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RESEARCH ARTICLE

Cloning and Interspecific Altered Expression of TPS Gene in Two Invasive Apple Snails, *Pomacea canaliculata* and *Pomacea maculata* (Gastropoda: Ampullariidae), Under Different Temperature

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ABSTRACT

Studies have demonstrated differences in temperature tolerance between two Pomacea species, *Pomacea canaliculata* and *Pomacea maculata*. To determine whether the trehalose-6-phosphate synthase (TPS) genes in the two species exhibited different expression profiles at different temperatures, we cloned the full-length cDNA of TPS in the two Pomacea species and analyzed the expression profile of TPS across a temperature gradient through real-time quantitative analysis. The TPS gene of both *P. canaliculata* (denoted by PcTPS) and *P. maculata* (denoted by PmTPS) contained 1908 bp and 1914 ORFs encoding 635 and 637 amino acids, respectively. The genes have a classical polyadenylation signal sequence AATAAA and poly(A), as well as two highly conserved motifs of the TPS protein family in deduced amino-acid sequences. Across the temperature gradient (from 3°C to 42°C), the onset temperature (Ton) or maximal temperature (Tmax) for inducing TPS expression in *P. maculata* was 3°C lower than that in *P. canaliculata*, and Ton was highly consistent with the upper temperature limits of the range of the two Pomacea species. All results revealed that the cloned genes were inducible TPS gene. Moreover, in terms of gene-expression level, P. maculata was more susceptible to cold temperature than *P. canaliculata*. The Ton (or Tmax) of TPS could represent the differences in temperature tolerance of the two Pomacea species. The study also provided useful molecular information on the ecological adaptability of invasive apple snails against extreme environmental stress.

KEYWORDS: Trehalose-6-phosphate synthase, *Pomacea canaliculata, Pomacea maculata*, Temperature gradient

INTRODUCTION

Golden apple snails are freshwater snails that are native to South America (Naylor, 1996). The snails were introduced to East and Southeast Asia in the 1980s for commercial production and as a dietary protein supplement (Cowie et al, 2006; Hayes et al, 2008; Mochida, 1991). However, commercial markets failed, and the discarded or escaped snails invaded rice paddies, thus, these snails have become an important rice pest in numerous countries (Hayes et al, 2008; Wada, 2004). Molecular biology renders more precise identification of apple snails (Rawlings et al, 2007; Hayes et al, 2008). Mitochondrial DNA analysis of samples collected from various countries in Asia and South America revealed four species of South American apple snails in Asia: *Pomacea canaliculata*, *P. maculata* (Perry, 1810) (previously known as *P. insularum*; Hayes et al, 2012), *P. scalaris*, and *P. diffusa* (Hayes et al, 2008). Among these species, *P. canaliculata* and *P. maculata* are widely distributed in rice ecosystems in Southeast and East Asia. These two species possess morphologically similar characteristics and have become a serious threat to rice production and ecosystems in Asia (Yoshida, 2014; Hayes et al, 2008). These species are distinct according to mitochondrial DNA (COI) analysis (Rawlings et al, 2007; Hayes et al, 2008; Matsukura et al, 2008), as well as shell anatomy and egg morphology (Hayes et al, 2012).

Trehalose is a non-reducing disaccharide and a main energy

source in prokaryotes, yeasts, plants, and invertebrates. In addition, trehalose is an important protectant of protein integrity and performs an important function in physiological and biochemical adaptations under extreme environmental conditions (Goyal et al, 2005; Zhang et al, 2012). Trehalose synthesis is the most widely distributed pathway, which involves the transfer of glucose from UDP-glucose to glucose-6-phosphate to form trehalose-6-phosphate and UDP via the trehalose-6-phosphate synthase (TPS) (Elbein et al, 2003). TPS, a protein in the biosynthesis of trehalose, is a glycosyltransferase that catalyzes the synthesis of α,α,α-1-trehalose-6-phosphate from glucose-6-phosphate by using a UDP-glucose donor (Elbein et al, 2003). *TPS* is noted in insects as a fused gene that codes two functional domains in tandem, namely, *TPS*, a homolog of Ost A of *Escherichia coli*, and trehalose-6-phophate phosphatase (*TPP*), a homolog of Ost B of *E. coli*. *TPS* and *TPP* are key proteins in trehalose biosynthesis (Becker et al, 1996; Chen et al, 2002). *TPS* is generally regarded as the key enzyme for catalyzing the first step in trehalose synthesis. In the long period of natural domestication, increase in trehalose content in insects may be due to the increasing expression of *TPS* gene (Clark et al, 2009). Trehalose has also been implicated as a key protective solute in a range of environmental stress responses, including low temperature (Lee, 2011) and hypoxia (Chen and Haddad, 2004), presumably by stabilizing cell membranes and proteins during periods of osmotic imbalance (Elbein et al, 2003). Trehalose appears to be particularly important during periods of extreme dehydration; for example, most invertebrates that are capable of anhydrobiosis use trehalose as the primary osmoprotectant (Clegg, 2001). This phenomenon implies that the *TPS* gene may perform an important function under environmental stressors.

Numerous studies have investigated the effect of temperature on apple snails (Matsukura et al, 2009; Wada and Matsukura, 2007). The activity of apple snails increases with increasing water temperature, and their crawling velocity is positively correlated with temperature (Song et al, 2014). Water temperature in the living habitat of snails also exhibits a critical effect on the growth, survival, development, and reproduction of apple snails. Yoshida (2014) found that different populations of apple snails exhibit varied resistance responses to low temperature. Therefore, temperature performs a critical function in the geographic distribution and spread of snails. In addition, *Pomacea* apple snails can resist pH 4.5-9.4 acid-based erosion and various types of chemical, bacterial, and parasitic threats. Thus, an increased understanding of the molecular mechanisms behind their invasion phenomenon is crucial.

P. canaliculata and *P. maculata* are widely distributed in more than 11 provinces in Southern China. These species may have expanded northward because of environmental adaptation and climate change, causing serious damage to humans and the environment (Li et al, 2009; Lv, et al, 2011; Song et al, 2014). Both species are tolerant to variable temperatures, salinities, and humidity, making them eminently suitable for successful dispersal (Cowie, 2002). However, the physiological mechanism of their distribution that extends further toward northern China has not been investigated in detail.

Numerous *TPS* genes have been cloned from animals, plants, bacteria, and fungi to better understand the structure and function of *TPS* at the molecular level. However, few data on molecular information of *TPS* in golden apple snails and their expression responses against environmental stressors are available. This study is the first to identify and characterize the *TPS* gene in apple snails. Quantitative polymerase chain reaction (PCR) was performed to analyze the differential expression of *TPS* in both *P. canaliculata* and *P. maculata* under different temperature treatments. The findings of this study reveal that the different temperature resistances of *P. canaliculata* and *P. maculata* result in different patterns of *TPS* expression, indicating that species-related differences likely exist in the temperature resistance of *Pomacea* apple snails. These results will provide valuable molecular information on increased tolerances against environmental stressors in *P. canaliculata* and *P. maculata,* as well as understanding the functional complexity of TPS in mollusks.

MATERIALS AND METHODS

Snails used

Golden apple snails were collected from a field of wild rice, *Zizania latifolia*, in Yuyao City (30°02′N, 121°10′E), Zhejiang Province. Snails were identified as *P. canaliculata* and *P. maculata* based on mitochondrial COI gene sequences, as described in previous studies (Rawlings et al, 2007; Hayes et al, 2008; Matsukura, et al 2008).

Rearing and temperature treatments

These snails were reared in a 40 cm × 25 cm × 28 cm tank that contained 10 L of freshwater at 26 ± 1°C. A dead cabbage was provided as basic food, and egg masses were obtained from above snails. Hatched juveniles were reared in an aquarium in a rearing room under a 16 h light: 8 h dark photoperiod at 26 ± 1°C. Juvenile snails with a shell height of 7.5-15.0 mm (about a month after hatching) were selected for experiments. For the temperature gradient treatment (3, 6, 9, 12, 15, 26, 30, 33, 36, 39, and 42°C), six *P. canaliculata* and six *P. maculate* individuals were selected and exposed to each temperature for 2 h, and then snails were returned to acclimation temperature (26°C) for 1 h before the expression test. For the temporal expression experiment (2, 6, 12, 24, 48, 72, 96, and 120 h), six *P. canaliculata* and six *P. maculate* individuals were sampled and decapitated in each group after the same temperature treatment (12°C).

Cloning the full-length cDNA of *TPSs*

Total RNA was extracted from 10 mg foot muscle of each sample using TRIzol© reagent (Invitrogen, USA), and 1 µg of the total RNA was used to generate cDNAs. Degenerate primers (Table 1) were used to amplify the partial segments of *TPSs*, and then 5′ and 3′ RACE were applied to obtain full cDNA lengths following the manufacturer's instructions (Smart™ RACE cDNA Amplification Kit, BD, Biosciences Clontech). Products were analyzed on a 1.2% agarose gel, and the objective bands were selected and purified with a gel extraction kit (Omega, Norcross, GA). The DNA fragment was then subcloned into the pMD18-T vector (TaKaRa, Tokyo, Japan). Following transfection into *E. coli* $DH_{5\alpha}$-competent cells, recombinants were identified using blue and white spot selection. Positive clones were sequenced by Sangon Biological Engineering Technology & Services Co., Ltd (Shanghai, China).

Sequence comparison and phylogenetic analysis

Table 1. Name, sequence, and technique for PCR primers used in the cloning and expression of *TPS* genes from *Pomacea* snails.

Primers	Sequence (5'-3')	Technique
TPS-F	TCCACgaytaycayyt	Amplification of TPS core region
TPS-R	CCTTGGCCACCAGGttcatnccrtc	Amplification of TPS core region
TPS-3-F	CGCTCAGGTCGTAGTAGCAGAAGGTC	3' RACE
TPS-3-R	TACCGTCGTTCCACTAGTGATTT	3' RACE
TPS-5-F	GGCTTCTTTCTTCACATTCCGTTCCC	5' RACE
TPS-5-R	CATGGCTACATGCTGACAGCCTA	5' RACE
18s-F	CCGTCCCTTTTGGTGACTCTG	Real-time PCR
18s-R	GGATGTGGTAGCCGTTTCTC	Real-time PCR
TPS-qF	GCTCGTGAAACCGATGATGTCGCAT	Real-time PCR
TPS-qR	ACTGCGTTTTGACTTACCCTAACG	Real-time PCR

Searches for nucleotide and amino-acid sequence similarities were performed with BLAST programs on the NCBI website (http://www.ncbi.nlm.gov/BLAST/). The open reading frame (ORF) was identified using the ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html), and the motif sequences were searched with the InterPro software (Hunter et al 2009). The resulting amino-acid sequence was predicted for theoretical molecular weight and isoelectric point using the ProtParam software (Gasteiger et al, 2005). Multiple sequence alignments and phylogenetic analysis were performed on amino-acid sequences of known TPS from other species using CLUSTALW and MEGA 6.0 (Tamura et al, 2013). The amino-acid sequences of TPS used in the phylogenetic study included TPS sequences of *A. simplex* (KJ560557.1), *C. sapidus* (EU679406.1), *D. melanogaster* (NM_134983.3), *S. exigua* (FJ792706.1), *P. haitanensis* (KF658272.1), *F. chinensis* (EU555435.1), *P. freudenreichii* (DQ356268.1), *C. elegans* (AJ811574.1), *C. bairdi* (EU910084.1), *B. antarctica* (JX462664.1), *P. savastanoi* (AY308798.1), *E. coli* (FJ895834.1), *L. migratoria* (EU131894.1), and *H. armigera* (DQ086235).

Quantitative real-time PCR analysis of *TPS* mRNA expression

The mRNA expression levels of the *TPS* gene from the foot muscle of each sampled individual were measured by using fluorescent quantitative PCR (qPCR). Amplifications were performed in a 25 µL reaction volume including 12.5 of 2X SYBR Premix Ex Taq™ master mix (TaKaRa, Japan), 1 µL each of gene-specific primers (Table 1) and 1 µL of 1:29 diluted cDNA. qPCR analysis was carried out on IQ™ 5 Multicolor Real-Time PCR detection system (Bio-Rad, USA). PCR conditions were as follows: 95°C for 1 min, followed by 45 cycles at 95°C for 10 s, 63°C for 25 s, and a plate read. At the end of each qPCR, melting curve analysis of amplification products was performed, and qPCR data were collected as *Ct* value. 18S RNA gene was cloned in both *P. canaliculata* and *P. maculate* and used as internal control. The relative expression levels of genes were calculated by $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). All data were expressed as the mean ± standard deviation (SD), and significant difference between the two *Pomacea* species at the same temperature or at the same time point was identified by an unpaired Student *t*-test. $P < 0.05$ (*) indicates significance, and $P < 0.01$ (**) was considered highly significant.

RESULTS

Molecular characteristics of the *TPS* gene

The full-length cDNAs of *TPS* in the two *Pomacea* species, namely, *P. canaliculata and P. maculate,* were obtained. The cDNAs contained 1908 bp and 1914 ORFs encoding 635 and 637 amino acids (Figures 1A and 1B), respectively. Both *Pomacea TPS* genes contain a classical polyadenylation signal sequence AATAAA and poly (A). *PcTPS* contained a 5′-UTR of 151 bp, a 3′-UTR of 95 bp, while *PmTPS* contained a 5′-UTR of 167 bp and a 3′-UTR of 102 bp. The molecular weights are 73842 and 73954 Da for *P. canaliculata and P. maculate*, respectively. The *PcTPS* aa sequence is highly homologous (99% identity) to that of *PmTPS*, and high shared identities with other TPSs were also observed: 93% to *C. elegans*, 85% to *L. migratoria*, 70% to *A. simplex*, 61% to *C. sapidus*, *78%* to *D. melanogaster*, 60% to *S. exigua*, 71% to *P. haitanensis*, 60% to *F. chinensis,* 65% to *P. freudenreichii*, 61% to *C. bairdi*, 59% to *B. antarctica*, 53% to *P. savastanoi,* 55% to *E. coli*, and 61% to *H. armigera*. The alignment of *TPS*s from insects, bacteria, yeast, fungi, nematodes, and plants also showed that two conserved motifs, namely, HDYHL and DGMNLV, were found in both *PmTPS* and *PcTPS*. These motifs may be the signature sequences of *TPS*s and putative catalytic domains (Figures 1A and 1B).

Phylogenetic analysis

Based on the amino-acid sequences of *Pomacea TPSs*, a phylogenetic tree was constructed by using MEGA 6.0 using neighbor joining method. Fourteen *TPSs* family members were selected for analysis. Sequences of *PcTPS* and *PmTPS* were clustered into a large group with *A. simplex*. The phylogenetic relationships displayed in the phylogenic tree were consistent with the traditional classification. (Figure 2)

Expression of *TPS* mRNA in response to temperature gradient shock stress

The relative mRNA levels of the two TPSs were quantified by real-time quantitative PCR at temperature gradients from 3°C to 42°C. Results revealed that the two TPS transcript levels were slightly decreased under high temperature (30°C, 33°C, 36°C, 39°C, and 42°C) compared with these at 26°C. Under cold treatment (3°C, 6°C, 9°C, 12°C, and 15°C), significant upregulation of the two TPSs were observed. The increase in *PmTPS* expression with temperature for *P. maculata* was less steep in compared with that in *PcTPS* for *P. canaliculata*. The highest expression level that was observed at 6°C was tenfold that at 26°C in *P. canaliculata*, whereas that at 9°C was six fold that 26°C in *P. maculata*. The maximal induction of *PcTPS* was significantly higher compared with *PmTPS*. After maximal induction, the expression level of the two TPSs significantly decreased in both *Pomacea* species (Figure 3).

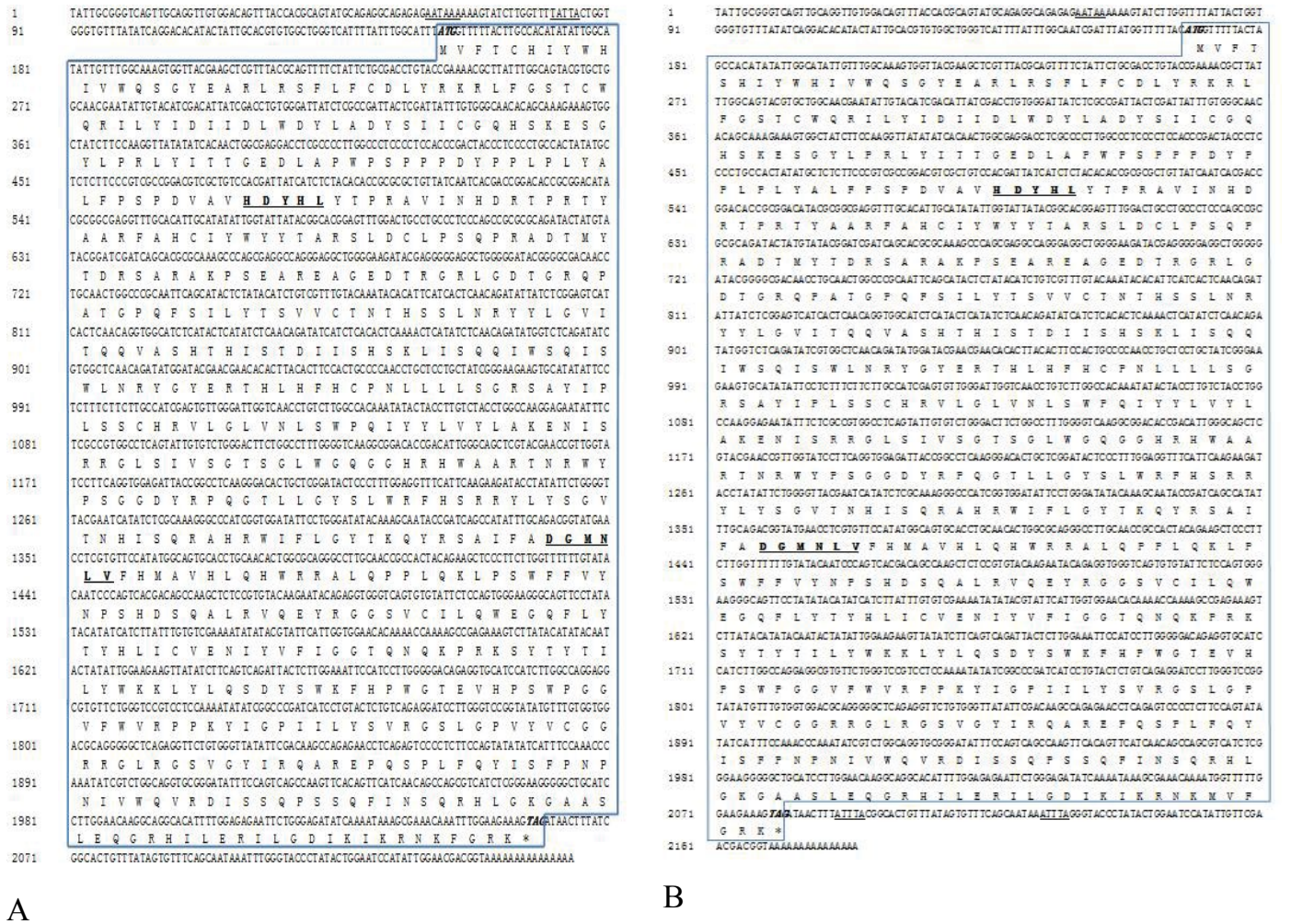

Figure 1. A: Nucleotide and deduced amino-acid sequences of *P. canaliculata PcTPS*. **B:** Nucleotide and deduced amino-acid sequences of *P. maculata PmTPS*. Both initiation codon and termination codon were indicated in bold font and italics. Motifs (or signature motifs) that are unique to trehalose-6-phosphate synthase (*TPS*) are underlined and bold.

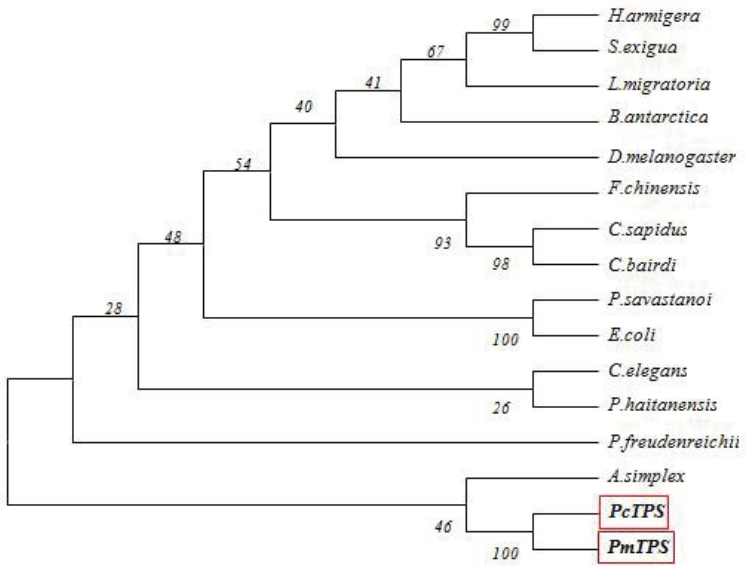

Figure 2. Phylogenetic relationships between the amino-acid sequences of *TPS* from both *Pomacea* species and other known *TPS*s. The tree was constructed by using MEGA 6.0 with neighbor joining method, and bootstrap confidence values (percentage of 1000 replicates) are shown at each branch point. The identified *Pomacea* snail *TPS* sequence is labeled with a box.

Interspecific differences of *TPS* gene expression

For determining the temperature for onset (T_{on}) and maximal (T_{max}) induction of TPS expression, the relative mRNA levels of TPSs were compared with each other, and an interspecific difference was observed in most comparisons. TPS started to be induced at 12°C in *P. canaliculata* and peaked at 6°C, whereas T_{on} and T_{max} in *P. maculate* were 15°C and 9°C, respectively.

Both T_{on} and T_{max} shifted by 3°C between the two *Pomacea* species under cold treatments. Under heat stress conditions, the *TPS* relative expression levels in both *Pomacea* species were slightly decreased in the foot muscle in comparison with that at normal temperature, and the species effect was not significant. (Figure 3)

Temporal expression profiles of *TPS* transcripts after a thermal challenge

RT-PCR was conducted to examine the time-dependent expression pattern of the two *TPS* genes in the foot muscle of *P. canaliculata* and *P. maculata* at 2, 6, 12, 24, 48, 72, 96, and 120 h after cold treatment (12°C), A clear time-dependent expression pattern of *TPS* was observed (Figure 4) in two *Pomacea species*. After cold treatment, the expression of the *PcTPS* gene was upregulated and reached the highest level at 48 h in *P. canaliculata*. However, the expression gradually decreased after 72 h and then returned to its baseline value after 96 h. The expression of *PmTPS* was induced after 2 h in *P. maculata* and peaked at 24 h. The expression significantly decreased after 48 h and then returned to its baseline level after 96 h. The highest expression level was markedly higher in *P. canaliculata* compared with *P. maculata* (Figure 4). The *TPS* expression levels at 12, 24, 48, and 72 h were significantly different between the two *Pomacea* species.

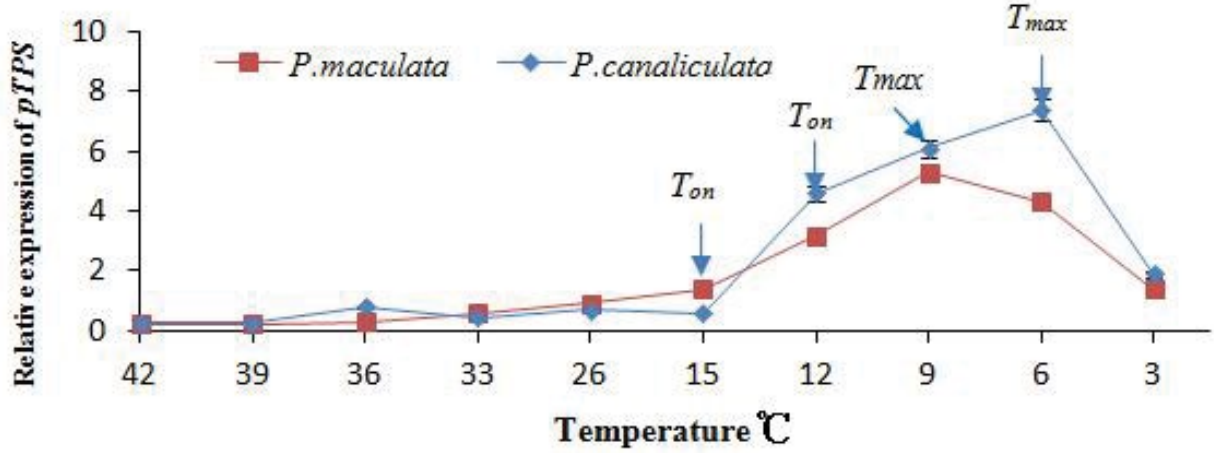

Figure 3. mRNA expression profiles of TPSs in two *Pomacea* species. The relative levels of *TPS* mRNAs were examined at temperature gradients. The first temperature at which the expression level was significantly higher than that of the 26 °C treated samples was described as the onset temperature (T_{on}) of the synthesis of a particular TPS, and the temperature at which the expression level was significantly higher than those of all the others was denoted as T_{max}. T_{on} and T_{max} are marked by the arrow " ", and marked temperature shifts of T_{on} and T_{max} are indicated on the curves. The letters "*T*" and "*t*" represent the temperature used in *P. canaliculata* and *P. maculate*, respectively. The values are expressed as mean ± SD.

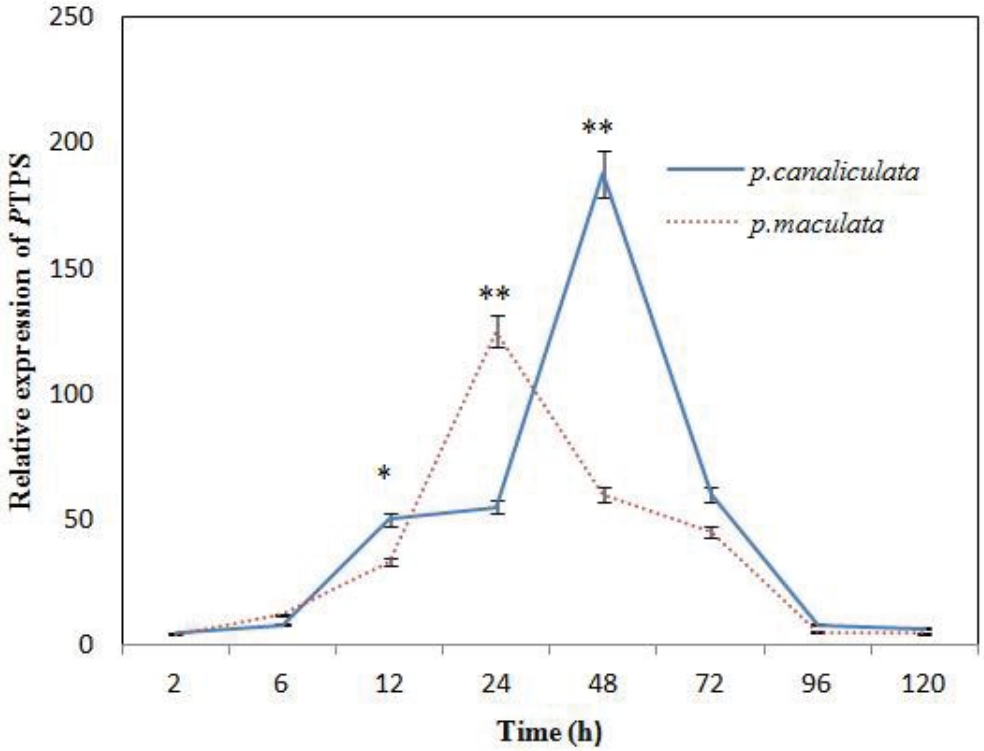

Figure 4. mRNA expression analysis of *TPSs* in two *Pomacea* species: *P. canaliculata* and *P. maculata* at different time points after cold challenge (12°C). Transcript levels for all samples were assessed by quantitative real-time PCR with SYBR Green. The values are expressed as the mean ± SD (n = 4). The asterisks above the lines indicate significant differences (*P < 0.05; **P < 0.01) between *P. canaliculata* and *P. maculata* in an unpaired Student *t*-test.

DISCUSSION

Golden apple snails of the genus *Pomacea* (Mollusca: *Ampullariidae*) are some of the largest freshwater snails. These snails damage various crops, with rice as the major host, through eating rice seedlings during the first three weeks after transplanting. Golden apple snails have been ranked in the top 100 of the worst invasive species worldwide (Lowe et al, 2000). Enormous snail populations have developed an ecologically adaptive mechanism in response to all types of stresses during invasion, and temperature adaptability of species is speculated to be one of the most important attributes (Yusa et al, 2006). Trehalose is a non-reducing disaccharide that is a primary energy source for prokaryotes, yeasts, plants, and invertebrates. In addition, trehalose is an important protectant of protein integrity, performing an important function in physiological and biochemical adaptations under extreme environmental conditions (Goyal et al, 2005; Zhang et al, 2012). *TPS*, which is a protein in the biosynthesis of trehalose, is regarded as the key enzyme to catalyze the first step in trehalose synthesis. Increasing evidence has demonstrated the importance of trehalose as molecular protectant in resistance to heat, cold, and a range of other biotic and abiotic stressors. Numerous TPS genes have been reported, including *S. exigua* (Tang et al, 2010), *A. aegypti* (GenBank: XM_001657763), *D. melanogaster* (GenBank: NM_134983), *H. armigera* (GenBank: DQ086235), and *F. chinensis* (Zhang et al, 2012).

The *TPS* mRNA expression in both *Pomacea* species significantly increased under cold treatments but slightly decreased under heat treatments. This finding suggests that a specific amount of *TPS* mRNA is present in foot muscle tissues under both stressed and non-stressed conditions, which may perform an important function in physiological processes and the prevention of cellular damage under temperature stressors. However, the expression level of *TPS* genes under heat stress were not significantly changed in comparison with that under normal temperature in both *Pomacea* apple snails. The same expression pattern was demonstrated in maize (Jiang et al, 2010), *Chilo suppressalis* (Zhang, 2007), and *Harmonia axyridis* (Qin et al, 2012). *TPS* may perform a lesser role in heat resistance. In the present study, we found that, under short-term cold treatment, *TPS* expression was markedly increased with decreasing temperature. Under long-term cold treatment, the TPS expression level initially increased and then decreased to baseline levels after 96 h in both *Pomacea* species. This result may be attributed to the conversion of trehalose to low molecular weight compounds, such as glycerol and amino acids, which could enhance cold hardiness in winter. This phenomenon is similar to those in insects.

The onset or maximal induction temperatures of *TPS* expression were used to interpret temperature tolerance. Under heat treatments, *TPS* could not be induced either in *P. canaliculata* or *P. maculata* at 26°C-42°C, revealing that no positive correlation was found between *TPS* expression levels and thermal tolerance. This result suggests that trehalose synthesis is not required for the induction of heat tolerance for *Pomacea* apple snails. Under cold treatment, the expression levels of *TPS* gene transcripts significantly increased in both *Pomacea* species and peaked at 6°C in *P. canaliculata* and 9°C in *P. maculata*, thereby suggesting that *P. maculata* was more susceptible to cold conditions than *P. canaliculata.* Previous studies revealed that a positive relationship exists between *TPS* expression levels and low temperature tolerance in organisms (Tang et al, 2010; Jiang et al, 2010; Zhang et al, 2012). However, further investigation is necessary to confirm this hypothesis because only a limited number of populations were examined in the present study. In particular, research that separates the influence of habitats on environmental stress from the influence of hybridization is required. High expression levels of the *TPS* gene enables organisms to survive during cold stress by repairing denatured proteins, and organisms could adapt to a wide range of temperature changes and resist cold stress. The maximal induction of *TPS* expression was 1.4 and 1.5 times higher in P. canaliculata compared with P. macu*lata* under different cold temperature and long-term cold treatment, respectively. This finding indicates that *P. canaliculata* may be more resistant to cold than *P. maculata*, as was consistent with observations that the *P. maculata* population may be less tolerant to cold weather than *P. canaliculata*. Thus, *P. canaliculata* snails may possess better ability to colonize Asian paddy ecosystems than *P. maculata* (Yoshida et al, 2014). Apple snail populations may have suffered short-term cold weather in winter, which may limit the expansion of apple snails.

Previous studies showed that different species and populations of apple snails exhibit varied resistances in response to low temperature (Yoshida et al, 2014; Matsukura et al, 2008). Thus, cold stress resistance is a complex trait. Numerous differentially expressed genes are assumed to be involved in the difference in cold resistance in *P. canaliculata* and *P. maculata*. Thus, identifying differentially expressed genes under cold conditions is necessary for further understanding the molecular mechanism underlying the cold resistance difference in both *Pomacea* snails.

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