
Pleurotus sajor-caju HSP100 complements a thermotolerance defect in *hsp104* mutant *Saccharomyces cerevisiae*

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A putative *Hsp100* gene was cloned from the fungus *Pleurotus sajor-caju*. mRNA expression studies demonstrated that this gene (designated *PsHsp100*) is highly induced by high temperature, induced less strongly by exposure to ethanol, and not induced by drought or salinity. Heat shock induction is detectable at 37°C and reaches a maximum level at 42°C. *PsHsp100* mRNA levels sharply increased within 15 min of exposure to high temperature, and reached a maximum expression level at 2 h that was maintained for several hours. These results indicate that PsHsp100 could work at an early step in thermotolerance. To examine its function, *PsHsp100* was transformed into a temperature-sensitive *hsp104* deletion mutant *Saccharomyces cerevisiae* strain to test the hypothesis that PsHSP100 is a protein that functions in thermotolerance. Overexpression of PsHSP100 complemented the thermotolerance defect of the *hsp104* mutant yeast, allowing them survive even at 50°C for 4 h. These results indicate that PsHSP100 protein is functional as an HSP100 in yeast and could play an important role in thermotolerance in *P. sajor-caju*.

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1. Introduction

In the natural environment, organisms are often exposed to abiotic stresses. Abiotic stresses affect the growth and development of the organisms both qualitatively and quantitatively. One of the common abiotic stresses is high temperature in growing season. At temperatures over the optimal temperature for growth, organisms usually display two major damages, oxidative stress and irreversible protein aggregation. Oxidative stress is the result of an imbalance between pro-oxidant species and the levels of the defenses resulting from the generation of reactive oxygen species (ROS) (Santoro and Thiele 1997; Mittler 2002). High temperature causes the misfolding and denaturation of many proteins, leading to irreversible aggregation. However, cells

respond to high temperature by expressing heat shock proteins (HSPs), including molecular chaperone proteins that promote refolding of misfolded and denatured proteins (Georgopoulos *et al* 1973; Nilsson and Anderson 1991; Getting and Sambrook 1992; Hartl *et al* 1992). Molecular chaperones also function in protein synthesis to prevent the aggregation of partially folded nascent and newly completed polypeptides (Hendrick and Hartl 1993). Another important function of certain HSPs is to promote the rapid degradation of abnormal proteins (Kandror *et al* 1994; Lee *et al* 1996; Sherman and Goldberg 1996) HSPs were first reported in *Drosophila* in the 1960s, and have been highly studied in various other organisms including bacteria, yeast, and humans (Lindquist 1986; Morimoto *et al* 1990; Nover 1991). It is now well-established that expression of HSPs

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Abbreviations used: HSPs, Heat shock proteins; MCM, mushroom complete medium; ORF, open reading frame; PCR, polymerase chain reaction, RT, reverse transcription; UTR, untranslated region.

is fundamental to thermotolerance, as well as surviving otherwise lethal high temperatures (Lindquist 1986; Vierling 1991). HSPs are grouped into classes based on their molecular masses (kDa), such as smHSPs (small molecular-mass HSPs), HSP60, HSP70, HSP90, and HSP100 (Craig *et al* 1993; Nover 1991; Neumann *et al* 1989).

The HSP100 family is an important component of the heat shock response in both prokaryotes and eukaryotes (Agarwal 2003). HSP100 is a member of HSP100/Clp (HSP100 family or caseinolytic proteases) subfamily of AAA proteins (Zolkiewski 1999; Cashikar *et al* 2002). The HSP100/Clp proteins share two large blocks of sequence homology (~200 amino acids) centered around two ATP-binding consensus elements and are part of a larger class of AAA⁺ chaperone-like ATPases. The ATP-binding domains are flanked with N-terminal, spacer, and tail domains (Gottesman *et al* 1990; Squires and Squires 1992). Squires *et al* (1992) used the spacer domain to define three subfamilies of the HSP100 family, namely ClpA, ClpB, and ClpC, with short, long, and intermediate spacers, respectively. The ClpB is inducible by heat and is required for thermotolerance in most cells. Some examples of the ClpB family are (note that the varied molecular weights result in varied names) GmHSP101 of *Glycine max* (Lee *et al* 1994), AtHSP101 *Arabidopsis thaliana* (Schirmer *et al* 1994), ZmHSP101 (Young *et al* 2001; Nieto-Sotelo *et al* 2002), ScHSP104 in *S. cerevisiae* (Young *et al* 2001), *Phycomyces* HSP100 of *Phycomyces blakesleeana* (Rodriguez-Romero and Corrochano Luis 2004), and hsp98 from *Neurospora crassa* (Vassilev *et al* 1992). Rodriguez-Romero and Corrochano Luis (2004) reported that *Phycomyces* HSP100 was induced by blue light and heat-shock in fungus. It was also reported that Hsp98 was synthesized during the heat-shock response of *N. crassa* (Vassilev *et al* 1992). In rice, the ClpB type HPS100 homology is SAP104 (Pareek *et al* 1995). Singla and Grover (1994) [28] reported that SAP104 was synthesized and accumulated in response to high temperature as well as various abiotic stresses. The SAP104 is therefore expected to act as a molecular chaperone to prevent protein folding and denaturation due to high temperature and other stresses, like its homologous (Lindquist and Craig 1988; Vierling 1991).

The ScHsp104 is perhaps the best studied of the eukaryotic Hsp100s. It prevents the irreversible aggregation of denatured proteins in yeast by binding to them and causing them to dissociate (Piper 1993; Ruis and Schüller 1995). ScHSP104 can be induced not only by high temperature but also by harmful levels of ethanol, suggesting it plays a role in tolerance to both stresses (Petko and Lindquist 1986; Piper *et al* 1994; Piper 1995; Aranda *et al* 2002). Exposure of yeast to sub-lethal high temperature and ethanol induce essentially identical stress responses (Piper 1995). These responses were characterized by the induction of heat shock proteins under high temperature stress above about 35°C or

under ethanol levels above a threshold level of 4-6% for strong induction. ScHSP104 also seems to control trehalose metabolism in relation to the thermotolerance (Iwahashi *et al* 1997, 1998; Singer and Lindquist 1998). It contributes to the simultaneous increase in both accumulation and degradation of trehalose. Iwahashi *et al* (1997, 1998) found that the activities of trehalose-synthesizing and hydrolyzing enzymes are low in the *hsp104⁻* mutant during heat shock. Sanchez and Lindquist (1990) revealed that ScHSP104 was not expressed under optimum growth temperatures but becomes a major product of protein synthesis shortly after a shift to high temperatures. Yeast *hsp104⁻* mutant cells do not acquire thermotolerance tolerance to a lethal high temperature, while wild-type yeast do (Sanchez and Lindquist 1990; Parsell *et al* 1994). These results demonstrate that the *Hsp100* Hsp104 plays a decisive role in cell survival at extreme temperatures in yeast.

In this study, we isolated a putative *Hsp100* gene, *PsHsp100*, from the fungus *Pleurotus sajor-caju*. The function of PsHSP100 in thermotolerance was tested by examining its ability to complement a *S. cerevisiae hsp104⁻* mutant.

2. Materials and methods

2.1 Strain and culture conditions

Pleurotus sajor-caju (ASI 2070) mycelia were obtained from the National Institute of Agricultural Science and Technology, Suweon, Korea. They were cultured for one week in a liquid mushroom complete medium (MCM; 0.2% yeast extract, 0.2% peptone, 2% glucose, 0.05% MgSO₄•7H₂O, 0.05% KH₂PO₄ and 0.1% K₂HPO₄) at 27°C.

2.2 Purification of genomic DNA and RNA from *P. sajor-caju*

P. sajor-caju mycelia were exposed to a high-temperature stress at 60°C for 20 min. After that, the stressed mycelia were harvested by filtration through Whatman No.2 filter paper and washed with deionized water. The washed mycelia were frozen in liquid nitrogen and ground to powder. Total RNA was extracted from powdered stressed mycelia using a Trizol reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) according to the manufacturer's instructions and was resuspended in RNasecure™ Resuspension solution (Ambion). RNA was quantified by absorbance at 260 nm. RNA (3 mg) was denatured in formamide-formaldehyde and separated on 1.2% formaldehyde agarose gels to confirm its quality and concentration. Genomic DNA was isolated from mycelia using a rapid extraction procedure as described by Graham *et al* (1994).

2.3 Construction of *P. sajor-caju* cDNA library and phage DNA isolation

Poly(A) RNA was isolated from the total RNA of the heat-shocked *P. sajor-caju* using the poly(A) RNA isolation kit (Stratagene®). cDNA was synthesized using the Lambda ZAP® II cDNA synthesis kit (Stratagene®). A cDNA library was constructed using the Gigapack® III Gold Packaging Extract kit (Stratagene®) according to the manufacturer's instructions. Lambda phage DNA was isolated from the cDNA library as described by Sambrook *et al* (2001).

2.4 Isolation of a putative Hsp100 gene

To isolate an *Hsp100* gene from *P. sajor-caju*, polymerase chain reaction (PCR) was performed using DNA from a heat-induced *P. sajor-caju* cDNA library as template. *Hsp100* specific primers were designed by analysing the conserved domains of *Hsp100* genes from other organisms. First, an *Hsp100* sense primer and the T7 primer reading into the library inserts from the vector sequence were used to amplify a fragment which was then cloned into pBluescript KS-II (+) (Stratagene®) and sequenced. Sequence analysis of that fragment allowed design of an *Hsp100* antisense primer that was used with the T3 primer (reading into library inserts from the opposite side as the T7 primer) to amplify the other end of the *Hsp100* cDNA which was also cloned into pBluescript KS-II (+) and sequenced. Based on these DNA sequences, a nested primer set was designed and used in PCR amplification to isolate full length *Hsp100* cDNA from the library.

2.5 Southern blot analysis

Total *P. sajor-caju* genomic DNA (5 mg per sample) was digested with *EcoRI*, *HindIII*, and *SalI*. The digested DNAs were then separated on 1.0% agarose gel and blotted to nitrocellulose membrane (Hybond-N+, Amersham Pharmacia Biotech). Southern hybridization was performed as described by Sambrook *et al* (2001) using a *HindIII* cDNA fragment corresponding to nucleotides 1,344 to 1,916 of the *PsHsp100* open reading frame (ORF) as the probe.

2.6 Imposition of stresses

P. sajor-caju mycelia which had been grown in MCM liquid medium in a 27°C incubator for seven days were used in these experiments. The mycelia were then exposed to various abiotic stresses as follows: drought (vacuum drying for 1 h), anaerobiosis (flooding in MCM broth for 4 h), alcohol (by agitating in MCM liquid medium with 10% ethanol for 4 h),

salinity (by soaking in MCM supplemented with 500 mM NaCl for 4 h), and acid (by shaking in MCM with acetic acid, glacial, pH 4.0, for 4 h). Since optimum temperature for the growth of *P. sajor-caju* is 27°C, all of stress treatments were carried out at 27°C except drought conditions, which were at ambient temperature. For the experiment examining temperature-dependent gene expression, mycelia were incubated at 22°C, 27°C, 32°C, 37°C, or 42°C for 4 h. For the time-course gene expression experiment, mycelia were heat shocked at 42°C for 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, 16 h, or 24 h. After stress treatment, samples were harvested by filtration through Whatman No. 2 filter paper. Mycelia were frozen in liquid nitrogen and ground to powder. Total RNA was extracted using Trizol reagent (Molecular Research Center, Inc., Cincinnati, OH, USA).

2.7 Reverse transcription-PCR and Northern blot analysis

Reverse transcription (RT) was carried out in 80 µl aliquots of reaction mixture containing 5x buffer (Promega, USA), 0.25 mM each of the four deoxyribonucleotides (dNTPs), 0.5 ml of 100 pmol *PsHsp100* specific anti-sense primer (5'-GTTGAAAGGAGAAATCGTGT-3'), 200 units of Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Promega, USA), and 8 ml DNase-treated RNA preheated at 72°C for 2 min. The reaction was performed at 37°C for 1 h. The 5 µl sample of first strand cDNA products was then employed as a template DNA for the PCR amplification of putative *PsHsp100* cDNA using *PsHsp100* specific sense (5'-ATGTCTGTTGATAATTCGA-3') and anti-sense primers. RT-PCR product was separated on 0.8% agarose gel, and stained with EtBr. For Northern blot analysis, total RNA samples (20 µg) were electrophoresed on agarose gel, and transferred to membrane and hybridized as described by Sambrook *et al* (2001) using a 562 bp *PsHsp100* cDNA fragment as a probe.

2.8 Introduction of the *PsHsp100* gene into *S. cerevisiae*

An expression vector containing the complete *PsHsp100* cDNA (pYES-DEST52/*PsHsp100*) was constructed by inserting the 2,709 bp full length *PsHsp100* cDNA under the control of a GAL promoter in the yeast expression vector, pYES-DEST52 (Invitrogen) using Gateway™ Technology. pYES-DEST52/*PsHsp100* was introduced into wild-type yeast strain INVSC2 (MAT α his3 Δ 1 leu2 trp1-289 ura3-52, from Invitrogen) and *hsp104* deletion mutant yeasts strain W303 Δ 104⁻ (Sanchez and Lindquist 1990; Sanchez *et al* 1992) as described by Jeong *et al* (2000). Transfer and expression of *PsHsp100* were confirmed by RT-PCR analysis.

2.9 Induction of PsHSP100 protein in yeast

The yeast cells harbouring the pYES-DEST52/*PsHsp100* plasmid were cultured for one day at 25°C in SC medium containing 2% raffinose. To induce transcription of *PsHsp100* (controlled by the *GAL1* promoter), raffinose was removed and galactose was added as a carbohydrate source. The cells were grown for 8 h at 25°C, and heated for 30 min at 25°C (control), 37°C, or 42°C. The cells were harvested, washed with deionized water, and frozen at -80°C. Overexpression of PsHSP100 protein was confirmed by SDS gel analysis after purification of using His-Bind[®] Resin (Novagen) column.

2.10 Heat, alcohol and acidic treatments

Transformants were grown to mid-log phase in SC-URA⁻ medium including 2% raffinose. Cells were collected by centrifugation and resuspended at a density of 8.0×10^6 cells ml⁻¹ in SC-URA⁻ medium with 2% galactose. After 8 h of induction in 2% galactose, cells were recounted and densities were adjusted to 8×10^7 cells ml⁻¹. Cells were distributed to 50 ml corning tubes (5 ml per tube) and heated in a 25°C, 37°C or 50°C water bath for 4 h. Samples were cooled on ice for 2 min and then diluted in serial 10-fold dilutions and spread onto YPD plates. After 2 days of incubation, plates were scored and photographed. To apply alcohol stress, 5 ml of cells were distributed to 50 ml corning tubes that were then filled with SC-URA⁻ liquid medium containing 10%, 20% or 30% ethanol. The samples were then incubated for 1 h or 2 h in a 25°C water bath. To apply acid stress, 5 ml of cells were distributed to 50 ml corning tubes and shifted from pH 4.2 to pH 3.0 in SC-URA⁻ liquid medium. The samples were then incubated for 0, 1 h or 4 h in a 25°C water bath. Samples treated with alcohol or acid were cooled on ice for 2 min and diluted to 2.5×10^4 . The cells were spread in YPD plates, and incubated at 25°C for 2 days and photographed. Each of these stress experiments was repeated three times.

3. Results and discussion

3.1 Isolation of a putative Hsp100 gene from *P. sajor-caju*

To isolate a full-length *Hsp100* cDNA from *P. sajor-caju*, we designed *Hsp100* sequence-specific sense and antisense primers to the conserved ATP-binding sites based on the sequences of *Hsp100* genes from *Glycine max*, *Trypanosoma brucei*, and *S. cerevisiae*. The PCR amplification from a heat-induced *P. sajor-caju* cDNA library using the sense primer and a T7 primer to vector sequences yielded a 2.3 kb cDNA which was cloned and sequenced. Sequencing

analysis showed that part of the deduced amino acid sequence of this cDNA fragment had high homology with HSP100 family members. To isolate the 5' fragment of the *Hsp100* cDNA from the cDNA library, PCR amplification was performed by using the *Hsp100* specific antisense primer and the T3 vector primer yielding a 0.6 kb cDNA fragment. Sequence analysis of the 0.6 kb cDNA showed that part of the deduced amino acid sequence had high homology with HSP100 family members from other organisms. These two fragments together appeared to contain a complete *Hsp100* gene. Based on the sequence data of these cDNA fragments, we designed a nested primer set to generate full length *P. sajor-caju Hsp100* cDNA. PCR amplification using this primer set yielded a 2.9 kb PCR product using the heat-induced cDNA library as template. The PCR product was purified and ligated into pBluescript KS-II.

3.2 Nucleotides and deduced amino acids sequences of the putative Hsp100 gene

Sequencing of the complete 2.9 kb insert showed that the putative *Hsp100* cDNA is 2,865 bp long, including 21 bp of 5' untranslated region (UTR), 115 bp of 3' UTR, an ATG translation initiation codon, a TAA stop codon, and a poly(A) tail, suggesting that the cDNA contains the full-length coding sequence (figure 1). The full-length cDNA of *Hsp100* contains an uninterrupted ORF of 2,709 nucleotides, which is predicted to encode a 100 kDa polypeptide consisting of 902 amino acid residues. This gene was designated *PsHsp100*. Figures 2 and 3 show the amino acid sequence alignment of *P. sajor-caju* HSP100 (PsHSP100) with HSP100 family members from other organisms. The deduced amino acid sequence of PsHSP100 contains the two ATP-binding domains that are highly conserved in members of the HSP101/HSP104/ClpB family (Gottesman *et al* 1990; Squires *et al* 1991; Squires and Squires 1992). PsHSP100 has high homology with *Arabidopsis* HSP101 (52.4%), maize HSP101 (51.3%), rice HSP101 (51.0%), soybean HSP101 (50.5%), and yeast HSP104 (47.2%). However, it has only weak homology (13.1%) with *Escherichia coli* ClpB (HSP104).

3.3 Determination of copy number of the PsHsp100 gene

To determine the copy number of *PsHsp100* gene in the *P. sajor-caju* genome, total genomic DNA was digested with three enzymes (*EcoRI*, *HindIII*, and *Sall*) and analyzed by Southern blot hybridization using a 562 bp cDNA fragment of *PsHsp100* gene as the probe. A single band of hybridization was detected in all three digests, indicating that the *PsHsp100* gene is present in single copy in the *P. sajor-caju* genome (figure 4).

1	GAATTCGGCAGGCCCCATCATGTCTGTTGATAATTTGACTTTACCGACAAGCTCGCCGACAGTACCCGCCGCTGTCCAACCTGGCAAAGGACTACGGCAATCGCAAGTTCAT	118
	M S V D N F D F T D K A R R T I T A A V Q L A K D Y A N A Q V H	
119	CCAGTTCATATCGCTTCGCTCTCTTGAATGATTCAGGAGAAGAGCAATCGTCAGGAGGAGCTGGGAGACGCTTCTCCAGGTCCCAATCCCTATTCACACTCAGCAATTTCA	236
	P V H I A S S L L N D S G E E E Q S S G G L G D A S S R S Q S L F H S A I S	
237	AAGGCTGGTGGTGATCCGGCAGTAACTAAACCGGGATACAGCGTGCAGTCTGTCAGGCTGCCTACGCAAGATCCCCCTCTGAAGAAGTTTACTTCAGCGGGCTCGGCTCAAAGTC	354
	K A G G D P A V V K R G I Q R A V V R L P T Q D P P P E E V Y F S G F A L K V	
355	CTGCGAGAAGCGCAGAGCTTGCAGAAGACAATGCACGACTCGTACATCGCTCAGGACCATCTACTCTCGCCGCTTGAAGGACGCGACCATCAAGCAGTTATCAAAGAAGCCGGG	472
	L R E A Q S L L Q K T M H D S Y I A Q D H L L L A A L K D A T I Q A V I K E A G	
473	CTTACTGAGGCTACCTTGAAGACTGCCATTGAACAAGCAGGAGAACCCGTCGATCGAATCGAAGCAGGCTGAACAAGTTTGTATGCCCTTCAAATAACCGCGCTCGACTGACC	590
	L T E A T L K T A I E Q A R G N R R I E S K T A E Q G F D A L Q K Y A V D L T	
591	GCTCTGGCAGGAAGAAAGATCGACCCAGTCATTTGGCCGTGACAATGAATTCGTCGCTCATCCGAATTCCTGCGGAAGAACAAGACAATCCGGTCTCATCGGTGAACCC	708
	A L A E E G K I D P V I G R D N E I R R V I R I L C R R T K N N P V L I G E P	
709	GGTGTAGGAAAACCTCGATCGCTGAGGCTCTCGCTCAAAGAATAGTCAAGAGGATGTACCAGCCGCTGATCTCTAGATTATACTCGCTCGACATGGGTGCTTTAATGGCAGCG	826
	G V G K T S I A E G L A Q R I V K R D V P A S L I S R L Y S L D M G A L M A G	
827	GCGAAGTAAAGGTGAATACGAAGCGTATCAAGGCTGTCTTGAACAGGTTGAGAAGCATCCGAAGATGGTGGCCAGGCGTTATTCCTTGTGACCAATTCGACCTCATT	944
	A K Y K G E Y E E R I K A V L E N V E K A S E D G G P G V I L F A D E L H L I	
945	ATGGTGGACGTGGCCGCAAGGAGGAGGATGGACGCTGCCAATCTCTTCAAACCTCTCTTGCCTGGCAGGCTTCGATGTATGGCGGACGACCCCTCGCCGAATATCGAAAA	1062
	M A G R G A E G G G M D A A N L F K P L L A R G K L R C I G A T T L A E Y R K	
1063	TACATTGAACTGCGGCACTCGAAGCTTCGATTCGCGCAAGTCTTAGTAAACGAAAGCCAGGCTGGGAACGATCAGTATCCCTCGAGGATCCGTGAGAGTACGAAGTCCAT	1180
	Y I E T D A A L E R R F A Q V L V N E P S V L E T I S I L R G I R E K Y E V H	
1181	CATGGTGCAGGATTTGGACGGCGCTGATACAAGCTGGACATTTGGACACCGCTACTGACATTCGCGCTGCCTGACCTGCTGATCTCTGCTGACCAATTCGACCTCATT	1298
	H G V R I L D G A L I Q A A T L A H R Y L T S R R L P D A A I D L V D E A C A	
1299	AGCGTACGTGTTAATCGCGAAACGGAGCCCGAGGCAATCGACAAGCTTCAGCGCAAGAGGCTTGAGCTTGAATAGAAATCCATGCCCTCGAGCGAGAGAAGGATGCTGCAAGCAAG	1416
	S V R V N R E T E P E A I D K L Q K R L E L E I E I H A L E R E K D D A S K	
1417	GAGCGTCCCAAGCTGCGAAGAAGCTATTCGACGCTAGACGAGGAGCTGCAACCTCCAGGCTGCGTTCGAGCAGAGAAGATGCGAGGCGATAACTCAACGTTCTCGCGCGGA	1534
	E R L Q A A K A I S D V D E E L Q P L Q A F Q A E K M R G D N S T F C G A G	
1535	AGATGGACGAACCTCAAGCGAAGATTGAAGAGGCTGAGCGAAGACCGGACATACCCTGCTCCGATTTGAAATTCATGCTGTCCCGAAGTCAAGCGGATACGACGAGCTA	1652
	R W T N S R R R L K R L S E D R D I T T A S D L K F Y A V P E V Q A R I E Q L	
1653	GAAGCAAAGAGCAGCAGAGGACGCTCCACCGGCTCTCGAGCTGATGGTAAACCCAGAAGCATTGCAGATATTGTTGGACGGTGGACCAACATCCCGCTCCGAGGCTCATG	1770
	E A K K A A C A E D A S T G S A A D V V T P E A I A I D I V G R W T N I P V S R L M	
1771	TCTCCGAAAGGAGAAGCTTAGGATGGAGCGGATTCACCGAAAAGCTGTTGGCGAGCCGAGGCTGAAGCTGTCGCGAATGCATCCGCTCTCCGAGTGGCTCGGCTG	1888
	S S E K E K L L R M E R I L T E N V V G Q P E A V K A V A N A I R L S R S G L	
1889	GCAAATGCTCAAAGGCAATTCGAACTCTCTTTCGAGGCCCCAGTGGCAGCGGCAAACTCTCTTCCAAAGACTCGGACACTTCTATTGACTCGCTGACGCTATGATC	2006
	A N A Q R P I A S F L F A G P S G T G K T L L S K T L A T L L F D S P D A M I	
2007	CGAATGATGGCTCCGACTACTCAGAGAAGCACTCCATTCGCTGTTGATCGGACCCCGAGGATACATCGGCATGATGAGGAGGACAACCTCACGGAATACGTTCCGCGCAAG	2124
	R I D G S E Y S E K H S I A R L I G A P P G Y I G H D A G G Q L T E Y V R R K	
2125	CCTACAGTATTATCTCATTGATGAATCGAGAAGCGCTCGAGAAATTTGCACCTCTCTCTGCAAGTCCCTGATGATGGCAGACTTACTGATCGCAAGGAGCTGCTGCTGAC	2242
	P Y S I I I L I E I E K A C R E F V T L F L D Q V L D D D G R L T D D G Q G R V D	
2243	TTCAGAACTCGGTTATCATCATGACATCCAACCTGGGCGCCCTATCTCAATGATATGGCGATGGACCTGTTGCTCGGAGACAAGAACACTAGTCATGGGCGCCATTCAGGT	2360
	F R N S V N R I I M T S N L G A A Y L L N D M G D G P V R P E T R T L V M G A I Q G	
2361	CATTCCCTCCGAGTTTGTGAATCGTATCGACGAGATTGTATCTTCCGTCGCTTTCGAGAAGACGTAAGACTCGTGGACATCCGCTCAAGGACTACTCGAGGCTCTT	2478
	H F P P E F V N R I D E I V I F R A L S Q K N V L K I V D I R L K E V L E R L	
2479	GCTGAACGAAAGATTACATCGACATCGACGCTGGCGAAGAGCTACCTCAGCTCAGCCGATATTCCTGCTGCTACGCGCCGCTCCACTGAATCGGGCCATCAAAGCGAATC	2595
	A E R K I T I D I D V A K S Y L S A G Y S P A Y G A R P L N R A I Q S E L	
2596	TTGAACCCCTTGTGATCATGATCTTGTCCGAACAAGTTCGCTTCGAGGCACTATAATCGCTGAATATCATCCGAATCAGGAACCCGAAACCGAAGGATGGATGGATGATG	2714
	L N P L S I M I L S E Q V R F D G P H N R L N I I P N H E A T E T Q G M D V D	
2715	TATGACGACGAGTATTGAAATTAAGAGATGGATTAAGGGTCACTGAAAAAGTAGTATCACTCCATTCATGTACAGTACAACGCATCAAACCTCAAATGTAGGATAGTATCA	2832
	Y D D D D I E I E E M D	
2833	TCACATGACTTTTATAACAGATTTCCTCTTCAACAAAAAAAAAAAAAAAAAA	

Figure 1. Nucleotide and deduced amino acid sequences of *P. sajor-caju Hsp100* cDNA. The *Hsp100* ORF consists of 2,709 bp nucleotides encoding a putative peptide of 902 amino acid residues (GenBank accession No. AF188207).

3.4 mRNA expression of *PsHsp100* gene under various abiotic stresses

Since *Hsp100* genes are usually induced by abiotic stresses, we analysed the mRNA expression pattern of the *PsHsp100* gene in response to various abiotic stresses. *P. sajor-caju* mycelia, which had been cultured in optimal growth conditions were imposed to stress conditions (see section 2) heat shock (a 42°C incubator for 4 h); drought (an evaporator for 1 h), salinity (50 mM NaCl solution for 4 h), anaerobiosis (flooding state for 4 h), alcohol (10% ethanol for 4 h), and acid (acetic acid, pH 4.0, for 4 h). Northern blot analysis (figure 5) showed that mRNA expression of the *PsHsp100* gene is highly induced by heat (10-fold), anaerobic (6-fold), or acid stress (5-fold), and slightly induced by alcohol treatment (2-fold). However, it did not show significant increase in mRNA level by drought or salt stress. These results suggest that *PsHSP100* might play a role in tolerance of abiotic stresses such as high temperature, anaerobic, and acidic conditions, but not drought or salt stress.

3.5 Temperature-dependent regulation of *PsHsp100* gene expression

Temperature-dependent regulation of the *PsHsp100* gene expression was examined. To investigate the effects of temperature on *PsHsp100* transcription, Mycelia were grown at 27°C and transferred directly to a second temperature for 4 h (22°C, 27°C, 32°C, 37°C, or 42°C). Mycelia were frozen in liquid nitrogen and RNA was isolated for Northern blot analysis. The results show that induction of the *PsHsp100* started from 37°C, and reached its maximum level at 42°C (figure 6).

3.6 Time-course expression of *PsHsp100* gene

Since the expression of the *PsHsp100* is highly increased at 42°C, we investigated time-course gene expression at 42°C. This gene showed basal level mRNA expression at optimal growth temperature (27°C). After heat treatment, the mRNA level increased proportionally with increasing time



Figure 2. Amino acid sequences comparison of *P. sajor-caju* HSP100 protein and HSP100 family members. The complete amino acid sequence of PsHSP104 (*P. sajor-caju*) was compared with AtHSP101 (*A. thaliana*), EcClpB (*E. coli*), OsHSP101 (*O. sativa*), GmHSP101 (*G. max*), SchHSP104 (*S. cerevisiae*), and ZmHSP101 (*Z. mays*). Amino acids conserved in at least six of the seven proteins are boxed. The first and second ATP-binding sites, ATP-1 and ATP-2, are indicated by arrows. Each of the ATP-binding regions is composed of two conserved sequence motifs, which are indicated by asterisks. Red and green boxes showed 75% and 100% homology domain, respectively.

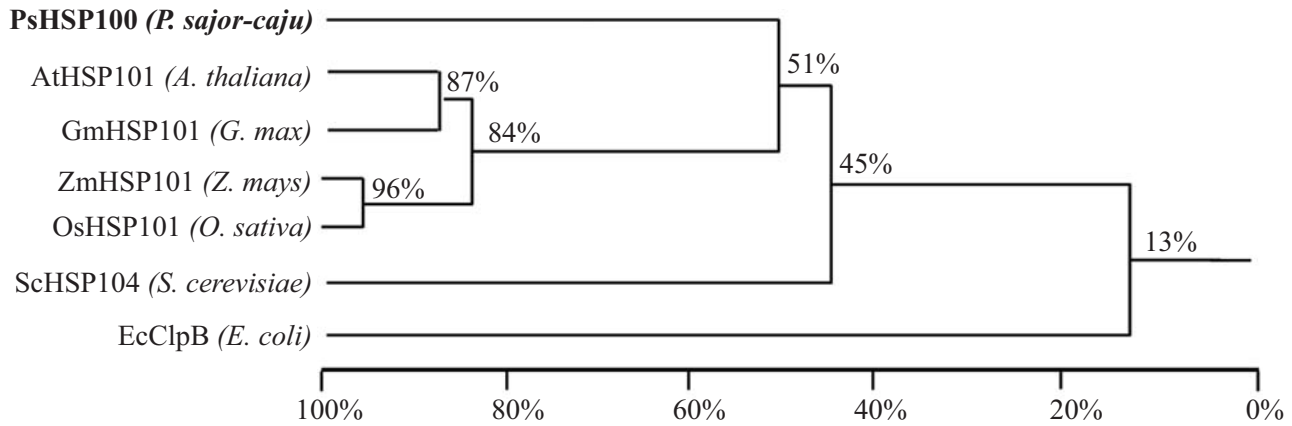


Figure 3. Phylogenetic tree of amino acid sequence alignment between *P. sajor-caju* HSP100 and other HSP100 proteins.

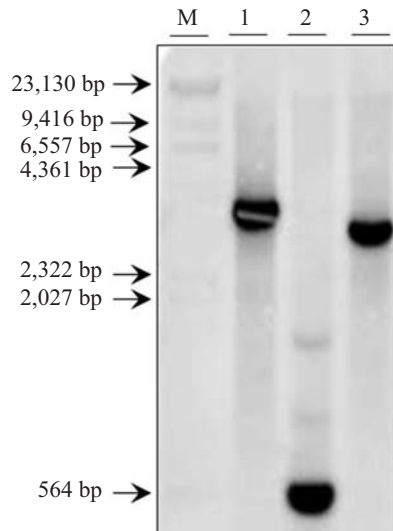


Figure 4. Determination of copy number of the *PsHsp100* gene. Southern blot analysis was performed using total genomic DNA isolated from *P. sajor-caju*. DNA was digested with *EcoRI* (lane 1), *HindIII* (lane 2), and *SalI* (lane 3). The blot was hybridized to the 562 bp internal cDNA fragment of the *PsHsp100* gene. M, molecular marker, λ HindIII.

(figure 7). The level of *PsHsp100* mRNA increased rapidly within 15 min after starting the treatment, and reached a maximum within 2 h that was maintained until 8 h. This rapid alteration in mRNA levels suggests that *PsHsp100* may work at an early step in thermotolerance.

3.7 Making the *PsHsp100* transgenic yeast for bio-functional assay

To investigate the ability of PsHSP100 to protect organism from high temperature stress and other abiotic stresses, the

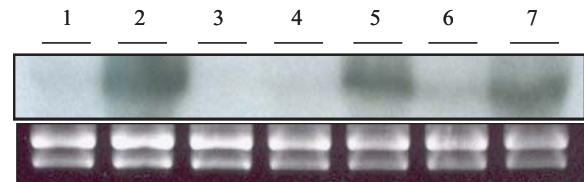


Figure 5. mRNA expression levels of the *PsHsp100* gene under various abiotic stresses. Northern blot analysis was performed using total RNA from *P. sajor-caju* mycelia exposed to various abiotic stresses: high temperature (lane 2), drought (lane 3), salinity (lane 4), anaerobiosis (lane 5), alcohol (lane 6), and acid stress (lane 7). Non-treated mycelia (lane 1) were used as a control.

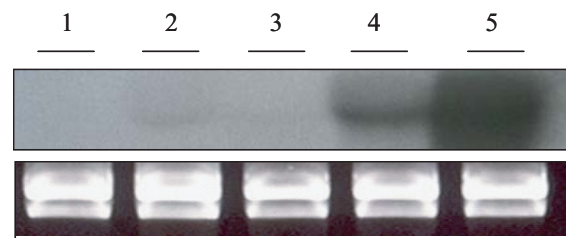


Figure 6. Temperature-dependant mRNA expression levels of the *PsHsp100* gene. Northern blot analysis was performed using total RNA from mycelia of *P. sajor-caju* which were exposed to various temperature for 4 h: 22°C (lane 1), 27°C (lane 2), 32°C (lane 3), 37°C (lane 4), and 42°C (lane 5).

complete cDNA encoding PsHSP100 was cloned into a galactose-regulated yeast overexpression vector (pYES-DEST52), and, transferred into a *S. cerevisiae* mutant lacking *hsp104* (*W303Δ104*⁻). This strain is temperature sensitive due to the lack of HSP104. For negative controls, yeast overexpression vectors without *PsHsp100* were transferred into wild-type yeast (INVSc1) and *hsp104* deletion

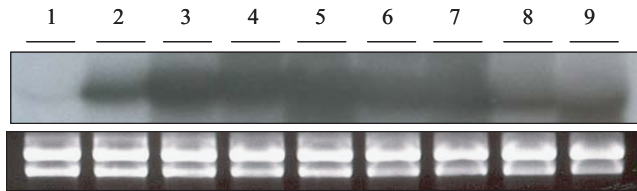


Figure 7. Time-course mRNA expression pattern of the *PsHsp100* gene at 42°C. Northern blot analysis was performed using total RNA from mycelia of *P. sajor-caju* which were exposed to 42°C for various times: 0 min (control, lane 1), 15 min (lane 2), 30 min (lane 3), 1 h (lane 4), 2 h (lane 5), 4 h (lane 6), 8 h (lane 7), 16 h (lane 8), and 24 h (lane 9).



Figure 8. Growth of the *hsp104* deleted yeast mutant cells (*W303Δ104*⁻) that were transformed with the *PsHsp100* overexpressing plasmid. Yeast strains INVSc1 (wild type yeast), *W303Δ104*⁻ (yeast *hsp104* deletion mutant), pYES-DEST52/INVSc1 (overexpression vector + wild type yeast), pYES-DEST52/*W303Δ104*⁻ (overexpression vector + yeast *hsp104* deletion mutant), and *PsHsp100/W303Δ104*⁻ (*PsHsp100* gene + *hsp104* deleted yeast mutant) were grown for 2 days in SC-URA⁻ selection medium at 25°C.

mutant (*W303Δ104*⁻). The presence of the plasmid was confirmed in transformants by both growth uracil drop-out selection medium (figure 8) and by PCR analysis from total DNA of transformants using the *PsHsp100* specific sense and antisense primer set (figure 9). The *PsHsp100* transgenic yeast was designated *PsHsp100/W303Δ104*⁻. To confirm that the *PsHsp100* gene is expressed in transgenic yeast, RT-PCR analysis was performed using *PsHsp100* specific primers. In SC-URA⁻ medium, little increase of *PsHsp100* mRNA was observed 1 h after galactose was added to induce expression, but after 3 h *PsHsp100* mRNA had accumulated (figure 10). Translation of the *PsHsp100* gene was confirmed by purification of PsHSP100 protein from the transgenic yeast cells, taking advantage of the histidine tag encoded by vector sequences. PsHSP100 protein

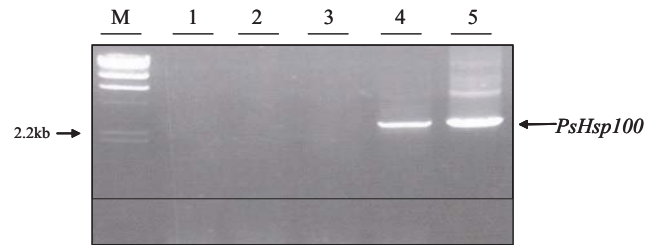


Figure 9. Confirmation of *PsHsp100*⁺ yeast transformants by PCR analysis. Total DNA isolated from pYES-DEST52/INVSc1 (lane 1), *W303Δ104*⁻ (lane 2), YES-DEST52/*W303Δ104*⁻ (lane 3), and *PsHsp100/W303Δ104*⁻ (lane 4) cells were used in PCR amplification. Plasmid DNA harbouring *Hsp100* cDNA was used as a positive control (lane 5).

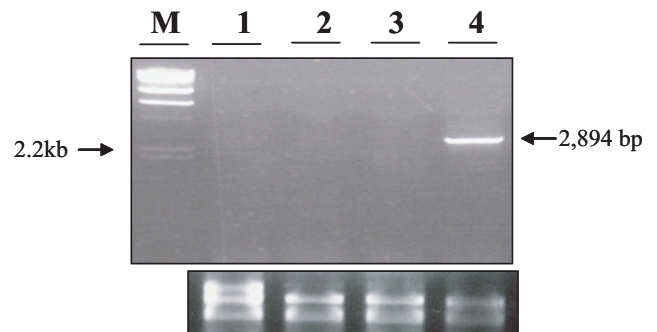


Figure 10. mRNA expression of *PsHsp100* in the transgenic yeast cells. RT-PCR analysis was performed using total RNA from the transgenic yeast cells (*PsHsp100/W303Δ104*⁻) grown in galactose induction media for 1 h (lane 3) and 3 h (lane 4). pYES-DEST52/INVSc1 (lane 1) and pYES-DEST52/*W303Δ104*⁻ (lane 2) yeast strains were used as negative controls.

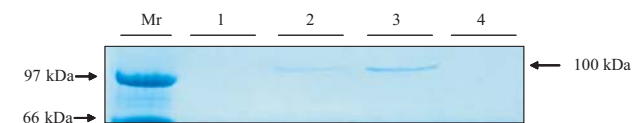


Figure 11. Synthesis of PsHSP100 protein in the transgenic yeast cells. SDS-PAGE analysis was performed to investigate the synthesis of PsHSP100 protein in the transgenic yeast cells. PsHSP100 proteins were purified by His-Tag column from the *PsHsp100* transgenic yeast lines (lanes 2 and 3) and were separated on a 6% polyacrylamide SDS gel. pYES-DEST52/INVSc1 (lane 1) and pYES-DEST52/*W303Δ104*⁻ (lane 4) yeast strains were used as negative controls. Mr, molecular weight standard (Amersham Biosciences).

was purified by using a His-Tag column, and then, confirmed by SDS-PAGE analysis. As shown in figure 11, the transgenic yeast produced significant amounts of PsHSP100 protein after 8 h of growth in galactose containing medium.

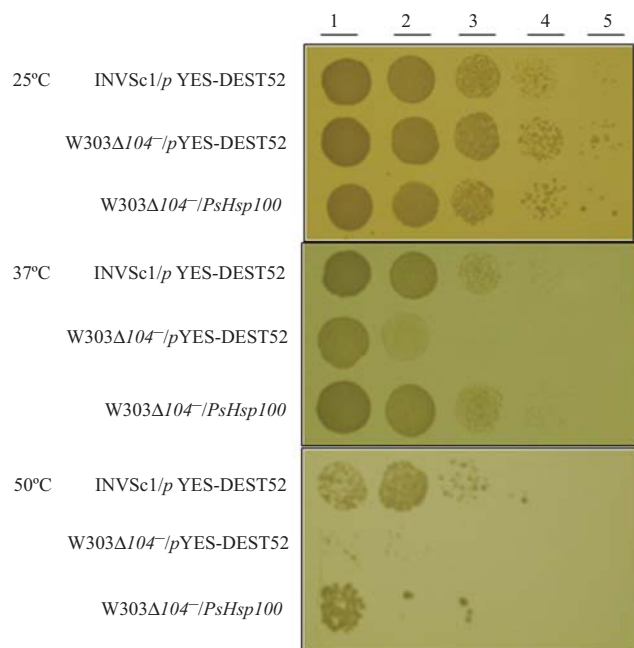


Figure 12. Complementation of the thermotolerance defect of the *hsp104* deleted yeast mutant cells by overexpressing *PsHsp100*. Complementation analysis was performed using wild type yeast cells (INVSc1), *hsp104* deleted mutant yeast cells (W303Δ104⁻), and the *hsp104* deleted mutant yeast cells overexpressing *PsHsp100* (PsHsp100). The cells exposed to 25°C, 37°C or 50°C for 4 h were diluted to 2.0×10^6 (lane 1), 2.0×10^5 (lane 2), 2.0×10^4 (lane 3), 2.0×10^3 (lane 4) or 2.0×10^2 (lane 5) and then spread onto YPD plates.

3.8 Bio-functional assay of the *PsHsp100* gene for thermotolerance

Complementation tests were performed to determine whether the expression of *PsHsp100* could provide thermotolerance to transgenic yeast cells that were deficient in thermotolerance due to the deletion of the *Hsp104* gene. Effects of exposure to high temperature on viability were assayed as follows. Yeast cells were cultured in a 25°C, 37°C or 50°C water-bath for 4 h with SC-URA⁻ liquid medium including 2% galactose. After 4 h, tubes were transferred to ice, and then 10-fold serially diluted and plated to YPD agar plates. Colonies were photographed after 2 days of incubation at 25°C. The results show that the *hsp104* mutant cells were killed by the 50°C exposure, although they were as viable as wild-type yeast at 25°C. However, transgenic *hsp104* mutant yeast which expressed PsHSP100 continuously were as tolerant wild-type yeast to the 50°C exposure indicating that PsHSP100 completely complemented the thermotolerance defect in the *hsp104* deletion mutant (figure 12). This result strongly suggests that the *PsHsp100* gene can confer thermotolerance.

3.9 Bio-functional assay of the *PsHsp100* gene for alcohol or acidic stress tolerance

Since the *PsHsp100* gene was induced by alcohol or acidic treatment, we also analysed whether it might confer tolerance to acidic or alcohol stresses in this *hsp104* mutant yeast assay system. The *PsHsp100* gene was induced by

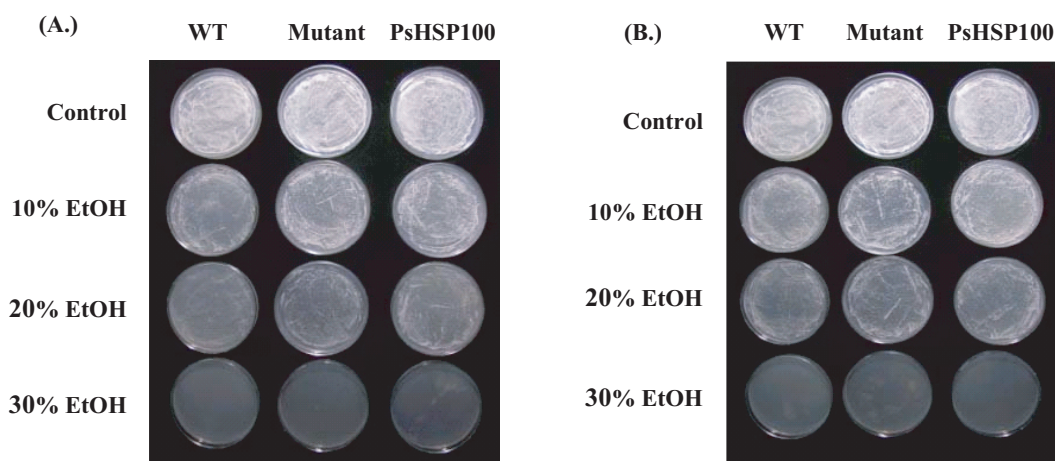


Figure 13. Biofunctional assay of the *PsHsp100* gene for alcohol stress tolerance. Wild type yeast cells (INVSc1), the *hsp104* deleted mutant yeast cells (mutant), and the *hsp104* deleted mutant yeast cells overexpressing PsHSP100 (PsHSP100) were exposed to 10%, 20%, or 30% alcohol and then spread onto YPD plates. (A) 1 h exposure to alcohol. (B) 2 h exposure to alcohol.

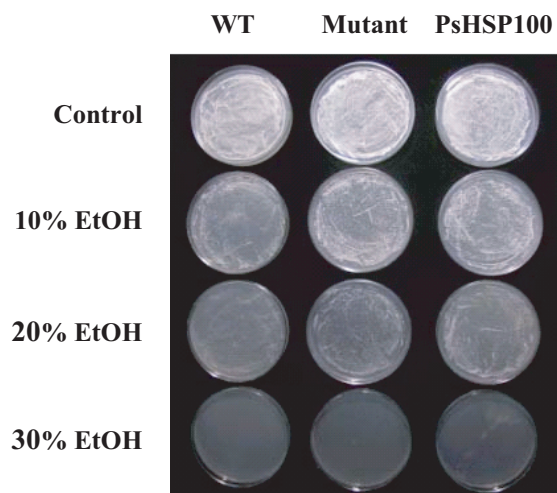


Figure 14. Biofunctional assay of the *PsHsp100* gene for acidic stress tolerance. Wild-type yeast cells (INVSc1), the *hsp104* deleted mutant yeast cells (mutant), and the *hsp104* deleted mutant yeast cells overexpressing PsHSP100 (PsHSP100) were exposed to 1 h or 4 h acidic stress (pH 3.0) and then spread onto YPD plates.

growing the cells for 8 h in SC-URA⁻ medium with 2% galactose at 25°C. The yeast cells were then stressed with 10%, 20% and 30% ethanol or acid (pH 3.0, acetic acid) treatments. For alcohol stress, cells were cultured for 1 h or 2 h in a 25°C water-bath, and spread in YPD plates at a concentration of 2.5×10^4 yields for analysing survival rate. The wild-type, *hsp104*⁻ mutant, and *PsHsp100* transgenic yeast grew well in 10% or 20% ethanol treatments, and they all died in 30% ethanol stress (figure 13). The strains all survived 10% ethanol somewhat better than 20% ethanol. There was no significant difference in survival rate among the wild-type, the *hsp104* deletion mutant, and the *PsHsp100* transgenic yeast at the same concentration of ethanol. Because yeast grows well in acidic conditions of about pH 4.2–4.3, the yeast medium was adjusted to pH 3.0 by adding acetic acid into the medium to impose acidic stress, and an experiment like that described above was performed to determine the effect on viability. Figure 14 shows that overexpression of PsHSP100 did not increase the survival rate of yeast cell under acidic conditions. These results suggest that there are other factors in addition to PsHSP100 for conferring alcohol or acidic stress tolerance in yeast cells.

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