Article

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Trehalose-6-Phosphate Synthase Contributes to Rapid Cold Hardening in Invasive Insect *Lissorhoptrus oryzophilus* (Coleoptera: Curculionidae) by Regulating Trehalose Metabolism

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Simple Summary: Trehalose plays crucial roles in the cold tolerance of insects. TPS is an important enzyme involved in trehalose synthesis. TPS genes have been demonstrated to be involved in cold resistant physiological processes. *Lissorhoptrus oryzophilus* is an important invasive pest of rice in China. To gain insight into the function of TPS in RCH, we cloned and characterized *TPS* gene of *L. oryzophilus* (*LoTPS*). Next, we examined changes of gene expression and trehalose level under low temperature after RCH treatment. We found *LoTPS* was a refused gene with conserved TPS and TPP domain. As expected, RCH increased the survival rate of *L. oryzophilus* adults under low temperature. Furthermore, it led to an increase in *TPS* gene expression and trehalose content. However, RCH efficiency disappeared when *LoTPS* gene was RNA interfered, causing no significant increase of survival rate, *TPS* gene expression and trehalose level. This study demonstrates the importance of TPS in in enhancing cold tolerance through RCH. *TPS* gene regulats trehalose synthesis and accumulation in adults, thus improving their survival rate under low temperature. Our findings contribute to a better understanding of the cold tolerance mechanisms of *L. oryzophilus* and invasiveness.

Abstract: RCH is known rapidly enhance cold tolerance of insects. Trehalose has been demonstrated to be one of the cryoprotectant in *L. oryzophilus*, an important invasive pest of rice in China. Trehalose synthesis mainly occurs through the TPS/TPP pathway in insects. In this study, *LoTPS* gene was cloned and characterized for the first time. Its expression and trehalose content changes elicited by RCH were investigated. Our results revealed that RCH not only increased the survival rate of adults but also upregulated the gene expression level of *LoTPS* and increased trehalose content under low temperature. We hypothesized upregulated *LoTPS* promoted trehalose synthesis and accumulation to protect adults from low-temperature damage. To further verify the function of *LoTPS* gene, we employed RNAi technology. Our findings showed that RCH efficiency disappeared and failed to increase the survival rate when the adults were fed dsRNA of *LoTPS*. Additionally, inhibiting *LoTPS* gene expression resulted in no significant difference in trehalose content between RCH and non-RCH treatments. Furthermore, the expression patterns of *TRET* and *TRE* were also affected. Collectively, these results indicate the critical role of *LoTPS* in *L. oryzophilus* cold resistance after RCH induction. *LoTPS* can enhance the survival ability by regulating trehalose metabolism. These findings contribute to further understanding of the role of *TPS* in insect cold resistance and invasiveness of *L. oryzophilus*. Moreover, RNAi of the *LoTPS* opens up possibilities for novel control strategies against *L. oryzophilus* in the future.

Keywords: trehalose-6-phosphate synthase; rapid cold hardening; trehalose metabolism; cold resistance; rice water weevil doi:10.20944/preprints202310.0479.v1

1. Introduction

Insects have developed various physiological mechanisms to cope with the detrimental effects of low temperatures. Among these, rapid cold hardening (RCH) is one of well-known types adaptive response in insects [1]. RCH occurs following a brief exposure to milder cold temperatures and provides rapid protection against sudden drops of temperature [2]. During spring and autumn, RCH can significantly enhance the survival rates of insects, particularly when temperatures are prone to fluctuations or sharp declines. Previous studies have demonstrated increased survival rates under low temperatures in different insect species following RCH, including *Agasicles hygrophila, Helicoverpa assulta, Solenopsis invicta, and Chilo suppressalis* [3–6].

Recent advances in metabolomics have confirmed the significance of low-molecular-weight sugars and polyols as crucial metabolites produced during RCH in many insect species. Although different insects accumulate different types of cryoprotectants like subitol, glucose, glycerol, and trehalose [7–10], trehalose has been implicated in RCH. Several studies have provided evidence linking increased trehalose levels to enhanced cold tolerance through RCH [9–13]. Specifically, trehalose accumulation in the hemolymph stabilizes proteins and maintains cell membrane integrity during RCH [2,8,14].

Trehalose is predominantly synthesized in the fat body in insects [15]. It is then transported to the haemolymph and further to other tissues by trehalose transporters (*TRET*) [16]. Eventually, trehalose is broken down into two glucose moieties by trehalase enzyme (*TRE*). Among the reported trehalose biosynthesis pathways in insects, trehalose is mainly synthesized by TPS/TPP or TPS pathway. In the TPS/TPP pathway, TPS transfers glucose from UDP-glucose to glucose-6-phosphate, yielding trehalose-6-phosphate and UDP. TPP converts trehalose-6-phosphate to trehalose. In the TPS pathway, the *TPS* gene is a fused gene encoding proteins with both TPS and TPP domains and exhibit both TPS and TPP enzyme activities [17,18]. *TPS* genes have been identified from different insect species [19–24]. However, *TPP* genes have only been found in certain insect species, predominantly in dipteran insects [25–27]. Three categories of TPS genes (TPS1, TPS2 and TPS3) have been discovered in insects to date [28,29]. Some insects have a single TPS gene [18,24,28,30], while others have two [31,32] or three TPS genes [29,33]. Interestingly, certain insects like *Drosophila melanogaster*, *Delia antiqua*, and *Plutella xylostella* also have distinct *TPS* and *TPP* genes [34–37]. However, the mode and functioning of *TPS* gene in *L. oryzophilus* has not been reported.

Rice water weevil (RWW), *Lissorhoptrus oryzophilus* Kuschel (Coleoptera: Curculionidae), is the most important invasive pest of rice (*Oryza sativa* L.) in China. It was initially introduced from Korea in 1988 and has since expanded its distribution to twenty-five provinces, with a continuing trend towards both northern and southern regions of the China. Due to its significant impact on agriculture, it has been designated as a national agricultural plant quarantine pest. The adult weevils feed on mesophyll, leading to distinctive longitudinal scars along the leaf blade [20,38]. However, the larvae cause more severe damage by feeding on the roots. Rice water weevil causes yield losses up to 25% in in the absence of prevention [39,40]. In northeast China, *L. oryzophilus* completes only one generation per year, with adult weevils entering diapause and overwintering at the base of perennial grasses on field bunds, levees, field margins, and other uncultivated areas. Studies on the diapause biology of rice water weevil have revealed significant mechanisms of cold hardiness, enabling their survival under relatively cold temperatures during winter. Trehalose has been identified as a major cryoprotectant in overwintering rice water weevils, contributing to their cold tolerance through increased trehalose content [41]. In regions like Changchun city in northeast China, where temperature fluctuations are common during early spring and autumn, adult weevils may experience sudden temperature drops without prior exposure to acclimating conditions. In such cases, RCH may play a crucial role. However, our understanding of the physiological mechanisms behind RCH in rice water weevils remains limited.

In this study, we aimed to investigate the role of the *TPS* gene in the cold resistance of *L. oryzophilus*. To begin, we cloned the *TPS* gene from *L. oryzophilus* adults using transcriptome data and characterized its properties. Next, we examined the potential of the rice water weevil to induce RCH during the adult stage. We analyzed changes in trehalose content and *TPS* gene expression profile in adult weevils after RCH to assess the role of *TPS* in the cold resistance response elicited by RCH. In order to further understand the function of the TPS gene in cold resistance, we employed RNAi technology to inhibit the expression of the *TPS* gene in adults of rice water weevil. These RNAi-treated weevils were subsequently exposed to low temperatures to evaluate the effectiveness of RCH, and trehalose content and relevant gene expression of trehalose metabolism were analyzed. These results will provide insight into the regulation to trehalose metabolism of *TPS* in insect cold resistance and lead to a new potential target for further control of *L. oryzophilus.*

2. Materials and Methods

2.1. The insects

Adults of L. oryzophilus were collected from rice fields near Changchun city, located in the Jilin province of northeast China (43°88′N, 125°35′E). The collection took place in late May, when the rice seedlings had just been transplanted for about one week and the weevils had started their spring feeding. Then these weevils were transferred to rice seedlings about 20cm in plastic box in laboratory. The conditions for feeding L. oryzophilus were as follows: temperature 25 ±1℃, photoperiod 16 h/8 h, and relative humidity of 70%. By providing a stable and controlled environment, we were able to conduct further investigations and analyses to understand the cold resistance mechanisms of L. oryzophilus.

2.2. Low temperature tolerance and discriminating temperature determination

Following the method of Yang et al. (2018) [42], we measured the discriminating temperature to assess the survival of adult *L. oryzophilus* after various pretreatments. To screen a standard cold shock exposure, adults were directly transferred from their rearing conditions to a range of subzero temperatures between -2℃ and -12℃ for 2 h in a refrigerator. This was achieved by placing the adults in petri dishes with a diameter of 10 cm, with each dish containing twenty adults. To ensure accurate temperature control, a thermometer was placed inside the refrigerator to monitor the temperature, and adjustments were made as necessary prior to each cold-shock treatment. The adults were exposed to the subzero temperatures for a duration of 2 hours. After the 2-hour exposure, the adults were returned to their rearing conditions, and their survival was assessed 2 hours later. Adult weevils were considered dead if their legs did not move when lightly touched with a brush.

2.3. Induction and detection of RCH

To assess the efficiency of RCH and determine the conditions for its induction, we followed a modified version of the method described previously [42]. Adult weevils of L. oryzophilus were placed in petri dishes with a diameter of 10 cm. We divided the weevils into different groups and subjected them to various temperature treatments. Firstly, the weevils were transferred from their rearing conditions at 25℃ to either 0℃ or 5℃ for different durations (1, 2, 3, or 4 hours). Subsequently, they were exposed to the discriminating temperature of -8℃ for 2 hours. After this exposure, the weevils were returned to their original rearing conditions at 25℃, and their survival was assessed after a 2-hour recovery period. We considered weevils that were able to move as having survived. For the control group, weevils were directly transferred from room temperature to the discriminating temperature of -8℃ for 2 hours. Each treatment group consisted of 20 individuals, and we replicated the experiment five times to ensure reliable results. To further analyze trehalose content and gene expression, all surviving adult weevils were immediately transferred to liquid nitrogen and preserved at -80℃ until further analysis.

2.4. Measurement of trehalose content

To determine the trehalose content in the samples, we utilized the Trehalose Content Kit from Solarbio, Beijing, China. Following the instructions provided in the kit, ten adults of *L. oryzophilus* were collected from both the control group and the RCH group. The collected weevils were weighed and transferred into a grinder for grinding. Each group was homogenized with 50 μL of extraction solution and left at room temperature for 45 minutes to allow for complete extraction. Then, samples were centrifuged at 8000 rpm for 5 min and cooled to room temperature. Next, 250 μL of the supernatant was mixed with 500 μL of the reaction reagent from the kit. This mixture was incubated in a 95℃ water bath for 10 minutes to react and release the trehalose. Following incubation, the solution was cooled to room temperature. Finally, the trehalose content was estimated by measuring the absorbance at 620 nm using a spectrophotometer. The absorbance reading provided an indication of the trehalose concentration in the sample. Three separate biological replicates were performed to ensure accuracy and reliability of the results.

2.5. Sequence determination and Bioinformatics Analysis of TPS gene

For the total RNA extraction and cDNA synthesis, we followed the same method as described in a previous study [43]. Total RNA was extracted from the whole bodies of *L. oryzophilus* adults using RNAiso Plus (Takara, Dalian, China), following the manufacturer's instructions. The concentration and quality of the extracted RNA were determined by measuring the absorbance at 260 nm with a spectrophotometer (NanoDrop2000, Wilmington, DE, USA). Purified RNA was stored at -20℃ for future experiments. First-strand cDNA synthesis was performed using the PrimeScript® RT reagent kit with gDNA Eraser (TaKaRa, Dalian, China) following the manufacturer's protocol.

The sequence of *LoTPS* was identified using our unpublished transcriptome data. The amino acid sequence of *LoTPS* was deduced using DNAMAN software. Additionally, we calculated the molecular weight and isoelectric points of the protein using the Compute pI/Mw tool (http://web.expasy.org/compute_pi/). The open reading frames (ORFs) were predicted using ORF finder (https://www.ncbi.nlm.nih.gov/orffinder/). Furthermore, we predicted signal peptides and transmembrane domains of the putative protein using the SignalP 5.0 server (http://www.cbs.dtu.dk/services/signalP). For multiple sequence alignments of TPS proteins, we employed DNAMAN 6.0 software (Lynnon Corporation, Pointe-Claire, Quebec, Canada). We compared the deduced amino acid sequence of TPS from *L. oryzophilus* with other insect TPS sequences. Finally, we constructed a phylogenetic neighbor-joining tree using MEGA-X software. This analysis aimed to understand the evolutionary relationships between the TPS proteins from different insect species.

2.6. RNAi of TPS

The dsRNA of *LoTPS* was synthesized according to the the manufacturer's recommendations of T7 RioMAX Express RNAi Sys-tem (Promega, San Luis Obispo, CA, USA). As a control, green fluorescent protein (GFP) dsRNA was also synthesized. Specific primers for the *LoTPS* dsRNA fragments were designed and synthesized. The primer sequences can be found in Table 1.

To prepare the rice leaves for dsRNA feeding experiments, leaves at the jointing stage were selected and cut into strips measuring 5.0 cm × 0.4 cm in size. The two ends of the strips were fixed with clamps and dried in a 40℃ oven for 20 seconds. The dsRNA was dissolved on ice and diluted with RNase-free water to 500 ng/ML. 200 μL of diluted dsRNA solution was packed into 10 mL EP tubes. The leaf strips dried were transferred into the EP tubes, ensuring that the leaf surface was coated with dsRNA. The base of the leaf was were immersed in dsRNA. After the leaf strips absorbed the dsRNA for 24 hours, they were used to feed the adults of rice water weevils. The weevils were starved for 48 hours before being transferred to the dsRNA-treated leaves. The EP tubes were sealed with gauze, and fresh rice leaves coated with dsRNA were replaced every 24 hours. The alive adult weevils feeding for 24 h, 48 h and 72 h were collected and stored at -80℃ for gene expression analysis. Each treatment contained 20 adults and repeated 3 times. The adults fed on normal leaves and treated with ds-GFP solution were used as CK and control.

Table 1. Primer sequences used in this study.

Prime name	Primer sequence（5′-3′）	Purpose
LoTPS	GCGTTTGGTGTGGATTGG /ATACGCTGACATCACCCC	ORF cloning
LoTPS	GCGTTTGGTGTGGATTGG /GATGATGTGCGAGGAGGT	RT-qPCR
TRET	ACCACGACTCAGGAAAAT/ACCAACGCATAAGATAGC	
TRE	AACCTGTGATTGTCCCTG/TCCTTTGGCTGTTTCGTG	
RpS18	GTAATGTTTGCCTTGACTG/ TTTCTACTTCCTCTTCGG	
dsTPS-F	taatacgactcactatagggGACAAAAAGCTTCCTCAGCG	RNAi
dsTPS-R	taatacgactcactatagggAGTGGAACGTTAACAACGCC	
dsGFP-F	taatacgactcactatagggTGTTCTGCTGGTAGTGGTCG	
dsGFP-R	taatacgactcactatagggTGTTCTGCTGGTAGTGGTCG	

2.7. Expression analysis using RT-qPCR

In this experiment, the effect of RCH (described in Section 2.3) on the transcript expression of the *LoTPS* gene was analyzed using qRT-PCR. Similarly, the effect of RNAi (described in Section 2.7) on key genes involved in trehalose metabolism, including *TPS*, *TRET*, and *TRE* genes, was also analyzed using qRT-PCR. Total RNA and first-strand cDNA synthesis were the same as in Section 2.6. Primer pairs for qPCR were designed using Primer 5 software, as shown in Table 1. *RPS18* was used as a reference gene for normalization. For each qPCR reaction, a total volume of 25 μL was prepared. This included 1 μL of template cDNA, 5 μL of SGExcel Fast SYBR Mixture, 0.4 μL of forward primer, 0.4 μL of reverse primer, and 18.2 μL of RNase-free water. qPCR was performed on an ABI 7500 Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) under the following conditions: initial denaturation at 95℃ for 3 min, followed by denaturation at 95℃ for 10 s and annealing at 60℃ for 30 s (40 cycles), and a dissolution curve at 65-95℃ with a 0.5℃rise per cycle. Standard errors and means were obtained from the mean of three biological replicates with three corresponding technical replicates. The relative expression values were calculated by the $2^{-\triangle\triangle Ct}$ method, which compares the threshold cycle (Ct) values of the target genes to the reference gene RPS18.

2.8. Rapid cold hardening assay

To evaluate the effect of knockdown of *LoTPS* gene on RCH efficiency, test adults were divided into four groups: CK with Non-RCH (directly transferred to -8°C for 2h), CK with RCH (exposed to 0°C for 4 h prior to -8°C for 2 h), dsTPS with Non-RCH (directly transferred to -8°C for 2 h after knockdown of TPS), dsTPS with RCH (exposed to 0°C for 4 h prior to -8°C for 2 h after knockdown of TPS). In the RCH bioassay, test adults were transferred in petri dishes and placed in refrigerator at the corresponding temperature. After the treatment period, the survival rates of the adults were determined following a 2-hour recovery at 25°C. The adults were considered alive if they were able to move. Each treatment was replicated five times with 20 adults per treatment. All alive adults were transferred directly to liquid nitrogen and placed at -80℃ until trehalose content and gene expression analysis.

2.9. Data analysis

All bioassays were performed in three independent replicates. Differences among the treatments were tested by one-way analysis of variance (ANOVA). All statistical tests were performed using Sigmaplot software. Data are presented as mean ± standard error and differences are considered significant at the $p < 0.05$ level.

3. Results

3.1. Identification and characterization of LoTPS

Based on the transcriptome database of *L. oryzophilus*, the complete ORF of the *LoTPS* was cloned and identified. The nucleotide sequence of *LoTPS* is 2499 bp long and encodes a putative protein consisting of 833 amino acids. The cDNA sequence has a theoretical isoelectric point of 6.31, indicating its overall charge at pH 7. *LoTPS* is a fusion gene that contains two conserved domains: the TPS domain (located at positions 24-474) and the TPP domain (located at positions 539-764). Within the TPS domain, two conserved motifs, HDYQL and RDGMNLN, were identified in the *LoTPS* sequence (Figure 1). No signal peptide or transmembrane regions were found in *LoTPS*. There are two potential N-glycosylation sites at position 122 and 571, respectively (Figure 1).

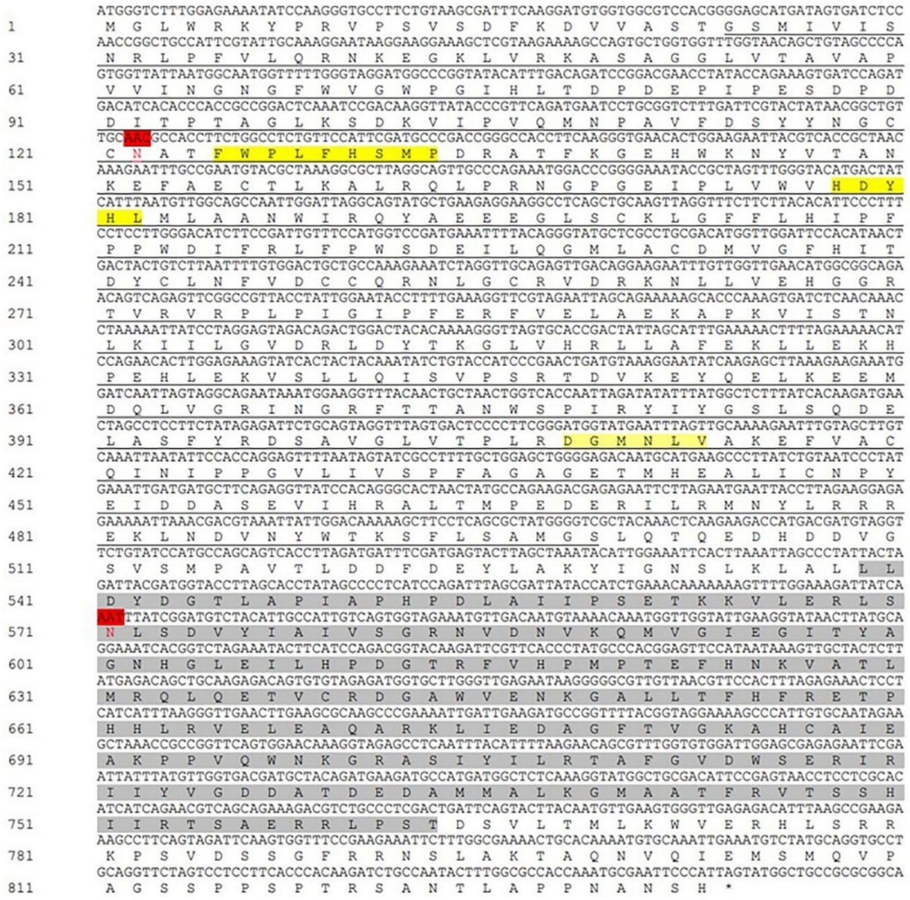

Figure 1. Nucleotide and deduced amino acid sequences of *LoTPS*. The TPS domain is represented by underlined nucleotides, and the TPP domain is shaded in gray. Motifs (or signature motifs) unique to trehalose-6-phosphate synthase (TPS) are shaded in yellow, and predicted N-glycosylation sites are shaded in red.

The deduced amino acid sequence of *LoTPS* was compared to *TPS*s from other species through sequence alignment. The highest similarity was observed with a *TPS* from the Coleoptera species *Rhynchophorus ferrugineus*, with a sequence identity of 93.23%. *LoTPS* also exhibited similarity to *TPSs* from *Dendroctonus ponderosae* (91.45% identity) and *Sitophilus oryzae* (91.08% identity) (Figure 2). The phylogenetic tree analysis further supported the homology among *TPS* genes and their evolutionary relationships with different species. *LoTPS* was found to cluster with other Coleoptera *TPS* genes in the phylogenetic analysis (Figure 3). This suggests that *LoTPS* shares a closer evolutionary relationship with *TPS* genes from other Coleoptera species compared to other insect species.

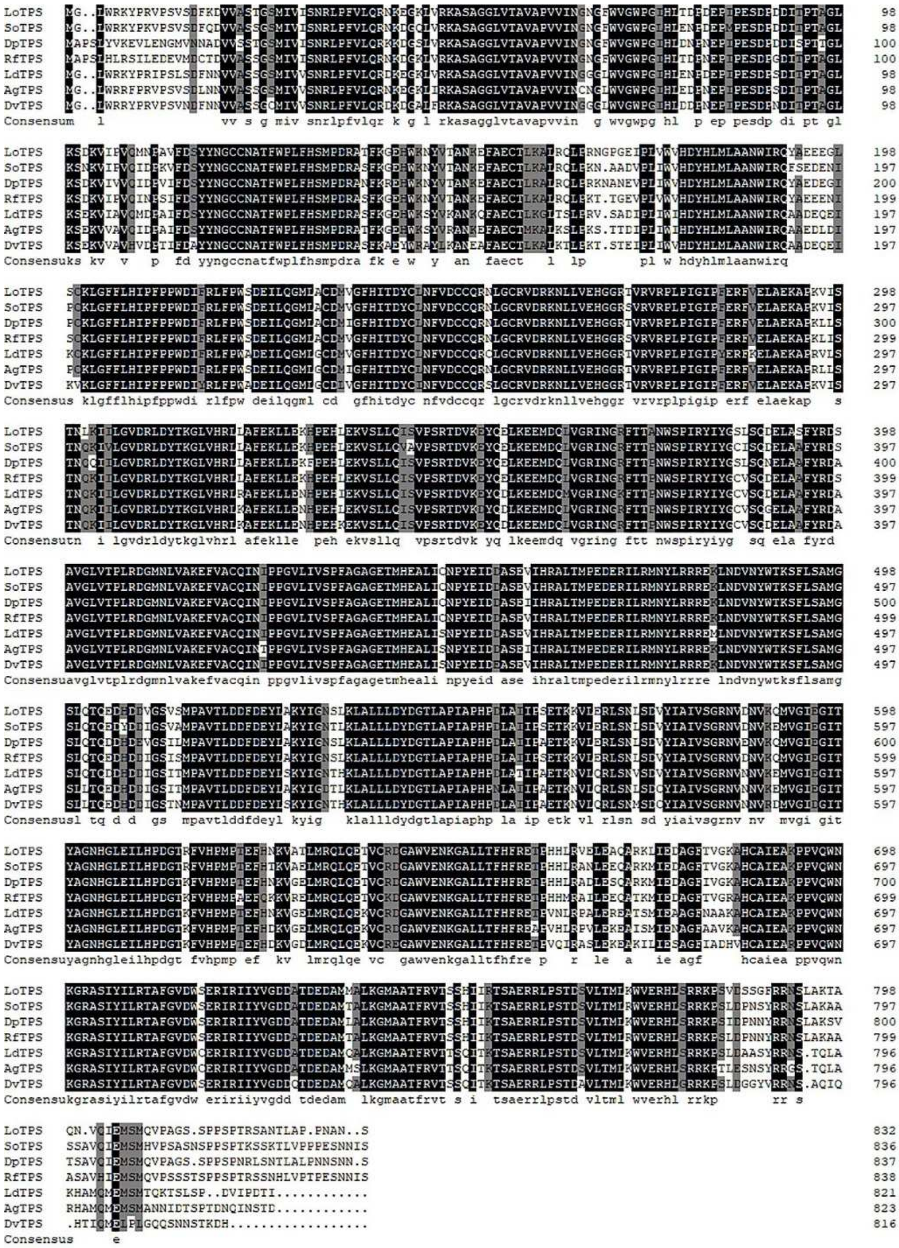

Figure 2. Multiple sequence alignment of trehalose-6-phosphate synthase (TPS) from *Lissorhoptrus oryzophilus* with known other Coleoptera insects. The fully conserved regions are shown in white and partly conserved regions in grey, the identical residues are shown in dark. So, *Sitophilus oryzae*; (XP_030760030); Dp, *Dendroctonus ponderosae* (XP_019761749.1); Rf, *Rhynchophorus ferrugineus* (KAF7280304.1); Ld, *Leptinotarsa decemlineata* (XP_023020816.1); Aa, *Anoplophora glabripennis* (XP_023311886.1); Dv, Diabrotica virgifera virgifera (XP_028127759.1).

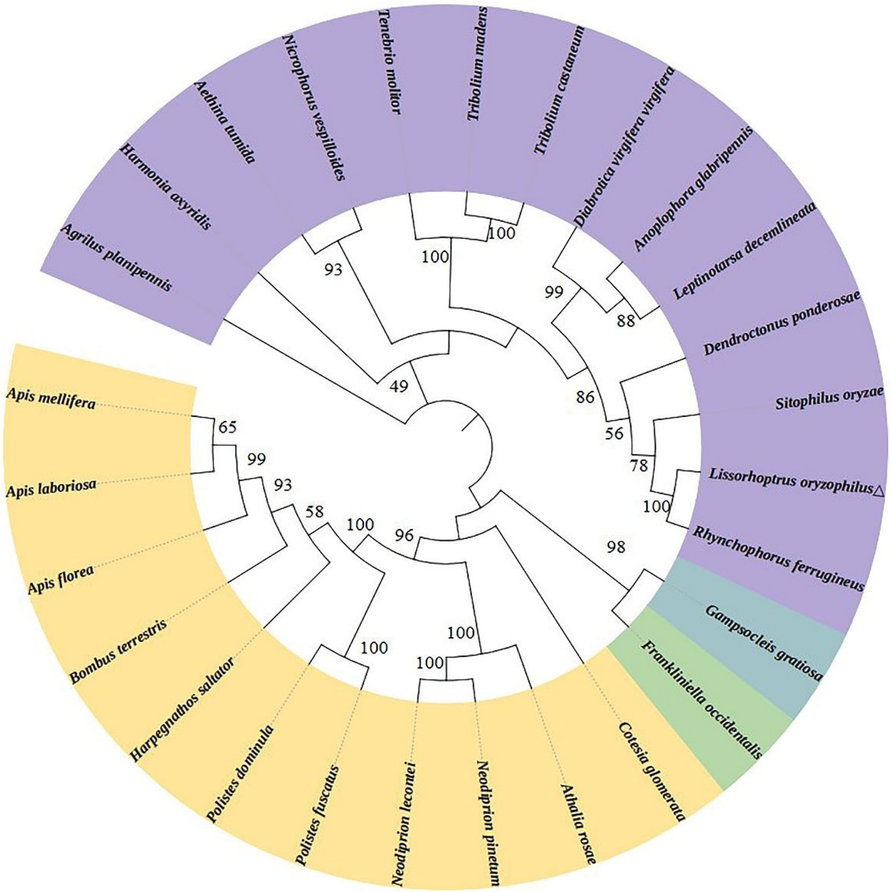

Figure 3. Phylogenetic tree of *LoTPS* gene. The phylogenetic analyses were constructed by the neighbor-joining method. Hymenoptera insects are marked in yellow, Thysanoptera insects are marked in green, Orthoptera insects are marked in blue, and Coleoptera insects are marked in purple. The numbers above the branches represent bootstrap values.

3.2. Induction of RCH in adult of L. oryzophilus

To determine the discriminating temperature, *L. oryzophilus* adults were directly exposed to subzero temperatures for 2 h from 25 °C. The survival rates of the adults after exposure to temperatures ranging from -2 to -12℃ for 2 hours are shown in Figure 3. As commonly observed in other insects, there was a significant decline in survivorship over a narrow range of temperatures. The survival of adults exposed to temperatures from-2 to -6℃ for 2 h was similar to that of the control, with over 90%. However, the survival rates dropped to about 20% at -8℃ and -10℃, and nearly all adults died after 2 hours at -12℃ (Figure 4A). Based on these results, 2 h at -8℃ was chosen as the discriminating temperature.

Then, RCH response and the optimal conditions for inducing RCH in *L. oryzophilus* adults were determined. The results indicated that pre-exposure to 0℃ or 5℃ over 1 to 4 h (RCH) significantly elevated cold tolerance of adults against -8℃ for 2 h (Figure 4B). The survival rates at the discriminating temperature were approximately twice as high for the groups pre-treated at 5℃ or 0℃ for 1 hour compared to those with no pre-treatment. The duration of pre-exposure had a significant effect on survival, with increased cold tolerance observed as the hardening period was extended up to 4 hours. Additionally, the temperature of pre-exposure (5℃ or 0℃) significantly affected survival at the discriminating temperature, with higher survival rates observed for induction at 0℃ compared to 5℃. Based on these findings, an exposure of 4 hours at 0℃ was selected as the condition for inducing RCH in the subsequent experiments.

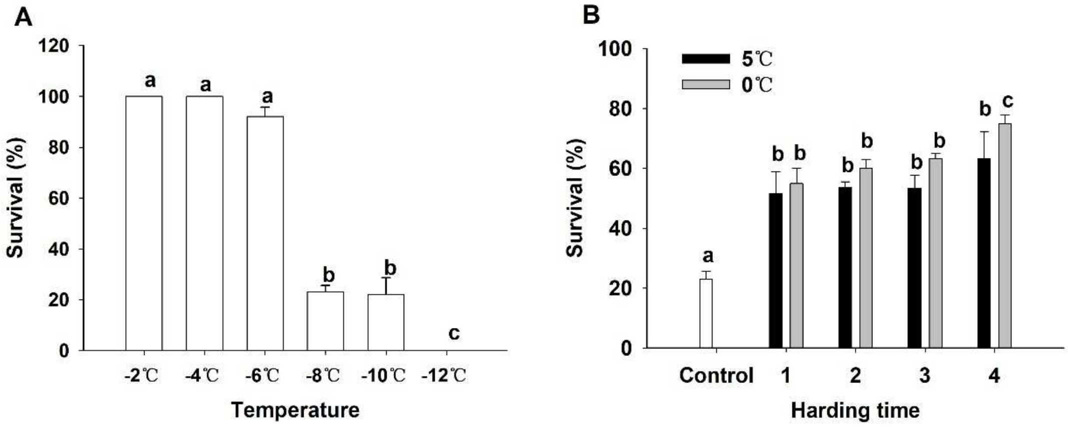

Figure 4. Rapid cold hardening (RCH) of *L. oryzophilus*. (A) Discriminating temperature determination. Survival (%, mean ± SE) of *L. oryzophilus* adult after 2 h exposure to sub-zero temperatures. Based on these results, -8℃for 2 h was selected as the discriminating temperature treatment. (B) RCH was induced and increased the survival of adult of *L. oryzophilus* by pre-exposure to a cool temperature (0°C and 5°C) for 1-4h. The control was no pretreatment to cool temperatures and the adults were directly transferred to discrimiating temperature from rearing temperature (25℃). Bars indicate standard errors. Means with different letters are significantly different (Tukey multiple comparison test at $p < 0.05$).

3.3. Trehalose content and LoTPS expression level increased during RCH

In order to understand the mechanism by which *TPS* regulates trehalose levels after RCH induction, the content of trehalose and the relative expression level of the *LoTPS* gene were investigated, as shown in Figure 5. Under low temperatures, the amount of trehalose was significantly altered, with an increase observed after exposure to -8°C for 2 hours, both in the treatment pre-exposed to 0°C for 4 hours (RCH) and that with no pre-exposure (Non-RCH). RCH treatment exhibited a maximum trehalose content level than Non-RCH and Control (Figure 5A). These findings suggest that trehalose plays a critical role in the cold resistance of rice water weevils after RCH. To identify the genetic factor(s) responsible for RCH and the increase of trehalose, *LoTPS* gene were predicted from the whole *L. oryzophilus* genome and analyzed for their function in trehalose biosynthesis. exThe expression of *LoTPS* at the discriminating temperature significantly increased after a short exposure to 0°C for 4 hours. However, there was no significant difference in expression between the Non-RCH and Control groups (Figure 5B). This indicates that the *LoTPS* gene is up-regulated and involved in regulating trehalose synthesis after RCH to enhance the cold resistance of weevil adults.

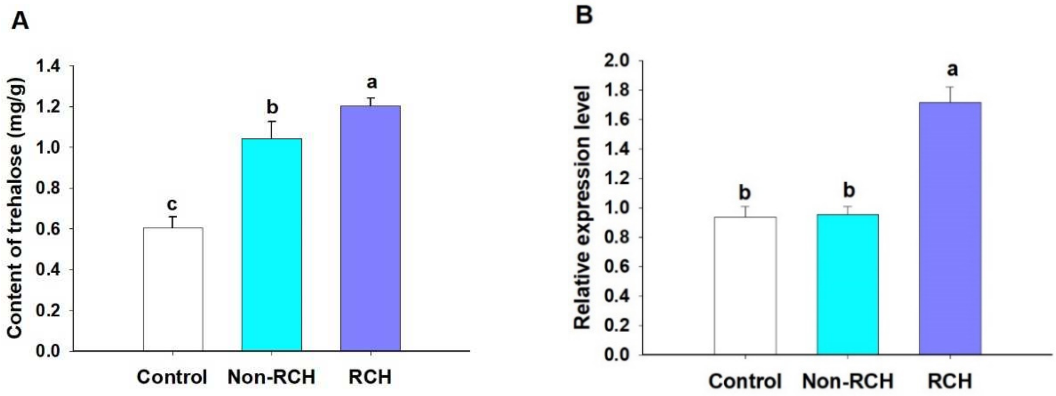

Figure 5. RCH increases the content of trehalose and the relative expression level of LoTPS in response to pre-exposure to 0°Cfor 4h. (A) The trehalose content and (B) the relative expression level of LoTPS of L. oryzophilus adults at -8℃ after RCH 0℃ for 4h. Data are means ± SD. Letter codes indicate statistically significant differences.

3.4. Knockdown of LoTPS expression reduces RCH efficiency.

To determine the optimal duration of *dsLoTPS* treatment for efficient RNAi, a series of time (24, 48, and 72h) were tested by feeding on the adults of *L. oryzophilus*. Quantitative real-time PCR was used to measure the expression of *LoTPS* after treatment with different times of *dsLoTPS*. The results showed that the expression of *LoTPS* was significantly down-regulated compared to the control and GFP treatment. The most effective RNAi efficiency was achieved after 24 hours of *dsLoTPS* feeding (Figure 6A).

In order to examine the effect of *LoTPS* suppression on RCH efficiency, a survival test under low temperature after RCH were performed. RNAi-treated adults were exposed to RCH conditions followed by the discriminating temperature. The survival rate of the CK with RCH induction was over 60%, while less than 40% of CK with Non-RCH individuals survived when adults were exposed to discriminating temperature. The RCH induction significantly increased survival rate in the CK. However, there was no RCH effect observed in the adults fed on *dsLoTPS*. Even when the adults were pre-exposed to RCH (dsLoTPS with RCH), the survival rate at discriminating temperatures did not increase significantly compared to the Non-RCH (Figure 6B).

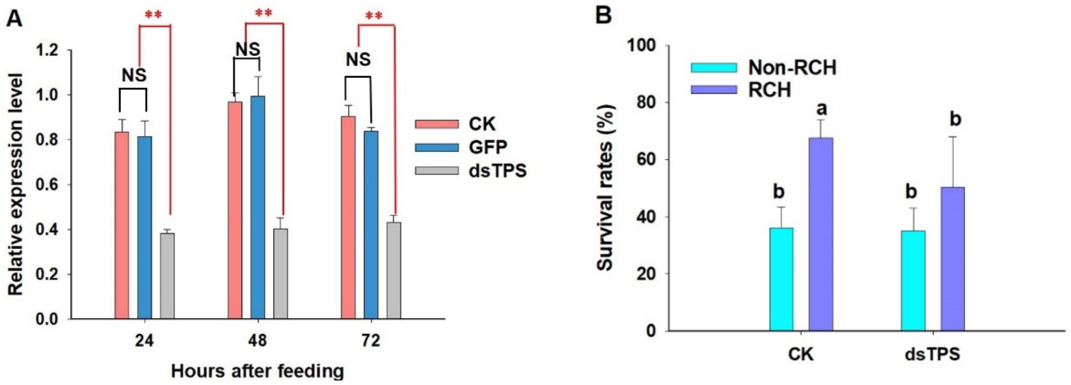

Figure 6. Suppression of *LoTPS* via RNA interference (A) and effect of *LoTPS* RNAi on survival rate after RCH treatment (B). (A)The relative expression level of *LoTPS* in adult was examined by quantitative qRT-PCR. From 24h to 72h after dsRNA feeding, TPS expression level exhibited statistically significant decrease, but the expression level was the lowest at 24h. Error bars represent the standard error of the calculated means based on three biological replicates. Different letters above the error bars indicate significant differences among treatments and the CK measured at the same time ($p < 0.05$). (B) The survival rates after suppression of *LoTPS* genes by RNAi and RCH induction at low temperature. Each treatment was replicated three times with 20 adult per replication. The different letter indicates the statistical difference ($p < 0.05$).

3.5. Roles of LoTPS on trehalose biosynthesis during RCH

To elucidate the molecular mechanisms underlying the loss of RCH in adult treated with ds*LoTPS*, the trehalose content and genes expression level of related genes was analyzed. It was observed that adults subjected to RNA interference of *LoTPS* did not exhibit an increase in trehalose content under low temperature conditions after RCH treatment. (Figure 7A). The suppression of *LoTPS* expression through RNAi significantly inhibited RCH and trehalose accumulation. There was no significant difference in trehalose content between Non-RCH and RCH treatments in adults fed on *dsLoTPS*. In contrast, effective RCH in *L. oryzophilus* was accompanied by trehalose accumulation in the control group (CK). As *LoTPS* expression was reduced by specific dsRNA in *L. oryzophilus* (Figure 7B), the expression levels of key genes associated with trehalose biosynthesis were measured by qRT-PCR. The expression patterns of these genes differed. The expression of the *TRET* gene was found to be upregulated in *L. oryzophilus* adults under low temperature after RCH induction and significantly higher than that of Non-RCH. However, after suppressing the expression of *LoTPS*, there was no significant difference in *TRET* expression between the RCH and Non-RCH treatments. (Figure 7C). On the other hand, the expression of *TRE* gene was downregulated under low temperature before and after feeding on *dsLoTPS*, and there was significant difference between RCH and Non-RCH treatments (Figure 7D). These results indicate that the RNAi-mediated suppression of *LoTPS* expression affects the expression of key genes involved in trehalose biosynthesis, leading to a loss of trehalose accumulation and compromised RCH in *L. oryzophilus* adults.

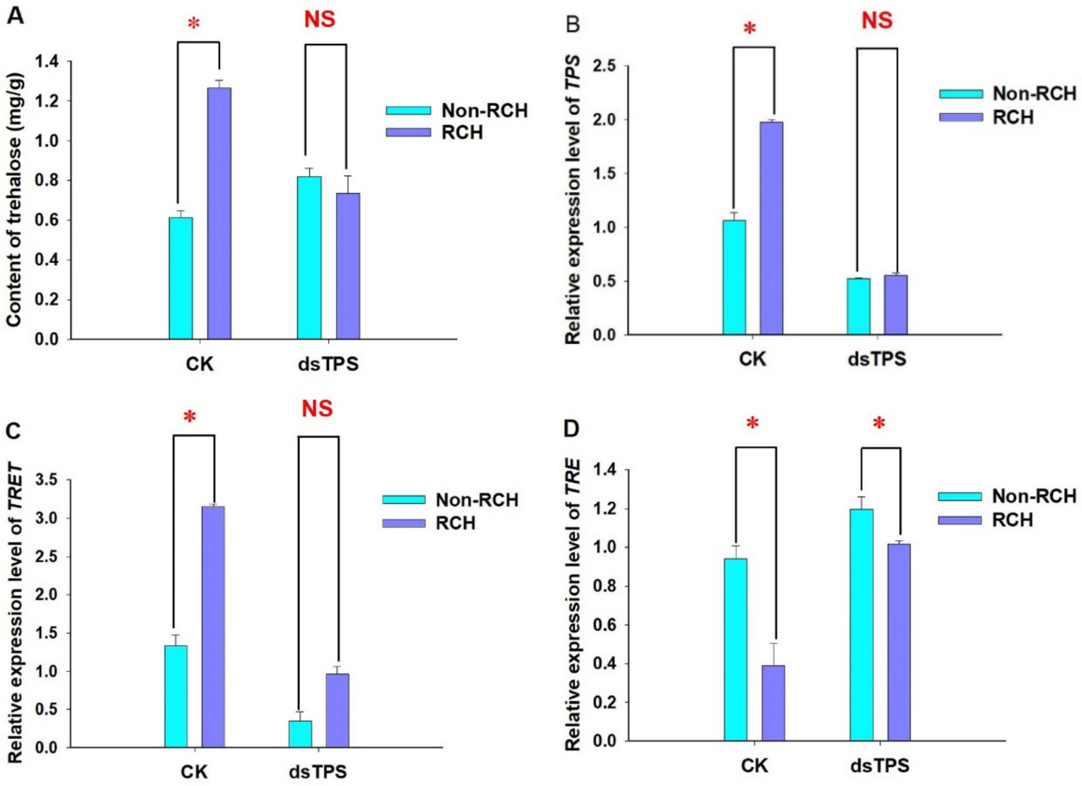

Figure 7. Effect of *LoTPS* RNAi on the content of trehalose (A) and expression levels of key genes related to trehalose biosynthesis after RCH induction including of *TPS* (B), *TRET* (C) and *TRE* (D) in *L. oryzophilus*. Error bars represent the standard error of the calculated means based on three biological replicates. Different letters on the error bars indicate significant differences ($p < 0.05$).

4. Discussion

In insects, trehalose plays crucial roles to tolerate low temperatures. It is synthesized as a cold-responsive or an anti-cold metabolite [44–46]. The metabolism of trehalose and its levels are crucial for insect survival during cold tolerance. Previous study has demonstrated that trehalose acts as a cryoprotectant in *L. oryzophilus*, with higher levels observed during overwintering [41]. In our research, we observed that RCH increased the survival rate and trehalose content in adult weevils under low temperature. This suggests that the enhanced cold resistance in *L. oryzophilus* adults following RCH induction may be associated with the increased trehalose levels. In insects, trehalose synthesis primarily relies on the TPS/TPP or TPS pathway [28]. And different species exhibit diverse evolutionary paths for genes involved in trehalose synthesis [47]. To gain insight into the characteristics and function of the TPS gene in *L. oryzophilus* adults in response to RCH induction, we cloned and characterized the *LoTPS* gene. Additionally, we measured changes in trehalose content and the expression of key genes involved in trehalose metabolism (TPS, trehalose transporters (TRET), and soluble trehalase (TRE)) in *L. oryzophilus* adults subjected to RNAi-mediated *LoTPS* suppression under low temperature after RCH induction. These will allow us to understand the specific roles of the TPS gene in *L. oryzophilus* adults responding to RCH induction.

In the TPP/TPS pathway of trehalose synthesis, the two key enzymes involved are trehalose-6-phosphate synthase (TPS) and trehalose-6-phosphate phosphatase (TPP) [21]. In our study, TPS gene was identified from transcriptome of *L. oryzophilus* adults. Similar to other known TPS genes in insects [28,34,48], the *LoTPS* gene is also a fused gene. The deduced *LoTPS* amino acid sequence codes two functional domains, an N-terminal TPS domain and a C-terminal TPP domain (one Glyco_transf_20 domain and one Trehalose_Ppase domain), and contains two signature motifs (HDYHL and DGMNLV). Through multiple sequence alignment, we found that the deduced amino acid sequence of *LoTPS* exhibits a higher level of identity with homologous proteins reported in other Coleoptera insects. Furthermore, the phylogenetic tree clearly indicates that TPS from different insects can be grouped into four major clades. As expected, *LoTPS* clusters together with TPS proteins from Coleoptera species, indicating a close evolutionary relationship between *LoTPS* and its homologues in Coleoptera insects.

RCH can offer protection in insect against acute cold stress [49]. In our study, we found that RCH treatment for 4 h at 0℃ significantly increased the cold tolerance capabilities of *L. oryzophilus* adults. This was evident by the increased survival rate when exposed to a discriminating temperature of -8℃. In the insect orders, RCH response has been observed in many insects, and prevents chilling injury by pr-exposing insects to non-lethal low temperatures [5,8,50,51]. Therefore, RCH is a widely used adaptation of insects to cope with thermally variable environments.

Cold tolerance in insects is a complex adaptive response that involves significant changes in biochemistry, gene expression, cell function, and endocrinological system. these adaptations allow for increased cell function and viability at low temperatures [2]. In our study, the accumulation of trehalose and up-regulated expression of *LoTPS* were observed compared to the control group without RCH. This suggests that RCH can enhance the cold resistance of these adults, with trehalose potentially playing a crucial role in the development of cold tolerance, while *LoTPS* is involved in RCH-induced trehalose accumulation. Previous research has already established a connection between RCH treatment and trehalose synthesis in insects [11,13]. The accumulation of trehalose plays central roles in the capacity of insects to survive freezing. The accumulation of trehalose is known to play a central role in the capacity of insects to survive freezing. Increased trehalose content is often associated with improved cold tolerance [44,48,52,53]. Trehalose biosynthesis genes have been observed to be upregulated following RCH induction in various insect species. Earlier studies on *Maruca vitrata* and *P. xylostella* have shown that RCH increased trehalose concentration in the hemolymph and the expression of *TPS* [5,13,54]. These findings support the idea that TPS is an essential factor contributing to RCH-induced cold tolerance.

To further comfirm the importance of *LoTPS* in low temperature tolerance after RCH, we examined the expression of LoTPS and the survival of RNAi-treated adults in response to a discriminating temperature. The results showed *LoTPS* expression was effectively suppressed by RNAi at 24 h post-feeding specific dsRAN against *LoTPS*. However, the survival rates of the RNAi-treated group were not improved at low temperature after RCH induction compared to the CK group. This suggests that the suppression of *LoTPS* expression had a negative impact on the ability of *L. oryzophilus* adults to survive at low temperatures after RCH induction. Similar findings were observed on *P. xylostella*, where RCH had no effect on larvae treated with dsRNA against *TPS*, resulting in significantly lower survival rates compared to those without RCH [54]. *TPS* genes are known to play a crucial role in trehalose synthesis, affecting trehalose levels through gene copies and expression levels. Their involvement in cold resistance has been well-defined [44,48,53]. The accumulation of trehalose has been associated with the induction of *TPS* in certain insects under extreme environmental stresses [44,48]. Based on these observations, we speculated that RNAi-mediated suppression of *LoTPS* gene expression affected the synthesis and accumulation of trehalose under low temperature conditions, thus preventing RCH from effectively increasing the survival of rice water weevils. Subsequent research indeed showed that RNAi of *LoTPS* significantly reduced trehalose accumulation in response to low temperature after RCH induction. These findings confirm that the suppression of *LoTPS* by RNAi inhibits trehalose biosynthesis and disrupts the protective effect of RCH. This aligns with our hypothesis that *TPS* plays a vital role in trehalose synthesis under low temperature conditions. *L. oryzophilus* adults rely on *TPS*-mediated regulation of trehalose as a cryoprotectant to enhance their survival ability at low temperatures after RCH induction. Overall, our results further highlight the importance of *LoTPS* in trehalose biosynthesis in adults during cold resistance after RCH induction. The findings support the critical role of *TPS* in the synthesis of trehalose under low temperature.

In a study on *H. axyridis* adults, it was observed that the expression of *TPS* mRNA initially increased and then decreased following a drop of temperature [55]. It was also found that higher *TPS* expression can increase the content of trehalose only if the activities of trehalase remain unchanged or decrease due to a decrease in *TRE* mRNA levels. Another study reported an increase in trehalose content when a single *TPS* gene was knocked down [19]. Furthermore, the rapid low-temperature stress of *H. axyridis* appeared to be more conducive to the accumulation of trehalose compared to gradual cooling, as *TRE2* activity remained high. *TPS*, *TRET* and *TRE* are key enzymes involved in trehalose metabolism in insects. Based on our hypothesis, these genes, including *TPS*, *TRET*, and *TRE*, co-regulate the changes in trehalose content. Therefore, we tested the expression levels of *TRET* and *TRE* genes after *TPS* RNA interference (RNAi). The results showed that silencing *TPS* genes not only reduced the expression level of the target gene, but also affected *TRET* and *TRE* genes. Specifically, RCH did not significantly increase the expression of *TRET* gene after *TPS* gene was interfered. The expression of *TRE* gene was downregulated under low temperature before and after feeding on *dsLoTPS*, and there was significant difference between RCH and Non-RCH treatments. These results suggest that the coordination of trehalose synthetic, transportation and degradation pathways is responsible for trehalose accumulation with RCH. We speculate that RNAi can effectively knock down the gene expression of *LoTPS*, thus disrupting trehalose metabolism and affecting the cold tolerance of *L. oryzophilus* adults. However, the molecular mechanisms underlying trehalose accumulation in this context require further study in the future.

5. Conclusions

In conclusion, our study has demonstrated that rapid cold-hardening (RCH) enhances the survival of *L. oryzophilus* adults at low temperatures. This improvement in survival is attributed to the up-regulation of *LoTPS* expression, which leads to increased trehalose biosynthesis. However, when *LoTPS* was suppressed through RNAi, the efficiency of RCH at low temperatures disappeared, indicating the essential role of *LoTPS* in trehalose biosynthesis. These findings highlight the significance of trehalose as a cryoprotectant for acquiring cold hardiness in *L. oryzophilus*, with *LoTPS* serving as a critical regulator of trehalose biosynthesis.

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References

1. Lee: R.E.; Chen, C.P.; Denlinger, D.L. A Rapid Cold-Hardening Process in Insects. *Science* **1987**, *238*, 1415-1417. https://doi.org/10.1126/science.238.4832.1415.
2. Teets, N.M.; Denlinger, D.L. Physiological Mechanisms of Seasonal and Rapid Cold-Hardening in Insects. *Physiol. Entomol.* **2013**, *38*, 105-116. https://doi.org/10.1111/phen.12019.
3. Yang, G.; Wen, J.; Han, Y.; Hou, M. Rapid Cold Hardening Confers a Transient Increase in Low Temperature Survival in Diapausing *Chilo suppressalis* Larvae. *Insects* **2018**, *9*, 53. https://doi.org/10.3390/insects9020053.
4. Vatanparast, M.; Sajjadian, S.M.; Park, Y. Glycerol Biosynthesis Plays an Essential Role in Mediating Cold Tolerance the Red Imported Fire Ant, *Solenopsis invicta*. *Arch. Insect Biochem. Physiol.* **2022**, *109*, e21861. https://doi.org/10.1002/arch.21861.
5. Cha, W.H.; Lee, D. Identification of Rapid Cold Hardening-Related Genes in the Tobacco Budworm, *Helicoverpa assulta*. *J. Asia-Pac. Entomol.* **2016**, *19*, 1061-1066. https://doi.org/10.1016/j.aspen.2016.09.007.
6. Pei, Y.; Jin, J.; Wu, Q.; Liang, X.; Lv, C.; Guo, J. Cold Acclimation and Supercooling Capacity of *Agasicles aygrophila* Adults. *Insects* **2023**, *14*, 58. https://doi.org/10.3390/insects14010058.
7. Michaud, M.R.; Denlinger, D.L. Shifts in the Carbohydrate, Polyol, and Amino Acid Pools during Rapid Cold-Hardening and Diapause-Associated Cold-Hardening in Flesh Flies (*Sarcophaga crassipalpis*): A Metabolomic Comparison. *J. Comp. Physiol. B* **2007**, *177*, 753-763. https://doi.org/10.1007/s00360-007-0172-5.

8. Mohammadzadeh, M.; Izadi, H. Cold Acclimation of *Trogoderma granarium* Everts Is Tightly Linked to Regulation of Enzyme Activity, Energy Content, and Ion Concentration. *Front. Physiol.* **2018**, *9*. https://doi.org/10.3389/fphys.2018.01427.
9. Izadi, H.; Mohammadzadeh, M.; Mehrabian, M. Cold Tolerance of the *Tribolium castaneum* (Coleoptera: Tenebrionidae), Under Different Thermal Regimes: Impact of Cold Acclimation. *J. Econ. Entomol.* **2019**, *112*, 1983-1988. https://doi.org/10.1093/jee/toz089.
10. Xie, Z.; Xu, L.; Zhao, J.; Li, N.; Qin, D.; Xiao, C.; Lu, Y.; Guo, Z. Rapid Cold Hardening and Cold Acclimation Promote Cold Tolerance of Oriental Fruit Fly, *Bactrocera dorsalis* (Hendel) by Physiological Substances Transformation and Cryoprotectants Accumulation. *Bull Entomol. Res.* **2023**, 113, 574-586. https://doi.org/10.1017/S0007485323000251
11. Overgaard, J.; Malmendal, A.; Sørensen, J.G.; Bundy, J.G.; Loeschcke, V.; Nielsen, N.Chr.; Holmstrup, M. Metabolomic Profiling of Rapid Cold Hardening and Cold Shock in *Drosophila* melanogaster. *J. Insect Physiol.* **2007**, *53*, 1218-1232. https://doi.org/10.1016/j.jinsphys.2007.06.012.
12. Zheng, X.L.; Zhou, L.J.; Lu, W.; Xian, Z.H.; Yang, Z.D.; Lei, C.L.; Wang, X.P. Cold-Hardiness Mechanisms in Third Instar Larvae of *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae). *Afr. Entomol*. **2014**, *22*, 863-871. https://doi.org/10.4001/003.022.0401.
13. Kim, Y.; Lee, D.W.; Jung, J.K. Rapid Cold-Hardening of a Subtropical Species, *Maruca vitrata* (Lepidoptera: Crambidae), Accompanies Hypertrehalosemia by Upregulating Trehalose-6-Phosphate Synthase. *Environ. Entomol.* **2017**, *46*, 1432-1438. https://doi.org/10.1093/ee/nvx153.
14. Kosˇta´l,V.; Korbelova´, J.; Rozsypal, J.; Zahradnı´cˇkova´, H.; Cimlova´, J. Long-Term Cold Acclimation Extends Survival Time at 0℃ and Modifies the Metabolomic Profiles of the Larvae of the Fruit Fly *Drosophila melanogaster*. *PLoS ONE* **2011**, *6*, e25025. https://doi.org/10.1371/journal.pone.0025025.
15. Arrese, E.L.; Soulages, J.L. Insect Fat Body: Energy, Metabolism, and Regulation. *Annu. Rev. Entomol.* **2010**, *55*, 207-225. https://doi.org/10.1146/annurev-ento-112408-085356.
16. Kanako, M.; Yasushi K.; Mika Fujita, Ke.I.; Daisuke, T.; Shingo, K.; Masahiko, W.; Richard, C.; Takashi, O.; Takahiro, K. Enzymatic Control of Anhydrobiosis-Related Accumulation of Trehalose in the Sleeping Chironomid, *Polypedilum vanderplanki*. *FEBS. J.* **2010**, *277*, 4215-4228. https://doi.org/10.1111/j.1742-4658.2010.07811.x.
17. Chen, Q.; Ma, E.; Behar, K.L.; Xu, T.; Haddad, G.G. Role of Trehalose Phosphate Synthase in Anoxia Tolerance and Development in *Drosophila* melanogaster. *J. Biol. Chem.* **2002**, *277*, 3274-3279. https://doi.org/10.1074/jbc.M109479200.
18. Jin, T.T.; Gao, Y.L.; He, K.L., Ge, F. Expression Profiles of the Trehalose-6-Phosphate Synthase Gene Associated with Thermal Stress in *Ostrinia furnacalis* (Lepidoptera: Crambidae). *J. insect sci*. **2018**, *18*, 1-10. https://doi.org/doi: 10.1093/jisesa/iex111.
19. Yang, M.; Zhao, L.; Shen, Q.; Xie, G.; Wang, S.; Tang, B. Knockdown of Two Trehalose-6-Phosphate Synthases Severely Affects Chitin Metabolism Gene Expression in the Brown Planthopper *Nilaparvata lugens*. *Pest Manag. Sci.* **2017**, *73*, 206-216. https://doi.org/10.1002/ps.4287.
20. Shi, J.F.; Xu, Q.Y.; Sun, Q.K.; Meng, Q.W.; Mu, L.L.; Guo, W.C.; Li, G.Q. Physiological Roles of Trehalose in Leptinotarsa Larvae Revealed by RNA Interference of Trehalose-6-Phosphate Synthase and Trehalase Genes. *Insect Biochem. Mol. Biol.* **2016**, *77*, 52-68. https://doi.org/10.1016/j.ibmb.2016.07.012.
21. Chen, J.X.; Lyu, Z.H.; Wang, C.Y.; Cheng, J.; Lin, T. RNA Interference of a Trehalose-6-Phosphate Synthase Gene Reveals Its Roles in the Biosynthesis of Chitin and Lipids in *Heortia vitessoides* (Lepidoptera: Crambidae). *Insect Sci.* **2020**, *27*, 212-223. https://doi.org/10.1111/1744-7917.12650.
22. Zhang, H.Z.; Wang, M.Q.; Xie, Y.Q.; Xiang, M.; Li, P.; Li, Y.Y.; Zhang, L.S. Gene Cloning and Expression Analysis of Trehalose-6-Phosphate Synthase, Glycogen Synthase and Glycogen Phosphorylase Reveal the Glycometabolism in the Diapause Process of *Aphidius gifuensis*. *J. Asia-Pac. Entomol.* **2020**, *23*, 641-645. https://doi.org/10.1016/j.aspen.2020.05.007.
23. Zhang, Y.; Wang, F.; Feng, Q.; Wang, H.; Tang, T.; Huang, D.; Liu, F. Involvement of Trehalose-6-Phosphate Synthase in Innate Immunity of *Musca domestica*. *Dev. Comp. Immunol.* **2019**, *91*, 85-92. http://dx.chinadoi.cn/10.1016/j.dci.2018.10.010.
24. Yang, H.J.; Cui, M.Y.; Zhao, X.H.; Zhang, C.Y.; Hu, Y.S.; Fan, D. Trehalose-6-Phosphate Synthase Regulates Chitin Synthesis in *Mythimna separate*. *Front. Physiol*, **2023**, *14*, 1109661. https://doi.org/10.3389/fphys.2023.1109661.
25. Kushwaha, S.; Singh, P.K.; Shahab, Mohd.; Pathak, M.; Bhattacharya, S.M. *In Vitro* Silencing of *Brugia Malayi* Trehalose-6-Phosphate Phosphatase Impairs Embryogenesis and *In Vivo* Development of Infective Larvae in Jirds. *PLoS Negl. Trop. Dis.* **2012**, *6*, e1770. https://doi.org/10.1371/journal.pntd.0001770.
26. Yoshida, M., Matsuda, H., Kubo, H. and Nishimura, T. Molecular Characterization of Tps1 and Treh Genes in Drosophila and Their Role in Body Water Homeostasis. *Sci. rep*. **2016**, *6*, 30582. https://doi.org/DOI: 10.1038/srep30582.

27. Wang, J.; Fan, H.; Li, Y.; Zhang, T.F.; Liu, Y.H. Trehalose-6-Phosphate Phosphatases Are Involved in Trehalose Synthesis and Metamorphosis in *Bactrocera minax*. *Insect Sci.* **2022**, *29*, 1643-1658. http://dx.chinadoi.cn/10.1111/1744-7917.13010.
28. Tang, B.; Wang, S.; Wang, S.G.; Wang, H.J.; Zhang, J.Y.; Cui, S.Y. Invertebrate Trehalose-6-Phosphate Synthase Gene: Genetic Architecture, Biochemistry, Physiological Function, and Potential Applications. *Front. Physiol.* **2018**, *9*, 30. https://doi.org/10.3389/fphys.2018.00030.
29. Wang, S.S.; Li. G.Y.; Liu, Y.K.; Luo, Y.J.; Xu, C.D.; Li, C.; Tang, B. Regulation of Carbohydrate Metabolism by Trehalose-6-Phosphate Synthase 3 in the Brown Planthopper, *Nilaparvata lugens*. *Front. Physiol.* **2020**, *11*, 575485. http://dx.doi.org/10.3389/fphys.2020.575485.
30. Gong, C.; Yang, Z.; Hu, Y.; Wu, Q.; Wang, S.; Guo, Z.; Zhang, Y. Silencing of the *BtTPS* Genes by Transgenic Plant-Mediated RNAi to Control *Bemisia tabaci* MED. *Pest Manag. Sci.* **2022**, *78*, 1128-1137. https://doi.org/10.1002/ps.6727.
31. Chen, Q.W.; Jin, S.; Zhang L.; Shen, Q.D.; Wei, P.; Wei, Z.M., Wang, S.G. Tang, B. Regulatory Functions of Trehalose-6-phosphate Synthase in the Chitin Biosynthesis Pathway in *Tribolium castaneum* (Coleoptera: Tenebrionidae) Revealed by RNA Interference. *B. Entomol. Res.* **2018**, *108*, 388-399. https://doi.org/10.1017/S000748531700089X.
32. Ding, Y.J; Li, G.Y.; Xu, C.D.; Wu, Y.; Zhou, Z.S.; Wang, S.G.; Li, C. Regulatory Functions of *Nilaparvata lugens* GSK-3 in Energy and Chitin Metabolism. *Front. Physiol*. **2020**, *11*, 518876. https://doi.org/10.3389/fphys.2020.51887.
33. Zhou, M.; Shen, Q.; Wang, SS.; Li, G.Y.; Wu, Y.; Xu, C.D.; Tang, B.; Li, C. Regulatory Function of The Trehalose-6-Phosphate Synthase Gene TPS3 on Chitin Metabolism in Brown Planthopper, *Nilaparvata lugens*. *Insect Mo.l Biol.* **2022**, *31*, 241-250. https://doi.org/10.1111/imb.12754.
34. Cui, S.Y.; Xia, Y.X. Isolation and Characterization of the Trehalose-6-Phosphate Synthase Gene from Locusta *migratoria manilensis*. *Insect Sci*. **2009**, *16*, 287-295. https://doi.org/10.1111/j.1744-7917.2009.01268.x.
35. Huang, Q.; Zhang, G.; Nan, J.; Cheng, W.; Zhu-Salzman, K. Characterization of Trehalose Metabolic Genes and Corresponding Enzymatic Activities during Diapause of *Sitodiplosis mosellana*. *J. Insect Physiol.* **2021**, *135*, 104324. https://doi.org/10.1016/j.jinsphys.2021.104324.
36. Guo, Q.; Hao, Y.-J.; Li, Y.; Zhang, Y.-J.; Ren, S.; Si, F.-L.; Chen, B. Gene Cloning, Characterization and Expression and Enzymatic Activities Related to Trehalose Metabolism during Diapause of the Onion Maggot *Delia antiqua* (Diptera: Anthomyiidae). *Gene.* **2015**, *565*, 106-115. https://doi.org/10.1016/j.gene.2015.03.070.
37. Dmitryjuk, M.; Łopieńska-Biernat, E.; Zaobidna, E.A. The *In Vitro* Effect of Ivermectin on the Activity of Trehalose Synthesis Pathway Enzymes and Their mRNA Expression in the Muscle of Adult Female *Ascaris suum* (Nematoda). *Sci. World J.* **2014**, *2014*, 936560. https://doi.org/10.1155/2014/936560.
38. Stout, M.J., Rice, W.C; Ring, D.R. The Influence of Plant Age on Tolerance of Rice to Injury by the Rice Water Weevil, *Lissorhoptrus oryzophilus* (Coleoptera: Curculionidae). *Bull. Entomol. Res*. **2002**, 92, 177-184. https://doi.org/10.1079/BER2001147.
39. Reay-Jones, F.P.F.; Way, M.O.; Tarpley L. Nitrogen Fertilization at the Rice Panicle Differentiation Stage to Compensate for Rice Water Weevil (Coleoptera: Curculionidae) Injury. *Crop Prot*, **2008**, 27, 84-89. https://doi.org/10.1016/j.cropro.2007.04.009
40. Aghaee, M. and Godfrey, L.D. A Century of Rice Water Weevil (Coleoptera: Curculionidae): A History of Research and Management with an Emphasis on the United States. *J. Integr. Pest Manag.* **2014**, *5*, 1-14. http://dx.doi.org/10.1603/IPM14011.
41. Lee, K.Y.; Chang, Y.D.; Kim, Y.G. Trehalose, A Major Sugar Cryoprotectant of the Overwintering Rice Water Weevil, *Lissorhoptrus oryzophilus* (Coleoptera: Curculionidae). *J. Asia-Pacific Entomol*. **2002**, *5*, 35-41. https://doi.org/10.1016/S1226-8615(08)60130-2.
42. Yang, S.; Zhang, X.X.; Wang, J.X; Wang S.; Pan Y.; Zhang J.H.; Xi J.H. Identification and Analysis of Up-Regulated Proteins in *Lissorhoptrus oryzophilus* Adults for Rapid Cold Hardening. *Gene* **2018**, *642*, 9-15. https://doi.org/10.1016/j.gene.2017.11.002.
43. Wang, Q.; Fang, K.; Qi, L.; Wang, X.; Pan, Y.; Li, Y.; Xi, J.; Zhang, J. Purification and Functional Characterization of a Soluble Trehalase in *Lissorhoptrus oryzophilus* (Coleoptera: Curculionidae). Insects **2022**, *13*, 867. https://doi.org/ 10.3390/insects13100867.
44. Wan, S.; He, J.; Chao, L.; Shi, Z.; Wang, S.; Yu, W.; Huang, Z.; Wang, S.; Wang, S.; Zhang, Z. Regulatory Role of Trehalose Metabolism in Cold Stress of *Harmonia axyridis* Laboratory and Overwinter Populations. *Agronomy* **2023**, *13*, 148. https://doi.org/10.3390/agronomy13010148.
45. Shukla, E.; Thorat, L.; Bendre, A.D.; Jadhav, S.; Pal, J.K.; Nath, B.B.; Gaikwad, S.M. Cloning and Characterization of Trehalase: A Conserved Glycosidase from Oriental Midge, *Chironomus ramosus*. *3 Biotech*. **2018**, *8*, 352. https://doi.org/10.1007/s13205-018-1376-y.
46. Shen, X.N.; Ji, S.X.; Wan, X.; Liu, W.X.; Guo, J.Y.; Lü, Z.C.; Wan, F.H. Molecular Charactristics of Three Cold Resistance Genes and Their Roles in Temperature Stress Response in Two *Bemisia tabaci* Cryptic Species. *Entomol. Gen*. **2021**, *41*, 317-328. https://doi.org/10.1127/entomologia/2021/0954.

47. Tellis, M.B.; Kotkar, H.M.; Joshi, R.S. Regulation of Trehalose Metabolism in Insects: From Genes to the Metabolite Window. *Glycobiology* **2023**, *33*, 262-273. https://doi.org/10.1093/glycob/cwad011.
48. Lü, X.; Han, S.; Li, Z.; Li, L.; Li, J. Gene Characterization and Enzymatic Activities Related to Trehalose Metabolism of In Vitro Reared *Trichogramma dendrolimi* Matsumura (Hymenoptera: Trichogrammatidae) under Sustained Cold Stress. *Insects* **2020**, *11*, 767. https://doi.org/10.3390/insects11110767.
49. Teets, N.M.; Gantz, J.D.; Kawarasaki, Y. Rapid Cold Hardening: Ecological Relevance, Physiological Mechanisms and New Perspectives. *J. Exp. Biol.* **2020**, *223*, jeb203448. https://doi.org/10.1242/jeb.203448.
50. Malekera, M.J.; Acharya, R.; Hwang, H. S.; Lee, K. Y. Effect of Cold Acclimation and Rapid Cold-Hardening on the Survival of *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) under Cold Stress. *J. Asia-Pac. Entomol.* **2022**, *25*, 101862. https://doi.org/10.1016/j.aspen.2021.101862.
51. Iqbal, J.; Zhang, X.-X.; Chang, Y.-W.; Du, Y.-Z. Differential Response of Leafminer Flies *Liriomyza trifolii* (Burgess) and *Liriomyza sativae* (Blanchard) to Rapid Cold Hardening. *Insects* **2021**, *12*, 1041. https://doi.org/10.3390/insects12111041.
52. Tamang, A.M.; Kalra, B.; Parkash, R. Cold and Desiccation Stress Induced Changes in the Accumulation and Utilization of Proline and Trehalose in Seasonal Populations of *Drosophila immigrans*. *Comp. Biochem. Physiol. A. Mol. Integr. Physiol.* **2017**, *203*, 304-313. https://doi.org/10.1016/j.cbpa.2016.10.011.
53. Yu, C.; Zhao, R.; Zhou, W.; Pan, Y.; Tian, H.; Yin, Z.; Chen, W. Fruit Fly in a Challenging Environment: Impact of Short-Term Temperature Stress on the Survival, Development, Reproduction, and Trehalose Metabolism of *Bactrocera dorsalis* (Diptera: Tephritidae). *Insects* **2022**, *13*, 753. https://doi.org/10.3390/insects13080753.
54. Cha, W.H.; Lee, D.W. RNA Interference of Trehalose Phosphate Synthase Inhibits Metamorphosis and Decreases Cold Tolerance in the Diamondback Moth, *Plutella xylostella* (L.). *J. Asia-Pac. Entomol.* **2018**, *21*, 1034-1039, https://doi.org/10.1016/j.aspen.2018.07.007.
55. Qin, Z., Wang, S., Wei, P., Xu, C.D., Tang, B.; Zhang, F. Molecular Cloning and Cold-Induced Expression of Trehalose-6-Phosphate Synthase Gene in *Harmonia axyridis* (Coleoptera: Coccinellidae). *Acta Entomol. Sin.* **2012**, *55*, 651-658. https://doi.org/10.16380/j.kcxb.2012.06.014.