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Trehalose Accumulation in a High-Trehalose-Accumulating Mutant of *Saccharomycopsis fibuligera* sdu Does Not Respond to Stress Treatments

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Abstract—The isolation of high-trehalose-accumulating mutant A11 from *Saccharomycopsis fibuligera* sdu has been previously described. In this paper, accumulation of trehalose under various stress conditions in *S. fibuligera* A11 was investigated. Neither activation of trehalose-6-phosphate synthase (SfTps1) nor change in trehalose content was observed under stress exposure of *S. fibuligera* A11 cells. A fragment of the *Sftps1* gene in this strain was also cloned by degenerate PCR using the CoDeHOP strategy and multiply-aligned Tps1 sequences. This sequence allowed us to investigate the expression of the *Sftps1* gene, which was also kept constant under the various stress conditions. Altogether, these results indicate that trehalose metabolism in *S. fibuligera* A11 in response to stress conditions clearly differs from that of *Saccharomyces cerevisiae* and most other fungi. The expression of the *Sftps1* gene was not responsive to different stress treatments.

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Key words: trehalose, accumulation, stress, response, yeast

In most eukaryotes, trehalose is produced from glucose-6-phosphate and UDP-glucose in two sequential steps: initially a glucosyl unit is transferred from UDP-glucose to glucose 6-phosphate to yield trehalose-6-phosphate. This reaction is catalyzed by trehalose-6-phosphate synthase (Tps1). Subsequent dephosphorylation by trehalose-6-phosphate phosphatase (Tps2) leads to formation of trehalose. This pathway has been extensively studied in *Escherichia coli* and *Saccharomyces cerevisiae* [1]. In *S. cerevisiae*, Tps1 activity and trehalose concentration are low under normal growth conditions. If the temperature rises above 37°C, almost all genes associated with the trehalose cycle are up-regulated [4]. Subsequently, the abundance of mRNA increases rapidly and strongly, the activity of Tps1 increases several-fold, and the amount of trehalose may rise to an amazing 1 g/g protein, with the final concentration depending on temperature [3].

Cells subjected to stresses develop genetic and metabolic responses that eventually lead to the acquisition of a "stress resistance" state within minutes [2]. Studies in many microorganisms have shown that numerous forms of stress induce trehalose accumulation, most often through regulation at the level of transcription. Transcription of the *tps1* gene is up-regulated under a variety of stress conditions [1-4].

Trehalose possesses several unique physical properties, which include high hydrophilicity and chemical stability, non-hygroscopic glass formation, and the absence of internal hydrogen bonds. These features account for the principal role of trehalose as a stress metabolite [5]. Trehalose is now being recognized as a crucial defense mechanism that stabilizes proteins and biological membranes under various stress conditions [2, 6]. Based on its unique properties, trehalose has become an important target for biotechnology, where it is produced for food manufacture, vaccine protection, pharmaceuticals, and cosmetic products [7]. In previous studies [8, 9], we found that the mutant A11 of *Saccharomycopsis fibuligera* sdu with low acidic and neutral trehalase activities could accumu-

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late more trehalose (22% dry weight) from soluble starch than its parent strain (18% dry weight) at the beginning of stationary phase during growth on starch as the sole carbon source. So far, little is known about trehalose biosynthesis in this mutant. Here we report the partial cloning of the *Sftps1* gene, and the effects of various stress conditions on its expression, SfTps1 activity, and trehalose content.

MATERIALS AND METHODS

Reagents and equipment. Absorbance measurements and spectral analysis were performed with an Ultrospec 2000UV spectrophotometer (Pharmacia Biotech, Sweden). The following reagents were used for enzyme assay: UDP-glucose, glucose-6-phosphate, NADH (Fluka, Germany); phosphoenolpyruvate, lactate dehydrogenase, pyruvate kinase, trehalase (Sigma, USA). For genetic engineering, we used enzymes from Promega (USA) and TaKaRa (Dalian, China). All other reagents were from Sigma.

Yeast strains. *S. fibuligera* A11, which is a mutant of *S. fibuligera* sdu with low acidic and neutral trehalase activities, was maintained at 4°C on YPS agar (w/v), which contains 1.0% yeast extract, 2.0% polypeptone, 2.0% soluble starch, and 2.0% agar. *Saccharomyces cerevisiae* Y108 was maintained on YPD medium (w/v), which contains 1.0% yeast extract, 2.0% polypeptone, and 2.0% glucose.

Enzyme, protein, and trehalose assay. The Tps1 activity in the cell extract was assayed according to the method described by Hottiger et al. [3]. Briefly, the mixtures were incubated at 37°C for 20 min. UDP formed in the supernatant was determined from the decrease in A_{340}. One unit of enzyme activity was defined as the amount of enzyme that produces 1 μmol of NAD^+ per minute at 37°C and pH 6.6. Protein concentration in the cell extract was measured by the method of Bradford [10]. The content of trehalose in cells was assayed according to the method described by Parrou et al. [11]. Briefly, trehalose was released after alkaline hydrolysis of cells and was converted into glucose by trehalase. The released glucose was determined using a glucose assay kit (Sigma).

Stress treatments. Stress treatments were carried out according to the methods described by Parrou et al. [11] with some modification. The cells were cultivated in YPD medium at 30°C to mid-exponential phase (A_{600} = 0.4-0.6). Stress was induced by addition to the yeast culture of either a tenfold concentrated solution of NaCl (final concentration 0.3 M) or sorbitol (final concentration 0.4 M), a 1000-fold concentrated solution of H_2O_2 (final concentration 0.4 mM), or 100% ethanol (final concentration 7.0% (v/v)). For heat shock and cold experiments, the cells were grown at 25°C and shifted to 37°C or put on ice (0°C).

Isolation of genomic DNA and cloning of the *Sftps1* and *Sfβ-actin* gene fragments. Genomic DNA from *S. fibuligera* A11 was isolated according to the method described by Adams et al. [12]. In order to isolate the fragments of *Sftps1* and *Sfβ-actin* gene from *S. fibuligera* A11, we used the Consensus Degenerate Hybrid Oligonucleotide Primer program (CoDeHOP) [13]. This program used as input a multiple sequence alignment of Tps1 sequences prepared from the Block Maker program (http://blocks.fhcrc.org/blocks/make_blocks.html). Two degenerated primer sets (Sf-tps1S1, 5′-TCTAATTCTATTTTATGGCCATTATTTcaytaycaycc-3′ and Sf-tps1S2, 5′-ACTAATTCATTAACAACAGCTCTTAAATATtgrtaytcytc-3′; Sf-actinP1, 5′-ATGGAAAAAATTTGGCATCACACTttytayaayga-3′ and Sf-actinP2, 5′-TCGTATTCCTGTTTAGATATCCAcatytgytgraa-3′) were selected from the output data and were used for PCR with genomic DNA of *S. fibuligera* A11 as template. The PCR conditions were as follows: 5 min at 94°C, then 15 cycles of Touchdown step (30 sec at 94°C, 45 sec at 57 to 52.5°C (−0.3°C/cycle), 1 min at 72°C), followed by a specific amplification at 60°C for 15 cycles. The PCR products were cloned into pGEM-T Easy Vector and sequenced. The phylogenetic tree was created with VectorNTI software (Invitrogen, USA), which used the Neighbour-joining Method from Saitou and Nei [14].

Total RNA isolation and RT-PCR. Total RNA was isolated from *S. fibuligera* A11 and *S. cerevisiae* Y108 with the RNeasy Kit from Qiagen (USA) and treated with RNase-free DNase during the isolation step according to the manufacturer's instructions. The isolated RNA was quantified by spectrophotometry at 260 nm. Equal amount of total RNA (1 μg) was used to analyze mRNA levels by RT-PCR. To synthesize the first cDNA strand of the genes, a Reverse Transcription System Kit from Promega was used with the protocol provided by the manufacturer. PCR amplification was performed using Taq DNA polymerase from Promega. Two specific primer sets (Sf-tps1RTP1, 5′-ATACTCCATTCCCGTCTT-3′ and Sf-tps1RTP2, 5′-TTTCCCGTTAATTCTACC-3′; Sf-actinRTA1, 5′-AGCCAAGTCAATTCTCAA-3′ and Sf-actinRTA2, 5′-AAACCCTAAATCCAACAG-3′) were designed to target the *Sftps1* fragment (Accession No. DQ364059) and *Sfβ-actin* fragment (Accession No. DQ420644), respectively, which were cloned in this study. Two specific primer sets (Sc-tps1RT1, 5′-TCAGGTGAGGAAGGACTTGC-3′ and Sc-tps1RT2, 5′-CGTATGTGTGGAACCCGACT-3′; Sc-actinRTP1, 5′-ACACGGTATTGTCACCAACTG-3′ and Sc-actinRTP2, 5′-GAAGAAGATTGAGCAGCGGTT-3′) were designed to target the *S. cerevisiae tps1* gene (Accession No. Z35995) and *β-actin* gene (Accession No. L00026).

RESULTS

Alignment of a partial amino acid sequence of Tps1. To isolate the *S. fibuligera* A11 homolog of the *S. cerevisiae*

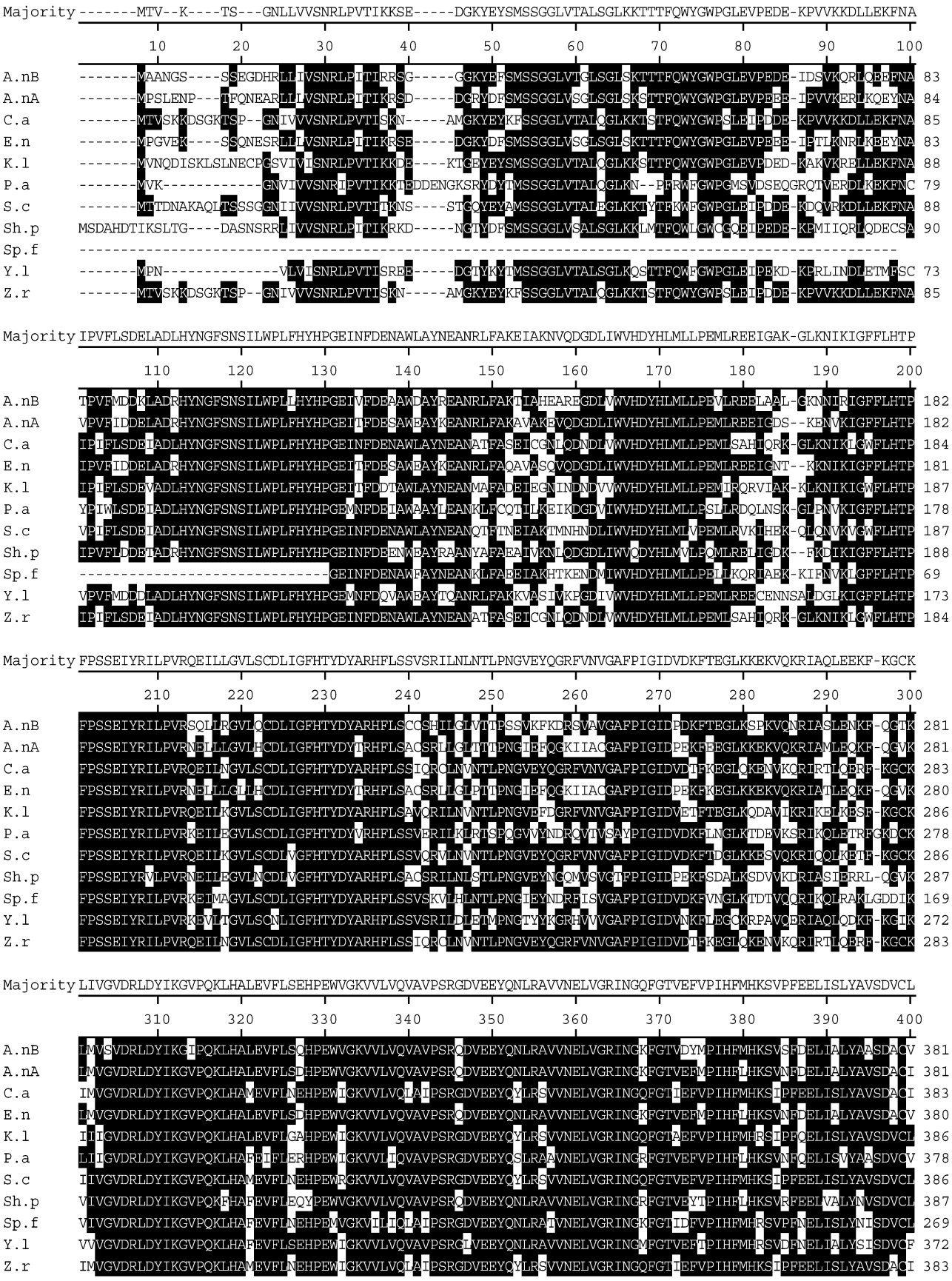

Fig. 1. (Legend on following page).

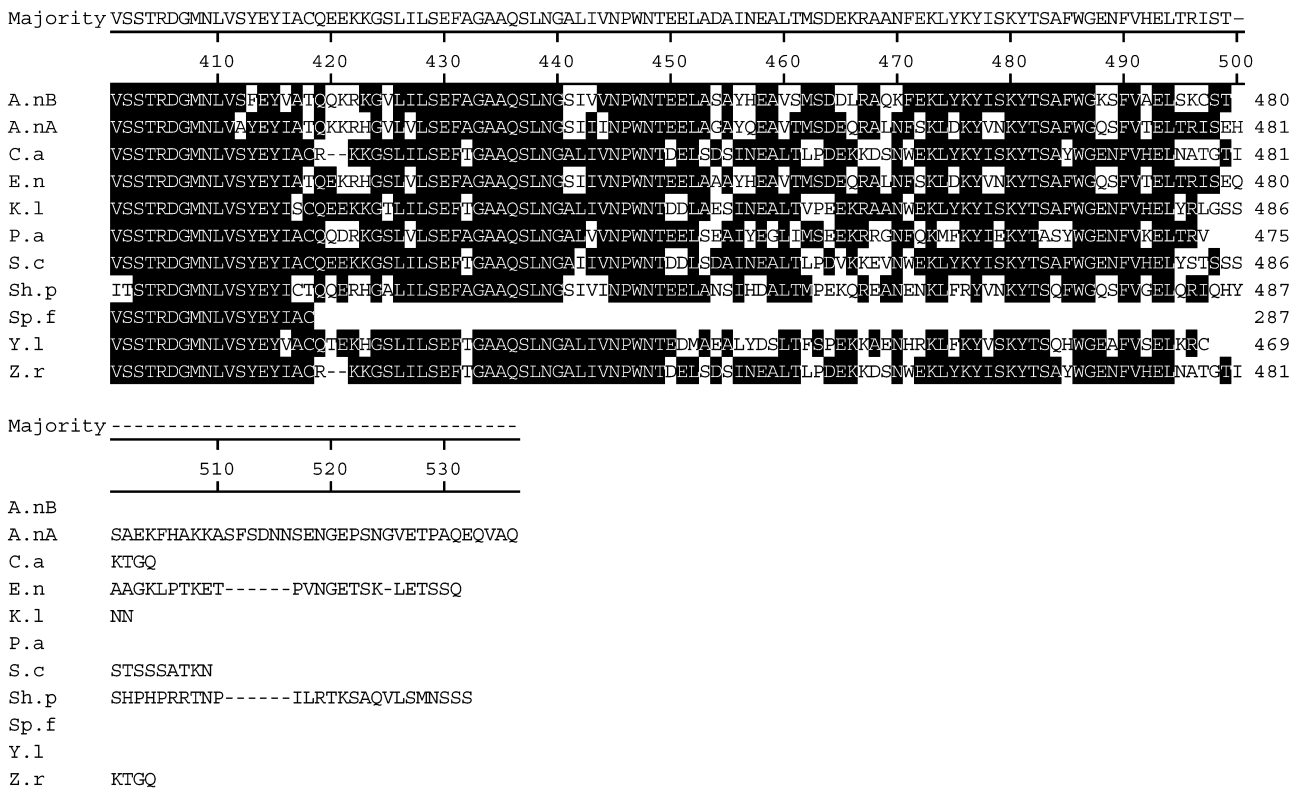

Fig. 1. Multiple alignments of the amino acid sequence deduced from partial *Sftps1* gene cloned from *S. fibuligera* sdu (Sp.f) with that of Tps1 from other fungi. The amino acid sequences of Tps1 from *S. cerevisiae* (S.c, No. CAA85083), *Kluyveromyces lactis* (K.l, No. XP_451921), *Schizosaccharomyces pombe* (Sh.p, No. CAB95998), *Candida albicans* (C.a, No. XP_711706), *Zygosaccharomyces rouxii* (Z.r, No. AAK69413), *Yarrowia lipolytica* (Y.l, No. CAA09463), *Emericella nidulans* (E.n, No. AAO72737), *Pichia angusta* (P.a, No. CAB38058), *Aspergillus niger* TpsA (A.nA, No. Q00075) and TpsB (A.nB, No. BAE56442) were aligned. This aliment was produced using Clustal W method of DNA Star. Perfectly conserved residues are indicated shaded.

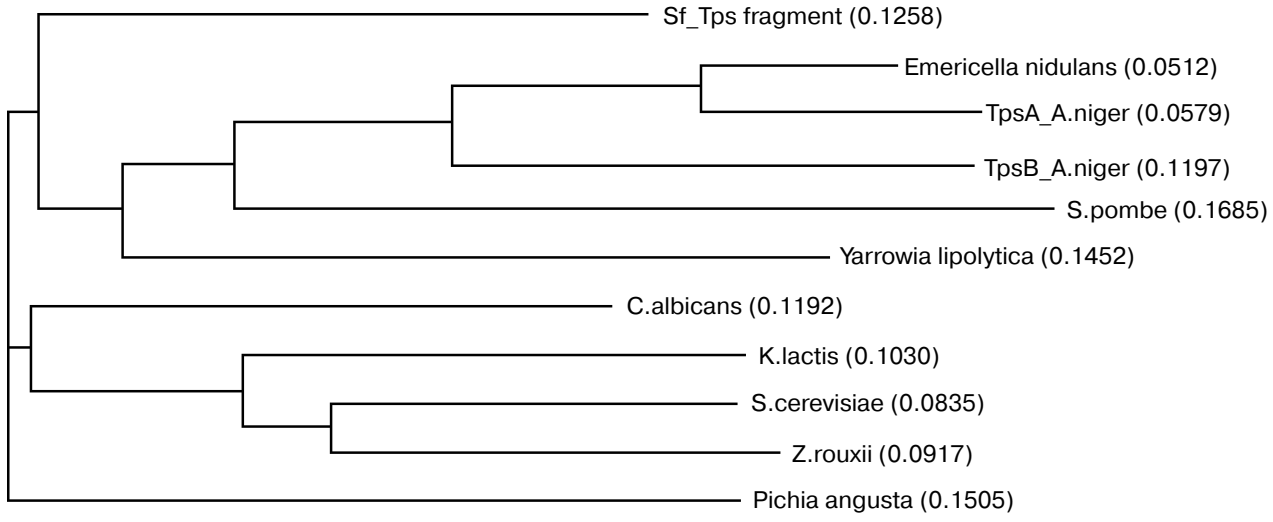

Fig. 2. Phylogenetic analysis of conserved region of SfTps1 from *S. fibuligera* sdu (Sf-Tps fragment) and those of other fungi. The proteins are identified by database accession numbers.

tps1 gene, we started from a multiple alignment of 10 Tps1 fungal sequences that allowed designing degenerate primers according to the CoDeHOP strategy [13]. We were able to amplify an 861 bp DNA fragment from *S. fibuligera* A11 genome after sequencing. The translated fragment had perfectly matched conserved regions in comparison with the enzymes from other fungi (Fig. 1), sequence identity with other Tps1 proteins lying between 67% (*S. pombe*) and 75% (*C. albicans*). Together with the fact that this strain is able to synthesize and accumulate trehalose, this result confirmed that we actually cloned the *Sftps1* gene in *S. fibuligera* A11. The phylogenetic analysis also showed that this putative amino acid sequence (Sp.f_Tps1 fragment) formed a separate family that was distantly related to *S. cerevisiae* Tps1 (Fig. 2).

Expression of the *Sftps1* gene under various stress treatments. Thanks to the previously cloned DNA fragment, we could investigate the quantitative response of

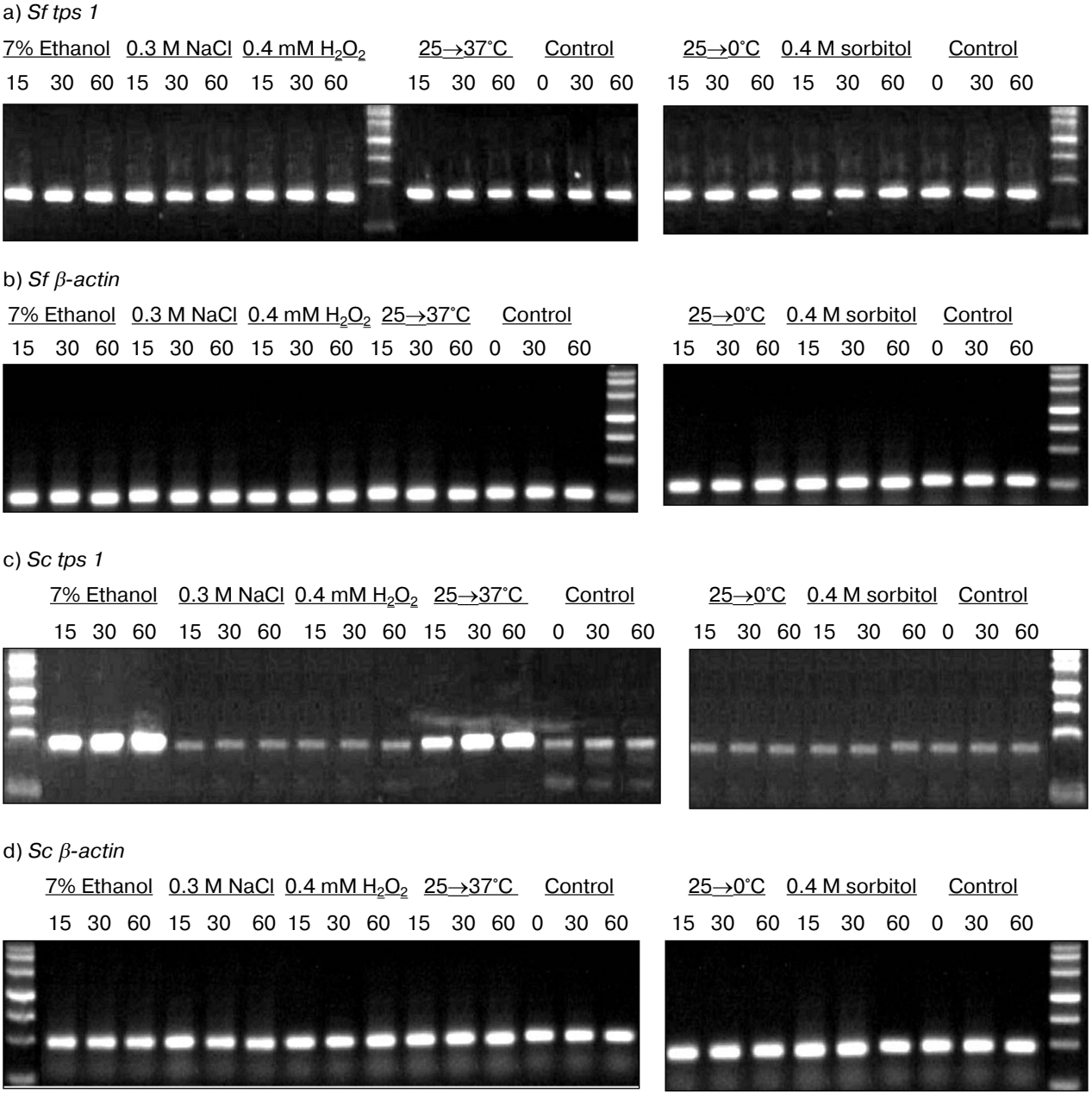

Fig. 3. Effects of various stress treatments on mRNA levels expressed by *tps1* gene in *S. fibuligera* sdu (a) (*Sftps1*) and *S. cerevisiae* Y108 (c) (*Sctps1*). Mid-exponential-phase cells (A_{600} = 0.4-0.6) growing on YPD medium were subjected to various stress treatments. At the indicated times, samples were taken and analyzed by RT-PCR for mRNA levels of *tps1* gene and β-*actin* gene as described in the methods. β-*Actin* gene was used as an internal standard (b, d).

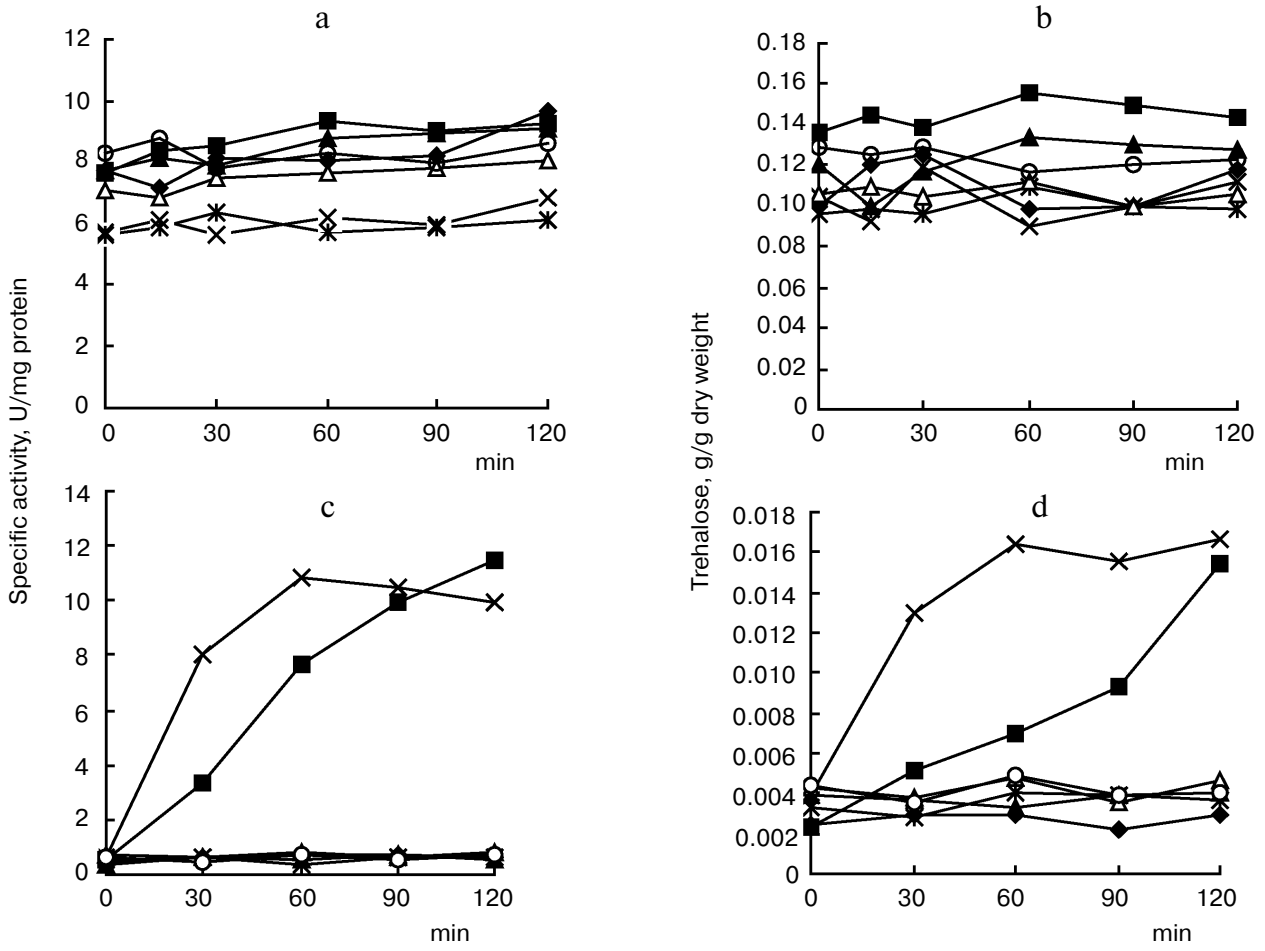

Fig. 4. Effects of various stress treatments on the SfTps1 activity (a, c) and trehalose content (b, d) of *S. fibuligera* sdu (a, b) and *S. cerevisiae* Y108 (c, d). Mid-exponential-phase cells (A_{600} = 0.4-0.6) growing on YPD medium were exposed to various stress treatments. Designations: control (closed rhombs), 25→37°C (closed squares), 0.3 M NaCl (closed triangles), 7% ethanol (×), 0.4 mM H_2O_2 (open squares), ice cold (open triangles), 0.4 M sorbitol (open circles).

the *Sftps1* gene to various stress conditions by RT-PCR. To this end, samples were taken before and 15, 30, and 60 min after cells were subjected to various stress conditions, and the *β-actin* gene was chosen as an internal reference gene. It was noticed that the expression of the *Sftps1* gene was not affected by the different stress treatments (Figs. 3a and 3b). In contrast, expression of the *tps1* gene in *S. cerevisiae* Y108 strain was induced by heat and ethanol shock (Figs. 3c and 3d).

Effects of various stress treatments on SfTps1 activity and trehalose content. To investigate the effects of various stress conditions on the activity of SfTps1 and the accumulation of trehalose, samples were taken at 15, 30, 60, 90, and 120 min after exposure to stress treatments. As shown in Figs. 4a and 4b, none of the stress treatments caused the activation of SfTps1 and change in trehalose content in the cells. However, in *S. cerevisiae* Y108, heat and ethanol shock caused significant activation of these two parameters (Figs. 4c and 4d). The results in Fig. 4 also show that SfTps1 activity and the accumulated trehalose in *S. fibuligera* A11 were significantly higher than those in *S. cerevisiae* Y108 in the absence of stress treatments.

DISCUSSION

In this study, we investigated the accumulation of trehalose in *S. fibuligera* A11 under various stress conditions. To get a complete view, from the transcriptional level to the accumulation of the product, we preliminarily isolated a genomic DNA fragment (*Sftps1*), which most probably encodes a Tps1 based on the high sequence identity with Tps1 of many different fungi. We found that stress treatments applied for 120 min did not increase the activity of SfTps1 and the concentration of trehalose in this strain. The results of RT-PCR also showed that none of the stress treatments applied for 60 min increased the expression of the *Sftps1* gene. In this study, we also found that trehalose

was present during growth of *S. fibuligera* A11 on YPD under non-stressed conditions in significant quantities (0.16 g/g dry weight) at the entrance to stationary phase. Many reports have demonstrated that the trehalose content and the Tps1 activity vary under different stress conditions and undergo drastic changes during the life cycle in *S. cerevisiae*, *Kluyveromyces lactis*, *Schizosaccharomyces pombe*, *Hanson polymorpha*, *Aspergillus niger*, and *C. albicans* [1, 15-18]. Hence, the stress response pattern of the *Sftps1* gene, the activation of SfTps1, and the accumulation of trehalose in *S. fibuligera* A11 were different from those in *S. cerevisiae* and other fungi.

According to the literature on *S. cerevisiae* Y108 strain, Tps1 activity and trehalose content were low but increased dramatically upon heat or ethanol shock. The expression of the *tps1* gene was also increased. It is known that the expression of the *tps1* gene in *S. cerevisiae* is a response to adverse environmental conditions, and its regulation shows a striking similarity with the expression patterns of a number of genes whose transcription is regulated by a DNA element called the stress responsive element (STRE) [2, 11, 19]. All these data favor the idea that trehalose is an important factor in the stress response in *S. cerevisiae*. Why is not synthesis of trehalose in *S. fibuligera* A11 a response to stress conditions? One possible explanation could be linked to the high level of trehalose already present in *S. fibuligera* A11 under non-stressed conditions, which may allow this yeast to live under stress conditions in natural environments, such as soil and air, with no need for further activation of SfTps1 and trehalose synthesis. However, elucidating the detailed mechanism for the lack of response to the stress conditions needs further investigation. To increase knowledge on trehalose synthesis in *S. fibuligera* A11, further research is directed to clone the full-length *Sftps1* gene by PCR techniques in this laboratory.

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