRNAs) affect opsin gene expression at both the transcriptional and post-transcriptional levels. In addition, it will be interesting to determine how the spatial structure of Rh6 opsin in the photoreceptor cell membranes of *B. minax* and *B. dorsalis* is shaped by these mechanisms. Such insights could deepen our understanding of the regulatory networks underlying the visual system of tephritid fruit flies, paving the way for practical applications in pest management. Based on these findings, gene drive technology, which primarily relies on CRISPR-Cas9, offers promising approaches to

controlling fruit fly populations [57]. By targeting visual-related genes, their transcription factors, or regulatory elements using CRISPR-Cas9, it could disturb host location, foraging and mating behavior of insect pests, ultimately suppressing populations. This strategy has the potential to facilitate effective and sustainable pest control, which will contribute to area-wide pest management. Overall, our findings help refine the understanding of TF regulation of opsin gene *Rh6* in insects and provide links between insect color vision and host location behavior based on visual cues.

Author contributions

All the authors contributed to and agreed to the published version of the manuscript. Haoran Wang: Conceptualization, Writing – original draft; Yaohui Wang and Guijian Zhang: Literature survey; Yi Liu and Shuai Cao: Figure generating; Bingbing Wei and Zhen Cao: Data collection; Marjorie A. Liénard: Writing – review & editing; Changying Niu: Writing – review & editing, Funding acquisition, Supervision.

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Disclosure

The authors declare no competing interests with this work.

Supporting information

Supporting information can be found in the online version of this article.

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Fig. 1. Activity levels of *BmRh6* and *BdRh6* promoter fragments. (A) Identification of the promoter of *BmRh6* using progressive deletion constructs between −1710 to +61 in dual luciferase reporter assays. (B) Progressive deletion constructs between −1792 to +69 were used to identify the *BdRh6* promoter in dual luciferase reporter assays. The luciferase activity was measured after 48 h of transfection. The relative activity of promoter was normalized against the Renilla luciferase activity. Data are represented as means ± SEM ($P < 0.05$, one-way ANOVA).

Fig. 2. Screening of transcription factor for *Rh6* in *B. minax* and *B. dorsalis*. (A) The Venn analysis of predictions from the JASPARS database and transcriptome identifications of *B. minax*. (B) The Venn analysis of predictions from the JASPARS database and transcriptome identifications of *B. dorsalis*. (C) Relative expression of *BmHmx* and *BmRh6* genes in newly emerged and sexually mature *B. minax*. (D) Relative expression of *BdPtx1* and *BdRh6* genes in newly emerged and sexually mature *B. dorsalis*. Significant differences were detected by Student's t-test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). Error bars indicate SEM.

Fig. 3. BmHmx and BdPtx1 directly regulate *Rh6* transcription. (A) Effects of BmHmx on the activity levels of the *BmRh6* promoter (BmRh6Pr) assessed by dual-luciferase assays in *Drosophila* S2 cells. (B) Identification of the putative BdPtx1 binding sites in the *BdRh6* promoter (BdRh6Pr). Bars labelled with different letters indicate significant difference with $P < 0.05$. The significance of differences was determined by a one-way ANOVA. (C) Exploring the direct interaction between BmHmx and BmRh6Pr via Y1H assay. (D) Investigating the interaction between BdPtx1 and BdRh6Pr by Y1H assay. Y1HGold yeast cells containing the pGADT7 and pAbAi recombinant vectors were selected on SD/−Leu media with or without AbA (100 ng/mL).

Fig. 4. The color preference behavior in both species is affected by the transcription of *Rh6* regulated by BmHmx and BdPtx1. (A) Interference efficiency of ds*BmHmx* treatment compared to ds*egfp* treatment in female *B. minax*. (B) After silencing *BmHmx*, relative expression of *BmRh6* in female *B. minax*. (C) Attraction rates of released *B. minax* female adults in the green-yellow two-color maze under ds*BmHmx* and ds*egfp* treatments. (D) Expression of *BmHmx* after RNAi. (E) After silencing *BmHmx*, expression of *BmRh6* in male *B. minax*. (F) Attraction rates of released male adults in the color maze under ds*BmHmx* and ds*egfp* treatments. (G, J)

Interference efficiency of ds*BdPtx1* treatment compared to ds*egfp* treatment in *B. dorsalis*. (H, K) Relative expression of *BdRh6* after silencing *BdPtx1*. (I, L) Attraction rates of released *B. dorsalis* adults in the color maze after silencing *BdPtx1*. Significant differences were detected by Student's t-test (ns, no significant difference, **P* < 0.05, ***P* < 0.01, ****P* < 0.001). Error bars indicate SEM.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.