Cloning and pattern of expression of trehalose-6-phosphate synthase cDNA from *Catantops pinguis* (Orthoptera: Catantopidae)

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**Abstract.** Trehalose is not only an important disaccharide, but also a key stress resistance factor in the development of many organisms, including plants, bacteria, fungi, and insects. To study the potential function of trehalose in development and behaviour, cDNA for a trehalose-6-phosphate synthase from *Catantops pinguis* (CpiTPS) was cloned and sequenced. Results revealed that the CpiTPS cDNA sequence contains an open reading frame of 2430 nucleotides encoding a protein of 809 amino acids with a predicted molecular weight of 91.13 kDa and a pI value of 6.25. Northern blot and RT-PCR analyses showed that CpiTPS mRNA expression was high in the fat body and testes, ovaries, Malpighian tubules, brain, trachea, rectum, and posterior extensor of *C. pinguis*. Northern blotting revealed that CpiTPS mRNA was expressed in the fat body at different developmental stages and was present at a high level in first instar larvae and adults. The results demonstrate that CpiTPS is a key gene in *C. pinguis* development.

**INTRODUCTION**

Abiotic stress is the most important limiting constraint on crop productivity and yield, and could compromise food supply for the ever-increasing human population in the future. Organisms have evolved different strategies to cope with abiotic stress (Avonce et al., 2006). First, some species that live in extreme environments can modify features of their metabolic capability such as optimal enzyme activity and membrane stability (Madigan & Oren, 1999). Second, some organisms have evolved a drastically different adaptation to cope with stress when exposed to extreme conditions. They evolved biosynthetic pathways for compounds such as mannitol, sorbitol, sucrose, and trehalose, which are utilized for survival until conditions become favourable again (Yancey et al., 1982). In the case of insects, they accumulate large amounts of trehalose, a blood sugar, in extreme environments.

Trehalose (α-D-glucopyranosyl-1,1-α-D-glucopyranoside) is an important disaccharide (Birch et al., 1963; Elbein, 1974; Ryu et al., 2005), which is widespread among bacteria, yeasts, fungi, nematodes, plants, insects and some other invertebrates (Crowe et al., 1984; Gadd et al., 1987; Strom & Kaasen, 1993; Benaroudj et al., 2001). In plants, trehalose plays a role as an anti-stress substance by protecting against drought, high salt, and low temperature (Garg et al., 2002; Elbein et al., 2003, Jang et al., 2003). It can stabilize dehydrated enzymes, proteins, and lipid membranes efficiently and protects biological structures against damage during desiccation (Fillinger et al., 2001; Garg et al., 2002; Elbein et al., 2003).

There are three main ways for organisms to synthesize trehalose in vivo (Kong et al., 2001). The first is via catalysis by trehalose synthase, as in some bacteria (*Thermus aquaticus*), which can convert maltose to trehalose by transglycosidation (Tsusaki et al., 1997; Kong et al., 2001). The second is via the reaction of β-glucose 1-phosphat-phosphate catalyzed by trehalose-6-phosphate phosphatase, as in *Micrococcus varians* (Kizawa et al., 1995). Many organisms have adapted the latter mechanism to synthesize trehalose via a two-enzyme system of trehalose-6-phosphate synthase (TPS) and trehalose-6-phosphate phosphatase (TPP), which requires a high-energy intermediate of glucose metabolism as a substrate (Kong et al., 2001).

In insects, trehalose is a major blood sugar. It is synthesized mainly by the fat body (an organ analogous in function to a combination of the liver and adipose tissue in vertebrates) and is released rapidly into the hemolymph and other tissues in larvae, pupae, and adults (Candy & Kilby, 1959, 1961; Elbein, 1974; Becker et al., 1996; Tang et al., 2008). It is reported that trehalose is synthesized from glucose phosphate. Two enzymes are directly involved in this: TPS forms trehalose-6-phosphate from glucose 6-phosphate and UDP-glucose, after which TPP removes the phosphate to release trehalose into the hemolymph (Candy & Kilby, 1959, 1961). However, molecular

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level evidence for this is lacking. During this process, TPS and TPP both play an important role in the regulation of trehalose production for growth and development (Elbein et al., 2003; Wang et al., 2005b). Chen et al. (2002) and Chen & Haddad (2004) report that trehalose (Elbein et al., 2003; Wang et al., 2005b). Chen et al. (2002, 2003; Chen & Haddad, 2004).

To date, although many TPS genes have been cloned or deduced from genomic sequences, there are few such studies on insects, mainly Drosophila melanogaster (Chen et al., 2002, 2003; Chen & Haddad, 2004). TPS and TPP genes have been cloned from plants, bacteria, and fungi, but none have been identified in mammals. In arthropods, TPS and TPP have been cloned or deduced from genomic sequences (according to GenBank) and are known to be very important for insect development; however, there are only a few studies on TPS genes and the details of protein characteristics in insects: D. melanogaster (Chen et al., 2002), Callinectes sapidus (Chung, 2008), Locusta migratoria (Cui et al., 2009), Helicoverpa armigera (Xu et al., 2009), and Spodoptera exigua (Tang et al., 2010). Catantops pinguis (syn. Diabocolatantops pinguis) is a worldwide pest of agriculture and is a very strong flyer which requires a lot of energy, so trehalose synthesis is important in C. pinguis. In the present study, TPS cDNA from C. pinguis was cloned (GenBank accession no. GQ389790) and the tissue distribution and pattern of expression of this gene investigated.

**MATERIAL AND METHODS**

C. pinguis were maintained in the laboratory over a period of 1 year. Larvae were reared at 26 ± 1°C, 65% humidity and an 14L : 10D photoperiod on a diet of wheatgrass. The developmental stages were synchronized at each moult by collecting new adult larvae. The brain, trachea, midgut, rectum, testes, ovaries, Malpighian tubules, posterior extensor, heart, body wall, and fat body of adults, and the fat body of different stages, from larvae to adults, were dissected in saline containing 0.75% NaCl and stored at –80°C until required (Kumar et al., 2008).

**RNA isolation and cDNA synthesis**

Total RNA was extracted from the fat body of adult C. pinguis using the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski & Sacchi, 1987). Fat body (100 mg) was homogenized in solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol) and placed on ice for 5 min, and then sodium acetate and chloroform/isomyl alcohol (49 : 1) were added. The mixture was centrifuged at 10,000 × g at 4°C for 20 min. The supernatant was transferred to a new tube and isopropanol added. After centrifugation, the RNA pellet was washed with 75% ethanol and then dissolved in ddH2O. A sample of 1 mg of total RNA was reverse transcribed at 42°C for 1 h in 10 µl of reaction solution containing reaction buffer, 10 mM DTT, 0.5 mM dNTPs, 0.5 µg of oligo-dT18, and 5 unit reverse transcriptase from avian myeloblastosis virus (AMV, Takara, Dalian, China).

**Primer design and PCR**

Four degenerate primers (TPS-DF1, TPS-DF2, TPS-DR1, and TPS-DR2; Table 1) were designed based on the conserved amino acid sequences of known TPS forms. The first PCR reaction was performed with primers TPS-DF1 and TPS-DR1 using the following conditions: three cycles of 40 s at 94°C, 40 s at 45°C, and 120 s at 72°C, followed by 30 cycles of 40 s at 94°C, 40 s at 48°C, and 120 s at 72°C. A second PCR was carried out using the nested primers TPS-DF2 and TPS-DR2 using the same conditions as for the first PCR (Rinehart et al., 2003; Tang et al., 2008). The PCR products were subjected to agarose electrophoresis. A strong DNA band corresponding to the expected size of approximately 1700 bp was excised from the agarose gel and purified using a DNA gel extraction kit (Takara, Dalian, China). The PCR products were cloned into the pMD18-T vector (Takara, Dalian, China) and sequenced using the dideoxynucleotide method (Takara, Dalian, China).

**Table 1. The primers for CpiTPS cDNA cloned, Northern blot, and RT-PCR.**

<table>
<thead>
<tr>
<th>PCR fragment</th>
<th>Name</th>
<th>Direction</th>
<th>Type</th>
<th>Nucleotide sequence (5′–3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intermediate fragments</td>
<td>TPS-DF1</td>
<td>F</td>
<td>D</td>
<td>TGG NNT NTG GGT NNG HTG G</td>
</tr>
<tr>
<td></td>
<td>TPS-DF2</td>
<td>F</td>
<td>D</td>
<td>GCT GYA ACG VNA CBT TYT GG</td>
</tr>
<tr>
<td></td>
<td>TPS-DR1</td>
<td>R</td>
<td>D</td>
<td>GCC ATW CCY TTS ARV GCC</td>
</tr>
<tr>
<td></td>
<td>TPS-DR2</td>
<td>R</td>
<td>D</td>
<td>ATC YTR ATN ATN ATC TCR CTC C</td>
</tr>
<tr>
<td>Probe</td>
<td>CpiTPS-FP</td>
<td>F</td>
<td>G</td>
<td>CTG CTG TGC GAA CAC GCC</td>
</tr>
<tr>
<td></td>
<td>CpiTPS-RP</td>
<td>R</td>
<td>G</td>
<td>CTT CAT AGT CCA GCA GCA</td>
</tr>
<tr>
<td>5′-RACE</td>
<td>CpiTPS-5R1</td>
<td>R</td>
<td>G</td>
<td>AGC AAA CAG TTC ATT CAC AC</td>
</tr>
<tr>
<td></td>
<td>CpiTPS-5R2</td>
<td>R</td>
<td>G</td>
<td>TGC TGT TGA CTG CTC CTC CA</td>
</tr>
<tr>
<td></td>
<td>CpiTPS-5R3</td>
<td>R</td>
<td>G</td>
<td>CTG TCT GAC CCA ATT TGC TG</td>
</tr>
<tr>
<td>3′-RACE</td>
<td>CpiTPS-3F1</td>
<td>F</td>
<td>G</td>
<td>CTC TGA TTG AGC AGG CAG GA</td>
</tr>
<tr>
<td></td>
<td>CpiTPS-3F2</td>
<td>F</td>
<td>G</td>
<td>AGC TGG ATC TGCC CAAG TCG TG</td>
</tr>
<tr>
<td></td>
<td>CpiTPS-3F3</td>
<td>F</td>
<td>G</td>
<td>GCA AAG CCT CCG GTA CAG TG</td>
</tr>
</tbody>
</table>

The full-length CpiTPS cDNA sequence was determined by sequencing two overlapping PCR fragments using fat body cDNA as a template. The probe primers were for Northern blot and RT-PCR experiment. *F* – Forward; *R* – Reverse; *D* – degenerate primer; *G* – gene specific primer; *A* – anchor primer.
**Rapid amplification of cDNA ends (RACE)**

For 5´- and 3´-RACE, cDNA was synthesized according to the manufacturer’s protocol (BD SMART RACE cDNA Amplification Kit., Clontech of Takara, Dalian, China). Specific primers (CpiTPS-5R1/CpiTPS-5R2 for 5´-RACE and CpiTPS-3F1/CpiTPS-3F2 for 3´-RACE; Table 1), were synthesized based on the cDNA sequence of the PCR fragment. 5´-RACE was performed using 2.5 µl of 5´-ready-cDNA with Universal Primer Mix (UPM, Clontech) and CpiTPS-5R1, then nested PCR was carried out with Nested Universal Primer A (NUP, Clontech) and CpiTPS-5R2. 3´-RACE was performed using 2.5 µl of 3´-ready-cDNA with UPM and CpiTPS-3F1, then with NUP and CpiTPS-3F2. The PCR conditions were as follows: 10 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 60°C, and 80 s at 72°C, then 10 min at 72°C (Wang et al., 2005a; Tang et al., 2010).

**Analysis of the CpiTPS cDNA sequence**

The CpiTPS cDNA sequence was compared with other TPS sequences deposited in the GenBank using the BLAST-N and BLAST-X tools on the National Center for Biotechnology Information (NCBI) website. The amino acid sequence of CpiTPS was deduced from the corresponding cDNA sequence using the translation tool on the ExPASy Proteomics website (http://expasy.org/tools/dna.html). Multiple alignment of insect TPS sequences was performed using the tool on the multiple sequence alignment website http://bioinfo.genotoul.fr/multalin/multalin.html.

**Northern blot and RT-PCR analysis**

CpiTPS mRNA expression at different development stages and different seasons was determined by Northern blot and RT-PCR analysis. Total RNA was extracted from brain, trachea, midgut, rectum, testes, ovaries, Malpighian tubules, posterior extensor, heart, body wall, and fat body of young adults. Samples of 1 mg of total RNA were reverse transcribed at 42°C for 1 h in a 10-µl reaction solution containing reaction buffer, 10 mM DTT, 0.5 mM dNTPs, 0.5 µg of oligo-dT18, and 5 unit reverse transcriptase from avian myeloblastosis virus (AMV, Takara, Dalian, China). The product was used as the RT-PCR template. Another 25 µg of total RNA was subsequently blotted onto a Hybond-N+ membrane (Amersham of GE Healthcare, Little Chalfont Buckinghamshire, UK). Membranes were pre-hybridized at 42°C for 1 h and then incubated with the α-32P-labelled CpiTPS probe (same probe as described for Southern blotting) at 42°C for 18 h in 5× SSPE containing 50% formamide, 0.5 µg of oligo-dT18, and 5 unit reverse transcriptase from avian myeloblastosis virus (AMV, Takara, Dalian, China). The product was used as the RT-PCR template. Another 25 µg of total RNA was subsequently blotted onto a Hybond-N+ membrane (Amersham of GE Healthcare, Little Chalfont Buckinghamshire, UK). Membranes were pre-hybridized at 42°C for 1 h and then incubated with the α-32P-labelled CpiTPS probe (same probe as described for Southern blotting) at 42°C for 18 h in 5× SSPE containing 50% formamide, 5× Denhardt’s solution, 0.1% SDS, and 100 µg/ml of Hybridization solution was then applied to the membrane.
salmon sperm DNA. After hybridization, the membrane was washed with 0.2× SSPE at 45°C, and exposed to X-ray film at –70°C for 24 h (Choo et al., 2007; Tang et al., 2008).

RT-PCR was performed with the CpiTPS-FP/CpiTPS-RP primers. cDNA from brain, trachea, midgut, rectum, testes, ovaries, Malpighian tubules, posterior extensor, heart, body wall, and fat body was used as templates under the following conditions: 30 cycles of 40 s at 94°C, 40 s at 55°C, and 60 s at 72°C. Each PCR product (5 µl) was subjected to electrophoresis and detected by ethidium bromide staining; the amount of S. exigua ß-actin per lane was used as a loading control (Tang et al., 2008).

**RESULTS**

**Sequence analysis of CpiTPS cDNA**

CpiTPS cDNA (GenBank accession no. GQ389790) was obtained by PCR and 5'- and 3' -RACE. CpiTPS cDNA has an open reading frame of 2430 nucleotides (Fig. 1), which encodes a protein of 809 amino acids with a predicted mass of approximately 91.13 kDa and a pI value of 6.25. CpiTPS is from 68–93% identical in structure to other known insect TPS forms (Fig. 2).
The deduced amino acid sequence of CpiTPS was aligned with TPS forms from other species, as shown in Fig. 2. CpiTPS is most similar to the TPS from Locusta migratoria (93% identity). It is also similar to TPS forms from Apis mellifera (79%), Nilaparvata lugens (76%), Acrithosiphon pismum (76%), Pediculus humanus corporis (76%), Tribolium castaneum (75%), Harmonia axyridis (73%), S. exigua (73%), Anopheles gambiae (73%), D. melanogaster (71%), Aedes aegypti (71%), Culex quinquefasciatus (71%), Drosophila erecta (71%), Drosophila grimshawi (71%), Drosophila mojavensis (71%), Drosophila pseudoobscura (71%), Drosophila virils (71%), Drosophila willistoni (71%), Drosophila yakuba (71%), Drosophila sechellia (69%), and Polypedilum vanderplanki (68%).

Conserved sequence analysis of TPS

Multiple sequence alignment of TPS proteins showed a high degree of conservation, particularly in the middle of the putative catalytic domain (Fig. 3). CpiTPS has two motifs (HDYHL and DGMNLV) that are conserved in TPS proteins, as well as many more conserved sequences, such as VVSNRLPF, ASAGGLVTA, FWPLFHSMP, FFLHIPFPPW, LGVDRLDYTKGLVHRL, LVTPLRDG.
Tissue distribution of CpiTPS

Tissue-specific expression of CpiTPS was determined by Northern blotting and RT-PCR. CpiTPS transcripts were detected in fat body, testes, ovaries, Malpighian tubules (Mt), posterior extensor (Pe), heart (He), body wall (Bw), and fat body (Fb). A CpiTPS specific probe was radiolabelled with [α-32P]-dCTP. Following hybridization and detection by autoradiography using the CpiTPS probes, membranes were stripped by boiling in 0.1% SDS. rRNA was used as a control.

Expression of CpiTPS during development

Northern blot experiments were carried out to analyze the expression patterns of CpiTPS in fat body during the development of C. pinguis from first instar larva to adult. The results showed that CpiTPS mRNA is expressed in the fat body at different levels at different developmental stages. CpiTPS transcripts were highly expressed in fat body in early first instar larvae, as well as in early and middle second instar larvae, late fifth instar larvae, and early sixth instar larvae and adults. Transcripts were present at a lower level in fat body of late first and second instar larvae, early, middle and late third and fourth instar larvae, early and middle fifth instar larvae, and middle sixth instar larvae (Fig. 5). These results suggest that CpiTPS mRNA was expressed in the fat body according to the need of the different developmental stages, but most markedly in the egg to first instar larva and last instar larva to adult stages.

Relative expression of CpiTPS in different seasons

RT-PCR experiments to measure CpiTPS expression in adult fat body in different seasons of the year (from 17 May to 29 October) revealed that CpiTPS mRNA levels varied. CpiTPS transcripts were highly expressed in fat body in early July and mid-October. Transcripts were present at a lower level in fat body in early and mid-June, mid-August, and early and late October. Transcripts were hardly ever expressed in mid- and late May, and late June and September (Fig. 6). The results suggest that CpiTPS mRNA is expressed at varying levels in the fat body depending on developmental and behavioural requirements.
DISCUSSION

It is reported that trehalose is exclusively synthesized by the fat body (Candy & Kilby, 1959, 1961) from glucose phosphates and UTP. Many TPS genes have been cloned and reported from plants, yeast, and bacteria. An insect TPS gene was first cloned from D. melanogaster, encoding a protein of 809 amino acids and containing conserved domains similar to both yeast Tps1 (OtsA) and Tps2 (OtsB) (Chen et al., 2002). These authors, however, did not analyze the condition of the signature motifs or some protein characteristics. But there are two signature motifs (HDYHL and DGMNLV) according to the alignment of TPS protein sequences for insects, plants, bacteria, fungi, and nematodes. In C. pinguis, CpiTPS not only contains these signature motifs (Fig. 1), but also has other conserved motifs, such as VVSNRLPF, ASAGGLVTA, FWPLFHSMP, FFLHIPFPPW, LGVDRLDYTKGLV HRL, LVTPLRDGMNLVAKEFVACQIN, KLALLLDY DGTLAPIA, TYAGNHGLEILHPD, and ENKGALL TFH (Fig. 3).

TPS and TPP genes have been cloned from bacteria, fungi, and plants. In the genome of the plant Arabidopsis thaliana, there are 21 genes homologous to TPS that may have various functions (Leyman et al., 2001). In insects, the TPS gene was first cloned from D. melanogaster (Chen et al., 2002). Soon after, many TPS and TPP genes were cloned or deduced from genomic sequences, with two functional conserved domains similar to yeast genes, namely OtsA (TPS) and OtsB (TPP) (Chen et al., 2002; Chung 2008). According to the GenBank database, TPS genes have been identified in several insects, including D. melanogaster (BT100029), Macconellicoccus hirsutus (EF092085), Polypedilum vanderplanki (AB490334), C. quinquefasciatus (XM_001660971), A. aegypti (XM_001660971), and A. gambiae (XM_317247). Although TPS is a remarkably conserved gene family, Fig. 2 shows that TPS forms from Crustacea and Insecta can be clearly differentiated. Moreover, TPS forms from different insect species can also be differentiated.

It is reported that SexTPS is specifically expressed in the fat body and ovaries in larval stages, which is consistent with the results of studies on H. armigera (Xu et al., 2009, Tang et al., 2010). In Locusta migratoria, TPS is expressed not only in the fat body, but also in gut, hemolymph, and leg muscle (Cui & Xia, 2009). However, in adult C. sapidus, CsaTPS is expressed in all tissues (Chung, 2008). In adult C. pinguis, TPS was not only expressed in fat body and ovaries, but also in the testes, Malpighian tubules, brain, trachea, rectum, and posterior extensor (Fig. 4). The distribution of CpiTPS in tissues is very similar to that of CsaTPS. These results suggest that the distribution of TPS in tissues may differ not only between larval and adult stages, but also among different arthropod species.

In non-diapausing and diapausing H. armigera, HarTPS mRNA expression was low and high, respectively (Xu et al., 2009). In addition, in S. exigua, semi-quantitative RT-PCR results reveal that SexTPS mRNA is expressed in fat body at different levels from fifth instar larvae to pupae (Tang et al., 2010). However, different patterns of expression of CpiTPS were found and studied (Fig. 5). The seasonal expression of CpiTPS from 17 May to 29 October was also investigated (Fig. 6). There are possibly two reasons for the variations in expression and lack of a regular pattern in different seasons. First, TPS expression is associated with development and behaviour. Second, TPS expression can be induced at particular times, just as TPS mRNA expression varies over the course of a day in nature.

In yeast, trehalose synthesis is mediated by a complex of TPS1 and TPS2. Trehalose has a dual function as a storage carbohydrate and a stress protection metabolite (Kwon et al., 2003; Thevelein, 1996). Transcriptional levels of TPS1 and TPS2 are increased by heat shock, cold or ethanol stress, salt stress, nutrient starvation, and other osmotic stresses (Winderickx et al., 1996; Wolschek & Kubicek, 1997; Soto et al., 2002; Pereira et al., 2001). Improving stress tolerance is a major goal in agriculture. Over expression of TPS or TTP genes from either E. coli or yeast in tobacco, potato, and tomato improved drought tolerance, but only a limited accumulation of trehalose is recorded (Holmström et al., 1996; Romero et al., 1997; Yeo et al., 2000; Cortina Culiáñez-Macià, 2005; Grennan, 2007). Miranda et al. (2007) report that transgenic Arabidopsis thaliana containing yeast TPS and TTP genes exhibited a significant increase in tolerance of drought, freezing, salt, and heat. Thus, a transgenic approach could be a potential tool for improving the stress tolerance of crops. Interestingly, the absence of TPS in Arabidopsis thaliana can result in the death of embryos (Eastmond et al., 2002; Gómez et al., 2006), demonstrating that AtTPS1 is essential for embryo development (Eastmond et al., 2002). In insects, particularly pests, suppressing the expression of the TPS gene inhibits development and may result in death. In D. melanogaster, a mutation in the TPS gene results in young larvae dying, which indicates that the TPS gene plays a critical role in insect development (Chen et al., 2002). SexTPS RNAi also suggest that interference with TPS expression results in the death of larvae (Tang et al., 2010). Thus, TPS is a potential key gene that might also be used to control insect pests.

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