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Role of *Tps1* in Yeast Stress Tolerance

TABLE 1

CEN.PK and BY background strains used in this study

Name in the text	Origin ^a	Genotype ^a	Reference	Source
<i>TPS1</i> ^c	CEN.PK	MATa <i>ura3-52</i>	JF1094	Ref. 25
<i>tps1Δ</i>	CEN.PK	MATa <i>ura3-52 tps1::hisG</i>	JF1476	Lab collection
<i>tps1-156</i>	CEN.PK	MATa <i>ura3-52 tps1::tps1-156 KanMx4</i>	JF118	This study
<i>tps2Δ</i>	CEN.PK	MATα <i>tps2::KanMx4</i>	JF248	This study
<i>tps1Δ tps2Δ</i>	CEN.PK	MATa <i>ura3-52 tps1::hisG tps2::KanMx4</i>	JF87	This study
<i>tps1-156 tps2Δ</i>	CEN.PK	MATa <i>ura3-52 tps1::hisG tps2::KanMx4, YCpLac33 tps1-156</i>	JF451	This study
<i>TPS1 hsf1Δ pTet-HSF1</i>	CEN.PK	MATa <i>ura3-52 trp1-289 leu2,3-112 hsf1::KanMx6, pTet-O₇ HSF1</i>	JF91	This study
<i>tps1Δ hsf1Δ pTet-HSF1</i>	CEN.PK	MATa <i>ura3-52 trp1-289 tps1::hisG hsf1::KanMx6, pTet-O₇ HSF1</i>	JF90	This study
<i>tps1-156 hsf1Δ pTet-HSF1</i>	CEN.PK	MATa <i>ura3-52 trp1-289 tps1::hisG hsf1::KanMx6, pTet-O₇ HSF1 YCpLac33 tps1-156</i>	JF101	This study
<i>tps1Δ pHSP104</i>	CEN.PK	MATa <i>leu2,3-112 tps1::hisG, pGP564 HSP104</i>	JF154	This study
<i>tps1Δ pHSP82</i>	CEN.PK	MATa <i>leu2,3-112 tps1::hisG, pGP564 HSP82</i>	JF155	This study
<i>tps1Δ pHSC82</i>	CEN.PK	MATa <i>leu2,3-112 tps1::hisG, pGP564 HSC82</i>	JF156	This study
<i>tps1Δ pSSA3</i>	CEN.PK	MATa <i>leu2,3-112 tps1::hisG, pGP564 SSA3</i>	JF160	This study
<i>TPS1</i> ^d	BY4741	MATa	JF1412	Euroscarf
<i>tps1Δ</i>	BY4741	MATa <i>tps1::hisG</i>	JF1927	Lab collection
<i>tps1-156</i>	BY4741	MATa <i>tps1::tps1-156 KanMx4</i>	JF173	This study

^a Strain genetic background. All the CEN.PK113-7D-derived strains bear the *MAL2-8c* and *SUC2* alleles.

^b The plasmids present in the strain are given in bold type.

^c Referred to as strain *TPS1 TPS2* in Fig. 2A.

^d This strain is the BY4741 reference strain. All BY strains bear the *his3-Δ1 leu2-Δ0 ura3-Δ0 met15-Δ0 ADE2 CAN1 LYS2 TRP1* alleles.

kinds, including heat, freezing shock, hydrostatic pressure, desiccation, and oxidative, ethanol and osmotic stresses (13–19). However, the loss of the *TPS1* gene is also accompanied by several other phenotypes, apparently unrelated to the lack of trehalose, such as the inability to grow on rapidly fermentable sugars, hyperaccumulation of glycogen, sporulation and respiratory defects (20–22). In the rice blast fungus *Magnaporthe oryzae*, Talbot's group (23, 24) succeeded in demonstrating that *Tps1*, in addition to trehalose-6P synthase enzyme, also possesses a regulatory function essential for growth of the fungus.

These observations led us to speculate that *Tps1* protein may have regulatory functions in addition to its enzymatic role in the synthesis of trehalose. To determine whether the trehalose accumulation phenotype is due to withstand stress exposure or to trehalose synthesis, we used catalytically inactive variants of *Tps1* that lack the trehalose-6P and trehalose. More recently, we used a CEN.PK strain (25) because it constitutes a prototrophic *MAL* constitutive strain (25), allowing the study of the Agt1-mediated trehalose import (26), allowing the study of trehalose levels in the absence of *Tps1* or any stress. Combining these strategies, we demonstrated that the tolerance of yeast cells to different stresses was largely independent of trehalose but relied on the *Tps1* protein itself, independently of its annotated catalytic function.

Experimental Procedures

Construction of Yeast Strains and Plasmids—The *S. cerevisiae* strains used in this study (Table 1) were derived from the CEN.PK113-7D, a prototrophic *MAL* constitutive strain (25), or from the BY4741, a *mal*[−] strain (EUROSCARF collection). Primers and plasmids used in this study are listed in Tables 2 and 3, respectively. The *TPS1* alleles were obtained by site-directed mutagenesis. As an overview of the construction process, 139-bp sequences corresponding to 5' and 3' ends of *hisG* were added by recombinant PCR to the extremities of *TPS1*. This mosaic PCR product was cloned into a yeast shuttle plasmid (pMP1, YCpLac33 backbone) and marked with the kanMX4 selection marker, yielding to pMP2. This vector was then used as template for site-directed mutagenesis by inverse

PCR, yielding to different *TPS1* alleles (*tps1-102* in pMP8, *tps1-111* in pMP7, and *tps1-156* in pMP6). As a final step, the kanMX4-tagged *TPS1* alleles were excised from the YCpLac33 backbone and inserted into the *URA3::hisG* locus of JF1476 strain via the flanking *hisG* sites. The *URA3* gene was replaced to catalytically dead *URA3* by using the In-Fusion system (Clontech) under the Tet-O₇ promoter in a yeast strain carrying the *URA3* gene and 66. *HSF1* gene was then deleted by using homologous recombination in cells carrying the *URA3* gene and pMP20, after amplification of the KanMx6 cassette on the pFA6a plasmid with primers 82 and 83. Strain genotypes were confirmed by PCR. Overexpression of HSPs was carried out using the 2μ plasmid from the yeast tilling collection (Open Biosystems, YSC4613 (28)) and subcloning of the gene of interest in the same pGP564 backbone vector.

Culture Conditions—Unless otherwise stated, yeast cells were cultivated in YN synthetic medium (yeast nitrogen base without amino acids and ammonium at 1.7 g/liter (Difco)), supplemented with ammonium sulfate at 5 g/liter, complete dropout when required (MP-Biomedicals CSM) and 2% (w/v) galactose (YN Gal) or 2% galactose plus 1% trehalose (YN GalTre medium). Cultures were conducted in shake flasks (0.5-liter Erlenmeyer flasks with a working volume of 100 ml) at 30 °C and 200 rpm on a rotary shaker. Cell plating for viability analysis (cfu assay) was performed on YP Gal medium (10 g/liter yeast extract, 10 g/liter bacto peptone, 20 g/liter bacto agar, and 20 g/liter galactose). For modulation of *HSF1* transcript levels using the Tet-O₇ promoter, cells were cultivated according to Ref. 27 in the presence of 0.5 μg/ml tetracyclin (Sigma, stock solution of 5 mg/ml in 50% ethanol).

Stress Tolerance Assays—All stress experiments were carried out using exponentially growing cells (*A*₆₀₀ 0.5–0.7, corresponding to 1 × 10⁷ cells/ml) cultivated on YN Gal or YN Gal-Tre medium at 30 °C, after inoculation of the culture with a cell density corresponding to *A*₆₀₀ 0.025. For mild heat shock (37 °C to 42 °C), cells were incubated in an oven after initial immersion

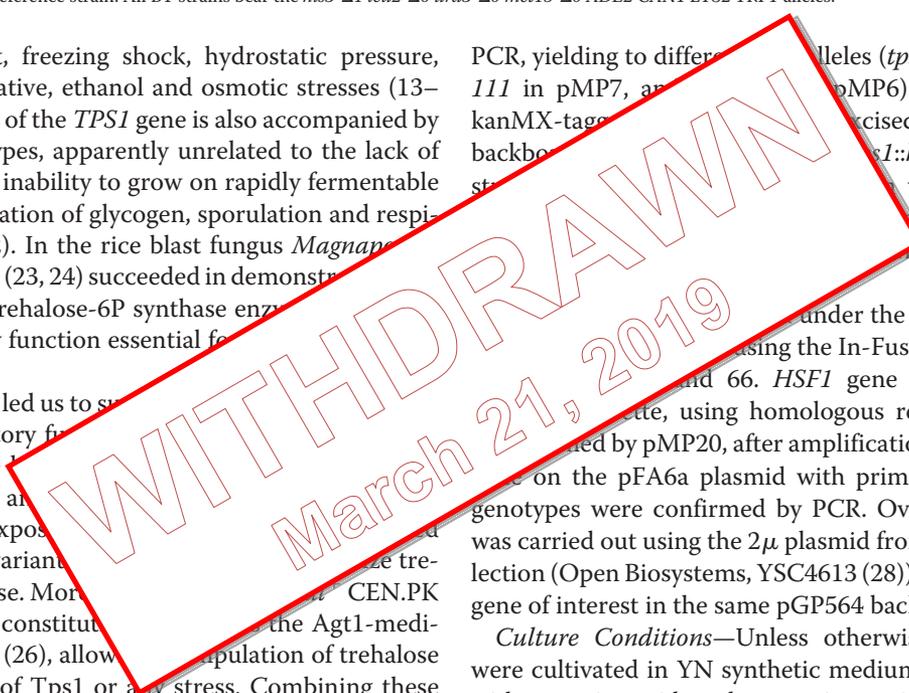


TABLE 2
Primers used in this study

	Name	Sequence ^a
1	hisG 1/24 (Sac1)	AGGAGCTCATGTTAGACAACACCCGCTTACGC
2	hisG 100/72 (TPS)	CGTCTCTGGCTCTATCGTAAAGGTACCAATTAATTTTTATGCCGACGCGGGCCAG
3	hisG 803/834 (TPS)	TCACCGTATAGGATATCGGATCCTCAGCAGCGAAACGTTGTTCTGGGAAACCATG
4	hisG 900/879 (Sph1)	TCCGATGCTCACTCCATCATCTTCTCGATC
5	TPS1 1021/1059 (hisG)	GCGGCATAAAAATTAATTGGTACCTTTACGATAGAGCCAGAGACGATGCCGACGAAGATGAAG
6	TPS1 3684/3656 (hisG)	ACAACGTTTCGCTGCTGAGGATCCGATATCCTATACGGTGAAAAAACTACTTGCGCAG
7	SP-TPS1-T304G-A305T	CGATGAAATCGTCGACTTACACGCTCAACGGGTTTCAGTAATTCCTATT
8	ASP-TPS1-T304G-A305T	AATAGAATTACTGAACCCGTTGACGTGTAAGTCGACGATTTCCATCG
9	SP-TPS1-G332C	ACAACGGGTCAGTAATTCCTATTCCTATCGCCGTTATTCCTATT
10	ASP-TPS1-G332C	AATGGAATAACGGCGATAGAATAGAATTAATCTGGACCCGTTGT
11	SP-TPS1-A467G	CGATTTAACCTAGGTGCATGGTTACCATTTTGATGTTGGTTC
12	ASP-TPS1-A467G	GAACCAACATCAAATGGTAACCATGCACCTAGGTTAAATCG
65	SP/HSF1-His-IF	TCAATTCGGGGATCCATGAATAATGCTGCAAAATACAGGGACGACC
66	ASP/HSF1-His-IF	TGCGGCCCTCCTGCAGCTAATGGTGTGATGGTGTATGATGTTTCTTAGC
82	SP/HSF1del-KanMX	CCAAATAGCAGTACTGTTCCGGTAGATAAAGGCAAAGAGTTAGAGATGGGTAAGGAAAAGACTCA
83	ASP/HSF1del-KanMX	GAAAACTCATCGAGCATCATTAATGATTATATACGCTATTTAATGACCTGCCTGTGTACTA

^a Restriction sites are underlined.**TABLE 3**
Plasmids used in this study

Identifying	Name	Description	References
pMP 6	YcpLac33 <i>tps1-156</i>	YCpLac33; CEN origin; <i>TPS1</i> promoter	This study
pMP 20	pTet- <i>HSF1</i>	pCM173 backbone (27); CEN origin; Tet- <i>O₇</i> promoter	This study
pMP 38	p <i>HSP104</i>	pGP564 backbone (28); 2 μ origin; native promoters	This study
pMP 39	p <i>HSC82</i>	Same as pMP 38	
pMP 40	p <i>HSP82</i>	Same as pMP 38	
pMP 41	p <i>SSA3</i>	Same as pMP 38	

in water bath set at the desired temperature. Cells were transferred and temperature increase. Temperature shock (45 °C to 52 °C) was carried out for 10 min to acquire thermotolerance. Cells were then transferred to 42 °C and then transferred to 25 °C. Cells were then transferred to ethanol, oxidized, and then transferred to the presence of 5% ethanol, oxidized, and then transferred, respectively. A short term experiment was carried out according to the vacuum method. Cells were incubated at 30 °C for 5 to 60 h. At the indicated time, cells were directly analyzed for viability by flow cytometry and cell wall content.

Analysis of Viability by Flow Cytometry—Flow cytometric analysis of the relative percentage of living and nonviable cells in the population was determined using the Guava[®] ViaCount[®] reagent (4000-0040, purchased from Millipore) and the Attune[®] Acoustic Focusing Cytometer (Life Technologies, Inc.). Exponentially growing cells (approximately 2×10^7 cells) were harvested and sonicated to ensure single cell suspensions (40 Hz, 10 s) and then mixed to the Guava[®] reagent according to manufacturer indications and incubated for 5 min at room temperature. Cytometer acquisition was run at 100 μ l/min to ensure high specificity, on a total number of 100,000 events/run. Excitation was performed from the 488-nm laser (blue). Voltage calibration of the cytometer was always performed by using the unstained, control sample of the WT and further checked on the *tps1* Δ strain. Routinely applied voltages were 2,000 mV for the forward scatter and 2,600 mV for the side scatter. For optimal positioning of the whole cell population within the autofluorescence black gate (signal below 10^3 thresholds for both the BL-1 (525 nm) and BL-3 (670 nm) filters), we set BL-1 and BL-3 to 1,200 and 2,100 mV, respectively.

In addition to the control strains, unstained samples from all strains of interest, before and after stress exposure, were analyzed to ensure that these operation settings for the side scatter-forward scatter and autofluorescence could apply. Sample analysis was carried out using a combination of two emission filters (BL-1 and BL-3) and the 670-nm filter. The 670-nm filter brightly stains dying cells. The data were then plotted (log scale) according to the manufacturer's instructions. The data were then processed using Cytometer software. The percentage of cells in the biological state was calculated from cell wall content (red and blue, respectively), relative to the number of cells determined from the forward scatter plot. Cytometry-based viability assays presented in this work were repeated at least three times, from independent cultures. The data were then exported to Excel for graphics preparation and to XL-Stat or Statgraphic software for statistical analysis. Viability was also determined by measuring cfu after 2 days on YP Gal plates at 30 °C to assess the correlation between the cytometry-based viability assay and cfu results.

Assay of Trehalose-6P Synthase—Crude extracts were prepared according to Ref. 29. Practically, 10 optical density units were collected in a centrifuge tube and washed once with sterile water, and cell pellets were frozen at -20 °C. For protein extraction, 200 μ l of extraction buffer (20 mM Hepes, pH 7.1, 1 mM EDTA, 100 mM KCl, 1 mM DTT, and 1 mM PMSF) and 0.3-g glass beads (0.5-mm diameter; BioSpec 11079105) were added to the cell pellets. Crude extracts were kept on ice before use, and total proteins were quantified using the Bradford assay (Bio-Rad protein assay dye reagent concentrate, 500-0006). The activity of trehalose-6P synthase was carried out according to Ref. 29.

Western Blot Analysis of *Tps1*—Crude extract preparations were carried out as for trehalose-6P synthase assay. For protein electrophoresis, 300 ng of proteins were loaded on Stain-Free precast SDS-PAGE gels 10% (Bio-Rad; 456-8033), and the migration was done at 180 V for 25 min. Transfer to nylon membrane (Bio-Rad; 170-4158) was done using the Trans-Blot[®] Turbo[™] blotting system. Membrane saturation was done in 50 ml of $10\times$ TBS and 0.1% Tween 20, plus 2.5 ml of

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blocking reagent for TBS (Thermo; 37535), for 1 h with gentle agitation and at room temperature. Primary antibodies were raised against the 56-kDa subunit of *Tps1* (KH-1142, rabbit serum, kind gift from J. Londesborough, Technical Research Center of Finland, Espoo, Finland (6)) and diluted 1/10,000 times in TBS Tween solution. Hybridization with secondary antibodies was performed with anti-rabbit alkaline phosphatase-conjugated antibodies (diluted 1/2,000 in 1× TBS) (Sigma; A3687). Revelation was monitored by 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate (Sigma; B3679).

Western Blot Analysis of *Hsf1*—For crude extract preparation, 50 optical density units were collected from cells grown to exponential phase, before (T0) and during heat shock to 40 °C at the indicated times. Cells were washed once with cold water and frozen in liquid nitrogen. The extraction was carried out immediately after at 4 °C in a buffer containing 50 mM Tris-HCl, pH 6, 0.5 M NaCl, 1 mM EDTA, pH 7, 0.1% SDS, 1% Triton, 1% mixture protease inhibitor, 20 mM NaF, 1 mM Na₂VO₃, 50 mM β-glycerophosphate, and 1 mM PMSF. For the control lane with phosphatase, the buffer contained 50 mM Tris-HCl, pH 6, 0.5 M NaCl, 1 mM EDTA, pH 7, 0.1% SDS, 1% Triton, and 10 units of alkaline phosphatase. The cells were broken in the presence of 0.5-g glass beads for 30 runs of 30 s at 4 °C. The samples were centrifuged for 5 min at 500 × *g*, and the supernatant transferred to 1.5-ml microcentrifuge tubes for an additional centrifugation (1 min, 13,000 × *g*). Crude extracts were stored on ice before their use, and total proteins were determined by Bradford assay (Bio-Rad protein assay reagent; 500-0006).

For Western blot analysis, 10 μg of total protein samples were loaded on 4–20% gradient SDS-PAGE gels (Bio-Rad; 456-8083), and transferred to Immobilon-P nitrocellulose membrane (Bio-Rad; 162-0150) by electrotransfer (Bio-Rad; 165-0180) at 100 V. Procedure was done according to the manufacturer's instructions. Revelation for *Hsf1* was performed using Clontech ECL Western Substrate (Pierce; 34075), using the Clontech ECL Western Substrate (Pierce; 34075), using the Clontech ECL Western Substrate (Pierce; 34075). Primary antibodies anti-*Hsf1* (kind gift from Dr. H. Sakurai, Graduate School of Medical Science, Kanazawa, Japan) were diluted 1/2,000-fold. Secondary antibodies used were anti-mouse peroxidase-conjugated antibodies (Thermo; 32430) and diluted 1/2,000-fold.

RNA Sampling and cDNA Synthesis for Quantitative PCR Assays—The cells were grown in YN Gal medium to A₆₀₀ ~1.0 and submitted to heat shock as described above. Samples (~10⁸ cells) were collected (2,200 × *g*, 4 °C, 3 min) before (T0 control, calibrator sample) and 15 and 30 min after the shift to 40 °C. The pellets were immediately transferred in 2-ml microcentrifuge tubes, washed with 1 ml of cold water, frozen in liquid nitrogen, and stored at -80 °C until RNA extraction. Frozen cells were mechanically disrupted for 3 min using the TissueLyserII apparatus from Qiagen, with one stainless steel bead per tube. Total RNA was extracted using the SV Total RNA purification kit from Promega. The quantification of the RNA samples was assessed by using the ND-1000 UV-visible light spectrophotometer (NanoDrop Technologies), whereas the Bioanalyzer 2100 with the RNA 6000 Nano LabChip kit (Agilent) was used to certify RNA integrity. Only RNA samples with a 260/280-nm wavelength ratio of ~2 and a 260/230-nm wave-

length ratio >2 were retained for analysis. One microgram of total RNA was reverse-transcribed into cDNA in a 20-μl reaction mixture, using the iScript cDNA synthesis kit (Bio-Rad).

Quantitative PCR Assay on the Biomark—High throughput quantitative PCR was performed using the Fluidigm® Biomark microfluidic system. Every sample-gene combination is quantified using 96.96 Dynamic Array™ integrated fluidic circuits (IFC), which ensure performing up to 9,216 parallel qPCRs³ in nanoliter scale volumes. Pre-amplification of cDNA samples, chip loading, and quantitative PCR were performed according to the manufacturer's protocols. cDNA sample preparation was as follows: 5 ng of each cDNA were initially pre-amplified (activation at 95 °C for 10 min and 14 PCR cycles (95 °C for 15 s and 60 °C for 4 min)) with PreAmp Master Mix (Fluidigm) and a primer mix at 200 nM containing all the primers targeting all the genes analyzed on the array. Pre-amplified samples were then diluted after an exonuclease treatment (NEB M02935). To finalize samples preparation for IFC loading, a reagent mix consisting of 440 μl of PreAmp Master Mix (Applied 430976), 44 μl of 20× sample loading reagent (Fluidigm; 100-1000), 100 μl of Biotium (Biotium; 31000) plus 133 μl of water. The reagent mix was divided into 6-μl aliquots were dispensed into the IFC. Two microliters of the reagent mix were added to this reagent mix and vortexed and centrifuged. For each gene, each pair of primers was tested in the assay loading reagent (each primer) in the assay loading reagent and dispensed into the 96 wells of the assay loading reagent. The priming of the Dynamic Array™ IFC in the assay loading reagent was performed by the IFC Controller HX, 5 μl of cDNA samples and primer sets were dispensed from the sample and assay plates, respectively, and dispensed into the inlets of the IFC. After loading the cDNA samples and primer sets into the IFC on the IFC Controller HX, the IFC was placed into the BioMark for thermal cycling with the following protocol: thermal mix steps at 50 °C for 2 min, 70 °C for 30 min, and 25 °C for 10 min and hot start at 50 °C for 2 min and 95 °C for 10 min, followed by 35 PCR cycles (95 °C for 15 s and 60 °C for 60 s). After completion of these cycles, melting analysis was performed to verify PCR specificity, contamination, and the absence of primer dimers.

qPCR Assays Quality Control and Data Analysis—Most of the primer sets for qPCR, especially the reference genes, were previously described and validated (30). New pairs of primers were designed using Vector NTI advance v11 (Life Technologies, Inc.). A BLAST analysis against the *S. cerevisiae* genome sequence was included for specificity confidence. Reaction efficiency for each primer pair was evaluated by the dilution series method using a mix of cDNA samples as the template. The absence of genomic DNA in RNA samples was checked by qPCR before cDNA synthesis. All these quality control assays were performed on a classical qPCR machine (MyIQ real time PCR system from Bio-Rad) as previously described (30). The qPCR assays on the Biomark were performed in technical duplicates. A negative control (No Template Con-

³ The abbreviations used are: qPCR, quantitative PCR; IFC, integrated fluidic circuit.

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trol) was incorporated in each assay. Assay efficiencies of all primer sets analyzed on the IFC were re-evaluated by the dilution series method and applied to final calculations of transcript levels.

For normalization purposes, the transcript levels of six putative reference genes were evaluated in each sample (data not shown). For data analysis, individual C_q values were exported from the Fluidigm real time PCR analysis software version 4.1.2. They were used to assess normalized fold change values, using the wild type (*TPS1*) strain before heat shock as the calibrator sample, and robust normalization factors calculated from the geometric averaging of the four most stable reference genes in our experimental setup (*i.e.* *TAF10*, *ALG9*, *IPP1*, and *UBC6*). Final fold-change values are given as means \pm S.D. from the two biological replicates (independent cultures) that were carried out for transcript level analysis in the CEN.PK strain background.

Other Analytical Procedures—Cell sampling and metabolites extraction were carried out as previously described (31), and ATP measurement was realized as described (32). The enzymatic measurement of ATP was carried out at 30 °C in Tris-HCl buffer with addition of 0.4 mM NAD^+ , 0.1 mM glucose, 4.5 units/ml glucose-6-phosphate dehydrogenase, and 1.5 units/ml hexokinase. NADH production was then assessed at 340 nm using a fluorescence spectrophotometer (Agilent G1103A). Trehalose measurement was done according to (Ref. 33).

Statistical Analysis of the Data—Statistical analysis of “data of viability” were conducted by using the “ANOVA” module of ICS Centurion 16 software. This analysis allowed to compare the Tukey’s test on the data. The Kruskal-Wallis procedure and whiskers plots to visualize the data.

Results

Construction and Validation of Catalytically Inactive Variants of the *Tps1* Protein—To construct catalytically inactive variants of the *S. cerevisiae* *Tps1* protein, we followed the strategy employed earlier by Wilson *et al.* (25) for *Magnaporthe grisea* *Tps1*. We mutated three key residues required for the interaction with glucose-6P substrate in the catalytic site of the protein, leading to *Tps1*^{Y102V}, *Tps1*^{W111S}, and *Tps1*^{D156G} variants, respectively. After transformation of a *tps1Δ* mutant with CEN plasmids carrying the corresponding *tps1* alleles, the loss of function of these strains relative to trehalose metabolism was validated in four ways. First, Western blotting against *Tps1* validated that expression and stability of *Tps1* variants were identical to wild type (not shown). Second, none of these strains exhibited measurable trehalose-6P synthase activity, whereas wild type (*TPS1*) cells led to a specific activity of 6.5 nmol/min/mg protein. Third, none of these strains could lead to trehalose-6P accumulation upon glucose addition to trehalose-grown cells according to experimental conditions described in (31). Contrary to the control strain that showed a burst of trehalose-6P 5 min after the glucose pulse (8 $\mu\text{mol/g DW}$), trehalose-6P was below the detection level in cells expressing the different *tps1* alleles. As a final proof for trehalose-6P synthase deficiency *in vivo*, we assessed the inability to produce trehalose

under conditions known to readily trigger the accumulation of this sugar, such as heat shock (6, 34, 35). When the cells were incubated for 2 h at 42 °C, which is the optimum temperature for *Tps1* catalytic activity and trehalose accumulation, wild type cells accumulated a huge amount of trehalose ($22 \pm 5 \mu\text{g eq-glucose}/10^7$ cells), whereas no trace of this disaccharide could be detected in cells expressing the catalytic variants. Because the three variants gave identical results, including for stress experiments carried out below (data not shown), we arbitrarily used and thoroughly present results obtained with the *Tps1*^{D156G} variant.

To directly assess the role of trehalose in response to various stresses (see below), we used the *MAL*⁺ CEN.PK strain, able to import trehalose from the medium by the constitutively expressed *Agt1* transporter (26, 36). The function of the catalytically inactive variants of *Tps1* in yeast survival to stress was therefore examined in this CEN.PK strain background, as well as in the *mal*⁻ BY4741 strain, unable to import trehalose. The impact of different stresses was assayed on exponentially growing cells in a galactose medium, permissive for growth of the *tps1* mutants, with or without 1% exogenous trehalose. The impact of trehalose on each strain was precisely measured by the ATP measurement method that allowed to assess the ATP content (Fig. 1). For each strain, a control experiment was realized and confirmed by flow cytometric method (not shown). Table 4 indicated a 30–40% decrease in ATP content of exponentially growing cells deleted for *TPS1* in the presence of the carbon sources used as the growth medium. This drop of viability was not prevented by preloading of the cells with trehalose. More remarkably, it was not prevented in *tps1-156* cells expressing the catalytically inactive *Tps1*^{D156G} variant.

***Tps1*, Not Trehalose, Is Important for Yeast Viability in Response to Various Kinds of Stresses**—In response to temperature upshift to 42 °C, the viability of the *tps1Δ* mutant sharply dropped by several decades, whereas the viability of the wild type *TPS1* strain remained almost unchanged as could be seen from this logarithmic graph (Fig. 2A). Interestingly, the viability pattern of the *tps1-156* strain was exactly the same as wild type. In none of these strains, preload of trehalose (see Table 5 for intracellular trehalose content) had a protective effect and changed strain sensitivity to heat. We then investigated the impact of the catalytically inactive *Tps1*^{D156G} variant on heat resistance of a *tps2Δ* mutant (Fig. 2A), defective in trehalose-6P phosphatase and also known to exhibit temperature-sensitive growth (37, 38). When exposed to 42 °C, we found that this mutant was much less sensitive than the *tps1Δ* mutant, whereas heat sensitivity of the *tps1Δ tps2Δ* double mutant was almost the same as *tps1Δ*. In contrast, upon expression of the *tps1-156* allele, the *tps2Δ* mutant recovered the survival rate of wild type *TPS1* cells. These data indicated that the heat sensitivity of a *tps2Δ* mutant strain was likely due to the overaccumulation of trehalose-6P during heat shock and not to the absence of the *Tps2* protein nor to the lack of trehalose.

We then analyzed cell viability in response to desiccation, another relevant adverse condition for yeast. As is shown in Fig. 2B, the loss of *TPS1* gene markedly increased the sensitivity of

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Role of *Tps1* in Yeast Stress Tolerance

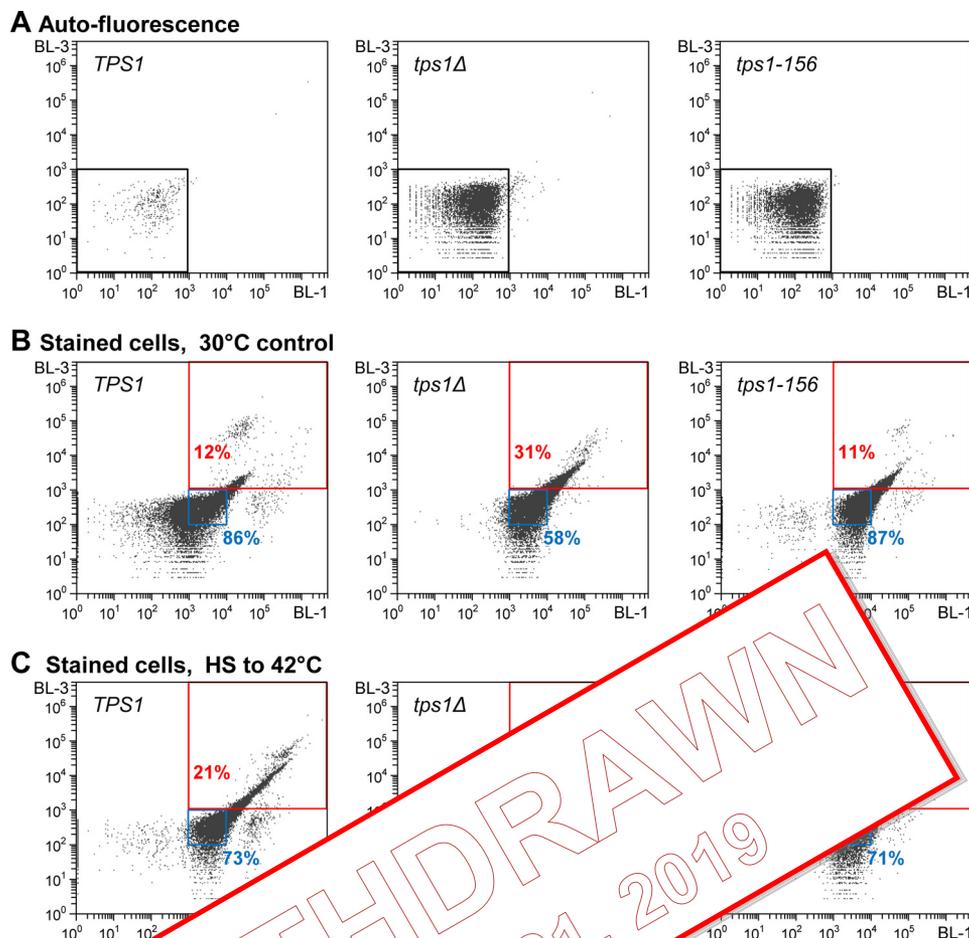


FIGURE 1. Monitoring cell viability. Flow cytometry plots (log scale, BL-1 (525-nm filter), BL-3 (670-nm filter)), showing gates positioning, after voltage calibration (Guava Technologies, Inc.). Plots obtained with exponentially growing cells ($A_{600} \sim 1.0$ unit) cultivated in YN synthetic medium supplemented with different carbon sources at 30 °C. The values are the means \pm S.D. of five independent experiments. Gal Tre, 2% galactose + 1% trehalose; Gly Lac Eth, 3% glycerol + 2% lactate + 2% ethanol; ND, not determined; NG, no growth. **A**, cell autofluorescence plots. Voltage calibration was set for optimal positioning of the cells to 42 °C for 2 h (C). **A**, cell autofluorescence plots. Voltage calibration was set for optimal positioning of the cells to 42 °C for 2 h (C). **B**, viability determination of the nuclear dye, which stains on “living” cells (blue gate) and “nonviable” cells (black gate), for both the x axis (BL-1, 525-nm filter) and the y axis (BL-3, 670-nm filter). **C**, viability determination of the nuclear dye, which stains on “living” cells (blue gate) and “nonviable” cells (black gate), for both the x axis (BL-1, 525-nm filter) and the y axis (BL-3, 670-nm filter). Numbers (in %) indicate the fraction of the population in the gate. **C**, viability determination of the nuclear dye, which stains on “living” cells (blue gate) and “nonviable” cells (black gate), for both the x axis (BL-1, 525-nm filter) and the y axis (BL-3, 670-nm filter). Legend is as in B.

TABLE 4

Influence of *TPS1* alleles on the viability of exponentially growing cells

Shown are results from flow cytometric analysis of viability of exponentially growing cells ($A_{600} \sim 1.0$ unit) cultivated in YN synthetic medium supplemented with different carbon sources at 30 °C. The values are the means \pm S.D. of five independent experiments. Gal Tre, 2% galactose + 1% trehalose; Gly Lac Eth, 3% glycerol + 2% lactate + 2% ethanol; ND, not determined; NG, no growth.

Carbon source	CEN.PK113–7D background			BY4741 background		
	<i>TPS1</i>	<i>tps1Δ</i>	<i>tps1-156</i>	<i>TPS1</i>	<i>tps1Δ</i>	<i>tps1-156</i>
Galactose 2%	91 \pm 4	63 \pm 10	89 \pm 4	86 \pm 5	61 \pm 12	83 \pm 6
Trehalose 2%	84 \pm 5	61 \pm 8	86 \pm 5	NG	NG	NG
Gal Tre	88 \pm 6	66 \pm 7	86 \pm 4	81 \pm 6	55 \pm 8	84 \pm 3
Gly Lac Eth	84 \pm 5	59 \pm 11	85 \pm 7	ND	ND	ND

yeast cells to dehydration, with less than 2% residual viability after 40 h. As for heat shock to 42 °C, this marked sensitivity was not observed in *tps1-156* cells. However, when the strains were loaded with trehalose prior to desiccation, a protective effect of the disaccharide was observed only in the *tps1Δ* mutant (p values $< 10^{-5}$).

These unpredicted results led us to extend investigation into the impact of the *Tps1*^{D156G} variant and/or of trehalose on the drop of viability in response to various stress conditions. As is

shown in Fig. 3, no statistically significant difference could be noticed between *TPS1* (wild type) and *tps1-156* cells. In none of these stresses, preload of trehalose had a protective effect, contrary to what was observed during desiccation for *tps1Δ* fragile cells. However, different patterns could be observed according to the stress applied. As previously seen in Fig. 2A, the drop of viability of a *tps1Δ* mutant was 5 times higher than that of wild type *TPS1* and *tps1-156* in response to temperature upshift to 42 °C (50% versus 10% drop of viability after 2 h). Likewise, the *tps1Δ* mutant was two times more sensitive than wild type *TPS1* and *tps1-156* cells, following a 5 mM H₂O₂ stress. Unexpectedly, we found that exposure to a mild temperature shock at 37 °C for 2 h was more detrimental than at 42 °C, even for the wild type *TPS1* strain. Also, this drop of viability at 37 °C was comparable between *TPS1*, *tps1-156* and *tps1Δ* cells. Similar results were obtained upon treatment of these three strains with 5% ethanol for 6 h.

Very similar profiles were obtained with strains from the BY4741 background (data not shown). A notable exception was the extreme sensitivity to desiccation stress of the BY derived

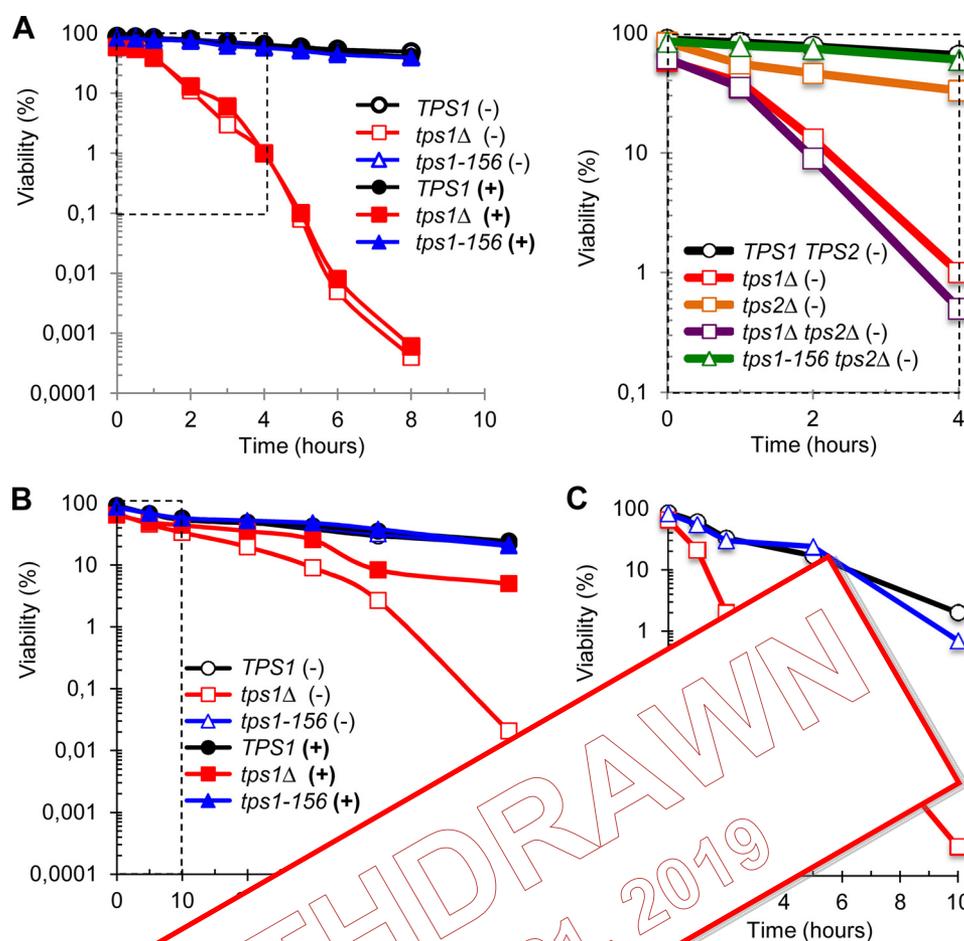


FIGURE 2. Time-dependent evolution of yeast viability. The figure shows the viability of CEN.PK background strains (A and B) in the absence of trehalose (–) and in the presence of trehalose (+) in YN Gal medium, (+). BY background strains (C) were only grown on YN Gal medium because the TPS2 gene is essential for growth on the medium. The data are represented as means \pm S.D. of at least three independent biological replicates. **A**, viability (log scale) as a function of time, in response to heat shock to 42 °C. The dotted line-delimited area in the left panel highlights the scaling for data presented in C. **B**, viability (log scale) as a function of desiccation time. After desiccation at 30 °C for the indicated time, the cells were rehydrated in PBS solution after 1 h of rehydration in PBS solution (see Ref. 18 for further details). Control at time 0 corresponds to exponentially growing cells. **C**, same as in B with BY background strains.

TABLE 5

Intracellular trehalose quantification in the CEN.PK113–7D background strains

–Tre and +Tre indicate whether growth was performed in YN Gal or in YN Gal+Tre medium, respectively. The values are the means \pm S.D. of three biological replicates and are expressed in $\mu\text{g eq}\cdot\text{glucose}/10^7$ cells.

Condition	<i>TPS1</i>		<i>tps1Δ</i>		<i>tps1-156</i>	
	–Tre	+Tre	–Tre	+Tre	–Tre	+Tre
2 h after upshift to the indicated temperature (unless otherwise stated in parentheses)						
Control (30 °C; T_0)	0	28 \pm 8	0	20 \pm 7	0	19 \pm 6
37 °C (1 h)	0	46 \pm 6	0	50 \pm 7	0	49 \pm 7
37 °C	0	40 \pm 7	0	45 \pm 5	0	42 \pm 8
42 °C (1 h)	35 \pm 6	45 \pm 6	0	47 \pm 8	0	41 \pm 9
42 °C	22 \pm 5	33 \pm 7	0	51 \pm 10	0	46 \pm 9
45 °C	13 \pm 4	36 \pm 7	0	25 \pm 9	0	32 \pm 7
48 °C	3 \pm 2	39 \pm 9	0	33 \pm 7	0	32 \pm 6
50 °C	0	22 \pm 8	0	8 \pm 7	0	15 \pm 6
52 °C	0	25 \pm 6	0	18 \pm 6	0	17 \pm 3
2 h after temperature upshift to 50 °C, following a preheat for 1 h at the given temperature						
Preheat at 37 °C	0	35 \pm 8	0	41 \pm 12	0	35 \pm 11
Preheat at 42 °C	3 \pm 2	21 \pm 8	0	26 \pm 9	0	31 \pm 5
Under peroxide, salt or ethanol exposure for the indicated time						
Control (30 °C; T_0)	0	25 \pm 7	0	23 \pm 11	0	27 \pm 8
50 mM H_2O_2 for 2 h	0	23 \pm 6	0	9 \pm 6	0	26 \pm 9
1 M NaCl for 2 h	0	26 \pm 5	0	19 \pm 4	0	23 \pm 5
5% EtOH for 6 h	8 \pm 3	9 \pm 6	0	4 \pm 3	0	7 \pm 5

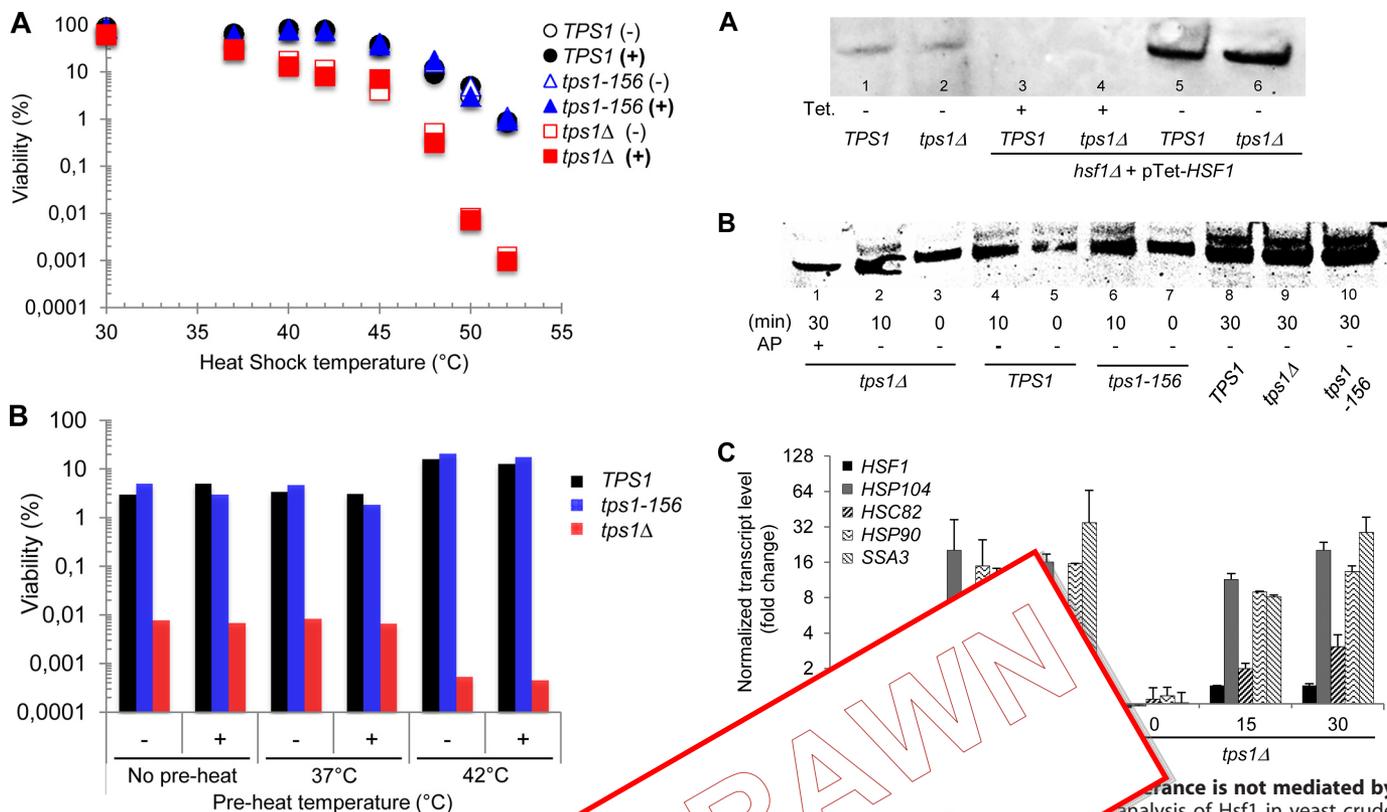
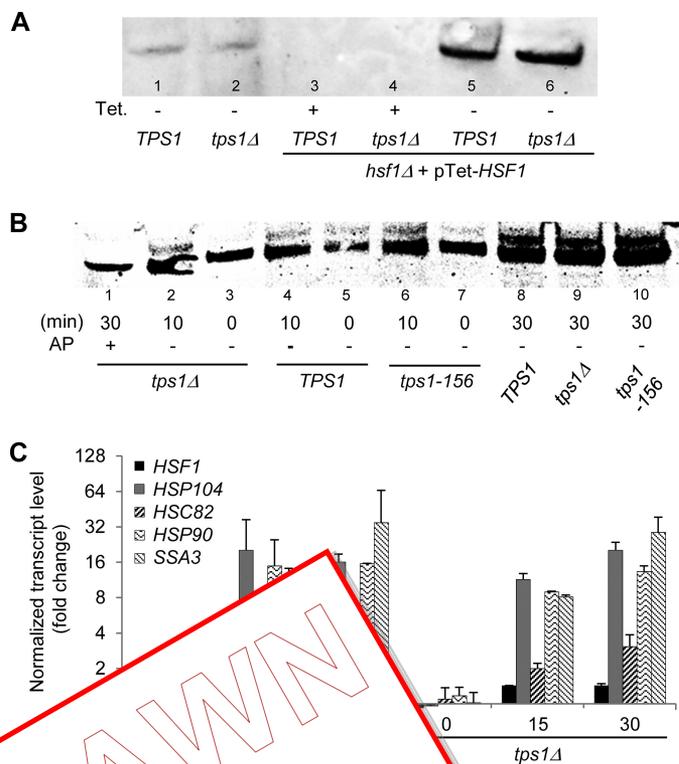


FIGURE 4. Contribution of the *Tps1* protein to thermally acquired thermotolerance.

Culture conditions were as described in the text. Cells growing exponentially in YN-Gal in the absence (+) of trehalose. The data represented are the means \pm S.D. values are not plotted. *A*, viability (log scale) in response to different heat shock temperatures. Viability was determined at 30°C, 45, 48, 50, or 52°C. *B*, viability after preheat for 1 h at 37 or 42°C followed by direct transfer to 50°C.

The Contribution of Tps1 to Thermally Acquired Thermotolerance is Independent of Hsf1-dependent Transcription

It is interesting that the *Tps1* protein played a major role in yeast cells to high temperature upshifts raised the question whether this role could be mediated through the heat shock transcriptional factor encoded by *HSF1*. *Hsf1* is an essential protein in yeast, ascribed to be the primary regulator for heat-induced transcription of heat shock proteins (HSP). It is also involved in diverse cellular processes such as protein degradation, detoxification, energy generation, carbohydrate metabolism, and maintenance of cell wall integrity (40–42). To investigate the interaction between *Hsf1* and *Tps1*, we used strains deleted for the essential *HSF1* gene and rescued by expression of this gene under the tetracyclin-Tet- O_7 -repressible promoter. Western blot analysis confirmed that *Hsf1* protein was no longer detected in cells treated with tetracyclin, whereas it was highly abundant in untreated cells because of the strength of Tet- O_7 promoter (27) (Fig. 5A). Then we revisited a previous report, suggesting that trehalose is a positive regulator of the heat-induced phosphorylation and activity of *Hsf1* (43, 44). Using yeast cells transformed with *pTet-HSF1*, we showed that a temperature upshift to 40°C for 10 and 30 min led to the appearance of two bands on the SDS-PAGE detected by the anti-*Hsf1* antibodies (Fig. 5B). As already shown, the upper band corresponded to the phosphorylated



form of this protein, because it disappeared after incubation of the cell extract with alkaline phosphatase prior to gel electrophoresis. The intensity of this upper, phosphorylated band of *Hsf1*, hardly visible in the control condition before heat shock, significantly raised with the time spent at 40°C, to be clearly and equally visible in all the strains, including the *tps1Δ* mutant. Therefore, our results indicated that, in contrast to a previous report (43), the phosphorylation state of *Hsf1* does not depend on trehalose. In agreement with this result and as shown in Fig. 5C, we found that the transcriptional induction of several *HSP* genes upon exposure of cells to 40°C for 15 and 30 min, a condition known to be *Hsf1*-dependent (45, 46), was not affected by the deletion of *TPS1*. Identical results were obtained with a *tps1Δ* mutant made in the BY4741 background (data not shown). Our results are therefore at variance to previous reports showing down-regulation of *HSP* genes in *tps1Δ* mutants (43, 47). The reason for this discrepancy could be explained by differences in methodology to quantify mRNA levels, which in our case employs a robust RT-qPCR protocol

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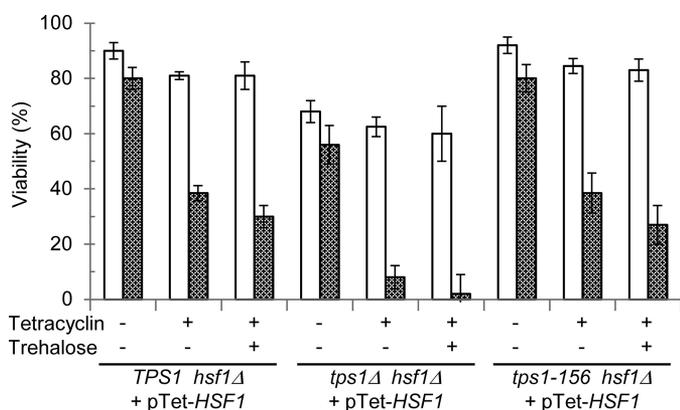


FIGURE 6. Genetic interaction between *TPS1* and *HSF1*. Viability of the *TPS1 hsf1Δ*, *tps1Δ hsf1Δ*, and *tps1-156 hsf1Δ* strains rescued by pTet-*HSF1* and exposed to 40 °C for 2 h. Viability measured before heat shock (30 °C, open bars) or after 2 h at 42 °C (gray bars). Tetracyclin, stress exposure in the absence (–) or presence (+) of tetracyclin. Trehalose, growth in YN Gal without (–) or with 1% trehalose (+). The data are represented as means ± S.D. of three independent biological replicates.

(30), whereas previous works were done by Northern blot analysis, a much less accurate and sensitive method.

Finally, we explored a genetic interaction between *TPS1* and *HSF1* on the cell viability in response to 40 °C heat shock (Fig. 6). The strong expression of Hsf1 (*TPS1 hsf1Δ* pTet-*HSF1* strain) led to similar results as for the *TPS1* strain (compare data in Figs. 3 and 6). Lowering the expression of Hsf1 in the *tps1Δ hsf1Δ* strain by the extinction of Tet-O₇ promoter with the anti-sense *HSF1* construct affected the viability of these cells at 30 °C (Fig. 6). This result sensitized them to heat shock as illustrated by the lower viability after 2 h at 40 °C. Interestingly, the *tps1-156 hsf1Δ* pTet-*HSF1* strain showed a similar sensitivity to heat shock as the control strain (*TPS1 hsf1Δ* pTet-*HSF1*). The overexpression of Hsf1 in the *tps1Δ hsf1Δ* strain sensitized them to heat shock, as illustrated by the lower viability in the *tps1Δ hsf1Δ* strain. The overexpression of Hsf1 in the *tps1Δ hsf1Δ* strain led to similar thermosensitivity than the single *tps1Δ* mutant. Finally, loading yeast cells with trehalose prior to heat shock had statistically no effect on their reduced viability upon *HSF1* extinction. Taken together, these results further indicated that trehalose had no role in thermotolerance and adaptive thermotolerance, which actually implicated Tps1, independently of the Hsf1 transcriptional activity.

***Tps1* Protects Cells from ATP Depletion upon Exposure to Heat Shock**—Based on the above results, we investigated whether overexpression of different heat shock proteins can suppress the *tps1Δ* sensitivity to heat shock. Accordingly, cell survival of this mutant after exposure to 42 °C for 2 h increased from 10 to ~70% upon overexpression of *HSP104*, *SSA3*, *HSP82*, and *HSC82* (Fig. 7A). Among the different HSPs found to suppress this *tps1Δ* phenotype, *HSP104* turned out to be the most efficient suppressor able to restore thermotolerance and adaptive thermotolerance similar to wild type cells (Fig. 7, A and B). However, upon longer exposure, the cell viability of the *tps1Δ* transformed with a high copy vector containing *HSP104* dropped significantly to reach less than 10% viable cells after 8 h (Fig. 8A). Because of the nature of the Hsp104 protein that

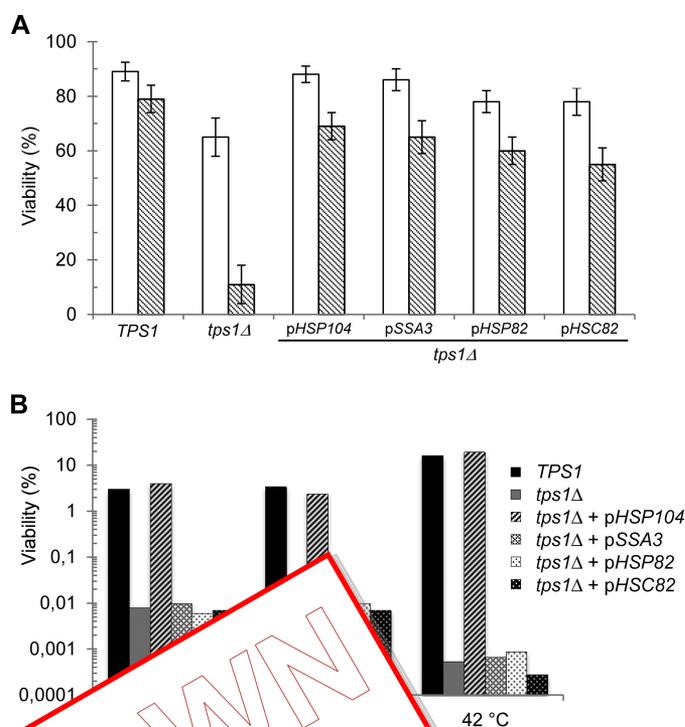


FIGURE 7. Viability of *tps1Δ* strains transformed with various HSPs. A, viability of the *tps1Δ* strain transformed with pTet-*HSF1* and various HSPs (*HSP104*, *SSA3*, *HSP82*, or *HSC82*). Control strains are transformed with pTet-*HSF1* and various HSPs from the CEN.PK background. Exponentially growing cells were exposed to 42 °C for 2 h. B, viability of the same strains preheated at 30 °C for 2 h and then exposed to 42 °C for 2 h. “No preheat” condition was used for the *tps1Δ* strain. The data are represented as means ± S.D. of three independent biological replicates.

belongs to the Clp/Hsp100 family of AAA+ proteins (ATPases associated with various cellular activities) (48) and because energy deficiencies in the *tps1Δ* mutant have been reported (49), we determined the ATP content in yeast cells during the heat shock response. Remarkably, we found that the *tps1Δ* mutant overexpressing or not *HSP104* became irreversibly depleted of its ATP content in less than 1 h at 42 °C (Fig. 8B). In contrast, in both wild type and *tps1-156* cells, the ATP transiently dropped by ~50% during the first 20 min to regain initial levels after 40–60 min. The complete loss of ATP coincided with the time when the viability of the *tps1Δ* overexpressing *HSP104* started to decline. However, this drop of viability was very slow as compared with that of *tps1Δ* alone, suggesting a protective effect of *HSP104* overexpression, independent of ATP levels.

Discussion

Trehalose has long been proposed to play an important role during stress response, mainly as a chemical chaperone (2, 50–52). However, doubts about this stress-protecting function have been recently raised from contradictory results, notably in thermotolerance and desiccation (51–53). A main reason for this ambiguity was the inability to directly assess the physiological function of trehalose in yeast cells without interfering with its metabolism, as well as with stress applied. In this report, we achieved this goal by exploiting our previous finding that yeast

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growing cells without exposure to any harmful conditions. Because these *tps1Δ* cells exhibited an ATP content similar to wild type cells in all permissive growth conditions for *tps1Δ* mutant (Ref. 49 and unpublished data), it is likely that Tps1 must have additional regulatory function(s) indispensable for cell viability in dividing cells. Moreover, Tps1 is a relatively low abundance protein in exponentially growing yeast cells on glucose (6, 8, 61), with similar levels to Pfk26, which synthesizes the glycolytic effector Fru-2,6-P₂ (62). We then favor the idea that Tps1, in addition to its catalytic function in trehalose synthesis, is a sensing/signaling intermediate with regulatory function(s), at least in energy homeostasis. Such function in preventing energy depletion should be essential to withstand adverse conditions.

To conclude, beyond the simple metabolic function of Tps1 via trehalose-6P and trehalose synthesis, our results may allow considering Tps1 as a new, attractive example of “moonlighting” proteins, which are characterized by their ability to perform completely unrelated tasks utilizing regions outside the active site for other functions, mostly regulatory and structural (63). Also, in light of this crucial role of Tps1 in stress resistance, probably through mechanisms that maintain energy homeostasis, our view of survival mechanisms to stress has to be revised and will likely be extendable to other organisms that express Tps1 homologs. These findings finally appear strategic for two relevant yet divergent perspectives. The first one fits with the current interest of many biotechnologically oriented researchers aiming at optimizing the microbial cell factories for industrial yeasts. In addition to their use in traditional fermentation processes, yeasts face growing interest in producing biofuels from C-sources, not only for bioethanol but also for biobutanol and white (synthetic) biotechnology. These microorganisms are sources of many additional products and are used as cell factories. Identifying the moonlighting functions of Tps1 in stress resistance is a prerequisite for the development of more robust strains, the interplay between metabolic and signaling homeostasis being an appealing target for metabolic engineering. The second perspective relates to the urgent need for new antifungal therapies. The trehalose synthesis pathway is widespread in the fungal kingdom where it can be part of the virulence system (23, 67–69), thus making Tps1 a potential antimicrobial target for weakening mammalian and plant pathogenic microorganisms.

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Note Added in Proof—The autofluorescence scatter plots shown in Fig. 1A were not correct in the version of this article that was published on May 1, 2015 as a Paper in Press. Specifically, data from the *TPS1* strain was mistakenly duplicated and used to represent results

from the *tps1-156* strain. Furthermore, the scatter plots shown in Fig. 1 (B and C) represented results of independent experiments with possible experimental variation. The corrected version of Fig. 1 presents data obtained for *TPS1*, *tps1Δ*, and *tps1-156* strains from experiments performed on the same day to avoid variations caused by the environment (medium and culture conditions). This correction does not affect the interpretation of the results or the conclusions.

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Role of Tps1 in Yeast Stress Tolerance

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Yeast Tolerance to Various Stresses Relies on the Trehalose-6P Synthase (Tps1) Protein, Not on Trehalose

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Withdrawal: Yeast tolerance to various stresses relies on the trehalose-6P synthase (Tps1) protein, not on trehalose.

Marjorie Petitjean, Marie-Ange Teste, Jean M. François, and Jean-Luc Parrou

This article has been withdrawn by Marie-Ange Teste, Jean M. François, and Jean-Luc Parrou. Marjorie Petitjean could not be reached. The corresponding author identified major issues and brought them to the attention of the Journal. These issues span significant errors in the Materials and Methods section of the article and major flaws in cytometry data analysis to data fabrication on the part of one of the authors. Given these errors, the withdrawing authors state that the only responsible course of action would be to withdraw the article to respect scientific integrity and maintain the standards and rigor of literature from the withdrawing authors' group as well as the Journal. The withdrawing authors sincerely apologize to the readers and editors.