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Molecular characterization of Hsf1 as a master regulator of heat shock response in the thermotolerant methylotrophic yeast *Ogataea parapolymorpha*[§]

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***Ogataea parapolymorpha* (*Hansenula polymorpha* DL-1) is a thermotolerant methylotrophic yeast with biotechnological applications. Here, *O. parapolymorpha* genes whose expression is induced in response to heat shock were identified by transcriptome analysis and shown to possess heat shock elements (HSEs) in their promoters. The function of *O. parapolymorpha HSF1* encoding a putative heat shock transcription factor 1 (OpHsf1) was characterized in the context of heat stress response. Despite exhibiting low sequence identity (26%) to its *Saccharomyces cerevisiae* homolog, OpHsf1 harbors conserved domains including a DNA binding domain (DBD), domains involved in trimerization (TRI), transcriptional activation (AR1, AR2), transcriptional repression (CE2), and a C-terminal modulator (CTM) domain. *OpHSF1* could complement the temperature sensitive (Ts) phenotype of a *S. cerevisiae hsf1* mutant. An *O. parapolymorpha* strain with an H221R mutation in the DBD domain of OpHsf1 exhibited significantly retarded growth and a Ts phenotype. Intriguingly, the expression of heat-shock-protein–coding genes harboring HSEs was significantly decreased in the H221R mutant strain, even under non-stress conditions, indicating the importance of the DBD for the basal growth of *O. parapolymorpha*. Notably, even though the deletion of C-terminal domains (ΔCE2, ΔAR2, ΔCTM) of OpHsf1 destroyed complementation of the growth defect of the *S. cerevisiae hsf1* strain, the C-terminal domains were shown to be dispensable in *O. parapolymorpha*. Overexpression of OpHsf1 in *S. cerevisiae* increased resistance to transient heat shock, supporting the idea that OpHsf1 could be useful in the development of heat-shock–resistant yeast host strains.**

Keywords: *Ogataea parapolymorpha*, heat shock transcription factor 1, heat stress response, thermotolerance

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Introduction

Sudden changes in the environment, such as heat shock, oxidative stress, and the introduction of heavy metals, can lead to the accumulation of abnormal proteins that are non-functional, posing a serious threat to cell survival (Daggett and Fersht, 2003). The heat shock response (HSR) is one of the main cellular responses towards such adverse conditions. Heat-shock transcription factors (Hsfs) are responsible for regulating eukaryotic gene expression in response to several stresses (Barna *et al*., 2018). The major role of the evolutionally conserved Hsfs is to mediate the HSR by increasing the transcription of genes encoding chaperones, such as HSP70. Some species harbor multiple Hsf isoforms; for example, there are six types of heat shock factors in humans (Xu *et al*., 2012) and multiple Hsf families in plants (Scharf *et al*., 2012), whereas only a single Hsf protein with functional equivalence to Hsf1 in humans is present in yeasts and invertebrates that have been studied (Verghese *et al*., 2012). Although Hsf1 plays a major role under conditions of stress, it is also essential for normal cellular growth; the deletion of *HSF1* has been shown to be lethal in the yeast *Saccharomyces cerevisiae* (Sorger and Pelham, 1988; Wiederrecht *et al*., 1988). In contrast to mammalian Hsf1, which is activated through several regulatory steps, including trimerization, nuclear localization, and DNA binding, *S. cerevisiae* Hsf1 is always present in the nucleus as a trimer, being engaged in both constitutive and stress-inducible binding to the heat shock elements (HSEs) (Verghese *et al*., 2012). Despite these differences, it is believed that hyperphosphorylation in response to heat shock is the key modification involved in activating Hsf proteins in yeast and animal cells (Xu *et al*., 2012).

Saccharomyces cerevisiae Hsf1 contains multiple discrete domains such as DNA-binding domain (DBD), and domains responsible for transcriptional activation (AR1, AR2), transcriptional repression (CE2), and C-terminal modulator (CTM), which are important in regulating its activity, along with the trimerization (TRI) domain. In *S. cerevisiae* Hsf1, the DBD has a winged helix-turn-helix structure that trimerizes during the binding of the protein to an HSE. The wing structure in the DBD comprises three helices, four β-sheets, and a flexible loop (Harrison *et al*., 1994; Vuister *et al*., 1994). The AR1 domain at the N-terminus of ScHsf1 regulates the activation and repression of ScHsf1 depending on non-shock temperatures. This domain is essential for the survival of yeast cells at heat shock temperature (Nieto-Sotelo *et al*., 1990). AR1 is also involved in the transient upregulation of ScHsf1 activity. In contrast, the AR2 domain at the

C-terminus regulates the sustained activity of ScHsf1. Different temperatures are shown to independently determine the degree of activation of ScHsf1, and whether the activation is transient or sustained. These AR domains are involved in the phosphorylation of ScHsf1 (Sorger, 1990). The CE2 domain, which is a yeast-specific heptapeptide located near AR2, binds CTM, and thereby sustains the repressed state of ScHsf1 at low temperature (Jakobsen and Pelham, 1991). CTM is rich in basic amino acids and plays a crucial role in upregulating the expression of heat-responsive genes containing non-canonical HSE, such as *HSP26* and *CUP1* in *S. cerevisiae* (Sakurai and Fukasawa, 2001). Studies on Hsf1 from *Kluyveromyces lactis*, which is closely related to *S. cerevisiae*, showed that the CE2 domain of Hsf1 is responsible for returning yeast Hsf1p to the inactive state after heat shock, and that deactivation can be enhanced by phosphorylation of serine residues adjacent to CE2 (Høj and Jakobsen, 1994).

Hansenula polymorpha is a methylotrophic yeast with high industrial potential, particularly due to its unusual thermotolerance up to 55°C. Consequently, it has attracted much attention as a host strain for biotechnological applications, including the production of recombinant proteins and bioethanol (Gellissen and Scheffers, 2005; Kurylenko *et al.*, 2014; Kim *et al.*, 2015; Manfrão-Netto *et al.*, 2019; Thak *et al.*, 2020). Three representative *H. polymorpha* strains of independent origin, including DL-1, CBS 4732, and NCYC 495, have been widely used in basic and applied research (Kurtzman, 2011; Kim *et al.*, 2018). In this work, we isolated and functionally characterized a Hsf1 homolog in *H. polymorpha* DL-1, which has been recently reassigned to *Ogataea parapolymorpha* (Yeon *et al.*, 2018; Yoo *et al.*, 2020). We constructed a series of *O. parapolymorpha HSF1* (*OpHSF1*) mutants and investigated their ability to function in the HSR. Our results indicate that OpHsf1 plays a critical role in mediating the defense response to heat shock stress in yeast, despite distinctive differences in the structural requirements for its activity. Overexpression of OpHsf1 holds great promise as a strategy to improve the capability of yeast host strains to withstand heat shock stress.

Materials and Methods

Yeast strains, plasmids, and culture conditions

Yeast strains and plasmids are listed in Supplementary data Tables S1 and S2. Yeast cells were cultured using 1% yeast extract, 2% peptone, 2% glucose (YPD) or synthetic complete medium containing 2% dextrose without uracil or tryptophan, (SC-URA and SC-TRP), consisting of 0.67% yeast nitrogen base without amino acids and drop-out amino acid mixture without uracil or tryptophan for *S. cerevisiae,* or 1.5× drop-out amino acid mixture without uracil for *O. parapolymorpha*. Synthetic minimal medium contained several amino acids necessary for the growth of the host strain (SD + HAU and SG + HAU), and consisted of 0.67% yeast nitrogen base without amino acids, 20 mg histidine/L, 20 mg adenine/L, 20 mg uracil/L, 2% dextrose or 2% galactose. In order to select for the pop-out of the *URA3* marker, yeast cells were cultivated on 5-fluoroorotic acid (5-FOA) medium, containing 0.67% yeast nitrogen base without amino acids, and an amino acid mixture containing 60 mg uracil/L for *S. cerevisiae* or 90 mg uracil/L for *O. parapolymorpha*, 2% dextrose and 0.5 mg 5-FOA/L. *Escherichia coli* TOP10 (Invitrogen) was used for general recombinant DNA techniques. *Escherichia coli* was transformed by the simple and efficient method (Inoue *et al.*, 1990), and the *E. coli* transformants were cultured in LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl) supplemented with 100 μg/ml of ampicillin.

Bioinformatic analysis

Multiple sequence alignments were performed with CLUSTALW (http://align.genome.jp) and shaded using Boxshade 3.21 (http://www.ch.embnet.org/software/BOX_form.html). Percentage identity and divergence of proteins were calculated by comparing sequence pairs in relation to the phylogeny constructed using the CLUSTAL-W module in the DNASTAR MegAlign program (Thompson *et al.*, 1994). Protein motif analysis was performed using CDD (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml), and phosphorylation sites of Hsf1 were predicted using the NetPhos 3.1 server (http://www.cbs.dtu.dk/services/NetPhos/).

Construction of *S. cerevisiae* expression vectors for wild-type, a H221R point mutant, and C-terminal deletion mutant forms of OpHsf1

In order to construct *S. cerevisiae* vectors expressing wild-type OpHsf1, pADH-OpHSF1, and pTEF-OpHSF1 (Supplementary data Table S2), the DNA fragment containing the open reading frame of *HpHSF1* was amplified from *O. parapolymorpha* genomic DNA using the primer set listed in Supplementary data Table S3 with Pyrobest DNA polymerase (Takara). For HA-tagging at the C-terminus of Hsf1, the PCR fragment was re-amplified with the forward primer OpHSF1PCR1_1F and the reverse primer HA_4B_Cla. The amplified DNA fragment encoding HA-tagged Hsf1 was confirmed by sequencing, digested with SpeI/EcoRI and subcloned into p414-ADH and p414-TEF vectors containing the *ADH1* and *TEF1* promoters, respectively (Mumberg *et al.*, 1995). The resultant overexpression vectors, pADH-OpHSF1 and pTEF-OpHSF1, were introduced into the PS145 strain harboring YCpGAL1-ScHSF1, using the modified lithium acetate/dimethylsulfoxide method (Hill *et al.*, 1991), and Trp^+ transformants were selected on SC-TRP plate. The YCpGAL1-ScHSF1 vector was selectively pop-out by cultivating PS145 on 5-FOA medium, generating *S. cerevisiae* strains expressing *OpHSF1* only, either from PS145m/pADH-OpHSF1 or from PS145m/pTEF-OpHSF1.

For generating the H221R amino acid substitution in OpHsf1, fusion PCR was carried out with the primer pairs PCR1 and PCR2 (Supplementary data Table S3). The PCR1 fragment from the first AUG codon to nucleotide 666 of *OpHSF1* and the PCR2 fragment from nucleotide 655 to nucleotide 1,095 of *OpHSF1* were amplified using Pyrobest DNA polymerase (Takara). The two PCR fragments were joined by subsequent fusion PCR, and the resultant PCR fragment was amplified for HA-tagging with the forward primer OpHSF1PCR1_1F and the reverse primer HA_4B_Cla. The amplified PCR product was digested with SpeI/BglII and subcloned into the partially digested pADH-OpHSF1 to generate the vector pADH-

OpHSF1H221R (Supplementary data Table S2). To construct a collection of C-terminal truncation mutant alleles of *OpHSF1*, a set of PCR fragments containing *OpHSF1* ORF with different C-terminal deletions, ΔCTM (1,887 bp), ΔAR2 (1,338 bp), and ΔCE2 (1,239 bp), were amplified using Pyrobest DNA polymerase (Takara) with the primer pairs listed in Supplementary data Table S3. The PCR fragments of ΔCTM, ΔAR2, and ΔCE2 were re-amplified for HA-tagging with the forward primer and the HA_4B_Cla primer. The PCR products encoding HA-tagged truncation mutant Hsf1 were digested with SpeI/ClaI and subcloned into the pT-OpHSF1-MUH221R 1.4k vector, generating pT-OpHSF1ΔCE2-URA(m), pT-OpHSF1ΔAR2-URA(m), and pT-OpHSF1ΔCTM-URA(m), respectively. The *OpHSF1* DNA fragments from pT-OpHSF1-ΔCE2-URA(m), pT-OpHSF1ΔAR2-URA(m), and pT-OpHSF1-ΔCTM-URA(m) were obtained by digestion with SpeI/ClaI and subcloned into the SpeI/ClaI digested pADH-OpHSF1, generating pADH-OpHSF1ΔCE2, pADH-OpHSF1ΔAR2, and pADH-OpHSF1ΔCTM, respectively.

Replacement of wild-type *OpHSF1* with mutant alleles at the chromosomal locus

Homologous recombination was used to replace the wild-type *OpHSF1* locus with the *OpHSF1* (H221R) mutant allele. An *OpHSF1*(H221R) mutant replacement cassette was constructed as follows. Two DNA fragments containing the 3′-UTR of *OpHSF1* with different lengths, i.e., Term and Term(ex), and the *OpURA3* gene fragment were amplified using Pyrobest DNA polymerase (Takara) with the primer pairs listed in Supplementary data Table S3. The PCR fragments were digested with ClaI/PstI, PstI/NotI, and NotI/NdeI respectively. The DNA fragment of *OpHSF1*(H221R) was digested with SpeI/ClaI. The digested PCR fragments were sequentially subcloned into pGEM easy T vector (Clontech), generating the vector pT-OpHSF1MUH221R-URA(m). To facilitate homologous recombination, the 1.4 kb DNA fragment containing the 5′-UTR of *OpHSF1* was amplified by PCR and subcloned into the pT-OpHSF1MUH221R vector digested with PsiI/SpeI, generating pT-OpHSF1MUH221R-1.4k (Supplementary data Table S2). The resultant vector OpHSF1MUH221R-1.4k was digested with PsiI/NdeI and introduced into the DL1-LdU strain. Ura^+ transformants were selected on SC-URA plates, and strains bearing the correct replacement with the mutant allele were confirmed by sequencing the *HSF1* locus. To recover uracil auxotrophs, pop-outs for the *ScURA3* gene were selected from the Ura^+ transformants by spreading cells on 5-FOA plates. To replace the wild-type *OpHSF1* on chromosomal DNA with the truncated alleles at the chromosomal locus, the *OpHSF1* mutant allele replacement vectors, pT-OpHSF1MUH221R-URA(m), pT-OpHSF1ΔCE2-URA(m), pT-OpHSF1ΔAR2-URA(m), and pT-OpHSF1ΔCTM-URA(m), were digested with PsiI/NdeI and introduced into the DL1-LdU strain. Ura^+ transformants were selected on SC-URA plate. PCR was used to ensure the correct replacement of the wild-type *HSF1* allele with the truncation mutant alleles. Primer pairs are listed in Supplementary data Table S3.

Complementation of *OpHSF1*(H221R) mutation by replacement with WT allele

To construct an *OpHSF1* complementation vector pT-OpHSF1MU-1.4k, the DNA fragment of *OpHSF1*(H221R) in the vector OpHSF1MUH221R-1.4k was replaced with the wild-type *OpHSF1* DNA fragment, which was amplified from the chromosomal DNA of the wild-type strain using Pyrobest DNA polymerase (Takara) with the primer pairs listed in Supplementary data Table S3. The *OpHSF1* complementation cassette was obtained by PsiI/NdeI digestion of OpHSF1MU-1.4k and introduced into the *OpHSF1*(H221R) mutant strain. Ura^+ transformants were selected on SC-URA plates.

RNA analysis by microarray and quantitative real-time PCR

Yeast cells which had been cultured overnight were inoculated at an initial OD_{600} of 0.2 and grown at 37°C to early logarithmic phase (OD_{600} = 0.5). For heat shock treatment, yeast cells were shifted to a water bath pre-warmed at 55°C. At the indicated cultivation times, the heat shocked cells were quickly treated with cold stop solution (EtOH:Phenol = 9:1) and thoroughly mixed. The harvested cells were washed twice with TE buffer (pH 8.0) and immediately frozen in liquid nitrogen. For microarray analysis, total RNA was isolated using the hot phenol extraction method (Schmitt *et al*., 2003) and purified with an RNeasy minikit (Qiagen), according to the manufacturer's instructions. cDNA was synthesized from 10 to 20 μg of total RNA using a special RT-dT primer kit (Genisphere), and labeled with fluorescent dyes using a 3DNA Submicro EX expression array detection kit (Genisphere), and hybridized with microarray slides (Gene Expression Omnibus [GEO] database accession number GPL4802), as described previously (Moon *et al*., 2015). The microarray data have been deposited in the GEO database with the accession number GSE135405. Quantitative real-time PCR was performed in a CFX96 Real-Time PCR detection system (Bio-Rad) using 1 ng of the synthesized cDNA as template and a set of gene-specific primers (Supplementary data Table S2) with SYBR Premix Ex Taq (Tli RNaseH Plus) (Takara). The specificity of amplification was confirmed by melting curve analysis with one single peak. Each sample was normalized to an endogenous control, i.e., the *OpACT1*-like gene.

Western blot analysis of HA-tagged OpHsf1 expression

Yeast cell lysates were obtained by vortexing cell pellets five times with glass beads in TNE lysis buffer (50 mM Tris-HCl; pH 7.5, 150 mM NaCl, 5 mM EDTA; pH 8.0, protease inhibitor cocktail: Promega) for 1 min intervals with alternating cooling on ice. Protein samples were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then subjected to immunoblot analysis using rat anti-hemagglutinin (HA) antibody (1:500, Sigma) and anti-rat-HRP (1:2,000, Sigma). Protein bands were detected by the ECL system (Amersham).

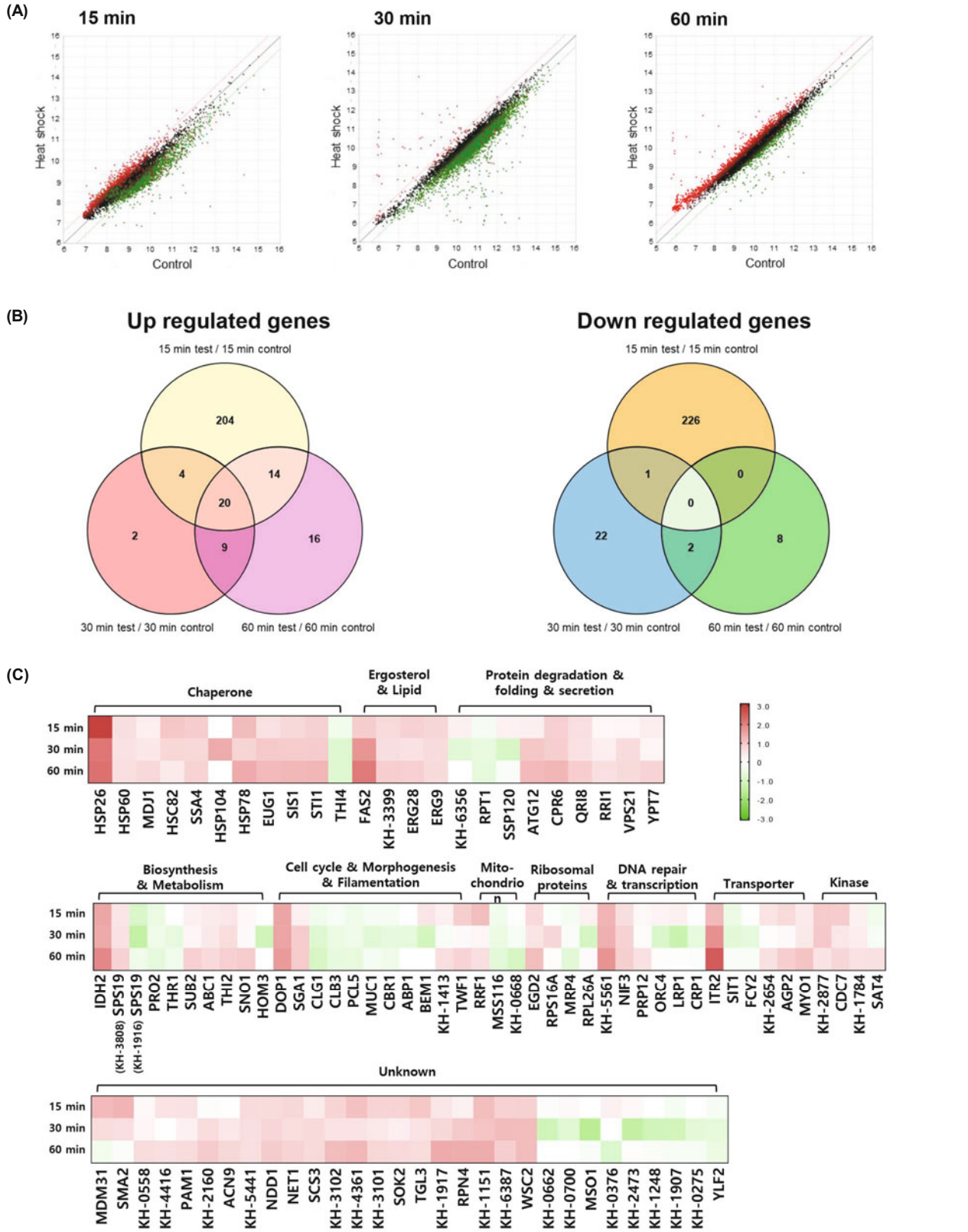

Fig. 1. Transcriptome profiles of *O. parapolymorpha* under conditions of heat shock stress. Yeast cells were exposed to heat shock at 55°C for 15, 30, and 60 min. At the indicated times, total RNAs were isolated and subjected to transcriptome analysis using the *H. polymorpha* cDNA microarray. (A) Scatter plots of differentially expressed genes under heat shock conditions. (B) Venn diagram of the genes with more than 1.5-fold change in expression at the indicated exposure time. Left, Upregulated genes; Right, Downregulated genes. (C) The heatmap of *O. parapolymorpha* genes exhibiting more than 1.5-fold change in expression at 30 or 60 min of heat shock.

Results

Ogataea parapolymorpha shows dynamic transcriptome profiles during heat shock stress

In an initial effort to obtain information on the transcriptome profiles of *O. parapolymorpha* under conditions of heat shock stress, we performed microarray analysis of RNA samples obtained from *O. parapolymorpha* cultured at 55°C for 15, 30, and 60 min (Fig. 1A). This analysis revealed that numerous genes were up-and downregulated at an early stage (15 min) of heat shock stress, while fewer genes exhibited changes in expression after 30 min and 60 min of heat shock stress. The dynamic transcriptome profiles indicate that different reaction mechanisms operate in cells during the early and late stages of heat stress exposure. At 15 min, the number of downregulated genes was greater than the number of upregulated genes, probably as a result of the sudden stress exposure causing the shut-down of several biological processes, such as protein synthesis and secretion. At 30 min, expression changes were observed in fewer genes, thus reflecting the recovery of yeast cells from short-term stress due to the early heat shock response. A similar pattern of dynamic transcriptome profiles was reported in a recent study examining the change in gene expression profiles in *S. cerevisiae* within the first 15 min of inducing 37°C heat shock stress (Mühlhofer *et al*., 2019). However, after exposure to heat stress for 60 min, a subset of genes was upregulated to a greater extent than at 30 min, probably to help the cell cope with the continued stress exposure. These results suggest that the early upregulated genes might be associated with an urgent stress defense mechanism whereas the later upregulated genes might be involved in a stress tolerance response. Thus, to identify the heat shock target genes responsible for the high thermotolerance of *O. parapolymorpha*, we selected genes showing more than 1.5-fold change in expression after 30 min or 60 min exposure to heat shock (Fig. 1B, Supplementary data Table S4). Out of a total of 98 *O. parapolymorpha* genes showing differential gene expression at 30 min or 60 min, 27 genes involved in protein folding, heat shock response, and ergosterol biosynthesis were upregulated by heat stress (Fig. 1C). Quite a few genes showing increased expression during heat shock exposure are grouped as hypothetical genes with suggested functions associated directly or indirectly with stress caused by heat shock.

Ogataea parapolymorpha heat shock genes possess putative HSEs in their promoters

Heat shock genes have been reported to be regulated directly or indirectly by Hsf1 during heat shock in *S. cerevisiae* (Shi *et al*., 1998; Amorós and Estruch, 2001; Grably *et al*., 2002). These genes generally contain heat shock elements (HSEs) that are recognized by Hsf1. HSEs bear a series of alternating, inverted repeats of the conserved sequence NGAAN and are classified into two types. Perfect-type HSEs have contiguous inverted repeats of 5-NGAAN-3 upstream of the heat shock target genes. Gap-type HSEs have a gap of 5 bp that separates two consecutive NGAAN units from another unit (Amin *et al*., 1988; Tamai *et al*., 1994; Yamamoto *et al*., 2005). In a previous study on the target genes of *S. cerevisiae* Hsf1 using three combined genome-wide approaches (NET-seq, RNA-

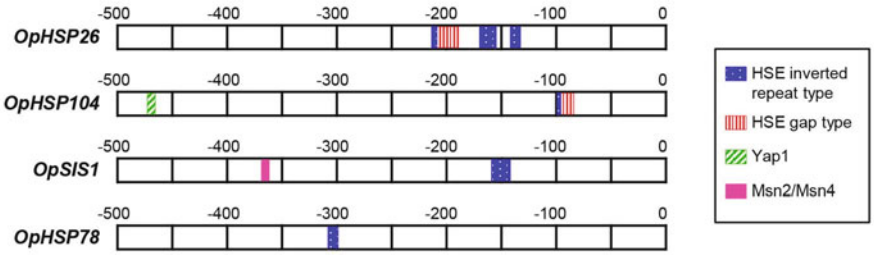

Fig. 2. Regulatory motif analysis of a subset of *O. parapolymorpha* candidate chaperone genes *OpHSP26*, *OpHSP104*, *OpSIS1*, and *OpHSP78*. *In silico* analysis of *O. parapolymorpha* HSP promoters was performed using the Regulatory Sequence Analysis Tools (http://www.rsat.eu/).

Promoter	Regulatory motif	Direction	Start	End	Sequence
OpHSP26	HSE inverted repeat type	DR	-162	-153	ctccAGAAAGTTCCtgcg
		DR	-142	-133	gcccAGAAATTTCGatgg
		DR	-208	-199	gagcTTTCGGGAACgctg
		DR	-166	-154	tgctCTCCAGAAAGTTCctgc
		DR	-212	-200	gatgGAGCTTTCGGGAAcgct
	HSE gap type	DR	-207	-190	agctTTCGGGAACGCTGGAGAAcagc
OpHSP104	HSE inverted repeat type	DR	-99	-90	tactAGAACTTTCTgtgc
	HSE gap type	DR	-98	-81	tctaGAGCCGCACAGAAAGTTCtagt
	Yap1	R	-473	-467	tattTTACAAAtcct
OpSIS1	HSE inverted repeat type	DR	-151	-142	ttctAGAATTTTCCattt
	Msn2/Msn4	D	-367	-363	actgAGGGGtacg
OpHSP78	HSE inverted repeat type	DR	-307	-298	ccctAGAAAATTCTagca

seq, and ChIP-seq), a small set of genes (total 18 genes) were defined as Hsf1-dependent genes (HDGs), whose promoters should be bound to Hsf1 to drive basal expression (Solís *et al.*, 2016). Out of the *O. parapolymorpha* genes whose expression was induced at a late stage of heat shock exposure, we selected the *O. parapolymorpha* homologs to *S. cerevisiae HSP78*, *HSP104*, and *SIS1*, which were defined as HDGs (Solís *et al.*, 2016), in order to test for the presence of putative HSEs in their promoter regions (Fig. 2). In addition, the *O. parapolymorpha HSP26* homolog was chosen for the promoter analysis based on its highest induction fold upon heat shock stress. The *O. parapolymorpha HSP26, HSP78*, *HSP104*, and *SIS1* exhibit 42%, 62.5%, 66.1%, and 59.3% identity, respectively, to its corresponding *S. cerevisiae* gene. We found that the four *O. parapolymorpha* genes possess HSEs almost identical to those of *S. cerevisiae*. They have perfect-type HSEs in common, whereas the gap-type HSE is present only in the promoter regions of *OpHSP26* and *OpHSP104*. In particular, the number of HSEs on the *OpHSP26* promoter appears to reflect its fast and strong response to the heat shock, as observed in the transcriptome profiles (Fig. 1C). Binding sites for other stress-response transcription factors, such as Yap1 and Msn2/Msn4, were identified in the promoters of *OpHSP104* and *OpSIS1*, respectively, consistent with a previous study reporting the involvement of Yap1 and Msn2/Msn4 in heat shock response in *S. cerevisiae* (Morano *et al.*, 2012).

Putative OpHsf1 has the functional domains conserved among yeast homologs

In order to identify an *O. parapolymorpha* ORF encoding heat shock factor 1 that can recognize HSEs in the promoters of heat shock protein genes, we performed a BLAST search using the *S. cerevisiae HSF1* as a query. This search identified only a single *HSF1* homolog in the entire *O. parapolymorpha* genome (Ravin *et al.*, 2013). The *O. parapolymorpha HSF1* homolog (*OpHSF1*) encodes a 648 amino-acid-long protein, exhibiting 26% identity to the 833 amino-acid-long ScHsf1 (Fig. 3A). Although OpHsf1 shows only low sequence identity to ScHsf1, OpHsf1 possesses most of the functional domains observed in ScHsf1, such AR1, DBD, TRI, CE2, AR2, and CTM. The CE2 of ScHsf1 contains potential phosphorylation sites (Jakobsen and Pelham, 1991), hence the CE2 region was predicted to be located between amino acids 414 and 446 in OpHsf1 by analyzing phosphorylation sites using NetPhos 3.1 server. Given that the CTM of ScHsf1 is rich in basic amino acid (Sakurai and Fukasawa, 2001), the region from residue 630 to residue 648 at the C-terminal end of OpHsf1 – which is also rich in basic amino acids – was predicted to be the CTM in OpHsf1. Overall, OpHsf1 has smaller

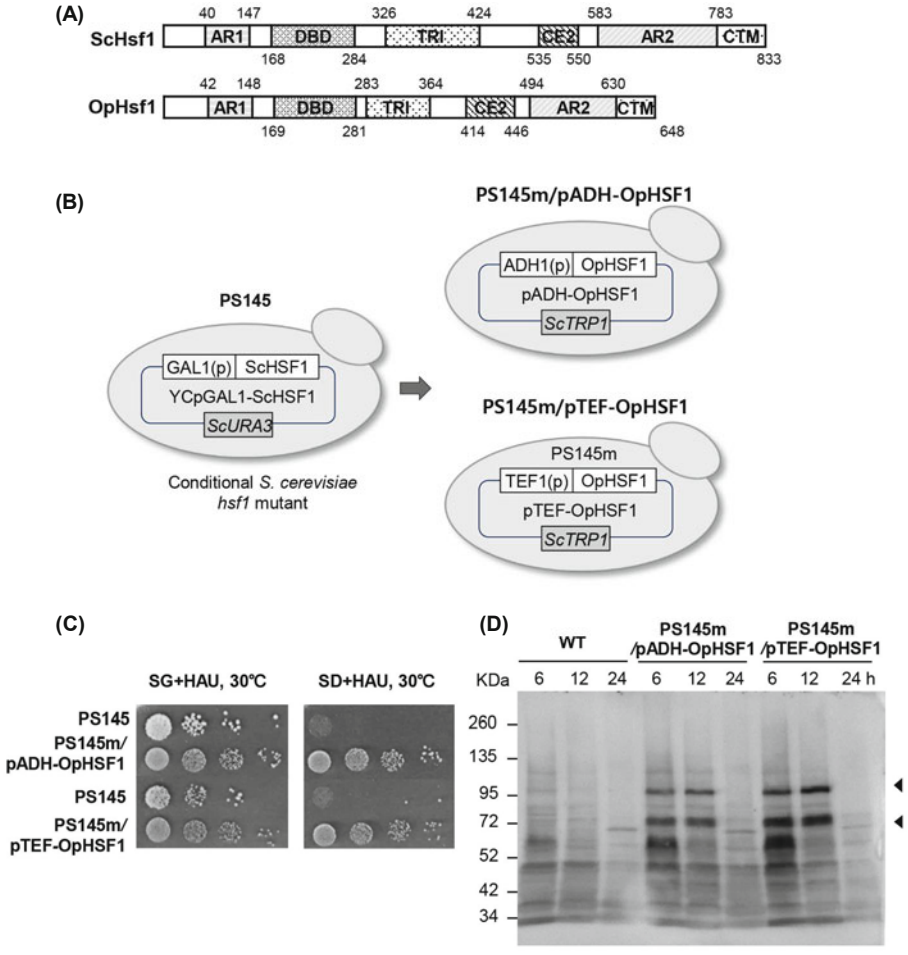

Fig. 3. Functional analysis of OpHsf1 using a conditional *hsf1* mutant *S. cerevisiae* strain. (A) Comparison of functional domains between *S. cerevisiae* (ScHsf1) and *O. parapolymorpha* (OpHsf1). (B) Schema of *S. cerevisiae hsf1* mutant strains harboring the OpHsf1 expression vectors, PS145m/pADH-OpHSF1 and PS145m/pTEF-OpHSF1. (C) Growth analysis of *S. cerevisiae* strains complemented with *OpHSF1*. (D) Western blot analysis of Hsf1 proteins expressed in the complemented *S. cerevisiae* strains. Total cell extracts of *S. cerevisiae* strains expressing OpHsf1 with HA tagging at its C-terminus were subjected to western blot analysis using an anti-HA antibody.

sized TRI, AR2 and CTM domains as compared to ScHsf1 (Fig. 3A). Comparison of the amino acid sequence of Hsf1 proteins from various other organisms revealed that the DBD and TRI domains are most highly conserved while other domains are comparatively less conserved (Supplementary data Fig. S1).

Expression of *OpHSF1* complemented growth defects of *S. cerevisiae hsf1* deletion mutation

In an effort to investigate the heat shock transcription factor function of OpHsf1, we constructed two *OpHSF1* expression vectors using the *S. cerevisiae* constitutive promoters, pADH-OpHSF1 and pTEF-OpHSF1, and introduced them into the *S. cerevisiae* conditional *hsf1* mutant PS145 strain (Sorger and Pelham, 1988). *Saccharomyces cerevisiae* PS145 is an *hsf1* deletion mutant strain harboring YCpGAL1-ScHSF1, in which the expression of *ScHSF1* is under the control of the *GAL10* promoter. Due to the essential function of Hsf1 for cell growth at normal temperature, the *S. cerevisiae* PS145 strain can grow only in the presence of galactose, even in the absence of heat shock. To generate *S. cerevisiae* strains expressing *OpHSF1* instead of *ScHSF1,* we popped out YCpGAL1-ScHSF1 in PS145 strains containing *OpHSF1* expression vectors, thereby generating PS145m/pADH-OpHSF1 and PS145m/pTEF-OpHSF1 (Fig. 3B). These strains were tested using a serially diluted spotting assay on SD and SG media containing essential amino acids at 30°C. Whereas the PS145 strain survived only on galactose medium, the PS145m stains containing only the *OpHSF1* expression vectors were able to grow on glucose medium (Fig. 3C). The results indicated that heterologous expression of *OpHSF1* complemented the growth defective phenotype of the *S. cerevisiae hsf1* deletion mutant, albeit incompletely, supporting the conclusion that OpHsf1 is a functional homolog of ScHsf1. We also confirmed by western blotting that HA-tagged OpHsf1 protein was expressed in the complemented *S. cerevisiae* strains. Noticeably, OpHsf1p was detected as two bands with molecular weights of ~72 kDa and ~100 kDa, respectively, in the total cell extracts generated after 6 h and 12 h of growth, but the signals

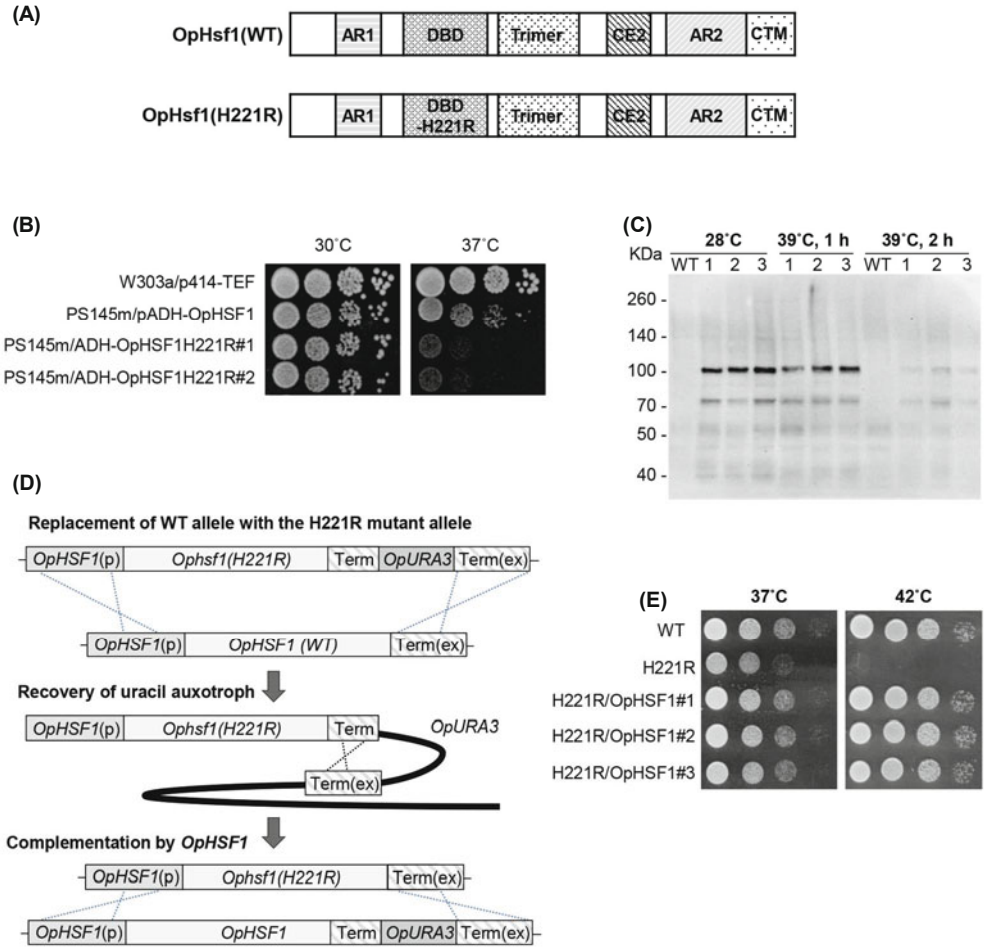

Fig. 4. Phenotypic analysis of the *Ophsf1*(H221R) mutation in *S. cerevisiae* and *O. parapolymorpha*. (A) Schema of *Ophsf1*(H221R) point mutant allele. (B) Growth analysis of the *S. cerevisiae* conditional *hsf1* mutant strain harboring the *Ophsf1*(H221R) expression vector by spotting assay on YPD plates. (C) Western blot analysis of OpHsf1 proteins in *S. cerevisiae* under heat shock conditions. Lanes: WT, wild type(w303a/p414-TEF); 1, PS145m/pADH-OpHSF1; 2, PS145m/pADH-OpHSF1H221R#1; 3, PS145m/pADH-OpHSF1H221R#2. (D) Generation and complementation of the *O. parapolymorpha hsf1*(H221R) mutant strains by homologous recombination. (E) Growth phenotype of the *Ophsf1*(H221R) mutant strain and the complemented strain generated by re-introducing wild-type *OpHSF1*.

for OpHsf1p disappeared in the sample grown for 24 h (Fig. 3D). We speculate that OpHsf1 protein might be subject to degradation in the stationary phase and the lower band of OpHsf1 might be a proteolytic cleavage product since the molecular mass is smaller than the expected size of OpHsf1. Considering that the open reading frame of *OpHSF1* specifies a protein with a calculated molecular mass of ~78 kDa, the OpHsf1 signal of ~100 kDa is much bigger than expected. This discrepancy was also reported for *S. cerevisiae* Hsf1, with an expected molecular mass of 93.3 kDa, and which was detected as a protein with a molecular weight of of 140–150 kDa on SDS-containing gels (Sorger and Pelham, 1988).

H221R mutation in the DBD domain of OpHsf1 generates a Ts phenotype both in *S. cerevisiae* and *O. parapolymorpha*

In *S. cerevisiae* Hsf1, the H220R and N222Y point mutations located in the turn region of DBD were reported to give rise to Ts phenotypes (Yamamoto and Sakurai, 2006). As a strategy to construct an *OpHSF1* mutant allele showing a Ts phenotype, we used site-directed mutagenesis to alter His-221 to Arg in the turn region of the DBD of OpHsf1 (Fig. 4A). To investigate whether this mutation can also cause a Ts phenotype in OpHsf1, we introduced this construct into the *S. cerevisiae* PS145 strain and eliminated the YCpGAL1-ScHSF1 vector by selection on 5-FOA medium. This generated an *S. cerevisiae* strain expressing the *Ophsf1*(H221R) mutant allele, designated PS145m/pADH-OpHSF1(H221R). Analysis of the PS145m/pADH-OpHSF1(H221R) strain revealed that it has an increased sensitivity to mild heat shock at 37°C, compared to the PS145m/pADH-OpHSF1 strain expressing the wild-type *OpHSF1* (Fig. 4B). This result indicates that the His221 residue is an important determinant of the role of OpHsf1 in the HSR, as is the case for His220 in *S. cerevisiae* Hsf1 (Yamamoto and Sakurai, 2006). To examine the expression of the *Ophsf1*(H221R) mutant protein under conditions of heat stress, we grew the PS145m/pADH-OpHSF1(H221R) strain in YPD-rich medium at 39°C for 1 h or 2 h. After exposure to heat shock for 1 h, the OpHsf1 (H221R) mutant proteins were detected, but the signals of OpHsf1 protein bands decreased greatly after 2 h of heat shock (Fig. 4C). An identical expression pattern was also observed with the wild-type OpHsf1 protein, indicating that OpHsf1 is inherently subject to rapid degradation in response to heat shock in the heterologous host *S. cerevisiae* and that the inability of the OpHsf1(H221R) mutation to complement the Ts phenotype of the *S. cerevisiae hsf1* deletion mutant does not result from the decreased stability.

To investigate the effect of the H221R mutation on the function of OpHsf1 in its endogenous host *O. parapolymorpha*, we constructed a *O. parapolymorpha hsf1*(H221R) mutant strain by replacing the wild-type *OpHSF1* locus on the chromosome with the H221R point mutation cassette containing the *OpURA3* blaster flanked by *OpHSF1* terminator sequences. Ura$^+$ transformants were screened by PCR and sequenced for the correct replacement generated by double homologous recombination. The *OpURA3* gene in the H221R

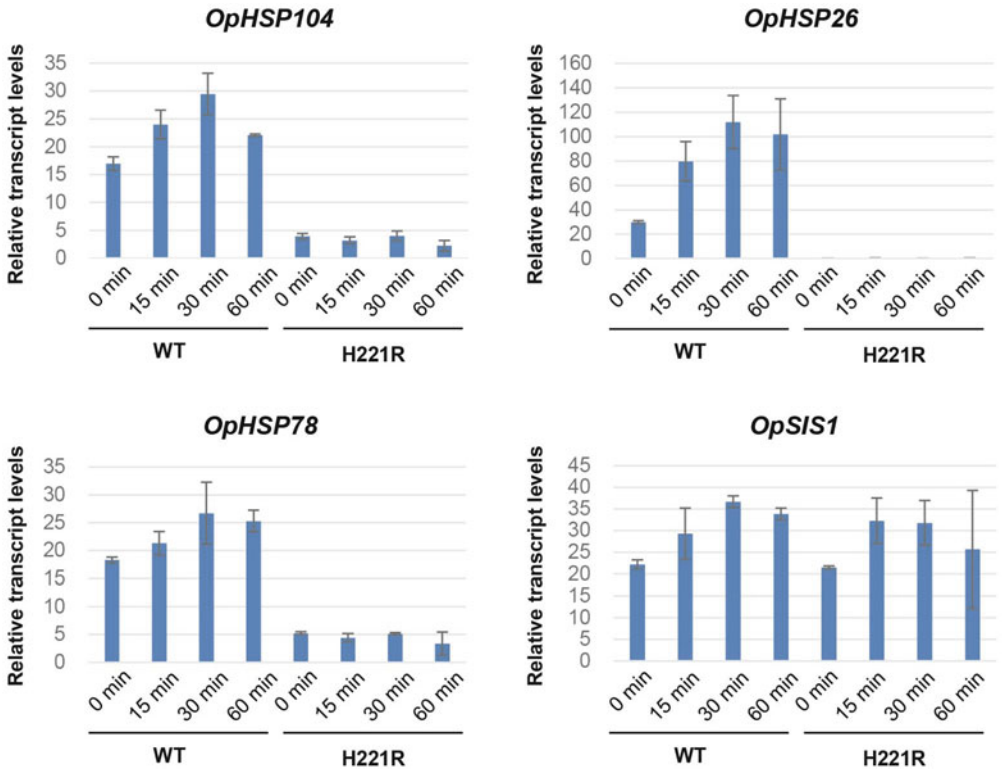

Fig. 5. Expression analysis of *O. parapolymorpha* chaperone genes involved in heat shock response in the WT and *Ophsf1*(H221R) mutant strains. Yeast cells were exposed to heat shock at 55°C for the indicated times. Transcript levels of *OpHSP96*, *OpHSP104*, *OpHSP78*, and *OpSIS1* were analyzed by qRT-PCR in duplicate and normalized against the transcript level of the endogenous *OpACT1*-like gene (control), which showed no apparent changes in the transcript level under conditions of heat shock stress.

point mutation cassette was popped out from the Ura$^+$ transformants, thereby generating an *O. parapolymorpha hsf1* (H221R) mutant strain with uracil auxotrophy. The complementation strain of *Ophsf1*(H221R), was generated by introducing the complementation cassette carrying the WT *OpHSF1* to *Ophsf1*(H221R) mutant (Fig. 4D). It is quite notable that the *O. parapolymorpha hsf1*(H221R) mutant showed impaired growth even at 37°C, which is the normal growth condition for this thermotolerant yeast. Further, this mutant could not grow at all even under mild heat shock conditions at 42°C (Fig. 4E). In contrast, the complemented strains showed normal thermotolerance. The finding that *O. parapolymorpha* with H221R-mutated OpHsf1 exhibited severe growth retardation of even under normal growth conditions indicates that the DBD domain is critical for the function of *HSF1,* not only for thermotolerance but also for basal growth of *O. parapolymorpha*.

The DBD of OpHsf1 is important not only for the HSR but also for the basal expression of HSP genes

To investigate whether the expression of heat shock genes carrying HSEs are under the control of OpHsf1 in *O. parapolymorpha*, we analyzed the mRNA levels of *OpHSP104*, *OpHSP26*, *OpHSP78*, *OpSIS1* in the WT and *Ophsf1*(H221R) mutant strains (Fig. 5). Total RNA samples were extracted from the WT and *Ophsf1*(H221R) strains cultured under normal or 55°C heat shock conditions for the indicated time (0, 15, 30, and 60 min) and subjected to quantitative real-time PCR (qRT-PCR). The transcript levels of these genes were generally increased in response to a temperature shift to 55°C in the WT strain. In contrast, the transcript levels of these genes, except *OpSIS1,* were not upregulated in the *Ophsf1*(H221R) strain. It is noteworthy that the basal levels of HSP genes were already extremely decreased in case of

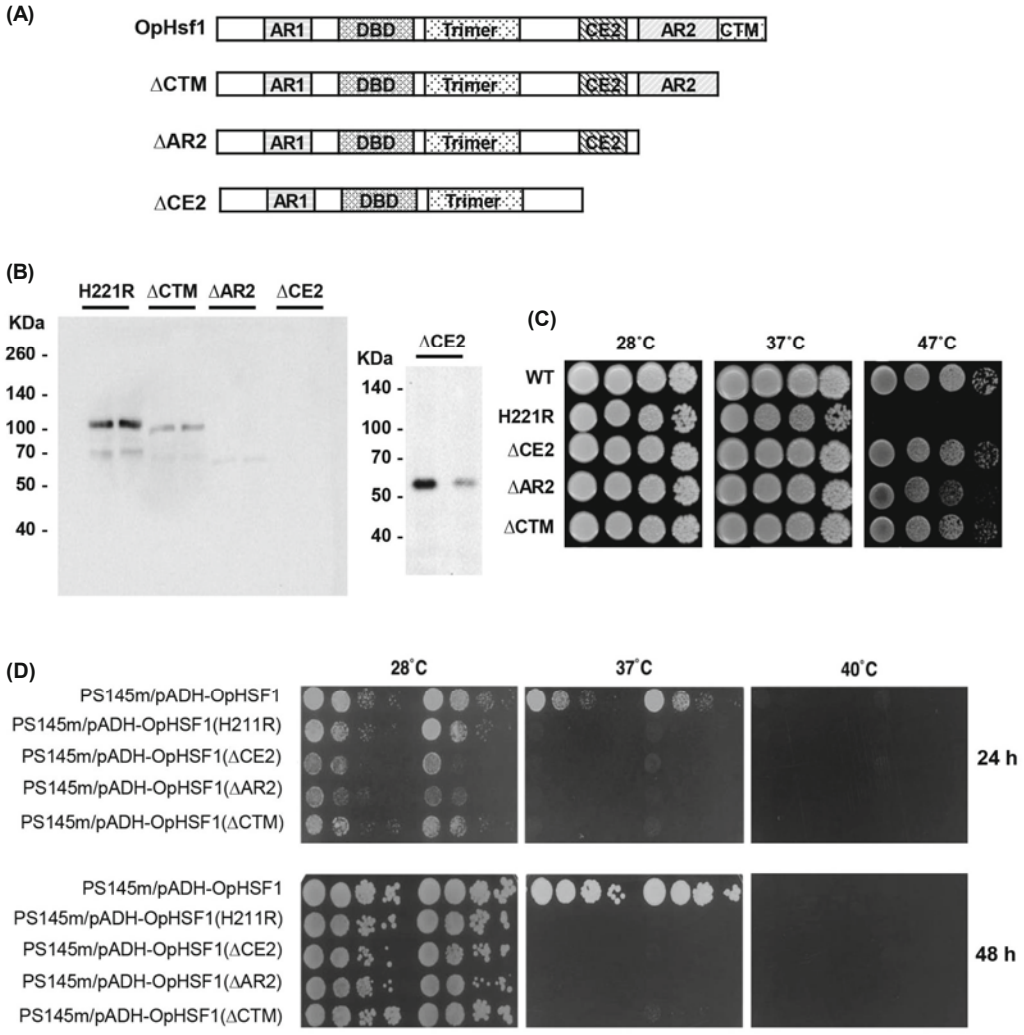

Fig. 6. Phenotypic analysis of *O. parapolymorpha* and *S. cerevisiae* strains expressing C-terminal truncated OpHsf1 proteins. (A) Schema of OpHsf1 proteins with C-terminal truncations (ΔCTM, ΔAR2, and ΔCE2). (B) Expression analysis of HA-tagged OpHsf1 proteins. Total intracellular cell extracts of *O. parapolymorpha* strains, *Ophsf1*(H221R), *Ophsf1*(ΔCTM), *Ophsf1*(ΔAR2), and *Ophsf1*(ΔCE2), were subjected to western blot analysis using an anti-HA antibody. The right panel is an overexposed western blot to detect the OpHsf1(ΔCE2) protein band. (C) Growth of *O. parapolymorpha* strains expressing OpHsf1 proteins under heat shock conditions, as assessed by spotting assay on YPD media. (D) Growth of *S. cerevisiae* strains expressing OpHsf1 proteins under heat shock conditions as assessed by spotting assay on YPD plate.

the *Ophsf1*(H221R) strain compared to those in the WT, even under non-heat shock conditions (Fig. 5). The results suggest that the binding of OpHsf1 to these HSP gene promoters is essential not only for the induction of HSR but also for the basal expression of these HSP genes. This strongly supports the hypothesis that OpHsf1 is required to maintain the basal growth of *O. parapolymorpha,* in addition to being responsible for the strong thermotolerance of this yeast.

The C-terminal domains of OpHsf1 are dispensable for the thermotolerance of *O. parapolymorpha*

To evaluate the function of domains located in the C-terminal part of OpHsf1, we generated a series of C-terminal truncation stains, including ΔCTM, ΔAR2, and ΔCE2 mutant strains. These strains express truncated forms of the Hsf1 protein in which the CTM domain, the region from CTM to AR2, and that from CTM to CE2, respectively were deleted (Fig. 6A). Expression of these C-terminal truncated OpHsf1 proteins was confirmed by western blotting. Intriguingly, the steady state expression levels of OpHsf1 were decreased based on the extent of truncation, indicating that C-terminal truncation decreased the stability of OpHsf1 protein or the detection of HA-tagged OpHsf1 by anti-HA antibody (Fig. 6B). Nevertheless, it was observed that all the C-terminal truncation mutant stains of *O. parapolymorpha* displayed a growth rate comparable to that of the WT stain and lacked any Ts phenotype even at 47°C, indicating that the C-terminal domains are dispensable for the function of OpHsf1 in *O. parapolymorpha*. This is in stark contrast to the *S. cerevisiae* mutant with a C-terminal deletion, which shows a severe Ts phenotype (Hashikawa and Sakurai, 2004). Only the *Ophsf1* (ΔAR2) strain showed a mild Ts phenotype at 47°C, while the *Ophsf1*(ΔCE2) strain – in which all three CTM, AR2, and CE2 domains were deleted – restored its ability (almost full) to function in complementing the Ts phenotype (Fig. 6C). Considering that the CE2 domain of ScHsf1 is associated with negative regulation (Jakobsen and Pelham, 1991), it could be speculated that the additional deletion of CE2 domain might enhance its function as a transcription factor. However, the C-terminal truncation mutant forms (ΔCE2, ΔAR2, ΔCTM) of OpHsf1 did not complement the growth defect of the *S. cerevisiae hsf1* strain (Fig. 6D). This implies that the C-terminal domains of OpHsf1 proteins are required for functionality in *S. cerevisiae*.

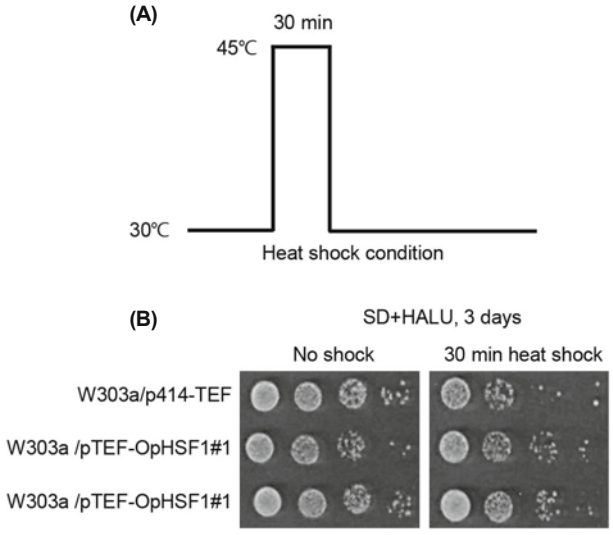

Fig. 7. Effect of *OpHSF1* overexpression on thermotolerance of *S. cerevisiae* upon transient heat shock. (A) *S. cerevisiae* cells cultivated at 30°C were subjected to heat shock at 45°C for 30 min and then returned to 30°C for the rest of cultivation time. (B) Growth of *S. cerevisiae* strains expressing wild-type OpHsf1 protein after 30 min heat shock as assessed by spotting on SD + HALU media and cultivation at 30°C.

OpHSF1 overexpression enhances thermotolerance of *S. cerevisiae*

In a previous study with *S. cerevisiae*, overexpression of *ScHSF1* was shown to prevent accumulation of misfolded proteins, thus improving protein secretion (Hou *et al*., 2013). To evaluate whether heterologous expression of *OpHSF1* could confer thermotolerance to *S. cerevisiae*, we tested the effect of *OpHSF1* overexpression on the HSR of a wild-type *S. cerevisiae* strain under heat shock conditions. During prolonged cultivation at 42°C, the *S. cerevisiae* cells expressing *OpHSF1* under control of the *TEF1* promoter (W303a/pTEF-OpHSF1) did not show any improvement in thermotolerance compared to the strain expressing only the empty vector (W303a/p414-TEF). We speculate that OpHsf1 was subjected to rapid degradation during prolonged heat shock exposure. As presented in Fig. 4C, the effects of OpHsf1 overexpression might be limited to only short exposures to heat shock. Thus, *S. cerevisiae* cells were shifted from 30°C to 45°C and cultivated for only 30 min at 45°C and then spotted on SD plates for subsequent cultivation at 30°C (Fig. 7A). The *S. cerevisiae* strains overexpressing *OpHSF1* strain showed slight but apparent improvement in thermotolerance compared to the wild type strain (Fig. 7B). These results suggest that *OpHSF1* is worthy of further exploration as a target gene to increase thermotolerance of other yeast species. Further optimization of OpHsf1 by improving protein stability or by inhibiting its proteolysis in *S. cerevisiae* might be required to confer persistent thermotolerance.

Discussion

Hsf1 is a master regulator in eukaryote cells, controlling the expression of a group of genes involved in mediating stress responses. This transcription factor directly binds to approximately 3% of the total genes in *S. cerevisiae* (Hahn *et al*., 2004). In particular, Hsf1 modulates the upregulation of specific heat shock genes, independently from other protective responses associated with the *MSN* genes (Eastmond and Nelson, 2006). The genes encoding Hsf1 have been studied in multiple eukaryotes, including several yeast species such as *S. cerevisiae*, *K. lactis*, *Candida albicans*, and *Cryptococcus neoformans* (Wiederrecht *et al*., 1988; Jakobsen and Pelham, 1991; Nicholls *et al*., 2011; Yang *et al*., 2017), and the findings indicate that Hsf1 plays essential roles not only in thermotolerance but also in other cellular processes. Here, we performed functional analyses on the *OpHSF1* gene from the thermotolerant methylotrophic yeast *O. parapolymorpha* and

showed that the *O. parapolymorpha* Hsf1 is a functional homolog of *S. cerevisiae* Hsf1, despite the low sequence identity between these two proteins (Fig. 3). In this study, two kinds of temperature-sensitive *O. parapolymorpha hsf1* mutants, i.e., *Ophsf1*(H221R) and *Ophsf1*(ΔAR2), were generated. The *Ophsf1*(H221R) mutation resulted in a severe Ts phenotype of *O. parapolymorpha* and decreased the basal expression of some HSP genes, such as *HSP104*, *HSP26*, and *HSP78*, even under normal culture conditions (Figs. 4 and 5). This indicates that binding of OpHsf1 to promoters harboring HSEs is essential not only for induction of the HSR, but also for the basal expression of HSP genes. In *S. cerevisiae* cells, Hsf1's essential function in the absence of stress was reported to drive basal gene expression of essential proteostasis factors (Solís *et al*., 2016). Thus, the loss of DNA binding activity of Hsf1 in DBD might shut down a housekeeping program required for proteostasis, thus eventually leading to loss of cell viability. Among three C-terminal truncated mutants (ΔCTM, ΔAR2, and ΔCE2), only the *Ophsf1*(ΔAR2) strain showed a mild Ts phenotype (Fig. 6), implying the C-terminal domains are dispensable for the function of OpHsf1. This is in contrast to that observed in case of ScHsf1.

The strength of Hsf1p-mediated transcriptional activation is shown to be dependent primarily on the number of HSEs within the promoter and secondarily on the sequence of the non-conserved "N" residues and the orientation of the binding site. In *S. cerevisiae*, *HSP78* and *HSP104* have the perfect-type of HSE (Grably *et al*., 2002; Yamamoto and Sakurai, 2006), whereas *HSP26* has both perfect- and gap-types of HSE (Chen and Pederson, 1993). The promoters of *O. parapolymorpha* HSP genes possess HSEs almost identical to those of *S. cerevisiae* (Fig. 2). Notably, *OpHSP26,* which has both types of HSEs in several sites, showed much higher levels of upregulation than *OpHSP104*, *OpHSP78*, and *OpSIS1*. The complementation of the *S. cerevisiae hsf1* mutation by OpHsf1 strongly supports the interpretation that *O. parapolymorpha* Hsf1 can recognize the same sequence elements as the *S. cerevisiae* Hsf1. It is intriguing that the expression of *OpSIS1* induced by heat shock is not strictly dependent on OpHsf1, implying that other transcription factors, probably the general stress response factors Msn2/Msn4, can induce the upregulation of *OpSIS1* in response to stress. We note the presence of binding sites for Msn2/Msn4 in the *OpSIS1* promoter. In *S. cerevisiae*, many heat-activated genes have stress response elements (STREs) that are the binding sites of the general stress response factors Msn2 and Msn4 (Treger *et al*., 1998). The most genes induced by thermal stress are controlled by Msn2/4 and are Hsf1-independent, although some are redundantly controlled by both the Msn2/Msn4 and Hsf1 proteins (Boy-Marcotte *et al*., 1999). Analysis of the stress-induced expression of *S. cerevisiae HSP26* and *HSP104* genes reveals that the contribution of Hsf1 and Msn2/4 is different depending on the gene and the stress condition (Amorós and Estruch, 2001). Therefore, it is expected that there are also a group of heat-inducible genes in *O. parapolymorpha*, whose promoters contain binding sites for both Msn2/4 and Hsf1. It will be an interesting subject to identify the putative target genes of OpHsf1, which are still induced by heat shock stress in the H221R mutation background, and to examine the presence of additional regulatory element like STRE, as the case of *OpSIS1*. Further analysis using ChIP-chip or ChIP-seq would provide more systematic information on how two different transcription factors, Hsf1 and Msn2/4, cooperate in the expression of heat shock-inducted genes in this thermotolerant non-conventional yeast.

Our data clearly show the critical role of OpHsf1 as a transcription activator, not only under conditions of heat shock but also under normal growth conditions. This is consistent with a previous report demonstrating that *S. cerevisiae* Hsf1 always exists in the nucleus as a trimer, and is engaged in both constitutive and stress-inducible binding to the HSEs (Verghese *et al*., 2012). Using a chemical genetics approach that allowed rapid inactivation of Hsf1, a recent study in *S. cerevisiae* provided strong evidence that rather than inducing the upregulation of genes coding for proteins involved in various cellular processes required for adaptation to thermal stress, Hsf1 controls a dedicated set of chaperone protein genes devoted to maintaining protein-folding homeostasis (Solís *et al*., 2016; Pincus, 2017). A recent study in the pathogenic yeast *C. albicans* also reported the importance of non-heat shock responsive roles of Hsf1, including essential roles under conditions of iron deprivation and in drug defense (Nair *et al*., 2017).

Regulation of *HSF1* activation confers diverse benefits in several organisms. In a mouse model, aggregates of misfold proteins formed as a result of expanded polyglutamine repeats were shown to cause neurodegenerative disease, but the formation of polyglutamine inclusions was efficiently reduced by the presence of active Hsf1 (Fujimoto *et al*., 2005). Multi-drug resistance of mammalian cells such as those derived from melanoma-associated skin cancer was increased upon overexpression of Hsf1, indicating that *Hsf1* activation can result in enhanced drug resistance (Vydra *et al*., 2013). *HSF1* activation was shown to increase ethanol production in sake yeasts (Noguchi *et al*., 2012) and to improve yeast protein secretion (Hou *et al*., 2013). In addition, a recent study reported that the expression of thermotolerant yeast *K. marxianus* Hsf1 promoted cell growth and ethanol fermentation of *S. cerevisiae* at high temperature (Li *et al*., 2017). We also showed that the overexpression of OpHsf1 in *S. cerevisiae* increased the resistance of the heterologous host to transient heat shock (Fig. 7). Our work indicates that although persistent thermotolerance may require further optimization of OpHsf1 by improving protein stability or by developing a constitutively activated form, *OpHSF1* can be exploited as a valuable resource for increasing thermotolerance of other yeast species used in industrial applications.

Acknowledgments

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Conflict of Interest

We have no conflicts of interest to report.

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