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Knockdown of a putative Halloween gene *Shade* reveals its role in ecdysteroidogenesis in the small brown planthopper *Laodelphax striatellus*

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ABSTRACT

Ecdysteroid hormone 20-hydroxyecdysone (20E) plays fundamental roles in insect development and reproduction, whereas the primary role of ecdysone (E) is the precursor for 20E. A cytochrome P450 monooxygenase (CYP), encoded by a Halloween gene *Shade* (*Shd*, *cyp314a1*), catalyzes the conversion of E into 20E in representative insect species in Diptera, Lepidoptera and Orthoptera. We describe here the cloning and characterization of *LsShd* in a hemipteran insect species, the small brown planthopper *Laodelphax striatellus*. LsSHD has five insect conserved P450 motifs, i.e., Helix-C, Helix-I, Helix-K, PERF and heme-binding motifs. Temporal expression pattern of *LsShd* was determined through the fourth-instar and the early fifth-instar stages by qPCR. *LsShd* showed two expression peaks in day 2 and day 5 fourth-instar nymphs, and two troughs in day 1 fourth and fifth instars. Dietary introduction of double-stranded RNA (dsRNA) of *LsShd* into nymphs successfully knocked down the target gene, decreased expression level of *ecdysone receptor* (*LsEcR*) gene, and caused nymphal lethality and delayed development. Ingestion of 20E did not increase *LsShd* expression level, but almost completely rescued *LsEcR* mRNA level, and relieved the negative effects on the survival and development in *LsShd*-dsRNA-exposed nymphs. In contrast, dietary introduction of E had little rescue effects. Thus, our data suggest that the ecdysteroidogenic pathway is conserved in insects, and LsSHD functions to regulate metamorphotic processes by converting E to 20E even in a hemipteran insect, *L. striatellus*.

1. Introduction

Ecdysteroid hormone 20-hydroxyecdysone (20E) regulates insect development and reproduction. A group of cytochrome P450 monooxygenases (CYPs), encoded by Halloween genes, are involved in ecdysteroidogenesis in immature insect stages. Among these CYPs, CYP307A1 (SPOOK) is involved in the conversion of 7-dehydrocholesterol into ketodiol, CYP306A1 (PHANTOM, 25-hydroxylase) converts ketodiol to ketotriol, CYP302A1 (DISEMBODIED, 22-hydroxylase) hydroxylates ketotriol to 2-deoxyecdysone, and CYP315A1 (SHADOW, 2-hydroxylase) catalyzes the conversion of 2-deoxyecdysone into ecdysone (E) in the prothoracic glands (PGs), or the prothoracic gland cells in the ring glands in dipterans. Finally, CYP314A1 (SHADE, SHD, ecdysone 20-monooxygenase, E20MO) catalyzes the conversion of E to 20E in the peripheral tissues, such as midgut, epidermis, fat body and Malpighian tubules (Iga and Kataoka, 2012; Luan et al., 2013). These CYP enzymes are characterized in Diptera *Drosophila melanogaster*, in Lepidoptera *Manduca sexta* and *Bombyx mori* (Chávez et al., 2000; Niwa et al., 2004, 2005; Warren et al., 2002, 2004), and in Orthoptera *Schistocerca gregaria* (Marchal et al., 2011, 2012). The description/prediction of the Halloween genes in other insect species is mostly based on sequence similarity (Christiaens et al., 2010; Iga and Smagghe, 2010; Yamazaki et al., 2011). In addition to insects, all Halloween genes are identified in the water flea *Daphnia pulex* (Rewitz and Gilbert, 2008) and some are reported in the spider mite *Tetranychus urticae* (Iga and Kataoka, 2012).

Up to now, however, involvement of Halloween genes in ecdysteroidogenesis has not been confirmed in hemipterans. The small brown planthopper, *Laodelphax striatellus* (Fallén) (Hemiptera: Delphacidae), is a pest of rice and wheat in China (Wang et al., 2010). It is now generally accepted that 20E is the principal molting hormone of arthropods and that the primary role of E is the precursor for 20E, although E plays other roles as well in the life of insects (Gilbert et al., 2002; Lafont et al., 2005). Therefore, efficient conversion of E into 20E to get a higher ratio of 20E/E is very important for insect in normal ecdysis (Petryk et al., 2003; Rewitz et al., 2006). In the present paper, we focus on the cloning and characterization of *Shd* gene in *L. striatellus*. Since ingestion of double-stranded RNA (dsRNA) could effectively knock down target genes in planthoppers (Chen et al., 2010; He et al.,

Abbreviations: PCR, polymerase chain reaction; RT-PCR, reverse transcriptase PCR; qPCR, quantitative real-time PCR; cDNA, complementary DNA; CYP, cytochrome P450 monooxygenase; dsRNA, double-stranded RNA; EcR, ecdysone receptor; E, ecdysone; 20E, 20-hydroxyecdysone; YLS, yeast-like symbionts; RNAi, RNA interference; ORF, open reading frame; ML, maximum-likelihood; SE, standard error; ANOVA, analysis of variance.

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2011; Li et al., 2011; Zha et al., 2011), we also detected the influence of dietary introduction of *LsShd*-dsRNA on nymphal performance.

Ecdysteroids act through their heterodimeric nuclear receptors, i.e., the ecdysone receptor (EcR) and the vertebrate retinoid × receptor homologue ultraspiracle. Mutations in and RNA interference (RNAi) against *EcR* cause phenotypic defects and lethality in *Tribolium castaneum* (Tan and Palli, 2008) and in *Laodelphax striatellus* and *Nilaparvata lugens* (Wu et al., 2012). Moreover, *EcR* expression is regulated by ecdysteroids through a positive feedback loop directly (Karim and Thummel, 1992) or indirectly (Varghese and Cohen, 2007) in *D. melanogaster*. In the present paper, therefore, we also determined whether dietary introduction of *LsShd*-dsRNA decreased *EcR* expression level to inhibit ecdysteroid signaling pathway. Our results revealed that knockdown of *LsShd* decreased expression level of *LsEcR*, caused nymphal lethality and delayed development. Ingestion of 20E almost completely rescued *LsEcR* mRNA level and overcame the negative effects on the survival and development in *LsShd*-dsRNA-exposed nymphs, in contrast to ingestion of E. Thus, our results suggest that LsSHD functions to regulate metamorphotic processes by converting E to 20E in *L. striatellus*.

2. Materials and methods

2.1. Insects and chemicals

L. striatellus was routinely reared on rice (*Oryza sativa*) variety (Taichung Native 1), using a protocol described recently (Yang et al., 2012). At laboratory, *L. striatellus* eggs hatched into nymphs within 13 days. Nymphs went through 5 instars, with the average periods of the first-, second-, third-, fourth- and fifth-instar stages of 6.0, 5.0, 5.0, 5.0 and 6.0 days, respectively. Upon reaching full size, the fifth-instar nymphs emerged as adults.

E and 20E were purchased from Sigma and were individually purified by reverse-phase HPLC before experiments.

2.2. Sequence assembly and homology searches

The transcriptome data sets of *L. striatellus* previously reported (Zhang et al., 2010) and recently constructed in our laboratory were obtained from mixed samples of *L. striatellus* whole bodies at different developing stages. The annotated SHD proteins from 4 representative insect species *Acyrthosiphon pisum*, *T. castaneum*, *M. sexta* and *D. melanogaster* were downloaded from NCBI reference sequence (RefSeq) database. These protein sequences were used for TBLASTN searches of *L. striatellus* transcriptome data sets to identify hits at a cut-off *e*-value of 1.0^{-5}. The nucleotide sequences of hits resulting from initial searches were annotated by blasting (BLASTX, *e*-values $<10^{-5}$) against a local protein database containing NCBI non-redundant proteins.

2.3. Molecular cloning

Total RNA was extracted from the fourth-instar nymphs using TRIzol reagent according to the manufacturer's instructions (Invitrogen) and was treated for 30 min at 37 °C with RNase free DNase I (Ambion, Austin, TX) to eliminate traces of chromosomal DNA. The purity and amount of RNA were determined by NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Rockland, DE, USA). First-strand cDNA was synthesized from the total RNA using the reverse transcriptase (M-MLV RT) (Takara Bio, Dalian, China) and an oligo $(dT)_{18}$ primer and was used as a template for polymerase chain reaction (PCR) to authenticate the sequence of the selected unigene. The primers based on the sequence were designed using Primer3 software (Rozen and Skaletsky, 2000) and listed in Table 1. Thermal cycling conditions were 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 45 s and 72 °C for 3 min. The last cycle was followed by final extension at 72 °C for 10 min. Each 50 μL PCR reaction contained 2 μL of cDNA template, 5 μL of 10× LA Taq buffer (Mg^{2+} Free), 4 μL of $MgCl_2$ (25 mM), 4 μL of dNTP mixture (2.5 mM/each), 1 μL of forward and 1 μL of reverse primers (10 μM), 0.5 μL of LA Taq polymerase (Takara Bio) (5 U/μL) and 32.5 μL of double distilled H_2O.

Table 1
Primers used in RT-PCR, 5′ and 3′ RACE, synthesizing dsRNA, and performing qPCR.

Primer	Sequence (5′ to 3′)	Amplicon size (bp)
Primers used in RT-PCR		
ShdFp	CATTTACATCCAACATCCAACC	1554
ShdRp	TCTTCTTCCGACCCCAAA	
Primers used in 5′-RACE		
ShdGSP	CGCCCTGTTCGTTTACTAGTCC	
ShdNGSP	TGTGCCACGTTTCGCCCTGTTC	
Primers used in 3′RACE		
ShdGSP	AGATGGCTGAGCAACAACGAGC	
ShdNGSP	CCCGTTTGGGGTCGGAAGAAGA	
Primers used in PCR for End to End		
ShdFp	CAAGGAGCGTCACAGAG	1693
ShdRp	ATTGGCGATACTAACTA	
Synthesizing the dsRNAs		
ShdFd	taatacgactcactataggg CGACCGATACACCAATG	470
ShdRd	taatacgactcactataggg TCTCGCTCCTCTGACAAT	
egfpup	taatacgactcactataggg AAGTTCAGCGTGTCCG	414
egfpdown	taatacgactcactataggg CTTGCCGTAGTTCCAC	
Performing the qPCR		
ShdFq	CGGAAAGATGGCTGAGCAACAAC	92
ShdRq	GCGTTTGGCGGGACACATTC	
EcRFq	AAACTGTTGCGCGAGGACCAAATC	187
EcRRq	AGAACTGCAACAGGTCTTCGACCA	
EF-1Fq	CCTTACCCATGTTGGATGCTTATT	95
EF-1Fq	TGCTTCTGTCTTCCTCTTTCTTCC	
ARFFq	TTGGACAGTATCAAGACCCATC	104
ARFRq	GCAGCAATGTCATCAATAAGC	

The 5′- and 3′-RACE Ready cDNAs were synthesized following the manufacturer's instructions, primed by oligo (dT) primer and the SMARTer II A oligonucleotide using the SMARTer RACE cDNA amplification kit (Takara Bio). Antisense and sense gene-specific primers (Table 1) corresponding to the 5′-end and 3′-end of the sequence obtained from above RT-PCR and the universal primer in the SMARTer RACE kit (Takara Bio) were used to amplify the 5′- and the 3′-ends. The components of reaction had been described above. Thermal cycling conditions were 94 °C for 3 min, followed by 5 cycles of 94 °C for 30 s, 72 °C for 5 min; and another 5 cycles of 94 °C for 30 s, 70 °C for 30 s, 72 °C for 5 min; and followed by 25 cycles of 94 °C for 30 s, 68 °C for 30 s, 72 °C for 5 min. The last cycle was followed by final extension at 72 °C for 10 min.

The amplified product was separated by 1.2% agarose gel and purified with Wizard DNA Gel Extraction Kit (Promega, Madison, Wis., USA), and then cloned into pGEM-T easy vector (Promega). Several independent subclones were sequenced on an Applied Biosystems 3730 automated sequencer (Applied Biosystems, Foster City, Calif., USA) from both directions.

After obtaining full-length cDNA, we designed a pair of primers (Table 1) to verify the complete ORF with the same PCR conditions outlined above. ORF was predicted using the EditSeq program of DNAStar (http://www.dnastar.com). The resulting sequence (*LsShd*) was submitted to GenBank (KC579461). The annotated SHD-like proteins from the 4 representative insect species mentioned above were aligned with the predicted LsSHD using ClustalW2.1 (Larkin et al., 2007).

2.4. Preparation of dsRNA

A 470 bp cDNA fragment of *LsShd* and a 414 bp fragment of enhanced green fluorescent protein gene *egfp* (control) were individually subcloned into pEASY-T3 vector (TransGen Biotech, Beijing, China), and the diluted plasmids were used as templates for amplification of these target sequences by RT-PCR, using specific primers (Table 1) conjugated with the T7 RNA polymerase promoter (5′-taatacgactcactataggg-3′).