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Ecdysone receptor controls wing morphogenesis and melanization during rice planthopper metamorphosis

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ABSTRACT

In this study, we cloned full-length *EcR* cDNAs from the small brown planthopper *Laodelphgax striatellus*, the brown planthopper *Nilaparvata lugens* and the white back planthopper *Sogatella furciferas*. This is the first reporting of *EcRs* from either *L. striatellus* or *S. furciferas*. The deduced amino acid sequences of the *EcRs* show high levels of similarity to each other. The highest transcriptional level of the *EcR* gene was detected in the mid-fifth instar nymphs of *N. lugens*. Silencing of *EcR* expression by *in vivo* RNAi generated phenotypic defects in molting and resulted in lethality in most of the treated nymphs. Intriguingly, apparent wing defects in morphogenesis and melanization occurred during *EcR* knockdown in *L. striatellus* nymphs.

1. Introduction

Ecdysone receptor (EcR) was discovered in arthropods and was found to play key roles in molting, metamorphosis, reproduction, innate immunity and many other aspects of insect life (Brown and Truman, 2009; Gautam and Tapadia, 2010; Konig et al., 2011; Liu et al., 2009; Mirth et al., 2009; Schwedes et al., 2011; Tian et al., 2010; Wang et al., 2011). EcR contains a highly conserved DNA-binding domain (DBD) and a structurally conserved ligand-binding domain (LBD) (Bain et al., 2007). This unique structure ingeniously combines a transcription factor with a nuclear receptor to effectively regulate transcriptional cascades (Cruz et al., 2006). EcR function is switched on and off by ecdysteroids; 20-hydroxyecdysone, also known as 20E, is generally the native hormone. Clarifying the functions of EcR in insects will aid in the development of gene switches to control important agricultural pests.

The rice planthoppers have become the most destructive insect pest in Asia. These insects cause serious damage to rice plants by sucking the phloem sap, blocking the phloem vessels and by acting as a virus vector (Wei et al., 2009). Currently, chemical control, using a variety of insecticides, is still the first choice for managing pest populations; however, the widespread use of insecticides has caused the rice planthopper to develop resistance, which has resulted in their resurgence and additional environmental risks (Cohen, 2006). Therefore, there is an urgent need to develop new strategies that function through target sites for the pest management. In recent years, RNA interference (RNAi)-based technology has shown great potential in protecting crops from agriculturally important insect pests; this is due to the wide range of potential targets for the gene suppression (Baum et al., 2007; Mao et al., 2007; Zhang et al., 2010). RNAi has been successfully used in studies to ascertain the function of *EcR* during the development of holometabolous insects, especially in *Droshophila melanogaster* (Lam and Thummel, 2000) and *Tribolium castaneum* (Xu et al., 2010), but has rarely been used in hemimetabolous insects, mainly due to the fact that these insects are not amenable to genetic analysis (Cruz et al., 2006).

In this study, we used the primitive hemimetabolous species *Nilaparvata lugens* and *Laodelphgax striatellus* as models to investigate the roles of *EcR* in molting and metamorphosis during development. We used an *in vivo* RNAi method that allowed us to efficiently disrupt *EcR* gene function in order to study its roles in coordinating the developmental processes in rice planthoppers. Our findings revealed that *EcR* regulation is a crucial mechanism for controlling wing morphogenesis and melanization during the molting and metamorphosis of the nymphal–nymphal transition in rice planthoppers.

2. Materials and methods

2.1. Insects

The *N. lugens*, *L. striatellus* and *Sogatella furciferas* insects were collected from a rice field located on the Huajiachi Campus of Zhejiang University. The nymphs were reared at 27 ± 0.5 °C with

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70% humidity on rice seedlings (xiushui 128) under a 16:8 h light: dark photoperiod. Newly molted *L. striatellus* fourth instar nymphs (the penultimate nymphal instar) and fifth instar nymphs (the last nymphal instar) of *N. lugens* and *S. furciferas* were used for these experiments.

2.2. Isolation of EcR cDNA clones of rice planthoppers

Based on the nucleotide sequence encoding the *N. lugens EcR* gene (GenBank accession no. FJ263049), we isolated an *EcR* cDNA clone containing a complete open reading frame (ORF) sequence from the fifth instar nymphs of *N. lugens*. The following gene-specific primers used: 5′-ATGGGTCATCATCTGCATCA-3′ and 5′-TTATGAAGTCGATGATGACA-3′. High-throughput transcriptome sequencing of *L. striatellus* in our laboratory generated an *EcR* homologous sequence; this sequence was 1239 bp in length but lacked some coding sequence at the 5′ and 3′ ends. To obtain the complete coding sequence of the *L. striatellus EcR*, we isolated the cDNA clone using the RACE (rapid amplification of cDNA ends) method, specifically the 3′ and 5′ Full RACE kit (TaKaRa, Dalian, China). The following primers were designed and used for the 3′ RACE experiment: outer primer 5′-AAAACTGCCCGACTTCCTAATGG-3′; inner primer 5′-ACGTGGATAATACTCAGTCGCAGTC-3′. The following gene specific primers (GSP) were used for 5′ RACE experiment: GSP1: 5′-GCAGGTGAGGGCATTGTAGTGAT-3′; GSP2: 5′- GCTCGATCTCCGCACACCAAA-3′ and GSP3: 5′-CAGAGTTCCTCCTGTTGCCTCG-3′. Based on the *N. lugens* and *L. striatellus EcR* gene sequences, we isolated a cDNA clone of the *S. furciferas EcR* gene by using the following primers in a RACE experiment: outer primer 5′-GGAAATCTACCTGGAAGCCCTGAA-3′ and inner primer 5′-CCGAACTTCTGTCTGTTCTCACC-3′ for obtaining the sequence at the 3′ end; GSP1: 5′-GACGCTGAAGTCGCACATCCTCT-3′; GSP2: 5′-TCATGCCGACACTGAGACACTTTT-3′ and GSP3: 5′-GTCGATCTCACAGTTATTGCCGTAC-3′ for extending the sequence at the 5′ end of the *EcR* gene.

2.3. Preparation of double stranded RNA (dsRNA)

A 468 bp of nucleotide sequence specific to the LBD of both the *N. lugens* and the *L. striatellus EcR* was cloned into the pGEM-T Easy Vector (Promega). *Aequorea victoria* green fluorescent protein (*GFP*) was used as a control. Specific dsRNAs (ds*EcR* and ds*GFP*) were synthesized by *in vitro* transcription using PCR-generated DNA templates that contained the T7 promoter sequence at both ends. The following primers were used for generating these DNA templates: *N. lugens EcR* (sense primer: 5′-TAATACGACTCACTATAGGGAGAGACCAGATTGTACTGCTCAA-3′; antisense primer: 5′-TAATACGACTCACTATAGGGAGACTCCATGAGGAAGTCGGG-3′), *L. striatellus EcR* (sense primer: 5′-TAATACGACTCACTATAGGGAGATGCAGCTCATCGTTGAGTTC-3′; antisense primer: 5′-TAATACGACTCACTATAGGGAGAAGTTCTGGTTGCCCAGTGTT-3′), *GFP* (sense primer: 5′-TAATACGACTCACTATAGGGAGAATGAGTAAAGGAGAAGAACTTTTCA-3′; antisense primer: 5′-TAATACGACTCACTATAGGGAGATTTGTATAGTTCATCCATGCCATGT3-′). The specific dsRNAs for each gene were synthesized using the MEGAscript T7 Transcription Kit (Ambion, Austin, TX) according to manufacturers' instructions. Following transcription, the DNA template was removed using TURBO DNase, and the dsRNAs were purified using the RNAqueous Kit (Ambion). The size of the dsRNA products was confirmed by electrophoresis on a 1% agarose gel that was run in TAE buffer.

2.4. RNA interference (RNAi) using microinjection

Newly molted fifth instar *N. lugens* nymphs and fourth instar *L. striatellus* nymphs were used in the RNAi experiments. Each nymph was anaesthetized with carbon dioxide for 5–10 s at PCO_2 = 5 mPa. Approximately 250 ng ds*EcR* or ds*GFP* was microinjected into each nymphal thorax between the mesocoxa and the hind coxa using the FemtoJet Microinjection System (Eppendorf, North America). The treated nymphs were reared at 27 ± 0.5 °C with 70% humidity on fresh rice seedlings under a 16:8 h light: dark photoperiod. The molting phenotypes and mortality rates were observed and calculated under a stereomicroscope (Leica S8AP0, Germany) every 24 h following dsRNA treatments. Ninety *N. lugens* nymphs were treated with ds*EcR* or ds*GFP*, respectively, and forty-five *L. striatellus* nymphs were treated with ds*GFP* or different doses of ds*EcR*, respectively.

2.5. Real time qPCR and reverse transcription PCR (RT-PCR) analysis

As the low quantity of an individual nymph or adult RNA, a mixture of six whole bodies of *N. lugens* or *L. striatellus* at each developmental stage was used as one sample, respectively. Total RNA was isolated from the whole body samples using Trizol reagent (Invitrogen) and treated with DNase I (TaKaRa). The concentration of DNase I-treated RNA was adjusted with DEPC-treated H_2O to 1 μg/μl, and 1 μg of RNA was reverse-transcribed in a 10 μl reaction system using the AMV RNA PCR Kit (TaKaRa). Real-time qPCR was performed on an ABI Prism 7300 real-time PCR system (Applied Biosystems, Foster City, CA, USA) using the SYBR *Premix Ex Taq* Kit (TaKaRa), according to the manufacturers' instructions. The first-strand cDNA (2 μl) and the no-template control (NTC, 2 μl) were used as templates in each 20-μl reaction mixture under the following conditions: denaturation at 95 °C for 30 s followed by 40 cycles of 95 °C for 5 s and 60 °C for 31 s. Fluorescence of the PCR products was detected by adding a heat-dissociation protocol (temperature range, 60–95 °C) during the last step of each cycle. Following amplification, melting curves were constructed, and data analysis was performed on an ABI 7300 system equipped with SDS software. Specific primer sets were designed for the *N. lugens EcR* gene (sense primer: 5′-GCTGTGAAGCGAAAGGA-3′; antisense primer: 5′-ATTCCACGCTGAAGTCG-3′) and the *L. striatellus EcR* gene (sense primer: 5′-ACTGGAGTCGGGCAACA-3′; antisense primer: 5′-CGAGTCGGTGAATGAGC-3′). As an internal control, the *N. lugens β-actin* gene (GenBank accession no. EU179846) was also analyzed using the following primers: 5′-TGGACTTCGAGCAGGAAATGG-3′ (sense primer) and 5′-ACGTCGCACTTCATGATCGAG-3′ (antisense primer). The specificity of the primers was confirmed using NCBI BLAST algorithms (http://www.ncbi.nlm.nih.gov/). The results were standardized to the expression level of *N. lugens β-actin*. An NTC sample was run to detect any contamination and to determine the degree of dimer formation. The $\Delta\Delta C_t$ method was used to analyze the relative differences in the transcript levels.

Reverse transcription PCR (RT-PCR) was carried out to further confirm *EcR* gene expression levels using the same first-strand cDNA templates. The following primers (sense and antisense) were used to amplify the cDNA fragments of the *N. lugens EcR* gene: 5′-GAAGGGTGCAAAGGTTTCTT-3′ and 5′-GCCACCTGACTGGGTAGATT-3′; and the *L. striatellus EcR* gene: 5′-TGCAGCTCATCGTTGAGTTCGCCAA-3′ and 5′-AGTTCTGGTTGCCCAGTGTTCTGAG-3′. *N. lugens β-actin* (5′-CCAAAGGCCAACCGTGAGAA-3′ and 5′-CAGGAAGGAAGGCTGGAACA-3′) was used as an internal control. The RT-PCR reaction was conducted under the following conditions: denaturation at 95 °C for 3 min followed by 30 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 45 s.

3. Results

3.1. Isolation and sequence analysis of EcR cDNA clones

A full-length cDNA clone encoding EcR was isolated from *L. striatellus* nymphs by the 3′ and 5′ RACE methods. The cDNA (GenBank