Identification of \textit{CDC25-P1306L}, a novel mutant allele of \textit{CDC25}, conferring tolerance to multiple stresses associated with food production on \textit{Saccharomyces cerevisiae}

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ABSTRACT

Mutations conferring tolerance to diverse stresses (i.e. multistress tolerance) on budding yeast \textit{Saccharomyces cerevisiae} are useful for industrial yeast strains such as baker’s and wine yeast. However, little is known about the mutations conferring multistress tolerance. Previously, we developed a simple method for isolating multistress-tolerant semidominant mutants of \textit{S. cerevisiae} by one-step selection under lethal hydrogen peroxide stress, which we named the LCH method. In this study, we applied a combination of genetics and next-generation sequencing (NGS) technology to identify the causal mutation for multistress tolerance of the mutant isolated using the LCH method. The haploid mutant strain was crossed with the wild-type strain and the resulting diploids were sporulated. The 20 haploid progeny strains showing multistress tolerance were mixed and subjected to DNA extraction for NGS. \textit{CDC25-P1306L}, a novel mutant allele of \textit{CDC25} encoding a Ras guanine nucleotide exchange factor, was detected 86 times; however, a wild-type \textit{CDC25} allele was not detected in the NGS data from the mixture of the multistress-tolerant progeny strains, suggesting that all of the progeny strains showing multistress tolerance have a \textit{CDC25-P1306L} allele instead of a wild-type \textit{CDC25} allele. Substitution of \textit{CDC25} in the wild-type strain with \textit{CDC25-P1306L} rendered the strain tolerant to ethanol, heat shock, freeze-thaw, chronological aging and high concentrations of glucose. These results indicate that \textit{CDC25-P1306L} is a multistress-tolerant mutation and is promising for breeding multistress-tolerant \textit{S. cerevisiae} strains for food production.

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Introduction

When the budding yeast \textit{Saccharomyces cerevisiae} is applied to fermentation processes in the production of foods such as bread, Japanese sake and wine, it is exposed to various stresses, such as high temperature [1,2], freeze-thaw [1,3], high concentrations of sugar [4,5], chronological aging [6,7] and ethanol [8,9]. Therefore, breeding of stress tolerant strains of \textit{S. cerevisiae} for these diverse stresses (i.e. multistress-tolerant strains) is important for improving the quality of the foods, improving the efficiency or reducing the costs of food production and developing novel types of breads and alcoholic beverages that are difficult to produce because of these stresses.

For breeding multistress-tolerant strains for food production, isolation of multistress-tolerant mutant strains from diploid industrial \textit{S. cerevisiae} strains is one of the most useful procedures. The main reason is that gene-recombination technologies, unfavourable for food production because of public unacceptability [10,11], are not required for isolation of the mutant strains. However, the mutant strains tend to lose their useful properties, because heterozygous mutations in the cells of diploid industrial strains are gradually lost by a phenomenon designated as ‘loss of heterozygosity’ in the course of subculture of the strains [12,13]. On the other hand, loss of heterozygosity does not occur in haploid strains and diploid strains harbouring homozygous mutations, because of an absence of the other allele. Therefore, identification of the mutated genes is useful for breeding genetically stable diploid industrial strains harbouring homozygous mutations. Previously, we have developed a simple method for isolating multistress-tolerant semidominant mutants of...
S. cerevisiae by one-step selection under lethal hydrogen peroxide (H₂O₂) stress condition, which we named the lethal concentration of H₂O₂ (LCH) method [14]. By using the LCH method on cells of a haploid laboratory S. cerevisiae strain mutagenised with ethyl methanesulfonate (EMS), we isolated a multistress-tolerant mutant strain of S. cerevisiae, in which a single gene mutation designated MLT2-1 is responsible for the multistress tolerance [14]. However, MLT2-1 has not yet been identified. Identification of the mutation conferring multistress tolerance is important for understanding the mechanism of multistress tolerance, and would make it possible to use this mutation for breeding multistress-tolerant and genetically stable diploid industrial strains of S. cerevisiae for food production harbouring homozygous mutations.

In this study, we attempted to identify the MLT2-1 mutation gene. The candidates for the causal mutation for multistress tolerance were successfully identified by a combination of genetics and next-generation sequencing (NGS) technology. Among the candidate mutations, we identified CDC25-P1306L, a novel mutant allele of CDC25, as the MLT2-1 mutation gene by substituting CDC25 in the parental wild-type strain to CDC25-P1306L. Since the strain harbouring CDC25-P1306L, instead of CDC25, was tolerant to ethanol, heat shock, freeze-thaw, chronological aging and high concentrations of glucose, CDC25-P1306L is promising for breeding industrial strains for production of foods, such as bread, Japanese sake and wine.

Materials and methods

Microorganisms and media

The S. cerevisiae strains used in this study are listed in Table 1. Escherichia coli strain JM109 [15] was used as a host for the propagation and manipulation of plasmid DNA. Yeast strains were grown in YPDA medium (1% yeast extract, 2% polypeptone, 2% glucose and 0.04% adenine) or synthetic dextrose (SD) medium (0.67% yeast nitrogen base without amino acids and 2% glucose) supplemented with 0.002% L-histidine-HCl-H₂O, 0.04% adenine, 0.002% uracil (if required), 0.002% L-tryptophan (if required) and 0.01% L-leucine (if required) [16] unless otherwise specified. Solid media were prepared using 2% agar. A diploid yeast strain was sporulated on minimal sporulation medium (1% potassium acetate and 2% agar) supplemented with 0.0005% L-histidine-HCl-H₂O, 0.01% adenine and 0.0005% uracil [16]. 5-Fluoroorotic acid (5-FOA) medium for selection of uracil auxotrophic strains was prepared as described by Akada et al. [17]. E. coli cells harbouring plasmid DNA were cultivated in Luria–Bertani (LB) medium (1% tryptone, 0.5% yeast extract and 0.5% NaCl) containing 50 μg/mL ampicillin.

Genetic, biochemical and physiological methods

Mating of haploid yeast strains was performed on YPDA solid medium and the resulting diploid strain was selected on SD solid medium supplemented with 0.002% L-histidine-HCl-H₂O, 0.04% adenine and 0.0002% uracil. Asci formed on the minimal sporulation medium by the diploid strain were treated with a filter-sterilised solution of 0.02% Zymolyase 100T (Seikagaku Corporation, Tokyo, Japan) for 10 min or more at room temperature. The tetrads in asci were dissected on YPDA solid medium using a micromanipulator Singer MSM Manual (Singer Instruments, Somerset, UK) in accordance with the manufacturer’s instructions. S. cerevisiae cells were transformed using a Frozen-EZ Yeast Transformation II Kit (Zymo Research, Irvine, CA, USA). Preparation of yeast chromosomal DNA was performed as described by Hereford et al. [18] with modifications. Briefly, the cells were incubated in solution A (0.2 mol/L Tris and 5% 2-mercaptoethanol) for 30 min at room temperature. Then, the cells were spun down and incubated in solution B (1.0 mol/L sorbitol, 40 mmol/L potassium phosphate buffer (pH 6.8) and 0.0125% Zymolyase 100T) for 1 h at 30 °C. Then, the spheroplasts were spun down and lysed by incubation for 30 min or more at 60 °C in solution C (50 mmol/L Tris-HCl (pH 8.5), 0.2 mol/L NaCl, 0.1 mol/L EDTA disodium salt and 5% SDS). Then, nucleic acids were purified by phenol-chloroform:isoamyl alcohol extraction [15]. The thread-like chromosomal DNA was recovered by ethanol precipitation [15], dried and dissolved in Tris EDTA (TE) buffer (pH 8.0) [15]. The chromosomal DNA was incubated for 1 h at 37 °C with 0.1 mg/mL of ribonuclease A. Then, the chromosomal DNA was purified, recovered and dissolved in TE buffer (pH 8.0) as described above. Genomic polymerase chain reaction (PCR) was performed using PrimeSTAR HS DNA polymerase (Takara Bio Inc., Shiga, Japan) in accordance with the manufacturer’s instructions. Restriction enzymes were purchased from New England Biolabs Japan Inc. (Tokyo, Japan) and used in accordance with the manufacturer’s instructions. DNA ligation was performed using T4 DNA ligase (Takara Bio Inc.) [15]. Plasmid DNAs were prepared from E. coli cells using LaboPass Plasmid Mini Purification Kit (Hokkaido System Science, Hokkaido, Japan). Spot assays for analyzing stress tolerance were performed as described previously [14], except that the assay for chronological
aging stress tolerance was performed after incubation of the cells in sterile distilled water at 30 °C for 7 days and that the assay for freeze-thaw stress tolerance was performed after the samples were subjected to freeze-thaw treatment (frozen at −80 °C for 24 h and thawed at 30 °C for 10 min) five times in 5 days. The plates for spot assays were incubated at 30 °C for 2 days, except for the plates containing 50% (w/v) glucose and 15% (v/v) ethanol, which were incubated for 5 days and 7 days before photography, respectively.

**Preparation of mixed genomic DNA and NGS sequencing**

The multistress-tolerant strain JSEO8 was crossed with the wild-type strain YN101 and the resulting diploid strain was sporulated and subjected to tetrad analyses as described previously [14]. Then the 20 segregants which exhibited stress tolerance to H₂O₂, ethanol, heat shock and high concentrations of glucose were collected as multistress-tolerant progeny strains. The cells of these strains were cultivated in 10 mL of YPDA medium independently, mixed, subjected to preparation of yeast chromosomal DNA as described above and designated as MLT2-1 Mix. The DNA samples of MLT2-1 Mix, the parental wild-type strain YN57 and the wild-type strain YN101 with opposite mating type were sequenced using an Illumina HiSeq 2000 sequencer (paired-end, 100 bp) by Macrogen Japan Corp. (Tokyo, Japan).

**Identification of candidates for the multistress-tolerant mutation**

The fastq.gz files of MLT2-1 Mix containing total bases of 2.1 Giga generated by NGS and the fastq.gz files of YN57 containing total bases of 2.6 Giga generated by NGS were uploaded to a browser-accessible bioinformatics tool called Mutation discovery (Mudi; http://naoii.nig.ac.jp/mudi_top.html) [19] as the mutant fastq.gz files and the parental fastq.gz files, respectively, by choosing *S. cerevisiae* as a reference genome. The fastq.gz files of the MLT2-1 Mix and the fastq.gz files of YN101 containing total bases of 2.7 Giga generated by NGS were similarly uploaded to Mudi as the mutant fastq.gz files and the parental fastq.gz files, respectively. Then, mutations in nuclear DNA that were commonly detected more than 20 times in both output data, but had no different variants in the same position as the NGS data for MLT2-1 Mix or in the same gene as the NGS data for YN57 or YN101, were listed as candidates for the multistress-tolerant mutation.

**Substitution of CDC25 in the parental strain to CDC25-P1306L**

Substitution of *CDC25* in the parental strain YN57 to CDC25-P1306L was performed by two-step gene replacement method [16] as follows. The 3'-terminal part of the CDC25-P1306L allele containing the mutation point was amplified by PCR using the chromosomal DNA of the multistress-tolerant strain JSEO8 as a template and oligonucleotides 5’-CTCGAATTCCTGGTGTCACATTATGAGA-3’ (EcoRI_CDC25 + 3582) and 5’-CTCGTGCACCTGGATCGATAAC TTAACTGG-3’ (SalI_CDC25 + 5020c) as the forward and reverse primers, respectively. The underlined sequences in the oligonucleotides EcoRI_CDC25 + 3582 and SalI_CDC25 + 5020c indicate the restriction sites of EcoRI and SalI, respectively. The amplified product was doubly digested with EcoRI and SalI, and cloned into the EcoRI-SalI gap in YIp5 [20] to obtain YIp5+ CDC25-P1306L. Then, the YIp5+ CDC25-P1306L was cleaved at the HindIII site in the CDC25-P1306L and integrated into the CDC25 locus of YN57 by transformation, resulting in an uracil prototrophic transformant carrying a duplication of the CDC25 region in which one duplicate is CDC25 and the other is CDC25-P1306L with the plasmid sequences in between. Strains that excised the plasmid by homologous crossovers between the *CDC25* and the CDC25-P1306L were selected using 5-FOA medium. The 5-FOA-resistant strains with uracil auxotrophy were analyzed by direct sequencing of the CDC25 region amplified by genomic PCR using oligonucleotides 5’-CCGTGTTGGTCAACATTATGAGA-3’ (CDC25 + 3582) and 5’-CGGTAGATTGGGGAGGAATA-3’ (CDC25 + 4232c) as the primers. Then, the strain that was verified to possess the CDC25-P1306L allele, but not the wild-type *CDC25* allele, was selected as the YN57 CDC25-P1306L strain (Table 1), which is isogenic to YN57, except for CDC25-P1306L.

**Results and discussion**

**Identification of candidates for the multistress-tolerant mutation MLT2-1 by a combination of genetics and NGS technology**

A multistress-tolerant semidominant mutant strain JSEO8 was previously isolated by using the LCH method in EMS-mutagenised cells of a laboratory *S. cerevisiae* strain YN57 with a W303-1A background [14]. Recently, NGS technology has become a powerful tool for finding mutated genes in mutant strains. However, it is difficult to identify the causal mutated gene responsible for the phenotypes of mutant strains.
treated with mutagen, because these strains have many other mutated genes unrelated to the phenotypes [19]. On the other hand, \textit{S. cerevisiae} is an ideal model organism to use classical genetics because it can proliferate as haploid cells (mating type a or α), can be easily manipulated to mate between opposite mating type cells and sporulate to produce ascospores for tetrad analysis [16]. Therefore, to identify candidates for the multistress-tolerant mutation MLT2-1, we applied a combination of genetics and NGS technology (Figure 1). First, we mated JSEOA8 with the wild-type strain YN101 of the opposite mating type. Then, cells of the resulting diploid strain were sporulated and the tetrads were analyzed. The 20 segregants which exhibited multistress tolerance were selected as multistress-tolerant progeny strains and cultivated independently in YPDA broth. Then each cultivated broth was mixed with the others and the cells in the mixture were collected and subjected to chromosomal DNA extraction. Chromosomal DNAs designated as MLT2-1 Mix were subjected to NGS. As a result, 2.1 Gb of DNA sequence of MLT2-1 Mix, which is more than 161-fold of the genome size (13 Mb) of laboratory haploid \textit{S. cerevisiae} strain [21], was obtained. On the other hand, 2.6 Gb of genomic DNA sequence of the wild-type strain YN57 and 2.7 Gb of genomic DNA sequence of YN101 were also obtained by NGS. These NGS data were uploaded to Mudi, a browser-accessible bioinformatics tool, and the candidates for the multistress-tolerant mutation MLT2-1 were listed (Table 2) using the output data as described in ‘Materials and methods’. As a result, one missense mutation was detected in the \textit{CDC25} gene. The other mutations were outside of the coding regions. The missense mutation in \textit{CDC25} was a G-to-A base substitution at position 753077 of chromosome XII and was predicted to result in a single amino acid substitution from proline to leucine at position 1306 in the Cdc25p protein. Therefore, we designated the mutant allele of \textit{CDC25} as \textit{CDC25-P1306L}. The \textit{CDC25-P1306L} allele was detected 86 times, but a wild-type \textit{CDC25} allele was not detected in the NGS data of the MLT2-1 Mix (Table 2), suggesting that all of the progeny strains showing multistress tolerance have a \textit{CDC25-P1306L} allele instead of a wild-type \textit{CDC25} allele.

\textbf{Identification of \textit{CDC25-P1306L} as the multistress-tolerant mutation MLT2-1}

Among the candidates for the MLT2-1 mutation listed in Table 2, a missense mutation in \textit{CDC25} designated as \textit{CDC25-P1306L} seemed to be most probable
Table 2. Candidates for the multistress-tolerant mutation MLT2-1 identified by a combination of genetics and NGS technology.

| Chr. | Position | Reference allele | Mutation allele | Reads^b | Read frequency^c (%) | Mutation type^d |
|------|----------|------------------|----------------|--------|----------------------|----------------|
| III  | 273025   | GAAAAAAAAAAAAAAAA | GAAAAAAAAAAAAAAA | 21     | 100                  |                |
| V    | 196609   | TAAAAAAAAAAAAAAA  | TAAAAAAAAAAAAAAA | 35     | 100                  |                |
| VII  | 272291   | GATATATATATATATAT | GATATATATATATATATATAT | 41     | 100                  |                |
| VII  | 321487   | CATATATATATATATATAT | CATATATATATATATATATATAT | 69     | 100                  |                |
| VII  | 325276   | GAA             | CAA            | 20     | 100                  |                |
| VII  | 736553   | TAAAAAAAAAAAAAAA | TAAAAAAAAAAAAAAA | 45     | 100                  |                |
| VIII | 39756    | GC              | GCC            | 128    | 100                  |                |
| XI   | 146920   | C               | A              | 64     | 100                  |                |
| XI   | 146921   | A               | C              | 64     | 100                  |                |
| XI   | 146922   | G               | T              | 62     | 100                  |                |
| XII  | 753077   | G               | A              | 86     | 100                  | Missense mutation of CDC25 (P1306L) |
| XII  | 1011391  | C               | G              | 56     | 100                  |                |
| XIV  | 107294   | GAAAAAAAAAAAAAAA | GAAAAAAAAAAAAAA | 34     | 100                  |                |

^a Chromosome number of S. cerevisiae.

^b Number of reads mapped in this position. ‘Reads’ means coverage depth at this position [19].

^c Ratio of reads with mutation allele among all mapped reads in this position.

^d Blank indicates mutation outside of genes.

because the other mutations were outside of any coding regions. To elucidate whether CDC25-P1306L is the causal mutation for multistress tolerance of the JSEOA8 strain, we substituted CDC25 in the parental wild-type strain YN57 to CDC25-P1306L by a two-step gene replacement method. The substituted strain was designated as YN57 CDC25-P1306L strain and was analyzed for stress tolerance by spot assays (Figure 2). The YN57 CDC25-P1306L cells grew well, like the YN57 cells on YPDA medium as no-stress controls. On the other hand, the YN57 CDC25-P1306L cells grew much better than the YN57 cells on the YPDA media after heat shock stress (50°C, 1 h), freeze-thaw stress (−80°C, 5 days), and chronological aging stress (30°C, 7 days). Furthermore, the YN57 CDC25-P1306L cells also grew much better than YN57 cells on theYPD media containing 50% (w/v) glucose and 15% (v/v) ethanol. From the results described above, CDC25-P1306L was demonstrated to be the causal mutation for the multistress tolerance of the JSEOA8 strain, namely MLT2-1.

How did the CDC25-P1306L strain, YN57 CDC25-P1306L, exhibit multistress tolerance? Cdc25p protein, the product of CDC25, is a Ras guanine nucleotide exchange factor (rasGEF), which promotes exchange of GDP bound to Ras1/2p (Ras) to GTP, and activates the cAMP-protein kinase A (PKA) pathway [22]. Cdc25p is a 1589-amino-acid-long protein and has a Src homology 3 (SH3) domain (65 to 129 aa region) [23], a cyclin destruction box (CDB) motif (149 to 157 aa region) [24] and a C-terminal catalytically active region (amino acids 1084 to 1589 aa region) [25] which contains three structurally conserved regions (SCRs) between rasGEFs of various species: SCR1 (1301 to 1324 aa region), SCR2 (1374 to 1416 aa region) and SCR3 (1454 to 1475 aa region) (Figure 3(A)) [26]. Interestingly, the mutated proline residue at position 1306 in Cdc25p, which is changed to leucine in the CDC25-P1306L strain, is located in the SCR1 region and conserved in rasGEFs of various species [26,27] such as Ste6 of Schizosaccharomyces pombe, protein Son of sevenless (SoS) of Drosophila melanogaster, Son of sevenless homologues of Caenorhabditis elegans (CeSos1), Mus musculus (MmSos1) and Homo sapiens (HsSos1) (Figure 3(B)). In S. cerevisiae, decreased activity of the cAMP-PKA pathway increases the stress tolerance of the cells [28,29]. Therefore, we propose the following mechanism of multistress tolerance of the CDC25-P1306L strain (Figure 4). In the wild-type CDC25 strain (Figure 4(A)), Cdc25p promotes exchange of GDP bound to Ras to GTP. GTP-bound Ras (Ras-GTP), an active form of Ras, activates adenylate cyclase Cyr1p which synthesizes cAMP from ATP. cAMP activates catalytic subunits of PKA (Tpk1/2/3p) by binding to regulatory subunits of PKA, resulting in inhibition of the stress tolerance of the cells [28,29]. On the other hand, in the CDC25-P1306L strain (Figure 4(B)), the mutated variant of the Cdc25p protein, Cdc25p, which probably has lower catalytic activity for the exchange of GDP bound to Ras to GTP than the wild-type Cdc25p, confers multistress tolerance on the S. cerevisiae strain probably by decreasing the activity of the cAMP-PKA pathway.

Previously, Folch-Mallol et al. [30] isolated heat-shock resistant mutants from a laboratory S. cerevisiae strain and found that the mutant strain harbouring cdc25-21, a mutant allele of CDC25 predicted to encode a fusion protein containing the N-terminal 977 amino acids of Cdc25p fused to 12 non-native amino acids at the C-terminus, or cdc25-22, a mutant allele of CDC25 predicted to result in a single amino acid substitution from histidine to proline at position 1363 in the protein, showed tolerance to heat shock, H2O2, high concentrations of sorbitol and LiCl and resistance
to lyticase digestion. Recently, Satomura et al. [31] isolated thermotolerant mutant strains from a laboratory S. cerevisiae strain by adaptation experiments under heat stress and found four kinds of one-point mutations in CDC25, designated as CDC25(T943P), CDC25(N1393T), CDC25(W1416C) and CDC25(G1459C), among the mutant strains. All the reconstructed mutant strains harbouring each one-point mutation exhibited thermotolerance [31], indicating that these mutant alleles of CDC25 confer thermotolerance on the S. cerevisiae strain. In this study, we identified CDC25-P1306L, a novel mutant allele of CDC25, as the causal mutated gene for multistress tolerance of the multistress-tolerant semidominant mutant of S. cerevisiae isolated by the LCH method using lethal concentration of H2O2 for the first screening. To the best of our knowledge, this is the first report showing that the specific allele of CDC25 confers stress tolerance to ethanol, freeze-thaw, chronological aging and high concentrations of glucose. It is interesting to know whether the alleles of CDC25 other than CDC25-P1306L identified in this study confer stress tolerance to ethanol, freeze-thaw, chronological aging and high concentrations of glucose, which are industrially important stresses for the production of foods, such as bread, Japanese sake and wine.

In the breeding of microorganisms for the food industry, mutation breeding is a useful procedure because gene-recombination technologies, which are unfavourable for food production, are not required for isolation of mutant strains [32]. If the LCH method is applied to the breeding of industrial yeast strains such as baker’s yeast and wine yeast, identification of the causal mutated gene by a combination of genetics and NGS technology would be difficult because most of these strains have poor sporulation and low spore viability [33]. In this study, CDC25-P1306L was identified for the first time as the causal mutated gene for multistress tolerance of the multistress-tolerant mutant isolated by using the LCH method from the laboratory.
S. cerevisiae strain, which is easy to perform genetic analysis on. This finding would accelerate the identification of previously unknown causal mutated genes for stress tolerance in the stress-tolerant industrial mutant stains for food production obtained by mutation breeding such as the LCH method. Furthermore, this finding would also be useful for breeding multi-stress-tolerant and genetically stable diploid industrial strains of S. cerevisiae for food production harbouring homozygous mutated alleles.

Conclusions
To our knowledge, this is the first report on the identification of the causal mutated gene for multistress tolerance of a multistress-tolerant mutant of S. cerevisiae isolated using the LCH method. The identified CDC25-P1306L gene, a novel mutant allele of CDC25, was shown to confer stress tolerance to ethanol, heat shock, freeze-thaw, chronological aging and high concentrations of glucose on a S. cerevisiae strain. Therefore, CDC25-P1306L is promising for breeding multistress-tolerant S. cerevisiae strains for production of foods, such as bread, Japanese sake and wine.

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