

## **RNA Interference Mediated Serine Protease Gene (Spbtry1) Knockdown Affects Growth and Mortality in the Soybean Pod Borer (Lepidoptera: Olethreutidae)**

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# RNA interference mediated serine protease gene (*Spbtry1*) knockdown affects growth and mortality in the soybean pod borer (Lepidoptera: Olethreutidae)

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## Abstract

The soybean pod borer, *Leguminivora glycinivorella* Matsumura (Lepidoptera: Tortricidae), is an economically significant soybean pest in northeastern Asia. Serine proteases are crucial enzymes responsible for protein digestion in herbivorous lepidopterans. In this study, a gene (*Spbtry1*) encoding a soybean pod borer serine protease was cloned from the organism's midgut. The *Spbtry1* open reading frame encoded a 269 amino acid protein with a predicted molecular mass of approximately 29 kDa. Alignment of *Spbtry1* with trypsins and chymotrypsins from other insects revealed a high degree of conservation in the putative catalytic domain region. Analysis by reverse transcriptase polymerase chain reaction indicated that *Spbtry1* was specifically expressed in the midgut, its transcript existed constitutively in the larval stage, and its expression was highest in the 3rd instar larval stage. RNA interference indicated that *Spbtry1* expression levels decreased on diets containing *Spbtry1* double-stranded RNA (dsRNA). Larvae had significantly lower body weight ( $18.7 \pm 0.25$  mg in the *Spbtry1* dsRNA-fed group versus  $30.1 \pm 0.78$  mg and  $29.9 \pm 0.88$  mg in the phosphate buffered saline (PBS) and green fluorescent protein (GFP) dsRNA-fed groups, respectively (Student's *t*-test,  $P < 0.01$ ) and higher mortality (43.8%) than the control groups (20.8% in PBS-treated and 19.5% in GFP dsRNA-treated) after 15 d, suggesting that *Spbtry1* is important for soybean pod borer larval growth and development.

Key Words: *Leguminivora glycinivorella*; gene expression; RNAi; larval development

## Resumen

El barrenador de la vaina de la soja, *Leguminivora glycinivorella* Matsumura (Lepidoptera: Tortricidae), es una plaga económicamente significativa de la soja en el noreste de Asia. Las serinas proteasas son enzimas cruciales responsables de la digestión de proteínas en los lepidópteros herbívoros. En este estudio, se clonó un gen (*Spbtry1*) que codifica una serina proteasa del intestino medio del organismo del barrenador de vaina de la soja. El marco de lectura abierto *Spbtry1* codificó una proteína de 269 aminoácidos con una masa molecular predicha de aproximadamente 29 kDa. La alineación de *Spbtry1* con tripsinas y quimotripsinas de otros insectos reveló un alto grado de conservación en la región del dominio catalítico putativo. El análisis por la reacción en cadena de la polimerasa de la transcriptasa inversa indicó que *Spbtry1* se expresó específicamente en el intestino medio, su transcripción existió constitutivamente en la fase larvaria, y su expresión fue más alta en la fase larvaria del 3er instar. La interferencia de ARN indicó que los niveles de expresión de *Spbtry1* disminuyeron en dietas que contenían ARN bicatenario *Spbtry1* (dsRNA). Las larvas tuvieron significativamente menor peso corporal ( $18,7 \pm 0,25$  mg en el grupo alimentado con dsRNA de *Spbtry1* frente a  $30,1 \pm 0,78$  mg y  $29,9 \pm 0,88$  mg en los grupos alimentados con dsRNA de PBS y GFP, respectivamente (prueba *t* de Student,  $P < 0,01$ ) y una mayor mortalidad (43,8%) que los grupos control (20,8% en PBS tratados y 19,5% en GFP dsRNA tratados) después de 15 d, lo que sugiere que *Spbtry1* es importante para la vaina de la soja barrenador de larvas de crecimiento y desarrollo.

Palabras Clave: *Leguminivora glycinivorella*; expresión de genes; ARNi; desarrollo de larva

The soybean pod borer, *Leguminivora glycinivorella* Matsumura (Lepidoptera: Tortricidae), is one of the most serious soybean pests in northeastern Asia, including northeastern China, Korea, and Russia (Zhao et al. 2008). It is a univoltine insect, and the mature larvae make cocoons in the soil from Oct throughout the winter, pupate in mid-Jul, and then adults emerge from late Jul to early Aug. The females oviposit on young bean pods. The hatched larvae enter the pod and actively feed on the immature beans, resulting in up to 40% yield loss. The

control of this insect is difficult, because when insecticide is applied to the plant, it does not make contact with the larvae inside the soybean pods (Wang et al. 2014).

Insecticides have been applied to control soybean pod borer adults for the last 3 decades and insecticide resistance in certain soybean pod borer populations is now a threat (Hu et al. 2013). Transgenic plants that over-express either insecticidal proteins or double-stranded RNA (dsRNA) specific to important insect genes might be a suitable alterna-

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tive. This has been demonstrated by transferring proteinase inhibitor oryzacystatin II (*OCII*) from rice to tobacco, which enhances tobacco resistance to the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae) (Cingel et al. 2015). Additionally, transgenic plants that express dsRNA specific to the cytochrome P450 gene (*CYP6AE14*) have improved resistance to the cotton bollworm, *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae) (Mao et al. 2007).

The serine proteases are critical targets for insect control because of their importance in food digestion (Srinivasan et al. 2006). Serine proteases contribute approximately 95% of the total digestive activity in the herbivorous lepidopteran larvae (Chougule et al. 2005). Moreover, serine proteases are involved in various essential processes, such as intra- and extra-cellular protein metabolism, immune responses (Loof et al. 2011), molting (Wei et al. 2007), blood coagulation, fertilization, and developmental regulation (Rao et al. 1998). The plant protease inhibitor (PI) interaction with serine proteases in insects from a stable and inactive acyl-enzyme distort the serine protease active site (catalytic triad). The loss of protease activity retards digestion and leads to negative effects on both larval survival (Srinivasan et al. 2005) and adult moth fertility and fecundity (DeLeo & Gallerani 2002). However, PIs are highly specific to a particular class of digestive enzymes. Insects could overcome the deleterious effect of PI ingestion by either overexpressing the existing proteases or synthesizing new proteases that are insensitive to the introduced PIs (Huma & Khalid 2007; Paulillo et al. 2000). RNAi is a promising tool in the control of insect pests. *Helicoverpa armigera* trypsin proteases are silenced when fed on diets containing dsRNA. RNAi against trypsin proteases inhibits insect growth and development and results in higher adult moth mortality (Chu et al. 2014). Trypsin- (*MsT*) and chymotrypsin-like (*MsCT*) serine protease expression levels decreased 76.7 and 86.2% after treatment with *MsT* and *MsCT* dsRNA, respectively, in *Mythimna separata* Walker (Lepidoptera: Noctuidae) (Zhou et al. 2016).

In this study, a serine protease (*Spbtry1*) gene was cloned from the soybean pod borer larvae midgut, and its transcription profiles in various developmental stages and tissues were determined. Moreover, *Spbtry1* gene silencing was carried out in the soybean pod borer via oral feeding to analyze the function of the serine protease gene in this organism.

## Materials and Methods

### INSECT REARING

Soybean pod borer eggs were originally collected from a naturally infested field at the experimental station at Northeast Agricultural University, Harbin, China. The soybean pod borer eggs were hatched at 26 °C. The larvae were reared on an artificial diet at 80 to 90% RH, 18:6 h L:D, and 26 °C until they reached adulthood. The adult moths were fed a 5% honey solution and allowed to oviposit on young bean pods.

### SPBTRY1 CLONING

To obtain full-length *Spbtry1* cDNA, soybean pod borer 3rd instar larvae gut tissues were dissected. The 3rd instar larvae were anesthetized for 40 s with ethyl ether. A drop of diethyl pyrocarbonate-treated phosphate buffered saline (PBS) was placed onto a glass slide fixed under a stereomicroscope (Nikon® SMZ1270, Nikon, Tokyo, Japan). The larvae were transferred onto the prepared slide by stabbing the thorax with a needle-tip probe. While the larvae were held down with the probe, forceps were used to grasp the 2nd to the 4th abdominal seg-

ment to gently pull off the larva's abdomen in a single motion. The gut should remain attached to the immobilized thorax. The abdomen was discarded, and forceps were used to detach the gut from the thorax (Supplementary Fig. S1; supplementary material is online at <http://purl.fcla.edu/fcla/entomologist/browse>). Total RNA was isolated from dissected gut tissues using Trizol® reagent (Invitrogen, Carlsbad, California). Genomic DNA was removed from the isolated RNA by adding 2 µL (1 mg/µL) DNaseI (Invitrogen). The 1st-strand cDNA was synthesized using reverse transcriptase AMV (Takara, Dalian, China). To amplify the major portion of *Spbtry1*, the following degenerate primers were designed according to the amino acid residues flanking the His and Ser of the serine proteases SP-F and SP-R active site (Table 1) (Wanick et al. 2005). The polymerase chain reaction (PCR) cycles were as follows: denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, and elongation at 72 °C for 10 min using Taq polymerase (Takara) for 35 cycles.

The 3'- and 5'-ends of the transcript were cloned using 3'- and 5'-rapid amplification of cDNA ends (RACE), according to the manufacturer's protocol (3'/5' RACE Kit, Takara, Japan) with 2 gene-specific primers (Table 1). The PCR products were cloned into the pMD18-T vector (Takara), and sequenced by the Sanger method. The resulting overlapping sequences were assembled to obtain the full-length *Spbtry1* cDNA sequence. To confirm the assembled cDNA sequence from the overlapping PCR products, the entire *Spbtry1* coding region was amplified by PCR reactions with the *Spbtry1* F and *Spbtry1* R primers (Table 1). The PCR conditions were as follows: denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and elongation at 72 °C for 10 min using Taq polymerase (Takara) for 40 cycles. The products were cloned into the pMD18-T vector (Takara) and sequenced from both directions as described above.

### cDNA AND PROTEIN ALIGNMENT ANALYSIS

The full-length *Spbtry1* cDNA obtained was analyzed using a basic local alignment search tool (BLAST) through the National Center for Biotechnology Information (NCBI) website (<http://www.ncbi.nlm.nih.gov/>). The *Spbtry1* amino acid sequence was deduced from the corresponding cDNA sequence using the translation tool at the ExPASy Proteomics website (<http://expasy.org/tools/dna.html>). Other protein sequence analysis tools used in this study, including molecular weight and isoelectric point, were verified from the ExPASy Proteomics website (<http://expasy.org/>). Multiple sequence alignments of deduced amino acid sequences were performed using the Multiple Alignment software (<http://www.ebi.ac.uk/clustalw/index.html>). The phylogenetic tree was constructed in MEGA 6 (Tamura et al. 2013) based on the amino acid sequences of known serine proteases. A bootstrap analysis with 1,000 permutations was carried out.

### SPBTRY1 GENE TRANSCRIPTION EXPRESSION PROFILES IN DIFFERENT TISSUES AND DEVELOPMENT STAGES

Total RNA was isolated from different tissues (ganglion, cuticle, salivary, midgut, ovary, testis, and fat body) and whole soybean pod borers at different developmental stages (eggs, larvae, pupa, and adult females and males) using the Trizol® reagent (Invitrogen) as described above. The cDNA prepared from total RNA was used as a template for quantitative real-time PCR (qPCR). The qPCR analysis was performed using a SYBR Green kit (Bio-Rad, Hercules, California) and a Bio-Rad iCycler IQs real-time PCR detection system. The primers used for qPCR analysis were designed using Primer-BLAST from NCBI. The efficiencies of the primer pairs were higher than 90% for all of the qPCR primer pairs used in this study (Table 1). The qPCR conditions were as follows: 95 °C for 5 min, 40 cycles at 95 °C for 30 s, 62 °C for 15 s, and 72 °C for

**Table 1.** Primers used in cloning, reverse transcriptase polymerase chain reaction (RT-PCR), double-stranded RNA (dsRNA) synthesis, and quantitative real-time polymerase chain reaction (qPCR).

Primer sequences for cloning and RT-PCR	
Gene Names	
SP-F	5'-GGGT (A/C/G/T)GT (A/C/G/T)AC (A/C/G/T)GC(A/C/G/T)GC(A/C/G/T)CA(T/C)TG
SP-R	5'-A(A/G) (A/C/G/T)GGG(T)CC(A/C/G/T)CC(A/C/G/T)(G/C)(A/T)(A/G)T-C(A/C/G/T)CC
<i>Spbtry13'</i> race GPS1	5'-CTACAATGTCGCCGATAACC
<i>Spbtry13'</i> race GPS2	5'-TGCCTGGCTC GCTACAAC
<i>Spbtry15'</i> race GPS inner Primer	5'-CGTTGTCAGGGTGTTC
<i>Spbtry15'</i> race GPS outer Primer	5'-ACTAAGAACGGCCATGCAACA
<i>Spbtry1F</i>	5'-AAGACAGTCA GCCTTAGAAC C
<i>Spbtry1R</i>	5'-CGGGTTTAAA AAGGACAATA TC
<i>Spbtry1-E-F</i>	5'-TCTCCCAAGCAATTCTCCACCC
<i>Spbtry1-E-R</i>	5'-TCGGCGACATTGTAGTAGCG
<i>Spbactin-E-F</i>	5'-GGCGACATAGCAGCTTCTC
<i>Spbactin-E-R</i>	5'-ATCCTCCGT CTGACTTGCC

primer sequences for dsRNA synthesis		Product length (bp)
Gene Names		
<i>Spbtry-70-T7-F</i>	5'-GGATCCTAATACGACTCACTATAGGGTCGCTGTAT TGATACT	70
<i>Spbtry-70-T7-R</i>	5'-GGATCCTAATACGACTCACTATAGGTTGGCTGGGGGTTTC	
<i>Spbtry-343-T7-F</i>	5'-GGATCCTAATACGACTCACTATAGGCCACCTTCGCCAACAGC	343
<i>Spbtry-343-T7-R</i>	5'-GGATCCTAATACGACTCACTATAGGGCCAGCAGACACAACA	
<i>Spbtry-41-T7-F</i>	5'-GGATCCTAATACGACTCACTATAGGTCGCGACCCAGTCGCTGGGTG	41
<i>Spbtry-41-T7-R</i>	5'-GGATCCTAATACGACTCACTATAGGGAATGAGGGGGCCGACAGAGTCAC	
<i>GFP-T7-F</i>	5'-GGATCCTAATACGACTCACTATAGGGTGTGCTACATACGGAAAG	370
<i>GFP-T7-R</i>	5'-GGATCCTAATACGACTCACTATAGGTTGTTGTCGCGCGTAT	

primer sequences for qPCR		Product length (bp)	slope	R2	Primer efficiency (%)
Gene Names					
<i>Spbtry1-Q-F</i>	5'-TCTGTGACCACCATCGAGAAC	154	-3.166	0.999	91.02
<i>Spbtry1-Q-R</i>	5'-CGGTTAGCAGCGGTGAAGTA				
<i>Spbactin-Q-F</i>	5'-GGCGACATAGCAGCTTCTC	149	-3.218	0.997	97.32
<i>Spbactin-Q-R</i>	5'-ATCCTCCGT CTGACTTGCC				

Underlined sequence corresponds to T7 promoter; F, forward; R, reverse.

45 s, followed by 95 °C for 1 min and 55 °C for 1 min. At the end of each qPCR experiment, a melt curve was generated to rule out the possibility of primer-dimer formation. Each experimental group contained 3 biological replications, and each biological replication had 2 technical replications. The relative expression analysis for qPCR was performed using the soybean pod borer actin gene as an internal reference. For PCR analysis, 25 cycles were used for the *Spbtry1* and actin genes, each cycle consisted of 94 °C for 30 s, 55 °C for 60 s, and 72 °C for 60 s. The forward and reverse *Spbtry1*-E primers and *LgActin*-E primers (Table 1) were used for the *Spbtry1* and actin genes, respectively.

### dsRNA SYNTHESIS

The complete serine protease coding sequence for soybean pod borer was sub-cloned into a pGEM-T vector and used as a template for the target sequence amplification. The *Spbtry1* dsRNA was designed based on the sequence similarity among *Spbtry1* and 13 homologous trypsin-like proteases from other lepidopteran insects (Supplementary Fig. S2). The dsRNA fragment target regions and sequences are illustrated in Supplementary Fig. S3. The target dsRNA nucleotide and protein sequences were analyzed using blastn and blastp on the NCBI website. The target sequence's E-value, similarity, and identity to other insects are shown in Supplementary Tables S1–S6. Three serine protease gene target sequences were amplified by reverse transcriptase PCR (RT-PCR) using specific primers conjugated with 23 bases of the T7 RNA polymerase promoter (Table 1). For the negative control, the green fluorescent protein (*GFP*) gene was amplified from the PCAMBIA1302 expression vector using gene-specific primers (Table 1). The RT-PCR products amplified for *Spbtry1* and *GFP* were used as templates for dsRNA synthesis using Promega T7 RiboMAX Express Large Scale RNA Production Systems (Promega, Wisconsin) according to the manufacturer's protocol. The dsRNA was purified by a phenol–chloroform extraction followed by ammonium acetate precipitation. The dsRNA was resuspended in ultrapure water and quantified spectrophotometrically at 260 nm. The product was stored at –80 °C.

### SOYBEAN POD BORER FEEDING BIOASSAY

The 2nd instar larvae were provided with dsRNA in an artificial diet. The dsRNA was carefully mixed with the artificial diet. The final concentration of dsRNA in the diet was 10 µg per ml. Control larvae were treated with either the same concentration of GFP dsRNA or the same volume of PBS. Each treatment was carried out on 50 larvae and conducted in triplicate; thus, 150 larvae were analyzed in each treatment. The larvae were reared at 26 °C, 80 to 90% humidity, and 16:8 h L:D for 15 days. The dsRNA artificial diet was replaced with a new one every 3 days. Soybean pod borer body weight and mortality were recorded every 3 days after they were fed the dsRNA artificial diet. Some feeding larvae were randomly selected, frozen in liquid nitrogen, and kept at –80 °C for future use.

### DETERMINATION OF SERINE PROTEASE GENE SILENCING

Six individual larvae were randomly collected at each time point from 1 to 15 days after feeding on *Spbtry1* dsRNA. Total RNA was extracted from 3 pools of 6 larvae using Trizol<sup>®</sup>. The extracted RNA samples were subjected to qPCR amplification as described above. To evaluate the off-target effect, soybean pod borer serine protease mRNA levels were determined and compared between the dsRNA serine protease treated insects and the controls. The qPCR experiment was performed in triplicate. Statistical significance between the controls and the serine protease dsRNA-treated group was determined by 1-way ANOVA test using SSPS version 17.0 (IBM, Hawthorne, USA). Post hoc analysis used

was the t-test to examine for significant differences. The statistical significance for all tests was set at  $P < 0.05$ . The RNAi effect of the serine protease in soybean pod borer was measured by larval body weight and mortality.

## Results

### SERINE PROTEASE cDNA CLONING AND SEQUENCE ANALYSIS

The full-length soybean pod borer serine protease cDNA sequence was obtained by RT-PCR and 5'/3' RACE. RT-PCR amplification of total RNA from the soybean pod borer 3rd instar gut tissues using degenerate primers (from the regions of conserved amino acids flanking the active site of the His75 and Ser 220 residues) amplified a 400 bp fragment. Sequence analysis of 6 clones from this fragment resulted in a unique sequence homologous to serine proteases in other lepidopteran species. The full-length serine protease cDNA was subsequently obtained using 5' and 3' RACE. The gene was 891 nt long, containing a 27 bp putative 5' untranslated region, an 84 bp 3' untranslated region, and a 780 bp open reading frame (NCBI accession number JQ340915). The cDNA sequence contained a stop codon (TGA) at position 805–807 bp and a polyadenylation signal (AATAAA) located 12 bp upstream of the poly (A) tail (Fig. 1). The cDNA sequence open reading frame encoded a putative protein of 259 amino acid residues with a predicted molecular mass of 29 kD and an isoelectric point of 5.64. Comparison of the deduced protein sequence with other sequences from the databases using Blastp showed 30 to 78% identity to trypsin-like proteases from other insect species (data not shown). Therefore, the protein was named *Spbtry1*.

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GAAAAAAGACAGTCAGCCTTAGAACCC
28 ATGCGTGTGCGTGTATTGATACTTGGAGTGGTGTGGCTGTGTCGGCCTTGCCAGGACC
   M R V A V L I L G V V V A V S A L P R T 20
88 GAACCCCCAGCAGCAAGGATCGTGGGAGGCTCTGTGACCACCATCGAGAACTATCCCTAC
   E P P S Q R I V G G S V T T I E N Y P Y 40
148 CAAGCGGCTCTGTTGTCGACAAAGCGACTTCAACTGGTATGGCCAAATTTGTGGTGGTTCC
   Q A A L L S T S D F N W Y G Q I C G G S 60
208 ATCC TCAACAACCGATCTGTGCTGACGCGCGTCACTGCTACTGGTACTTCAACGGCTGCT
   I L N N R S V L T A A C Y W Y F T A A 80
268 AACCGTTCCCGCGTCCGTGTTGGTTCCACCTTCGCGCAACAGCGGTGGCGTCTGCTCCACGCT
   N R F R V R V G S T F A N S G V V H A 100
328 GTCTCCCAAGACATTCTCCACCCAGCTTTAACCGCAACACCTGGGACACGACATCGCC
   V S Q N I L H P S F N R N T L D N I A 120
388 GTCTGCGCGCTGTGCTCCACCATTTGTCTTCAACAACAGGCTCGTGGGCTCCATCGCT
   V V R L S S T I V F N N Q A R A G S I A 140
448 GCGCGTAATACATGTCGCGGATAACCGAGGTGTCTGGGCTGTCGGATGGGTACCACT
   G A N Y N V A D N Q A V W A V G W G T T 160
508 TCGTCTGGAGGTTTGTCTTCTGAGCAACTTCGTCACGTCACAAATCTGGACTGTAAACAG
   S S G G F A S E Q L R H V Q I W T V N Q 180
568 GCAACCTGCGTGGCTCGCTACAACGAGCTCCGCAAGGTGGTCAACCAATAGCATGTTGTGC
   A T C V A R Y N E L R K V V T N S M L C 200
628 TCTGCTGGCTCGACGTCGCGGTCGCGACCAAGTGCCTGGGTGACTCTGGCGGCCCTC
   S G W L D V G G R D Q C L G D G G P L 220
688 ATTCACAAACGAGTCGTCATCGGTGTGCTCTTGGGGAACAGTGTGCCCTTCCACAG
   I H N G V V I G V C S W G E Q C A L P R 240
748 TATCTGGCGTTAACGCTAGGGTGTCTGAGTTACACTTCTCGATCCAGTCCCAATGCTTAA
   Y P G V N A R V S S Y T S W I Q S N A * 259
808 GCGGATATTGCTCTTTTAAACCGATTCTGATTTGTTCTCATCTTAAACCAATGAATAA
868 AGAAATACTACATATAAAAAA

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**Fig. 1.** Nucleotide and deduced amino acid sequences of *Spbtry1* cDNA cloned from *Leguminivora glycinivorella*. The numbers on the left and right refer to the amino acid sequence. The putative signal peptide of 17 amino acids is in bold and the cleavage site is indicated by an arrow. The cleavage site for the putative propeptide removal is indicated by an arrowhead. The stop codon TAA is indicated by an asterisk. The putative polyadenylation signal (AATAA) is underlined. The putative serine protease conserved motif (GDSGGPL) is boxed. The 3 amino acid residues (His72, Asp118, and Ser216) of the catalytic triad for serine protease activity are indicated with white letters on a gray background. GenBank accession no. JQ340915.



## MULTIPLE SEQUENCE ALIGNMENT AND PHYLOGENETIC ANALYSIS

Multi-sequence alignment of *Spbtry1* and 13 homologous trypsin-like proteases from other lepidopteran insects are shown in Supplementary Fig. S3. The cleavage site (RIVGG) for removal of the propeptide from the mature protein was identical in all of the proteins, except for the European corn borer, *Ostrinia nubilalis* Hübner (Lepidoptera: Crambidae) (AAX62030), in which the Val residue at the 3rd place was substituted by Trp. The catalytic motif (GDSGGPL) was also highly conserved among the sequences. There were 81 amino acid residues that were identical in all of the aligned sequences, particularly in the AAHC regions and the catalytic motif (GDSGGPL), implying that these amino acids might be essential for either secretory or functional activity in these serine proteases. The 3 catalytic triads (His72, Asp118, and Ser216) for serine protease activity were highly conserved in these trypsin-like proteases. The residues, aspartate (210), glycine (218), and glycine (228), from the substrate-binding pocket were conserved in the trypsin-like proteases.

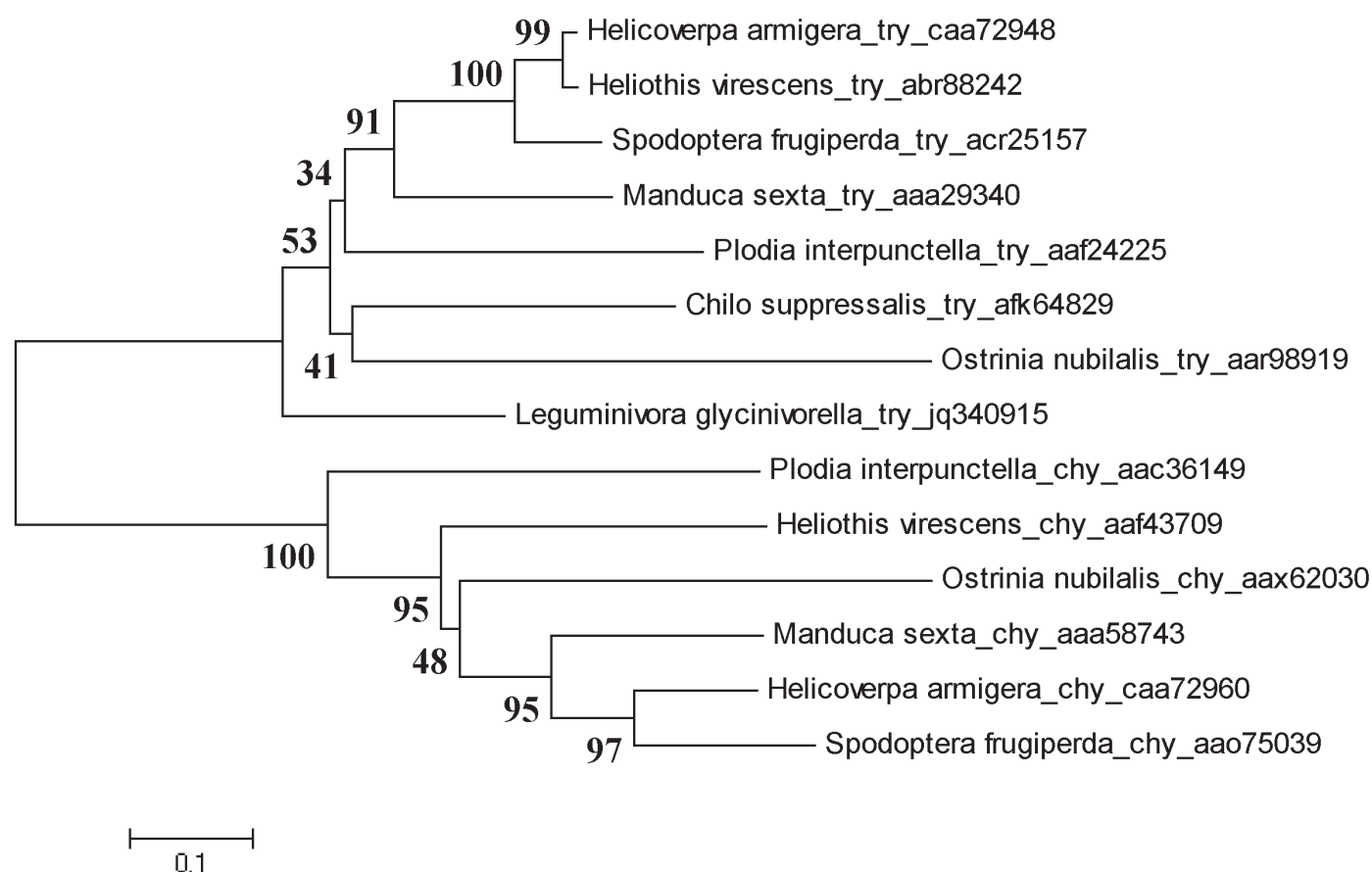
A phylogenetic tree was constructed for *Spbtry1* and 13 homologous serine proteases from other insects (Fig. 2). Two major groups were formed for the insect serine proteases, and the deduced *Spbtry1* amino acid sequence had 33 to 62% identity at the amino acid level to the lepidopteran species homologues. Chymotrypsins and trypsins are the major serine protease enzymes present in the gut of most insect species. *Spbtry1* belongs to the 2nd group and this group includes *Chilo suppressalis* Walker (Lepidoptera: Crambidae) trypsin-

like proteinase (AFK64829), *Manduca sexta* L. (Lepidoptera: Sphingidae) trypsin (AAF29341), *Spodoptera frugiperda* Smith & Abbot (Lepidoptera: Noctuidae) trypsin (ACR25157), *H. armigera* trypsin-like protease (CAA72948), *Heliothis virescens* F. (Lepidoptera: Noctuidae) trypsin T2a (ABR88242), *O. nubilalis* trypsin-like protease T23 precursor (AAR98919), and *Plodia interpunctella* Hübner (Lepidoptera: Pyralidae) trypsin-like PiT2b precursor (AF173495). This group had more conserved sequences for the signal peptides, propeptides, catalytic motif region, and substrate-binding pocket than the members of the 1st group (Fig. 2).

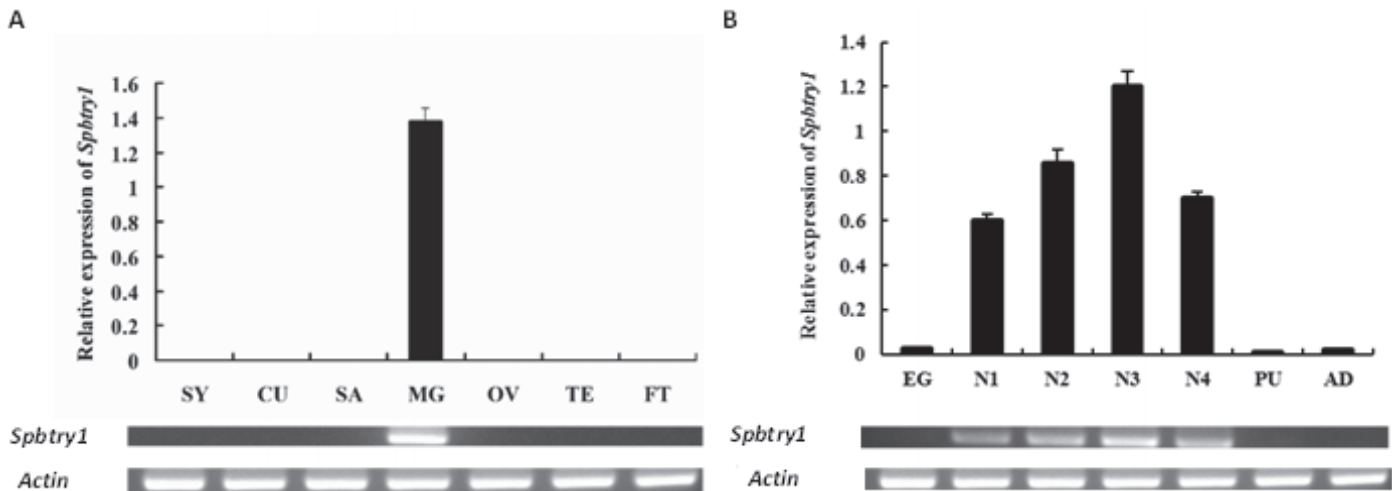
TISSUE AND DEVELOPMENT STAGE DEPENDENT *SPBTRY1* EXPRESSION

The *Spbtry1* gene transcript levels in different soybean pod border larvae tissues were determined by RT-PCR and qPCR. The RT-PCR analysis showed that *Spbtry1* was specifically expressed at the transcript level in the midgut, but was not detectable in the ganglion, cuticle, salivary, ovary, testis, and fat body. The qPCR analysis of *Spbtry1* tissue-specific expression resulted in the same expression pattern (Fig. 3A).

RT-PCR and qPCR were carried out to analyze *Spbtry1* expression patterns during different *L. glycinivorella* developmental stages. The results indicated that the *Spbtry1* transcript was expressed throughout different feeding stages, with the highest expression level in the 3rd instar larvae, no expression was detected in eggs, pupae, and adults (Fig. 3B).



**Fig. 2.** Phylogenetic tree analysis of *Spbtry1* and 13 homologues of other lepidopteran trypsin- and chymotrypsin-like serine proteases. The phylogenetic tree analysis was performed using the neighbor-joining algorithm to estimate evolutionary distances in MEGA 6.method at a gap penalty of 10, a gap length penalty of 0.2, and a bootstrap value of 1,000 iterations.

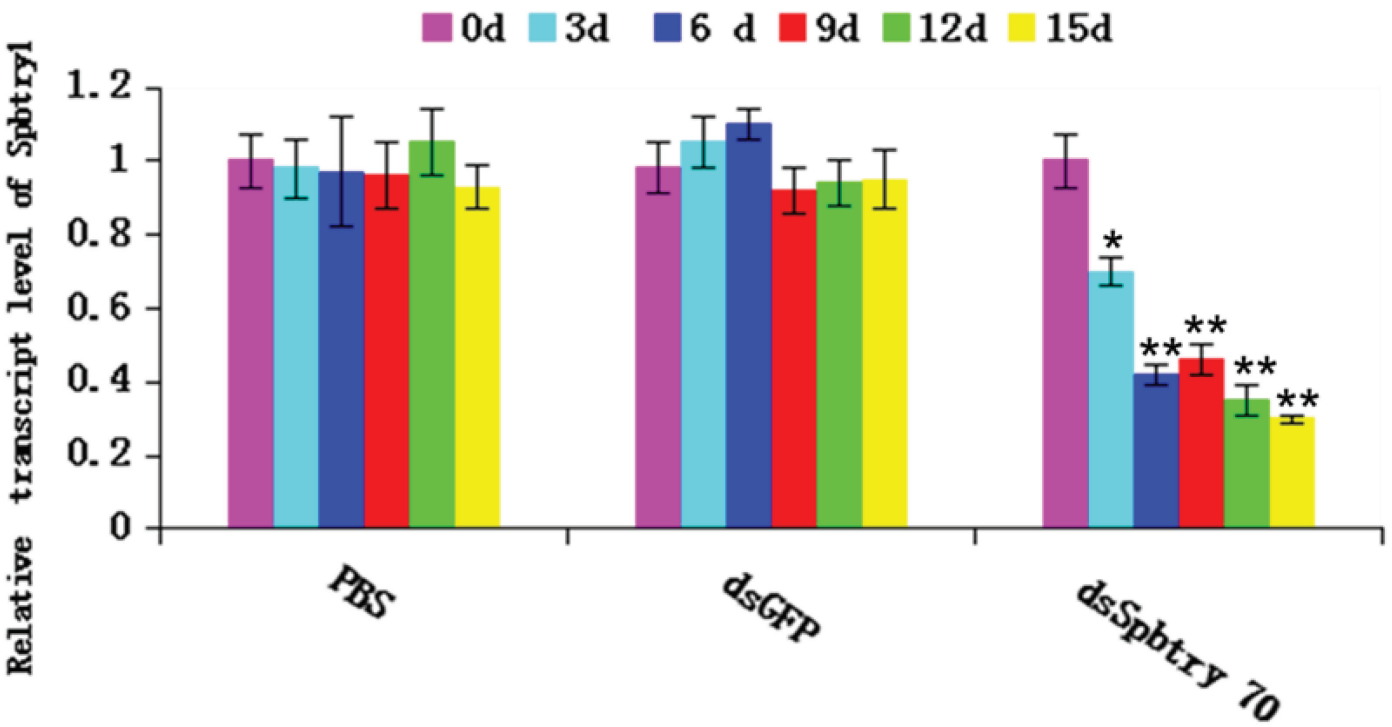


**Fig. 3.** A) Relative *Spbtry1* gene expression levels was determined by qPCR (histograms) and RT-PCR (gel pictures) in the synganglion (SY), cuticle (CU), salivary (SA), midgut (MG), ovary (OV), testis (TE), and fat body (FT) in the 3rd instar soybean pod borer larvae. Actin was used as an internal reference gene. (B) Relative trypsin gene (*Spbtry1*) expression levels as determined by qPCR (histograms) and RT-PCR (gel pictures) in soybean pod borer eggs (EG), 1st (N1), 2nd (N2), 3rd (N3), 4th (N4) instar larvae and pupae (PU), and adults (AD). Actin was used as an internal reference gene. Relative *Spbtry1* gene expression was analyzed by MJ Opticon Monitor Software Version 3.1.

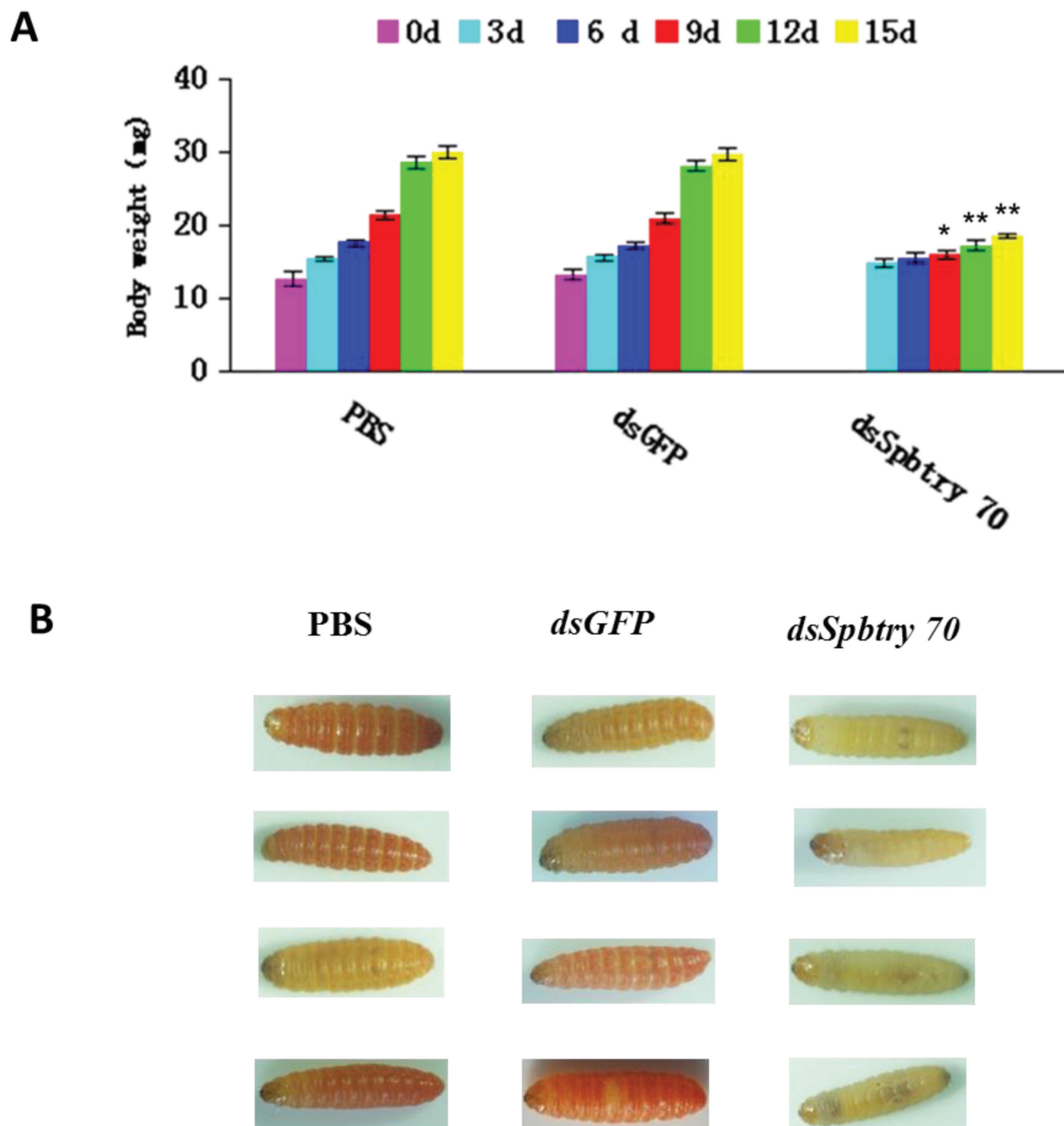
IMPACT OF *SPBTRY1* SILENCING ON *L. GLYCINIVORELLA* LARVAL DEVELOPMENT AND SURVIVAL

Three days after the 2nd instar larvae were fed *Spbtry1* dsRNA, the *Spbtry1* gene transcript decreased compared to the control larvae fed either GFP dsRNA or PBS. This decrease in the *Spbtry1* transcript level continued until the 15th day (Fig. 4). The larvae fed either GFP dsRNA or PBS exhibited a greater increase in body size than those fed *Spbtry1* dsRNA after 15 days (Fig. 5B). The body weight of larvae from the 3 groups was recorded before feeding. The average weight

of unfed larvae from the fed-*Spbtry1* dsRNA group ( $12.8 \pm 0.82$  mg) was not significantly different from that of either the PBS fed group ( $12.8 \pm 0.98$  mg) or the GFP dsRNA-fed group ( $13.4 \pm 0.67$  mg). The body weight of the PBS and GFP dsRNA-fed groups increased significantly with increased feeding time, but body weight did not increase significantly in the *Spbtry1* dsRNA-fed group. There was a significant difference in body weight between the *Spbtry1* dsRNA-fed and the control groups after 15 days ( $18.7 \pm 0.25$  mg in the *Spbtry1* dsRNA-fed group vs.  $30.1 \pm 0.78$  and  $29.9 \pm 0.88$  mg in the PBS and GFP dsRNA-fed groups, respectively, Student's *t*-test,  $P < 0.01$ ) (Fig. 5A).



**Fig. 4.** Relative trypsin gene (*Spbtry1*) expression levels as determined by qPCR at different time points. Actin was used as an internal reference gene. (\*Student's *t*-test,  $n = 3$ ,  $P < 0.05$ ; \*\*Student's *t*-test,  $n = 3$ ,  $P < 0.01$ ).



**Fig. 5.** Effect of *Spbtry1* RNAi on *Leguminivora glycinivorella* larval development. (A) The body weight of larvae fed on an artificial diet with added dsRNA at different time points. (B) Pictures of the larvae showing reduced body size and developmental stage after 15 days on an artificial diet with added dsRNA. (\*Student's *t*-test,  $n = 3$ ,  $P < 0.05$ ; \*\*Student's *t*-test,  $n = 3$ ,  $P < 0.01$ ).

The cumulative larval mortality increased significantly after 3 days in the *Spbtry1* dsRNA-fed group. The final mortality rate in the larvae fed *Spbtry1* dsRNA was 43.8%, this mortality rate was significantly higher than the 20.8% and 19.5% in the PBS and GFP dsRNA-fed larvae (Fig. 6).

## Discussion

The RT-PCR and qPCR analysis results revealed that the *Spbtry1* transcript is predominately expressed in the midgut and the larval feeding stages, whereas, the transcript was not detected during the



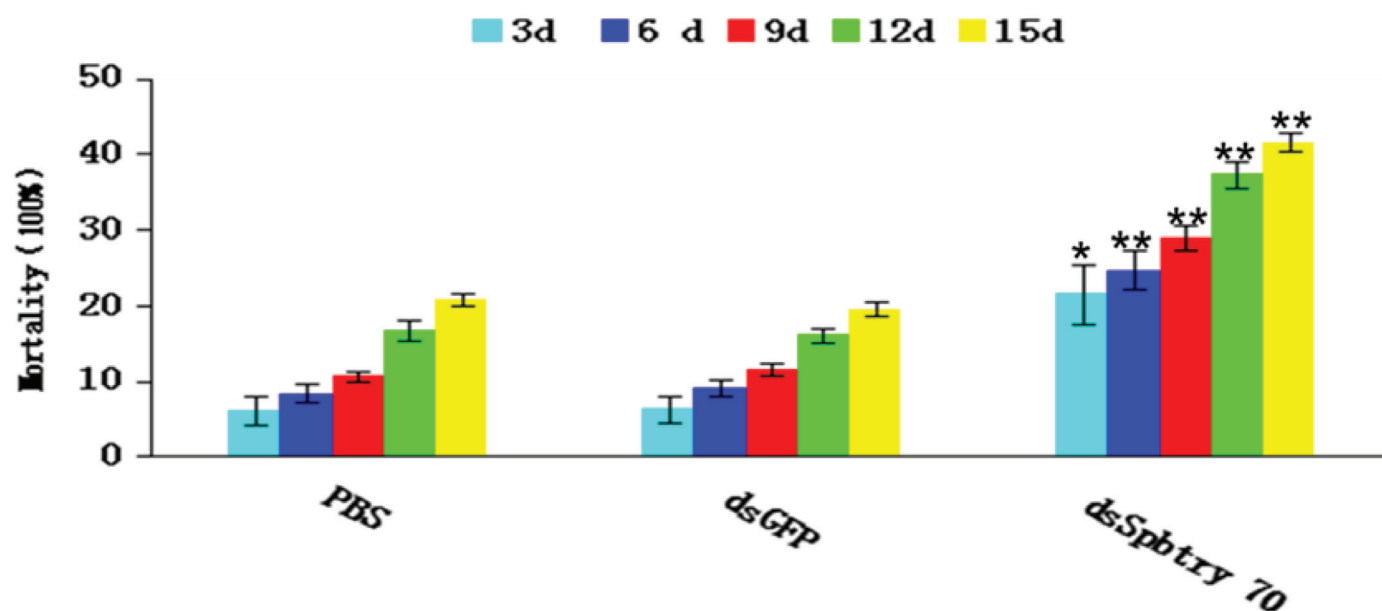


Fig. 6. The mortality of the larvae feed on an artificial diet with added dsRNA. (\*Student's *t*-test,  $n = 3$ ,  $P < 0.05$ ; \*\*Student's *t*-test,  $n = 3$ ,  $P < 0.01$ ).

non-feeding egg, pupae, and adult stages. Similar results have been reported in *Spodoptera litura* F. (Lepidoptera: Noctuidae) (Zhan et al. 2011), *M. sexta* (Felföldi et al. 2011), *S. frugiperda* (Rodríguez-Cabrera et al. 2010), and *Tribolium castaneum* Herbst (Coleoptera: Tenebrionidae) (Oppert et al. 2005). Furthermore, the *Spbtry1* gene deduced amino acid sequence had a putative signal peptide and an activation site, indicating that it is a secretory protein synthesized in the midgut and activated after secretion into the midgut lumen, where it likely digests dietary proteins (Zhang et al. 2010a). The tissue- and stage-dependent expression pattern of the *Spbtry1* gene implied that it might be involved in protein digestion in the *L. glycinivorella* midgut during the feeding stages.

RNA interference (RNAi), 1st characterized in the nematode *Caenorhabditis elegans* (Nematoda: Rhabditidae), has been the most powerful method for the rapid analysis of gene function in organisms (Whyard et al. 2009); the use of dsRNA-mediated RNAi to control insect pests of agriculturally important crops has been investigated (Zhang et al. 2015). When western corn rootworm, *Diabrotica virgifera virgifera* J. L. LeConte (Coleoptera: Chrysomelidae) larvae were fed transgenic plants expressing dsRNA specific to a vacuolar ATPase subunit, the transgenic corn plants were less damaged by western corn rootworm larvae than the control (Baum et al. 2007). The gap gene *hunchback* (*hb*) is of crucial importance in insect axial patterning, and *hb* knockdown is lethal to the next generation. When *Myzus persicae* Sulzer (Hemiptera: Aphididae) nymphs were fed *Mphb* dsRNA-expressing tobacco, the transgenic diet reduced *Mphb* mRNA levels in the aphids and inhibited insect reproduction (Mao et al. 2014). These studies suggest that genes encoding proteins with essential functions in insects are the best RNAi targets for increasing mortality and deformity (Liu et al. 2015).

In this study, we performed RNAi for *Spbtry1* by orally feeding dsRNA to assess the use of dsRNA approaches to control the soybean pod borer. The insect serine proteinase gene family has 8 to 11 members (Saikia et al. 2011; Ge et al. 2013). Three dsRNA fragments were synthesized (Supplementary Fig. S3) and incorporated into the soybean pod borer diet to silence the *Spbtry1* gene. After 15 days, the relative expression of *Spbtry1* in soybean pod borer fed dsRNAs to dsSpbtry70, dsSpbtry343, and dsSpbtry41 were between 27% and 36%, and no sig-

nificant differences were observed. Whereas the *Spbtry41* dsRNA from the conserved region resulted in greater mortality and lower body weight than the *Spbtry70* and *Spbtry343* dsRNA from variable regions (Supplementary Fig. S4). These results suggest that *Spbtry41* dsRNA targeted *Spbtry1* and other serine protease genes, while *Spbtry70* and *Spbtry343* dsRNA had lower impact because they only target the *Spbtry1* gene. Furthermore, the target *Spbtry70* nucleotide and protein sequences shared no similarity or identity with those of other insects, except for *Cimex lectularius* L. (Hemiptera: Cimicidae) (Supplementary Tables S1 and S2). The *Spbtry70* dsRNA was specific to *L. glycinivorella* and had no effect on other insects. Based on this experiment, *Spbtry70* dsRNA caused moderate suppression of *Spbtry1* expression in the soybean pod borer. Treated larvae continued to demonstrate developmental stunting, which was not observed in the PBS and GFP controls, suggesting that larval digestion was conspicuously blocked by *Spbtry1* knockdown. The RNAi-mediated gene silencing also led to high mortality (43.8% of the larvae fed *Spbtry1* dsRNA) in *L. glycinivorella*, which is similar to RNAi in *Locusta migratoria manilensis* (Meyen) (Orthoptera: Acrididae) (Whyard et al. 2009; Zhang et al. 2010b). These results further indicated that *Spbtry1* is involved in protein digestion in the *L. glycinivorella* midgut, which subsequently affects larval growth and development rates. Thus, our results indicate that *Spbtry1* is important for *L. glycinivorella* growth and development, and *Spbtry1* gene silencing is lethal to the soybean pod borer. These results support the potential use of RNAi technology, by transferring *Spbtry1* gene dsRNA into soybeans, to control the soybean pod borer.

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