Identification of a novel gene encoding the trehalose phosphate synthase in the cotton bollworm, Helicoverpa armigera

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Introduction

Trehalose (α-D-glucopyranosyl-[1,1]-α-D-glucopyranoside) is a nonreducing disaccharide sugar consisting of two glucose molecules linked by an α, α-1, 1-linkage. It is widespread in a large range of organisms, including bacteria, fungi, yeast, invertebrates, and plants (Elbein 1974). Trehalose serves as a source of carbon and energy in many organisms; it can also be a signaling molecule to regulate certain metabolic pathways or even to affect growth in yeast and plants (Vogel et al. 1998; Muller et al. 1999; Arguelles 2000; Bonini et al. 2000; Paul et al. 2001). In addition, trehalose can protect proteins and cellular membranes from inactivation or denaturation under a variety of stress conditions, including heat, cold, desiccation, and oxidation (Elbein et al. 2003).

Insects are the most successful animal group in terms of numbers of species and biomass. Although many factors must contribute to the success of insects, a key element is probably their developmental plan (Yamashita 1996). Most insect species have evolved a period of developmental arrest, called diapause, which ensures their survival through seasonal periods of adversity and increases their chance of mating by synchronizing the growth rates of populations. It is well known that the diapause program is induced by environmental signals, such as photoperiod, temperature, and nutrition; these signals are then transduced via endogenous chemical messengers, such as neurohormones (Denlinger et al. 2005). Neurohormones bring about the phase change from development to diapause through a metabolic shift in the target organs. When insects enter an overwintering diapause, some substances, such as fat, carbohydrate, polyol, and certain proteins, accumulate to enhance the insect’s survival during a long period of adversity. The mechanism by which metabolism is regulated at the molecular level in diapause is not yet known, however.

Trehalose is the major sugar in the blood for insect growth and development; it is also thought to be involved in response to environmental stress, especially at low temperature. Singer and Lindquist (1998) have demonstrated that trehalose stabilizes the membrane and native proteins and facilitates protein refolding by cellular chaperones after the stress condition is terminated. Trehalose concentrations in diapausing insects are considerably higher than in their nondiapausing counterparts, and its function contributes to cold resistance (Hahn and Denlinger 2007). Therefore, trehalose and trehalose metabolism are crucial for insect survival. The best-known pathway for trehalose synthesis involves trehalose-6-phosphate synthase (TPS), which converts two glucose molecules from uridine-5′-diphosphoglucose and glucose-6-phosphate to trehalose-6-phosphate, which turns into free trehalose via the catalysis of trehalose-6-phosphate phosphatase (TPP) (Eastmond and Graham 2003; Elbein et al. 2003).

Keywords: expression/gene structure/ Helicoverpa armigera/ pupal diapause/trehalose-6-phosphate synthase

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In this study, we investigated the changes in the levels of trehalose and TPS activity during diapause- and nondiapause-destined larval–pupal development of *Helicoverpa armigera* (Har), an agriculturally important noctuid moth that enters diapause at the pupal stage. Trehalose was found to accumulate in diapause-destined individuals, and TPS activity is able to respond to trehalose biosynthesis. We then focused on Har-TPS, as it is a regulator of metabolism. We characterized the Har-TPS cDNA and its expression patterns, paying particular attention to the fate of diapause that could be influenced by trehalose and TPS. The Har-TPS protein can be successfully overexpressed in the *Bombbyx mori* baculovirus expression system; its catalytic activity is approximately 5-fold higher in *B. mori* blood infected with the recombinant baculovirus than the control. When *H. armigera* pupal diapause is broken, the trehalose content drops sharply, while the glucose content increases. Thus, Har-TPS regulates *H. armigera* pupal diapause through trehalose biosynthesis.

**Results**

**Changes in the trehalose content during larval–pupal development**

The changes in the trehalose content in the hemolymph of larvae and pupae were measured, and the results are shown in Figure 1. In nondiapause-type individuals, the trehalose content was found to increase on day 0 of the sixth larval instar and reach a peak on day 4; it then dropped at a late stage of the sixth larval instar. After pupation, the content increased to the highest level on day 0 before starting to decrease on day 2; a low level was maintained during pupal–adult development (days 6–8). In diapause-destined individuals, the trehalose content rose gradually and reached a higher level at a late stage of the sixth larval instar. After pupation, the trehalose content decreased slightly, and then increased on day 4. The trehalose content reached the highest level on day 8; it was then maintained at this high level until the pupae entered diapause (days 20–100). Apparently, trehalose heavily accumulates in diapause-type pupae for overwintering.

**TPS activity during larval–pupal development**

As described in the Introduction, TPS is a key enzyme for trehalose biosynthesis. Therefore, we measured TPS activity during *H. armigera* larval–pupal development (Figure 2). In nondiapause-type individuals, the activity was low on day 0 of the sixth larval instar; it increased on day 2, and then remained constant until day 4 of the pupae. The activity decreased rapidly to a low level on day 6 and was maintained at this low level in the pharate-adult stage (day 8). In diapause-destined individuals, the TPS activity increased continuously at the early stage of the sixth larval instar, reaching its highest peak on day 2 after pupation before declining gradually. When the pupae entered diapause (day 20), the activity was relatively low until day 100. The pattern of Har-TPS activity is consistent with the levels of trehalose in diapause- and nondiapause-destined individuals.

**Cloning and characterization of Har-TPS cDNA**

A 260 bp fragment was obtained by polymerase chain reaction (PCR) amplification using two degenerate primers, TF and TR, which were designed to recognize the conserved sequences of TPS cDNA that were previously reported for *Drosophila melanogaster*, *Escherichia coli*, and *Saccharomyces cerevisiae*. This fragment showed 55% identity at the amino acid level to the corresponding region of *D. melanogaster* TPS. Based on the 260 bp cDNA fragment, two specific primers, TPSPF and TPSPR, were synthesized for 3′- and 5′-RACE (Figure 3). With 3′- and 5′-RACE amplification, products were obtained of approximately 2200 bp at the 3′-end and 760 bp at the 5′-end. The 3′- and 5′-cDNA fragments shared overlapping sequences; the sequences and corresponding amino acids are shown in Figure 3. The nucleotide sequence data that are reported here have been submitted to GenBank under the accession number EU878265.

The cDNA has an open reading frame (ORF) encoding a polypeptide of 826 amino acids. The protein contains at the amino-terminus the conserved domains of OtsA, which is the TPS of *E. coli*, suggesting that this sequence may encode a trehalose-6-phosphate synthase in *H. armigera* (Har-TPS). By
Fig. 3. Nucleotide and deduced amino acid sequences of a cDNA encoding TPS. The suggested start ATG and stop TAA codons are shown by boxes. Arrows over the nucleotide sequences represent the positions of the different synthetic primers used in PCR. Degenerate primers TF and TR and specific primers TPSPF, TPSPR, TPSBa, and TPSNt were used for cDNA amplification.

homology, the ORF is most identical to the homolog from Spodoptera exigua (96%) (GenBank no. ABM66814), followed by Apis mellifera (78%) (GenBank no. XP392397), Tribolium castaneum (77%) (GenBank no. XP975776), Locusta migratoria (GenBank no. ABV44614), Anopheles gambiae (76%) (GenBank no. XP317243), Culex pipiens (73%) (GenBank no. XP001850998), D. melanogaster (73%) (Chen et al. 2002), and Acyrthosiphon pisum (72%) (GenBank no. XP001945523).

Compared with TPS genes reported in yeast, and plant, Har-TPS possesses a TPS2 domain that encodes TPP at the carboxy-terminus (Figure 4). Therefore, the Har-TPS cDNA encodes a precursor polypeptide that contains both TPS and TPP.
Tissue distribution of Har-TPS mRNA
Northern blot analysis was performed on total RNA (25 µg) from various tissues of day 1 pupae. Using Har-TPS cDNA as a hybridization probe, hybridization signals were detected in the fat body and ovary; a weak signal was also detected in the midgut but not in the brain or Malpighian tubules (Figure 5). These positive bands were approximately 2.8 kb, indicating that the characterized cDNA represents the full-length mRNA. A weak hybridization signal at 1.9 kb was also found in the RNA of the fat body; this band could be either a part of the smeared band of the 2.8 kb product or a spliced RNA.

Developmental changes in Har-TPS mRNA
The expression of the Har-TPS mRNA was examined during larval–pupal development using the combined methods of quantitative reverse transcription-polymerase chain reaction (RT-PCR) and southern blot (Xu et al. 1995; Zhang et al. 2004). In the nondiapause type, the expression of the Har-TPS mRNA was low with a little change. In contrast, the expression of Har-TPS mRNA was higher in the diapause-type individuals than in nondiapause type, especially at the late stage of the sixth
Fig. 6. Developmental changes in Har-TPS mRNA. Total RNA was extracted from the fat body, subjected to RT-PCR amplification with 20 cycles, and then southern blotted with hybridization probes. The Arabic numerals represent the days of sixth instar larvae and pupae. The rabbit globin (RG) cDNA was used as an internal standard.

Fig. 7. Overexpression of Har-TPS using the Bombyx mori baculovirus expression system. (A) SDS–PAGE analysis of recombinant TPS. The 60 kD recombinant TPS is indicated by the arrow. (B) Activity analysis of recombinant TPS.

larval instar (day 8) and the early pupal stage (days 0–10). When pupae entered diapause, the amount of TPS mRNA decreased and was maintained at a constant level from day 20 to day 100 (Figure 6).

Bioassay of re-TPS
To evaluate the authenticity of the putative Har-TPS, recombinant viruses were constructed to express Har-TPS. Hemolymph samples of the silkworm larvae that were infected with the recombinant viruses were submitted to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). An additional 60 kD protein could be easily detected in the sample from the silkworm infected with the recombinant viruses but not in the mock-infected samples, which were infected with viruses containing a foreign fragment inserted into the vector instead of Har-TPS (Figure 7A). The TPS activity in the re-TPS sample was measured and shown to be about 5-fold higher than the mock and control (Figure 7B).

Trehalose content at diapause termination
To investigate the specificity of trehalose for responding to diapause, we measured the trehalose content when diapausing pupae (28 days after pupation) were injected with 100 pmol of diapause hormone to break the pupal diapause (Zhang et al. 2004). The trehalose content dropped significantly on the third day after injection, and the levels of glucose, which is used to construct the trehalose molecule, increased rapidly on the fourth day after injection (Figure 8). Apparently, there is a close relationship between trehalose and diapause, as trehalose accumulates before the entry into diapause, and is degraded quickly after diapause termination.

Discussion
Previous studies have demonstrated that polyhydric compounds serve as antifreeze substances in cold-adapted species (Zachariassen 1985). For example, B. mori accumulate glycerol and sorbitol in diapause eggs to bridge the winter (Chino 1957). It is unknown, however, whether the diapause-destined pupa accumulates polyols to resist low temperatures to survive. Here, we first investigated the polyhydric compounds in H. armigera hemolymph by a high-performance liquid chromatography (HPLC) assay and showed that trehalose is a prominent component of H. armigera diapause-programmed pupae. We then measured the activity of TPS, which catalyzes the conversion of glucose molecules into trehalose during larval–pupal development; the change in TPS activity was found to correlate well with the expression pattern of trehalose. Further, we cloned the
Har-TPS cDNA and examined its tissue distribution and developmental expression. We also successfully expressed Har-TPS in *B. mori* larvae with an infection of recombinant viruses. The Har-TPS activity was much higher than the control. Thus, it is clear that the cDNA indeed encodes the TPS enzyme for trehalose biosynthesis. Finally, trehalose levels decreased rapidly, while glucose levels increased, when diapause was broken. This result shows that trehalose is relevant to diapause.

Actually, we detected several polyols, such as glycerol, sorbitol, and glucose. Glycerol, which accumulates in many cold-adapted insects, could not be detected in *H. armigera*. The amount of glucose in diapause-type pupae is much lower than in nondiapause-type pupae (see supplementary Figure 1). The changes in sorbitol levels in the hemolymph during larval–pupal development are similar to those of trehalose; the level in diapause-type individuals is much higher than in nondiapause-type individuals, implying that sorbitol is also involved in regulating pupal diapause (see supplementary Figure 2). Trehalose, however, seems to be more important than sorbitol because the levels of trehalose are more significantly different between diapause- and nondiapause-destined individuals in *H. armigera*.

In the present paper, 2858 bp of *H. armigera* TPS cDNA was cloned, and a northern blot showed that the gene is mainly expressed in the fat body. Since the fat body has been reported to be the major tissue for metabolism in *Bombbyx* (Murphy and Wyatt 1965), trehalose biosynthesis is assumed to occur in the fat body by TPS regulation; then, it is released into the hemolymph for pupal diapause.

We tested the developmental expression of the TPS gene in the larval–pupal stage by semi-quantitative RT-PCR. The expression in diapause-type individuals was found to be much higher than in nondiapause-type individuals. A high level of TPS mRNA in diapause-destined individuals was found to be present in prepupae and in the early stage of pupae (days 0–10). This expression pattern is consistent with the changes in TPS activity, indicating that the increased TPS activity in *H. armigera* may be due to upregulated transcription.

TPS genes in insect species have been reported to exist as a single copy in a haploid genome and encode TPS and TPP together. In *E. coli*, TPS and TPP are encoded by *otsA* and *otsB*, respectively. In *S. cerevisiae*, TPS1 encodes only TPS, and TPS2 encodes TPS and TPP. In *Arabidopsis thaliana*, there are 11 TPS genes and one TPP gene: TPS1–4 encodes only TPS, TPS5–11 encodes TPS and TPP, and TPSA–B encodes only TPP (Leyman et al. 2001). By northern blot, the full-length 2.8 kb cDNA containing TPS and TPP and a 1.9 kb fragment of the cDNA were detected in *H. armigera* (Figure 5). The 1.9 kb cDNA has been sequenced (GenBank no. AAY87162) and shown to encode only TPS. It seems that TPP can be excised by transcriptional splicing. Recently, trehalose-6-phosphate has been shown to be a signaling molecule for sugar metabolism in yeast and plants (Eastmond and Graham 2003). Apparently, the 2.8 kb mRNA containing TPS and TPP is expressed for trehalose biosynthesis. Interestingly, the 1.9 kb mRNA containing only TPS is present in *H. armigera*, implying that trehalose-6-phosphate may be a regulatory molecule for sugar metabolism. This hypothesis remains to be confirmed by further experiments, though.

Trehalose, which has multiple functions, has been reported to be an energy and carbon reserve, a stabilizer and protector of proteins and membranes, a structural component of the cell wall, or a sensing compound and/or growth regulator (Elbein et al. 2003). In pupal diapause, trehalose may play two roles: (1) abundant trehalose accumulates in the hemolymph of diapause-programmed individuals and assists organs in resisting cold temperature for overwintering, since all organs contact the insect blood and (2) trehalose is as an energy and carbon reserve for postpupal development when diapause is terminated the following year. Based on our preliminary experiments, pupal diapause cannot be induced by the injection of trehalose into nondiapause pupae (data not shown). Therefore, trehalose does not serve as a sensing compound and/or growth regulator for developmental arrest.

**Method and materials**

**Insects**

*H. armigera* were maintained over 5 years in our laboratory. Larvae were reared on an artificial diet at 22°C and a light 10 h:dark 14 h photoperiod; more than 95% of the individuals entered diapause. When larvae were reared at 22°C and an L14:D10 photoperiod, all pupae developed without entering diapause.

The developmental stages were synchronized at each molt by collecting new larvae or pupae. The fat body, brain, ovary, midgut, and Malpighian tubules were dissected in insect saline containing 0.75% NaCl and stored at −70°C until use.

**Preparation of hemolymph**

Hemolymph was collected from each larva or pupa. The hemolymphs from 30 individuals were mixed as a sample for measuring trehalose titer. Three samples were tested at each point. Hemolymph was centrifuged at 5000 × g for 5 min to remove free cells; then a 20 µL sample was mixed with 50 µL of distilled water, 5 µL 15% K3Fe(CN)6, and 5 µL 30% ZnAc2. This sample was then centrifuged at 12,000 × g for 30 min at 4°C. The supernatant was transferred into another Eppendorf tube for derivation, similar to the method described by Schwarzenbach (1977) with modification as follows: 20 µL of 20% NaOH and 10 µL of benzoyl chloride were added; the mixture was well shaken at 4°C for 10 min, and then extracted with 250 µL of chloroform and ether (2:1) twice. The extract was washed with 500 µL of 5% NaHCO3 three times and 500 µL of 5% CH3COOH once and dried using a nitrogen stream. The residue was resolved with 70% CH3CN.

**Analysis of high-performance liquid chromatography**

The hemolymph preparation was carried out on a Shimadzu LC-10AVP Plus HPLC system with an ultraviolet detector set to 254 nm and a Waters C18 column equilibrated with 100% methanol. Elution was performed with acetonitrile/water (70/30) at a flow rate of 1 mL/min. The quantity of polyols was determined from the peak areas of the chromatogram.

**Bioassay of trehalose-6-phosphate synthase**

The activity of trehalose-6-phosphate synthase was determined as reported previously (Cabib and Leloir 1958) with some modifications. Fifty microliters of hemolymph was mixed with 50 µL of 500 mM phosphate buffer (pH 7.2) containing 0.5 µmol uridine-5′-diphosphoglucose (Sigma), 1 µmol glucose-6-phosphate (Sigma), 2.5 µmol MgSO4, and 0.1 µmol glucose-6-phosphate synthase.
EDTA; this mixture was incubated at 37°C for 15 min, heated for 3 min at 100°C, and cooled on ice for 3 min. After the addition of 50 µL of 500 mM phosphate buffer containing 0.5 µmol of phosphopyruvate (Sigma, China) in 20 units of pyruvate kinase (Sigma, China), the tube was incubated again for 15 min at 37°C. Cold 10% trichloroacetic acid in a volume of 1 mL was added to the tube. After centrifuging at 14,000 × g for 20 min at 4°C, 600 µL of the supernatant was added to 200 µL of 2M HCl containing 0.1% 2,4-dinitrophenylhydrazine; the sample was incubated at 25°C for 5 min, and then extracted with 600 µL of benzene and 1.2 mL of 10% sodium carbonate, respectively. The optical density at 520 nm was measured for 1 mL of extract mixed with 1 mL 1.5 M NaOH as reported by Friedemann and Haugen (1943).

RNA extraction and RT-PCR
Total RNA was extracted from the fat body of H. armigera by the single-step method of acid guanidinium thiocyanate-phenol-chloroform extraction according to Chomczynski and Sacchi (1987). One microgram of total RNA was reverse transcribed at 42°C for 1 h in a volume of 25 µL with the AMV reverse transcription system (Takara, Dalian, China). One microliter of the reaction mixture was added to 50 µL of PCR reaction system. PCR amplification was performed using the degenerate primers TF (5′-GCC SYT STT YCA YTC GAT GCC-3′) and TR (5′-GGR ATG TGC AGR AAG AA-3′), which were designed for the conserved TPS cDNA sequences from D. melanogaster (Chen et al. 2002), E. coli (Kaasen et al. 1992), and S. cerevisiae (De Virgilio et al. 1993), and under the following reaction conditions: 1 min at 94°C, 1 min at 45°C, 1 min at 72°C with 30 cycles, and then 10 min at 72°C.

Amplification of the full-length TPS cDNA
Based on the partial sequence of Har-TPS cDNA amplified by primers TF and TR, specific primers, TPSPF (5′-CTC AAC TCG CTG TCT AAT AAC-3′) and TPSPR (5′-CAC TCC ACG TCG CTG TCT AAT AAC-3′) for the 3′- and 5′-cDNA ends, respectively, were designed and are shown in Figure 4. PCR amplification was performed according to the manufacturer’s protocol (SMART™ kit, Clontech) under the following conditions: 30 cycles of 1 min at 94°C, 1 min at 60°C, 1 min at 72°C, and then 10 min at 72°C.

The PCR products were separated on a 1.5% agarose gel, purified, ligated into the pMD18-T vector (Takara), and amplified in E. coli. Multiple sequencing reactions were run by the Takara company (Dalian, China) using both M13F and M13R primers.

Northern blot analysis
To start, 25 µg of total RNA was extracted from various tissues of day 1 pupae and separated on a 1.2% agarose gel containing 0.22 mol/L formaldehyde and 0.5 µg/mL ethidium bromide. The separated proteins were then subsequently transferred to a nylon membrane (Hybond N+, Amersham). The Har-TPS cDNA was labeled with [α-32P]dCTP using a random primer method and a commercial kit (Takara). The nylon membrane was pre-hybridized for 4 h, and then a radiolabeled probe was added and incubated for 18 h at 42°C in 5 × SSPE (1 × SSPE = 180 mM NaCl, 10 mM sodium phosphate, pH 7.7, 1 mM EDTA) containing 50% formamide, 5 × Denhardt’s solution, 0.1% SDS, and 100 µg/mL salmon sperm DNA. After hybridization, the membrane was washed with 0.2 × SSPE at 45°C and finally exposed to the X-ray film for 20 h at −70°C.

Developmental expression
A 1 µg sample of total RNA extracted from the fat body containing 0.1 ng of rabbit globin (RG) mRNA (Gibco BRL) as an internal standard was reverse transcribed at 42°C for 1 h in a 1 × buffer (50 mM Tris/HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂), 10 mM DTT, 0.5 mM dNTP, 1 µg of oligo dT₁₈, and AMV (Takara) to a final volume of 25 µL. The reaction was terminated by heating the sample to 75°C for 10 min.

PCR amplification was performed using primers TPSB (5′-ATG AGT GGA ACG GAC AGC-3′) and TPSN (5′-TTA GTC AAG TAG TAA TGC CAG-3′) under the following conditions: 20 cycles of 1 min at 94°C, 1 min at 55°C, 1.5 min at 72°C, followed by 10 min at 72°C. RG cDNA was amplified with the primers RP1 (5′-CAG TTC GAC ACC CAC GG-3′) and RP2 (5′-TCA GCA CGG TGC TCA GTG TG-3′), corresponding to 372–391 and 742–761 bp of the RG cDNA sequence. The PCR products were electrophoresed on a 1.2% (w/v) agarose gel, transferred on to a Hybond N+ membrane, and hybridized with the corresponding probes labeled with [α-32P]dCTP using a random primed DNA labeling kit (Takara).

Construction of the TPS baculovirus transfer vector
An expression transfer vector of TPS was constructed as follows. The opening reading frame of Har-TPS was amplified by PCR using primers TPSBa (5′-GTA GGA TCC AAC AAT GGA ACG GAC AGC-3′) and TPSPR (5′-CTC AAC CAC TCG CTG TCT AAT AAC-3′) for the 3′- and 5′-cDNA ends, respectively, and introduced into the pMD18-T vector. After sequencing, the insert cut from the T vector by BamHI and NotI was cloned into the transfer vector pVL1393 that was digested with the same endonucleases.

Generation of recombinant baculovirus
The original baculovirus Bm-BacPAK6, a genetically modified B. mori nuclear polyhedrosis virus (BmNPV), was kindly provided by Professor X-F Wu (Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences). Bm-5 cells were cultured at 27°C in a TC-100 insect medium, supplemented with 10% heat-inactivated (56°C, 30 min) fetal bovine serum (Invitrogen, Carlsbad, CA, USA). After the Bm-BacPAK6 viral DNA was digested with Bsu36 I, the linearized viral DNA and the transfer plasmid DNA containing the TPS gene were cotransfected into Bm-5 cells mediated by Lipofectin (Invitrogen, Carlsbad, CA, USA). After being incubated at 27°C for 4–5 days, the cotransfection supernatant was subjected to plaque assays to screen individual plaques. Finally, the pure recombinant viruses were used to generate high titer viral stocks for protein expression.

Expression of TPS in silkworm larvae
Day 1 fifth-instar silkworm larvae were injected with 1 × 10⁵ pfu recombinant baculovirus and reared for 4–5 days until the hemolymph was collected by puncturing the abdominal legs. After being centrifuged at 5000 × g for 10 min to remove blood cells, the hemolymph samples were stored at −20°C until use.
References

Abbreviations
Har, Helicoverpa armigera; HPLC, high-performance liquid chromatography; ORF, open reading frame; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription-PCR; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TPP, trehalose-6-phosphate synthase/phosphatase complex in Saccharomyces cerevisiae, causes accumulation of trehalose-6-phosphate and loss of trehalose-6-phosphate phosphatase activity. Eur J Biochem. 212:315–323.

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