Molecular cloning and RNA interference-mediated functional characterization of a Halloween gene *spook* in the white-backed planthopper *Sogatella furcifera*

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**Abstract**

**Background:** Ecdysteroid hormones ecdysone and 20-hydroxyecdysone play fundamental roles in insect postembryonic development and reproduction. Five cytochrome P450 monooxygenases (CYPs), encoded by Halloween genes, have been documented to be involved in the ecdysteroidogenesis in insect species of diverse orders such as Diptera, Lepidoptera and Orthoptera. Up to now, however, the involvement of the Halloween genes in ecdysteroid synthesis has not been confirmed in hemipteran insect species.

**Results:** In the present paper, a Halloween gene *spook* (*Sfspo, Sfcyp307a1*) was cloned in the hemipteran *Sogatella furcifera*. *SfSPO* has three insect conserved P450 motifs, i.e., Helix-K, PERF and heme-binding motifs. Temporal and spatial expression patterns of *Sfspo* were evaluated by qPCR. *Sfspo* showed three expression peaks in late second-, third- and fourth-instar stages. In contrast, the expression levels were lower and formed three troughs in the newly-molted second-, third- and fourth-instar nymphs. On day 3 of the fourth-instar nymphs, *Sfspo* clearly had a high transcript level in the thorax where PGs were located. Dietary introduction of double-stranded RNA (dsRNA) of *Sfspo* into the second instars successfully knocked down the target gene, and greatly reduced expression level of ecdysone receptor (*EcR*) gene. Moreover, knockdown of *Sfspo* caused lethality and delayed development during nymphal stages. Furthermore, application of 20-hydroxyecdysone on *Sfspo*-dsRNA-exposed nymphs did not increase *Sfspo* expression, but could almost completely rescue *SfEcR* expression, and relieved the negative effects on nymphal survival and development.

**Conclusion:** In *S. furcifera*, *Sfspo* was cloned and the conservation of *SfSPO* is valid. Thus, *SfSPO* is probably also involved in ecdysteroidogenesis for hemiptera.

**Keywords:** *Sogatella furcifera*, Halloween gene, Ecdysteroidogenesis, RNA interference, Lethality, Development

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**Background**

20-Hydroxyecdysone (20E), an active form of ecdysteroid, regulates insect postembryonic development and reproduction. Because of the absence of the enzymes involving in squalene synthesis, insects cannot synthesize 20E de novo, and must obtain precursor sterols from their food [1], or their associated yeasts or fungi [2]. Rice planthoppers reportedly harbored yeast-like symbionts (YLSs), mainly in mycetocytes formed by abdominal fat body cells [3-8]. The YLSs synthesize ergosta-5,7,24(28)-tri-enol [9-12]. Ergosta-5,7,24(28)-tri-enol is then converted into cholesterol in planthoppers [10,11].

The ecdysteroid biosynthesis in the prothoracic glands (PGs) begins from conversion of cholesterol into 7-dehydrocholesterol (7dC), mediated by a Rieske oxygenase *Neverland* [13,14]. The conversion of 7dC into 2,22,25-trideoxyecdysone (ketodiol) is a series of hypothetical and unproven reactions, and is called ‘Black Box’ [15]. In *Drosophila melanogaster* and *Bombyx mori*, CYP307A1/A2 (SPOOK/SPOOKIER, SPO/SPOK) [16,17] and CYP6T3...
Results
Molecular cloning and sequence analysis
Complete coding sequence of *S. furcifera* Halloween gene Sfspo (spo, cyp307a1) was obtained. Its open reading frame (ORF) encoded a putative protein with the length of 510 amino acid residue (Figure 1).

SPO sequence is similar to those from other insects. Insect CYPs have five insect conserved P450 motifs, i.e., WxxRxR (Helix-C), GxEx/DTT/S (Helix-I), ExRxR (Helix-K), PxxFxE/DRF (PERF motif) and PfxxGxxCxxG/A (heme-binding domain), where ‘x’ means any amino acid [40]. For SfSPO, Helix-C and Helix-I are not conserved. Helix-C had the amino acid sequence of H/YxxPR, and the amino acid sequence of Helix-I was GGHSA/V (Figure 1).

In insects, SPO belongs to CYP2 family. The N-terminus of SfSPO has one of the common characters in microsomal P450s, consisting many hydrophobic residues followed by a proline/glycine (P/G) rich region (Figure 1).

Temporal and spatial transcript profiles
At our experiment temperature, *S. furcifera* second-, third- and fourth-instar nymphs lasted an average of 2.0, 2.0 and 3.0 days. Sfspo showed three expression peaks in day 2 of second-instar, day 2 of third-instar and day 3 of fourth-instar nymphs. In contrast, the expression levels were lower and formed three troughs in the newly-molted second-, third- and fourth-instar nymphs (Figure 2A).

The spatial distribution of Sf spo on day 3 of the fourth-instar nymphs was also tested using qPCR. Sf spo clearly had a high transcript level in the thorax where PGs were located. Moreover, trace amounts of transcripts were found in the head and abdomen (Figure 2B).

Dietary ingestion of dsRNA on expression of Sfspo and EcR genes
During 6 days of continuous exposure to dsRNA-contained diet and 1 day after experiment, mRNA abundance of Sf spo in the surviving nymphs was examined by q-PCR. The mRNA level of Sf spo in treated nymphs respectively reduced by 63.0%, 87.8%, 76.2%, 93.9%, 81.8%, 92.2% and 94.5%, respectively, comparing to that in dsRNA-exposed controls (Figure 3A). This indicated that the RNAi-mediated knockdown of Sf spo was successful.

Since SfSPO is expected to act in other genes in the same signaling pathway, the possible effect of Sf spo knockdown was examined on the transcript level of SfEcR, which was one of 20E heterodimeric nuclear receptors and was regulated by 20E through a positive feedback loop directly [29] or indirectly in *D. melanogaster* [30]. As expected, during 6 days of continuous exposure to dsRNA-contained diet and 1 day after experiment, SfEcR expression levels in nymphs decreased by 76.0%, 88.3%, 71.7%, 88.8%, 82.1%, 85.9% and 89.2% respectively, when compared with that in dsegfp-ingested planthoppers (Figure 3B).

Effect of dsRNA on nymph survival
Six day ingestion of dsRNA-contained diet caused nymphal lethality. The mortality reached up to 20% in nymphs that
had ingested dsSfspo. In most cases, nymphs died during the period of ecdysis. In contrast, less than 5% of the planthoppers on normal or egfp-dsRNA-contained diets died (Figure 3C).

Effects of dsRNA on nymph development

Six day period of continuous exposure to dsRNA-contained diet significantly delayed nymphal development. 100% of the nymphs on normal and egfp-dsRNA-contained diets became the fourth instars after experiment. In contrast, 24% of the individuals on Sfspo-dsRNA-contained diets remained in the third-instar (Figure 3D).

Rescue experiment

Application of 300 pg of 20E did not affect the expression level of Sfspo. In contrast, 20E application almost completely rescued SfEcR expression at mRNA level. Moreover, 20E application to Sfspo-dsRNA-exposed nymphs almost completely overcame the negative effects on the survival and the development (Figure 4).
Discussion
Since the fundamental phenomena such as molting and metamorphosis are conserved during arthropod evolution, the Halloween genes are expected to be well conserved in insects [23,25,26,41-44], and in other arthropods [1,45]. In the present paper, the presence of Sfspo was demonstrated in S. furcifera. The primary structure of SfSPO has three insect conserved P450 motifs, i.e., Helix-K, PERF and heme-binding motifs. Similar structural characters have been documented in SPO- and SPOK-like proteins from other insect species of diverse orders such as Diptera [16,17,20], Coleoptera [21], Hymenoptera [22], Lepidoptera [17,23], Orthoptera [25], and Hemiptera [26]. The N-terminus of SfSPO has one of the common characters in microsomal P450s, consisting many hydrophobic residues followed by a proline/glycine (P/G) rich region. Consistent with the structural features, SPO is detected in endoplasmic reticulum (ER) when the corresponding gene is transfected to Drosophila S2 cells [17,46]. Moreover, Sfspo showed three expression peaks in late second-, third- and fourth-instar stages. In contrast, the expression levels were lower and formed three troughs in the newly-molted second-, third- and fourth-instar nymphs. In the fourth-instar nymphs of the brown planthopper N. lugens [47] and in the sixth-instar larvae of a lepidopteran species S. littoralis, the level of ecdysteroid showed a peak in the later instar stage. In D. melanogaster larval stage, expression patterns of Dmspo gene undergoes dramatic fluctuations, consistent with circulating ecdysteroid quantity in the haemolymph: being high in late seconds, low in early third and high in late thirds [17]. Furthermore, we found in this study that Sfspo clearly had a high transcript level in the thorax where PGs were located. Similarly, Dmspo is expressed primarily in the PG cells of the ring gland in larval and adult stages [17]. Thus, the structural features and temporal and spatial expression patterns suggest that SfSPO might be involved in the ecdysteroidogenesis in S. furcifera.

The suggestion is further confirmed by three lines of experimental evidence in the present paper. Firstly, RNAi-mediated knockdown of Sfspo in S. furcifera reduced the expression level of SfEcR at the mRNA level. In other insect species, mutations in or RNAi against the Halloween enzymes caused a decrease in ecdysteroid titers [23,25,26,44,46,48-51]. Moreover, the expression of EcR gene was regulated by ecdysteroids through a positive feedback loop in D. melanogaster [29,52]. Accordingly, it can be hypothesized that RNAi-mediated knockdown of Sfspo negatively affects ecdysteroidogenesis in S. furcifera, and subsequently down-regulated SfEcR expression in S. furcifera. Consistent with the hypothesis, our rescue experiment revealed that 20E application almost completely rescued SfEcR expression in nymphs that had ingested dsSfcyp307a1.

The second line of experimental evidence is that RNAi-mediated knockdown of Sfspo in S. furcifera caused phenotypic defects similar to insects whose ecdysteroid synthesis was disturbed or whose ecdysteroid-mediated signaling had been inhibited [53,54]. In the present paper, we found that ingestion of dsSfspo caused nymphal lethality and developmental delay. Since the average second- and third-instar periods of the nymphs in our experimental conditions was respectively about 2 days and the deaths mainly occurred in the sixth day after dsRNA exposure, it means that the nymphs died during the third ecdysis. In fact, we also observed many abnormal and lethal ecdisis individuals on Sfspo-dsRNA contained diet, whereas most of the larvae on control normally molted. Similar phenomena have been observed in other two rice planthoppers, L. striatellus and N. lugens, in which silencing of EcR expression by in vivo RNAi to inhibit ecdysteroid-mediated signaling generated phenotypic defects in molting and
resulted in lethality in most of the treated nymphs. Intriguingly, apparent wing defects in morphogenesis and melanization occurred in L. striatellus nymphs subjected to dsEcR microinjection [54].

It has long been known that topical application of 20E could trigger physiological response such as regulation of diapause in the fourth-instar planthopper nymphs [55]. In the present paper, we tested whether 20E could rescue the negative effects of Sfspo-dsRNA ingestion on nymphs. Our results revealed that 20E application to Sfspo-dsRNA-exposed nymphs almost completely relieved the negative effects on the survival and the development. Thus, we provided the third line of evidence to support the suggestion that SfSPO plays critical roles in ecdysteroidogenesis in S. furcifera.

Conclusions
In the present paper, we cloned Sfspo and found that the conservation of SfSPO is valid in S. furcifera. Thus, SfSPO is probably also involved in ecdysteroidogenesis for hemiptera.

Methods
Insect culture and chemicals
S. furcifera adults were collected from Nanjing (32.0° N, 118.5° E), Jiangsu Province in China in 2010. The strain has been reared routinely on rice (Oryza sativa), in an insectary under controlled temperature (28 ± 1°C), photoperiod (16 h light/8 h dark) and relative humidity (more than 80%) since then, with wild stock injections every summer. Rice variety (Taichung Native 1) was grown in soil at 30–35°C under a long day photoperiod (16 h light/8 h dark) in a growth incubator. The planthoppers were transferred to fresh seedlings every 10–14 days to assure sufficient nutrition.

At laboratory reared by above protocol, S. furcifera eggs hatched into nymphs within 7 days. Nymphs went through 5 instars, with the average periods of the first-, second-, third-, fourth- and fifth-instar stages of 2.5, 2.0, 2.0, 3.0 and 3.0 days, respectively. Upon reaching full size, the fifth-instar nymphs emerged as adults.

20E was purchased from Sigma, and was purified by reverse-phase HPLC before experiments.

Sequence assembly and homology searches
Raw nucleotide reads of S. furcifera were downloaded from the NCBI Sequence Read Archive (SRA) database with its accession number SRP009194, and assembled into unigenes using Trinity software [56]. The annotated SPO from 4 representative insect species A. pisum, T. castaneum, M. sexta and D. melanogaster were downloaded from NCBI reference sequences (RefSeq) database. These protein...
sequences were used for TBLASTN searches of *S. furcifera* transcriptome data to identify hits at a cutoff E-value of $10^{-5}$. 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.

**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 sequences of the selected

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**Figure 4** Relative expression level (REL) of *spo* and *EcR* gene, mortality and percentage of third-instar nymphs in *S. furcifera* nymphs subjected to both ds*Sfspo* exposure and 20E application. The nymphs were continuously ingested dsRNA from the second-instar through the early fourth-instar stage. Two-days after dsRNA exposure, the nymphs received 0.03 μL acetone or 300 pg of 20E in 0.03 μL acetone. The values represent averages with vertical bars indicating SE, which topped with the same letters are not statistically different at $P = 0.05$. 

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The graphical representation shows the relative expression levels (REL) of *spo* and *EcR* genes, mortality, and percentage of third-instar nymphs under different treatment conditions.
unigenes. The primers based on the sequences were designed using Primer3 software [57]. Once initial Sfspo unigenes were authenticated, they were aligned to the full cDNA sequence of the gene from the 4 representative insect species mentioned above. Some short sequence gaps between two aligned unigenes were found. Specific primers were designed based on the two unigenes between each gap, and the gaps were filled by PCR. The final cDNA sequence was authenticated using the primers listed in Table 1. Thermal cycling conditions were 94°C for 5 min, followed by 35 cycles of 94°C for 30 sec, 55°C for 45 sec 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 (Mg2+ Free), 4 μL of MgCl2 (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 H2O.

The 5’- and 3’-RACE Ready cDNA were synthesized following the manufacturer’s instructions, primed by oligo (dT) primer and the SMART II A oligonucleotide using the SMARTer RACE cDNA amplification kit (Takara Bio.). Antisense and sense gene-specific primers (Table 1) corresponding to the 5’- and 3’-end of the sequence obtained above, and the universal primers in the SMARTer RACE kit (Takara Bio.) were used to amplify the 5’-end and the 3’-end. The components of reaction have been described above. Thermal cycling conditions were 94°C for 3 min; followed by 5 cycles of 94°C for 30 sec, 72°C for 5 min; and another 5 cycles of 94°C for 30 sec, 70°C for 30 sec, 72°C for 5 min; and followed by 25 cycles of 94°C for 30 sec, 68°C for 30 sec, 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 full-length cDNA was obtained, we designed primers (Table 1) to verify the complete ORF with the same PCR conditions outlined above. ORF was predicted using the edisseq program of DNAStar (http://www.dnastar.com) and the features of the protein were determined by TargetP. The resulting sequence was submitted to GenBank (KC579454). The annotated SPO-like proteins from the 4 representative insect species mentioned above were aligned with the predicted LsSPOK using ClustalW2.1 [58].

Preparation of dsRNA
A 415 bp cDNA sequence of Sfspo 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 PCR, using specific primers (Table 1) conjugated with the T7 RNA polymerase promoter (5’-taatacactacactattag-3’) and the PCR conditions described above. The PCR products were purified with Wizard H SV Gel (Promega) and used as templates for dsRNA synthesis with the T7 Ribomax TM Express RNAi System, according to the manufacturer’s instructions (Promega). The reaction products were treated with RNase and DNase I to degrade single-strand RNA and DNA template, respectively, at 37°C for one hour, following manufacturer’s directions. The synthesized dsRNA was isopropanol precipitated, resuspended in Nuclease-free water, and quantified by a spectrophotometer (NanoDrop TM 1000) at 260 nm. The purity and integrity were determined by agarose gel electrophoresis. The dsRNA stocks can be stored for several weeks at −80°C until use.

### Table 1 Primers used in RT-PCR, 5’ and 3’ RACE, synthesizing dsRNA, and performing qRT-PCR

| Primer | Sequence (5’ to 3’) | Amplicon size (bp) |
|--------|---------------------|--------------------|
| **Primers used in RT-PCR** | | |
| spoFp | ACGGCCGAGTCCATTTCAG | 344 |
| spoRp | TGTTGGATGAGCAGTCCG | |
| **Primers used in 5’-RACE** | | |
| spoGP | GATGCCAAATGAGGTTGGAT | |
| spoNGSP | TCGTGAGAAGTTCGGTTCG | |
| **Primers used in 3’-RACE** | | |
| spoGSP | ACGGCCACATTCGCCGTTTGT | |
| spoNGSP | CGATGCCGCTGTCGTCACCTT | |
| **Primers used in PCR for End to End** | | |
| spoFp | CGTCGTGAAACACCCTTAT | 2090 |
| spoRp | GCCCGGATACCATTTATCTT | |
| **Synthesizing the dsRNAs** | | |
| spoFd | CTTGCCGACATTCGCCGTTT | 415 |
| spoRd | TCGGGTTTTTTTATGTTC | |
| egfpup | AAGTTCAGCGTGTCCG | 414 |
| egfpdown | CTTGCCGACATTCGCCGTTT | |
| **Performing the qPCR** | | |
| spoFq | CAACCTCCATACATACATCAAGCCACTG | 119 |
| spoRq | ACCGACGACCACCATATCTCTGAAC | |
| EcRFq | AATGGTAGGCCGAGCACCCTAGCCGA | 129 |
| EcRRq | AATGTTGCATTCCGCTGAGTGTGGCG | |
| RPL9Fq | TGTGTGACCACCGAGAACAAC | 131 |
| RPL9Rq | ACAGTACGCTGTCAGTG | |
| ARFFq | CACAATATACGCCGACTTGGGAT | 141 |
| ARFRq | CAGATCGACCGTCCGTAACC | |

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Bioassay
Previously reported dietary dsRNA-introducing procedure [38,39] was used, with small modifications. Briefly, glass cylinders, 12 cm in length and 2.8 cm in internal diameter, were used as feeding chambers. Twenty first-instar nymphs were carefully transferred into each chamber and pre-reared for one day to the second-instar stage, on liquid artificial diet (according to Dr. Fu et al. [59]) between two layers of stretched Parafilm M (Pechiney Plastic Packaging Company, Chicago, IL, USA) that was placed at both ends of the chamber. The artificial diet containing one of the dsRNAs at the concentration of 0.5 mg/ml [38,39] were then used to feed the second-instar nymphs. The diet was changed and dead nymphs were removed daily.

Two experiments were carried out. The first had three treatments including non-dsRNA diet (blank control), dsegfp diet (negative control) and dsSfspo diet. The experiment lasted for 6 days. The second bioassay was a rescue experiment. Since topical application of 300 pg of 20E was enough to trigger physiological response in the fourth-instar planthopper nymphs [55], 300 pg of 20E was used in the second bioassay. After exposed to dsRNA for 2 days, the nymphs were anesthetized with carbon dioxide. A 0.03 μL aliquot of acetone with or without 300 pg of 20E was topically applied to the dorsal thoracic surface of the nymphs with a 10-μL microsyringe connected to a microapplicator (Hamilton Company, Reno, NV). And then, the nymphs were continuously exposed to dsRNA for another 4 days. There were four treatments including: (1) nymphs on non-dsRNA diet and applied acetone (blank control); (2) nymphs on dsegfp diet and applied acetone (negative control); (3) nymphs on dsSfspo diet and applied acetone; (4) nymphs on dsSfspo diet and applied 20E. All treatments in both experiments were replicated 25 times (25 chambers), and a total of 250 nymphs in each treatment were used (100 nymphs for bioassays and 150 nymphs for q-PCR).

Mortality was recorded daily. The surviving nymphs after bioassay were collected and frozen. The instars of the surviving nymphs were identified by head capsule width after bioassay were collected and frozen. The instars of the nymphs in each treatment were used (100 nymphs for bioassays, using SV Total RNA Isolation System Kit (Promega). Each sample contained 10 nymphs and repeated in biological triplicate. Purified RNA was subjected to DNase I to remove any residual DNA according to the manufacturer’s instructions. In a preliminary experiment, we estimated the expression stability of four house-keeping genes (Actin; ADP-ribosylation factor, ARF; ribosomal protein RPL9; translation elongation factor 1α EF1α), and found that ARF and RPL9 were the most stable house-keeping genes and selected as internal controls. The primers of the Halloween genes Sfspo, EcR gene, ARF and RPL9 were designed with Beacon Designer 7 (Table 1). Putative mRNA abundance of Sfspo the Halloween and EcR genes in each nymphal sample was estimated by qPCR using SYBR Premix Ex Taq™ (Perfect Real Time) (Takara Bio.) and ABI 7500 Real-Time PCR System (Applied Biosystems) according to the manufacturer's instruction. The reaction mixture consisted of 2 μL of cDNA template (corresponding to 50 ng of the starting amount of RNA), 10 μL of SYBR Premix Ex Taq (Takara Bio.), 1 μL of forward primer (10 μM), 1 μL of reverse primer (10 μM), 0.4 μL of Rox Reference Dye (50×) in a final reaction volume of 20 μL. A reverse transcription negative control (without reverse transcriptase) and a non-template negative control were included for each primer set to confirm the absence of genomic DNA and to check for primer-dimer or contamination in the reactions, respectively. The following standard qPCR protocol was used: denaturing at 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec and 60°C for 34 sec. After amplification, the melting curves were determined by heating the sample up to 95°C for 15 sec, followed by cooling down to 60°C for 1 min, and heating the samples to 95°C for 15 sec.

The generation of specific PCR products was confirmed by sequencing and gel electrophoresis. Each primer pair was tested with a 10-fold logarithmic dilution of a cDNA mixture to generate a linear standard curve (crossing point CP plotted vs. log of template concentration), which was used to calculate the primer pair efficiency. All experiments were repeated in technical triplicate. Data were analyzed by the 2-ΔΔCt method [62], using the geometric mean of ARF1 and RP18 for normalization according to the strategy described previously [62,63].

Data analysis
The data were given as means ± SE, and were analyzed by ANOVAs or a repeated measures ANOVA followed by the Tukey-Kramer test, using SPSS for Windows (SPSS, Chicago, IL, USA).

Abbreviations
PCR: Polymerase chain reaction; RT-PCR: Reverse transcriptase PCR; qRT-PCR: Quantitative real-time PCR; cDNA: Complementary DNA; CYP: Cytochrome P450 monoxygenase; 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.

Competing interests
The authors declare that they have no competing interests.
Authors’ contributions
SJ and P.W. performed most of the experimental procedures, and data analysis. LTZ and L.L.W. performed partial experiments, assisted in manuscript revising and provided helpful discussions. G.Q.L. wrote the manuscript, conceived and supervised the research. All authors read and approved the final manuscript.

Acknowledgments
This research was supported by the National Basic Research Program of China (973 Program, No. 2010CB126200). We thank Drs Z. Han, and S. Dong of our laboratory for useful discussions during the course of this research.

Received: 22 March 2013 Accepted: 26 August 2013 Published: 4 September 2013

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Cite this article as: Jia et al. Molecular cloning and RNA interference-mediated functional characterization of a Halloween gene spook in the white-backed planthopper Sogatella furcifera. BMC Molecular Biology 2013 14:19.