Knockdown of Cs-Spook induces delayed larval molting in rice striped stem borer Chilo suppressalis

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KNOCKDOWN OF *CS-SPOOK* INDUCES DELAYED LARVAL MOLTING IN RICE STRIPED STEM BORER *Chilo suppressalis*

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Spook has essential roles in the biogenesis of the molting hormone 20-hydroxyecdysone (20-E). The function of spook in the rice striped stem borer (SSB) Chilo suppressalis *remains unclear, prompting our hypothesis that it exerts actions similar to those reported for other insect species. Here we amplified the full-length transcript of spook (*Cs-Spook*) in SSB by 5′ and 3′ rapid amplification of cDNA ends.* Cs-Spook *has conserved P450 motifs such as Helix-C, Helix-I, Helix-K, and PERF motif (*PxxFxPxRF*). It was highly expressed in late instar larvae but less so in newly molted larvae.* Cs-Spook *was highly expressed in prothoracic glands.* Cs-Spook *was knocked down by dsRNA treatments. Compared with controls, the gene expression level was reduced to 9% at 24 h post injection (PI), 33% at 48 h PI, and 24% at 72 h PI. The ecdysteroid titer decreased significantly in the dsRNA-treated group (*$P < 0.05$*), resulting in delayed larval development. The delayed development in dsRNA-treatment group was rescued by treating with 20-E. Our work demonstrates that* Cs-Spook *participates in the biogenesis of 20-E and regulates the molt of SSB, as seen in other species.* © *2014 Wiley Periodicals, Inc.*

Keywords: *Chilo suppressalis*; Halloween genes; development; *spook*

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INTRODUCTION

Insect molting and metamorphosis are controlled by the pulses of steroid hormones (Yamanaka et al., 2013), mainly the ecdysteroid, 20-hydroxyecdysone (20-E), that is derived from ecdysone (E). Ecdysone is synthesized via a series of hydroxylation and oxidation steps in the prothoracic gland, which is afterward converted to 20-E by 20-hydroxylase present in the peripheral tissues (Lafont et al., 2012). The 20-E biosynthesis begins from the conversion of cholesterol into 7-dehydrocholesterol (7dC) mediated by a conserved rieske oxygenase DAF-36/Neverland (Yoshiyama et al., 2006; Yoshiyama-Yanagawa et al., 2011). The Halloween genes are responsible for the synthesis of 20-E (Brown et al., 2009; Christiaens et al., 2010).

CYP307A1/2 (*spook/spookier* [*spo/spok*]) are involved in the synthesis of 20-E (Namiki et al., 2005; Ono et al., 2006; Ou et al., 2011). Spo- and/or Spok-like proteins were found in a variety of insects such as *Tribolium castaneum* (Hentze et al., 2013), *Schistocerca gregaria* (Marchal et al., 2011), *Acyrthosiphon pisum* (Christiaens et al., 2010), *Drosophila melanogaster* (Ono et al., 2006), *Bemisia tabaci* (Luan et al., 2013), *Apis mellifera* (Yamazaki et al., 2011), *Bombyx mori* (Namiki et al., 2005), *Spodoptera littoralis* (Iga and Smagghe, 2010), *Manduca sexta* (Ono et al., 2006), and *Holcocerus hippophaecolus* (Zhou et al., 2013). Dipterans have two spook-like genes, *spo* (*CYP307A1*) and *spok* (*CYP307A2*). However, lepidopterans only have one *spo* gene, *CYP307A1* (Ono et al., 2006), and the honeybee *A. mellifera* has only one ortholog, *spookiest* (*spot, CYP307B1*). Function analysis indicated that insect *spos* are involved in metamorphosis. RNAi-mediated knockdown of *spok* in the prothoracic *glands* induced arrest of molting in *D. melanogaster* (Ono et al., 2012).

The rice striped stem borer (SSB), *Chilo suppressalis* (Walker), is a lepidopteran rice pest in East Asia (Zhu et al., 2007). SSB larvae enter into the rice stem and feed on rice, causing severe yield loss (Beevor et al., 1990). *Spo* remains understudied in the SSB. Here, we cloned an SSB *spo* (*Cs-Spo*) and studied its expression in different developmental stages. The function of *spo* was also investigated, showing that knockdown of *spo* delayed metamorphosis development of SSB.

MATERIALS AND METHODS

Insects

The SSB larvae were collected in 2011 from a rice field in Nanjing, Jiangsu province of China (32.0° N, 118.5° E). All insects were reared on rice seedlings under temperature of 28 ± 1°C, RH of 70 ± 5%, and the photoperiod of 16:8 (L: D). The SSB larvae were transferred to fresh seedlings every 3–5 days to ensure sufficient nutrition.

Total RNA Isolation and cDNA Synthesis

Total RNA was extracted with TRIzol reagent following the manufacturer's recommended procedure (Invitrogen, Grand Island, NY, USA). Fourth instar larvae (~10 mg/larva) were frozen with liquid nitrogen. The samples were homogenized in a tissue grinder using 1 ml TRIzol reagent. To prevent DNA contamination, an additional RNase-free DNase I treatment was used following the DNA-free kit's protocol (Roche Diagnostics, Mannheim, Germany). RNA integrity was assessed on a 1.2% agarose gel and the purity was measured

Table 1. Primers Used for RT-PCR, qRT-PCR, RACE, and dsRNA Synthesis

	Primers	*Sequence (5′ to 3′)*	*Product size (bp)*
RT-PCR	Spo-RT-F	TGGGTGGACATTCTTCTG	468
	Spo-RT-R	TCACGGATACAACCTCTTT	
5′-RACE	Spo-GSP-5	CGATTCGTGCTTGCCCAAAAGGTGTA	
	Spo-NGSP-5	CCCGGTGCCGTCCGAAGCGTAT	
3′-RACE	Spo-GSP-3	TACCACATTTCCTCCCCTTCAGCATCG	
	Spo-NGSP-3	CCGCACCTTCATACTGTCAAGAATCGTC	
End-to-end PCR	Spo-end-F	TCCTTGCCGTCGTAATA	1,980
	Spo-end-R	TTTGAACTCCAGCCACTT	
dsRNA synthesis	Spo-dsRNA-F	taatacgactcactataggTGGGTGGACATTCTTCTG	468
	Spo-dsRNA-R	taatacgactcactataggTCACGGATACAACCTCTTT	
	GFP-dsRNA-F	taatacgactcactataggAGTTCAGCGTGTCCG	414
	GFP-dsRNA-R	taatacgactcactataggCACCTTGATGCCGTTC	
qRT-PCR	Spo-qRT-F	AAGACATCCGCACCTTCATAC	215
	Spo-qRT-R	ACAGCAGCTAGACACAACATAA	
	actinA1-qRT-F	GTCGCTTCCCAAATTACATC	153
	actinA1-qRT-R	CTCCATATCGTTCCAGTCG	

using the NanoDrop. PrimeScript™ 1st Strand cDNA Synthesis Kit (Takara Bio., Dalian, China) was used to synthesize cDNA template.

PCR

Cs-Spo transcript was obtained from the SSB draft genome (Yin et al., 2014). Primer Premier 5.0 was used to design PCR primers (all reported in Table 1; Ren et al., 2004). PCRs were performed in a 25 μl reaction mixture containing 2 μl of dNTP (2.5 mmol/l), 2.5 μl of 10× LA Taq buffer (Mg^{2+} free), 2 μl of $MgCl_2$ (25 mmol/l), 1 μl of forward and 1 μl of reverse primers (10 mmol/l), 0.5 μl of cDNA template, 0.25 μl of LA *Taq* polymerase (5U/μl; Takara Bio., Dalian, China). The PCR program included an initial denaturation step for 3 min at 94°C, followed by 30 cycles of 94°C for 30 sec (denaturation), 55°C for 30 sec (primer annealing), and 72°C for 1 min (elongation), then at 72°C for 10 min for final extension. Aliquotes of the PCR products were assessed on a 1.5% w/v agarose gel run in TAE buffer (40 mmol/l Tris-acetate, 2 mmol/l $Na_2EDTA{\cdot}2H_2O$). Ethidium bromide staining was used to visualize the resulting bands. Bands of the expected size were cut and purified from the gel with Wizard DNA Gel Extraction Kit (Promega, Madison, WI), and then cloned into a pGEM-T easy vector (Invitrogen). Recombinant plasmids with primers for both strands were sequenced (GeneScript, Nanjing, China).

Rapid Amplification of cDNA Ends (RACE)

For RACE-PCR, 3′ and 5′ cDNA templates were synthesized using SMARTer™ RACE cDNA Amplification Kit (Takara, Japan). Primer Premier 5.0 was used to design RACE-PCR primers (Table 1). Standard procedures for RACE-PCRs at an annealing temperature of 55–65°C were performed with 2 μM of each primer and 2 μl Takara LA Taq DNA polymerase. To obtain specific amplification of the desired target, nest RACE-PCRs were carried out. In the nested PCRs, 1 μl of the 100× diluted first-round RACE-PCR product was used as the template, 1 μl of nested universal primer (provided with the kit), 1 μl of nested gene-specific primers, and 2 μl Takara LA Taq DNA polymerase was used in

the reactions. Standard procedures were used in the nested RACE-PCRs. The amplified products were purified with Wizard DNA Gel Extraction Kit), and then cloned into pGEM-T easy vector (Promega). Transcript was verified with end-to-end PCRs. The resulting sequence was submitted to GenBank (accession number: KF701128).

Sequence Analysis

Sequence identities and orthologs analysis were performed using BLASTP (http://blast.ncbi.nlm.nih.gov/) with e-value cutoff of BLASTP = 0.00001. The spo sequences of different insects were downloaded from the GenBank database. The deduced amino acids sequences of Cs-Spo were aligned by MUSCLE (Edgar, 2004) and were viewed using GeneDoc (http://www.nrbsc.org). For phylogenetic analysis, we first used ClustalX to align the protein sequences and then constructed an evolution tree by MEGA version 5.0 with the neighbor-joining method (Saitou and Nei, 1987). The bootstrap value was 1,000 (Tamura et al., 2011). To ensure the reliability, only those P450 proteins with >300 amino acids were used for phylogenetic analysis.

qPCR

The insects were collected every 24 h from the fourth to sixth instar larvae and pupae. Total RNA purification, DNase I treatment, and cDNA synthesis were performed as described above. The expression stability of the reference gene, *actin-A1*, was reported in our previous study (Teng et al., 2012). Primers (Table 1) for *Cs-Spo* and *actin-A1* for real-time PCR were designed using an online tool PrimerQuest (http://www.idtdna.com/Scitools/Applications/Primerquest/). To calculate the primer pair efficiency, each primer pair was tested with a 10-fold logarithmic dilution of a cDNA template. qPCR was conducted in 20 μl reactions, containing 2 μl of cDNA template, 10 μl of SYBR Premix Ex Taq, 1 μl of forward primer (10 μM), 1 μl of reverse primer (10 μM), 0.4 μl of Rox Reference Dye (50×). A standard protocol was used in qPCR, including denature 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 to 95°C for 15 sec, followed by cooling to 60°C for 1 min, and heating the samples to 95°C for 15 sec. All the samples were repeated in independent biological triplicate. Data were analyzed using $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008).

Preparation of dsRNA and Microinjection

Double-strand RNA (dsRNA) was synthesized using T7 Ribomax^{TM} Express RNAi System (Promega). cDNA of *Cs-Spo* and green fluorescent protein (GFP) were cloned into pEASY-T3 vector (TransGen Biotech, Bejing, China). Diluted plasmids were used as the templates using gene-specific primers conjugated with T7 promoter sites for PCR (Table 1). The PCR products were purified with Wizard HSV Gel (Promega) and used as the templates for dsRNA synthesis with the T7 Ribomax^{TM} Express RNAi System according to manufacturer's instructions. Two complementary RNA transcripts were made and hybridized to form dsRNA. Single-strand RNA and DNA were removed by treating the reaction product with RNase and DNase I at 37°C incubation for 30 min. The synthesized dsRNA was precipitated by isopropanol and resuspended in nuclease-free water. The dsRNA quantity was measured by spectrophotometer NanoDrop TM 1000 at 260 nm. Aliquotes of the dsRNA products were assessed on a 1.2% w/v agarose gel run in TAE buffer (40 mmol/l

Tris-acetate, 2 mmol/l $Na_2EDTA{\cdot}2H_2O$). Ethidium bromide staining was used to visualize the resulting bands.

The final concentration of 2400 ng/μl of dsRNA was prepared by adding nuclease-free water. Two microliters of dsRNA was injected between the third and fourth segments of day 3, fourth instar larvae using the Eppendorf InjectMan NI 2 microinjection system (Eppendorf, Hamburg, Germany). The needles were pulled from glass capillaries (1.0 mm outer diameter and 0.50 mm inner diameter) using a micro pipette puller (Model P-87, Sutter Instruments Co., Novato, CA). Needles were kept at the injection points for 30 sec to avoid dsRNA leakage from the insect body.

Relative Expression Level of Cs-Spo in dsRNA-Treated Insects

The dsRNA was injected into the forth instar larvae at 3 days after molting. There are two groups. Both groups have two treatments, dsGFP injection (negative control) and ds*Cs-Spo* injection. One group was set up for phenotype observation (30 individuals for each treatment). The other group was used for estimating mRNA abundance after dsRNA injection (40 individuals for each treatment). Samples for estimating mRNA abundance were collected at 24, 48, 72, 96, and 120 h after dsRNA injection. The abundances of *Cs-Spo* in the dsRNA-treated group were normalized with the data at the same time point in the control group. The expression of *Cs-Spo* in the control group was normalized as 1. All experiments were repeated in independent biological triplicates.

Total RNA isolation and cDNA synthesis were carried out as described above. *Actin-A1* (ChiloDB accession number: CSUOGS101387-TA) was used as the reference gene (Teng et al., 2012; Yin et al., 2014). The mRNA abundances were determined by qPCR using an ABI PRISM 7300 (Applied Biosystems) with Fast Start Universal SYBR Green Master (Roche, Germany). The mortality rate and molting rate were calculated at 3 days PI. The data are presented as means ± SE, and were analyzed by ANOVAs using SPSS for Windows (SPSS, Chicago, IL, USA). All graphs were made using GraphPad Prism 5.

20-E Titer Measurements

The dsRNA-treated insects were collected at 48 h PI. Whole larval bodies were homogenized with 70% methanol, heated to 60°C for 10 min, and centrifuged at 10, 000 × g for 10 min. The resulting pellets were extracted twice with 70% methanol. Supernatants were collected and dried under reduced pressure. To remove apolar lipids, hexane and 70% methanol were added to dried residues (Dinan and Rees, 1981). Hexane phase was discarded. The lower methanolic phase was desiccated and redissolved in 300 μl 70% methanol. Ecdysteroids was measured by Agilent HPLC system on XDB-C18 column (4.6 × 250 mm, 5μm; Agilent, CA). The injection volume of the sample was 20 μl. 20-E (Sigma, St. Louis, MO USA) was used as the standard.

Rescue Experiments

We used 20-E to rescue the dsRNA phenotype. A 0.25 μl aliquot of acetone with or without 20-E (1.0 mg/ml) was topically applied to the thoracic surface of the dsRNA-treated larvae. Each group contains 30 individuals. There were three treatments: (i) larvae injected with GFP dsRNA and treated with acetone, (ii) larvae injected with *Cs-Spo* dsRNA and treated with acetone, and (iii) larvae injected with *Cs-Spo* dsRNA and treated with 20-E. All treatments were repeated three times.

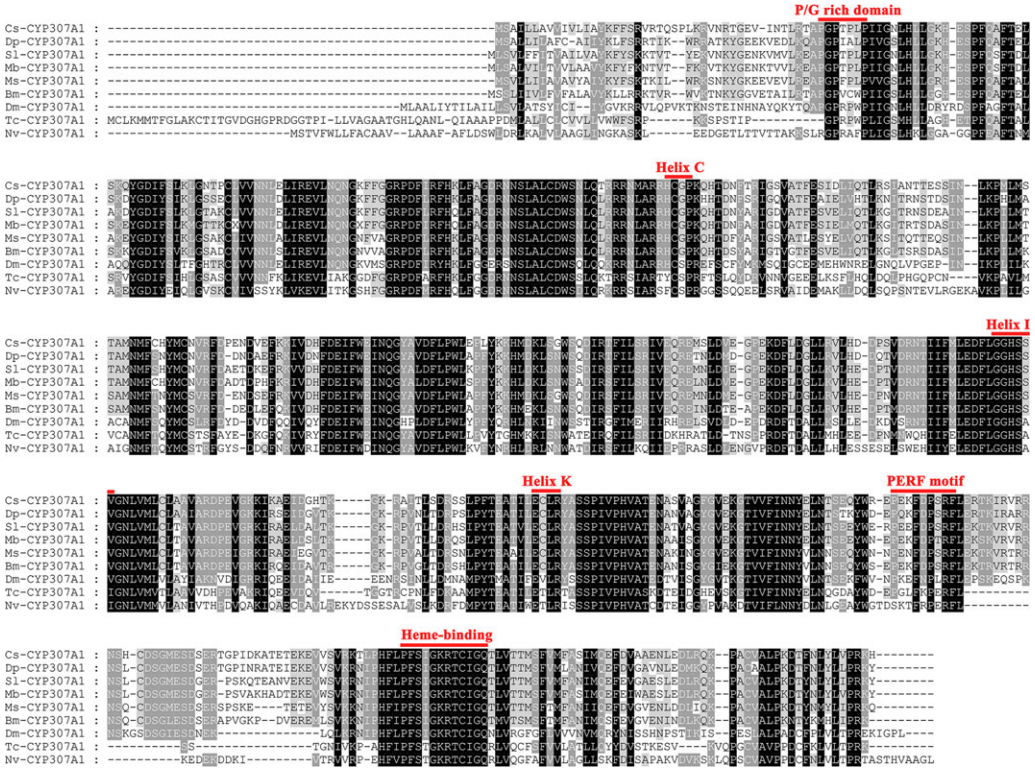

Figure 1. Alignment of spo from nine insect species. Spo from Dp: *Danaus plexippus* (AGBW01008433), Sl: *S. littoralis* (FJ981602), Mb: *M.a brassicae* (AB649120), Ms: *M. sexta* (DQ899315), Bm: *B. mori* (AB124841), Dm: *D. melanogaster* (NM139718), Tc: *T. castaneum* (XM964494), Nv: *Nasonia vitripennis* (XM_001603385). Amino acids with 100, 80, and 60% sequence similarity are shaded in black, dark gray, and light gray. The characteristic P450 structure, membrane-targeting hydrophobic segment, Helix C, Helix I, Helix K, PERF motif, and heme-binding domain are shown in the figure.

RESULTS

Molecular Cloning and Characterization of Cs-Spo

The *Cs-Spo* sequence was submitted to GenBank (accession number: KF701128). Its open reading frame (ORF) encodes a putative protein with 540 amino acids. Cs-Spo contained typical features of microsomal cytochrome P450s (CYPs), consisting hydrophobic residues followed by a proline and glycine rich domain at N-terminus (van den Broeck et al., 1996). Alignment of Cs-Spo amino acid sequences with other previously well characterized insects revealed that insect CYPs have conserved P450 motifs such as WxxxR (Helix-C), GxE/DTT/S (Helix-I), ExxR (Helix-K), PxxFxPE/DRF (PERF motif), and PFxxGxRxCxG/A (heme-binding domain), where "x" means any amino acid (Fig. 1).

Phylogenetic Analysis

An evolutional tree was constructed using amino acid sequences by neighbor-joining method with a bootstrap value of 1,000. The result revealed that spos within the same order were grouped together (Fig. 2), suggesting *spo* is a conserved and ancient gene

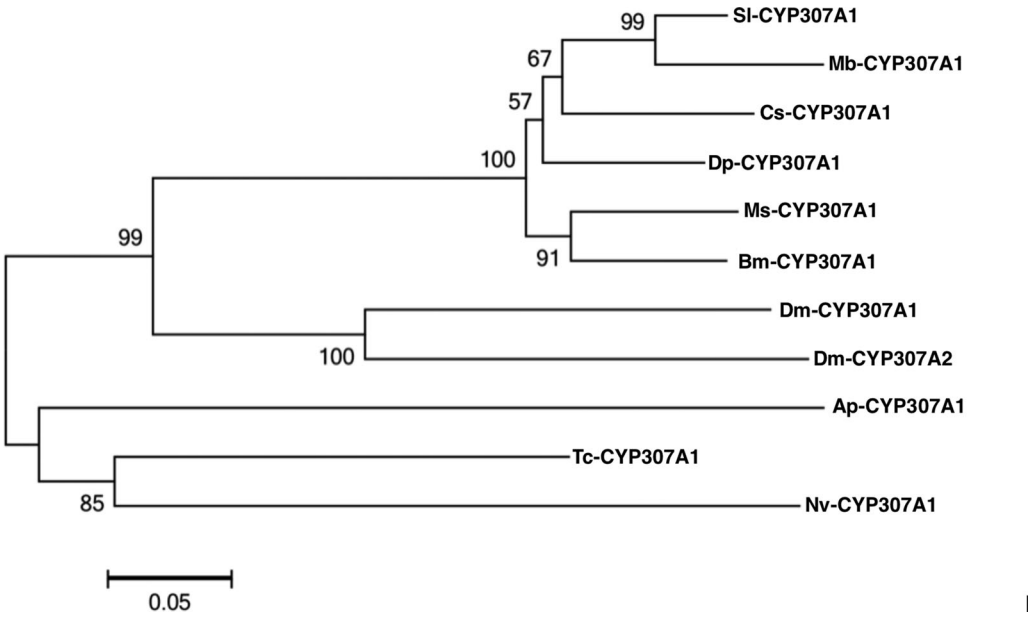

Figure 2. Phylogenetic tree of Cs-Spo. The tree was constructed using amino acid sequences by neighbor-joining method using ClustalX program with a bootstrap value of 1,000 trials for each branch position, excluding the gap position. The indicated numbers are bootstrap value as percentage of a 1,000 replicates and the scale bar indicates 0.05 change per residue. Sl: *S. littoralis*, Mb: *M. brassicae*, Dp: *D. plexippus*, Ms: *M. sexta*, Bm: *B. mori*, Dm: *D. melanogaster*, Ap: *A. pisum*, Tc: *T. castaneum*. Nv: *N. vitripennis*.

in insects. As expected, Cs-Spo fell into a lepidopteran branch with *S. littoralis*, *Mamestra brassicae*, *Danaus plexippus*, *M. sexta*, and *B. mori*.

Expression Profile of Cs-spo

At 28°C, the fourth, fifth, and sixth instar SSB larvae last an average of 5, 5 and 7 days, respectively, 6 days for pupae. Results showed that *Cs-Spo* expression level was the lowest at the first day of the fourth instar larvae, followed by a small increase until day 4 and the major peak at fifth day. After rapid decline, the expression at the first day of the fifth instar larvae was the lowest. The similar expression change was observed in the sixth instar larvae, with the expression increase at the prepupal stage. After the transition of pupae, the transcript level decreased and was low in first 5 days of the pupal stage, then peaked at day 6 (Fig. 3A).

The spatial expression pattern of *Cs-Spo* transcript levels was measured in various tissues, prothoracic gland (PG), midgut (MG), fat body (FB), epidermis (EP), and Malpighian tubules (MT). *Cs-Spo* was predominantly expressed in PG (Fig. 3B).

dsRNA Knockdown of Cs-Spo

Our results indicate that dsRNA efficacy of *Cs-Spo* were robust (Fig. 4A). The mRNA abundance decreased to 9% of the control level at 24 h PI, 33% at 48 h PI, and 24% at 72 h PI. The dsRNA effect diminished on the fifth day PI (Fig. 4A). dsRNA knockdown of *Cs-Spo* did not lead to high mortality (Fig. 4B), but the survivors were significantly delayed

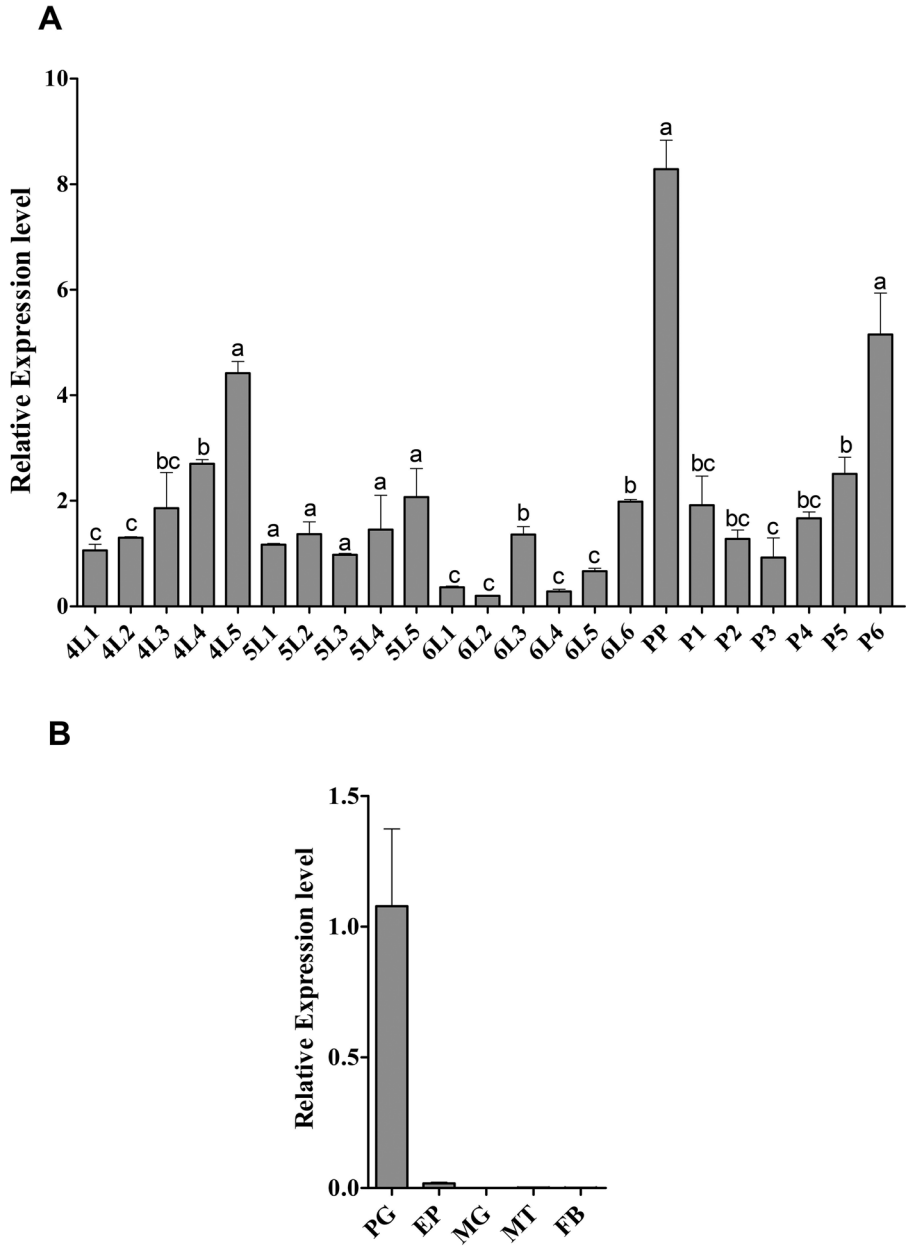

Figure 3. Graphic representation of the relative *Cs-Spo* Temporal and spatial transcript profiles. (A) *Cs-Spo* transcript levels measured at the fourth, fifth, sixth instar larvae and pupae at 24-h intervals. Three biological replicates were conducted, and the relative transcript levels were calculated using the $2^{-\Delta\Delta Ct}$ method. The columns represent averages with vertical bars indicating SE. The averages topped with the same letters are not statistically different at $P = 0.05$. (B) *Cs-Spo* relative transcript levels measured in various tissues, prothoracic gland (PG), epidermis (EP), midgut (MG), malpighian tubules (MT), and fat body (FB) of fourth instar larvae. For each sample, three independent pools of 10–15 larvae were measured in technical triplicate using qRT-PCR. The values were calculated using the $2^{-\Delta\Delta Ct}$ method. Each bar represents the mean SE.

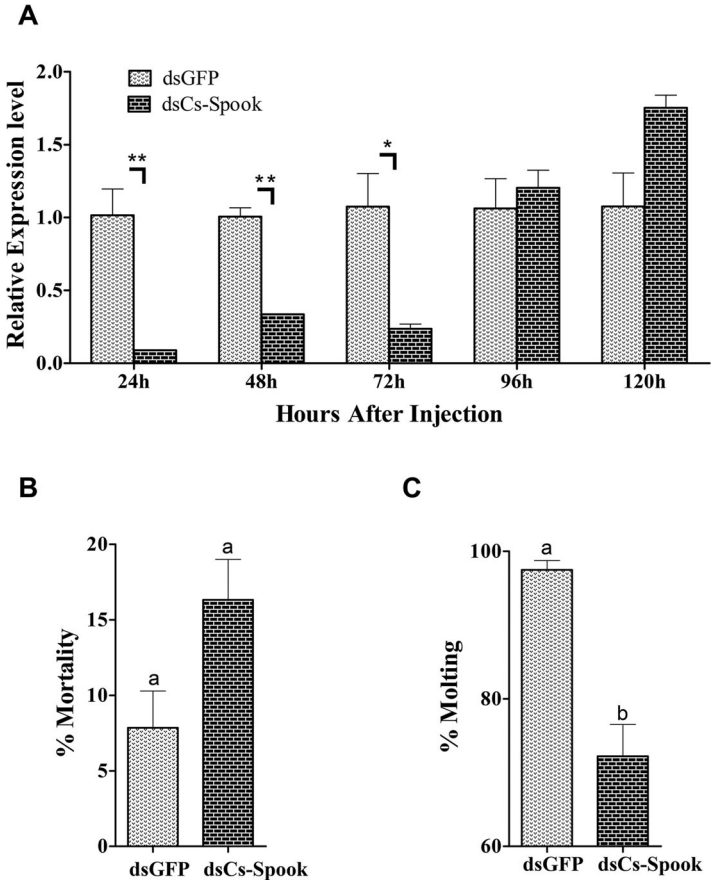

Figure 4. Effect of *Cs-Spo* dsRNA injection on (A) relative *Cs-Spo* transcript levels, (B) lethality, and (C) larval development of *Chilo suppressalis*. Day 3 forth instar larvae were injected with dsRNA. The relative *Cs-Spo* transcript level was measured at 24, 48, 72, 96, and 120 h after dsRNA injection. The abundances of *Cs-Spo* in the dsRNA-treated group were normalized with the data at the same time point in the control group. Each sample contained two larvae and repeated in biological triplicate. The percentage of mortality and molting was calculated from those repeated in three batches and ~30 larvae in each batch. The columns represent averages with vertical bars indicating SE. The paired means in the same day marked with * and ** are statistically different at $P < 0.05$ and $P < 0.01$, respectively. The averages topped with the same letters are not statistically different at $P = 0.05$.

in larval development. At 3 days PI, 25.3% of the individuals failed in molting to the fifth instar larvae, significantly higher than the control group (Fig. 4C).

Ecdysteroid Titer was Reduced in the Cs-Spo dsRNA-Treated Insects

Insects treated with dsRNA had significantly reduced level of ecdysteroid compared with the GFP control animals ($P < 0.05$, Fig. 5), indicating that *Cs-Spo* activity is required for ecdysteroid biosynthesis in PG.

20-E Rescues the dsRNA Phenotype

To address whether the larval arrest recorded in dsRNA-treated insects is due to reduced ecdysteroid titers, we treated the larval arrested phenotype with topical application of

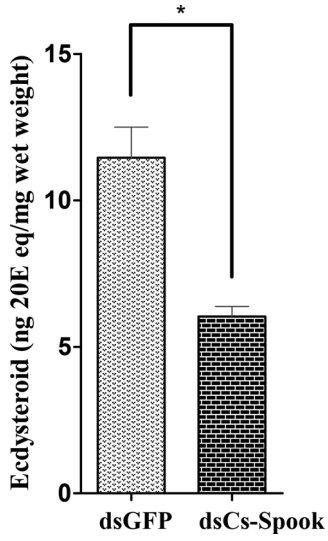

Figure 5. *Cs-Spo* dsRNA animals show reduced ecdysteroid titer. Larvae were collected after 48 h of dsRNA injection. Ecdysteroid titers of dsRNA-GFP and the *Cs-Spo* dsRNA animals were determined by HPLC. The results are depicted as nanograms of 20-E equivalents per milligram initial body weight on the vertical axis. Each bar represents the mean ± SEM from three independent samples. $^{*}P < 0.05$ by Student's *t*-test.

20-E at 24 h PI. In the ds*Cs-Spo*-treated insects, there were 23.3% animals showing delay in molting, which was significantly higher than the control group (7.8%). The percentage of delayed individuals in the 20-E rescue group was 11.1%, which was similar with the control group (Fig. 6).

DISCUSSION

It has been shown that the *spo* codes for enzymes involved in ecdysteroidogenesis in several model insects (Namiki et al., 2005; Ono et al., 2006). However, little has been understood about its function in rice SSB. Here, we cloned a Halloween gene *Cs-Spo* in SSB. We confirmed that *Cs-Spo* plays an essential role in ecdysteroid biosynthesis in SSB. Spo is a microsomal enzyme, detected in ER when *spo* is transfected into *Drosophila* S2 cells (Ono et al., 2006). Consistent with the structural features, Cs-Spo has the character of microsomal enzymes at the N-terminal part of the sequence. Insect P450 structural motifs such as Helix-K, PERF, and heme-binding have been reported in diverse orders (Namiki et al., 2005; Ono et al., 2006; Christiaens et al., 2010; Iga and Smagghe, 2010; Marchal et al., 2011; Yamazaki et al., 2011; Hentze et al., 2013; Luan et al., 2013). These conserved motifs were also found in Cs-Spo, suggesting that Cs-Spo has a similar function. In situ hybridization studies in various insects have found the Halloween genes *spo*/*spok* to be predominantly expressed in in the prothoracic cells of the ring gland and in the PG during immature insect stages (Namiki et al., 2005; Ono et al., 2006; Iga and Smagghe, 2010). Consistent with previous studies, *Cs-Spo* was predominantly expressed in PG tissues that synthesize ecdysteroids.

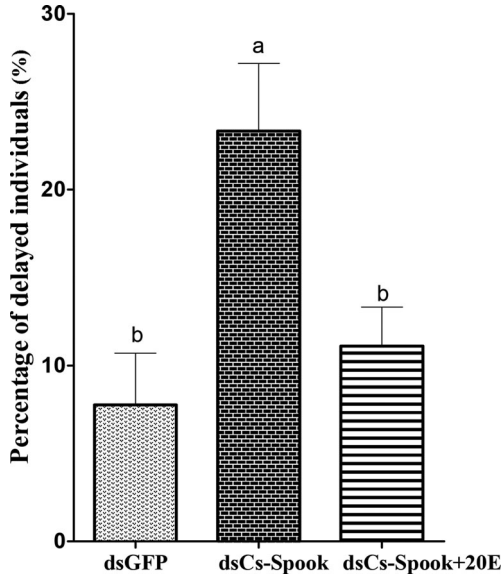

Figure 6. Percentage of delayed individuals subjected to ds*Cs-Spo* and 20-E application. Day 3 fourth instar larvae were injected with dsRNA. After 24 h of dsRNA injection, a 0.25 μl aliquot of acetone with 1.0 mg/ml 20-E concentration was used in this rescue experiment. The columns represent averages with vertical bars indicating SE. The averages topped with the same letters are not statistically different at $P = 0.05$.

Relative expression of *Cs-Spo* dramatically increased at late stage of the fourth, fifth, and sixth larval instars. The same expression trend was also observed in pupa stage. The temporal expression pattern of *Cs-Spo* was in line with earlier studies of *spo* in *D. melanogaster* (Ono et al., 2006), *T. castaneum* (Hentze et al., 2013), and *Sogatella furcifera* (Jia et al., 2013).These results suggest that *Cs-Spo* involved in the ecdysteroidogenesis in *C. suppressalis*.

Cs-Spo dsRNA animals showed significantly delayed metamorphosis phenotypic defects compared with the control group. We also observed abnormal phenotypes, such as growth retardation and lethal ecdysis in *Cs-Spo* dsRNA animals. Similar phenotypic defects have been observed in insects whose ecdysteroid synthesis had been disturbed or whose ecdysteroid-mediated signaling had been inhibited (Tan and Palli, 2008). We also measured the ecdysteroid titer of *Cs-Spo* dsRNA insects using HPLC. The ecdysteroid titers were significantly reduced. It has been reported that loss of Halloween enzymes caused a decrease in ecdysteroid titers (Warren et al., 2002, 2004; Niwa et al., 2004; Christiaens et al., 2010; Iga and Smagghe, 2010; Marchal et al., 2011; Yoshiyama-Yanagawa et al., 2011). 20-E could rescue the negative effects of *Cs-Spo* dsRNA injection. In all, we presented evidence that *Cs-Spo* plays pivotal role in metamorphosis development in *C. suppressalis* by participating in the ecdysteroidogenesis, as expected from the reports on other insects.

At present, controlling of SSB mainly relies on chemical pesticides, which cause severe environmental and food safety problems. Finding an alternative method of pest control is an important task. Regulating insect development is one of the feasible ways. Since *Cs-Spo* has an essential role in regulating metamorphosis in the SSB, it can be used as a potential effective target for pest control by knocking down its normal expression via transgenic plant or genetically modified microbes.

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