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Two novel soluble trehalase genes cloned from *Harmonia axyridis* and regulation of the enzyme in a rapid changing temperature

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ABSTRACT

In previous studies, we have cloned two soluble trehalase genes (*HaTreh1–1* and *HaTreh1–2*) from the harlequin ladybird *Harmonia axyridis*. Here, we obtained the other two novel genes (*HaTreh1–3* and *HaTreh1–4*) by transcriptome sequencing and rapid amplification of cDNA ends. Generally, anabolism enhancement and catabolism inhibition together contribute to accumulation of trehalose, and trehalase is the key enzyme to start the catabolism of trehalose. To characterize the metabolism of trehalose in *H. axyridis* and how these trehalase genes are regulated under cold stress conditions, a comparison of trehalose content and trehalase levels in two different rapidly changing temperature environments was carried out to explore the regulation of these genes. We found that an accumulation of trehalose could be observed at 5 °C, 0 °C and −5 °C and trehalase was suppressed in these temperature points during a gradually cooling environment. Then, in a gradually warming environment, trehalose levels increased slightly from −5 °C to 15 °C and then decreased at 25 °C; however, no significant negative association was observed between trehalase and trehalose. Additionally, we found that glycogen could be converted into trehalose to help the individual resist the low temperature. Analysis of the expression of soluble trehalase showed that *HaTreh1–1*, *HaTreh1–2*, *HaTreh1–3* and *HaTreh1–4* were involved in trehalose metabolism; but the gene *HaTreh1–4* plays the most important role in the cooling process, and *HaTreh1–2* and *HaTreh1–4* play the most important role in the warming process. Finally, we found that 5 °C might be a temperature signal for *H. axyridis*; prior to this temperature, individuals must make enough physical preparations to resist cold stress during the winter.

1. Introduction

The harlequin ladybird *Harmonia axyridis* (Coleoptera: Coccinellidae), which is native to a large part of Asia, was introduced on several occasions during the last century into both North America and Europe (Iperti and Bertand, 2001; Koch, 2003; Gordon, 1985). This beetle is an important biological control agent of aphids and coccid pests in Asia, but is notorious for invasiveness in Europe (Adriaens et al., 2008; Brown et al., 2008a, 2008b; Koch, 2003). The cold hardiness of the harlequin ladybird *H. axyridis* represents an important characteristic that has been widely studied in both biological control applications and aspects of invasiveness (Bazzocchi et al., 2004; Berkvens et al., 2010; Pervez and Omkar, 2006; van Lenteren et al., 2008). To date, these studies have focused on physiological adaptations and winter survival of overwintering populations or other field populations (Iperti and Bertand, 2001; Watanabe, 2002; Zhao et al., 2008). However, the molecular mechanisms involved remain poorly understood.

Trehalose (α-D-glucopyranosyl-α-D-glucopyranoside) is found in organisms as diverse as bacteria, yeast, fungi, nematodes, plants, insects, and some other invertebrates, but is absent in mammals (Elbein et al., 2003; Tang et al., 2008; Wingler, 2002). In these organisms, trehalose may act as a source of energy, as a carbohydrate depot, or as an agent for protecting proteins and cellular membranes upon exposure to environmental extremes, such as desiccation, dehydration, heat, freezing, or oxidation (Crowe et al., 1984; Elbein et al., 2003; Garg et al., 2002). In insects, trehalose is the main blood sugar, which has an important role in physiological adaptation to the environment (Bin et al., 2012; Thompson, 2003; Wyatt, 1961). Notably, an extremely high concentration of trehalose has been detected during the winter (Bale and Hayward, 2010; Khani et al., 2007; Shimada, 1984).

Trehalase (Treh) (EC 3.2.1.28) is an enzyme that catalyzes 1 mol trehalose → 2 mol glucose. Changes in trehalase activity are closely related to shifts in physiological conditions and developmental events; thus, trehalase is deeply involved in both homeostasis and development (Chen et al., 2010a; Silva et al., 2004; Terra and Ferreira, 1994). Trehalase has been cloned, characterized, and purified from many insect species, and each of these enzymes has both membrane-bound and soluble forms (Chen et al., 2010a; Tang et al., 2008; Tatun et al., 2008; Terra and Ferreira, 1994; Thompson, 2003). *H. axyridis*, as a kind of natural enemy, the research mainly focused on biological control application, such as: how to be more effective save and prolong this species lifetime

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using the method of low temperature storage (Chen et al., 2008; Teng and Xu, 2005; Wang and Shen, 2002). However, limited data about the molecular mechanism of the low temperature storage, especially, trehalase genes or proteins from *H. axyridis* have been reported.

Previously, it has been reported that two soluble trehalase genes can be found in *H. axyridis* (Tang et al., 2014). Subsequently, we identified other soluble trehalase sequence segments during transcriptome sequencing of *H. axyridis*. In this present report, we cloned the full-length cDNA of these new soluble trehalase genes from *H. axyridis*. When faced with a cold environment, trehalose catabolism should be suppressed, help the ladybird to accumulate trehalose in order to resist low temperature injury. Therefore, to clarify how do these trehalase genes regulate the trehalose metabolism of *H. axyridis* during cold exposure, we examined changes in enzymatic activity and measured trehalose and glycogen content after two different cold-induction regimens. Molecular regulation of the enzyme was also examined at the mRNA level by tracking changes in expression of the soluble trehalase genes.

2. Materials and methods

2.1. Experimental animals

H. axyridis were established in our laboratory using insects collected from the Lab of Natural Enemy Research, Beijing Academy of Agriculture and Forestry Science. Non-melanic and melanic populations were each set up and maintained at 25 °C, 70% relative humidity, and a 16:8 h (light:dark) photoperiod. Insects were fed *Aphis medicaginis*. The developmental stages were synchronized at each molt by collecting new larvae, pupae, or adults. All of the abdominal tissues from different developmental stages were dissected in insect saline containing 0.75% NaCl and stored at −80 °C until required. For all cold treatments, 7-day-old adults of the non-melanic group were used.

2.2. Cold shock

Lee (1991) found that the rapid cooling was useful for enhancing cold-hardiness in arthropods, such as: gradual cooling over a range of temperatures. Furthermore, the coming of winter or spring is a process of gradual cooling or warming, so we design a series of different temperatures and test much measure responses about trehalose across temperature transients. A total of six temperatures were tested: 25 °C, 15 °C, 10 °C, 5 °C, 0 °C, and −5 °C. Individual *H. axyridis* were placed into one of the following rapid changing temperature environments: (i) from 25 °C to −5 °C, or (ii) from −5 °C to 25 °C. Specifically, treatment (i): hundreds of individuals were placed in plastic fruit fly tubes sealed with a sponge (ten individuals per tube), and tubes were then maintained at 25 °C, then tubes were cooled rapidly to 15 °C after 2 h exposure at 25 °C, and then cooled to 10 °C after 2 h exposure to 15 °C, and finally were cooled to −5 °C; treatment (ii) involved a similar procedure, but the starting temperature was set at −5 °C. A total of 100 ladybird adults were cultured at 25 °C without any cold stimulation as control before treatment (ii). We randomly sampled experimental animals in every temperature point and three pieces of abdominal tissues as a repeat were analyzed at each sampling point. The above treatments repeated three times. The soluble trehalase activities, trehalose and glycogen contents, and gene expression levels were measured at each temperature.

2.3. Measurements of trehalose content

From adults, three pieces of abdominal tissue were placed in a 1.5 ml Eppendorf tube. After adding 200 μl 20 mM phosphate buffered saline (PBS, pH 6.0), tissues were homogenized at 0 °C (TGrinder OSE-Y20 homogenizer, Tiangen Biotech Co., Beijing, China), and followed by sonication for 30 s (VCX 130PB, Sonics, Connecticut, USA). Homogenates were centrifuged at 12,000 ×*g* at 4 °C for 10 min after adding 800 μl PBS. Precipitates were removed and aliquots of supernatant were assayed to determine the amount of protein content using a protein–dye binding method (Bio-Rad, Hangzhou, China) with bovine serum albumin as the standard. Then, 500 μl supernatant was added to a 1.5 ml tube and then boiled, after which the solution was centrifuged at 12,000 ×*g* for 10 min to remove any residual protein. Supernatants were processed for the measurement of trehalose. Trehalose content was estimated using a modified version of a protocol that was described previously (Ge et al., 2011). A total of 50 μl supernatant was put into a 1.5 ml tube, 50 μl 1% H_2SO_4 was added, and the tube was incubated in 90 °C water for 10 min to hydrolyze glycogen, after which it was cooled on ice for 3 min. Then, the supernatant was again incubated in 90 °C water for 10 min after the addition of 50 μl 30% potassium hydroxide solution to decompose glucose. Now, the supernatant only contained trehalose without other carbohydrates or proteins. Next, four volumes of 0.2% (M/V) anthrone (Sigma, Shanghai, China) in 80% H_2SO_4 solution were added after it was cooled on ice for 3 min, and the supernatant was boiled for 10 min. After cooling, 200 μl reaction solution was placed into a 96-well plate and the absorbance at 620 nm was determined using a SpectraMax M5 (Molecular Device, California, USA). Trehalose content was calculated based on a standard curve and compared how many trehalose under per gram of total protein. Finally, the result was expressed as mg trehalose per g total protein.

2.4. Measurements of glycogen content

From adults, three pieces of abdominal tissues were placed in a 1.5 ml Eppendorf tube. After adding 200 μl 20 mM phosphate buffered saline (PBS, pH 6.0), tissues were homogenized at 0 °C (TGrinder OSE-Y20 homogenizer, Tiangen Biotech Co., Beijing, China), and followed by sonication for 30 s (VCX 130PB, Sonics, Connecticut, USA). Homogenates were centrifuged at 12,000 ×*g* at 4 °C for 10 min after adding 800 μl PBS. Precipitates were removed and aliquots of supernatant were assayed to determine the amount of protein content using a protein–dye binding method (Bio-Rad, Hangzhou, China) with bovine serum albumin as the standard. Then, remainder supernatant was added to a 1.5 ml tube and then boiled, after which the solution was centrifuged at 12,000 ×*g* for 10 min to remove any residual protein. Then, supernatants were directly subjected to a glycogen content assay. Glycogen content was measured as described by Santos et al. (2008). Supernatant (100 μl) was incubated for 4 h at 37 °C in the presence of 20 μl (1 U) amyloglucosidase (EC 3.2.1.3, Sigma) diluted in 100 mM sodium acetate (pH 5.5) to hydrolyze glycogen. The amount of glucose generated from glycogen was determined using a Glucose Assay Kit (GAGO20-1KT, Sigma) following the manufacturer's instructions. Controls were prepared in the absence of enzyme, and the amount of glycogen was calculated by follows: total glucose minus endogenous glucose, then divided by total protein. Finally, the result was expressed as mg glucose per g total protein.

2.5. Trehalase activity assay

To determine soluble trehalase activity, a previously described method was used (Tatun et al., 2008). Three abdominal tissues of adults in a 1.5 ml tube were homogenized at 0 °C (TGrinder OSE-Y20 homogenizer, Tiangen) after adding 200 μl of 20 mM phosphate buffered saline (PBS, pH 6.0), followed by sonication for 30 s (VCX 130 PB, Sonics). The homogenates were centrifuged at 1000 ×*g* at 4 °C for 10 min after adding 800 μl PBS, and the cuticle debris was removed and centrifuged at 105,000 ×*g* at 4 °C for 60 min (CP100MX, Hitachi, Tokyo, Japan). The supernatant was directly used to measure the activity of soluble trehalase. The amount of protein in each sample was determined prior to the trehalase assay using a protein–dye binding method (Bio-Rad) with bovine serum albumin as a standard. For the trehalase activity assay, the reaction mixture (250 μl) consisted of 62.5 μl 40 mM trehalose (Sigma) in 20 mM PBS (pH 6.0), 50 μl soluble trehalase fraction, and

137.5 μl PBS. The mixture was incubated at 37 °C for 30 min, and the reaction was stopped by heating in boiling water for 5 min. Coagulated protein was removed by centrifugation at 12,000 ×*g* at 4 °C for 10 min, and an aliquot of the resulting supernatant was used to measure the amount of glucose using a Glucose Assay Kit (GAGO20-1KT, Sigma) following the manufacturer's instructions; data were expressed as mg glucose/g protein/min.

2.6. Cloning of two novel trehalase genes

Total RNA was isolated from *H. axyridis* using TRIzol reagent (Invitrogen, Shanghai, China). Synthesis of first-strand cDNA was carried out using a PrimeScript RT® with gDNA Eraser kit (TaKaRa, Dalian, China). The full-length cDNA of *HaTreh1–4* and the partial sequence of *HaTreh1–3* had been obtained by transcriptome sequencing of *H. axyridis* (Hiseq 2000, Illumina, Beijing, China). The full-length cDNA for *HaTreh1–3* was obtained using the rapid amplification of cDNA ends (RACE) method using the SMART™ RACE cDNA Amplification Kit (Clontech, Dalian, China). For *HaTreh1–3*, two reverse primers, 5-Treh1-3R1 (5′-GGGACTGTACAGTGTGGAGCA-3′) and 5-Treh1-3R2 (5′-CTGGTAATATGGAGATTGGACTG-3′), were used for 5′-RACE, and two forward primers, 3-Treh1-3F1 (5′-GTGTTGACTGATGTTCTTGTTGC-3′) and 3-Treh1-3F2 (5′-CCTTCGAGGCATGGGACTTCAC-3′), were used for 3′-RACE.

PCR products were separated by electrophoresis on 1.0% agarose gels, and cDNA fragments of interest were purified using a DNA gel extraction kit (OMEGA, Hangzhou, China). Purified DNA was ligated into the pMD18-T vector (TaKaRa) and sequenced by the dideoxynucleotide method.

2.7. Sequence analysis

The nucleic acid sequence of *H. axyridis* in the FASTA format was used to query the sequence database of the National Center for Biotechnology Information (NCBI) to identify proteins with primary sequence similarity to *HaTreh1–3* and *HaTreh1–4*. A multiple sequence alignment of *HaTreh1–3*, *HaTreh1–4*, and other trehalases was constructed using ClustalW (Julie et al., 2002). A phylogenetic tree was constructed using the neighbor-joining (NJ) method with the Mega 5.2 software (Naruya and Masatoshi, 1987). *Escherichia coli* periplasmic trehalase (TrehA) (Tanaka et al., 1999) was used as an out-group, and the stability of the tree was assessed via bootstrapping with over 1000 replicates.

2.8. Quantitative real-time PCR

Total RNA was isolated from *H. axyridis* adults after cold induction and 1 μg total RNA was used for synthesis of first-strand cDNA using the method described above. The expression levels of all known soluble trehalase genes from *H. axyridis*, including *HaTreh1–1*, *HaTreh1–2*, *HaTreh1–3*, and *HaTreh1–4*, were estimated by real-time PCR using a Bio-Rad CFX96™ system (Bio-Rad) and SsoFastTM EvaGreen® Supermix (Bio-Rad). Then, real-time PCR was performed in a 20 μl total reaction volume containing 1 μl cDNA sample, 1 μl (10 μmol/μl) of each primer, 7 μl RNase-free and DNase-free water, and 10 μl SsoFastTM EvaGreen® Supermix. Primers were replaced with H_2O as negative controls and *Harp49* (*H. axyridis ribosomal protein 49 gene*, AB552923) was used as an endogenous control. The primers were as follows: *Harp49-qF* (5′-GCGATCGCTATGGAAAACTC-3′) and *Harp49-qR* (5′-TACGATTTTGCATCAACAGT-3′) (Osanai-Futahashi et al., 2012). Primers for the four soluble trehalase genes of *H. axyridis* were designed to target their own unique regions and the annealing temperature for each pair of primers is shown (Table 1). The target amplification efficiency was the same as that of the reference amplification for each annealing temperature. The cycling parameters were 95 °C for 3 min for initial denaturation, followed by 40 cycles at 95 °C for 10 s, 56 °C–62.5 °C for 30 s; melting curve analysis at 65 °C–95 °C was performed to ensure that only a single product was amplified. Data were analyzed using a relative quantitative method ($\Delta\Delta C_t$) (Livak and Schmittgen, 2001).

Table 1
Primers sequences used for real-time PCR.

Primer name	Sequence (5′–3′)	Nucleotide nos.	T_m (°C)
HaTreh1–1qF	CTTCGCCAGTCAAATCGTCA	369–388	59.5
HaTreh1–1qR	CCGTTTGGGACATTCCAGATA	548–568	
HaTreh1–2qF	TGACAACTTCCAACCTGGTAATG	267–289	56
HaTreh1–2qR	TTCCTTCGAGACATCTGGCTTA	417–438	
HaTreh1–3qF	ACAGTCCCTCAGAATCTATCGTCA	120–143	59.5
HaTreh1–3qR	GGAGCCAAGTCTCAAGCTCATC	286–307	
HaTreh1–4qF	TTACTGCCAGTTTGATGACCATT	371–393	62.5
HaTreh1–4qR	CATTTCGCTAATCAGAAGACCCT	536–558	

Abbreviations: Ha, *Harmonia axyridis*; Treh, Trehalase. Tm means the annealing temperature. F and R represent forward and reverse primer, respectively.

2.9. Statistical analysis

Each temperature point has three repeats. Data were evaluated for normality and homogeneity of variance. Trehalose content, glycogen content, and trehalase activity in cooling or heating process were all analyzed using one-way analysis of variance (ANOVA) with Statistica 7.0 software (Oklahoma, USA) and multiple comparisons of means were conducted using Tukey's test. The experiment and control in heating treatment were analyzed using ANOVA and multiple comparison. Differences between means were deemed to be significant when $P \le 0.05$.

3. Results

3.1. Molecular cloning of HaTreh1–3 and HaTreh1–4

Two novel trehalase genes were identified in the *H. axyridis* transcriptome (Hiseq 2000, Illumina): one was a full length cDNA and another was a partial of cDNA fragment. To identify both genes, we performed 5′- and 3′-RACE using two primer sets. Then, using TMHMM Server v.2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/), we found that no transmembrane regions existed in these genes, suggesting that both genes possibly encode soluble trehalase (Treh1). Additionally, two soluble trehalase genes—*HaTre1–1* (HM056038) and *HaTreh1–2* (FJ501961)—had been published previously (Tang et al., 2014). Thus, these two genes were named *HaTreh1–3* (JX514372) and *HaTreh1–4* (KP318742), respectively. The whole *HaTreh1–3* cDNA consisted of 2336-bp, including a 1644-bp open reading frame (ORF) encoding a 547 amino acid (aa) with an estimated molecular mass of 65.02 kDa and a pI of 8.88 (Supplementary file 1A). The deduced amino acid sequence indicated that aa 1–20 comprise a signal peptide, whereas aa 162–175 correspond to trehalase signatures 1 (PS00927; Henrissat and Bairoch, 1993). The protein also contained four potential *N*-glycosylation sites (Asn-X-Thr/Ser). The entire *HaTreh1–4* gene consisted of 2084-bp, including an ORF of 1677-bp and a 558 predicted aa protein with an estimated molecular mass of ~64.93 kDa and a pI of 6.32 (Supplementary file 1B). The deduced protein indicated that aa 1–21 comprise a signal peptide, whereas aa 162–175 and 464–473 correspond to trehalase signatures 1 and 2, respectively (PS00927 and PS00928; Henrissat and Bairoch, 1993). Five potential N-glycosylation sites were also identified in the sequence.

The primary sequences encoded by *HaTreh1–3* and *HaTreh1–4* were then compared with those of other soluble trehalase genes (Supplementary file 2). *HaTreh1–3* and *HaTreh1–4* shared 40% identity. *HaTreh1–3* had a relatively low identity compared with other known trehalase family members and was mostly similar to the trehalase encoded by the genome of *Tenebrio molitor* (40%; AGO32658). It only showed 34% and 35% identity compared with *HaTre1–1* and *HaTreh1–2*, respectively; lower homology was detected with *Omphisa*

fuscidentalis (32%; EF426724). Similarly, *HaTreh1–4* showed high homology with *T. molitor* (51%; AGO32658) and *Tribolium castaneum* (52%; XP 973919), moderate homology with *HaTre1–1* (45%; HM056038) and *HaTreh1–2* (46%; FJ501961), and relatively low homology with *Bombyx mori* (42%; D86212). Base on their strong sequence identities with known soluble trehalase genes (*Treh1*), we deduced that the cDNA sequences of *Treh1–3* and *Treh1–4* both encoded a soluble trehalase of *H. axyridis*.

3.2. Phylogenetic analysis

Phylogenetic analyses showed that *HaTreh1–3* and *HaTreh1–4* were assigned into same subgroup (Fig. 1). Thus, all trehalases identified from insects so far have been classified as either a soluble or a membrane-bound trehalase with a transmembrane domain near the C-terminus. Currently, we have identified a total of four trehalases in *H. axyridis*—*Hatreh1–1*, *HaTreh1–2*, *HaTreh1–3*, *HaTreh1–4*—but they all belong to the soluble trehalase cluster and form a well-supported common clade (99% bootstrap value) that is associated with *TmTreh* and *TcTreh1*.

3.3. Developmental expression pattern of HaTreh1–3 and HaTreh1–4

The expression patterns of the two genes during *H. axyridis* development from day 1 of the fourth instar larva through pupa stages to adults were determined by quantitative real-time PCR. The *HaTreh1–3* and *HaTreh1–4* genes were continuously expressed from the fourth instar to the day 3 of adult (Fig. 2). The expression of *HaTreh1–3* increased steadily from the end of fourth instar, until day 2 of adult, and then decreased at day 3 of adult. *HaTreh1–4* showed higher expression in pupa and adults (except for day 2 pupa) than in larva, and the expression peaked at day 1 of adult, then, gradually declined at day 2 and day 3 of adult. The different temporal expression patterns suggested distinct physiological roles for the two genes, such as: regulation of chitin metabolism, which relates to insect molting, and energy metabolism.

3.4. Effects of low temperature on trehalose and glycogen content

In order to ensure the role of trehalose in *H. axyridis* and whether happen conversion between energy materials when facing a environmental extreme. The comparison of trehalose content and glycogen content in the two different rapidly changing temperature environments was respectively performed. In the environment cooled from 25 °C to −5 °C, an increase in the absolute concentration of trehalose occurred at 5 °C, 0 °C, and −5 °C in *H. axyridis* ($F_{5,\ 12} = 35.604$, $P < 0.001$) (Fig. 3A). By contrast, we found opposite trends for glycogen content in this process ($F_{5,12} = 196.028$, $P < 0.001$) (Fig. 3B). These findings indicted that glycogen might be converted into trehalose when exposed to a low temperature environment. In the environment heated from −5 °C to 25 °C, the highest level of trehalose content, 145.8 ± 21.3 μg trehalose/mg protein, was observed after exposure for 2 h at −5 °C (Fig. 3C), which was significantly higher than the content in the control group (98.2 ± 11.3 mg trehalose/g protein) ($F_{6,\ 14} = 49.14$, $P < 0.001$). From 0 °C to 15 °C, the level of trehalose was almost as high as that in the control group (CK) and decreased to a low level (43.0 ± 7.3 mg trehalose/g protein) at 25 °C (Fig. 3C). Similarly, changes of glycogen content were opposite to those observed for trehalose ($F_{6,\ 14} = 93.396$, $P < 0.001$) (Fig. 3D). However, there was no glucose be observed at each temperature in both treatment groups.

3.5. Effects of low temperature on soluble trehalase activity

The activity of soluble trehalase was measured, and we found significant differences between the cooling and warming environments. In the environment cooled from 25 °C to −5 °C, there was a positive correlation between the changes in soluble trehalase activity and temperature. Specifically, enzyme activity decreased following the reduction in temperature, but significant differences were only found after 2 h exposure to −5 °C in the cooling treatment ($F_{5,\ 12} = 5.631$, $P = 0.0067$) (Fig. 4A). In the environment warmed from −5 °C to 25 °C, enzyme activity was significantly lower than that of the control group (CK) at −5 °C, and the highest activity was observed at 0 °C ($F_{6,\ 14} = 43.559$, $P < 0.001$) (Fig. 4B). From 0 °C to 15 °C, the activities of trehalase showed a negative correlation with temperature, but then were enhanced at 25 °C (Fig. 4B).

3.6. Effects of low temperature on the expression levels of four soluble trehalase genes

The mRNA levels of soluble trehalase genes identified from *H. axyridis*, including *HaTreh1–1*, *HaTreh1–2*, *HaTreh1–3*, and *HaTreh1–*

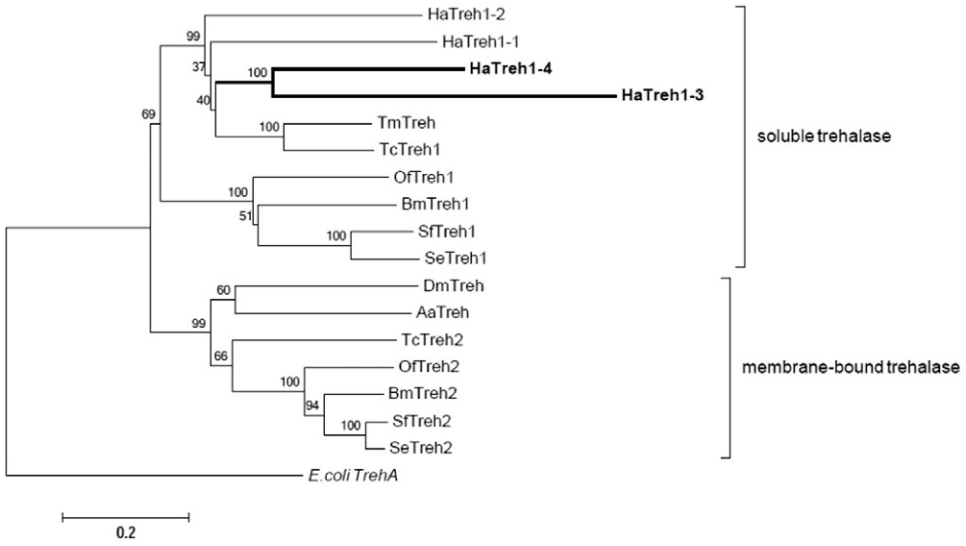

Fig. 1. Phylogenetic tree showing arthropod trehalase, constructed by the neighbor-joining method. Bootstrap values for 1000 trials are indicated at each node. Scale bar = 0.2 PAM. Abbreviations: Ha, *Harmonia axyridis* (HM056038 for *Treh1–1*, FJ501961 for *Treh1–2*, JX514372 for *Treh1–3*, and KP318742 for *Treh1–4*); Tm, *Tenebrio molitor* (AGO32658); Sf, *Spodoptera frugiperda* (DQ447188 for *Treh1* and EU872435 for *Treh2*); Se, *Spodoptera exigua* (EU427311 for *Treh1* and EU106080 for *Treh2*); Dm, *Drosophila melanogaster* (Q9W2M2); Aa, *Aedes aegypti* (Q16V81); Tc, *Tribolium castaneum* (XP 973919 for *Treh1* and XM_967517 for *Treh2*); Of, *Omphisa fuscidentalis* (EF426724 for *Treh1* and EF426723 for *Treh2*); Bm, *Bombyx mori* (D86212 for *Treh1* and NM_001043445 for *Treh2*).

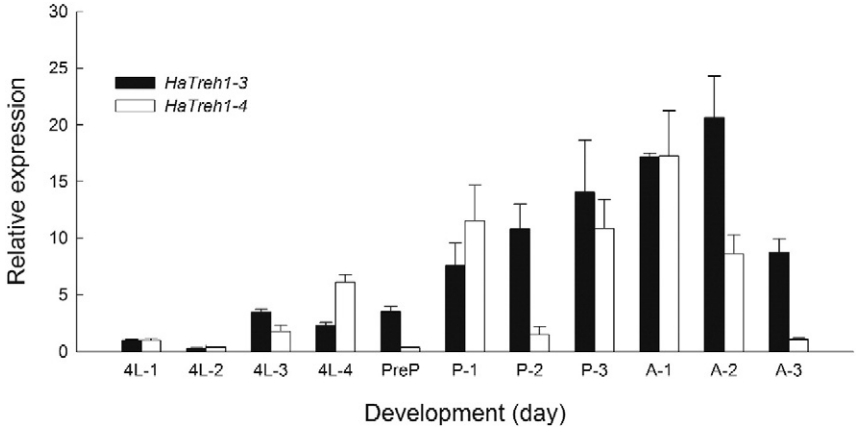

Fig. 2. Developmental changes in *HaTreh1–3* and *HaTreh1–4* mRNA levels from day 1 of the fourth instar larva to the adult stage. Expression levels of both *HaTreh1–3* and *HaTreh1–4* mRNA relative to those of *Harp49* (*H. axyridis* ribosomal protein 49 gene) were measured by real-time PCR. Abbreviations: L, larvae; PreP, prepupal; P, pupae; and A, adult. The letter and preceding number represent the age of the beetle, and the number after each letter represents days, such as 4L-1: the 1st day of the fourth instar larva. Data are presented as means ± s.d. (n = 3).

4, were calculated both in cooling and warming environments. Complex expression patterns were observed under cooling (treatment i) conditions (Fig. 5). Levels of the four genes were all reduced at 15 °C, 10 °C, and 5 °C, but only *HaTreh1–1* was relatively enhanced at 10 °C and 5 °C (Fig. 5A). Levels of *HaTreh1–1*, *HaTreh1–2* and *HaTreh1–3* were high (Fig. 5A, B, C), but *HaTreh1–4* mRNA expression was low at 0 °C (Fig. 5D). The mRNA expression levels of *HaTreh1–1* and *HaTreh1–2* increased (Fig. 5A, B), whereas those of *HaTreh1–3* and *HaTreh1–4* were silenced at −5 °C (Fig. 5C, D). By contrast, expression levels of the enzyme showed more consistent trends under warming (treatment ii) conditions (Fig. 6). When the temperature was increased, the expression levels of the four genes decreased and only *HaTreh1–2* was enhanced at 25 °C (Fig. 6A, B, C, D). The gene expression levels were lower than CK group, excluding *HaTreh1–2*, after exposure for

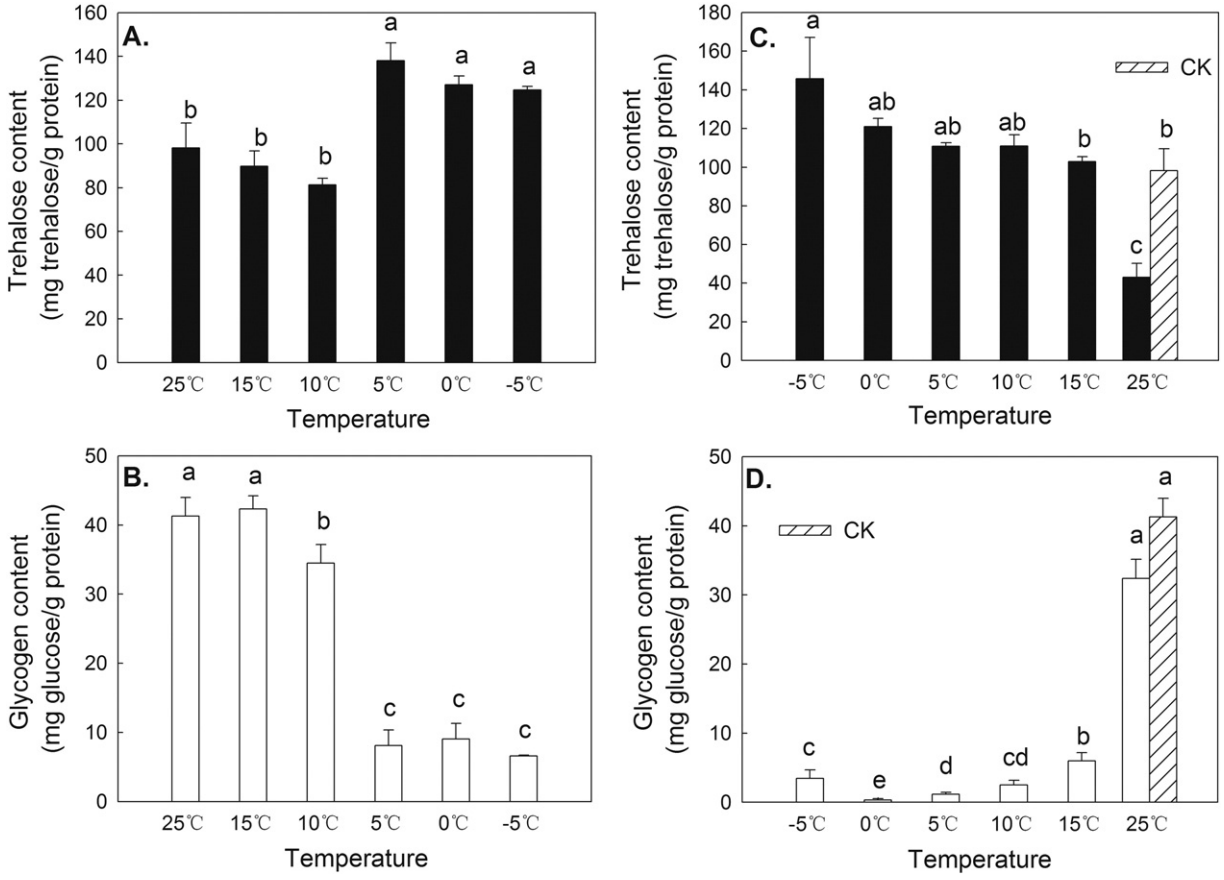

Fig. 3. Changes in trehalose and glycogen content during cooling and warming. Trehalose content was examined in (A) an environment that was gradually cooled from 25 °C to −5 °C and (B) an environment that was gradually warmed from −5 °C to 25 °C. Glycogen content was determined during both the (C) cooling and (D) warming processes. During the warming process, CK represents the control group, which included adults that were reared at an optimum temperature (25 °C) without any cold stimulation. Bars with different letters indicate significant differences ($P < 0.05$). Data are presented as means ± s.d. (n = 3).

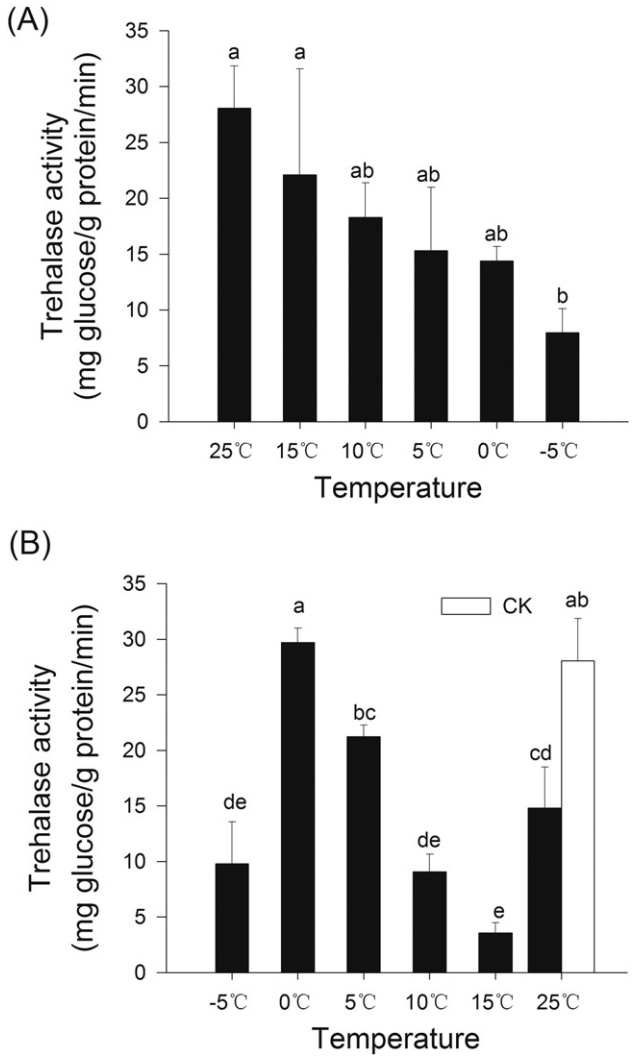

Fig. 4. Trehalase activities in *H. axyridis* during cooling (A) and warming (B) conditions. Trehalase activities were examined in (A) an environment that was gradually cooled from 25 °C to −5 °C and (B) an environment that was gradually warmed from −5 °C to 25 °C. During the warning process, the control group (CK) represents adults reared at an optimum temperature (25 °C) without any cold stimulation. Bars with different letters indicate significant differences ($P < 0.05$). Data are presented as means ± s.d. ($n = 3$).

2 h at 25 °C (Fig. 6B). However, *HaTreh1–4* consistently exhibited a lower expression level than CK under warming conditions from −5 °C to 25 °C (Fig. 6D).

4. Discussion

The gene cloning, protein purification and trehalase activity studies showed that there are two different genes that encode two different types of trehalase in insects, as indicated by the trehalase transmembrane regions, the first is soluble trehalase (Treh1) (Kamimura et al., 1999; Su, 1993; Takiguchi et al., 1992; Tatun et al., 2008), and the second is membrane-bound trehalase (Treh2) (Chen et al., 2010a; Mitsumasu et al., 2005; Tang et al., 2008; Tatun et al., 2008). However, insects with more than two types of trehalase enzymes have been identified, including more than two soluble trehalase genes, such as *T. castaneum*, *T. molitor*, *Dendroctonus ponderosae*, *Apis mellifera*, and *Acyrthosiphon pisum*. Additionally, four soluble trehalase genes have been identified in *T. castaneum* and *D. ponderosae* (Tang et al., 2014).

Previously (Tang et al., 2014), we identified two genes (*HaTreh1–1* and *HaTreh1–2*) encoding soluble trehalase from *H. axyridis*. Subsequently, we found two novel soluble trehalase genes (*HaTreh1–3* and *HaTreh1–4*) by transcriptome sequencing and RACE. Prosite analysis of the deduced amino acid sequences of HaTreh1–3 indicated the presence of a trehalase signature 1 consensus sequence, P-G-G-R-F-x-E-x-Y-x-W-D-x-Y. However, Prosite analysis of HaTreh1–4 indicated the presence of both a trehalase signature 1 and signature 2 consensus sequence (Q-W-D-x-P-x-[GAV]-W-[PAS]-P). These signatures are specific to trehalase and are not found in any other protein. Moreover, transmembrane analysis indicated that no transmembrane helical regions were detected near the HaTreh1–3 and 1–4 C-terminus. The predicted molecular masses of HaTreh1–3 (65.02 kDa) and HaTreh1–4 (64.93 kDa) were similar to the molecular weight of the homologous enzymes from other insects, such as BmTreh-1 (64.9 kDa) and OfTreh-1 (65.2 kDa) (Su, 1993; Tatun et al., 2008). These sequence characteristics indicate that these novel genes encode distinct soluble trehalase enzymes.

The predicted pIs of HaTreh1–3 and HaTreh1–4 were 8.88 and 6.32, respectively, which were significantly different from HaTreh1–1 (4.82) and HaTreh1–2 (4.91) (Tang et al., 2014). These findings suggest that there are big differences between the amino acid composition of HaTreh1–3, HaTreh1–4, and HaTreh1–1, HaTreh1–2. These differences also are reflected by their distinct functions. For *HaTreh1–1* and *HaTreh1–2*, expression is most abundant in the larval developmental stages (Tang et al., 2014), whereas the peak mRNA expression levels of *HaTreh1–3* and *HaTreh1–4* can be observed during the adult stages. Therefore, *HaTreh1–1* and *HaTreh1–2* might be related to a greater requirement for chitin and energy during larva–larva metamorphosis. By contrast, *HaTreh1–3* and *HaTreh1–4* might be related to a greater requirement for chitin and energy during pupa–adult metamorphosis, most notably because *HaTreh1–4* has similar changes to *SeTre-1* (*Spodoptera exigua*) (Chen et al., 2010a), and as *HaTreh1–4* showed temporal increase at day 1 pupae compared to pre-pupae and day 2 pupae (Fig. 2), suggesting its potential role in molting from pre-pupae to pupae. These findings demonstrate that the gene regulation for soluble trehalase might be quite complex, and this combination of four genes could determine the enzyme activity. However, there is no evidence to prove whether membrane-bound trehalase genes exist in *H. axyridis*.

Previous studies on the protective function of trehalose under different stress conditions have also been reported, including desiccation, dehydration, heating, freezing, and oxidation (Bale and Hayward, 2010; Crowe et al., 1984; Elbein et al., 2003; Khani et al., 2007; Shimada, 1984). In this present study, trehalose levels were compared between cooling and warming conditions. We observed that a significantly increased concentration of trehalose occurred at 5 °C, 0 °C, and −5 °C during the cooling process (Fig. 3A). Our findings show that trehalose can also protect *H. axyridis* from cold stress. Meanwhile, we observed that trehalase activities could be gradually suppressed by the reduction of temperature that occurs during this process (Fig. 4A). The reduction of trehalase activity inhibits the trehalose catabolism, which indirectly helps the trehalose accumulation. Meanwhile, Qin et al. found that trehalose 6-phosphate synthase (TPS) activity had a maximum value at 5 °C in a similar cooling process (Zi et al., 2012). These findings explain why the accumulation of trehalose begins at 5 °C. Although trehalose content was high at low temperatures during warming conditions, a reduction occurred from −5 °C to 5 °C (Fig. 3B), and trehalase activity peaked at 0 °C and 5 °C (Fig. 4B). A possible explanation for this finding is that trehalose might transform other anti-cold substances, such as glycerol, which is a very important substance for protecting an organism from cold injury (Lee et al., 1987). Generally, negative relationships are observed between trehalase and trehalose (Ge et al., 2011; Tatun et al., 2008). However, this relationship is ambiguous during the warming process, resulting from TPS, which is highly involved in this process (Zi et al., 2012). TPS and trehalose 6-phosphate phosphatase are two key enzymes for trehalose synthesis in insects (Becker et al., 1996; Chen et al., 2010b).

Most insects, such as *O. fuscidentalis* and *Nilaparvata lugens*, only have one soluble trehalase gene, and regulation of gene expression is

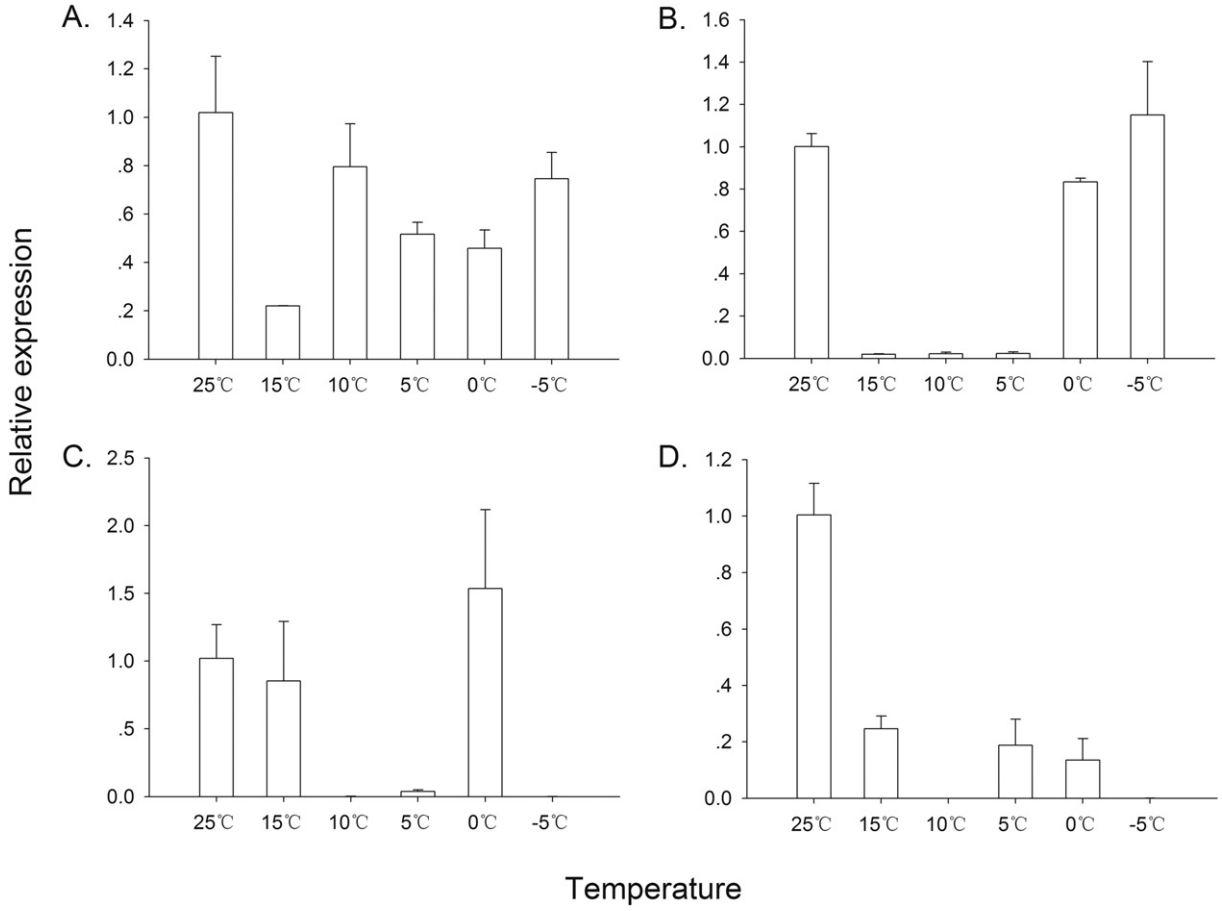

Fig. 5. Expression of four soluble genes during cooling condition. The relative expression of *HaTreh 1–1* (A), *HaTreh 1–2* (B), *HaTreh 1–3* (C), and *HaTreh 1–4* (D) identified from *H. axyridis* was tested by real-time PCR in an environment gradually cooled from 25 °C to −5 °C. All mRNA levels were measured relative to the *Harp49* (*H. axyridis* ribosomal protein 49 gene) mRNA levels. Data are presented as means ± s.d. (n = 3).

a key way to control enzyme activities (Ge et al., 2011; Gu et al., 2009; Tatun et al., 2008). The regulation of soluble trehalase gene expression levels in these insects is simple and unambiguous, but for *H. axyridis*, four genes that encode soluble trehalase are involved in its regulation. Therefore, it is difficult to determine how each gene that can regulate trehalase interacts, especially as the different trehalase proteins are often co-expressed. During the cooling process, although the four genes all affected trehalase activity, the expression of *HaTreh1–4* was most similar to the overall changes in trehalase activity (Fig. 4A and Fig. 5D). Therefore, the gene *HaTreh1–4* might play an important role in cold induction. Similarly, trehalase activity was determined by four genes that acted together during the warming process, but *HaTreh1–2* and *HaTreh1–4* might be the most important of these genes. For example, trehalase activity was low at −5 °C compared with CK (Fig. 4B), but the mRNA expression levels of *HaTreh1–1*, *HaTreh1–2*, and *HaTreh1–3* were elevated (versus CK) at −5 °C (Fig. 6A, B, C), whereas only *HaTreh1–4* was much lower than CK (Fig. 6D). These findings suggested that *HaTreh1–4* had a critical role at −5 °C. However, trehalase activity was enhanced at 25 °C, which strictly correlated with the expression of *HaTreh1–2* (Fig. 6B). Interestingly, trehalase activity peaked at 0 °C (Fig. 4B), but the mRNA expression levels of the four genes were decreased, which is confusing, other modes of regulation of its activity could be involved.

Conversion from glycogen to trehalose during the cooling and warming process was feasible, as shown in our study (Fig. 3C, D). This phenomenon is well-known to occur in various insects and the conversion mostly depends upon the trehalose synthesis pathway (Elbein et al., 2003; Hayakawa and Chino, 1982; Kunieda et al., 2006; Montooth et al., 2003; Tang et al., 2012; Zou et al., 1983). From glycogen to trehalose, it mainly involves glycogen phosphorylase (GP), which regulates glycogen decomposition, and TPS; but from trehalose to glycogen, it mainly involves glycogen synthase (GS), which controls glycogen synthesis, and trehalase. Generally, there are mutual constraints in these two pathways (Becker et al., 1996; Elbein et al., 2003; Hayakawa and Chino, 1982). Therefore, inhibition of the trehalose catabolic pathway also contributed to the accumulation of trehalose. Consequently, trehalase activities gradually decreased as the temperature dropped (Fig. 4A). Because glucose, degraded by trehalase, is middle metabolite from trehalose to glycogen, we also tested the levels of glucose at every temperature point. But in our present study, glucose was constantly absent throughout the changes in temperature. Therefore, trehalose could immediately be transformed into glycogen. This rapid transformation model between glycogen and trehalose might be a critical pathway used by *H. axyridis* to adapt to a disadvantageous environment. Unfortunately, we did not measure the levels of glucose 1-phosphate, which is a common metabolite from both glycogen synthesis and decomposition, so we cannot judge whether this transformation from glycogen to trehalose is quick.

Overwintering cold acclimation can be induced over several weeks or months, but Lee et al. found that the rapid cooling was useful for establishing cold-hardiness in arthropods; this could be induced by a short exposure (several minor h) to low temperatures (from 0 °C to 5 °C) or through gradual cooling (from 0.1 °C to 1 °C min^{-1}) over a range of temperatures (Lee et al., 1987; Lee, 1991). We designed the courses of cooling to occur gradually from 25 °C to −5 °C, and those of warming from −5 °C to 25 °C, which represent the two processes that *H. axyridis* must face at the beginning and end of winter. Indeed, the accumulation of trehalose peaked at 5 °C (124.61 ± 1.72) during

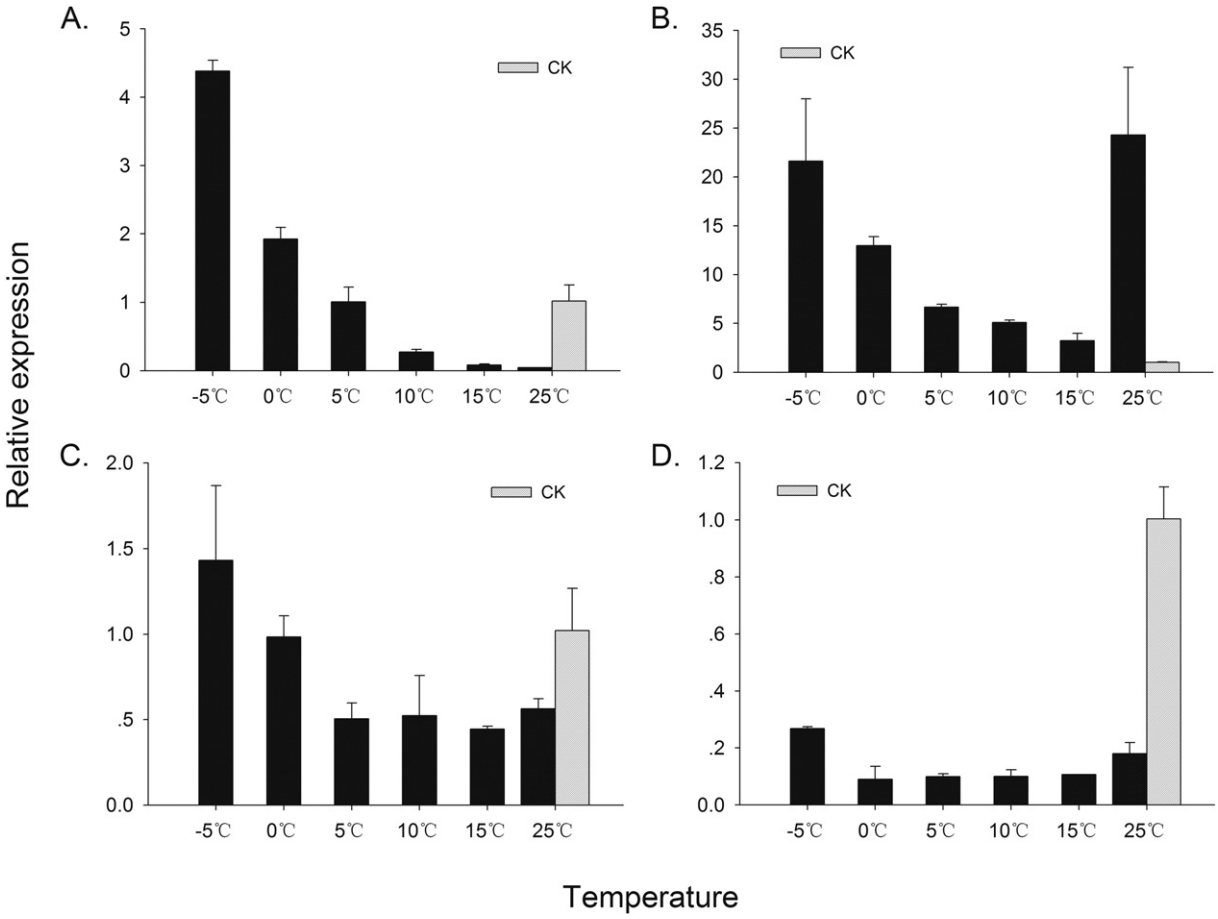

Fig. 6. Expression of four soluble genes during cooling condition. The relative expression of *HaTreh 1–1* (A), *HaTreh 1–2* (B), *HaTreh 1–3* (C), and *HaTreh 1–4* (D) identified from *H. axyridis* was tested by real-time PCR in an environment gradually warmed from −5 °C to 25 °C. All mRNA levels were measured relative to the *Harp49* (*H. axyridis* ribosomal protein 49 gene) mRNA levels. White sets represent the control group, which were adults reared at an optimal temperature (25 °C) without any cold stimulation. Data are presented as means ± s.d. (n = 3).

the cooling conditions (Fig. 3A), and thereafter remained significantly increased. However, the cold stimulation carried out over a short period of time, so the physiological changes in the experimental population were not equal to that of the field population. In this present study, trehalose did not increase gradually and its accumulation became obvious suddenly beginning at 5 °C (Fig. 3A), suggesting that 5 °C might be a temperature signal for *H. axyridis*. For field populations, to avoid the danger represented by extreme cold winter temperatures, the preparation for winter began in autumn prior to drops in temperature to 5 °C. These preparations include the following: migrations into cracks and crevices of rocks, caves, or sometimes in leaf litter, and into houses or other artificial structures in autumn (Kidd et al., 1995; LaMana and Miller, 1996; Nalepa et al., 1996; Obata, 1986); accumulation of myo-inositol starting in early November (Watanabe, 2002); increased fat and decreased water content at the end of October (Zhao et al., 2008); and aggregative behavior prior to overwintering, which can create a protective microclimate, thereby helping the insects experience less extreme temperature variations than in the surrounding areas (Obata, 1986; Wang et al., 2011; Schaefer, 2003).

In conclusion, four soluble trehalase genes were identified from *H. axyridis*. We found that trehalose content could be enhanced in the face of freezing, and glycogen could be converted into trehalose when the ladybird encountered a cold stimulus. Based on the changes in soluble trehalase activity, the physiological relationship to a warming environment is more complex than the response to a cooling environment. Additionally, the transcriptional control of soluble trehalase genes was found to be complex and involving four genes. But there are still some deficiencies in the experiment that need to be improved. For example, GP, GS and TPS activities need to be tested in order to clarify the relationship of enzymes and metabolites in these conditions, and how do these metabolites transform, such as trehalose and glycogen.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cbpb.2016.03.002.

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