Expression Profiles of the Trehalose-6-Phosphate Synthase Gene Associated With Thermal Stress in *Ostrinia furnacalis* (Lepidoptera: Crambidae)

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**Abstract**

Trehalose is the major blood sugar in insects. Physiological significance of this compound has been extensively reported. Trehalose-6-phosphate synthase (TPS) is an important enzyme in the trehalose biosynthesis pathway. Full-length cDNAs of TPS (*Oftps*) and its alternative splicing isoform (*Oftps_isoformI*) were cloned from the Asian corn borer (ACB), *Ostrinia furnacalis* (Guenée; Lepidoptera: Crambidae) larvae. The *Oftps* and *Oftps_isoformI* transcripts were 2913 and 1689 bp long, contained 2529 and 1293 bp open reading frames encoding proteins of 842 and 430 amino acids with a molecular mass of 94.4 and 48.6 kDa, respectively. Transcriptional profiling and response to thermal stress of *Oftps* gene were determined by quantitative real-time PCR showing that the *Oftps* was predominantly expressed in the larval fat body, significantly enhanced during molting and transformation; and thermal stress also induced *Oftps* expression. Gene structure analysis is indicating that one TPS domain and one trehalose-6-phosphate phosphatase (TPP) domain were located at the N- and C-termini of *Oftps*, respectively, while only the TPS domain was detected in *Oftps_isoformI*. Three-dimensional modeling and heterologous expression were developed to predict the putative functions of *Oftps* and *Oftps_isoformI*. We infer that the expression of *Oftps* gene is thermally induced and might be crucial for larvae survival.

**Key words:** alternative splicing isoform, transcriptional analysis, heterologous expression

Trehalose is a nonreducing disaccharide in which two glucose molecules are linked together through a 1,1-glycosidic linkage. This molecule is widespread throughout the biological world, such as in bacteria, yeast, fungi, insects, and plants (Elbein et al. 2003). In insects, trehalose is predominantly synthesized in the fat body. Serves as the major hemolymph sugar, trehalose released into the hemolymph, and uptake by other tissues. Among the reported biological trehalose synthesis routes (Kong et al. 2001), the OtsA-OtsB route is commendably broad studied and is conserved in insects, plants, fungi, and bacteria. In this pathway, trehalose-6-phosphate synthase (TPS, a homolog of *OtsA* in *Escherichia coli*) and trehalose-6-phosphate phosphatase (TPP, a homolog of *OtsB* in *E. coli*) are involved. TPS transfers glucose from UDP-glucose to glucose-6-phosphate to form trehalose-6-phosphate and UDP. TPP dephosphorylates trehalose-6-phosphate to yield trehalose in *E. coli*, the *otsA* and *otsB* genes present on the same operon (McDougall et al. 1993); in *Spodoptera exigua* (Hübner; Lepidoptera: Noctuidae) (Tang et al. 2010) and *Helicoverpa armigera* (Hübner; Lepidoptera: Noctuidae) (Xu et al. 2009), the *tps* gene is a fused gene, in which the TPS domain locates at the N-terminal and the TPP domain locates at the C-terminal. Alternatively spliced isoform encoding only the TPS domain has been cloned from *S. exigua* (FJ792706.1) and *H. armigera* (DQ086235.2). The genes encoding TPP have been cloned from *Delia antiqua* (Meigen; Diptera: Anthomyiidae) (KJ130467) (Guo et al. 2015), *Drosophila simulans* (Matsumura; Diptera: Drosophilidae) (XP002078502) and *Glossina morsitans morsitans* (ADD19820).

Accumulated trehalose has been indicated as a stabilizer and a protectant of proteins and membranes that guards creatures from adverse environmental stresses, such as dehydration (Watanabe et al. 2002), heat (De Virgilio et al. 1994), desiccation (Reina-Bueno et al. 2012), freezing (Wharton et al. 2000), and anoxia (Chen et al. 2002). Research on the soil bacterium *Rhizobium etli* reported that compared with wild-type organisms, the *otsA* mutant, which is unable to synthesize trehalose in minimal medium, showed impaired growth...
at high temperatures, indicating the importance of trehalose synthesis in the thermotolerance of soil bacteria (Reina-Bueno et al. 2012). In insects, trehalose has been considered as a significant cryoprotectant (Storey and Storey 2012), and may determine overwinter survival indirectly through serve as a fuel at the end of winter (Sinclair 2015).

Trehalose metabolism is associated with many important physiological processes of insects (Becker et al. 1996), including development, molting, metamorphosis (Chen et al. 2010), reproduction (Kamei et al. 2011), and flight (Ge et al. 2011). In Drosophila melanogaster, trehalose plays a critical role during development; a mutant of Tps1 failed to have metamorphosis, the larvae exhibit diet-dependent phenotypes of growth, survived into the late pupal period and died before eclosion (Matsuda et al. 2015). In S. exigua, the survival rate of the 5th instar larvae was decreased after dsSeTPS injection (Tang et al. 2010). In S. exigua, the metamorphosis was disrupted by the RNA silencing of trehalase (Chen et al. 2010). Both TPS and trehalase have been taken into account as potential targets for insect control through RNA interference. In Bombyx mori (L.; Lepidoptera: Bombycidae), the contribution of trehalose to sugar accumulation in the oocytes for the sustenance of development and the viability of embryonic stages has also been reported (Kamei et al. 2011). In Nalaparvata lugens, enhanced flight capacity was achieved after increasing trehalase activity as a result of the administration of sublethal doses of three insecticides (Ge et al. 2011).

The Asian corn borer (ACB), Ostrinia furnacalis (Gueneé; Lepidoptera: Crambidae), is a key insect pest of maize in China (Wang et al. 2000). Yield losses to this insect are estimated at 10–100% and might even result in no harvest in an outbreak year (Wang et al. 2000). In the present study, to extend the current knowledge on trehalose metabolism in ACB, we focused on the Oftps gene to elucidate its structural features, spatio-temporal expression patterns and transcriptional regulations in response to thermal stress. Furthermore, heterologous expression was developed in E.coli, and the enzyme activities of these purified recombinant proteins were determined.

**Materials and Methods**

**Insect Culture and RNA Preparation**

The insects used in this research were provided by Ms. Chunying Liu from the Institute of Plant Protection, Chinese Academy of Agricultural Sciences. This population was originally collected from Huxian, Shaan’xi Province (China, situated at 34°06’35” North latitude, 108°35’36” East longitude) in 2000 and was maintained under laboratory conditions using a semi-artificial diet at 28 ± 1°C, 70–80% relative humidity, and a photoperiod of 16:8 (L:D) h, as previously described (Zhou et al. 1992).

For the temporal expression pattern study, the larvae were collected at the feeding stage (second day after hatching or molting) of each instar and were distinguished by comparing the sizes of larval heads with the samples of larval head capsules of the 1st to 5th instar larvae. New-molting larvae were collected before the head capsule darkening of the 5th instar. Pre-molting and pre-pupae larvae were collected at the wondering stage of the 4th and 5th instar. During the pupal stage, in which the average duration is approximately 7 d in ACB, the samples were collected at three times: new pupae with light brown color and soft epidermis were collected just after pupation; pupae were collected on the fourth day after pupation; and pre-eclosion pupae were collected on the sixth day after pupation. The lifespan of ACB adults is approximately 7 d, and the male/female moths (the wings were removed) were respectively harvested. New moths were collected within 12 h after emergence, and the post-mating moths were collected five days after emergence. To determine the spatial expression pattern, the heads, foregut, midgut, hindgut, fat body, epidermis and trachea were dissected in saline solution (0.75% NaCl) from 5th instar larvae; the hemolymph was gathered. For thermal stress research, the fat body was dissected from 5th instar feeding larvae with similar body sizes (approximately 40 mg/larvae) after exposure to 10°C, 37°C, 39°C, 41°C, and 43°C for 2 h. A set of normally reared larvae was used as controls. All samples were immediately preserved in liquid nitrogen and stored at –80°C until total RNA extraction. One biological sample included 12 individuals. Total RNA was isolated from each sample using the TRIZol reagent (Life Technologies, Carlsbad, CA), and the concentration and purity were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA integrity was confirmed using formaldehyde agarose gel electrophoresis.

**Cloning of Full-Length cDNA Sequence of Oftps**

Degenerate primers DF/DR (Table 1) were designed using DNAMAN v6 (Lynnon Corporation, Vaudreuil-Dorion, Quebec, Canada) based on the conserved nucleotide sequences derived from the alignment of tps cDNA sequences of H. armigera, S. exigua and S. littura (GenBank accession Nos. EU878265.1, EF051258.2 and GU211889.1, respectively). Total RNA (1 μg) extracted from the 5th instar larval fat body

| Primers Position Primer sequence (5'-3') |
|----------------|------------------------------------------|
| For cDNA cloning DF 238 GCAACAGCAAAGGGGMCATGA |
| DR 921 ACGAGTARTCGTTATRTGGA |
| 5'GSP 773 TTCTACTTTCTGTCTGATCC |
| 3'GSP1 218 CAGTGCCAGTCGTTCGTCGTGCAACAG |
| 3'GSP2 625 AGATCAACAGGAGCTTCGCCAGAAG |
| For qPCR qO/TPS-F 2171 GTCTGGAGCTTGCTGAGAGGAT |
| qO/TPS-R 2338 CGAGGACTCGTTCGTGTGGAAGA |
| O/rapL8-F 2338 AAGCCGAGGAAAATCATGC |
| O/rapL8-R 2338 GGTCTTTGCCACCAAGAAT |
| For heterologous expression TPS-F-EcoRI 249 CCGGAATTCCTGAGAGGATATTGTGTTGT |
| TPS-R-NorI 1688 TTATAAGCGGCGCTTACGAGTTCATGCGCCTGAGGAA |
| TPP-F-EcoRI 1800 CCGGAATTCCTCGTACTGAGGAA |
| TPP-R-NorI 2477 TTATAAGCGGCGCTTACGAGTTCATGCGCCTGAGGAA |
was used as template, and the first-strand cDNA was synthesized using TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix Kit (TransGen, Beijing, China). Combined with degenerate primers, Oftps sequence was amplified by high-fidelity PCR polymerase (PrimeSTAR HS, Takara, Dalian, China). Amplification included an initial denaturation at 95°C for 2 min, followed by 35 cycles of 98°C for 10 s, 56°C for 5 s and 72°C for 1 min, and a final elongation at 72°C for 5 min. The PCR product was purified, cloned into the EASY-T1 vector (pEASY-T1 simple cloning kit; TransGen, Beijing), and sequenced. The nucleotide sequence encoding putative Oftps was identified using the BLAST program (http://blast.ncbi.nlm.nih.gov/BLAST.cgi). Gene-specific primers (GSPs, Table 1) were designed based on the sequence. 5′- and 3′-RACE-ready cDNAs were synthesized according to manufacturer instructions (SMART kit, Takara, Dalian, China). RACE PCRs were performed, and the products were individually cloned and sequenced. The full-length Oftps cDNA was assembled using DNAMAN v6.

Sequence Analysis and Molecular Modeling

The gene’s open reading frame (ORF) was identified using DNAMAN v6. Subsequently, the amino acid sequences were deduced. These sequences were scanned for motifs using SMART (Simple Molecular Architecture Research Tool, http://smart.embl-heidelberg.de/) (Letunic et al. 2015). The presence of a signal peptide was confirmed using the SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/) (Petersen et al. 2011). Other parameters were computed using the ProtParam tool (http://web.expasy.org/prot-param/) (Gasteiger et al. 2003). N-glycosylation sites were identified using the NetNGlyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc/). Multiple sequences were aligned using ClustalW, implemented in MEGA 5.10 (Tamura et al. 2011). A maximum likelihood phylogenetic tree was subsequently constructed with a bootstrap of 1,000 replicates. The percentage identities between the aligned TPSs and the Oftps amino acid sequence were calculated using the multiple sequence alignment program Clustal2.1 from MUSCLE analysis (http://www.ebi.ac.uk/Tools/msa/muscle/) (Edgar 2004).

Homology modeling was created using the SWISS-MODEL server (http://swissmodel.expasy.org/) (Biasini et al. 2014). High-quality models were obtained for the Oftps-TPS-TPS and -TPP domain sequences using the structures of E. coli K-12 TPS (PDB code: 1gz5.1.A) and Thermoplasma acidophilum T6PP (PDB code: 1u02.1.A) as templates. The models were visualized using WebLab ViewerLite 5.0 software (Accelrys, Inc., San Diego, California, USA).

Analysis of Oftps mRNA Expression Profiles

Expression levels of Oftps cDNA were examined using quantitative real-time PCR (qPCR). O. furnacalis ribosomal protein L8 (Ofrpl8) was utilized as an internal standard to normalize the expression levels (Liu et al. 2014). The primers (Ofrpl8-F/R and O/Oftps-F/R, Table 1) were validated according to Livak and Schmittgen (Livak and Schmittgen 2001), and were used in both qPCR and semi-quantitative PCR (sqPCR); O/Oftps-F/R were designed to amplify a 168-bp fragment located in the Oftps-tpp domain to ensure that Oftps isoform was not amplified.

qPCR was performed on a PikoReal Real-time PCR thermocycler (Thermo Fisher Scientific, Waltham, MA) using a SYBR premix Ex Taq Kit (Takara, Dalian, China) with three technical replicates and two independent biological replicates. The thermal cycling program included denaturation at 95°C for 60 s, followed by 40 cycles of 95°C for 5 s and 60°C for 34 s. After the cycling protocol, a melting curve analysis from 60°C to 95°C was applied to verify a single PCR product. Relative transcript abundance was calculated based on the difference in threshold cycle (Ct) values between Oftps and Ofrpl8 using the 2^ΔΔCt method (Livak and Schmittgen 2001). The 1st instar larvae, head and the fat body from normally reared 5th instar larvae served as reference samples for temporal, spatial and thermal stress expression profiling analyses. The gene expression levels were analyzed using ANOVA, and the means were separated using Fisher’s protected Least Significant Difference (LSD) test for significance in the SAS program (SAS Institute, Inc.).

sqPCR was performed using Premix Taq (Takara, Dalian, China) with an annealing temperature at 56°C for 30 cycles in parallel with a spatial expression profiling experiment to ensure the housekeeping gene was expressed at relatively constant levels in all tissues. The products were then electrophoresed on 1% agarose gels, and the ratio of the average intensities of the Ofrpl8 and Oftps mRNA signals was considered as the relative expression value.

Heterologous Expression of OftPS Proteins

The primer pairs, TPS-F-EcoRI, TPS-R-NoI and TPP-F-EcoRI, TPP-R-NoI (Table 1), containing endonuclease restriction sites EcoRI and NoI, respectively, were utilized to amplify the sequences encoding for Oftps-TPS-TPS domain (G34^-S513) and Oftps-TPS-TPP domain (L551^-D776). TPS-F-EcoRI and TPS-R-NoI were used to amplify the sequence encoding for both Oftps-TPS-TPS domain (G34^-D776). PrimeSTAR HS (Premix) (Takara, Dalian, China) was used to amplify these fragments. PCR products were purified, digested with EcoRI and NoI restriction endonuclease (NEB, Beijing, China), cloned into the expression vector pET30a (+) (Novagen, Darmstadt, Germany) using the DNA Ligation Kit Ver.2.1 (Takara, Dalian, China) and transformed to Trans1-T1 Phage Resistant Chemically Competent Cells (Transgen, Beijing, China) following the instructions. The recombinant plasmids were sequenced and transformed to Trans BL21 (DE3) Chemically Competent Cells (Transgen, Beijing, China). The target proteins were expressed in E. coli (ArtMedia Protein Expression; Transgen, Beijing, China) and affinity purified on a Ni-Agarose column. The purified proteins were concentrated using Centricon-10 centrifugal filters (Millipore, Ireland), separated on 10% SDS-PAGE gel electrophoresis and detected by staining with Coomassie Brilliant Blue, desalted using Zeba Desalt Spin Columns (Thermo Scientific, Uppsala, Sweden), and the concentration was determined using the Easy Protein Quantitative Kit (Transgen, Beijing, China).

Determination of Enzyme Activity

Enzyme activity of purified proteins was measured as described previously (Mitsumasa et al. 2010) with some modification. The reaction mixture, containing purified protein, either 0.1 μmol Oftps-TPS-TPS-TPP domain (G34^-D776) or mixture of 0.1 μmol Oftps-TPS-TPS domain (G34^-S513) and 0.1 μmol Oftps-TPS-TPP domain (L551^-D776), 20 mM phosphate buffer (PBS, pH 5.8), 2.5 mM glucose-6-phosphate, 2.5 mM UDP-glucose and 2.5 mM MgCl₂, and the volume was brought up to 100 μl with PBS buffer. Incubated at 37°C for 1 h, terminated by boiling for 5 min and centrifuged at 12,000g for 10 min at 4°C, the supernatant was transferred to a new Eppendorf tube for trehalose content test. 28 μl of 1% H₂SO₄ was added, and the tube was boiled for 10 min; 28 μl of 30% NaOH was added after the sample cooled on ice for 3 min; subsequently, the sample was boiled for 10 min with 0.7 ml of developer, in which 0.014g anthrone (Sigma, Milwaukee, Wisconsin, USA) was added, and the tube was boiled for 10 min; 0.1 μmol of 80% H₂SO₄ was added, and the absorbance at 630 nm was determined in a UV755B Spectrometer (Shanghai Precision Instrument Co., Ltd., Shanghai, China). A standard curve was generated using a series of
diluted trehalose solutions in parallel with the experiment, and the amount of trehalose was calculated using a regressive equation. The enzyme activity was defined as the amount of trehalose produced min⁻¹ μmol⁻¹ protein.

Results
cDNA Sequences of Oftps and Oftps_isoformI
A 684 bp cDNA fragment was obtained through degenerate primer amplification, a 773 bp cDNA fragment was obtained using 5’-RACE PCR, and two products (2289 bp and 1065 bp) were obtained using 3’-RACE PCR. The assembled full-length cDNAs were 2913 bp for Oftps (GenBank accession No. KX013356) and 1689 bp for Oftps_isoformI (GenBank accession No. KX013357). Oftps cDNA contains an ORF of 2529 nucleotides, encoding a protein of 842 amino acids with a predicted mass of approximately 94.4 kDa and a pI value of 6.58. Oftps_isoformI cDNA contains an ORF of 1293 nucleotides, encoding a protein of 430 amino acids, with a predicted mass of approximately 48.6 kDa and a pI value of 8.65. Oftps cDNA encoding a TPS domain (a.a.G34–S513, E = 2.7e–130, Pfam accession No. PF00982) and a TPP domain (a.a.L51–D776, E = 2.6e–33, Pfam accession No. PF02358). Oftps_isoformI cDNA encoding a TPS domain (a.a.G34–E130, E = 1.7e–106) (Fig. 1). No signal peptide was detected in either Oftps or Oftps_isoformI using the SignalP 4.1 server. Three N-glycosylation sites were predicted in Oftps using the NetNGlyc 1.0 server (17NMSG, 133NGTF, and 213NMSD); only the first two sites were observed in Oftps_isoformI (Fig. 1).

Amino acid sequence of Oftps was aligned with TPS sequences from insects, bacteria, and plants (Fig. 2). Conservation was observed in the middle region of the Oftps-TPS domain, and both the N- and C-termini displayed diversity. 7 residues (42R, 64G, 128Y, 193D, 221H, 324R, and 328K) in Oftps, which are homologous to the key residues in the E. coli TPS catalytic center (Rao et al. 2006), were conserved in all TPS sequences, as were two TPS signature motifs. Three conserved motifs of the HAD superfamily active site existed in the aligned insect TPS sequences. Phylogenetic sequence analysis with 22 other TPS sequences from insects, nematodes, plants, and bacteria (Fig. 3) revealed that Oftps showed 22.44–92.48% identity with other known TPS sequences and is most closely related to the TPS from S. exigua.

![Fig. 1. Deduced amino acid sequence analysis of Oftps and Oftps_isoformI. The amino acid sequences of Oftps and Oftps_isoformI were deduced from the cloned cDNAs of Oftps and Oftps_isoformI (GenBank accession nos. KX013356 and KX013357). Signature motifs (or conserved motifs) unique to trehalose-6-phosphate synthase (TPS) (residues 192–196 and 422–427) and trehalose-6-phosphate phosphatase (TPP) (residues 553–557, 593–595 and 711–742) are shaded in black. The TPS (residues 34–513) and TPP (residues 551–660) domains are indicated with blue and red boxes. Potential N-glycosylation sites are shaded in gray.](image-url)
Molecular Modeling

Two Rossmann-fold motifs comprised the model structure of the Of/TPS-TPS domain, consistent with the template 1gz5.A. The catalytic center was existed in the fissure, in which key residues were indicated in both the template and the model (Fig. 4). A total of 9 α-helices, 8 from the N-terminus and 1 folded back from the C-terminus, were flanked by 11 β-sheets in Rossmann-fold motif I, and 7 α-helices were flanked by 4 β-sheets in Rossmann-fold motif II. The total amino acid residues in Rossmann-fold motif I was 275, while the counterpart in 1gz5.1.A was 244. One extra loop, consisting of two β-sheets in the Of/TPS-TPS domain, extended outside the molecule (Position 1 in Fig. 4A). In addition, the first two β-sheets were linked by two α-helices in the Of/TPS-TPS domain compared with one helix in the template (Position 2 in Fig. 4A and A1). The total amino acid residues in the Of/TPS-TPP domain Rossmann-fold motif II was 204, with 212 in the template; this difference could reflect the missing α-helix in position 3 of Fig. 4A.

The three-dimensional (3D) model of the Of/TPS-TPP domain consisted of 6 α-helices and 10 β-sheets, with one less α-helix near the C-terminus compared to the template 1u02.1.A (Fig. 4). Moreover, the loop at position 1 of Fig. 4B was obviously larger than the template, and nine more amino acid residues (170RTAFGLDWC178, 720–728 in the whole TPS sequence) were detected compared with the counterpart in the template. The key residues in the active site according to Rao et al. (2006) are illustrated in both the model and the template (Fig. 4B and B1).

Fig. 2. Alignment of TPS proteins from Ostrinia furnacalis (OfTPS) (KX013356), Anopheles gambiae (AgTPS) (XP_317243.4), Apis mellifera (AmTPS) (XP_392397.3), Tribolium castaneum (TcTPS) (XP_975776.2), Arabidopsis thaliana (AtTPS) (AAD30578.1) and Escherichia coli (EcTPS) (ABU24467.1). Identical residues are shaded in red (=100%) and pink (≥75%). The start and end residues of the OfTPS-TPS and OfTPS-TPP domains are indicated with arrows. Signature motifs (or conserved motifs) unique to trehalose-6-phosphate synthase (TPS) and trehalose-6-phosphate phosphatase (TPP) are underlined with blue and red lines, respectively. The key residues involved in the E. coli OtsA (TPS) catalytic center are indicated with a solid triangle. The residues in the red boxes within TPP motifs I and III are possibly only identical in insect TPS proteins.
Expression in ACB

The Of tps mRNA expression was up-regulated 12.5-fold in the fat body (Fig. 5A) versus the control and was weakly expressed in the epidermis and tracheae; it was only slightly expressed in other tissues. The reference gene rpL8 was constantly expressed in each tissue (Fig. 5A).

Of tps mRNA was constantly expressed in the larvae feeding stage (Fig. 5B). During the larval-larval molting, larval-pupal transformation and pupal-adult metamorphosis, the expression level increased significantly. In newly emerging moths, the expression level was higher in males than that in females. After mating and reproduction, Of tps mRNA expression decreased in male moths, but no significant difference was detected in female. The lowest point appeared in the pupal stage (4 d after pupation).

Transcriptional Response of Of tps mRNA Expression to Thermal Stress

As indicated in Fig. 5C, Of tps mRNA expression was gradually increased with ascending temperature from 37 to 43°C, and was up-regulated to the highest level after exposure to 10°C for 2 h.

Enzyme Activity Analysis of the Purified Of TPS Proteins

Three recombinant proteins containing Of/TPS-TPS domain (G14–S511, PTP), Of/TPS-TPP domain (L551–D776, PTP) and Of/TPS-TPS-TPP domain (G14–D776, PT–T) were expressed in E.coli and purified; His-tag was presented at the N-terminal. The predicted molecular mass of PTPS, PTPP and PT–T was 59.8, 30.1 and 88.7 kDa, respectively, and matched each proteins (Fig. 6). The enzyme activity of PT–T was 1.33 U/μmol, approximately 8-fold higher than that of the mixture of PTPS and PTPP (mol ratio = 1:1), 0.16 U/μmol.

Discussion

In insects, trehalose is synthesized from UDP-glucose and glucose-6-phosphate through a two-step reaction catalyzed by TPS and TPP. Similar to other lepidopteran insect TPS proteins, Of TPS contains a TPS domain near the N-terminus and a TPP domain near the C-terminus (Fig. 1), suggesting that the insect tps is a fused gene. However, only the TPS domain was identified in Of TPS_isoformI. Two signature motifs (HDYHL and RDGMNLN) unique to TPS (Gibson et al. 2002) and three conserved motifs, DXXX(T/V), (S/T)GX (Collet et al. 1998) and K(X)16–30(G/S)(D/N) (Morais et al. 2000), which form the active site of the HAD (larger L-2-haloacid dehalogenase) superfamily, were identified in the Of TPS-TPS and -TPP domains (Fig. 1). Thus, Of tps cDNA encodes a precursor polypeptide that contains both TPS and TPP domains, while Of tps_isoformI cDNA encodes TPS domain.

Key residues in the catalytic center in E. coli OtsA, according to Gibson et al. (2002), and their homologous residues in the Of/TPS_isoformI proteins were compared with those in E. coli OtsA and T6PP in T. acidophilum (Rao et al. 2006) and matched each proteins (Fig. 6). The enzyme activity of PT–T was 1.33 U/μmol, approximately 8-fold higher than that of the mixture of PTPS and PTPP (mol ratio = 1:1), 0.16 U/μmol.
might employ similar catalytic mechanisms; when combined with the identification of the signature motifs of the TPS and HAD superfamily in the OfTPS amino acid sequence, the OfTPS-TPS and -TPP domain was speculated to possess TPS and TPP activity. Both the OfTPS-TPS and OfTPS-TPP domains were distinguished from the 3D model template by an extra loop extending outside the molecular domains (position 1 in Fig. 4A and B). Multiple polar amino acids (143RDSKEK148 in TPS domain; 176DWCERI180 in TPP domain, sequence based on the domain) were detected among the residues in both loops (Fig. 4A and B), and the existence of electrostatic interactions between these loops was suspected to maintain OfTPS stability. In addition, the catalytic centers of the OfTPS-TPS and OfTPS-TPP domains might be placed more reasonable to enhance the catalytic efficiency, and the enzyme activity of P T–T was approximately 8-fold higher than the mixture of P TPS and PTPP (mol ratio = 1:1) further confirmed our conjecture.

An Oftpsisoform cDNA was also cloned from the 5th instar larval fat body, and was shown to encode the OfTPS-TPS domain. In H. armigera, a 1.9-kb mRNA encoding only TPS was detected in the fat body of pupae using Northern blotting (Xu et al. 2009). Both trehalose-6-phosphate and trehalose have been reported as signaling molecules for sugar metabolism (Eastmond et al. 2003, Suárez et al. 2008); the insect fat body serves as the center of energy metabolism regulation (Arrese and Soulages 2010), suggesting that trehalose-6-phosphate acts as a regulatory molecule for sugar metabolism in lepidopteran insects. Intensive future studies on these biological functions might shed additional light on Oftps and its alternative splicing isoform.

The deduced amino acid sequence of OfTPS displayed identities to 16 other known insect TPSs that ranged from 58.32 to 92.48%. OfTPS was the most similar to the lepidopteran TPS (ScTPS) and was far from the isopteran and Blattodea TPSs (ZnTPS and BaTPS1,
Based on the phylogenetic analysis, the insect TPSs could clearly be distinguished from those of plants, bacteria and nematodes. In addition, the 17 insect TPSs were clustered into two branches: ZnTPS and BaTPS1 formed one branch, while the remaining TPSs comprised the other branch. Furthermore, two TPSs have been cloned from Blattella germanica and were each allocated to different branches, likely suggesting that more than one TPS subfamily exists in insects.

Oftps mRNA in the 5th instar ACB larvae is predominately expressed in the fat body, inconsistent with the findings for Hatps mRNA from H. armigera (Xu et al. 2009) and Setps mRNA from S. exigua (Tang et al. 2010); the weak expression detected in the epidermis and tracheae might result from remaining fat bodies attached to the tissues.

In S. exigua, when trehalose utilization was inhibited through RNAi of two trehalases, the mortality rates during the larval-pupal transition and the pupal-adult transition were significantly increased (Chen et al. 2010), indicating that trehalose catabolism is essential for insect metamorphosis. Setps mRNA expression was increased during the larval-pupal transformation, the down-up-down expression pattern was observed in the pupal stage, and Setps mRNA expression was reduced in both the middle and end stages in the pupae (Tang et al. 2010). In contrast, in H. armigera, Hatps mRNA was expressed with little change in the last instar larvae and pupae of

Fig. 5. Spatio-temporal expression patterns and transcriptional responses of OFTPS mRNA to thermal stress. Spatial expression pattern of OFTPS mRNA were measured using sqPCR (A) and qPCR (A1). Abbreviation: H, head; Hc, hemocytes; Fg, foregut; Mg, midgut; Hg, hindgut; T, trachea; FB, fat body and Ep, epidermis. The temporal expression pattern of OFTPS mRNA measured using qPCR (B). Abbreviation: 1L-5L, 1st–5th instar larvae; 4W and 5W, wandering larvae in the 4th and 5th instar; NP, new pupae; P, pupae and PM, pre-molting pupae; NM, newly emerging moths and MM, post-mating moths. Transcriptional responses to thermal stress of OFTPS mRNA measured using qPCR (C). Each data point represents the mean ± standard error of results from three technique replicates. Different letters indicate significant differences (P < 0.05).
TLPS and TPS domains (G34–S513); Line 2, TPS-TPS domain (L551–D776); Line 3, TPS-TPP domain (G34–S513); Line 2, TPS-TPS domain (L551–D776); Line 3, TPS-TPSTPP domain (G34–D776).

nondiapause individuals (Xu et al. 2009). Our research revealed that Oftps mRNA expression levels were significantly elevated during insect metamorphosis, consistent with findings for S. exigua, and different from those for H. armigera, suggesting that tps transcriptional regulation during insect metamorphosis might vary among insect species. Furthermore, we also found that Oftps mRNA was constantly expressed at the feeding stage of the larval period and was significantly enhanced during larval-larval molting. Taken together, these findings suggest that Oftps mRNA was transcriptionally up-regulated to meet the energy requirements and to supply the initial substrate trehalose in the chitin biosynthesis pathway in ACB molting and metamorphosis. Constant Oftps expression at the larval feeding stage might be crucial for larvae survival, making OFTPS a potential target gene for ACB control.

The role of trehalase as a thermoprotectant has been well documented in microorganisms, such as yeast (De Virgilio et al. 1994), E. coli (Hengge-Aronis et al. 1991), Salmonella enterica serovar Typhimurium (Cánovas et al. 2001) and the halophilic bacterium Chromohalobacter salexigens (Vargas et al. 2008). A recent study showed that TPS1, not trehalase, plays an essential role in yeast thermotolerance and classified TPS1 as a ‘moonlighting’ protein (Petitjean et al. 2015). Although the underlying mechanism is still far from revealed, Oftps is a transcriptionally thermo-regulated gene, and itself, or its downstream product might contribute to thermotolerance in ACB.

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