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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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A B S T R A C T

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.

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## 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

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