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Full Length Research Paper

Construction of bottom-fermenting yeasts that over express the ubiquitin ligase *RSP5* gene and their effects on high-gravity fermentation

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It has been reported that over expression of the ubiquitin ligase *RSP5* gene conferred tolerance to various stresses in laboratory yeast *Saccharomyces cereivisiae*. We constructed strains of bottom-fermenting yeast, natural hybrid, in which the *S. cerevisiae* (SC)-*RSP5* or *Saccharomyces bayanus* (SB)-*RSP5* is highly expressed and used for high-gravity fermentation. The SB-*RSP5*-overexpressing yeast showed a higher fermentation rate than the parent strain. In contrast, the fermentation rate of the SC-*RSP5*-overexpressing yeast was slower than the parent strain. These results suggest significant functional differences in brewing between the SC-*RSP5* and SB-*RSP5* genes.

Key words: High-gravity fermentation, *RSP5* gene, stress tolerance, bottom-fermenting yeast, natural hybrid.

INTRODUCTION

In brewing, high-gravity fermentation is an attractive method that can reduce the energy cost by increasing production efficiency. However, this method causes problems, such as a significant decrease in the fermentation rate, incomplete fermentation (a large amount of fermentable sugar, especially maltotriose, remains in the final fermented wort) and reduced vitality of the post-fermentation yeast. For these reasons, yeast strains with a sufficiently high fermentation rates and high post-fermentation cell vitality for high-gravity fermentation have been desired. Huuskonen et al. (2010) selected yeast variants suitable for high-gravity fermentation, but the mechanism of the accelerated fermentation was not clear and the reproducibility of obtaining the yeast variants seemed to be low. It is considered that yeast cells are exposed to high osmotic pressure and ethanol duringhigh-gravity fermentation. In the laboratory yeast *Saccharomyces cerevisiae* cells, it has been shown that over expression of the *RSP5* gene, encoding an essential ubiquitin ligase, conferred tolerance to high gravity (simulated by a medium containing 2.5 M sorbitol) and high ethanol concentrations (simulated by a medium containing 12% ethanol) (Haitani et al., 2009; Hiraishi et al., 2006). The ubiquitin ligase Rsp5 (the *RSP5* gene product) is suggested to be a key enzyme involved in the degradation and repair of stress-induced abnormal proteins for yeast cell growth under stress conditions (Haitani and Takagi, 2008; Hiraishi et al., 2009; Hoshikawa et al., 2003). Therefore, we constructed bottom-fermenting yeast strains in which the *RSP5* gene is highly expressed.

The bottom-fermenting yeast *Saccharomyces pastorianus* is a natural hybrid of *S. cerevisiae* (SC) and *Saccharomyces bayanus* (SB) (Tamai et al., 1998; Vaughan-Martini and Martini, 1998; Yamagishi and Ogata, 1999) and has both SC- and SB-types of genes and chromosomes. However, some of the SC- and SB-type orthologues have different functions (Fujii et al., 1996; Iijima and Ogata, 2010; Verstrepen et al., 2003; Yamagishi et al., 2010). In this study, bottom-fermenting yeast strains over expressing SC-*RSP5* or SB-*RSP5* were constructed to examine whether they have different fermentation effects on the high-gravity fermentation condition. In this experiment, the uracil-requiring strain of the meiotic segregant of bottom-fermenting yeast was

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Table 1. DNA primers used in this study.

Primer name	Sequence (5' – 3')	Remark
A	CTGGCGCAGACCAGCCGCACAGTCACGTGA	SGD* Chr.V 409495-409524
B	gaaaagctgtggcccgggcgcgccaatccaTTTCTTTTTTCTTTCCTTTCTGTTACTTTA	Lower-case letters; AB628198 1-30 complementary strand Upper-case letters; SGD Chr.V 410155-410184 complementary strand
C	agtttcgaataaaaatcaatcatcacataaAAAAATGCCTTCATCCATATCCGTCAAGTT	Lower-case letters; AB628198 1771-1800 Upper-case letters; SGD Chr.V 410185-410214
D	GGAAGGATGAGACGTTGAATGATTACGGGT	SGD Chr.V 410705-410734 complementary strand
E	tggattggcgcgcccgggccacagcttttc	AB628198 1-30
F	ttatgtgatgattgatttttattcgaaact	AB628198 1771-1800 complementary strand
G	TGGCACGCTTCTGCCGCACAGTCACGTGAT	ABPO01000008 10191-10220
H	gaaaagctgtggcccgggcgcgccaatccaTTGTTTCACTCTATGTTTGTTTTGTTCTTT	Lower-case letters; AB628198 1-30 complementary strand Upper-case letters; ABPO01000008 10862-10893 complementary strand
I	agtttcgaataaaaatcaatcatcacataaAAAATGCCTTCATCAATATCTGTCAAGCTA	Lower-case letters; AB628198 1771-1800 Upper-case letters; ABPO01000008 10894-10923
J	AGGAGTACTGTCTTGTGCCGCTAGAAGCGG	ABPO01000008 11534-11563 complementary strand

SGD: Saccharomyces Genome Database http://www.yeastgenome.org.

used as the host strain for transformation. In the fermenting test simulation high-gravity brewing, the high-sugar medium (20% maltose, 1% yeast extract and 2% peptone) was used.

MATERIALS AND METHODS

Yeast strains

For the transformation host, we used W34:70-1B-ura3 (*SC-ura3Δ SB-ura3Δ*), the uracil-requiring strain of the meiotic segregant W34:70-1B of bottom-fermenting yeast Weihenstephan34/70 (Iijima and Ogata, 2010; Ogata et al., 2011).

DNA manipulation

DNA primers used in this study were described in Table 1. To construct the *RSP5*-overexpressing yeast, we used deoxyribonucleic acid (DNA) sequences deposited in the SGD (Saccharomyces Genome Database, http://www.yeastgenome.org/), because the DNA sequence of the SC-type gene of bottom-fermenting yeast is almost the same as the laboratory strain *S. cerevisiae* S288C. The SB-type gene is available from the draft sequence submitted by Nakao et al. (2009) (Genbank/EMBL/DDBJ project no. ABPO01000000). The open reading frame (ORF) of the SB-*RSP5* gene was sequence no. 10897 to 11707 of Contig10.3 (Genbank/EMBL/DDBJ accession no. ABPO01000008). Strains W34:70-1B/P_{TDH3}-SCRSP5 and W-1B/P_{TDH3}-SBRSP5, which overexpress the *RSP5* gene, were constructed by replacing the *TDH3* promoter with the SC-*RSP5* gene and SB-*RSP5* gene by fusion PCR using pST106, a plasmid with the *URA3* gene (selection marker) and the *TDH3* promoter (high-expression promoter), as a template (Cha-aim et al., 2009).

To construct the SC-*RSP5*-overexpressing yeast, the DNA fragment was prepared by the following three PCR reactions (PCR 1, PCR 2, and PCR 3) (Figure 1). In PCR 1, using the genomic DNA of Weihenstephan34/70 as a template and primers A and B, the PCR fragment consisting of a region (-694 to -5) upstream of the translation initiation

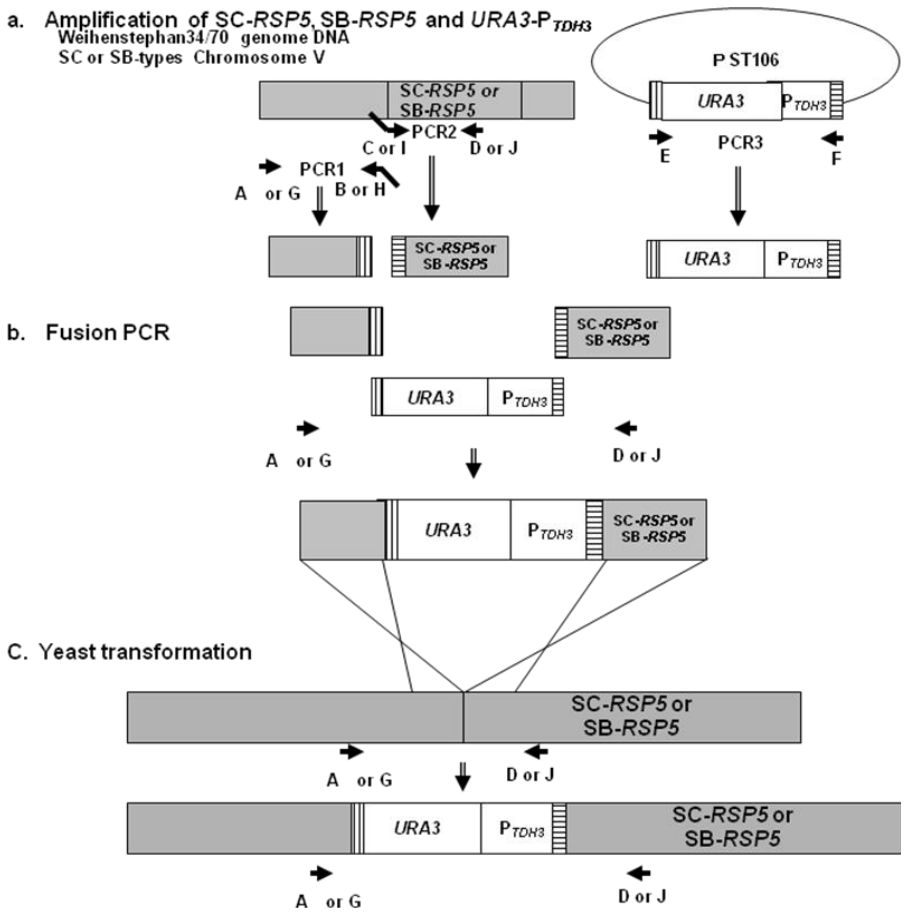

Figure 1. Schematic diagram of the construction of the SC-*RSP5* and SB-*RSP5*-overexpressing yeast strains. Details are described in the text.

site of the SC-*RSP5* gene (SGD, Chr.V 409495-409524) and the DNA sequence of pST106 (Genbank/EMBL/DDBJ accession no. AB628198, bases 1 to 30) was obtained. In PCR 2, using the genomic DNA of Weihenstephan34/70 and primers C and D, the PCR fragment consisting of a region (-4 to +546) near the translation initiation site of the SC-*RSP5* gene (SGD, Chr.V 410155 to 410214) and the DNA sequence of pST106 (Genbank/EMBL/DDBJ accession no. AB628198, bases 1771 to 1800) was obtained. In PCR 3, using pST106 as a template and primers E and F, the polymerase chain reaction (PCR) fragment made up of the DNA sequence of pST106 (Genbank/EMBL/DDBJ accession no. AB628198, bases 1 to 1,800: a fragment containing the *URA3* gene [marker gene] and the *TDH3* promoter [high-expression promoter]) was obtained. Fusion PCR was performed using these three PCR fragments as a template (Kuwayama et al., 2002). Since both the PCR fragments from PCR 1 and PCR 3 and the PCR fragments from PCR 2 and PCR 3 have a 30-bp overlapping sequence at their ends, a fragment containing the *URA3* gene (marker gene), *TDH3* promoter (high-expression promoter), and the translation-initiation-site surrounding the SC-*RSP5* gene at both ends was obtained by fusion PCR using primers A and D. This PCR fragment was introduced into *S. pastorianus* W34:70-1B-ura3, the uracil-requiring strain of a meiotic segregant from bottom-fermenting yeast Weihenstephan34/70 (Iijima and Ogata, 2010, Ogata et al., 2011). In the transformant obtained by homologous recombination between the SC-*RSP5* sequence at both ends of the PCR product and the SC-*RSP5* sequence on the host genome, the promoter sequence of the SC-*RSP5* gene should be replaced with the high-expression *TDH3* promoter, making the host a uracil autotroph. Whether intended homologous recombination has occurred or not, was confirmed by PCR with primers A and D. As a result, we obtained the SC-*RSP5*-overexpressing bottom-fermenting yeast meiotic segregant *S. pastorianus* W-1B/P_{TDH3}-SCRSP5.

The SB-*RSP5*-overexpressing yeast was also constructed using the same method (Figure 1). In PCR 1, using the genome DNA of Weihenstephan34/70 as a template and primers G and H, the PCR product consisting of a region, bases -707 to -4, upstream of the translation initiation site of SB-*RSP5* (Genbank/EMBL/DDBJ accession no. ABPO01000008, bases 10191 to 10962) and the DNA sequence of pST106 (Genbank/EMBL/DDBJ accession no. AB628198, bases 1 to 30) was obtained. In PCR 2, using the genomic DNA of Weihenstephan34/70 as a template and primers I and J, the PCR product consisting of a region, bases -3 to +666, near the translation initiation site of the SB-*RSP5* gene (Genbank/EMBL/DDBJ accession no. ABPO01000008, bases 10894 to 11534) and the DNA sequence of pST106 (Genbank/EMBL/DDBJ accession no. AB628198, bases 1771 to 1800) was obtained. In PCR 3, using pST106 as a template and primers E and F, the DNA sequence of pST106 (Genbank/EMBL/DDBJ accession no. AB628198, bases 1 to 1800) was amplified. Fusion PCR was performed using these three PCR products as a template and primers G and J, and the PCR product obtained was introduced into *S. pastorianus* W34:70-1B-ura3, the

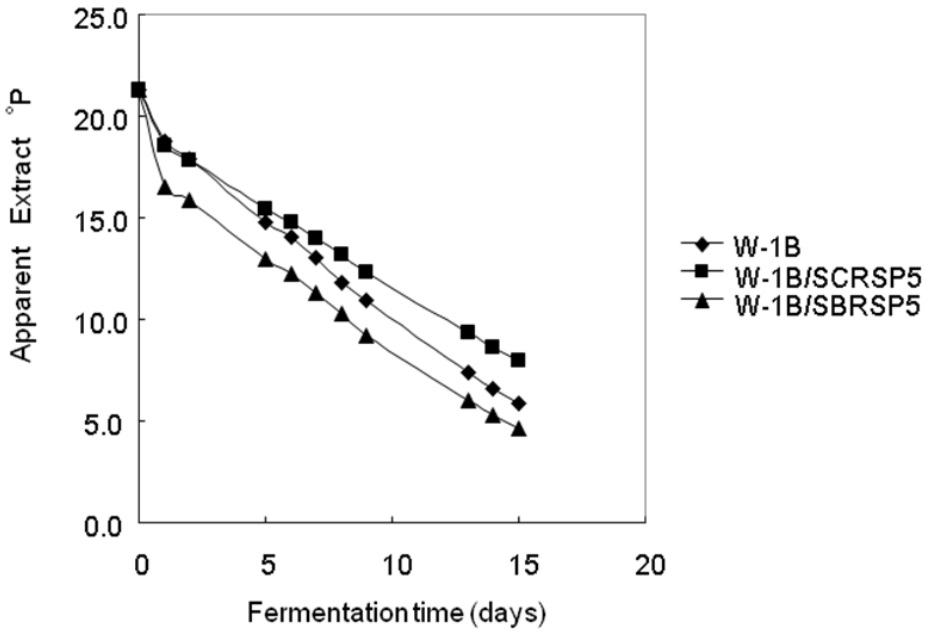

Figure 2. Time courses of high-gravity fermentation using the SC-*RSP5* and SB-*RSP5*-overexpressing strains and their parent strain. Values are means of results from two independent experiments.

uracil-requiring bottom-fermenting yeast meiotic segregant. From this, we obtained the transformant *S. pastorianus* W34:70-1B/P_{TDH3}-SBRSP5.

Fermentation experiment

The fermentation experiment simulating high-gravity brewing was performed as follows: W34:70-1B (the parent strain) and two transformants were added to the high-sugar medium (1% Bacto-yeast extract, 2% Bacto-peptone, 20% Maltose) at 25°C. Fermentation was started at the initial concentration of 2.0×10^7 cells/ml. A portion of fermenting medium was sampled daily to measure apparent extract. The apparent extract was measured using a Density/Specific Gravity Meter DA-510 (Kyoto Electronics Manufacturing Co. Ltd., Kyoto, Japan).

RESULTS

The apparent extract from the parent strain *S. pastorianus* W-1B was reduced to 5.8%, 15 days after the start of fermentation. Interestingly, we found the fermentation rate of SB-*RSP5*-overexpresisng W34:70-1B/P_{TDH3}-SBRSP5 was higher than W34:70-1B and the apparent extract were reduced to 4.7% after 15 days. This result suggests that the SB-*RSP5*-overexpressing strain is suitable for high-gravity fermentation. However, the SC-*RSP5*-overexpressing strain W34:70-1B/P_{TDH3}-SCRSP5 showed a lower fermentation rate than W34:70-1B and the apparent extract were reduced only 8.0% after 15 days (Figure 2). It appears that the differences in the apparent extract are significant among the strains based on the results from two independent experiments. This result suggests that the SB-*RSP5*-overexpressing bottom-fermenting yeast showed a higher fermentation rate than the parent strain. On the other hand, the fermentation rate of the SC-*RSP5*-overexpressing bottom-fermenting yeast may be slower than the parent strain.

DISCUSSION

During high-gravity fermentation, yeast cells are exposed to environmental stresses, including high osmotic pressure, low temperature, and high ethanol concentrations. Such stresses induce protein denaturation, generate abnormal proteins, and lead to growth inhibition or cell death. Hence, stress tolerance is of crucial importance in brewing yeast. The ubiquitin ligase Rsp5 (the *RSP5* gene product) is suggested to be a key enzyme involved in the degradation and repair of stress-induced abnormal proteins for yeast cell growth under stress conditions (Haitani and Takagi, 2008; Hiraishi et al., 2009; Hoshikawa et al., 2003). Previously, laboratory yeast cells overexpressing the *RSP5* gene showed tolerance to both high gravity and ethanol (Haitani et al., 2009; Hiraishi et al., 2006). Rsp5 is necessary for ubiquitination of the maltose transporters Mal61 and Mal21 (Hatanaka et al., 2009). The SC-*RSP5* and SB-*RSP5* genes share very similar DNA sequences; the identity of the deduced amino acid sequence between the two genes is approximately 97%. In particular, there is no difference in the WW domains required for substrate recognition. Since over expression of the SC-*RSP5* and SB-*RSP5* genes

exhibited the opposite effects to bottom-fermenting yeast during high-gravity fermentation, it is intriguing to see which residues are involved in the function of Rsp5 and how Rsp5 participates in stress adaptation of yeast cells during brewing. Another possibility is that the expression levels of these two genes are different in bottom-fermenting yeast. Our results suggest engineering of Rsp5 is promising for efficient beer production using novel yeast strains that are tolerant to various stresses.

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