Appl Entomol Zool (2014) 49:255–264
DOI 10.1007/s13355-014-0244-4

ORIGINAL RESEARCH PAPER

Trehalase in *Harmonia axyridis* (Coleoptera: Coccinellidae): effects on beetle locomotory activity and the correlation with trehalose metabolism under starvation conditions

Bin Tang · Zi Qin · Zuo-Kun Shi · Su Wang · Xiao-Jun Guo · Shi-Gui Wang · Fan Zhang

Received: 10 July 2013 / Accepted: 29 December 2013 / Published online: 29 January 2014

Abstract Trehalose is important in the energy metabolism of insects and protects them from extreme environmental conditions. To study the synthesis and degradation of trehalose, which is hydrolyzed to glucose by trehalase, two soluble trehalase cDNAs of the beetle *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae) (*HaTreh1-1* and *HaTreh1-2*) were cloned. The *HaTreh* mRNA expression levels were higher during the larval than adult stages. The locomotory behavior of the beetle and its mRNA expression of *HaTreh1-1* and *HaTreh1-2* under starvation were then investigated. Sprint speed and maximum moving distance were increased, and the pause frequency decreased in *H. axyridis* adults starved for 8 h compared with control (0 h) adults. In contrast, the mRNA expression of both *HaTrehs* increased quickly in adults starved from 8 to 18 h, with *HaTreh1-1* in particular being expressed 289-fold in adults starved for 18 h versus control. These results show that stored food reserves can provide energy to sustain vital activities for 8 h in *H. axyridis* adults. However, the beetles require trehalose and other energy sources to provide energy to find food and for vital activities when starved for more than 8 h.

Keywords Soluble trehalase · Trehalose · Short-term starvation · Locomotor activity · *Harmonia axyridis*

B. Tang · Z. Qin · S. Wang · X.-J. Guo · F. Zhang (✉)
Institute of Plant and Environment Protection, Beijing Academy of Agriculture and Forestry Sciences, Beijing 100089, China
e-mail: zhangf6131@yahoo.com

B. Tang · Z.-K. Shi · S.-G. Wang
Hangzhou Key Laboratory of Animal Adaptation and Evolution, College of Life and Environmental Sciences, Hangzhou Normal University, Hangzhou 310036, Zhejiang, China

Introduction

Trehalose is a non-reducing sugar comprising two glycosidically linked glucose units that is found in many organisms, including bacteria, yeast, fungi, nematodes, plants, insects and some other invertebrates, but not mammals (Elbein 1974; Elbein et al. 2003; Frison et al. 2007; Wingler 2002). Trehalose acts as both a carbohydrate store and an agent for protecting proteins and cellular membranes from a variety of environmental stressors, including desiccation, dehydration, heat, freezing and oxidation (Becker et al. 1996; Friedman 1978; Thompson 2003; Wyatt 1967). Also, in insects, trehalose is the main metabolic source of energy and has an important role in the physiological adaptation to the environment (Qin et al. 2012; Tang et al. 2012a).

The synthesis and utilization of trehalose in insects are unique in that trehalose is synthesized mainly in the insect fat body and is rapidly released into the hemolymph (Tang et al. 2010). Trehalase not only participates in homeostasis and development (Friedman 1978; Silva et al. 2004; Terra and Ferreira 1994; Thompson 2003), but also has roles in locomotion, flight metabolism, cold tolerance and so on (Clegg and Evans 1961; Tatun et al. 2008a; b). Trehalose can be rapidly used as an energy source when the insect is hungry (Thompson 2003). Trehalose and glycogen are the main sources of glucose and glucose-6-phosphate, and might be key intermediate regulatory products (Tang et al. 2012b). Based on the previous reports (Elbein et al. 2003; Kunieda et al. 2006; Montooth et al. 2003; Rockstein 1978; Tang et al. 2012a, b), trehalose and glycogen can be mutually transformed by trehalose 6-phosphate synthase (TPS) and glycogen synthase, based on changes in the surrounding environment or in extreme environmental conditions.

The results of gene cloning, protein purification and trehalase activity studies have shown that there are two different genes encoding two different types of trehalase (Treh) in insects, depending on whether the trehalase has transmembrane helices. The first is soluble trehalase (Treh1) (Kamimura et al. 1999; Parkinson et al. 2003; Su et al. 1993, 1994, 1997; Takiguchi et al. 1992), and the second is membrane-bound trehalase (Treh2), which has more than one potential transmembrane helix (Chen et al. 2010; Lee et al. 2007; Mitsumasu et al. 2005, 2008, 2010; Tang et al. 2008). Treh1 and Treh2 were first found in *Tenebrio molitor* (Linné) (Coleoptera: Tenebrionidae) (Gomez et al. 2013; Takiguchi et al. 1992) and *Bombyx mori* (Linnaeus) (Lepidoptera: Bombycidae) (Mitsumasu et al. 2005), respectively. Treh1 is localized in the goblet cavity, which suggests a possible function of trehalase on the apical surface of the brush-border membranes of the mammalian small intestine (Mitsumasu et al. 2005; Ruf et al. 1990). Predominant localization of Treh2 on visceral muscular cells indicates that Treh2 is involved in incorporating trehalose from the blood into muscular cells and then providing the energy required for visceral muscles to strongly support the peristaltic movement of the midgut for active feeding (Azuma and Yamashita 1985a; Mitsumasu et al. 2005). However, the gene regulatory mechanisms behind such changes are poorly known, particularly given that the amount of trehalose changes as soon an insect starts to fly (Van der Horst et al. 1978), as well during short-term starvation.

The beetle *Harmonia axyridis* (Pallas) is able to survive in a variety of environmental conditions and has a worldwide distribution. This beetle is an important natural enemy that is a focus of pest control strategies in agriculture and forestry production in China. Such predatory insects can survive and be vigorous even under starvation conditions with few insect pests as prey in the field. Thus, would these starvation conditions affect the physiological activities of this beetle and affect its trehalose synthesis and degradation as well? The trehalose-6-phosphate synthase (TPS) gene in *H. axyridis* (GenBank accession no. FJ501960) has previously been cloned and studied (Qin et al. 2012). In the present study, we first cloned two cDNAs of *Treh* genes from *H. axyridis* (GenBank accession nos. HM056038 and FJ501961; *HaTreh*). Here, we report on the outcomes of experiments investigating the expression of one *TPS* and two *Treh* genes, recording the locomotory activities to test our hypothesis.

Materials and methods

Insect cultures

Harmonia axyridis was collected from Wangjiayuan village, Beijing, China. The experimental population had been maintained in our laboratory for more than 2 years, fed with *Aphis medicaginis*. In the experiment on the developmental stages and starvation, all of the insects were put in a feeding box under the following conditions: 25 ± 1 °C, L14:D10 photoperiod and 50–75 % relative humidity. The light condition started at 8:00 am and ended at 22:00 pm (L14:D10 photoperiod). The developmental stages were synchronized at each molt by collecting new larvae, pupae or adults by giving fresh *A. medicaginis* daily.

RNA extraction, cDNA synthesis and PCR

Total RNA was extracted from the abdominal tissues and at different developmental stages (from larvae to adult) using the Trizol (Invitrogen, USA) method. First-strand cDNA synthesis was carried out using a PrimeScript® RT reagent Kit With gDNA Eraser (TaKaRa, Japan) according to the manufacturer's instructions. First-strand cDNA (1 μl) was used as the template for the polymerase chain reaction (PCR).

Treh1-DF1 and Treh1-DR1, Treh1-DF2 and Treh1-DR2, Treh2-DF1 and Treh2-DR1, and Treh2-DF2 and Treh2-DR2 (Table 1) were designed based on the conserved amino acid sequences of the two known forms of Treh. The first PCR reaction was performed with the primers Treh1-DF1/Treh2-DF1 and Treh1-DR1/Treh2-DR1 using the following conditions: three cycles of 40 s at 94 °C, 40 s at 45 °C and 60 s at 72 °C, followed by 30 cycles of 40 s at 94 °C, 40 s at 48 °C and 60 s at 72 °C. A second PCR was carried out using the nested primers Treh1-DF2/Treh2-DF2 and Treh1-DR2/Treh2-DR2 using the same conditions as for the first PCR (Tang et al. 2008).

The PCR products were subjected to agarose gel (1.2–1.5 %) electrophoresis. The true electrophoretic DNA bands corresponding to the expected size of approximately 600–800 base pairs (bp) were excised from the agarose gel and purified using a DNA gel extraction kit (Omega, USA). Purified DNA was ligated into the pMD18-T vector (TaKaRa) and sequenced using the dideoxynucleotide method (Tang et al. 2008).

Rapid amplification of cDNA ends

For 5′- and 3′-rapid amplification of cDNA ends (RACE), cDNA was synthesized according to the manufacturer's protocol (SMART™ kit, Clontech, USA). Specific primers [HaTreh1-5RA, HaTreh1-5RB/HaTreh2-5RA or HaTreh2-5RB for 5′-RACE; and HaTreh1-3FA or HaTreh1-3FB/HaTreh2-3FA or HaTreh2-3FB for 3′-RACE (Table 1)] were synthesized based on the cDNA sequence of the PCR fragment. 5′-RACE was performed using 5′-ready-cDNA (2.5 μl) with Universal Primer Mix (UPM, Clontech) and

Table 1 Primer sequences used for amplification of *HaTPS*, *HaTreh1-1*, *HaTreh1-2* and *18SrRNA*

PCR fragment	Primer name	Nucleotide sequences (5′-3′)
Degenerate primers	Treh1-DF1	GGA RWT YTA YTA CTG GGA
	Treh1-DF2	TGG ATH RTB RAA GGK CT
	Treh1-DR1	CCR TTB SWC CAY CCG AA
	Treh1-DR2	CGC RTC RTA YTT CTC RAA CAT
	Treh2-DF1	TAC TGG GAY TCB TAC TGG A
	Treh2-DF2	ACG GNG GMM GVA THT ACT A
	Treh2-DR1	GCC AVG CRT TSG GRT AGT CC
	Treh2-DR2	CCA SRC NCC BAC NTC BTC GTG C
RACE	HaTreh1-3FA	CCA GAG TCA GCT CAA GAG
	HaTreh1-3FB	CAC AGG AGA ACA ATG GGA C
	HaTreh1-5RA	GTC CCA TCC ACT TTC TGC
	HaTreh1-5RB	CCT TGT AGT TCA CTT CG
	LaTreh2-3FA	TGG GCT GAA CTA TAC GAA GAA CC
	LaTreh2-3FB	GGA GTT TCC AGC AGG AGT ACC GA
	LaTreh2-5RA	CTG AGT AAC GGG CAA GAG TGT GC
	LaTreh2-5RB	GGT TGC TTT GAG ATA CTC GTG TG
	NUP	AAG CAG TGG TAA CAA CGC AGA GT
QRT-PCR	QTreh1-1F	AGA GAC TTC GCC AGT CAA ATC
	QTreh1-1R	GAG ACC TTC AAT AAT CCA GAA GC
	QTreh1-1P	CCT GTG CCA AAT ACC TTC GTG GT
	QTreh1-2F	TGG ACA TCC AGA GGC TAC AA
	QTreh1-2F	GAG GTC GGT ACT CCT GCT G
	QTreh1-2P	CAA TCT TGC TCC TCT ATG GGC TGA A
Reference gene	18SrRNA-F	ACG GAC TTC GGT AGG ACG
	18SrRNA-R	CGC AGA CAA TCC CGA AA
	18SrRNA Probe	ACG TTG TGC GAC GCC CGT TA

HaTreh1-5RA/HaTreh2-5RA; the nested PCR was then carried out with Nested Universal Primer A (NUP, Clontech) and HaTreh1-5RB/HaTreh2-5RB. 3′-RACE was performed using 3′-ready-cDNA (2.5 μl) with UPM and HaTreh1-3FA/HaTreh2-3FA, then with NUP and HaTreh1-3FB/HaTreh2-3FB. The PCR conditions were as follows: 10 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at 60 °C and 150 s at 72 °C, then 10 min at 72 °C.

Analysis of the *HaTreh* cDNA and protein sequences

HaTreh cDNA sequences were compared with other trehalase sequences deposited in GenBank using the BLAST-N and BLAST-X tools on the National Center for Biotechnology Information (NCBI) website. Multiple sequence alignment of insect trehalases was performed using the tool at the MultiAlin website (http://bioinfo.genotoul.fr/multalin/multalin.html). HaTreh protein sequences and other analysis tools used in this study, including MW, pI and topology, were deduced from the corresponding cDNA sequences using the translation tool on the ExPASy Proteom prediction tools ICS website (http://expasy.org/tools/dna.html).

Locomotory activity of *H. axyridis* during periods of short-term starvation

Adult *H. axyridis* were used in this experiment 3 days following eclosion; plenty of prey was provided to the beetles before the experiment. Six treatments were set up corresponding to different periods of starvation: 0 (control), 4, 8, 12, 18 and 24 h. Thus, in the experiment, different experimental groups of insects were kept without food for different lengths of time, and the other rearing condition is consistent with the normal feeding condition.

In this experiment, a polymethyl methacrylate (PMMA) pipe (300 × 4 cm) (Fig. 1) was used to detect the sprint speed, pause frequency and maximum moving distance of *Harmonia axyridis* under the different periods of starvation. This polymethyl methacrylate pipe was set up in a phytotron maintained at 25 ± 1 °C and 50–75 % relative humidity. Adult *H. axyridis* were put in this pipe to initiate insect movement; a single stimulation by gentle brushing was required. We then recorded the locomotory activities over a 20-min period. Three indexes were used to record the locomotion: (1) sprint speed (SS): movement speed over the first 10 cm; (2) pause frequency (PF): the number of pauses per minute; (3) maximum moving distance (MMD): the distance of continual exercise per minute. Each treatment was replicated 12 times.

Expression levels of *HaTrehs* and *HaTPS* during developmental stages and periods of starvation in *H. axyridis*

Total RNA was isolated from *H. axyridis* daily throughout its life cycle, including adults subjected to starvation treatments. Total RNA (1 μg) was used as a template to

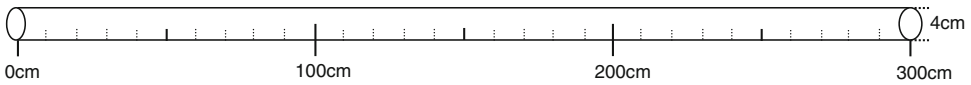

Fig. 1 The polymethyl methacrylate pipe for the locomotory activity experiment

demonstrate the stability of *Ha18srRNA*. PCR reactions were performed with the primers 18SrRNA-F and 18SrRNA-R using the following conditions: 95 °C for 5 min, 28 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, followed by a final extension at 72 °C for 10 min. Expression of *HaTrehs* was estimated by real-time fluorescence quantitative PCR (qRT-PCR) using a Bio-Rad CFX96^{TM} system (Bio-Rad, USA) and SsoFast ^{TM} EvaGreenSupermix (Bio-Rad). Each reaction took place in a final volume (20 µl) containing the cDNA sample (or standard), (1 µl), primer [1 µl (l0 µmol/µl)], RNase-free and DNAase-free H_2O (7 µl) and SsoFast ^{TM} EvaGreen-Supermix (10 µl). After 3 min of initial denaturation at 95 °C, the cycling protocol comprised 40 cycles of denaturation at 95 °C for 5 s, annealing at 55–62.5 °C for 20 s and finally making the melt curve at 65–95 °C, according to the manufacturer's instructions. Standard curves were obtained using a tenfold serial dilution of pooled total RNA.

Statistical analysis

The statistical significance of differences in sprint speed, pause frequency and maximum moving distance were determined by one-way analysis of variance (ANOVA) and Tukey-Kramer HSD test using the JMP software (version 7.0.1 for Macintosh; SAS Institute Inc., Cary, NC).

Results

Sequence analysis of *HaTreh* cDNAs

Two *HaTreh* cDNAs were obtained by PCR, and the encoded amino acids were submitted to GenBank. Based on results from the TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/), both Trehs were shown to be Treh1 rather than Treh2 and therefore were named *HaTreh1-1* and *HaTreh1-2*, respectively. *HaTreh1-1* (HM056038) and *HaTreh1-2* (FJ501961) cDNA had open reading frames of 1665 and 1539 bp, respectively. *HaTreh1-1* encoded a protein of 554 amino acids with a predicted mass of approximately 63.7 kDa and a p*I* of 4.82; *HaTreh1-2* cDNA encoded a protein of 512 amino acids with a predicted mass of approximately 59.1 kDa and a p*I* of 4.91 (Fig. 2). HaTreh1-1 and HaTreh1-2 had six and four *N*-glycosylation sites, respectively. In addition, both proteins had a signal peptide of 20 amino acids.

Developmental expression pattern of *HaTreh1-1 and HaTreh1-2*

The daily expression patterns of *HaTreh1-1* and *HaTreh1-2* mRNA from first-instar *H. axyridis* to 3-day-old adults are shown in Fig. 3. The daily expression of *H. axyridis* 18SrRNA was used to standardize the relative expression levels of two *HaTreh* genes. The mRNA levels of both genes were relatively high during the larval development stages compared to the adult stages (Fig. 3). The expression of *HaTreh1-1* mRNA was high in the first-instar larvae to the pre-pupae stages, but relatively low in the 1-day-old pupae to 3-day-old adults (Fig. 3a). The developmental expression patterns of *HaTreh1-2* mRNA differed from those of *HaTreh1-1*, being high during the first-, second- and third-instar larval stages (Fig. 3b), but low on day 4 of the fourth-instar larva, in pre-pupae, 1-day-old and 3-day-old pupa, and in the 1–3-day-old adults (Fig. 3b). Moreover, *HaTreh1-2* mRNA was highly expressed during the second day of the pupal stage.

Locomotory activity of *H. axyridis* during periods of starvation

The SS of *H. axyridis* adults fluctuated under the different starvation periods; adults fluctuated, but significantly less than adults starved for 8 h ($P < 0.05$, Fig. 4a). The SS of *H. axyridis* adults was inversely correlated with the length of time for which they had been starved. The PF was higher in control adults than in those subjected to different periods of starvation. The PF curve first decreased then increased as the length of starvation time increased, reaching a minimum in the group starved for 8 h. The PF of *H. axyridis* adults starved for 4 h was the same as in those starved for 18 h, and significantly less than in those starved for 0 or 24 h ($P < 0.05$, Fig. 4b). Compared with the PF, the MMD showed the reverse pattern. The MMD of control *H. axyridis* adults was more than that demonstrated by adults starved for 18 or 24 h; the maximum MMD was recorded in adults starved for 8 h (Fig. 4c).

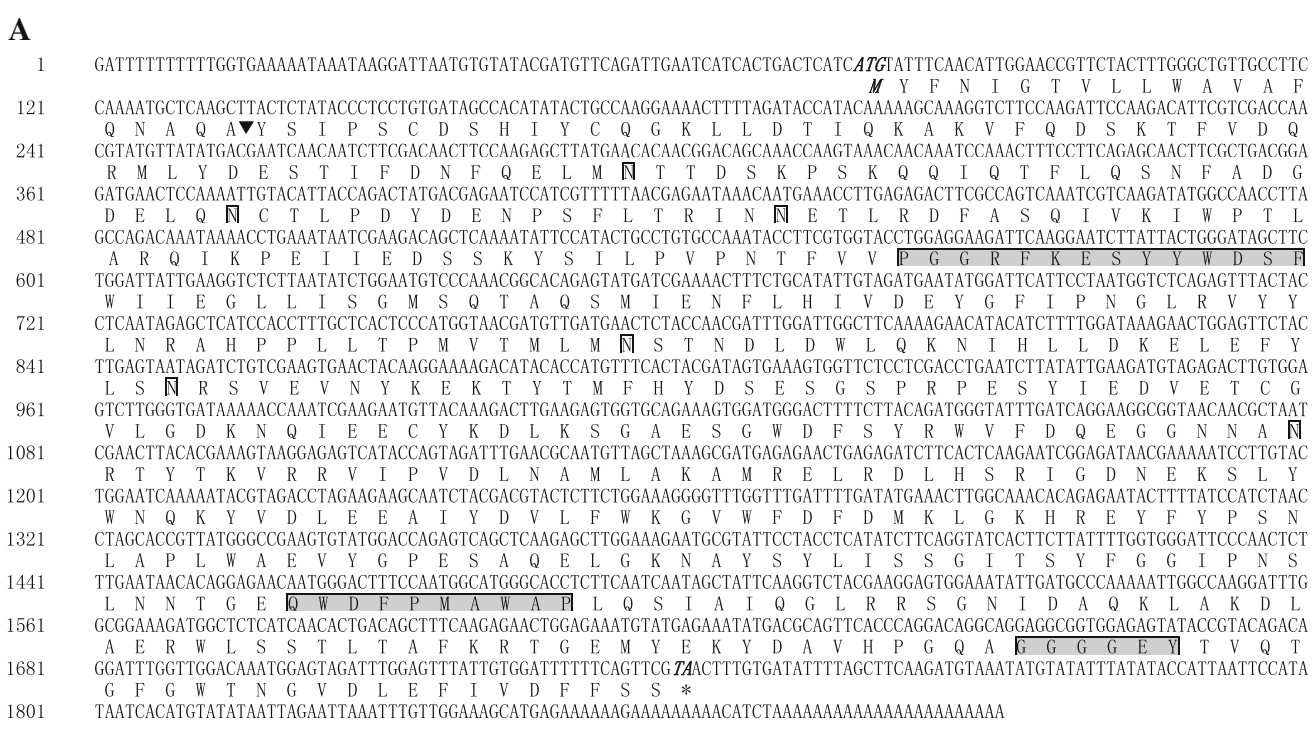

Fig. 2 Nucleotide and deduced amino acid sequences of genes encoding trehalases from *Harmonia axyridis* (*HaTreh*). Both initiation and termination codons are indicated by *bold italics*; the termination codon before the first Met is also indicated by *bold italics*. A highly conserved glycine-rich (GGGGEY) region is highlighted in gray. **a** HaTreh1-1: *underlined* amino acid residues (1–20) and the *arrowhead* represent the signal peptide and putative cleavage site, respectively. Trehalase signatures (amino acid residues 162–175 and 462–471) are highlighted in *gray*. Potential *N*-glycosylation sites (amino acid residues 73, 100, 118, 233, 258 and 335) are outlined. The nucleotide sequence reported in this study has been submitted to GenBank (accession number). **b** HaTreh1-2: *underlined* amino acid residues (1–21) and the *arrowhead* represent the signal peptide and putative cleavage site, respectively. Trehalase signatures (amino acid residues 162–175 and 461–470) are highlighted in *gray*. Potential *N*-glycosylation sites (amino acid residues 217, 335, 393 and 507) are outlined. The nucleotide sequences reported in this article have been submitted to GenBank (accession nos.: HM056038 and FJ501961, respectively)

Changes in the expression levels of Treh and TPS mRNA during periods of starvation

Our results showed that the trend in expression levels of TPS mRNA was similar to that in the PF of *H. axyridis* adults in that low levels of expression correlated with increasing levels of starvation (Fig. 5a). The expression of TPS mRNA in starved individuals was lower than in the controls, being lowest in those starved for 12 h. By contrast, the expression of mRNA of the two *HaTrehs* differed according to the length of the starvation period. The expression of *HaTreh1-1* mRNA was increased more than 20-fold in 8 h compared with controls and more than 289-fold in adults starved for 18 h versus controls. Furthermore, the expression of HaTreh1-1 mRNA over 24 h was more than 200-fold that of controls (Fig. 5b). Similarly, the expression of *HaTreh1-2* mRNA in starved adult *H. axyridis* was also higher than in the control group. However, the expression of *HaTreh1-2* mRNA increased 3.83-fold compared with the control group or normal group in 18 h (Fig. 5c). Both *HaTrehs* mRNAs showed the highest expression in adults starved for 18 h.

Discussion

Trehalase is an anomer-inverting α-trehalose-1-D-glucosidase that hydrolyzes a trehalose molecule into two glucose molecules. To date, two forms of trehalase, Treh1 and Treh2, have been found and cloned in many insect species, including *Tenebrio molitor* (Linné) (Coleoptera: Tenebrionidae), *Pimpla hypochondriaca* (Retzius) (Hymenoptera: Ichneumonidae), *B. mori* (Linnaeus) (Lepidoptera: Bombycidae), honeybee (*Apis mellifera* L) (Hymenoptera: Apidae) and *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) (Gomez et al. 2013; Lee et al. 2007; Mitsumasu et al. 2005; Parkinson et al. 2003; Takiguchi et al. 1992; Tang et al. 2008; Tatun et al. 2008a). There are many reports on the characteristics of two forms of trehalase. Southern blot analysis has determined that there are two trehalase genes in the DNA of insects and that *SeTreh-2* from *S. exigua* might be a single-copy gene (Tang et al. 2008). The protein structure of the gene encoding Treh2 from *B. mori* and alignment results clearly demonstrated that *BmTreh-2* is completely different from the gene encoding Treh1 (Su et al. 1993, 1994). In the current study, we found that *H. axyridis* contains two genes encoding trehalase. Although the degenerate primers used in the study were designed based on the conserved sequences of Treh1 and Treh2, the sequencing results showed that the genes both encode Treh1 (Fig. 2). Therefore, some insects are likely to contain more than two Treh1, and it is not clear whether all insects contain Treh2.

It is well known that insect trehalases share similar characteristics, including a signal peptide leader, a highly conserved glycine-rich (GGGGEY) region and two conserved 'signature motifs,' PGGRFREFYYWDSY and QWDYPNAWPP (Mitsumasu et al. 2005; Tang et al. 2008). The two trehalases of *H. axyridis* also have one signal peptide sequence and one highly conserved glycine-rich (GGGGEY) region (Fig. 2). In addition, HaTreh1-1 and HaTreh1-2 proteins also have two signature motifs, although they differ from those of the *B. mori* trehalase. The sequence of the two signature motifs of HaTreh1-1 are PGGRFKESYYWDSF and QWDFPMAWAP (Fig. 2a), where those of HaTreh1-2 are PGGRFREIYYWDSY and QWDLPNAWPP (Fig. 2b).

In *S. exigua*, the *SeTreh2* transcript was expressed in the fat body with a higher expression level in the larvae and pre-pupae, but none in pupae (Tang et al. 2008). In addition, *SeTreh1* and *SeTreh2* transcripts were expressed relatively stably throughout the body of *S. exigua*, with *SeTreh1* transcript showing high expression in 1-day-old pupae (Chen et al. 2010). However, the two *HaTreh1* transcript expression patterns differed from those of the *SeTreh1* transcript in that expression of the *SeTreh1* transcript was stable from the larval through to the pupal stage and was highly expressed during all of these stages (Chen et al. 2010). *HaTreh1-1* mRNA expression was higher in larvae but lower in pupae and adult beetles; *HaTreh1-2* mRNA expression was also higher in larvae and in pupae on the second day of the pupal stage, which was unexpected (Fig. 3). It is well known that degradation of the fat body and formation of adult organs occurs during the pupal stages and that these processes consume a lot of energy, Therefore, *HaTreh1-2* might be involved in degrading the trehalose to glucose and providing the energy for these processes. These results suggest that *Treh1* genes have different functions depending on species of insects and the different developmental stages.

Glucose can be converted to trehalose and glycogen for energy storage in insects, and both can be converted back to glucose to release the energy (Tang et al. 2010). It has been suggested that an important function of trehalase is to facilitate the uptake and utilization of trehalose from the blood (Azuma and Yamashita 1985a, b; Su et al. 1993, 1994). From studies of *BmTreh-2*, it was shown to be involved with incorporating trehalose from blood into muscular cells and then providing the energy required for visceral muscles to support the peristaltic movement of the midgut for active feeding (Azuma and Yamashita 1985a; Mitsumasu et al. 2005). To maintain normal physiological activities during periods of starvation, *H. axyridis* needs trehalose or other sources of energy for survival. Our results showed that the SS of adults starved for 4 h was lower than in those starved for 8 h; therefore, it might be

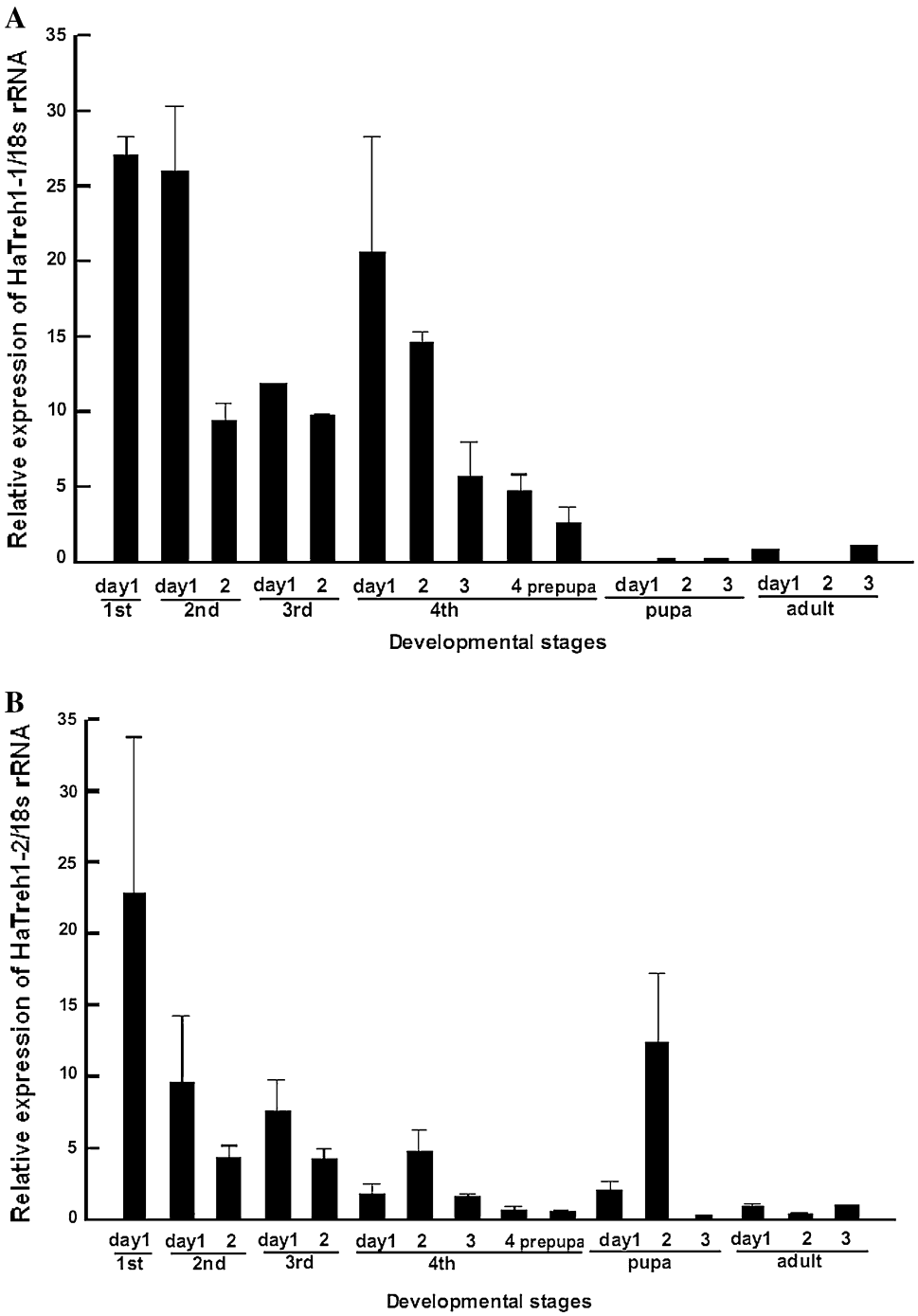

Fig. 3 Developmental profiles of the mRNAs of the two genes encoding trehalases from *Harmonia axyridis* (*HaTreh*) during the larva-pupa-adult transition. The expression levels of *HaTreh* mRNA relative to the 18srRNA expression level were measured with qRT-PCR. Each point represents the mean ± standard deviation from three independent experiments. **a** and **b** represent the developmental expression pattern of *HaTreh1-1* and *HaTreh1-2* cDNA, respectively

that *H. axyridis* adults take a longer time under starvation conditions. With increasing starvation, the SS decreased further, suggesting that the energy levels of the insects were also decreasing (Fig. 4a). This result was consistent with the changes in expression of *HaTPS*, *HaTreh1-1* and *HaTreh1-2* (Fig. 5). In other words, the expression of *HaTPS* decreased, whereas that of *HaTreh1-1* and *HaTreh1-2* from 4 to 24 h of starvation increased compared with their expression at 0 h starvation. In *S. exigua*, the mRNA expression of glycogen synthase and glycogen phosphorylase increased gradually after a short starvation period, reaching the same levels as recorded in adults before starvation began (Tang et al. 2012b); this suggests that trehalose and glycogen provide energy during periods of starvation. The results for the expression levels of *HaTPS* and *HaTrehs* in adults starved for 12–24 h showed that the *HaTPS* expression level was lower and that the *HaTrehs* expression level was higher than in those at 0–8 h, especially for *HaTreh1-1* (Fig. 4). However, the expression of *HaTPS* decreased during the initial starvation

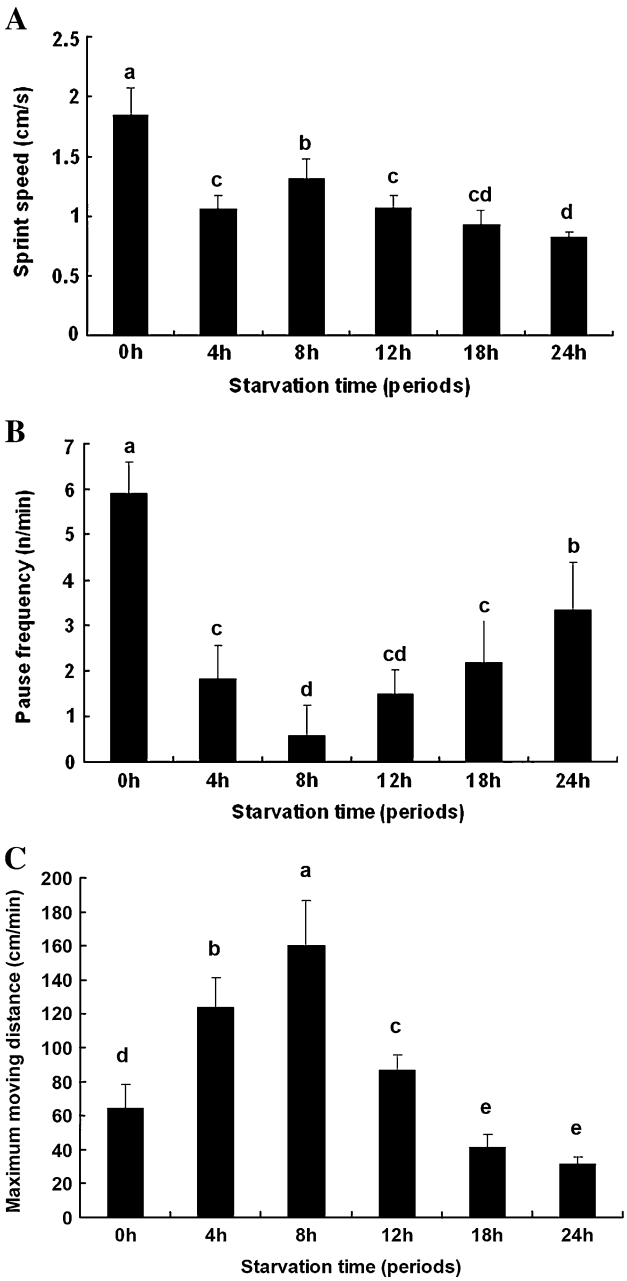

Fig. 4 The sprint speed (SS) (**a**), pause frequency (PF) (**b**) and maximum moving distance (MMD) (**c**) of *Harmonia axyridis* under different periods of starvation (0–24 h). All values in the figure are represented as mean ± standard deviation. Different small letters above the histograms indicate values that differ significantly between treatments ($P < 0.05$)

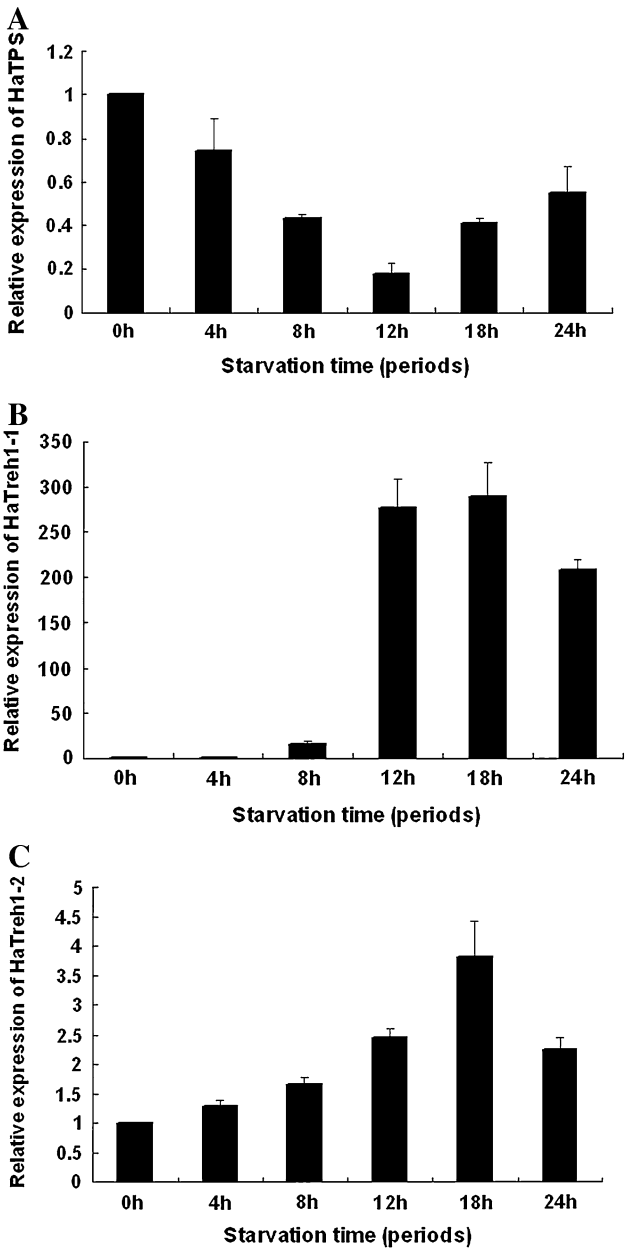

Fig. 5 The expression of *Harmonia axyridis* trehalose-6-phosphate synthase (*HaTPS*) and *H. axyridis* trehalase (*HaTreh1-1* and *HaTreh1-2*) mRNA under different starvation periods (0–24 h). The levels of these genes relative to the 18srRNA expression level were measured with qRT-PCR. Each point represents the mean ± standard deviation from three independent experiments with four individuals in each replicate. (**a**) The mRNA levels of the *HaTPS* gene. (**b**) The mRNA levels of the *HaTreh1-1* gene. (**c**) The mRNA levels of the *HaTreh1-2* gene

period and then increased in adults starved for 12–24 h, which suggests that accumulating levels of trehalose enable adult *H. axyridis* to survive periods of starvation.

The PF results showed control *H. axyridis* adults had a higher PF than those that were starved, but that adults starved for 8 h rarely stopped (Fig. 4b). This suggests that starvation drives the movement of insects and increases rapidly as the period of starvation increases, possibly because there is a lack of energy restrictions on movement as the animal attempts to search for food. Compared with PF, the trend in MMD was very different. The MMD in control adults was only slightly more than in those starved

for 18 or 24 h; adults starved for 8 h recorded the maximum MMD (Fig. 4c). Johnson et al. (2010) showed that a reduction in AMP kinase (AMPK) mRNA levels leads to hypersensitivity to starvation conditions, as measured by lifespan and locomotor activity; in addition, locomotor levels in flies with reduced AMPK function were lower during unstressed conditions, but starvation-induced hyperactivity, an adaptive response to encourage foraging, was significantly higher than in wild-type flies. This result also indicated that locomotory ability was associated with the desire to find food and that energy limitations also impact directly on the desire to find food. These results indicate that trehalose stores are degraded by trehalase, especially *HaTreh1-1*, especially given that trehalase functions to facilitate the uptake and utilization of trehalose from the food or blood (Friedman 1985; Ishihara et al. 1997; Oesterreicher et al. 1998, 2001; Sumida and Yamashita 1983; Yaginuma et al. 1996).

Adults of the invasive ground beetle *Merizodus soledadinus* (Guerin-Meneville). (Coleoptera, Carabidae) can go without food for more than 60 days and feed only every 3.76 days on average when food is available; the main reason for such behavior is that the beetles increased their hydrolyzation of sugars and triglycerides significantly during periods of food deprivation and restored their levels of such compounds after refeeding (Laparie et al. 2012). In the present study, the results showed that *HaTreh1-1* is a key gene in regulating energy metabolism and providing glucose and that *HaTreh1-2* and *HaTPS* might be balancing trehalose use during periods of starvation. This suggests that the *HaTreh* genes have a role in the regulation of insect function under starvation conditions.

Acknowledgments This work was supported by the National Basic Research Program of China (grant nos. 2012CB127605 and 2009CB119206), National Natural Science Foundation of China (grant nos. 31071731 and 31371996) and the Program for Excellent Young Teachers in Hangzhou Normal University (grant no. JTAS 2011-01-031).

References

Azuma M, Yamashita O (1985a) Cellular localization and proposed function of midgut trehalase in the silkworm larva, *Bombyx mori*. Tissue Cell 17:539–551

Azuma M, Yamashita O (1985b) Immunohistochemical and biochemical localization of trehalase in the developing ovaries of the silkworm, *Bombyx mori*. Insect Biochem Mol Biol 15:589–596

Becker A, Schloer P, Steel JE, Wegener G (1996) The regulation of trehalose metabolism in insects. Experientia 52:433–439

Chen J, Tang B, Chen HX, Yao Q, Huang XF, Chen J, Zhang DW, Zhang WQ (2010) Differential functions of the soluble and membrane-bound trehalase genes of a lepidopteran pest *Spodoptera exigua* for chitin biosynthesis revealed by RNA interference. PLoS One 5(4):e10133

Clegg JS, Evans DR (1961) Blood trehalose and flight metabolism in the blowfly. Science 134:54–55

Elbein AD (1974) The metabolism of α, α-trehalose. Adv Carbohydr Chem Biochem 30:227–256

Elbein AD, Pan YT, Pastuszak I, Carroll D (2003) New insights on trehalose: a multifunctional molecule. Glycobiology 13:17R–27R

Friedman S (1978) Trehalose regulation, one aspect of metabolic homeostasis. Annu Rev Entomol 23:389–407

Friedman S (1985) Carbohydrate metabolism. In: Kerkut GA, Gilbert LI (eds) Comparative insect physiology, biochemistry, and pharmacology, vol 10. Pergamon, Oxford, pp 43–76

Frison M, Parrou JL, Guillaumot D, Masquelier D, Francois J, Chaumont F, Batoko H (2007) The *Arabidopsis thaliana* trehalase is a plasma membrance-bound enzyme with extracellular activity. FEBS Lett 581(21):4010–4016

Gomez A, Cardoso C, Genta FA, Terra WR, Ferreira C (2013) Active site characterization and molecular cloning of Tenebrio molitor midgut trehalase and comments on their insect homologs. Insect Biochem Mol Biol 43(8):768–780

Ishihara R, Taketani S, Sasai-Takedatsu M, Kino M, Tokunaga R, Kobayashi Y (1997) Molecular cloning, sequencing and expression of cDNA encoding human trehalase. Gene 202:69–74

Johnson EC, Kazgan N, Bretz CA, Forsberg LJ, Hector CE, Worthen RJ, Onyenwoke R, Brenman JE (2010) Altered metabolism and persistent starvation behaviors caused by reduced AMPK function in *Drosophila*. PLoS One 5(9):e12799

Kamimura M, Takahashi M, Tomita S, Fujiwara H, Kiuchi M (1999) Expression of ecdysone receptor isoforms and trehalase in the anterior silk gland of *Bombyx mori* during an extra larval molt and precocious pupation induced by 20-hydroxyecdysone administration. Arch Insect Biochem Physiol 41:79–88

Kunieda T, Fujiyuki T, Kucharski R, Foret S, Ament SA, Toth AL, Ohashi K, Takeuchi H, Kamikouchi A, Kage E, Morioka M, Beye M, Kubo T, Robinson GE, Maleszka R (2006) Carbohydrate metabolism genes and pathways in insects: insights from the honey bee genome. Insect Mol Biol 15(5):563–576

Laparie M, Larvor V, Frenot Y, Renault D (2012) Starvation resistance and effects of diet on energy reserves in a predatory ground beetle (Merizodus soledadinus; Carabidae) invading the Kerguelen Islands. Comp Biochem Physiol A: Mol Integr Physiol 161(2):122–129

Lee JH, Saito S, Mori H, Nishimoto M, Okuyama M, Kim D, Wongchawalit J, Kimura A, Chiba S (2007) Molecular cloning of cDNA for trehalase from the European honeybee, *Apis mellifera* L., and its heterologous expression in *Pichia pastoris*. Biosci Biotechnol Biochem 71(9):2256–2265

Mitsumasu K, Azuma M, Niimi T, Yamashita O, Yaginuma T (2005) Membrane-penetrating trehalase from silkworm *Bombyx mori*. Molecular cloning and localization in larval midgut. Insect Mol Biol 14:501–508

Mitsumasu K, Azuma M, Niimi T, Yamashita O, Yaginuma T (2008) Changes in the expression of soluble and integral-membrane trehalases in the midgut during metamorphosis in *Bombyx mori*. Zoolog Sci 25(7):693–698

Mitsumasu K, Kanamori Y, Fujita M, Iwata K, Tanaka D, Kikuta S, Watanabe M, Cornette R, Okuda T, Kikawada T (2010) Enzymatic control of anhydrobiosis-related accumulation of trehalose in the sleeping chironomid, *Polypedilum vanderplanki*. FEBS J 277:4215–4228

Montooth KL, Marden JH, Clark AG (2003) Mapping determinants of variation in energy metabolism, respiration and flight in Drosophila. Genetics 165:623–635

Oesterreicher TJ, Nanthakumar NN, Winston JH, Henning SJ (1998) Rat trehalase: cDNA cloning and mRNA expression in adult rat tissues and during intestinal ontogeny. Am J Physiol 274:1220–1227

Oesterreicher TJ, Markesich DC, Henning SJ (2001) Cloning, characterization and mapping of the mouse trehalase (Treh) gene. Gene 270:211–220

Parkinson NM, Conyers CM, Keen JN, MacNicoll AD, Smith I, Weaver RJ (2003) cDNAs encoding large venom proteins from the parasitoid wasp *Pimpla hypochondriaca* identified by random sequence analysis. Comp Biochem Physiol 134C:513–520

Qin Z, Wang S, Wei P, Xu CD, Tang B, Zhang F (2012) Molecular cloning and expression in cold induction of trehalose-6-phosphate synthase gene in *Harmonia axyridis* (Pallas). Acta Entomologica Sinica 55(6):651–658

Rockstein M (ed) (1978) Biochemistry of insect. Academic Press New York, San Francisco, London

Ruf J, Wacker H, James P, Maffia M, Seiler P, Galand G, von Kieckebusch A, Semenza G, Matei N (1990) Rabbit small intestinal trehalase. Purification, cDNA cloning, expression, and verification of glycosylphosphatidylinositol anchoring. J Biol Chem 265(25):15034–15039

Silva CPM, Terra RW, Ferreira C (2004) The role of carboxyl, guanidine and imidazole groups in catalysis by a midgut trehalase purified from an insect larvae. Insect Biochem Mol Biol 34:1089–1099

Su ZH, Sato Y, Yamashita O (1993) Purification, cDNA cloning and Northern blot analysis of trehalase of pupae midgut of the silkworm, *Bombyx mori*. Biochim Biophys Acta 1173:217–224

Su ZH, Ikeda M, Sato Y, Saito H, Imai K, Isobe M, Yamashita O (1994) Molecular characterization of ovary trehalase of the silkworm, *Bombyx mori* and its transcriptional activation by diapause hormone. Biochim Biophys Acta 1218:366–374

Su ZH, Itani Y, Yamashita O (1997) Structure of trehalase gene of the silkworm, *Bombyx mori* and phylogenic relationship of trelalases. Nihon Sanshigaku Zasshi 66:457–465

Sumida M, Yamashita O (1983) Purification and some properties of soluble trehalase from midgut of pharate adult of the silkworm, *Bombyx mori*. Insect Biochem Mol Biol 13:257–265

Takiguchi M, Niimi T, Su ZH, Yaginuma T (1992) Trehalase from male accessory gland of an insect, *Tenebrio molitor*. cDNA sequencing and developmental profile of the gene expression. Biochem J 288:19–22

Tang B, Chen XF, Liu Y, Tian HG, Liu J, Hu J, Xu WH, Zhang WQ (2008) Characterization and expression patterns of a membrane-bound trehalase from *Spodoptera exigua*. BMC Mol Biol 9:51

Tang B, Chen J, Yao Q, Pan ZQ, Xu WH, Wang SG, Zhang WQ (2010) Characterization of a trehalose-6-phosphate synthase gene from *Spodoptera exigu*a and its function identification through RNA interference. J Insect Physiol 56(7):813–821

Tang B, Wei P, Chen J, Wang SG, Zhang WQ (2012a) Progress in gene features and functions of insect trehalases. Acta Entomologica Sinica 55(11):1315–1321

Tang B, Xu Q, Zou Q, Fang Q, Wang SG, Ye GY (2012b) Sequencing and characterization of glycogen synthase and glycogen phosphorylase genes from *Spodoptera exigua* and analysis of their function in starvation and excessive sugar intake. Arch Insect Biochem Physiol 80(1):42–62

Tatun N, Singtripop T, Tungjitwitayakul J, Sakurai S (2008a) Regulation of soluble and membrane-bound trehalase activity and expression of the enzyme in the larval midgut of the bamboo borer *Omphisa fuscidentalis*. Insect Biochem Mol Biol 38:788–795

Tatun N, Singtripop T, Sakurai S (2008b) Dual control of midgut trehalase activity by 20-hydroxyecdysone and an inhibitory factor in the bamboo borer *Omhisa fuscidentalis* Hampson. J Insect Physiol 54:351–357

Terra WR, Ferreira C (1994) Insect digestive enzymes: properties, compartmentalization and function. Comp Biochem Physiol 109B:1–62

Thompson SN (2003) Trehalose—the insect 'blood' sugar. Adv Insect Physiol 31:203–285

Van der Horst DJ, van Doorn JM, Beenakkers AMTh (1978) Dynamics in the haemolymph trehalose pool during flight of the locust, *Locusta migratoria*. Insect Biochem Mol Biol 8(6):413–416

Wingler A (2002) The function of trehalose biosynthesis in plants. Phytochemistry 60:437–440

Wyatt GR (1967) The biochemistry of sugars and polysaccharides in insects. Adv Insect Physiol 4:287–360

Yaginuma T, Mizuno T, Mizuno C, Ikeda M, Wada T, Hattori K, Yamashita O, Happ GM (1996) Trehalase in the spermatophore from the bean-shaped accessory gland of the male mealworm beetle, *Tenebrio molitor*: purification, kinetic properties and localization of the enzyme. J Comp Physiol Part B Biochem Syst Environ 166:1–10