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Superior thermotolerance of *Saccharomyces cerevisiae* for efficient bioethanol fermentation can be achieved by overexpression of *RSP5* ubiquitin ligase

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ABSTRACT

The simultaneous saccharification and fermentation process requires thermo-tolerant yeast to facilitate the enzymatic hydrolysis of cellulose. In this paper, we describe a Htg$^+$ strain that exhibits confluent growth at high temperature (41 °C) and resistance to heat shock, ethanol, osmotic, oxidative and DNA damage stresses. *HTG6*, one of the six genes responsible for the thermotolerant phenotype was identified to be the gene *RSP5* encoding a ubiquitin ligase. The *RSP5* allele of the Htg$^+$ strain, designated *RSP5-C*, possessed five, one and two base changes in the promoter, open reading frame and terminator region, respectively. The base changes in the promoter region of the *RSP5-C* allele were found to be responsible for the thermotolerant phenotype by strongly increasing transcription of the *RSP5* gene and consequently causing a rise in the ubiquitination of cell proteins. Overexpression of the *RSP5-BY* allele present in the *htg6* host strain (Htg$^-$) conferred thermotolerance at 41 °C, to this strain as in the case of *RSP5-C* allele. We also discovered that an Htg$^+$ strain overexpressing the *RSP5-C* allele exhibits a more robust Htg$^+$ phenotype against higher temperature (43 °C). The data presented here also suggest that overexpression of *RSP5* could be applied to raise the upper limit of thermotolerance in *S. cerevisiae* strain used for industrial bioethanol production.

1. Introduction

Eco-friendly bioethanol is a renewable alternative fuel that can be used in unmodified gasoline engines with their current fuelling infrastructure (Rass-Hansen et al., 2007). Among several microorganisms, *Saccharomyces cerevisiae* has attracted considerable attention in recent years for the production of bioethanol from agricultural wastes owing to its higher tolerance to both ethanol and inhibitors present in hydrolysates of lignocellulosic materials (Bettiga et al., 2009). Its potential to achieve simultaneous saccharification and fermentation (SSF) at high rates and high ethanol yields via enzymatic hydrolysis coupled with yeast fermentation in the same vessel has been discussed in many previous publications (Bertilsson et al., 2009; van Zyl et al., 2011). One of the problems associated with the SSF process is the different optimum temperatures for the enzymatic hydrolysis of cellulose (around 50 °C) and the ethanol fermentation of released sugars by yeast (30 to 35 °C). The fermentation efficiency of *S. cerevisiae* at high temperatures (>35 °C) is very low owing to an increase in fluidity in membranes, to which the yeast responds by changing its fatty acid composition (Suutari et al., 1990). The hydrolysis rate of cellulose in SSF process is slower at lower temperature due to the higher (45–50 °C) optimum temperature of commercial cellulases. Therefore, use of thermotolerant yeast strains to conduct SSF at temperatures closer to optimum values for commercial cellulases can be a solution to achieve higher ethanol production with faster cellulose hydrolysis rates and shorter SSF times (Zhao and Bai, 2009). Despite the large number of studies that have been performed on bioethanol production at high temperature, the theme continues to be of interest through the breeding and introduction of new yeast strains with resistance to elevated temperatures and increased performance in fermentation (Abdel-banat et al., 2010; Edgardo et al., 2008; Marullo et al., 2009).

Acquisition of thermotolerance is largely controlled through the activation and regulation of specific stress-related genes involved in the synthesis of specific compounds that protect the organism from high-temperature stress (Edgardo et al., 2008). Elucidation of the function of these genes and/or proteins will give insight into the various mechanisms underlying yeast response to high-temperature stress, providing useful information to improve bioethanol production at higher temperature. For that purpose, the isolation and characterization of new yeast strains from nature, capable of growing at high temperature with high ethanol yields during fermentation in specific conditions, continues to be of great interest from a practical point of view. A high-temperature growth phenotype (Htg$^+$) was recently categorized as a quantitative

Abbreviations: Htg$^+$, thermotolerant phenotype; Htg$^-$, thermosensitive phenotype (<41 °C); *RSP5-C*, *RSP5* allele in thermotolerant strain; QTL, quantitative trait loci; Ub, ubiquitin; E3, Ub-ligating enzyme; EMS, ethane methyl sulfonate.

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trait that is controlled by multiple genes (Steinmetz et al., 2002). Although some studies have focused on the role of a small class of candidate genes in some aspects of resistance to thermal stress and the great power of QTL mapping (Marullo et al., 2009; Sinha et al., 2006; Steinmetz et al., 2002), this approach has not been performed to genetically dissect any aspect of thermotolerance as yet.

High-temperature stress causes multiple changes in the cell that ultimately affect protein structures and function, generate abnormal proteins, and lead to growth inhibition or cell death (Goldberg, 2003). These denatured and aggregated proteins are mainly degraded via the proteasome pathway as a defense strategy to ensure survival (Haas, 2010). Ubiquitination is the primary signal used to target cellular proteins for destruction by 26S proteasomes. Ubiquitin is induced by diverse types of stresses in reflection of the need for more extensive protein turnover in stressed cells (French et al., 2009), and also it is an important non-proteolytic signal that regulates protein function by non-degradative mechanisms, including modulating protein–protein interactions in numerous biological pathways (Huang and D'Andrea, 2006). Ubiquitination plays a main regulatory role in most eukaryotic cellular processes such as receptor endocytosis, intracellular signaling, cell-cycle control, transcription, DNA repair, gene silencing, and stress response (Kaliszewski and Zoładek, 2008; Kwapisz et al., 2005). This process evolved as a thiol-ester cascade of reactions catalyzed by three enzymes consisting of ubiquitin-activating enzyme (E1), ubiquitin conjugating enzyme (E2), and a ubiquitin ligase (E3) that produces a Ub-modified protein. Although the attachment of a single ubiquitin moiety can occur, in general this reaction occurs repeatedly, leading to the attachment of Ub to several internal lysines of ubiquitin and/or proximal lysine residues on the substrate. E3 enzymes contain the primary determinants for substrate recognition. In *S. cerevisiae*, *RSP5* encoding an essential E3 ubiquitin ligase has a key role in regulating the trafficking, sorting, and eventual degradation of a large number of proteins in multiple cellular compartments (Krsmanović and Kölling, 2004). Moreover, it was recently assigned to other cellular events such as modifying gene expression, DNA repair, and RNA transport (Cardona et al., 2009). However, one important question still remains: how does E3 ubiquitin ligase regulate ubiquitination under stress conditions? Although recent studies have uncovered the molecular mechanisms underlying proteasome or vacuolar proteolysis pathways, as of yet this question cannot fully be answered.

In the previous studies, we found that a thermotolerant *S. cerevisiae* strain, C3723 isolated in Thailand, exhibited confluent growth at 41 °C (Htg^+ phenotype). Results of classical genetic analysis suggested that the Htg phenotype is dominant and approximately six genes, designated *HTG1* to *HTG6*, are responsible for conferring this phenotype. *htg1*, *htg2*, *htg4* and *htg6* host strains were successfully constructed and finally we selected HE120-12A strain (*htg6*) which exhibited clear Htg^- phenotype compared to the other *htg* host strains to study *HTG6* in this work. *CDC19* encoding pyruvate kinase has already been identified as *HTG2* (Benjaphokee et al., 2011). The objective of this work is to further elucidate the molecular mechanisms underlying the thermotolerance of this strain, which will contribute to the breeding of robust stress-tolerant strains with properties tailored for efficient ethanol fermentation at temperatures above 40 °C. Here, we cloned the *HTG6* gene which is identical to *RSP5*, and characterized a new thermotolerant allele, *RSP5-C*, that has higher transcription levels, consequently leading to an increased ubiquitination of proteins. In addition, we found that overexpression of the *RSP5-C* allele confers a more robust Htg^+ phenotype on the Htg^+ strain and therefore can be considered as a convenient way to develop thermotolerance in yeast strains.

2. Material and methods

2.1. Media, strains and plasmids

Standard methods for growth, maintenance, and transformation of yeast and bacteria for the manipulation of DNA were used throughout (Sherman, 1991). All yeast *S. cerevisiae* strains and plasmids used in this study are listed in Table 1.

Yeast strains were propagated in YPDA nutrient medium (2% glucose, 1% Bacto yeast extract, 2% Bacto peptone [Difco Laboratories, Detroit, MI] and 0.02% adenine [Wako Pure chemical Industries, Osaka, Japan]), YPGal media containing 2% galactose instead of glucose in YPDA, or synthetic dextrose medium (SD) consisting of 2% glucose, 0.67% Bacto yeast nitrogen base containing ammonia (Difco Laboratories, Detroit, MI), supplemented with appropriated amino

Table 1
List of *S. cerevisiae* strains and plasmids used in this study.

	Description	Source/reference
S. cerevisiae strain		
C3723	Wild type (Htg^+)	Isolated from long-gong fruits, Thailand
C3723-8B	Wild type (Htg^+)	Meiotic segregant of C3723
BY4741	*MAT***a** *met15Δ0 his3Δ1 leu2Δ0 ura3Δ0* (Htg^-)	Brachmann et al. (1998)
BY4742	*MATα lys2Δ0 his3Δ1 leu2Δ0 ura3Δ0* (Htg^-)	Brachmann et al. (1998)
HB8-3Aα	*MATα his3Δ1 ura3Δ0 leu2Δ0 HTG1HTG2HTG3 HTG4 HTG5 HTG6* (Htg^+)	This work
HB8-3A	*MAT***a** *his3Δ1 ura3Δ0 leu2Δ0 HTG1HTG2HTG3 HTG4 HTG5 HTG6* (Htg^+)	This work
HC1-5D	*MAT***a** *His3Δ1 ura3Δ0 leu2Δ0 htg1 HTG2 HTG3 HTG4 HTG5 HTG6* (Htg^-)	This work
HE6-8D	*MAT***a** *His3Δ1 ura3Δ0 leu2Δ0 HTG1 htg2 HTG3 HTG4 HTG5 HTG6* (Htg^-)	This work
HE1-10D	*MAT***a** *His3Δ1 ura3Δ0 leu2Δ0 HTG1 HTG2 HTG3 htg4 HTG5 HTG6* (Htg^-)	This work
TM2-8D	*MAT***a** *His3Δ1 ura3Δ0 leu2Δ0 HTG1 HTG2 HTG3 HTG4 htg5 HTG6* (Htg^-)	This work
HE120-12A	*MAT***a** *His3Δ1 ura3Δ0 leu2Δ0 HTG1 HTG2 HTG3 HTG4 HTG5 htg6* (Htg^-)	This work
HE120-4B	*MATα His3Δ1 ura3Δ0 leu2Δ0 HTG1 HTG2 HTG3 HTG4 HTG5 htg6* (Htg^-)	This work
Plasmid		
pH2720	YCp50 harboring a 4.7-kb fragment containing the *RSP5-C* gene and small parts of *DSE1* and *NSA2*.	This work
pRSP5-C	YCp50+ *RSP5-C*	This work
pRSP5-BY	YCp50+ *RSP5-BY*	This work
BYP5166	YCp50 + HO	NBRP-Yeast, Japan[a]
BYP555	pRS303 (*HIS3* YIp type)	NBRP-Yeast, Japan
BYP353	$G418^R$;*TRP1*; Amp^R	NBRP-Yeast, Japan
pYES2	Galactose-inducible yeast expression vector; p*GAL*; *URA3* Amp^R	Invitrogen, CA
pOVRSP5-B	*RSP5-BY* overexpression vector; p*GAL*; *URA3* Amp^R	This work
pOVRSP5-C	*RSP5-C* overexpression vector; p*GAL*; *URA3* Amp^R	This work

[a] National Bio-Resource Project-Yeast, Japan: http://yeast.lab.nig.ac.jp/nig/index_en.html.

acids if needed. For tetrad analysis, sporulation medium composed of 1% potassium acetate was used. To make solid agar medium, 2% agar was added into each liquid medium. *Escherichia coli* DH5α was used for plasmid construction, and the *E. coli* recombinant strains were grown in Luria-Bertani (LB) complete medium containing 100 μg/ml ampicillin. Sequencing was performed by the dideoxy chain termination method with a BigDye Terminator ver3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) and the ABI PRISM 310NT genetic analyzer (Applied Biosystems).

To construct heterothallic Htg^+ strain HB8-3A, HG12-1B, an Htg^+ heterothallic segregant from a diploid formed between BY4742 (Htg^-) and C3723-8A (Htg^+ meiotic segregant from C3723), was crossed with BY4741(Htg^-). HG15-4C, heterothallic segregant of resultant diploid, was transformed with a plasmid BYP353, and the resultant transformant was crossed with a spore of C3723-8B (a meiotic segregant from C3723) using direct spore-to-cell mating. The repeated back-cross between Htg^+ heterothallic segregants carrying BYP353 from this hybrid and spores of C3723-8B was performed three times until HB8-3A strain was obtained that showed growth ability at 41 °C to the same extent as C3723-8B. Besides, a strain HB8-3Aα with the opposite mating type to HB8-3A was subsequently constructed by transforming HB8-3A with plasmid BYP5166 harboring the *HO* gene encoding restriction endonuclease for mating-type switching (Benjaphokee et al., 2011).

We constructed strain HE120-12A (Htg^-) carrying only one *htg* gene by five times back-crossing Htg^- segregants from diploids of HB8-3A (Htg^+) and BY4742 (Htg^-) with HB8-3A (Htg^+) or HB8-3Aα (Htg^+). A single gene difference between HE120-12A and HB8-3Aα was detected by a 2+:2− segregation pattern of the Htg phenotype at 41 °C in tetrads of diploids resulting from crosses. A complementation test to confirm that *htg6* is distinct from other *htg* genes was done by crossing HE120-12A to other Htg^- strains constructed in our laboratory (Benjaphokee et al., 2011). In addition, a recombination test was done by tetrad analysis of the resultant hybrids and finally the gene was designated as *htg6*.

Plasmid pH2720 was digested by *Hin*dIII to remove a fragment containing *NSA2* and re-ligated to construct plasmid pH2720-10. Plasmid pH2720-12, which contains *DSE1*, was made by digesting pH2720 with *Bam*HI and removing the fragment surrounding *NSA2* and part of *RSP5*. A fragment containing *DSE1* was removed from plasmid pH2720 by *Eco*RV digestion to produce pH2720-14. These plasmids were introduced into strain HE120-12A to find the gene responsible for the Htg^+ phenotype.

Plasmids pRSP5-C and pRSP5-BY, harboring the *RSP5-C* and *RSP5-BY* alleles, respectively, were constructed by first amplifying a 3.95-kbp DNA fragment containing 1000 bp upstream and 520 bp downstream of the *RSP5* gene using the primers listed in Table 2 and genomic DNA from strain HB8-3A (Htg^+) and HE120-12A (Htg^-), respectively, and then cloning the amplified fragment into the *Nru*I gap of the YCp50 shuttle vector.

2.2. Construction of a C3723-8B genomic DNA library

Yeast genomic DNA from strain C3723-8B was prepared as described by Burke et al. (2000). Total genomic DNA isolated from C3723-8B was mechanically random-sheared and separated by agarose gel electrophoresis. After agarose gel purification, DNA fragments longer than 5 kb were treated with T4 DNA polymerase to convert both ends into blunt ends. These DNA fragments were ligated with BAP-treated YCp50, which had been digested by *Nru*I restriction enzyme. After ligation, the DNA mixture was introduced into electrocompetent *E. coli* DH10B cells (Invitrogen, Carlsbad, CA) by electroporation with GenePulser (Bio-Rad Laboratories Inc.). Ampicillin-resistant transformants were selected on solid LB medium containing 100 μg/ml ampicillin. About 10,000 clones were scraped up and pools of plasmid DNAs were prepared.

2.3. Screening for Htg^+ transformants from the C3723-8B genomic DNA library

Strain HE120-12A (Htg^-), which harbors the *htg6* gene, was used as a host strain to clone *HTG6*. After transformation with the C3723-8B (Htg^+) genomic DNA library, cells were spread on SD plates supplemented with the required amino acids except for uracil and plates were incubated at 30 °C for 4 days. After colonies had formed, the Ura^+ colonies were replica-plated onto SD plates and incubated at 41 °C for 48 h. Finally, cosegregation of the Ura^+ phenotype and the Htg^+ phenotype was tested to confirm selected Htg^+ candidates.

2.4. Integration mapping of RSP5-C

To construct plasmid pRS303 harboring the *RSP5-C* allele, a 4.89-kbp DNA fragment containing 1200 bp upstream and 757 bp downstream regions of the *RSP5* locus from plasmid pH2720 was digested by *Cla*I and inserted into the *Cla*I site of pRS303. The resultant plasmid, designated pRS303 [*RSP5-C*], was linearized by *Nru*I digestion and introduced by transformation into HE120-12A (Htg^-) and HB8-3A (Htg^+). Proper integration was confirmed in both cases by Southern blot analysis and PCR using the genomic DNA of several His^+ transformants, and finally transformants with correct integration were selected.

2.5. Construction of a chimeric allele of RSP5

RSP5 single-allele replacement experiments were conducted in HE120-12A (*MAT*a *his3Δ1 ura3Δ0 leu2Δ0 htg6*). Different parts of the thermotolerant and wild-type *RSP5* alleles were amplified by PCR using

Table 2
List of primers.

Primer name	Sequence (5′ -3′)	Description
RSP5-A	F:GTTAGAGCTACAAGAGTCGA R:GAGGGATTGTCTAGTACGCT	Primers to amplify the *RSP5* allele plus the 1000 bp upstream and 520 bpdownstream regions, respectively.
RSP5-R1	GAGGGATTGTCTAGTACGCT	Reverse primer to amplify the *RSP5* allele plus the bp downstream region
ChRsp5-F1	CCCTTTCGTCTTCAAGAATTCGCACCCACCCAGCTATGTCG	Forward primer to amplify the regulatory region for *RSP5*-chimeric I and II (*Eco*RI site underlined)
ChRsp5-R1	CAGAACAGCAAACGGATCCGGGGAA	Reverse primer to amplify the regulatory region of *RSP5*-chimeric I and II
ChRsp5-F2	ACGTATTCCGTTCCCCGGATCCGTT	Forward primer to amplify the ORF and terminator domain of *RSP5*
ChRsp5-R2	TAAACTACCGCATTAAAGCTTCGTTTCGGAATTGGGGATGG	Reverse primer to amplify the ORF and terminator domain for *RSP5*-chimeric I and II and only the terminator domain for *RSP5* chimeric IV (*Hind*III site underlined)
ChRsp5-F3	CGGTGGTATTGCGGAAATTGACATT	Forward primer to amplify a fragment containing the mutation in ORF for *RSP5*-chimericIII
ChRsp5-R3	GAGGCAGAAAGAATGTTGGGAAAAC	Reverse primer to amplify a fragment containing the base changes in the ORF for *RSP5*-chimericIII
ChRsp5-F4	TATGGTGTCCGTTTTCCCAACATTC	Forward primer to amplify the terminator region for *RSP5*-chimeric III and IV
ChRsp5-R4	TCCAATCTTCAATGTCAATTTCCGC	Reverse primer to amplify a fragment containing the promoter and 2090 bp of the *RSP5* open reading frame to make chimeric III
RSP5-OV	F: AGGGAATATTAAGCTTCCCTTTTCTTTGTTAGCTTGGG R: TAGATGCATGCTCGAGCCTCCTCTTATGGCGAACTTG	Primers to amplify *RSP5* for overexpression in plasmid pYES2 (*Hind*III and *Xho*I sites underlined in forward and reverse primer, respectively)

the genomic DNA of strain HB8-3A (Htg$^+$) and HE120-12A (Htg$^-$) and cloned into the *Eco*RI and *Hin*dIII restriction site of the YCp50 shuttle vector by using PCR Cloning, In-Fusion™ Advantage Kits (Clontech, Mountain View, CA). All PCR primers and the conditions for PCR amplification are represented in Table 2. The position of primers, as well as the amplified fragments that were used to make all chimeric genes, is shown in Fig. 5B. A control replacement with the respective *RSP5* allele from BY4742 was performed in parallel to rule out background mutations during the transformation process. Two independent transformants for each single replacement were analyzed and found to behave similarly. The allele replacements were verified by sequencing. Chimeric alleles were transformed into strain HE120-12A (Htg$^-$) and the high-temperature growth performance of the resulting transformants was examined at 41 °C in YPDA liquid medium. Samples were collected at 2-hour intervals for 24 h.

2.6. Stress sensitivity assays

Yeast cells were cultured in YPDA or SD (uracil omission) medium under normal growth conditions to an OD_{660} of 1, and then suspensions containing equal cell numbers were prepared on the basis of the OD_{660} value. Ten-fold serial dilutions were spotted onto plates and exposed to different stresses: cold (3 days at 13 °C) and high (2 days at 41 °C) temperature; heat shock (5 min at 55 °C); ethanol (12%vol/vol for 2 days at 30 °C); drug (Congo red at a final concentration of 100 μg/ml for 2 days at 30 °C); oxidative stress ($CdSO_4$ at a final concentration of 200 μM for 2 days at 30 °C); osmotic stress (NaCl at a final concentration of 500 mM for 1 day at 30 °C); and DNA damage stress (EMS at a final concentration of 0.08% and hydroxyurea at a final concentration of 0.3 M for 3 days at 30 °C).

2.7. Gene expression analysis

After the yeast culture had been continuously grown in SD (uracil omission) medium at 30 °C (OD_{660} = 0.8–1.0) or shifted from 30 °C to 41 °C for 1.5 h, total RNA was isolated by the hot-acid phenol method (Spellman et al., 1998) with modifications. Two micrograms of total RNA was reverse-transcribed by using a High-Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA) in accordance with the manufacturer's guidelines and used as a template for quantitative analysis

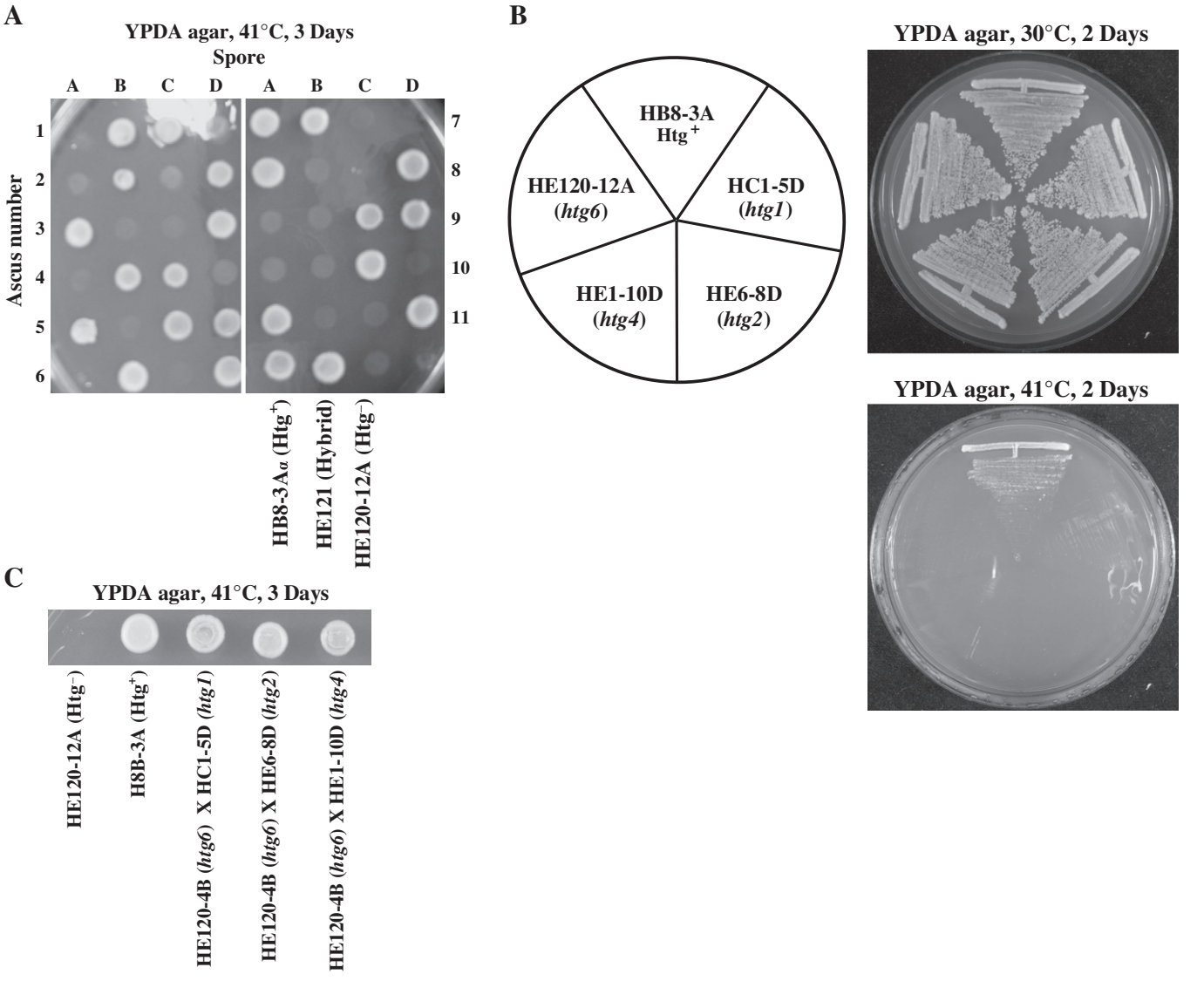

Fig. 1. Construction of the *HTG6* host strain. A) Tetrad analysis of HE121, the hybrid formed between HB8-3Aα (Htg$^+$) and HE120-12A, the *htg6* host strain (Htg$^-$), showed that the *htg6* host strain gave rise to 2+:2− segregation of the Htg$^+$/Htg$^-$ phenotype at 41 °C. A–D represent single spores derived from the same ascus. B) Growth phenotype of the *htg6* host strain in comparison to other host strains. The cells were streaked on YPDA agar plate. The plates were photographed after incubation for 48 h at 30 °C and 41 °C. C) Result of the complementation test of the Htg phenotype for pairs of hybrids formed between *htg6* and other *htg* allele-harboring strains.

of mRNA content by real-time (RT)-PCR in a 7300 Real Time PCR system (Applied Biosystems). *PDA1* encoding the α-subunit of pyruvate dehydrogenase was used as an internal standard for evaluating yeast mRNA in the RT-PCR assay (Dell Aguila et al., 2003).

2.8. Overexpression of RSP5

To construct plasmids for overexpression of *RSP5-BY* and *RSP5-C* alleles (pOVRSP5-B and pOVRSP5-C), a fragment containing the *RSP5* open reading frame and its terminator sequence was amplified by PCR using Takara Ex Taq, the primers listed in Table 2, and genomic DNA from strains HE120-12A (Htg^-) and HB8-3A (Htg^+), respectively, as a template, and the amplified fragment was cloned into the *Hin*dIII-*Eco*RI restriction site under the *GAL*1 promoter of the pYES2 vector (Invitrogen, Carlsbad, CA) by using PCR Cloning, In-Fusion™ Advantage Kits (Clontech, Mountain View, CA). HE120-12A (Htg^-) and HB8-3A (Htg^+) strains were transformed with the *RSP5-BY* and *RSP5-C* overexpression plasmids, which have *ura3* as an auxotrophic marker and a galactose-inducible promoter. The pYES2 vector was used as a control. After transformation, cells were spread on SD plates supplemented with the required amino acids except for uracil and plates were incubated at 30 °C for 4 days. Resultant Ura^+ transformants were examined for growth at 41 °C, 42 °C and 43 °C for 48 h on YPGal plates containing galactose to induce expression of the *RSP5* gene. YPDA medium was used as a control. Moreover, the growth behavior of the HB8-3A (Htg^+) transformant harboring the *RSP5-C* overexpression plasmid at 43 °C was assessed for 45 h in both YPGal and YPDA liquid media.

2.9. Western blot analysis

A 5-ml culture of yeast was grown either continuously at 30 °C (OD_{660} = 0.8–1.0) or shifted from 30 °C to 41 °C for 2 h in YPDA medium. Cells were collected by centrifugation, washed and then treated with 20% trichloroacetic acid (vol/vol). Next, cell pellets were suspended in 20% trichloroacetic acid (vol/vol) and disrupted with glass beads. The whole-cell protein extract was cleared by centrifugation. The crude extract samples (5 μg) were mixed with equal amounts of sample buffer, incubated at 95 °C for 5 min, and resolved by electrophoresis through 12% polyacrylamide–sodium dodecyl sulfate (SDS) gels. The concentration of purified protein was estimated on gels stained with Coomassie brilliant blue (using BSA as a standard). For western blot analysis, 3 μg of solubilized protein was loaded on a 12% SDS polyacrylamide gel by Laemmli's system and transferred to Millipore membrane filters. The filters were incubated with 1% skim milk in TBST buffer (10 mM Tris–HCl [pH 8.0], 0.15 M NaCl, and 0.05% Tween 20 [Bio Rad Laboratories]) for blocking overnight at 4 °C, washed three times with 10 ml of TBST buffer for 5 min each, and incubated with anti-ubiquitin monocolonal antibody (Santa Cruz Biotechnology; P4D1) at a 1:1000 dilution. Next, the membranes were washed three times with 10 ml of TBST buffer for 5 min each, and incubated with 10 ml of TBST buffer containing 5 μl of anti-mouse IgG peroxidase-linked secondary antibody. After three washes with 10 ml of TBST buffer for 5 min each, ubiquitinated proteins were detected using ECL, Western lightning™ Chemiluminescence (Perkin Elmer LAS, Inc). Ubiquitinated proteins were quantified by measuring the density of the band using a bio-imaging system (ChemiStage CC-16 mini, Kurabo, Osaka, Japan).

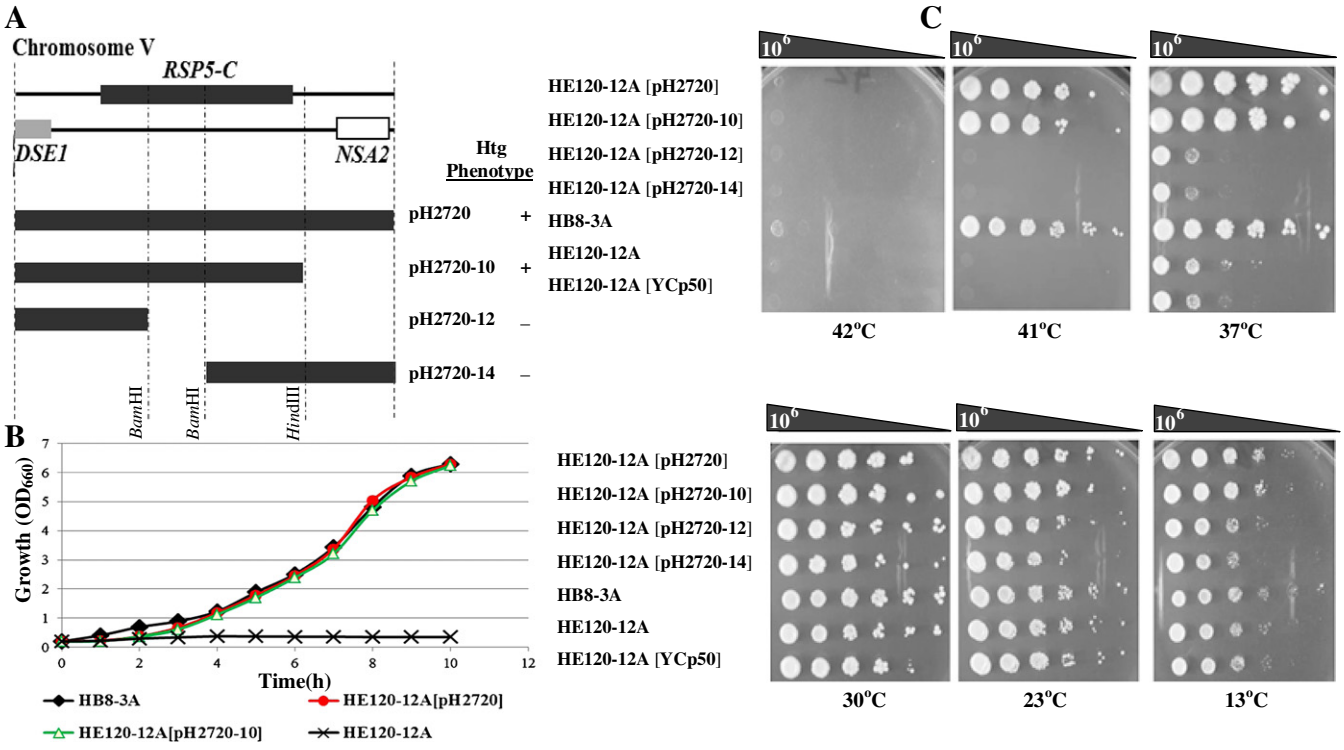

Fig. 2. Delimitation of the gene responsible for the Htg^+ phenotype. A) Sequence analysis revealed that plasmid pH2720 harboring a DNA fragment containing the *RSP5* gene and small parts of *DSE1* and *NSA2* could recover the Htg^+ phenotype in strain HE120-12A. Plasmid pH2720 was digested with *Hin*dIII, *Bam*HI and *Eco*RV to construct pH2720-10, pH2720-12 and pH2720-14 plasmids, which contain *RSP5*, part of *DSE1*, and part of *NSA2*, respectively. These plasmids were introduced into strain HE120-12A to find the gene responsible for the Htg^+ phenotype. B) Growth rate of HB8-3A (Htg^+), HE120-12A (Htg^-) and transformants containing plasmid pH2720 or pH2720-10. Growth at 41 °C in liquid YPDA culture, starting with an initial OD_{660} of 0.2, was examined by monitoring the OD_{660} at indicated times. C) Growth phenotype of HB8-3A (Htg^+), HE120-12A (Htg^-) and transformants containing plasmid pH2720, pH2720-10, pH2720-12, pH2720-14 or vector YCp50 at different temperatures.

3. Results

3.1. RSP5 is one of the genes responsible for thermotolerant phenotype and is identical to HTG6

Previously, we successfully constructed three Htg$^-$ host strains each harboring only one *htg* allele (designated as HC1-5D [*htg1*], HE6-8D [*htg2*] and HE1-10D [*htg4*]) by the repeated back-crossing of Htg$^-$ segregants from hybrids formed between HB8-3A (Htg$^+$) and BY4742 (Htg$^-$) to the HB8-3A (Htg$^+$) strain (Benjaphokee et al., 2011; unpublished data). The Htg$^-$ host strain HE120-12A, which has only one *htg* allele (naturally existing allele in Htg$^-$ strain) but an *HTG* allele for other five genes, was successfully constructed by the same method. As shown in Fig. 1A, HE120-12A gave a 2+:2− segregation pattern of the Htg phenotype at 41 °C when it was crossed with HB8-3Aα (Htg$^+$), demonstrating that these two strains genetically differed only in a single allele for the Htg phenotype. In addition, we found that this *HTG* allele is dominant owing to the Htg$^+$ phenotype of the hybrid. Growth performance of HE120-12A at normal and high temperature (41 °C) was comparable to other *htg* host strains, as shown in Fig. 1B. In addition, each of the four strains was crossed with one another to see whether they have different *htg* gene or not. Complementation of the Htg$^-$ phenotype in all pairs of hybrids formed between the *htg* allele-harboring strains (Fig. 1C), in addition to the irregular segregation pattern of the Htg phenotype at 41 °C in tetrads of these diploids (data not shown), revealed that the *htg* alleles in each Htg$^-$ strain were alleles of different genes. Finally, we designated this gene as *htg6* and HE120-12A was assigned as a host strain for cloning the *HTG6* gene.

Because the Htg$^+$ phenotype was dominant, we constructed an Htg$^+$ genome library from the C3723-8B strain to clone the *HTG6* gene (see Material and methods), and introduced it into strain HE120-12A (*his3Δ1 ura3Δ0 leu2Δ0 HTG1 HTG2 HTG3 HTG4 HTG5 htg6*) by transformation. Twenty-five Ura$^+$ transformants were screened for the Htg$^+$ phenotype, and eventually four transformants showing this phenotype were obtained. The plasmids in these transformants were recovered and the insert DNAs were subjected to sequence analysis. The result revealed that the cloned plasmid harboring a DNA fragment of 4.7 kb contains the *RSP5* gene and small parts of *DSE1* and *NSA2* (Fig. 2A). Delimitation of the gene responsible for the Htg$^+$ phenotype was conducted by constructing plasmids pH2720-10, pH2720-12 and pH2720-14, which contain one of 3 different segments in the cloned DNA fragment, and introducing them into the *htg6* host strain (HE120-12A) (Fig. 2A). Since we found that only the plasmid H2720-10 containing full length *RSP5-C* gene could confer the Htg$^+$ phenotype on the Htg$^-$ host strain HE120-12A, we conclude that *RSP5* was a candidate for the *HTG6* gene (Fig. 2B and C).

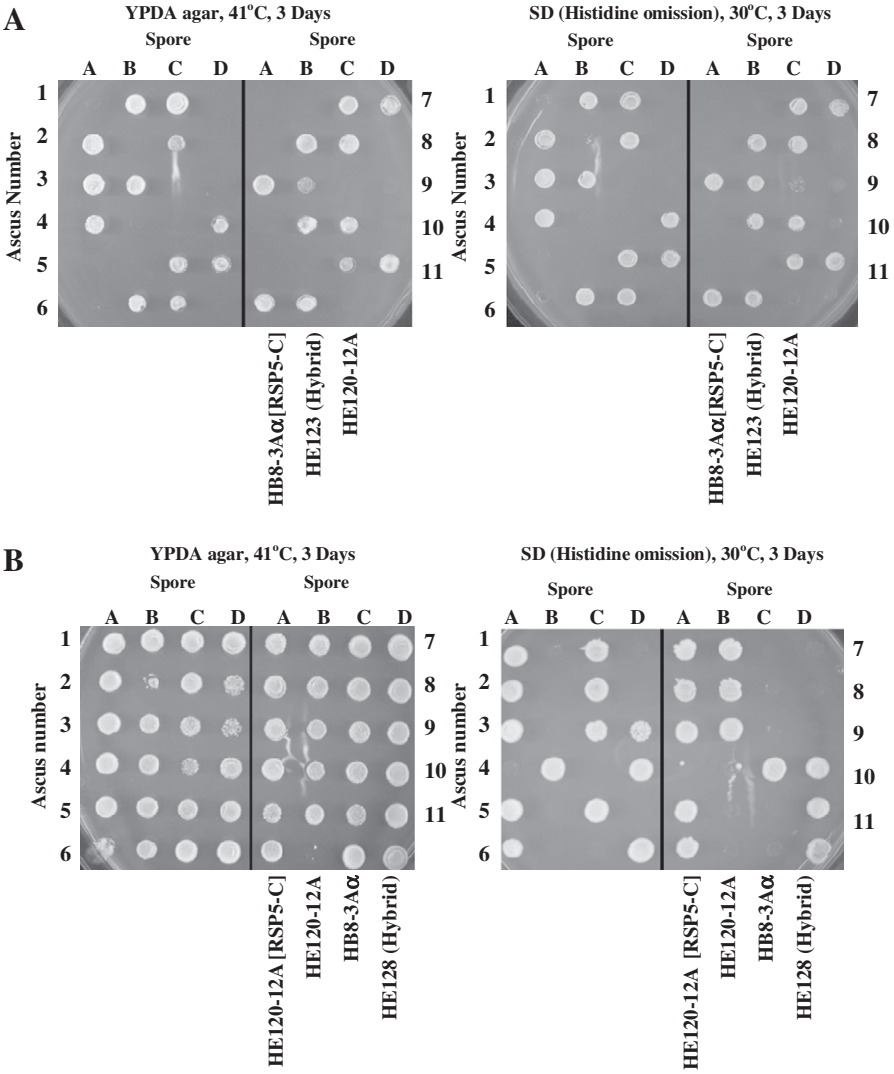

Fig. 3. Integration mapping of the *HTG6* gene. A) A hybrid of HB8-3Aα [*RSP5-C*](Htg$^+$) and HE120-12A(Htg$^-$) gave rise to a 2+:2− segregation pattern at the high temperature condition (41 °C). B) A hybrid of HE120-12A [*RSP5-C*] and HB8-3Aα (Htg$^+$) gave rise to 4+:0− segregation at the high temperature condition (41 °C).

A fragment containing 1000 bp upstream, which has been reported as a regulatory domain (Harbison et al., 2004), and 520 bp downstream of the open reading frame (ORF) of *RSP5* was amplified by using the genomic DNA of C3723-8B (Htg^+) and HE120-12A (Htg^-), and the *RSP5* allele contained in the amplified fragment was designated as *RSP5-C* and *RSP5-BY*, respectively. To verify whether *RSP5* is identical to *HTG6*, HB8-3A (Htg^+) was transformed with an integrative plasmid that had been linearized by *Nru*I digestion for homology-oriented integration and that harbored the Htg^+ *RSP5-C* allele marked with *HIS3*, and the His^+ transformant was crossed with HE120-12A (*htg6* host strain). The resulting diploid was subjected to tetrad analysis. As shown in Fig. 3A, the segregation pattern of the hybrid was 2+:2− on the Htg phenotype, and the Htg^+ phenotype co-segregated with the *HIS3* marker, indicating that the Htg^+ phenotype of the segregants was due to the integrated *RSP5-C* allele and that the integration event occurred at *HTG6* locus. For further confirmation, an integrative plasmid harboring the Htg^+ *RSP5-C* allele marked with *HIS3* was introduced into HE120-12A (*htg6* host strain), and the His^+ transformant was crossed with HB8-3A (Htg^+). Tetrad analysis of this hybrid showed a 4+:0− segregation pattern under the high-temperature condition (41 °C), but a 2+:2− segregation for auxotrophic markers (Fig. 3B). Taken together, these results revealed that *RSP5* is identical to the *HTG6* gene.

3.2. RSP5-C can protect the cell from DNA damage and ethanol, cell wall, osmotic, oxidative and heat stresses

To investigate the role of the *RSP5-C* allele, we tested several phenotypes of HE120-12A (*htg6* host strain) in the presence or absence of the *RSP5-C* allele. First, we examined the growth of strain HE120-12A containing either the YCp50-*RSP5-C* or YCp50 vector on SD (uracil omission) medium containing 100 μg/ml Congo red, a reagent that causes cell wall stress. Strain BY4742 containing the YCp50 vector was used as a control. Congo red mainly affects the stability of the cell-wall architecture and thereby increases the aggregation of cell membrane proteins (Shamrock et al., 2009). Whereas growth of the HE120-12A (*htg6* host strain, Htg^-) containing the YCp50 vector was inhibited by the presence of Congo red, transformant containing the *RSP5-C* allele grew even in the presence of this reagent (Fig. 4), suggesting that the *RSP5-C* allele plays a role in maintaining the stability of the cell wall structure. Resistance to Congo red in the presence of the *RSP5-C* allele in the Htg^+ strain might occur as a result of facilitating both the repair and the degradation of damaged cell-wall proteins (Goldberg, 2003). In addition, the specific interaction of Rsp5 and endoplasmic reticulum membrane proteins such as Rcr1 has been previously shown to protect the cell in response to Congo red (Imai et al., 2007), and therefore the *RSP5-C* allele might cause an increment in cell-wall stability by reducing the cell-wall chitin content through the ubiquitination of Rcr1 in the Htg^+ strain.

Protein conformations are labile, particularly at temperatures higher than 37 °C, and are easily damaged by highly reactive small molecules found in cells, especially oxygen radicals, which are continuously generated by intermediary metabolism (Goldberg, 2003). It is also reported that an enhanced ubiquitination occurs in oxidant-damaged proteins, especially newly synthesized proteins, during heat stress, followed by their proteasomal degradation (Medicherla and Goldberg, 2008). To elucidate the difference between the Htg^+ and Htg^- strains in the elimination of post-synthesis oxidant-damaged proteins as potentially toxic proteins, we studied the effects of exposure to $CdSO_4$, a reactive oxygen species generator in *S. cerevisiae*. As shown in Fig. 4, the BY4742 (Htg^-) and HE120-12A (Htg^-) strains containing the YCp50 vector both showed a sensitive phenotype to oxidative stress, whereas HE120-12A transformant harboring the *RSP5-C* allele (Htg^+) grew well in this condition.

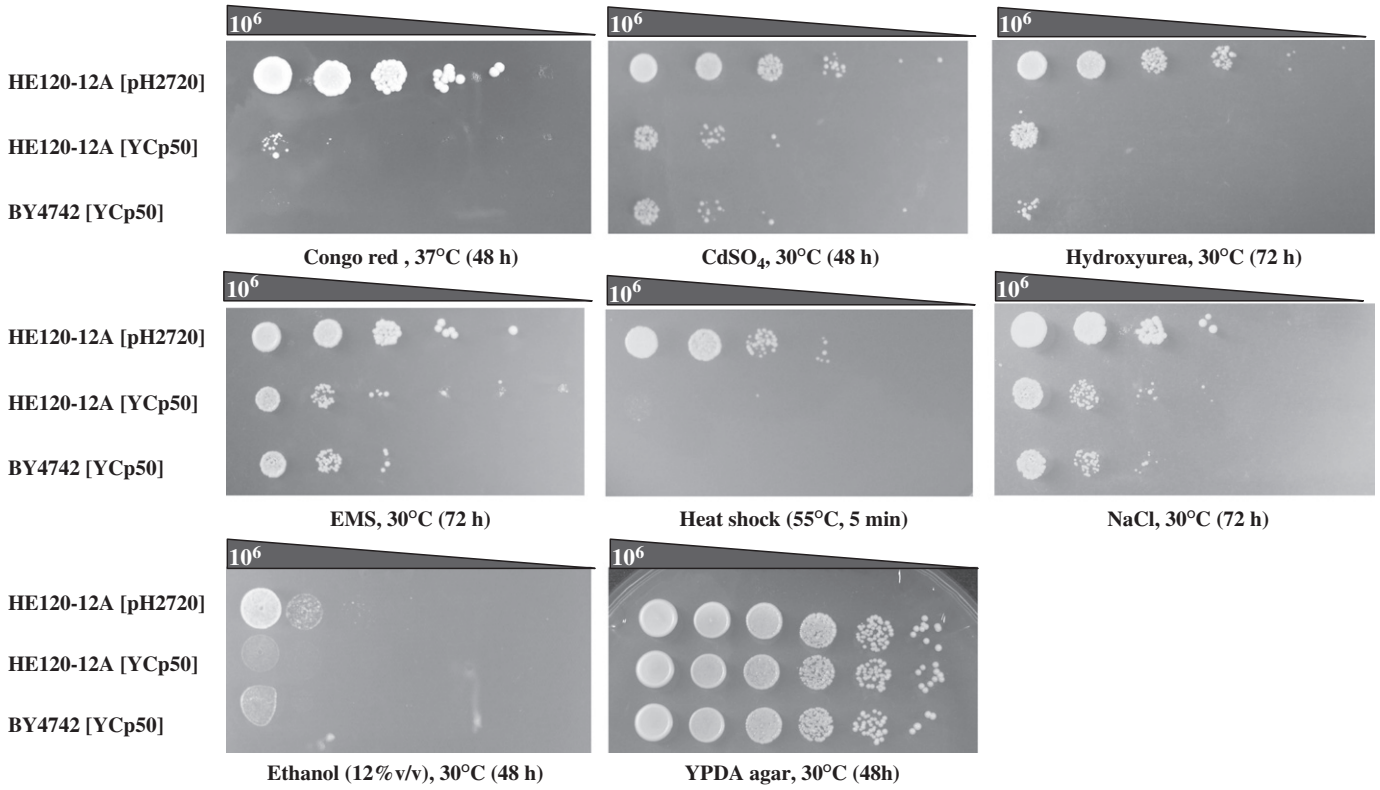

Fig. 4. Effect of different stress on the viability and growth of the Htg^+ and Htg^- strains. Exponentially growing yeast cultures of HE120-12A (Htg^-) and BY4742 (Htg^-) containing YCp50 or the *RSP5-C* allele (pH2720 vector) were spotted as a 10-fold serial dilution onto SD (uracil omission)-plates containing Congo red (100 μg/ml), hydroxyurea (0.3 M), CdSO4 (200 μM), EMS (0.08%), ethanol (12% vol/vol) and 0.5 M NaCl, and their viability was examined. In addition, their growth was assessed after exposure to heat shock at 55 °C (5 min), followed by incubation at 30 °C for 24 h. Transformant containing the *RSP5-C* allele (Htg^+) showed more tolerance to Congo red, DNA damage, oxidative stress and heat shock.

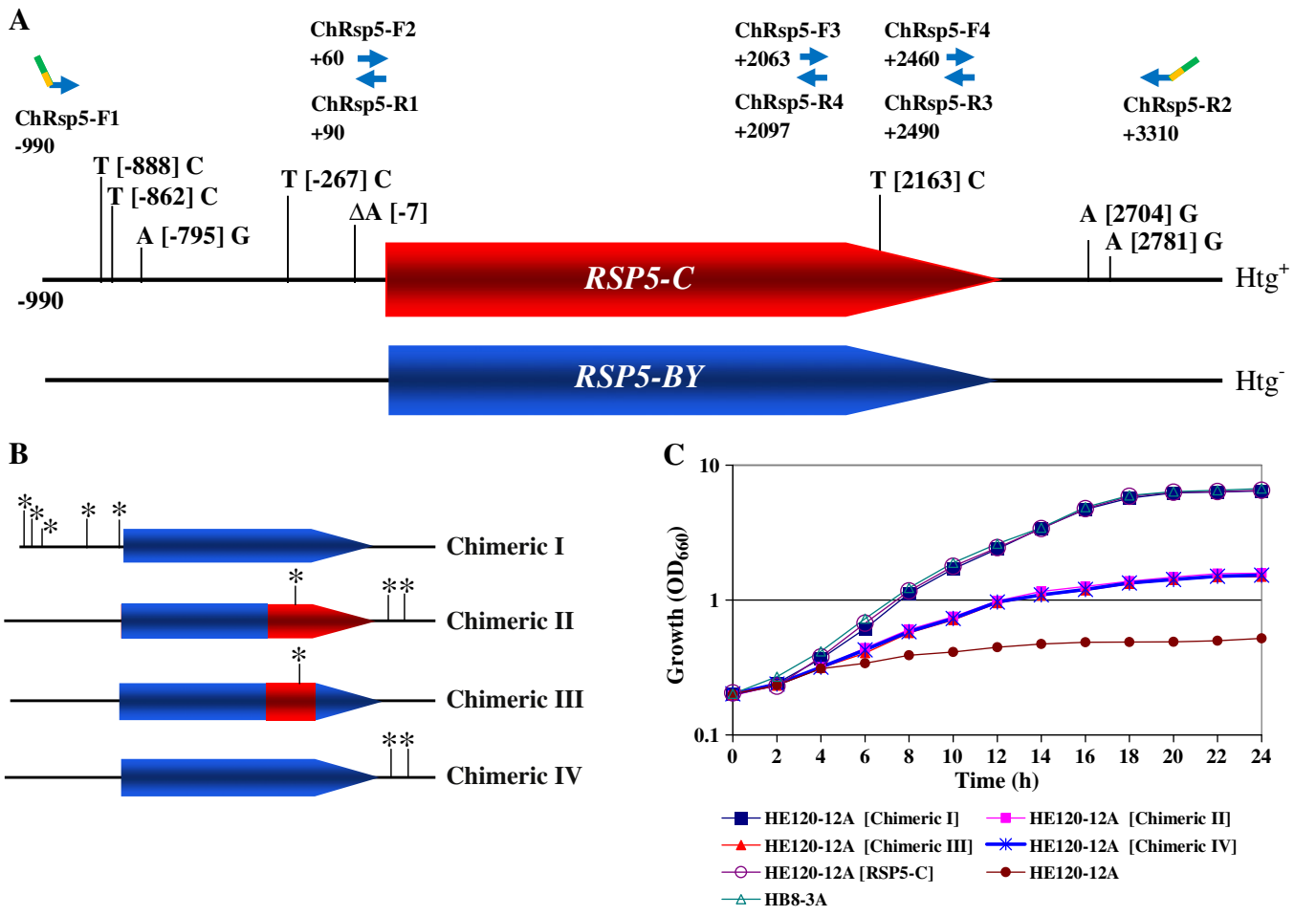

Fig. 5. Mutations located at the promoter region of *RSP5-C* affect the thermotolerance phenotype. A) The *RSP5-C* allele in the Htg$^+$ strain has five base changes in its promoter, one silent mutation in its coding sequence and two base changes in the terminator region. Arrows show the position of the primers that were used to amplify different parts of *RSP5*. B) Schematic representation of chimeric *RSP5* constructs. Relevant parts of the Htg$^-$ and Htg$^+$ *RSP5* alleles containing the base changes in *RSP5* were amplified by PCR using the genomic DNA of strains HB8-3A and HE120-12A, and cloned into the *Eco*RI-*Hin*dIII gap of the YCp50 vector. The black and gray rectangular bars represent the fragments originating from the Htg$^+$ and Htg$^-$ strains, respectively, that were used to make the chimeric genes. Asterisks demonstrate the positions of base changes originated from *RSP5-C* allele in Htg$^+$ strain that existed in each chimeric gene. C) Growth rate of the respective strains was examined at 41 °C in liquid YPDA culture, starting with an initial OD_{660} of 0.2, by monitoring OD_{660} at 2-hour intervals for 24 h.

Previous studies demonstrated that *RSP5* thermosensitive mutants (FW1808, YXW29, JYL01 and JYL04) showed no resistance to DNA damage stress (Jeong et al., 2005; Lu et al., 2008). In this study, we examined the cells for tolerance to hydroxyurea (a specific inhibitor of ribonucleotide reductase) and EMS, which induce the DNA damage response through inhibition of DNA synthesis and DNA alkylation, respectively (Fig. 4). Intriguingly, we found that HE120-12A transformant containing the *RSP5-C* allele (Htg$^+$) showed significant resistance to hydroxyurea, while *RSP5-C* allele did not make a significant difference in terms of resistance against EMS. This result indicated that the Htg$^+$ strain might have greater ability to repair DNA damage.

Increasing ethanol concentration during the fermentation process can induce protein misfolding in yeast cell. *RSP5* thermosensitive mutants show higher sensitivity to ethanol stress as compared with the wild-type strains (Hoshikawa et al., 2003). Here, we investigated the ethanol-stress response of HE120-12A transformant harboring the *RSP5-C* allele or YCp50 vector on medium containing 12% (vol/vol) ethanol. Fig. 4 shows that the *RSP5-C* allele (Htg$^+$) conferred a higher ethanol-tolerant phenotype, whereas strains BY4742 (Htg$^-$) and HE120-12A (Htg$^-$), which have an *RSP5-BY* allele, were less tolerant to ethanol. We concluded from these experiments that the Htg$^+$ strain can acclimatize more quickly to ethanol stress as compared with the Htg$^-$ strains.

On the one hand, the exposure of *S. cerevisiae* cells to high external osmolarities can change gene expression and consequently reduce viability, methionine uptake and protein biosynthesis (Mager and Siderius, 2002). On the other hand, it has been reported that osmosensitivity is reduced during growth at elevated temperatures (Wojda et al., 2003). We therefore, compared the osmostress response of transformants harboring *RSP5-C* and *RSP5-BY* in the presence of 0.5 M NaCl. The *RSP5-C* allele conferred a higher osmoresistant phenotype, whereas transformants harboring the *RSP5-BY* allele (Htg$^-$) showed more sensitivity to osmotic stress.

S. cerevisiae grows optimally at temperatures from 25 °C to 30 °C but cannot grow normally at temperatures higher than 40 °C. We evaluated growth behaviors of the Htg$^+$ and Htg$^-$ strains in the temperature range 13 °C to 42 °C. As shown in Fig. 2C, the HB8-3A (Htg$^+$) and HE120-12A (Htg$^-$) strains displayed the same growth behavior at the low temperatures of 13 °C and 23 °C. Although the Htg$^+$ and Htg$^-$ strains both can grow at 30 °C and 37 °C, HB8-3A (Htg$^+$) grew well at 41 °C, whereas the Htg$^-$ strains did not. However, HB8-3A showed very slow growth at 42 °C (Fig. 2C). Furthermore, to test whether the *RSP5-C* and *RSP5-BY* alleles respond to short exposure of high-temperatures (52–55 °C) stress, which would normally lead to rapid cell death, we compared the cell viability and growth of strains HE120-12A (*RSP5-BY*) [YCp50], HE120-12A (*RSP5-BY*) [YCp50 + *RSP5-C*], and BY4742 (*RSP5-BY*) [YCp50] after 5 min

exposure to 55 °C, followed by incubation at 30 °C. Fig. 4 shows that the *RSP5-C* allele has a significant role in the higher survival rate compared with the *RSP5-BY* allele in Htg$^-$ strains.

3.3. Base changes at the promoter region of the RSP5-C allele have a major effect on the thermotolerance phenotype

We compared the sequence of the *RSP5* allele in the HE120-12A (Htg$^-$) and BY4742 (Htg$^-$) lab strains with the cloned *RSP5-C* allele from C3723-8B (Htg$^+$). We found that in the Htg$^+$-*RSP5-C* allele there are five base changes located in the upstream region, one base change resulting in a silent mutation at the C terminus of the Rsp5 protein, and two base changes in its terminator region (Fig. 5A) while RSP5 allele in the HE120-12A (Htg$^-$) was same as that of BY4742 (Htg$^-$). We next investigated which base changes in the *RSP5-C* allele lead to the Htg$^+$ phenotype in the thermotolerant strain. To answer this question, we made four chimeric *RSP5* alleles containing either the base changes in the promoter region or the silent mutation at the open reading frame and the base changes at the terminator region, respectively (Fig. 5B), and compared the phenotype of these transformants harboring each of these alleles under the high-temperature condition (41 °C). The base changes located in the promoter region of *RSP5-C* had a major effect on the Htg$^+$ phenotype of the C3723-8B strain because only chimeric allele I gave a robust thermotolerant phenotype (Fig. 5C). Although chimeric allele I showed the same growth behavior as Htg$^+$ strains at high temperature (41 °C), transformants containing chimeric alleles II, III and IV also showed a faster growth rate than the host strain (HE120-12A) in liquid media at high temperature (Fig. 5C). This observation suggests that the other base changes may have a minor effect on Htg$^+$ phenotype through the mechanisms such as increased stability of an mRNA or translated efficiently. On the basis of these findings, we concluded that the five base changes present in the regulatory region of *RSP5-C* allele are mainly responsible for conferring the Htg$^+$ phenotype on strain HE120-12A.

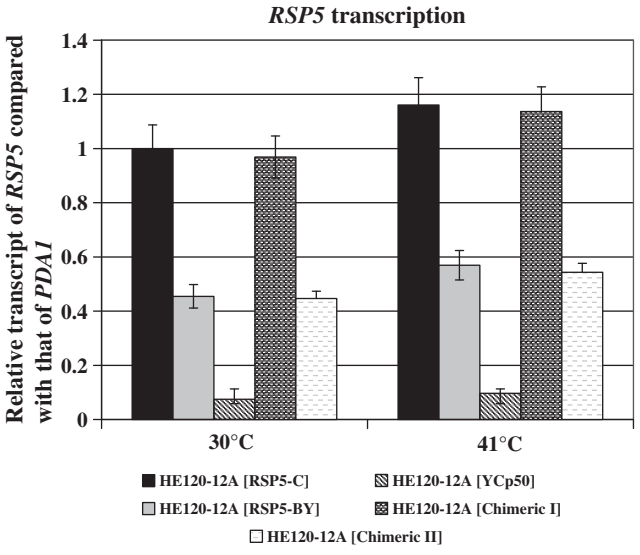

Fig. 6. Expression level of the *RSP5-C* transcript is higher than wild-type *RSP5*. *RSP5* gene transcription was assayed quantitatively by real time RT-PCR of RNA extracted from HE120-12A (Htg$^-$) transformants containing the *RSP5-C* allele, the *RSP5-BY* or chimeric *RSP5* alleles at normal (30 °C) and high (41 °C) temperatures. Strain HE120-12A containing the YCp50 vector was used as a control. *PDA1* was used as an internal standard. Total RNA was isolated after the yeast cells were continuously grown in SD (uracil omission) medium at 30 °C (OD_{660} = 0.8–1.0) or shifted from 30 °C to 41 °C for 1.5 h.

3.4. The RSP5-C allele has greater transcriptional activity than the RSP5-BY allele

To evaluate whether the base changes found in the *RSP5-C* allele cause differences in the levels of *RSP5* transcript, real-time RT-PCR was used to analyze mRNA levels in HE120-12A (*RSP5-BY*), HE120-12A (*RSP5-BY*) [*RSP5-BY*], and HE120-12A (*RSP5-BY*) [*RSP5-C*] (where the *RSP5* allele in the curved bracket represents the intrinsically present genomic allele of *RSP5*; and the *RSP5* allele in the square bracket represents the *RSP5* allele introduced by transformation) containing a single copy of *RSP5-BY*, a double copy of *RSP5-BY*, and a single copy each of *RSP5-BY* and *RSP5-C*, respectively (Fig. 6). At both normal (30 °C) and high temperatures (41 °C), the *RSP5-C* allele had higher transcript levels as compared with the *RSP5-BY* allele, suggesting that the base changes found in the *RSP5-C* allele are associated with an increase in the transcriptional activity of *RSP5*. Although overexpression of ubiquitin-related enzymes has been reported to enhance a cell's ability to grow at 39 °C by Hiraishi et al. (2006), here we found that a naturally-occurring *RSP5-C* allele with a higher *RSP5* transcription level confers resistance against higher temperature at 41 °C.

It has been reported that promoter architecture plays a major role in determining the level of transcription (Sanchez et al., 2011). To validate the hypothesis that base changes found in the promoter region of the *RSP5-C* allele are responsible for increasing the transcription of *RSP5* and the Htg$^+$ phenotype, we also conducted quantitative transcription analysis of two chimeric *RSP5* constructs by real-time RT-PCR. Chimeric I-*RSP5*, which contains only five base changes at the upstream region of *RSP5*, conferred a higher level of *RSP5* transcript, whereas chimeric II-*RSP5*, which contains the silent mutation at the open reading frame and the base changes in the terminator region, did not show any positive effect on the level of *RSP5-BY* transcription (Fig. 6). This finding confirmed that base changes present in the regulatory region of the *RSP5-C* allele have a major role in acquisition of the Htg$^+$ phenotype by increasing *RSP5* transcription.

3.5. A more robust Htg$^+$ phenotype can be achieved by overexpression of RSP5 in the Htg$^+$ strain

Based on the above results of the transcription of the *RSP5-C* allele, we hypothesized that overexpression of the wild-type *RSP5* allele by using a strong promoter might also confer the Htg$^+$ phenotype on HE120-12A (*htg6* host strain) as in the case of the *RSP5-C* allele. To verify this possibility, we overexpressed the wild-type *RSP5* allele in HE120-12A by using the galactose-inducible promoter *GAL1* on the multiple-copy plasmid pYES2, and examined the growth phenotype of the resultant transformants at high temperature (41 °C). The HE120-12A (Htg$^-$) strain harboring the overexpression plasmid for the wild-type *RSP5* allele (pOVRSP5-B) grew at 41 °C, whereas transformation with empty vector did not confer the thermotolerance phenotype in YPGal medium (Fig. 7A).

We also considered the possibility that overexpression of the *RSP5* gene might confer a more vigorous Htg$^+$ phenotype on the Htg$^+$ strain at higher temperatures. Strain HB8-3A (Htg$^+$) was transformed with a plasmid overexpressing the *RSP5-C* allele (pOVRSP5-C), and the growth phenotype of the resultant transformants was assessed in higher temperatures (42 °C to 43 °C) in the presence of glucose and galactose as a sole carbon source in the medium. Surprisingly, we found that overexpression of *RSP5-C* in the Htg$^+$ strain improved tolerance to higher temperature at 43 °C in YPGal medium, whereas growth of the Htg$^+$ transformant containing *RSP5-C* overexpression plasmid was inhibited in YPDA at 43 °C (Fig. **7** A and B).

3.6. High-temperature resistance correlates with ubiquitination status

Ubiquitin signals are recognized by a diverse set of ubiquitin-binding domains (UBDs) found within a variety of proteins that participate in

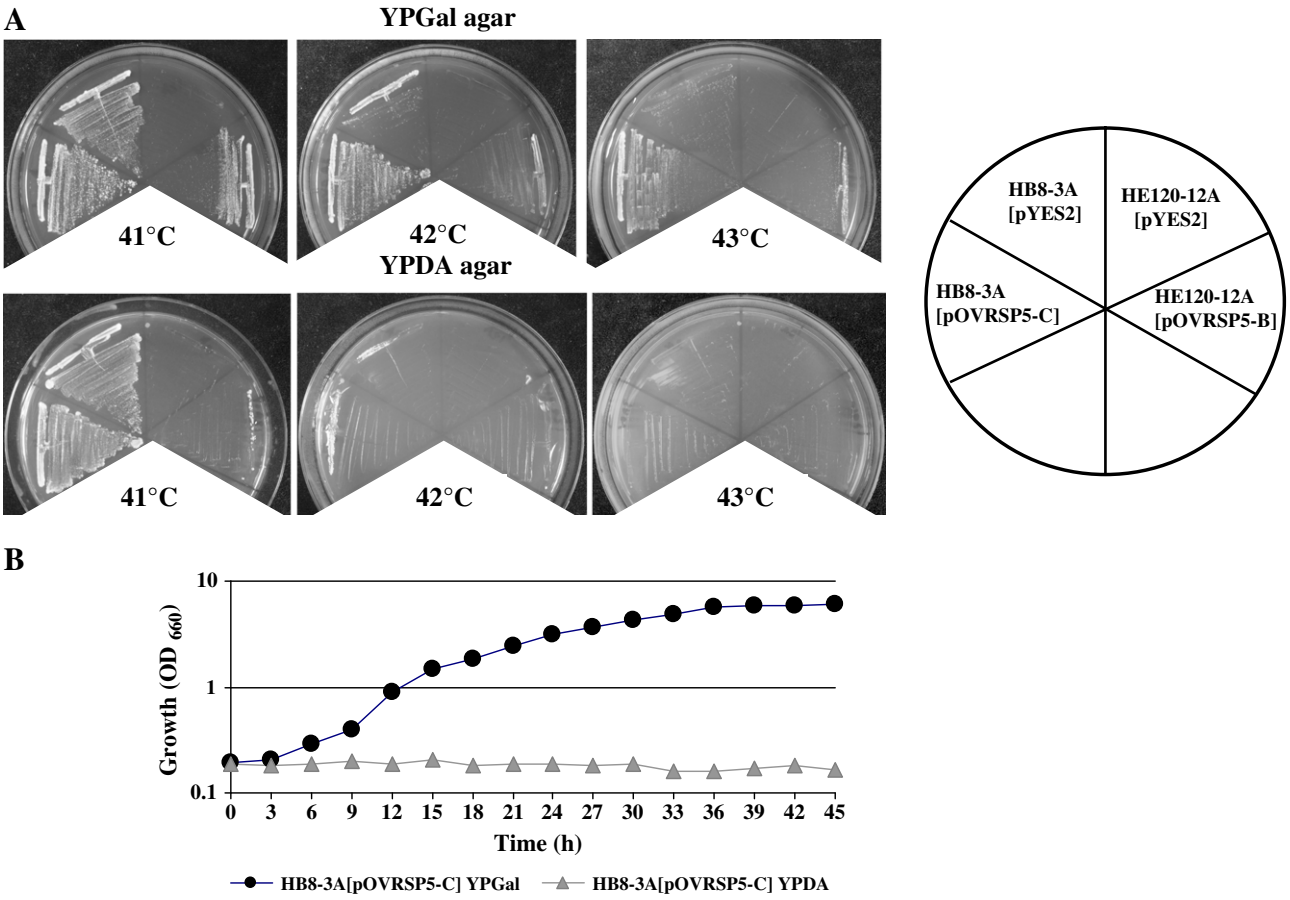

Fig. 7. Overexpression of *RSP5* can improve the Htg$^+$ phenotype. (A) Growth phenotype of HB8-3A (Htg$^+$) and HE120-12A (Htg$^-$) transformants containing plasmid overexpressing *RSP5-C* and *RSP5-BY* (pOVRSP5-C and pOVRSP5-B) under the high-temperature condition on YPGal and YPDA plates. The multiple-copy plasmid pYES2 was used as a control. Expression of the *RSP5* gene in transformant yeast cells was induced by galactose in the medium. The plates were photographed after incubation for 48 h. (B) The growth of HB8-3A (Htg$^+$) transformant containing the *RSP5-C* overexpression plasmid at 43 °C in liquid YPGal and YPDA, starting with an initial OD_{660} of 0.2, was examined by monitoring OD_{660} at indicated times.

numerous cellular functions. High temperature specifically accelerates the degradation of proteins especially 10% to 13% of newly synthesized proteins that at 30 °C would normally have become long-lived components (Medicherla and Goldberg, 2008). The above results showed that, as compared with Htg$^-$ strains, the *RSP5-C* allele results in increased transcription of the *RSP5* gene in the Htg$^+$ strain and may in turn lead to increased Rsp5 protein. We predicted that producing a greater amount of E3 ubiquitin ligase (Rsp5 protein) might lead to increased ubiquitination of *RSP5* substrates. To test this hypothesis, we determined the proportion of the total protein that was ubiquitinated for strains HB8-3A (Htg$^+$), HE120-12A (Htg$^-$) and BY4742 (Htg$^-$) by western blotting using anti-ubiquitin antibody under conditions of thermal stress (Fig. 8).

The total amount of ubiquitinated proteins in the HB8-3A (Htg$^+$) strain carrying the *RSP5-C* allele was significantly higher than that in the HE120-12A (Htg$^-$) and BY4742 (Htg$^-$) strains having a wild-type *RSP5* allele under the high-temperature condition (41 °C). Polyubiquitin linkages via lysine 48 (K48) or lysine 63 (K63) can differentially target damaged proteins for 26S proteasomal degradation or endosome trafficking to the lysosomes (French et al., 2009). As illustrated in Fig. 8, the intensity of high molecular weight ubiquitinated proteins was clearly greater in strain HB8-3A (Htg$^+$) than in Htg$^-$ strains. Thus, the *RSP5-C* allele has more ability to ubiquitinate heat-denatured proteins and facilitates their degradation or modifies cell membrane structure as compared with the wild-type *RSP5* allele.

4. Discussion

Thermo-tolerant yeast is a prerequisite for ethanol fermentation under high temperature conditions to facilitate the hydrolysis for the SSF process as well as to save the capital investment and operation cost of the cooling system.

Although previous studies reported that Htg$^+$ strains of *S. cerevisiae* were isolated from nature, or developed by protoplast fusion, evolutionary engineering, genome shuffling and mutagenesis, our knowledge about the genetic basis of the complex Htg trait is still unclear (Zhao and Bai, 2009). The impact of a new allele on an organism's fitness and thus its role in evolution is likely to depend on the high-temperature environment. It has been proposed that environmental stresses such as high temperature can induce random mutations in the genome of microorganisms such as *S. cerevisiae*, in a process called “adaptive evolution” that is central to the survival of robust strains (Galhardo et al., 2007). In the case of strain C3723, which was isolated from a tropical climate, high temperature might have acted as a trigger for such a mutation to enable the yeast to adapt to its high temperature niche.

Heat stress adversely affects organisms by causing loss of membrane integrity, production of reactive oxygen species (ROS), inactivation and denaturation of proteins, and metabolic and cellular disequilibria, which ultimately lead to cell death (Lindquist, 1992). The ubiquitin–proteasome pathway is one of the cell functions that prevents the accumulation of aggregated proteins and promotes the

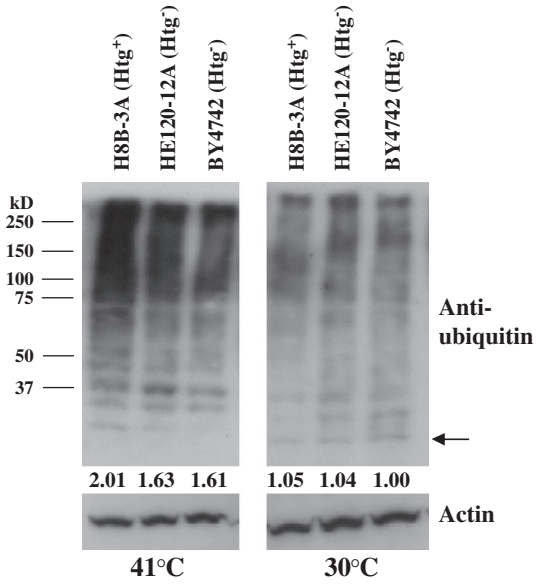

Fig. 8. Effect of temperature up-shift on the total amount of ubiquitinated proteins in Htg^+ and Htg^- strains. Total ubiquitin conjugates were analyzed using crude cell extracts of *S. cerevisiae* HB8-3A (Htg^+), HE120-12A (Htg^-) and BY4742 (Htg^-) strains grown at normal (30 °C) and high (41 °C) temperature for 2 h by immunoblotting with anti-ubiquitin antibody. Standard markers are shown on the left. Arrow shows the position of free ubiquitin. Comparative density values were expressed relative to that of the BY4247 strain at 30 °C (defined as 1.0) at the bottom of each line.

refolding of misfolded proteins. The key common requirement of both processes is that misfolded proteins, in various locations within the cells, are recognized as such by an appropriate ubiquitin ligase. The results presented here indicate that the *RSP5-C* allele is associated with the superior thermal resistance of *S. cerevisiae* (Figs. 2 and 3) and this association is primarily directed by increased transcription (Fig. 6) and ubiquitination of proteins (Fig. 8). Conversely, several temperature-sensitive *rsp5* mutants showed less ubiquitination as compared with the wild-type strain (Torres et al., 2009). Our result is also consistent with the finding of Hicke and Dunn (2003), which showed that the kinetics of lysosomal degradation of a particular membrane protein is tightly correlated with the extent of its ubiquitination. In addition, our finding that the *RSP5-C* allele showed dominance is consistent with the previous results of Sinha et al. (2006), which showed that the Htg^+ alleles in their Htg^+ strains of *S. cerevisiae* were dominant.

In this study, we investigated whether the Htg^+ *RSP5-C* allele provides protection against heat stress by both transcription analysis (Fig. 6) and a ubiquitination assay (Fig. 8). Because the ubiquitination of abnormal proteins is necessary under high-temperature stress conditions for the cell to facilitate degradation of damaged proteins or DNA repair, the increased transcription level of the *RSP5* gene would stimulate ubiquitination of its substrates, followed by endocytosis and vacuolar or proteasomal degradation. Diverging expression is a major driver of evolutional change and seems to be enriched for some particular genes, mainly those that interact with the environment (Tirosh et al., 2009). We found that the high-temperature-tolerant phenotype of our Htg^+ strain might be a result of higher transcription of the *RSP5-C* allele. We also discovered that superior temperature resistance could be achieved in *S. cerevisiae* by the overexpression of *RSP5* in Htg^+ strains (Fig. 7A and B). The activation domains of certain stress transcription factors, such as Msn2, Msn4 and Hsf1, have been demonstrated to serve as direct targets of ubiquitination (Haitani and Takagi, 2008); thus, it is possible that the increase in Rsp5 protein modulates these activation domains by increasing the ubiquitination level and regulating the gene transcription of target genes in response to environmental stresses. Moreover, the largest subunit of RNA polymerase II has been reported to be one of the substrates of Rsp5 ubiquitin ligase (Huibregtse et al., 1997) and, at the level of the general transcription machinery, *RSP5* stimulates its phosphorylation and ubiquitination, as well as the destruction of RNA polymerase II in response to DNA damage stress (Max et al., 2007). Rsp5 protein is also involved in the pathways responsible for the regulation of chromatin function and transcription, and ultimately controls gene expression in nutrient-limited conditions (Cardona et al., 2009). An increase in Rsp5 ubiquitin ligase might regulate the transcription of some genes and induce the heat stress response through the ubiquitination apparatus. The different pattern of protein ubiquitination observed in the thermotolerant strain C3723 (Htg^+) as compared with the wild-type Htg^- strain might be explained by this mechanism (Fig. 8).

On the other hand, because the Htg^+ phenotype of *S. cerevisiae* is considered to be associated with both the DNA repair system (Lu et al., 2008) and the maintenance of cellular integrity mechanisms (Lesage and Bussey, 2006; Winkler et al., 2002), we further tested these two properties, and found that the Htg^+ strain displayed more resistance to DNA damage caused by hydroxyurea as compared with the BY4742 (Htg^-) strain (Fig. 4). This finding is consistent with a previous report that *RSP5*-dependent ubiquitination of *RNR2*, which encodes ribonucleotide reductase, contributes to resistance to hydroxyurea by regulating the subcellular localization of the Rnr2 protein (Lee and Elledge, 2006). *RNR2* encodes a highly conserved protein that coverts nucleotides to deoxynucleotides and is localized to the nucleus. In response to DNA damage, Rnr2–Rnr4 enters the cytoplasm, where it combines with its regulatory subunit, Rnr1 to form an active complex (An et al., 2006). *RSP5* might regulate localization of the RNR complex present in the cytoplasm at high temperature to protect cell from damage and to facilitate recovery from such heat stresses by increasing the production of deoxyribonucleotides needed for DNA synthesis. The crucial role of the Rsp5 protein in the cellular content of ergosterol and the cell wall structure, in addition to activation of the *HOG1* MAPK pathway, following stresses such as high temperature, has been reported previously (Sato et al., 2003). In yeast, *RSP5* can trigger activation of the high-osmolarity glycerol mitogen-activated protein kinase (MAPK) pathway by release from repression through increasing ubiquitination of proteins such as protein tyrosine phosphatases (Ptp2 and Ptp3) under the high-temperature condition (Winkler et al., 2002).

The presence of *RSP5-C* allele reduced the sensitivity of yeast cells to Congo red, a cell-wall-damaging agent (Fig. 4). This finding confirms the regulatory effect of *RSP5* on cell wall permeability (Kamińska et al., 2002) and is consistent with the idea that the Htg^+ phenotype is acquired through changes in its structure and composition. Moreover, *RSP5*-mediated ubiquitination has been reported to have a major role in translation accuracy (Kwapisz et al., 2005), and ubiquitin ligase might regulate the expression of heat stress proteins via post-translational modification of stress response transcription factors such as MSN2/MSN4 (Haitani et al., 2006). Further studies are, however, required to clarify the precise mechanism of the ubiquitin machinery in controlling the transcription and translation of some of genes involved in the stress response under the high-temperature condition; moreover, identification of the complete set of *HTG* genes will be essential. Determination of the distinct mechanisms by which *HTG* genes are responsible for contributing to the Htg^+ phenotype is necessary to understand how the thermotolerance phenotype is conferred to Htg^+ strains by these genes and gene products.

5. Conclusions

In this study, we found that *RSP5-C*, a new allele of the *RSP5* gene that encodes E3 ubiquitin ligase, is associated with the high-temperature resistant phenotype in the *S. cerevisiae* thermotolerant strain C3723 and its derivatives. The transcription level of the *RSP5-C* allele from the Htg^+ strain was higher than that of the *RSP5-BY* allele originating from the

htg6 host strain (Htg^-) owing to base changes present in the upstream region of *RSP5-C*. We also revealed that the increase in ubiquitination of proteins was higher in the Htg^+ strain than Htg^- strains after exposure to temperature up-shift (41 °C). Overexpression of the wild-type *RSP5* allele in Htg^- strains conferred thermotolerance at 41 °C as in the case of the *RSP5-C* allele. Moreover, we found that an Htg^+ strain with overexpressed *RSP5-C* exhibits improved ability to tolerate higher temperatures (43 °C). This research has shown that overexpression of *RSP5* has much potential as a simple technique to develop thermotolerance in *S. cerevisiae* strains that are currently used in industrial fermentation.

Conflict of interest

The authors declare that they have no conflict of interest.

Authors' contributions

HS participated in the design of the study, performed the experimental work and wrote the draft manuscript. MS and YK participated in the experimental design, checking the results and reviewed the manuscript. CB commented on the manuscript. SH participated in the design of the research platform, contributed to the manuscript edition, and obtained funding for the work. All authors read and approved the final manuscript.

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