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cDNA cloning and characterization of two trehalases from *Spodoptera litura* (Lepidoptera; Noctuidade)

Q. Zou*, P. Wei*, Q. Xu, H.Z. Zheng, B. Tang and S.G. Wang

Hangzhou Key Laboratory of Animal Adaptation and Evolution,
Hangzhou Normal University, Hangzhou, Zhejiang, China

*These authors contributed equally to this study.
Corresponding author: S.G. Wang
E-mail: sgwang@mail.hz.zj.cn

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ABSTRACT. The oriental leafworm moth, *Spodoptera litura*, is a major agricultural pest in southeast Asia and nearby Pacific regions. Two distinct trehalases have been identified in insects: soluble trehalase (Treh1) and membrane-bound trehalase (Treh2), although there is currently no information on these genes in *S. litura*. To characterize the distribution and function of *treh*, cDNAs of Treh proteins were cloned from *S. litura*. *SpoliTreh1* cDNA has an open reading frame of 1758 nucleotides, which encodes a protein of 585 amino acids, with a predicted mass of approximately 67.07 kDa and an isoelectric point of 4.86. *SpoliTreh2* cDNA has an open reading frame of 2325 nucleotides, encoding a protein of 645 amino acids, a mass of approximately 73.62 kDa, and an isoelectric point of 5.90. Northern blotting analysis revealed that *SpoliTreh1* transcripts are in the midgut, fat body, tracheae, and epidermis, but not in the brain and Malpighian tubules of *S. litura* larvae, whereas *SpoliTreh2* transcripts were found in all 6 tissues. *SpoliTreh1* transcripts were highly expressed in the fat body of the pre-pupal stage, and *SpoliTreh2* transcripts were highly expressed in the fat body of 3-day-old larvae of the 6th instar and during the 1st 6 days of the pupal stage, except the 2nd day. Both *SpoliTreh1* and *SpoliTreh2*

were highly expressed in third-instar larvae.

Key words: Trehalase; Cloning; Characterization; Expression pattern; *Spodoptera litura*

INTRODUCTION

Carbohydrates are a primary source of energy for the maintenance of metabolism in insects (Mariano et al., 2009), and trehalose is the main carbohydrate in insects. Trehalose is present in almost all forms of life except mammals (Lee et al., 2001; Elbein et al., 2003; Frison et al., 2007; Silva et al., 2010). It protects cellular membranes and proteins from oxidation, dehydration, heat, freezing, desiccation, and cold owing to its higher glass transition temperature compared with those to other sugars (Crowe et al., 1984; Eleutherio et al., 1993; Sun and Davidson, 1998; Davidson and Sun, 2001).

Trehalase (encoded by 2 genes, *Treh1* and *Treh2*) is an anomer-inverting α-trehalose-1-D-glucosidase, which plays an important role in the use of carbohydrates by hydrolyzing trehalose molecules into 2 glucose molecules (Tang et al., 2008). To date, 2 forms of trehalase have been found and cloned in several insect species (Wyatt, 1967), including *Apis mellifera* (Lee et al., 2007), *Bombyx mori* (Mitsumasu et al., 2005), *Omphisa fuscidentalis* (Tatun et al., 2008), *Pimpla hypochondriaca* (Parkinson et al., 2003), *Spodoptera exigua* (Tang et al., 2008), and *Tenebrio molitor* (Takiguchi et al., 1992). Initial studies of trehalose and protein purification have found that the main distribution of soluble trehalase (Treh1) and membrane-bound trehalase (Treh2) sometimes differed (Tang et al., 2008), which might indicate different functions.

In the silkworm *B. mori*, *Treh1* is predominantly expressed in the pre-pupal and pupal midgut, whereas *Treh2* expression is almost constant in the midgut, although some fluctuations do occur (Yamashita et al., 1974; Sumida and Yamashita, 1977; Mitsumasu et al., 2008). Ujita et al. (2011) found that the oligosaccharide chains of insect trehalase are essential for soluble trehalase expression and enzymatic activity. Treh2 is mainly found in mitochondrial membranes as well as in brain, cuticle, and midgut and is believed to be a transmembrane enzyme with an active site on the outside of the cell membrane (Mitsumasu et al., 2008; Tang et al., 2008). Wegener et al. (2003) suggested that Treh2 comprises an overt form and a latent form that can be activated by destroying membrane integrity in flight muscle cells.

Treh1 and Treh2 use different sources of trehalose. Treh1 is located in intracellular hydrolyzing endogenous trehalose, and Treh2 is a transmembrane enzyme involved in the assimilation of exogenous trehalose to supply energy (de Almeida et al., 2009). Chen et al. (2010) found that the expression of trehalose is increased and that of glucose is reduced with Treh1 reduction, whereas Treh2 occurs in *S. exigua* through the RNA interference.

S. litura is an insect pest that belongs to the order Lepidoptera. It can devour crops in a surprisingly short time and migrates long distances. In this study, *Treh1* and *Treh2* in *S. litura* were cloned to evaluate their transcriptional regulation. The study determined that trehalase activity in *S. litura* tissues varied during every developmental period, which suggested that gene expression was diverse. Comparison and analysis characterized these genes and their tissue expression, revealing their relationship and the method through which they regulate the catabolic pathways of trehalose. In addition, this study may contribute to the development of new agents that inhibit trehalose by blocking catabolic pathways through which trehalose acts on trehalases. These agents could replace insecticides for the control of *S. litura*.

MATERIAL AND METHODS

Insect cultures

S. litura have been maintained in our laboratory for more than 3 years. Larvae were reared on an artificial diet at 25° ± 1°C under a 14-h:10-h light-dark photoperiod. The developmental stages were synchronized at each molt by collecting new larvae or pupae. The brain, midgut, fat body, epidermis, and other tissues from first-instar larvae to pupae were dissected in saline solution (0.75% NaCl) and stored at -80°C for future analysis.

RNA extraction, cDNA synthesis, and polymerase chain reaction (PCR)

Total RNA was extracted from the fat body of *S. litura* pupae using the Trizol (Invitrogen) method. The fat body (100 mg) was added into a tube containing liquid nitrogen and quickly ground. The homogenates were transferred to a new tube containing 1 mL Trizol, vortexed for 3 min, and incubated at room temperature for 5 min. Chloroform (200 µL) was added, and the mixture was incubated at room temperature for an additional 5 min. The mixture was centrifuged at 12,000 *g* for 15 min at 4°C. The supernatant was transferred to a new tube, 500 µL isopropanol was added, and the mixture was incubated at room temperature for 10 min. After centrifugation at 12,000 *g* for 10 min at 4°C, the supernatant was discarded, and the RNA pellet was washed with 75% ethanol, dissolved in diethylpyrocarbonate double-distilled water and stored at -80°C until further use. A 1-µg sample of total RNA was reverse-transcribed at 42°C for 1 h in a 10-µL reaction solution containing reaction buffer, 10 mM dithiothreitol, 0.5 mM deoxyribonucleotide triphosphates, 0.5 µg oligo-d(T)18, and reverse transcriptase from avian myeloblastosis virus.

Four pairs of primers were designed for *Treh1* and *Treh2* (Treh1-DF1 and Treh1-DF2, Treh1-DR1 and Treh1-DR2, Treh2-DF1 and Treh2-DF2, and Treh2-DR1 and Treh2-DR2; Table 1) based on the conserved nucleotide sequences of known trehalase proteins. An initial PCR was performed with primers Treh1-DF1 (or Treh2-DF1) and Treh1-DR1 (or Treh2-DR1) according to the following conditions: 3 cycles of 30 s at 94°C, 30 s at 45°C, and 120 s at 72°C, followed by 28 cycles of 30 s at 94°C, 30 s at 48°C, and 70 s at 72°C. A second PCR was carried out using the nested primers Treh1-DF2 (or Treh2-DF2) and Treh1-DR2 (or Treh2-DR2) under the same conditions as those described for the initial PCR (Yamoah et al., 2008). The products were subjected to agarose gel electrophoresis. The true electrophoretic DNA bands corresponding to the expected size of approximately 800-1000 bp were excised from the agarose gel and purified using a DNA gel extraction kit. These PCR products were cloned into a pMD18-T vector and sequenced using the dideoxynucleotide method.

Rapid amplification of cDNA ends (RACE)

Specific primers for 5'-RACE (SpoliTreh1-5RA/SpoliTreh2-5RA and SpoliTreh1-5RB/SpoliTreh2-5RB) and 3'-RACE (SpoliTreh1-3FA/SpoliTreh2-3FA and SpoliTreh1-3FB/SpoliTreh2-3FB; see Table 1) were synthesized based on the cDNA sequence of the PCR fragment. 5'-RACE was performed using 2.5 µL 5'-ready cDNA with Universal Primer Mix and SpoliTreh1-5RA/SpoliTreh2-5RA. Nested PCR was then carried out with Nested Universal Primer A and SpoliTreh1-5RB/SpoliTreh2-5RB. 3'-RACE was performed using 2.5 µL 3'-ready cDNA with Universal

Table 1. Cloning strategy for *Spolitreh* cDNA and probe primers for Northern blotting.

PCR fragment	Primers			
	Name	Direction	Type	Nucleotide sequence (5'-3')
1	Treh1-DF1	F	D	GGA RWT YTA YTA CTG GGA
	Treh1-DF2	F	D	TGG GAT HRT BRA AGG KCT
	Treh1-DF3	F	D	GAG AGY GGY TGG GAY TTC TC
	Treh1-DR1	R	D	CCR TTB SWC CAY CCG AA
	Treh1-DR2	R	D	CGC RTC RTA YTT CTC RAA CAT
	Treh1-DR3	R	D	GCC ADG CGT TRG GGW AGT CC
	Treh2-DF1	F	D	TAC TGG GAY TCB TAC TGG A
	Treh2-DF2	F	D	ACG GNG GMM GVA THT ACT A
	Treh2-DF3	F	D	TCC CVG TNG ACY TGA ACG C
	Treh2-DR1	R	D	GCG TCG TAC TTY TCR ARC AT
	Treh2-DR2	R	D	GCC AVG CRT TSG GRT AGT CC
2	Treh2-DR3	R	D	CCA SRC NCC BAC NTC BTC GTG C
3'-RACE	SpoliTreh1-3FA	F	G	GAG TGG AGA GCA ATG GGA C
	SpoliTreh1-3FB	F	G	GAG TAT TGT AGT AAA CGC CA
	SpoliTreh1-3FC	F	G	GGA ACA CCT GAG GCG AGT G
	SpoliTreh2-3FA	F	G	GTC GGG ATT ACT TCT GCC
	SpoliTreh2-3FB	F	G	GGA TTA CTA CGT GAA CAG AG
3	SpoliTreh2-3FC	F	G	GAC ATA TTC GAC GGA GGC A
5'-RACE	SpoliTreh1-5RA	R	G	GTG GCT ACT ACC TTC TTT CTC
	SpoliTreh1-5RB	R	G	CCA GCC AGA AGT CCA TTT CC
	SpoliTreh1-5RC	R	G	GTG TCG TTG GTG TAT TGG
	SpoliTreh2-5RA	R	G	GGT CCT TGT GAT TGG TCG
	SpoliTreh2-5RB	R	G	GAC TTC AAT GGT GTG GTT GG
4	SpoliTreh2-5RC	R	G	TGG ATG TGT TGT CTG AGG
Probe	SpoliTreh1-PFA	F	G	CTC TGA TGA AGG CGA ACT CG
	SpoliTreh1-PFB	R	G	CAT TTG GCC TCT TCT TGC CCG
	SpoliTreh1-PR	F	G	CCA ACC ACT CTC AGC TGC AC
	SpoliTreh2-PFA	R	G	CAG TTG GGC AGG AAG ATG AAG C
	SpoliTreh2-PFB	F	G	CTG TTA TTG TAC CTG GTG G
	SpoliTreh2-PR	R	G	CAC CTG TTC AAT GGC ATC C

The full-length *Treh* cDNA sequences were determined by sequencing two overlapping PCR fragments 1 using fat body cDNA as a template. PCR fragments 2 and 3 were obtained from 3'- and 5'-RACE, respectively. PCR fragment 4 was used as a probe for Northern blot. F = forward; R = reverse; D = degenerate primer; G = gene-specific primer; A = nested universal primer O: 3'-RACE CDS Primer.

Primer Mix and SpoliTreh1-3FA/SpoliTreh2-3FA, and then with Nested Universal Primer and SpoliTreh1-3FB/SpoliTreh2-3FB. The PCR conditions were 10 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 60°C, and 90 s at 72°C, and then 10 min at 72°C (Tang et al., 2008).

The PCR products were subjected to electrophoresis. Two strong DNA bands corresponding to the expected size of approximately 1000 bp were detected with *SpoliTreh1* 5'-RACE. Only 1 strong DNA band at approximately 700 bp was detected with *SpoliTreh1* 3'-RACE. For *SpoliTreh2* 5'- and 3'-RACE, 2 strong DNA bands corresponding to the expected size of approximately 830 and 990 bp were observed, respectively. All 5 bands were excised from the agarose gel and purified using a DNA gel extraction kit, cloned into a pMD18-T vector, and sequenced using the dideoxynucleotide method.

Analysis of SpoliTreh cDNA sequences

SpoliTreh1 and SpoliTreh2 cDNA sequences were compared with other trehalase sequences deposited in GenBank using Basic Local Area Search Tool-N and Basic Local Area Search Tool-X via the National Center for Biotechnology Information website. SpoliTreh1 and SpoliTreh2 amino acid sequences were deduced from the corresponding cDNA sequences using the translation tool on the ExPASy Proteomics website [http://expasy.org/tools/dna.html (accessed

August 5, 2012)]. The neighbor-joining method was used to construct a phylogenetic tree with the Molecular Evolutionary Genetics Analysis 3.1 software based on the amino acid sequences of trehalase. A bootstrap analysis was carried out, and the robustness of each cluster was verified using 1000 replicates. Other protein sequence analysis tools used in this study were obtained from the ExPASy Proteomics website [http://expasy.org/ (accessed August 5, 2012)]. Multiple sequence alignment of insect *Treh* was performed using a tool at the MultAlin multiple sequence alignment website [http://bioinfo.genotoul.fr/multalin/multalin.html (accessed August 5, 2012)].

Northern blot analysis of *Treh* tissue distribution

Samples of 20 μg total RNA were extracted from fat body, brain, midgut, epidermis, tracheae, and spermary of fifth-instar larvae and separated on a formaldehyde agarose gel containing ethidium bromide. The RNA was subsequently blotted onto a Hybond-N^+ membrane. Two *Treh* cDNA fragments of approximately 562 (*SpoliTreh1*) and 695 bp (*SpoliTreh2*) were labeled with digoxin and used as probes (Figure 1). Template DNA (1-3 μg) was added to an Eppendorf tube and diluted with double-distilled water to a final volume of 16 μL. After the DNA was denatured through heating in a 100°C water bath for 10 min, the solution was quickly cooled in an ice bath. At the same time, 4 μL thoroughly mixed Dig-High Primer was added to the denatured DNA, mixed, and centrifuged to collect remnants clinging to the tube wall and force the flow to the bottom of tube. The mixture was incubated at 37°C in a water bath for 20 h and then heated to 65°C in a water bath for 10 min to stop the reaction. After hybridization, the membrane was washed with 0.2X SSPE (0.03 M NaCl, 0.002 M NaH_2PO_4, 0.0002 M ethylenediaminetetraacetic acid, pH 7.4) at 45°C and exposed to X-ray film at -70°C for 24 h (Tang et al., 2008; Tatun et al., 2008). Ribosomal RNA was used as a control under the same conditions.

Developmental expression of *Spolitrehs*

Samples of total RNA (20 μg) isolated from 3 to 5 fat bodies of viable larva from day 1 of the 6th instar to day 6 of the pupal stage and whole body from the 1st instar to 5th instar were separated on a formaldehyde agarose gel containing ethidium bromide. The RNA was subsequently blotted onto a Hybond-N^+ membrane. Northern blotting was performed as described above.

RESULTS

Sequence analysis of *SpoliTreh* cDNAs

Two *SpoliTreh* cDNAs were obtained with 5'- and 3'-RACE. *SpoliTreh1* cDNA (GU211891) had an open reading frame of 1758 nucleotides, which encoded a protein of 585 amino acids with a predicted molecular mass of approximately 67.07 kDa and an isoelectric point of 4.86 (Figure 1A). *SpoliTreh2* cDNA (GU211890) had an open reading frame of 2325 nucleotides, which encoded a protein of 645 amino acids with a predicted molecular mass of approximately 73.62 kDa and an isoelectric point of 5.90 (Figure 1B). The 2 types of Treh had the same conserved amino acid sequence (GGGGEY). *SpoliTreh* showed a 36-96% identity to other known *Treh* family genes, and *Treh1* could be clearly distinguished from *Treh2* between different insects (Figure 2; Tables 2 and 3). *SpoliTreh1* showed the highest identity (92%)

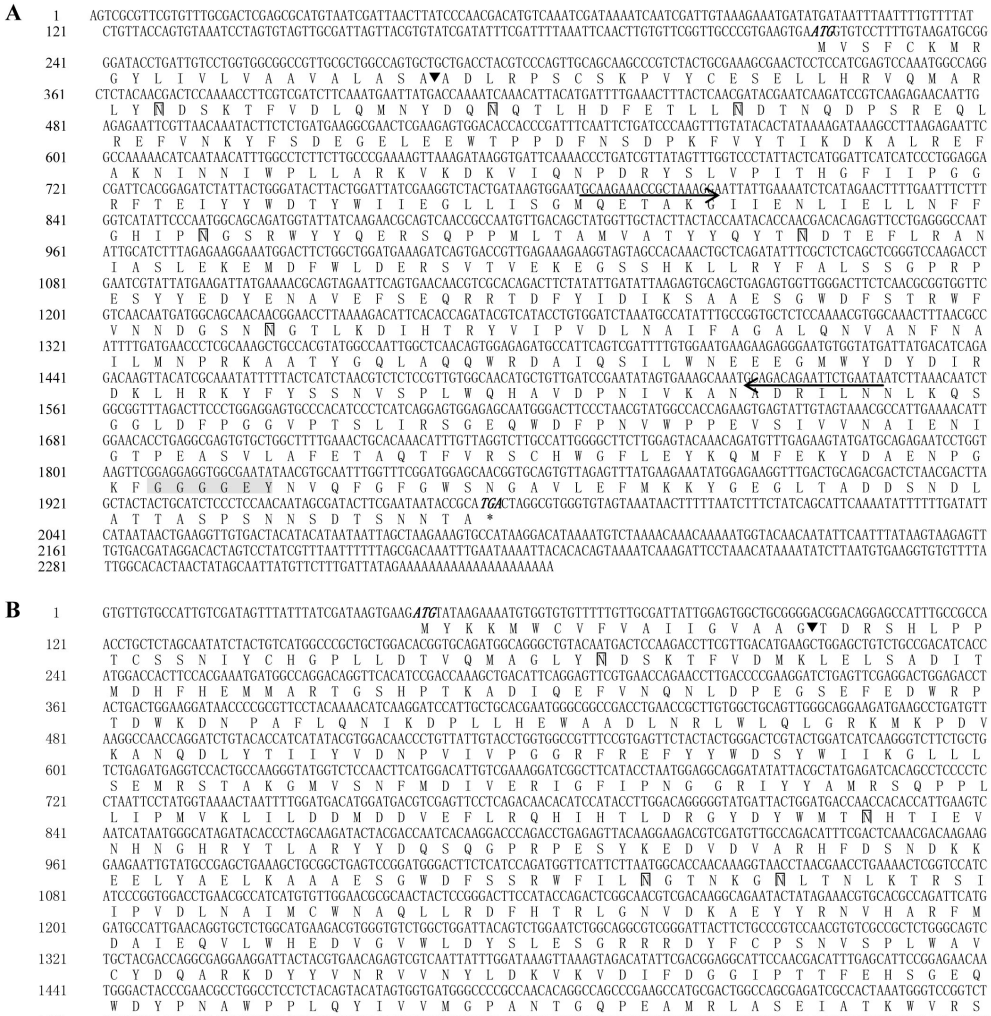

Figure 1. A. Amino acid residues (1-23) are underlined and the arrowheads represent the signal peptide and putative cleavage site, respectively. Potential N-glycosylation sites (amino acid residues 51, 66, 77, 213, 240, and 336) are boxed. The initiation and termination codons are indicated in bold italic font. The probe sequence is between the arrowheads. The conserved sequence is shaded. **B.** Amino acid residues (1-18) are underlined and the arrowheads represent the signal peptide and putative cleavage site, respectively. Potential N-glycosylation sites (amino acid residues 49, 261, 331, and 337) are boxed. The initiation and termination codons are indicated in bold italic font. The probe sequence is between the arrowheads. The putative transmembrane region (residues 586-608) is shaded and boxed. The conserved sequence is shaded.

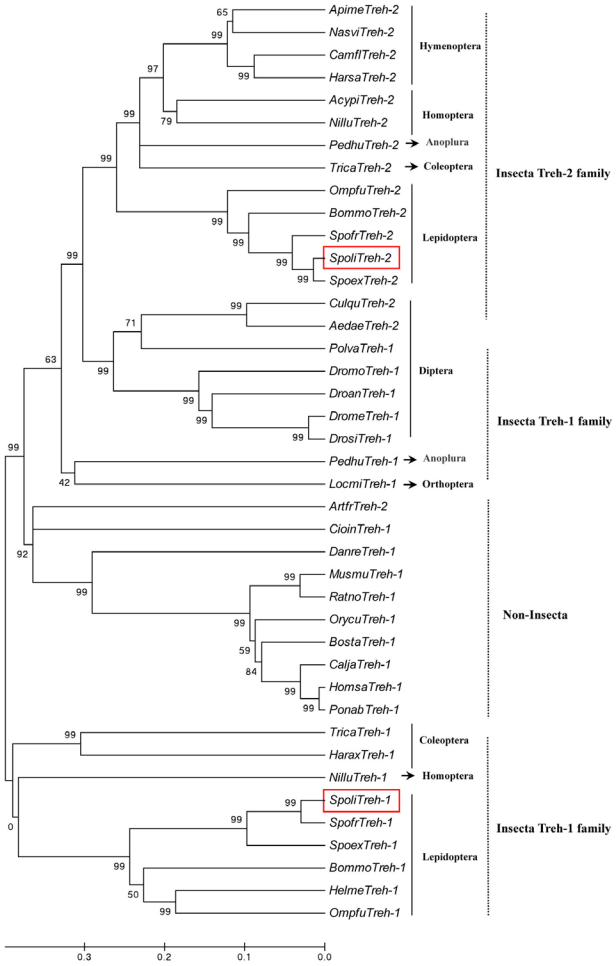

Figure 2. A phylogenetic tree was constructed based on the amino acid sequences of known Treh proteins. Full-length amino acid sequences were aligned using the Mega 3.1 program. A bootstrap analysis was carried out and the robustness of each cluster was verified using 1000 replicates. Values at the cluster branches indicate the results of the bootstrap analysis. Treh proteins were from *Acyrthosiphon pisum* (Acypi, XM_001949424), *Aedes aegypti* (Aedae, XM_001660244), *Apis mellifera* (Apime, NM_001112671), *Artemia franciscana* (Artfr, AB059268), *Bombyx mori* (Bommo, NM_001043993 and NM_001043445), *Bos taurus* (Bosta, NM_001192570), *Callithrix jacchus* (Calja, XM_002754462), *Camponotus floridanus* (Camfl, GL434492), *Ciona intestinalis* (Cioin, XM_002131746), *Culex quinquefasciatus* (Culqu, XM_001847882), *Danio rerio* (Danre, XM_001336151), *Drosophila ananassae* (Droan, XM_001959628), *D. melanogaster* (Drome, NM_166423 and DQ864058), *D. mojavensis* (Dromo, XM_002005742), *D. simulans* (Drosi, DQ864075), *Harmonia axyridis* (Harax, HM056038), *Harpegnathos saltator* (Harsa, GL448204), *Heliconius melpomene* (Helme, HM056038), *Homo sapiens* (Homsa, NM_007180), *Locusta migratoria* (Locmi, FJ795020), *Mus musculus* (Musmu, NM_021481), *Nasonia vitripennis* (Nasvi, XM_001602129), *Nilaparvata lugens* (Nillu, FJ790319 and GQ397451), *Omphisa fuscidentalis* (Ompfu, EF426724 and EF426723), *Oryctolagus cuniculus* (Orycu, NM_001082290), *Pediculus humanus* (Pedhu, XM_002426623 and XP_002433202), *Polypedilum vanderplanki* (Polva, AB490335), *Pongo abelii* (Ponab, XM_002822558), *Rattus norvegicus* (Ratno, NM_001136141), *Spodoptera exigua* (Spoex, EU427311 and EU106080), *Spodoptera frugiperda* (Spofr, DQ447188 and EU872435), *Spodoptera litura* (Spoli, GU211890 and GU211891), and *Tribolium castaneum* (Trica, XM_968826 and XM_967517).

to *S. frugiperda Treh1* (see Table 2). *SpoliTreh2* and *S. exigua Treh2* shared 96% sequence homology. All *Treh2* genes had 1 or 2 transmembrane domains, including some species with a potential transmembrane helical domain in *Treh2* (see Table 3). The deduced amino acid sequence of *SpoliTreh1* was aligned with *Treh1* and *Treh2* genes from other species (Figure 3).

Table 2. Related information of the *treh1* gene family and the identity (%) to *Spodoptera litura*.

Gene family	Species	No. of amino acids	GenBank No.	Identity (%) to *S. litura*
Treh1	*Spodoptera litura*	585	GU211891	-
	Spodoptera frugiperda	587	DQ447188	92
	Spodoptera exigua	585	EU427311	83
	Heliconius melpomene	554	HM056038	56
	Bombyx mori	579	NM_001043993	58
	Omphisa fuscidentalis	581	EF426724	58
	Pediculus humanus corporis	609	XP_002433202	45
	Tribolium castaneum	553	XM_968826	45
	Nilaparvata lugens	546	FJ790319	45
	Locusta migratoria manilensis	561	FJ795020	44
	Drosophila mojavensis	544	XM_002005742	43
	Drosophila melanogaster	596	NM_166423	41
	Polypedilum vanderplanki	578	AB490335	41
	Callithrix jacchus	579	XM_002754462	40
	Homo sapiens	583	NM_007180	40
	Oryctolagus cuniculus	578	NM_001082290	40
	Harmonia axyridis	554	HM056038	40
	Drosophila melanogaster	596	NM_166423	40
	Drosophila simulans	596	DQ864075	40
	Mus musculus	576	NM_021481	40
	Rattus norvegicus	576	NM_001136141	40
	Bos taurus	579	NM_001192570	40
	Pongo abelii	583	XM_002822558	40
	Danio rerio	577	XM_001336151	39
	Ciona intestinalis	585	XM_002131746	36

Table 3. Related information of the *treh2* gene family and the identity (%) to *Spodoptera litura*.

Gene family	Species	No. of amino acids	GenBank No.	Identity (%) to *S. litura*	Transmembrane domain analysis
Treh2	*S. litura*	645	GU211890	-	585-607
	S. exigua	645	EU106080	96	585-607
	S. frugiperda	647	EU872435	91	587-609
	Bombyx mori	642	NM_001043445	76	582-604
	Omphisa fuscidentalis	648	EF426723	74	581-603
	Apis mellifera	626	NM_001112671	53	13-32 and 594-616
	Tribolium castaneum	632	XM_967517	52	575-597
	Nilaparvata lugens	665	GQ397451	51	598-620
	Acyrthosiphon pisum	625	XM_001949424	51	Potential transmembrane helical domain
	Camponotus floridanus	665	GL434492	50	13-32 and 599-621
	Harpegnathos saltator	668	GL448204	50	12-34 and 606-625
	Pediculus humanus corporis	650	XM_002426623	49	Potential transmembrane helical domain
	Nasonia vitripennis	671	XM_001602129	48	600-622
	Culex quinquefasciatus	586	XM_001847882	46	Potential transmembrane helical domain
	Aedes aegypti	618	XM_001660244	46	12-34 and 590-612
	Artemia franciscana	703	AB059268	41	Potential transmembrane helical domain

Structure of SpoliTreh proteins

We chose 5 species of animals to ensure the reliability of the alignment. Multiple

sequence alignment of Treh proteins revealed a high degree of conservation (Figure 3). Alignment of proteins from insects revealed that Treh proteins have many conserved motifs, including KTFVD, PGGRFR (I)EFYYWDS(T)YWI, PRPESY, AESGWDFSSRW, MF(Y)EKY, and GGEYEVQTGFGWS(T)NG (see Figure 3). SpoliTreh1 has 6 potential N-glycosylation sites (residues 51, 66, 77, 213, 240, and 336) and SpoliTreh2 has 4 sites (residues 49, 261, 331, and 337). The first 23 amino acids were identified as the signal peptide, in which SpoliTreh2 displayed a signal peptide of 18 amino acids. In addition, the alignment of SpoliTreh2 with other insect trehalases showed that it contained 2 signature motifs in the trehalase and a putative transmembrane domain (residue 586-608), which is an important characteristic of Treh2 (see Table 2).

Figure 3. Alignment of DromeTreh-1, LocmiTreh-1, NilluTreh-1, PedhuTreh-1, SpoliTreh-1, TricaTreh-1, AedaeTreh-2, ApimeTreh-2, NilluTreh-2, PedhuTreh-2, SpoliTreh-2, and TricaTreh-2 proteins using the Vector NTI 9.0 software. Highly conserved regions are shown in yellow and sky-blue.

Treh tissue distribution in *S. litura*

SpoliTreh tissue-specific expression was determined with Northern blotting. *SpoliTreh1* transcripts were detected in midgut, fat body, trachea, and epidermis. The highest expression of *SpoliTreh1* occurred in the midgut and decreased rapidly in nearly all other tissues, whereas it was absent in the brain and Malpighian tubules of *S. litura* larvae. *SpoliTreh2* transcripts were detected in all 6 of the sampled tissues, including the fat body, midgut, Malpighian tubules, tracheae, brain, and epidermis of *S. litura* larvae. The expression in the midgut, Malpighian tubules, and epidermis was higher than that in other tissues but lower than *SpoliTreh1* expression in the midgut (Figure 4).

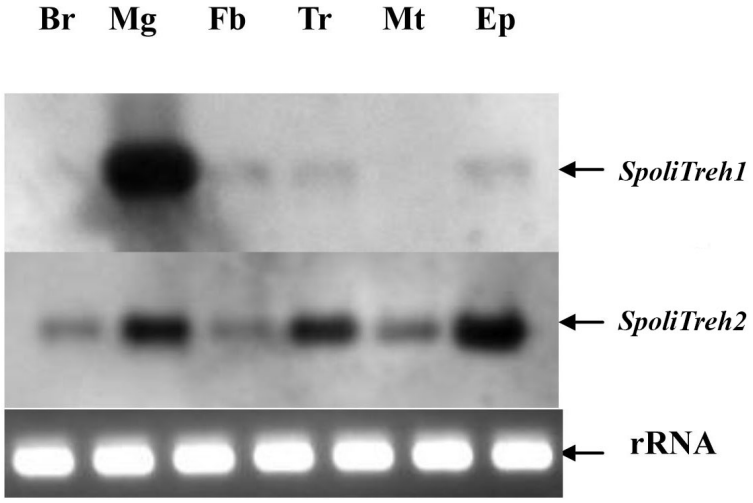

Figure 4. Northern blot analysis of *Spolitreh* transcripts in different tissues from 3-day-old larvae of 5th instar. Total RNA was extracted from various tissues: brain (Br), midgut (Mg), fat body (Fb), tracheae (Tr), Malpighian tubules (Mt), and epidermis (Ep). *Treh*-specific probes were marked with digoxin. Following hybridization and detection using colored substrate solution, the membrane was stripped by boiling in 0.1% SDS. rRNA was used as a control.

Developmental SpoliTreh expression

Northern blot experiments were carried out to analyze *SpoliTreh* expression patterns in the fat body from the 6th-instar larval stage to the pupal stage and in the whole body from the 1st- to 5th-instar larval stage of *S. litura*. *SpoliTreh* mRNAs were expressed at different levels in the fat body and the whole body. Compared to expression in other stages, *SpoliTreh1* transcripts were expressed more highly in the fat body in the pre-pupal stage, and *SpoliTreh2* transcripts were highly expressed in the fat body on the 3-day-old 6th-instar larval stage and in 1-, 3-, 4-, 5-, and 6-day-old pupae (Figure 5A).

Figure 5B shows the expression in the larval stages from the 1st to the 5th instar. Expression results of the whole body revealed that *SpoliTreh1* and *SpoliTreh2* mRNA was highly expressed in the 3rd-instar larval stage. *SpoliTreh1* mRNA was highly expressed on day one of the 2nd-instar larval stage. *SpoliTreh2* was highly expressed on day 2 of the 1st- and 2nd-instar larval stage and on days 1 and 2 of the 4th-instar larval stage (Figure 5B).

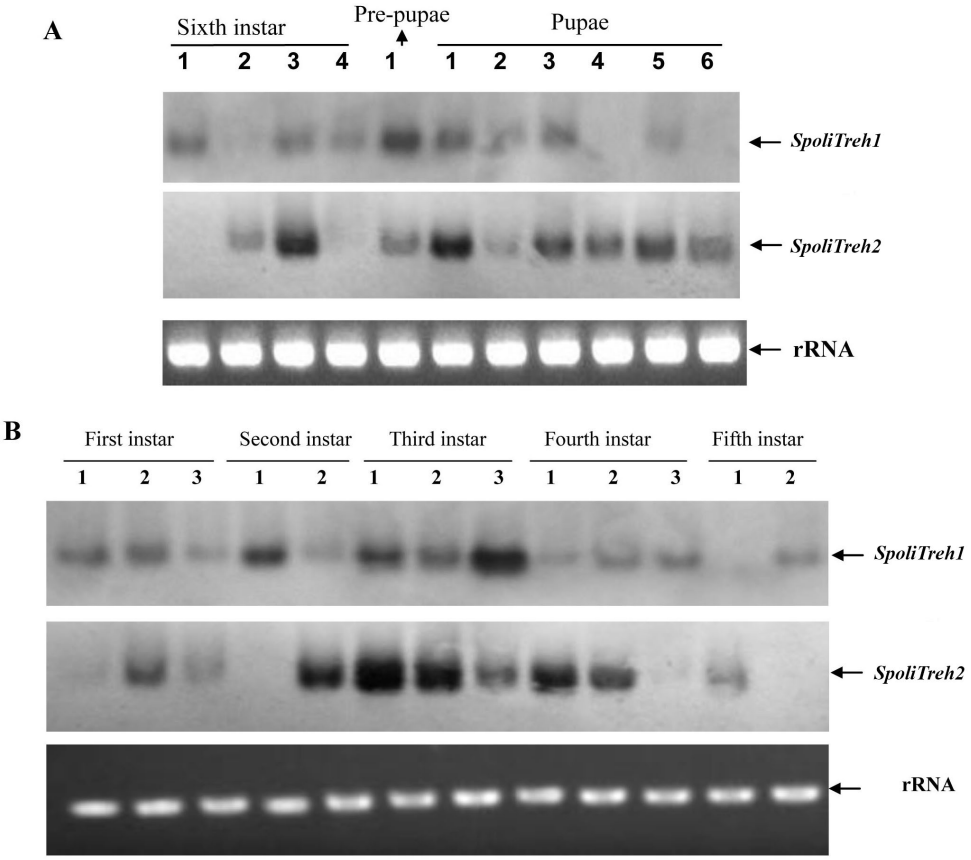

Figure 5. A. Developmental expression of *Spolitreh1* and *Spolitreh2* in the fat bodies of 6th-instar larvae, pre-pupae and pupae. rRNA was used as a control. Numbers 1-6 denote days 1-6 of the developmental stage, respectively. **B.** Developmental expression of *Spolitreh1* and *Spolitreh2* in the whole body of 1st-, 2nd-, 3rd-, 4th-, and 5th-instar larvae. rRNA was used as a control. Numbers 1-3 denote days 1-3 of the developmental stage, respectively.

DISCUSSION

The first insect trehalase gene was cloned in *T. molitor*, and *B. mori* trehalase genes were cloned soon thereafter (Takiguchi et al., 1992; Su et al., 1993). In this study, we cloned the cDNA sequences of *Treh1* and *Treh2* genes in *S. litura*, and the protein sequence translation analysis indicated that *Treh1* and *Treh2* coded for soluble and membrane-bound trehalase, respectively (GenBank accession Nos. GU211891 and GU211890). *SpoliTreh1* cDNA has a predicted mass of approximately 67.07 kDa (Figure 1A), which is similar to the molecular weights of the soluble trehalase purified from the pupal midgut of *B. mori* (67-70 kDa) and the larval midgut of *O. fuscidentalis* (65.2 kDa) (Sumida and Yamashita, 1983; Tatun et al., 2008). The molecular mass of *SpoliTreh2* cDNA is similar to the molecular mass of *Bmtreh-2* (71 and 74 kDa). Our results are almost identical to those of Tang et al. (2008).

In this study, comparison and analysis of *Treh* homology based on the DNA and proteins of several orders of insects found that *SpoliTreh* genes shared close homology with Lepidoptera, and the same types of *Treh* showed higher homology than that between *Treh1* and *Treh2* (Figure 2). Results from software analysis of each gene include a figure and corresponding description, the figures from software analysis of *Acyrthosiphon pisum Treh2*, *Artemia franciscana Treh2*, *Culex quinquefasciatus Treh2*, and *Pediculus humanus corporis Treh2* revealed that they have 1 or 2 potential transmembrane helical domains, indicated by a red crest (*O. fuscidentalis* has no potential transmembrane helical domains as a contrast in order to see clearly); however, their corresponding description from software analysis do not show what they do (Figure S1). The potential transmembrane helical domain may be related to species evolution or have unknown functions that deserve further investigation.

Trehalases in all insects have some common features: they contain a leading signal peptide area, a field coil, a highly conserved glycine-rich region, and 2 conserved signal sequences. SpoliTreh2 displayed 4 potential N-glycosylation sites, whereas SpoliTreh1 had more. In addition, the alignment of SpoliTreh2 with other insect trehalases showed that it contained 2 signature motifs and a putative transmembrane helical domain (residues 585-607) that was absent in SpoliTreh1 but was an important characteristic of Treh2. This result was consistent with a study of trehalase genes cloned in *S. exigua* (Tang et al., 2008). The comparison of 2 trehalase mRNAs and proteins revealed their respective characteristics, which may indicate differences in function between the 2 trehalases and lay the foundation for further study of trehalose metabolism regulation.

Almost all of the genes encoding the soluble trehalase were mainly expressed in the epidermis tissue, midgut, Malpighian tubules, and ovary during the larval stage (Su et al., 1993, 1994; Kamimura et al., 1999; Parkinson et al., 2003). Other studies have determined that *Treh2* and *Treh1* genes in *T. molitor* and *B. mori* were completely different (Su et al., 1993, 1994; Sato et al., 1997). In this study, we adopted Northern blotting hybridization combined with reverse transcription PCR to determine tissue distribution. Results of these analyses showed that fifth-instar larval *S. litura Treh1* was expressed in the midgut, fat body, tracheae, and epidermis but not in the brain and Malpighian tubules. *S. litura Treh2* expressed in each of these tissues, whereas previous research results have shown that in *S. exigua*, *Treh2* distribution was confined to the midgut, fat body, and Malpighian tubules and was not detected in the tracheae, epidermis, and brain (Tang et al., 2008). This finding indicated that *Treh2* in 2 species differed in expression and tissue distribution even though the species belong to the same order. The results also indicated that the 2 trehalases may serve different functions in these 6 tissues during the development of these Lepidopteran insects.

We do not know whether the expression pattern and function of the 2 trehalases are different or play several functions in all Lepidopteran insects. *Treh1* expression in the midgut was higher compared to that in other tissues, which was consistent with other studies and indicated its characteristic function (Mitsumasu et al., 2005; Tatun et al., 2008). The consistent but different levels of *Treh2* expression in the 6 tissues indicate that it plays an irreplaceable role in hydrolyzing the trehalose consumed by larvae from food because *Treh2* is an exogenous enzyme. *Treh1* and *Treh2* were distributed differently in tissue, which indicated that they have tissue-specific expression and play distinct roles.

We wanted to examine the dynamic expression of trehalases from the larval to the pupal stages, but many organs of the pupae degenerated; in fact, *S. litura* pupae had many fat bodies, so only fat bodies from the 6th-larval stage to the pupal stage were selected. In this study, *SpoliTreh1*

and *Treh2* in the fat body of the pre-pupal stage were highly expressed. This dramatic increase in expression is needed to prepare sufficient energy for pupation and hydrolyze trehalose for chitosan biosynthesis. These findings agreed with those of Chen et al. (2010) that *Treh1* regulates the chitosan biosynthesis in the epidermis. The reduction of *Treh1* was related to the fact that storage was sufficient and the insect required less energy in pupal stage (Figure 5A). *Treh2* in the fat body was expressed more highly in the pupal stage, which may promote chitosan in gut consistent with the findings of Chen et al. (2010) that *Treh2* regulates chitosan biosynthesis in the gut. *Treh1* and *Treh2* were both highly expressed in 3rd-instar larvae. *Treh1* on day 1 of the 2nd-larval stage showed high expression, as did *Treh2* on day 2 of the 2nd-larval stage and on days 1 and 2 of the 4th larval stage (Figure 5A). The expression of the 2 trehalases was similar to that in *S. exigua* (Chen et al., 2010). These results also demonstrated that *SpoliTreh1* and *SpoliTreh2* had different functions at different developmental stages. Treh1 and Treh2 hydrolyzed trehalose into glucose to provide the larvae with energy for growth, but they may have other roles. Our study found that *Treh1* influenced the expression of chitin synthase A, which is responsible for chitin synthesis in the cuticle and tracheae, whereas *Treh2* influenced the expression of chitin synthase B, which is responsible for chitin synthesis in the midgut in *S. exigua* (Chen et al., 2010). The larvae synthesized a high level of chitin synthase A for several ecdyses during the larval stages, so the expression of *Treh1* was constant except for some fluctuations and decreased in pupae when *S. litura* did not undergo ecdyses. *Treh2* contributed to the development of the midgut. Treh1 and Treh2 also hydrolyzed trehalose into energy for the insect growth. Meanwhile, the dynamic expression level of 2 trehalases was related to the regulation of trehalose and glucose.

Accordingly, we concluded that *Treh1* and *Treh2* had their own characteristic as well as some similar functions, regulating trehalose concentration together in some tissues and individually in others. They regulate trehalose together in the life of insect such that Treh1 mainly hydrolyzes trehalose intracellularly, and Treh2 acts on intracellular and extracellular trehalose. However, the precise function of these genes remains unknown and worth further research. For its important role in *S. litura*, trehalases that can block trehalase enzyme synthesis when its expression is high enough may provide a new target for pest control without environmental pollution.

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Supplementary material

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