Background: The chitin biosynthesis pathway starts with trehalose in insects and the main functions of trehalases are hydrolysis of trehalose to glucose. Although insects possess two types, soluble trehalase (Tre-1) and membrane-bound trehalase (Tre-2), very little is known about Tre-2 and the difference in function between Tre-1 and Tre-2.

Results: To gain an insight into trehalase functions in insects, we investigated a putative membrane-bound trehalase from Spodoptera exigua (SeTre-2) cloned from the fat body. The deduced amino acid sequence of SeTre-2 contains 645 residues and has a predicted molecular weight of ~74 kDa and pI of 6.01. Alignment of SeTre-2 with other insect trehalases showed that it contains two trehalase signature motifs and a putative transmembrane domain, which is an important characteristic of Tre-2. Comparison of the genomic DNA and cDNA sequences demonstrated that SeTre-2 comprises 13 exons and 12 introns. Southern blot analysis revealed that S. exigua has two trehalase genes and that SeTre-2 is a single-copy gene. Northern blot analyses showed that the SeTre-2 transcript is expressed not only in the midgut, as previously reported for Bombyx mori, but also in the fat body and Malpighian tubules, although expression patterns differed between the midgut and fat body. SeTre-2 transcripts were detected in the midgut of feeding stage larvae, but not in pupae, whereas SeTre-2 mRNA was detected in the fat body of fifth instar larvae and pupae.

Conclusion: These findings provide new data on the tissue distribution, expression patterns and potential function of membrane-bound trehalase. The results suggest that the SeTre-2 gene may have different functions in the midgut and fat body.
conditions, including desiccation, dehydration, heat, freezing and oxidation [5,6]. In plants, trehalose not only has an impact on some metabolic processes and affects plant development as a signaling molecule, but also serves as an anti-stress substance to protect plants from drought, high salt and low temperature [2,7]. In insects, unlike in mammals, trehalose is the main blood sugar and is present in the hemolymph of larvae, pupae and adults [1,8-11]. It is the main reserve sugar in the hemolymph of flying insects and is also indispensable for thermotolerance in larvae.

Trehalose is synthesized mainly in the insect fat body and is rapidly released into the hemolymph and other tissues. To utilize blood trehalose, insect tissues contain trehalases (EC 3.2.1.28) that catalyze the hydrolysis of one mole of trehalose to two moles of glucose. Thus, for uptake or utilization of trehalose in the blood, trehalases are essential enzymes in insects and are thought to be located on the cell membrane or within cells [8,12-15]. The first insect trehalase, a soluble trehalase, was reported in 1992 [16]. Although insects are believed to have two types, soluble trehalase, a soluble trehalase, was reported in 1992 [16]. HsTre (45%). The insect trehalase-2 from *B. mori* (BmTre-2; 77% identity) and *Ostrinia furnacalis* (OfTre-2; 76%). It is also similar to SeTre-1 (40%), SfTre-1 (41%), BmTre-1 (44%), OfTre-1 (43%), PhTre-1 (44%), AmTre-2 (54%), AmTre-1 (44%), TcTre-2 (54%), NvTre-2 (50%), AgTre-2 (50%), AaTre-2 (47%), DmTre-2 (45%), DsTre-2 (45%), TmTre-1 (44%), TcTre-1 (46%), RnTre (44%), MmTre (44%) and HsTre (45%). The insect *Tre-2* gene is highly conserved, particularly in the middle of the putative catalytic domain (Figures 2 and 3).

The deduced amino acid sequence of trehalase from *S. exigua* was aligned with the corresponding sequences of other insect trehalases (Figure 2). *SeTre-2* is most similar to lepidopteran trehalase-2 from *B. mori* (BmTre-2; 77% identity) and *O. furnacalis* (OfTre-2; 76%). It is also similar to *SeTre-1* (40%), *SfTre-1* (41%), *BmTre-1* (44%), *OfTre-1* (43%), *PhTre-1* (44%), *AmTre-2* (54%), *AmTre-1* (44%), *TcTre-2* (54%), *NvTre-2* (50%), *AgTre-2* (50%), *AaTre-2* (47%), *DmTre-2* (45%), *DsTre-2* (45%), *TmTre-1* (44%), *TcTre-1* (46%), *RnTre* (44%), *MmTre* (44%) and *HsTre* (45%). The insect *Tre-2* gene is highly conserved, particularly in the middle of the putative catalytic domain (Figures 2 and 3).

**SeTre-2 cDNA and protein sequence analysis**

The deduced amino acid sequence of *SeTre-2* contains two trehalase signature motifs, PVGRFREFYWDSY (residues 165–178) and QWDYPNWWPP (466–475) (Figures 1 and 2) and five other conserved motifs: DSKTFVDMK (residues 50–58), IPNQQQVW (210–218), RSQPPLL (221–227), GPRPSKEDV (284–294) and ELKAAAESGWDFSSRWFI (312–329). Residues 1–18 are a signal peptide leader and residues 530–536 correspond to a glycine-rich region (Figure 1). Residues 585–607 were found to comprise a putative transmembrane domain. N-terminal to this domain, residues 573–575 (Ser-Gly-Ala) are identical to amino acids 570–572 (Ser-Gly-Ala) in BmTre-2. However, this is not identified as an omega site by the big-PI predictor used to predict glycosylphosphatidyl inositol modification sites [21,23]. Five potential N-glycosylation sites (amino acids 48, 63, 260, 330 and 336) are present in BmTre-2, but six potential N-glycosylation sites were found in *SeTre-2*, five sites (amino acids 49, 64, 261, 331 and 337) homologous to those in BmTre-2 and an additional site at amino acid 419.

**Structure of SeTre-2**

We amplified the *SeTre-2* genomic DNA sequence, which is approximately 26 kb long. The exon/intron composition of the gene was determined by comparing the genomic sequence with the *SeTre-2* cDNA sequence. The *SeTre-2* gene consists of 13 exons separated by 12 introns of different lengths and exon-intron splice junctions following the GT-AG rule (Figure 4). The first intron is the longest, at 5.5 kb. Exons 1–13 correspond to nucleotides...
Figure 1

**Nucleotide and amino acid sequences of Tre-2 from the beet armyworm *S. exigua***. Underlined amino acid residues (1–18) and the arrowhead represent the signal peptide and putative cleavage site, respectively. Trehalase signature motifs (amino acid residues 165–178 and 466–475) are double underlined. The highly conserved glycine-rich region is shaded. The putative transmembrane region (residues 585–607) is shaded and boxed. Potential N-glycosylation sites (amino acid residues 49, 64, 261, 331, 337 and 419) are boxed. The nucleotide sequence reported in this paper has been submitted to GenBank under accession number EU106080.
Figure 2
Alignment of deduced amino acid sequences coded by the Tre-2 gene in insects. Alignment of deduced amino acid sequences coded by Ag-2 (GenBank accession no. XP_320471), Ao-2 (AJT38444), Se-2 (EU106080), Brm-2 (ABH06695), Nv-2 (XP_001602179), Of-2 (EF426723), Am-2 (XP_394271), Tc-2 (XP_972610), Ds-2 (ABH06710) and Dm-2 (ABH06695) using Vector NTI 9.0 multiple sequence alignment software. Highly conserved regions are highlighted in yellow and blue.

| Gene | Amino Acid Sequence |
|------|---------------------|
| Se-2 | (30) ENVCHPPLD1XAMG- |
| Bm-2 | (29) ENVCHPPLD1XAMG- |
| Of-2 | (26) ENVCHPPLD1XAMG- |
| Tc-2 | (29) ENVCHPPLD1XAMG- |
| Am-2 | (55) ENVCHPPLD1XAMG- |
| Nv-2 | (44) ENVCHPPLD1XAMG- |
| Aa-2 | (55) ENVCHPPLD1XAMG- |
| Dm-2 | (53) ENVCHPPLD1XAMG- |
| Da-2 | (53) ENVCHPPLD1XAMG- |
| Ag-2 | (1) -------------- |

Se-2   (30) SNIYCHGPLLDTVQMAG--LYNDSKTFVDMKLKLSANITMDHFHEMMART
Bm-2   (29) SMIYCHGPLLNTVQMAG--LYNDSKTFVDMKIKMSPNITLEHFYDMMSRT
Of-2   (26) SEIYCHGPLLDTVQMAA--LFNDSKTFVDMKIRYSPNITMEHFKQMMNRT
Tc-2   (29) SDIYCYGPLLHTIQMER--IYEDSKTFVDMKMRFEPNITLIKFNEFMVIN
Am-2   (55) SDVYCRGELLHTIQMAS--IYKDSKTFVDMKMKRPPDETLKSFREFMERH
Nv-2   (44) SEVYCHGELLHTIQMAS--IYTDSKTFVDMKMRQPTEATLGLFREFMNRT
Aa-2   (55) SEIYCHGKLLDTVQMAH--IYPDSKTFVDMKMKKTPNETLSAFNDFMEQK
Dm-2   (53) CKIYCEGNLLHTIQTAVPKLFADSKTFVDMKLNNSPDKTLEDFNAMMEAK
Ds-2   (53) CKIYCEGNLLHTIQTAVPKLFADSKTFVDMKLNYSPDKTLEDFNAMMETK
Ag-2   (1) --------------MSE--IYPDSKTFVDMKMRKSPNETLDSFHEFMVAQ

Se-2   (78) GSHPTKADIQEFVNQNFDPEGSEFEDWRPTDWKDNPAFLQNIKDPLLHEW
Bm-2   (77) DSNPTKADIQEFVNQNFDPEGSEFEDWRPSDWKHNPGFLAKIKDPLLHKW
Of-2   (74) DSRPTKAEIMEFVQNNFDPEGSEFEEWVPTDWKEQPKFLKDIKDPLLNKW
Tc-2   (77) NNKPSKNATRAFVNENFEPAGQEFEEWDPEDWVKHPKYIDGIQDDEFKQW
Am-2   (103) EQMPTRYQIERFVNDTFDPEGSEFEDWDPDDWTFRPKFLSRILDDDLRNF
Nv-2   (92) AGAPTRSQIEKFVNETFEPAGSEFTDFDPKDWVAQPKFLRKVLDPELRKF
Aa-2   (103) KEAPTTAELKAWVESMFEKPGAEFEEWIPDDWIDSPRFLNNIKDLDLRGF
Dm-2   (103) NQTPSSEDLKQFVDKYFSAPGTELEKWTPTDWKENPSFLDLISDPDLKQW
Ds-2   (103) NQTPSSEDLKQFVDKYFSAPGTELEKWTPTDWKENPSFLDLISDPDLKQW
Ag-2   (35) DNSPSKAKLKEWVELNFEKPGAEFENWTPDDWTASPKFLARIKDEDLRGF

Se-2   (128) EAVNRLPAQLGRKKMDKLASSPTLYEIPYNGGRKREFYYNDS
Bm-2   (127) EAVNRLPAQLGRKKMDKLASSPTLYEIPYNGGRKREFYYNDS
Of-2   (124) EAVNRLPAQLGRKKMDKLASSPTLYEIPYNGGRKREFYYNDS
Tc-2   (127) AEKLNLPAQLGRKKMDKLASSPTLYEIPYNGGRKREFYYNDS
Am-2   (153) EAVNRLPAQLGRKKMDKLASSPTLYEIPYNGGRKREFYYNDS
Nv-2   (142) EAVNRLPAQLGRKKMDKLASSPTLYEIPYNGGRKREFYYNDS
Aa-2   (153) EAVNRLPAQLGRKKMDKLASSPTLYEIPYNGGRKREFYYNDS
Dm-2   (153) EAVNRLPAQLGRKKMDKLASSPTLYEIPYNGGRKREFYYNDS
Ds-2   (153) EAVNRLPAQLGRKKMDKLASSPTLYEIPYNGGRKREFYYNDS
Ag-2   (85) EAVNRLPAQLGRKKMDKLASSPTLYEIPYNGGRKREFYYNDS

Se-2   (178) YWINQGTEEVSESTAVARMFELMSLAPQGTYAARRCPFP
Bm-2   (177) YWINQGTEEVSESTAVARMFELMSLAPQGTYAARRCPFP
Of-2   (174) YWINQGTEEVSESTAVARMFELMSLAPQGTYAARRCPFP
Tc-2   (177) YWINQGTEEVSESTAVARMFELMSLAPQGTYAARRCPFP
Am-2   (203) YWINQGTEEVSESTAVARMFELMSLAPQGTYAARRCPFP
Nv-2   (192) YWINQGTEEVSESTAVARMFELMSLAPQGTYAARRCPFP
Aa-2   (203) YWINQGTEEVSESTAVARMFELMSLAPQGTYAARRCPFP
Dm-2   (203) YWINQGTEEVSESTAVARMFELMSLAPQGTYAARRCPFP
Ds-2   (203) YWINQGTEEVSESTAVARMFELMSLAPQGTYAARRCPFP
Ag-2   (135) YWINQGTEEVSESTAVARMFELMSLAPQGTYAARRCPFP
Figure 3
Phylogenetic analysis of insect trehalases based on amino acid sequences. Full-length amino acid sequences were aligned using the Mega 3.1 program to generate a phylogenetic tree (1, class 1 gene of soluble trehalase; 2, class 2 gene of membrane-bound trehalase). A bootstrap analysis was carried out, and the robustness of each cluster was verified in 1000 replications. The scale on the x-axis represents estimated branch lengths and numbers indicate bootstrap values. Trehalases were from Aedes aegypti (Aa), Anopheles gambiae (Ag), Apis mellifera (Am), Bombyx mori (Bm), Drosophila melanogaster (Dm), Drosophila simulans (Ds), Homo sapiens (Hs), Mus musculus (Mm), Nasonia vitripennis (Nv), Omphisa fiscidentalis(Ph), Pimpla hypochondriaca (Ph), Rattus norvegicus (Rn), Spodoptera exigua (Se), Spodoptera frugiperda (Sf), Tribolium castaneum (Tc) and Tenebrio molitor (Tm). GenBank accession numbers (DNA) are as follows: Aa-2, EAT38444; Ag-2, XP_320471; Am-1, XM_393963; Am-2, XP_394271; Bm-1, BAA13042; Bm-2, AB162717; Dm-2, ABH06695; Ds-2, ABH06710; Hs, NM_007180; Mm, NM_021481; Nv-2, XP_001602179; Of-1, EF426742; Of-2, EF426723; Ph-1, Q8MMG9; Rn, CH473975; Se-1, EU427311; Se-2, EU106080; Sf-1, ABE27189; Tc-1, XP_973919; Tc-2, XP_972610; Tm-1, P32359.
1–364, 5855–5995, 6306–6421, 7873–8032, 8668–8864, 9592–9746, 11,029–11,222, 15,869–16,038, 17,074–17,250, 19,002–19,217, 20,226–20,336, 22,507–22,649, and 25,518–25,751 in the genomic sequence, respectively. Compared to the seven exons and six introns of the *A. mellifera* Tre-2 gene [22] and the nine exons and eight introns of *AmTre-1* (data from NCBI), *SeTre-2* has more exons and introns.

**Southern blot analysis**

Gene copy number can be determined by Southern blot analysis. Genomic DNA was obtained from *S. exigua* pupae and approximately 15 μg of DNA was digested with *Hind*III, *Sal*I and *Xho*I, electrophoresed and transferred to a nylon membrane, and then probed with a *SeTre-2* cDNA fragment (~770 bp) generated using *SeTreFP* and *SeTreRP* primers. The probe was designed to be specific for a highly conserved region so that it would hybridize to both *SeTre-1* and *SeTre-2*. One strong and one faint band were detected when genomic DNA was digested with *Sal*I and *Xho*I (Figure 5) whereas three strong and two faint bands were observed for *Hind*III treatment. The latter is attributed to the presence of two *Hind*III sites in the *SeTre-2* genomic sequence that correspond to the probe sequence. A *Hind*III site may also be present in the *SeTre-1* genomic sequence corresponding to the *SeTre-2* probe.

**SeTre-2 tissue distribution**

Tissue-specific expression *SeTre-2* was determined by Northern blotting. *SeTre-2* cDNA was cloned from *S. exigua* fat body, suggesting that *SeTre-2* mRNA is expressed in this tissue. This was confirmed by Northern blot analysis (Figure 6A). In addition, *SeTre-2* transcripts were also detected in the midgut, but not in the brain or cuticle (Figure 6A). *SeTre-2* mRNA may also be expressed in Malpighian tubules, since a faint band was observed. To determine the expression of *SeTre-2* transcripts in Malpighian tubules, RT-PCR was performed and a product of the size predicted for the *SeTre-2* transcript was observed.

**Figure 4**

*Trehalase gene in S. exigua.* The *SeTre-2* gene comprises 13 exons (boxes with numbers) and 12 introns. The positions of the start and termination codons located 276–278 of the first exon and 129–131 of the last exon in the nucleic acid sequence, respectively. The length of the 13 exons is 364, 141, 116, 160, 167, 155, 194, 171, 177, 216, 111, 143 and 237 bp, separated by 12 introns of various lengths of 5490, 310, 635, 757, 1282, 4755, 1034, 1751, 1008, 2170 and 2808 bp, respectively.

**Figure 5**

*Southern blot analysis of S. exigua genomic DNA.* Samples (15 μg) of *S. exigua* genomic DNA were digested with *Hind*III, *Sal*I or *Xho*I. DNA fragments were separated by electrophoresis, transferred onto a nylon membrane, and hybridized with an [α-32P]dCTP-labeled *SeTre-2* cDNA fragment. The strong and faint bands correspond to the *SeTre-2* and *SeTre-1* genes, respectively. The numbers on the left are DNA ladder sizes.
Northern blot (A) and RT-PCR (B) analyses of the SeTre-2 transcript in different tissues of fifth instar larvae of *S. exigua*. (A) For Northern blot analysis, total RNA was extracted from various tissues: brain (1), fat body (2), cuticle (3), Malpighian tubules (4), and midgut (5). Probes specific for SeTre-2 were radiolabeled using [α-\(^{32}\)P]dCTP. (B) RT-PCR analysis of DL2000 marker (M), midgut (1), brain (2), Malpighian tubules (3), cuticle (4), fat body (5), trachea (6), and spermary (7). β-Actin was used a loading control and visualization was by ethidium bromide staining.
Figure 7
Developmental expression of *S. exigua* Tre-2 mRNA in the midgut (A) and fat body (B). [$\alpha^{32}$P]dCTP-labeled SeTre-2 cDNA was amplified using specific primers SeTreFP and SeTreRP and used as a probe. $\beta$-Actin was labeled with [$\alpha^{32}$P]dCTP as a control. (A) RNA was extracted from the midgut third instar (3L), fourth instar (4L), fifth instar (5L) and wandering (pre-pupae) larvae (W) and from pupae (P). (B) RNA was extracted from the fat body of fifth instar (5L) and wandering (pre-pupae) larvae (W) and pupae (P).
in Malpighian tubules. Sequencing results confirmed the RT-PCR result (Figure 6B), demonstrating that SeTre-2 is expressed in the fat body, midgut and Malpighian tubules.

**Developmental SeTre-2 expression**

Semi-quantitative RT-PCR experiments were carried out to determine SeTre-2 expression patterns in the midgut and fat body during different developmental stages of *S. exigua*. SeTre-2 transcripts were detected in the midgut of larvae throughout the feeding stage, with higher expression levels in day-1 fourth instar (Figure 7A, lane 3) and day-4 fifth instar larvae (lane 8). However, no SeTre-2 expression was observed in the midgut of day-1 and day-3 pupae (lanes 10 and 11). Furthermore, SeTre-2 expression was observed in the midgut of pre-pupae (Figure 7A). In fat body, SeTre-2 expression patterns were different. SeTre-2 mRNA was detected in the fat body of fifth instar larvae and pupae (Figure 7B). Furthermore, higher SeTre-2 expression levels were observed in the fat body of day-4 fifth instar larvae (lane 4), as well as day-4 and day-7 pupae (lanes 9 and 12). Lower SeTre-2 expression levels were observed in the fat body of day-1 and day-2 fifth instar larvae, pre-pupae, and day-1 and day-2 pupae. However, SeTre-2 expression was not observed in the fat body of day-3 fifth instar larvae and day-3, day-5 and day-6 pupae.

**Discussion**

Two types of trehalase, soluble (Tre-1 or acid trehalase) and membrane-bound trehalase (Tre-2 or neutral trehalase), have been purified from a variety of organisms, and the corresponding genes have also been cloned. Trehalases facilitate the uptake and utilization of trehalose from food or blood [10-12,24-29]. Insects also have two types of trehalases [16-19,21,22,30,31]. The presence of two trehalase genes in *S. exigua* was verified by Southern blotting (Figure 5). We cloned one trehalase gene and protein sequence analysis suggested that it codes for a soluble trehalase. *S. exigua* trehalase transcript expression in fat body, but its function in this tissue is still unknown. We also cloned the trehalose-6-phosphate synthase gene (GenBank accession no. EF051258) from *S. exigua*, which is expressed mainly in fat body and not in midgut, and found that its expression levels showed the same trend as trehalose levels in hemolymph of *S. exigua* (unpublished data). This demonstrates

Insect trehalases have several common characteristics, namely, a signal peptide leader, a coiled-coil domain, a highly conserved glycine-rich (GGGGEY) region, and two conserved signature motifs (Figures 1 and 2) [21]. In addition, Tre-2 also has some unique characteristics, such as a transmembrane helical region and two conserved motifs (DAKTFVDMK and LGRKM; Figure 2), but Tre-2 does not have a putative transmembrane region. Based on the genomic sequence of *S. exigua* obtained in this study, the exons and introns of Tre-2 are reported for the first time.

Trehalases are important enzymes in insects as they catalyze the hydrolysis of trehalose to glucose [13,14,17,18]. It has been reported that *B. mori* midgut contains two trehalases, BmTre-1 and BmTre-2 [21]. Tre-2 is involved in incorporating trehalose from the blood into muscular cells and then providing the energy required by visceral muscles to support peristaltic movement of the midgut for active feeding [13,21]. The chitin biosynthesis pathway starts with trehalose, which is mainly synthesized by trehalose-6-phosphate synthase in the fat body and released into the hemolymph in insects [20,34,35]. According to the SeTre-2 expression patterns observed in the midgut and fat body (Figure 7), Tre-2 may have different functions in these two tissues. This is the first report of trehalase transcript expression in fat body, but its function in this tissue is still unknown. We also cloned the trehalose-6-phosphate synthase gene (GenBank accession no. EF051258) from *S. exigua*, which is expressed mainly in fat body and not in midgut, and found that its expression levels showed the same trend as trehalose levels in hemolymph of *S. exigua* (unpublished data). This demonstrates
that both Tre-2 and trehalose-6-phosphate synthase are synthesized in the fat body. Thus, the Tre-2 gene may have a crucial function in regulating the balance of trehalose in hemolymph.

The relative importance of Tre-1 and Tre-2 in the chitin biosynthesis pathway in \textit{S. exigua} is currently being investigated in our laboratory.

\textbf{Conclusion}

We have demonstrated that two trehalase genes exist in \textit{S. exigua}. \textit{SeTre-2} transcripts are expressed not only in the midgut, but also in fat body and Malpighian tubules. Furthermore, there are different \textit{SeTre-2} expression patterns between midgut and fat body. This suggests that \textit{SeTre-2} may have different functions in these different tissues.

\textbf{Methods}

\textbf{Insect cultures}

\textit{S. exigua} larvae were reared at 26 ± 1°C under a L14:D10 photoperiod on an artificial diet [36]. The developmental stages were synchronized at each molt by collecting new larvae or pupae. The midgut and fat body from larvae to pupae and the brain, cuticle, tracheae and Malpighian tubules from larvae were dissected in ice-cold saline, and stored at -80°C for later use.

\textbf{RNA isolation, cDNA synthesis and PCR}

Total RNA was isolated from fat body of \textit{S. exigua} pupae using an acid guanidinium thiocyanate-phenol-chloroform method [37,38]. Fat body (100 mg) was homogenized in solution D (solution D: 4M guanidinium thiocyanate, 0.025M sodium citrate, 0.1M mercaptoethanol, 0.05%C sarsosyl), placed on ice for 5 min and then sodium acetate and chloroform/isoamylalcohol (49:1) were added. The sample was centrifugated at 10,000×g at 4°C for 20 min and the supernatant was transferred into a new tube, and isopropanol was then added. After centrifugation, the RNA pellet was washed in 75% ethanol and then dissolved in ddH\textsubscript{2}O. A sample of 1 μg of total RNA was reverse-transcribed at 42°C for 1 h in a 10-μl reaction mixture containing reaction buffer, 10 mM DTT, 0.5 mM dNTP, 0.5 mg of oligo-dT\textsubscript{18}, and reverse transcriptase from avian myeloblastosis virus (AMV, Takara, Japan) [39].

Three degenerate primers, \textit{SeTre}-F1 (5'-CTA YTG GGA CDS WTA YTG G-3'), \textit{SeTre}-F2 (5'-GCY GAR AGC GGC TGG GAC TT-3') and \textit{SeTre}-R (5'-ACG CCR TTC GWC CAY CCG-3'), were designed based on the conserved amino acid sequences of known trehalases. The first PCR amplification was performed with primers \textit{SeTre}-F1 and \textit{SeTre}-R under the following conditions: 3 cycles of 40 s at 94°C, 40 s at 45°C, and 90 s at 72°C, then 28 cycles of 40 s at 94°C, 40 s at 48°C, and 90 s at 72°C. A second PCR was carried out using nested reverse primers \textit{SeTre}-F2 and \textit{SeTre}-R under the same conditions as for the first PCR. After PCR products were electrophoresed, a weak DNA band corresponding to the expected size of approximately 700 bp was excised from the agarose gel and purified using a DNA gel extraction kit (Takara, Japan). These PCR products were cloned into the pMD18-T vector (Takara) and sequenced by the dideoxynucleotide method (Takara).

\textbf{Rapid amplification of cDNA ends (RACE)}

For 5'- and 3'-RACE, cDNA was synthesized according to the manufacturer’s protocol (SMART\textsuperscript{™} kit, Clontech). Specific primers \textit{SeTre}-R1 (5'-CGG AGA AGC TGG GCG TTC C-3') and \textit{SeTre}-R2 (5'-GGC ATT CAG GTG TAC TGG C-3') for 5'-RACE, and \textit{SeTre}-F1 (5'-GGG CTA TCC GAA TGC CTT GC-3') and \textit{SeTre}-R2 (5'-CCA AAT GGG TCA GTT CG-3') for 3'-RACE were synthesized based on the cDNA sequence obtained from the PCR product. 5'-RACE was performed on 2.5 μl of 5'-ready-cDNA with Universal Primer Mix (UPM, Clontech) and \textit{SeTre}-R1, then nested PCR was carried out with Nested Universal Primer (NUP, Clontech) and \textit{SeTre}-R2. 3'-RACE was performed on 2.5 μl of 3'-ready-cDNA with UPM and \textit{SeTre}-F1, then with NUP and \textit{SeTre}-F2. PCR conditions were 10 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 55°C, and 90 s at 72°C, then 10 min at 72°C. After PCR products were electrophoresed, DNA bands corresponding to approximately 1200 bp from the 5'-RACE and ~600 bp from the 3'-RACE were excised from the agarose gel and purified using a DNA gel extraction kit (Takara, Japan). These PCR products were cloned into the pMD18-T vector (Takara) and sequenced by the dideoxynucleotide method (Takara). The resulting overlapping sequences were assembled to obtain the full-length \textit{SeTre-2} cDNA sequence. To confirm the assembled cDNA sequence from overlapping PCR products, the entire coding regions of \textit{SeTre-2} were amplified by PCR with the forward and reverse primers \textit{SeTre}-F5 (5'-CAT TGT CGA TAG TTT ATT TGT CG-3') and \textit{SeTre}-R3 (5'-CAC TCA CGT TCC ACC GGT CGA G-3'). PCR was performed as follows: denaturation at 94°C for 30 s, annealing at 55°C for 30 s and elongation at 72°C for 3 min using Takara \textit{Taq} polymerase for 30 cycles.

\textbf{cDNA and protein sequence analyses}

The sequence of \textit{SeTre-2} cDNA was compared with sequences of other trehalases deposited in GenBank using the BLAST-N and BLAST-X tools available from the National Center for Biotechnology Information (NCBI) web site. A phylogenetic tree was constructed using MEGA 3.1 software based on the amino acid sequences of known trehalases. A bootstrap analysis was carried out, and the robustness of each cluster was verified in 1000 replications. The amino acid sequence of \textit{SeTre-2} was deduced...
from the corresponding cDNA sequence using the translation tool at the ExPASy Proteomics website (please see Availability & requirements). Other protein sequence analysis tools used in this study, including molecular weight, pl, and N-glycosylation sites were also obtained from the ExPASy Proteomics website. The transmembrane helices of Trehalase proteins were analyzed using TMHMM v.2.0 (please see Availability & requirements). Multiple sequence alignments of deduced amino acid sequences were made using Vector NTI 9.0 software.

**Genomic DNA sequencing and gene structure analysis**

To obtain the SeTre-2 gene, genomic DNA was extracted from the fat body of fifth instar larvae using a Genomic DNA Purification Kit (Promega) according to the manufacturer’s instructions. Overlapping PCR fragments were obtained using pairs of gene-specific primers designed from the corresponding cDNA sequence of SeTre-2 and genomic DNA as a template. Cloning and sequencing of these PCR products were carried out in the same manner as described above [36]. Exons and introns were identified by comparing and analyzing the cDNA and genomic DNA sequences of SeTre-2.

**Southern blot analysis**

Genomic DNA was prepared from fresh *S. exigua* pupae and was purified after complete digestion with HindIII, *SalI* or *XhoI*. The digested DNA (15 μg per lane) was separated on a 0.8% agarose gel in TAE buffer (40 mM Tris acetate, 2 mM EDTA) After electrophoresis, DNA was transferred to Hybond-N+ nylon membranes (Amersham) in 20× SSC [Au: What is SSC?] for 17 h [40]. DNA was fixed to the membrane by baking at 120°C for 30 min. A cDNA fragment of 770 bp (using primers SeTreFP 5′-AGG ATC TGA GTT CGA GGA CTG G-3′ and SeTreRP 5′-GGC ATT CAG GTC TAC TGG G-3′) was labeled with [α-32P]dCTP-labeled probe as described above [41]. RT-PCR was performed using SeTreFP and SeTreRP primers and total RNA from midgut, brain, Malpighian tubules, cuticle, fat body, tracheae and spermary as templates for 30 cycles of 40 s at 94°C, 40 s at 55°C, and 60 s at 72°C. A 5-μl sample of each PCR product was electrophoresed and detected by ethidium bromide staining. β-Actin was used as a loading control.

**Developmental SeTre-2 expression in fat body and midgut**

The fat body of fifth instar larvae, pre-pupae and pupae, and the midgut of third, fourth and fifth instar larvae, pre-pupae and pupae were dissected. Total RNA was isolated from the fat body of 12 stages and the midgut of 11 stages. Then 1 μg of total RNA from each sample was reverse-transcribed at 42°C for 1 h in a 10-μl reaction mixture containing reaction buffer, 10 mM DTT, 0.5 mM dNTP, 0.5 mg of oligo-dT18, and reverse transcriptase from avian myeloblastosis virus (AMV, Takara, Japan). PCR reactions were performed using primers SeTreFP, SeTreRP and SeActinF/R for 22 cycles of 40 s at 94°C, 40 s at 55°C, 60 s at 72°C. A 5-μl sample of each PCR product was electrophoresed and transferred to a Hybond-N+ membrane and then hybridized with [α-32P]dCTP-labeled probes as described above. The amount of *S. exigua* β-actin loaded per lane is indicated as a control.

**Availability & requirements**

ExPASy Proteomics: [http://expasy.org/tools/dna.html](http://expasy.org/tools/dna.html)

TMHMM v.2.0: [http://www.cbs.dtu.dk/services/TMHMM-2.0/](http://www.cbs.dtu.dk/services/TMHMM-2.0/)

**Authors’ contributions**

BT carried out most of experiments and co-wrote the manuscript. XC participated in *SeTre*-2 gene cloning and acquisition of data. YL performed data analysis and interpretation. HT performed protein sequence and data analysis. JL participated in the study design. JH have made substantial contributions to conception and design. WX have been involved in drafting the manuscript or revising it critically for important intellectual content. WZ conceived the project, supervised the experiments and co-wrote the manuscript. All authors read and approved the final manuscript.

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References

1. Elbein AD: The metabolism of α,α-trehalos. Adv Carbohydr Chem Biochem 1974, 30:227-256.
2. Elbein AD, Pan YT, Pastuszak I, Carroll D: New insights on trehalose: a multifunctional molecule. Gecyology 2003, 13(4):17R-27R.
3. Wingler A: The function of trehalose biosynthesis in plants. Phytochemistry 2002, 60:437-440.
4. Frison M, Parrou JL, Guillamot D, Masquelier D, Francois J, Chau mort F, Batore H: The Arabidopsis thaliana trehalase is a plasma membrane-bound enzyme with extracellular activity. FEMS Letters 2007, 581(21):4010-4016.
5. Crowe J, Crowe L, Chapman D, Chapman D: Preservation of membranes in anhydrobiotic organisms: the role of trehalose. J Appl Microbiol 2002, 93:721-730.
6. Eleutherio EC, Araujo PS, Panek AD: Role of the trehalose carrier in dehydration resistance of Saccharomyces cerevisiae. Biochim Biophys Acta 1993, 1156:263-266.
7. Garg AK, Kim JK, Owens TG, Ranwala AP, Do Choi Y, Kochian LV, Wu RJ: Trehalose accumulation in rice plants confers high tolerance levels to different abiotic stresses. Proc Natl Acad Sci USA 2002, 99:15898-15903.
8. Wyatt GR: The biochemistry of sugars and polysaccharides in insects. Adv Insect Physiol 1967, 4:287-360.
9. Friedman S: Trehalose regulation, one aspect of metabolic homeostasis. Ann Rev Enolomol 1978, 23:389-407.
10. Becker A, Schloer P, StelleJE, Wegener G: The regulation of trehalose metabolism in insects. Experientia 1996, 52:433-439.
11. Thompson SN: Trehalose – the insect ‘blood’ sugar. Adv Insect Physiol 2003, 31:203-285.
12. Yaginuma T, Mizuno T, Mizuno C, Ikeda M, Wada T, Hattori K, Yaginuma T, Mizuno T, Ikeda M, Wada T, Hattori K: Molecular cloning, sequencing and developmental profile of the gene expres sion. J Insect Physiol 2007, 53:2256-65.
13. Azuma M, Yamashita O: Immunohistochemical and biochemical localization of trehalase in the developing ovaries of the silkworm, Bombyx mori. Tissue Cell 1985, 17:539-551.
14. Matsu M, Yamashita O: Immunohistochemical and biochemical localization of trehalase in the developing ovaries of the silkworm, Bombyx mori. Insect Biochem 1985, 15:589-596.
15. Valatis AP, Bowers DP: Purification and properties of the soluble midgut trehalase from the gypsy moth, Lymantria dispar. Insect Biochem Mol Biol 1993, 23:599-606.
16. Takiguchi M, Niimi T, Su ZH, Yamashita O: Trehalase from male accessory gland of an insect, Tenebrio molitor: cDNA sequencing and developmental profile of the gene expression. Biochem J 1992, 280:19-32.
17. Su ZH, Ikekda M, Sato Y, Saito H, Imai K, Isobe M, Yamashita O: Molecular characterization of ovary trehalase of the silkworm, Bombyx mori and its transcriptional activation by dia pause hormone. Biochim Biophys Acta 1994, 1218:366-374.
18. Su ZH, Sato Y, Yamashita O: Purification, cDNA cloning and Northern blot analysis of trehalase of pupa midgut of the silkworm, Bombyx mori. Biochim Biophys Acta 1993, 1173:217-224.
19. Su ZH, Itani Y, Yamashita O: Structure of trehalase gene of the silkworm, Bombyx mori and phylogenetic relationship of treha lases. Nihon Sashigaku Zasshi 1997, 66:457-465 [http://www3.interscience.wiley.com/cgi-bin/fulltext/61501585/PDFSTART].
20. Kramer KJ, Koga D: Insect chitin: physical state, synthesis, degradation and metabolic regulation. Insect Biochem 1986, 16:851-877.
21. Mitsu matsu K, Azuma M, Niimi T, Yamashita O, Yaginuma T: Membrane-penetrating trehalase from silkworm Bombyx mori. Molecular cloning and localization in larval midgut. Insect Molecular Biology 2005, 14:501-508.
22. Lee JH, Sato S, Mori H, Nishimoto M, Okuyama M, Kim D, Wangcha-walit J, Kimura A, Chiba S: Molecular cloning of cDNA for treha lase from the European honeybee, Apis mellifera L., and its heterologous expression in Pichia pastoris. Biosci Biotechnol Biochem 2007, 71(9):2256-65.
23. Eisenhaber B, Bork P, Yuan Y, Loseffler G, Eisenhaber F: Automated annotation of GPI anchor sites: case study C. elegans. Trends Biol Sci 2000, 25:340-341.
24. Sumida M, Yamashita O: Purification and some properties of soluble trehalase from midgut of pharate adult of the silk worm, Bombyx mori. Insect Biochem 1983, 13:257-265.
25. Friedman S: Carbohydrate metabolism in the silkworm. In Comparative Insect Physiology, Biochemistry, and Pharmacology Volume 10. Edited by: Kerkut GA, Gilbert LI. Oxford, Pergamon: 1985:43-76.
26. Terra WR, Ferreira C: Insect digestive enzymes: properties, compartmentalization and function. Comp Biochem Physiol 1994, 109B:1-62.
27. Ishihara R, Takekai S, Sasa-Takedas M, Kino M, Tokunaga R, Kobayashi Y: Molecular cloning, sequencing and expression of cDNA encoding human trehalase. Gene 1997, 202:69-74.
28. Oesterreicher TJ, Nandakumar NN, Winston JH, Henning SJ: Rat trehalase: cDNA cloning and mRNA expression in adult rat tissues and during intestinal ontogeny. Am J Physiol 1998, 274(5 Pt 2):R1220-7.
29. Oesterreicher TJ, Markesich DC, Henning SJ: Cloning, characteri zation and mapping of the mouse trehalase (Treh) gene. Gene 1997, 210:1-220.
30. Parkinson NM, Conyers CM, Keen JN, MacNicol AD, Smith I, Weaver RJ: cDNAs encoding large venom proteins from the parasitoid wasp Pimpla hypochondriaca identified by random sequence analysis. Comp Biochem Physiol C Toxicol Pharmacol 2003, 134(4):453-520.
31. Kamimura M, Takahashi M, Tomita S, Fujiwara H, Kuchi M: Expression of eddyson receptor isoforms and trehalase in the anterior silk gland of Bombyx mori during an extra larval molt and preoviposition period induced by 20-hydroxyecdysone adminis tration. Arch Insect Biochem Physiol 1999, 41:79-88 [http://www3.interscience.wiley.com/cgi-bin/fulltext/61501585/PDFSTART].
32. Yamashita O, Sumida M, Hasegawa K: Developmental changes in midgut trehalase activity and its localization in the silkworm, Bombyx mori. J Insect Physiol 1974, 20:1079-1085.
33. Sato K, Komoto M, Sato T, Enei H, Kobayashi MM, Yaginuma T: Bac lovirus-mediated Expression of a Gene for Trehalase of the Mealworm Beetle, Tenebrio molitor, in Insect Cells, SF-9, and Larvae of the Cabbage Armyworm, Mamestra brassicae. Insect Biochem Mol Biol 1997, 27(12):1007-1016.
34. Cohen E: Chitin synthesis and inhibition: a revisits. Pest Manag Sci 2001, 57:946-950.
35. Merzendorfer H, Zimoch L: Chitin metabolism in insects: structure, function and regulation of chitin synthases and chitinases. The Journal of Experimental Biology 2003, 206:4393-4412.
36. Chen XF, Yang X, Kumar NS, Tang B, Qiu XM, Hu J, Zhang WQ: The class A chitin synthase gene of Spodoptera exigua : molecular cloning and expression patterns. Insect Biochem Mol Biol 2007, 37:409-417.
37. Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987, 162:156-159.
38. Aguila ED, Dutra MB, Silva JT, Paschoalini VM: Comparing protocols for preparation of DNA free total yeast RNA suitable for RT-PCR. BMC Molecular Biology 2005, 6:9.
39. Cui SY, Wu VH: Molecular characterization and functional distribution of N-ethylnmaleimide-sensitive factor in Helicoverpa armigera. Pesticides 2006, 27:1226-1234.
40. Sambrook J, Fritsch EF, Maniatis T: Molecular Cloning: A Laboratory Manual 1989 [http://books.google.com/books?hl=hi&lr=&id=YTtXxWWUVUS&oe=UC&oi=fnd&pg=PR2&dq=Molecu lar+Cloning:&q=9780716783538+&f=false].
41. Hoogerwerf WA, Hellmich HL, Micci MA, Winston JH, Zhou L, Pasricha PJ: Molecular cloning of the rat protease-activated receptor 4 (PAR4). BMC Molecular Biology 2002, 3:2.