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Functional analysis of γ-glutamyl kinase mutants in baker's yeast

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Abstract

Previous studies have shown that proline is a good hydroxyl-radical scavenger, chemical chaperone, osmolyte and cryoprotectant. Proline is accumulated in many bacterial and plant cells in response to osmotic stress. However, yeast cells induce glycerol or trehalose synthesis instead of proline during various stresses. Previously, Takagi *et al*. isolated the conventional mutant from among the proline toxic analogue-resistant mutants, and that the *PRO1* gene was mutated in the proline toxic analogue-resistant mutants. Interestingly, the proline-accumulating mutant was more tolerant to various stresses, including freezing and desiccation, than the wild-type strain. Hence, it is possible to engineer industrial baker's yeast to accumulate proline. Previous studies reported that self-cloning diploid baker's yeast strains that accumulate proline retained a higher level of fermentation abilities in both frozen and sweet doughs compared to the wild-type strain. According to the Japanese government guidelines, self-cloning yeasts do not have to be treated as genetically modified yeasts, but the conventional methods for breeding baker's yeasts are more acceptable to consumers than the use of self-cloning yeasts.

On the other hand, traditional baking uses a sourdough starter for dough fermentation and this method has several disadvantages, such as the difficulty in keeping the sourdough starter healthy. However, modern food technology has allowed the development of frozen doughs and dried yeasts for use by the bread industry and home bakers. In addition, bakery products using sweet dough have become popular among consumers. During bread-making processes, cells of baker's yeast (usually *Saccharomyces cerevisiae*) are exposed to baking-associated stresses, such as freeze-thaw, air-drying, and high-sucrose concentrations. Therefore, in order to increase the efficiency of yeast used in making frozen dough, dried yeast, and sweet dough, food scientists and engineers have developed several technologies, such as increasing the cellular trehalose level in yeast for frozen dough or isolating stress-tolerant strains from natural sources.

In this study, I isolated mutants resistant to the proline analogue azetidine-2-carboxylate (AZC) derived from diploid baker's yeast of *S. cerevisiae* by the conventional mutagenesis method. Some of the mutants accumulated a greater amount of intracellular proline, and among them, 5 mutants showed higher cell viability than that observed in the parent wild-type strain under freezing or high-sucrose stress conditions. It appears that most of these mutants also enhanced the fermentation ability in both frozen dough and sweet dough. Two clones from these mutants, namely

A6 and a22, carried novel mutations in the *PRO1* gene encoding the Pro247Ser or Glu415Lys variant of GK, the key enzyme in proline biosynthesis in *S. cerevisiae*, respectively.

Interestingly, these mutations were found to result in increased turnover for substrate and desensitization to proline feedback inhibition of GK, leading to intracellular proline accumulation and AZC-resistance. Moreover, baker's yeast cells expressing the *PRO1*^{P247S} and *PRO1*^{E415K} genes were more tolerant to freezing stress than cells expressing the wild-type *PRO1* gene. Pro247Ser and Glu415Lys are distal substitutions, i.e., they are not in the active site or allosteric site on either the primary amino acid sequence or the structural model of the protein. Homology modeling of the *S. cerevisiae* GK using the *E. coli* GK (pdb code: 2j5t) as a template demonstrated that Pro247 in the kinase domain forms a rigid bend that might be responsible for the unfavorable conformation. Therefore, its substitution to Ser may be responsible for the improved enzymatic properties. On the other hand, Glu415 located in the pseudouridine synthase and archaeosine transglycosylase (PUA) domain, which is a highly conserved RNA-binding motif that was widely found in proteins involved in RNA metabolism, is a surface residue that does not reside in the interacting interface of the protein, and hence its role is still unclear.

Previous studies involving self-cloning yeast cells expressing GK variants with mutations at the partially overlapping active and allosteric site, Ile150Thr and Asp154Asn, showed higher AZC-resistance and baking-associated stress tolerance than the wild-type cells. Here, I studied the collaborative effect of the active site and distal mutations. For this purpose, I constructed a library of plasmids harboring double, triple and quadruple variants of GK with all possible combinations of the substitutions Ile150Thr, Asp154Asn, Pro247Ser, and Glu415Lys. All cumulative mutants showed AZC-resistance and their intracellular proline levels were further increased in the double and substitution mutants compared to the single substitution mutants. However, proline accumulation in the triple and quadruple substitution mutants was comparable to that in the double substitution variants. The excessive proline in the cell did not seem to enhance the stress tolerance, suggesting that the proline-glutamate cycle and proline localization in the cell are also important in stress tolerance in addition to the intracellular proline level.

In conclusion, I successfully obtained diploid baker's yeast mutants that accumulate proline, A6 and a22, and demonstrated that these strains are tolerant to freezing stress, which is one of the major baking-associated stresses. Also, I identified some mutations in the target *PRO1* gene and proved that these novel mutations are responsible for the beneficial phenotype in A6 and a22 strains.

Cumulative mutation of GK could be used to further improve the proline accumulation in yeast cells. The conventional mutagenesis approach developed in this study for the selection of AZC-resistant mutants could be a practical method for the breeding of proline-accumulating baker's yeasts with higher tolerance to baking-associated stresses.

1. Introduction

1.1. Baker's yeast and stress

Baking is one of the oldest food cultures in human history, and the use of yeast in leavening bread dough dates back to ancient Egypt. A sourdough starter is used in traditional bread making, and the maintenance of a stable starter presents a major challenge. As opposed to traditional baking, modern technologies offer various opportunities to economize and adjust to the convenience and taste demands of consumers. Modern bread-making techniques include using dry-yeast for the starter, frozen dough and sweet dough. However, during the bread-making process the baker's yeast strains (usually *Saccharomyces cerevisiae*) face various baking-associated stresses (Shima and Takagi, 2009), such as air-drying, freeze-thaw, and high-sucrose. Such stresses decrease the fermentation ability of baker's yeast and affect the properties of the dough, resulting in poor taste and quality of the final products (Pérez-Torrado et al., 2010; Ribotta et al., 2001). Industrial baker's yeast strains are derivatives of domesticated yeast strains, which have been crossbred for their beneficial traits, such as robustness, larger cell size, and high growth rate. Although the genes responsible for these traits are not well-determined, industrial baker's yeast prefers ploidy rather than genetic diversity. However, such a ploidy shift is unfavorable under stress conditions, and commercial baker's strains perform poorly for sweet dough and frozen dough, in which cells are exposed to high osmotic stress. The evolutionary adaptation of polyploidy strains to these stress conditions leads to a decrease in genome size. Regulating genome size is one strategy in baker's yeast, while yeast in general have several mechanisms for adapting to stress, including the accumulation of compatible solutes such as trehalose. It has been well documented that proline functions as a stress protectant in bacteria and plants, and proline accumulation is a natural response to stress in these organisms. On the other hand, proline has been shown to confer stress tolerance to mutants of baker's yeast that was genetically engineered to accumulate proline in the cell (Shima and Takagi, 2009). Previous reports showed that self-cloning baker's yeast strains that accumulate proline are tolerant to various baking-associated stresses, including freeze-thaw, air-drying, and high-sucrose (Kaino et al., 2008; Sasano et al., 2012a, 2012b).

Proline has several protective functions in the cell. In bacterial and plant cells, proline acts as a salinity-related osmoprotectant, along with trehalose, glycerol, and betaine (Takagi, 2008). Exogeneous proline has a cryoprotective effect in yeast that is nearly as potent as the cryoprotective

effects of glycerol or trehalose (Takagi et al., 1997). Moreover, proline stabilizes proteins and the cell membrane (Hayat et al., 2012) and lowers the melting temperature (Tm) of DNA (Rajendrakumar et al., 1997). Proline has also been shown to scavenge reactive oxygen species (ROS) (Kaul et al., 2008; Smirnoff and Cumbes, 1989; Wang et al., 2009). Interestingly, proline significantly reduces ROS levels and increases the survival rate of yeast cells in the stationary phase under ethanol stress conditions (Takagi et al., 2016). Also, proline biosynthesis and proline are shown to play fundamental roles in endoplasmic reticulum (ER) stress protection (Liang et al., 2014).

1.2. Proline metabolism

Proline metabolism and its evolutionary conservations and variations in the three major domains of life have been well reviewed (Fichman et al., 2015). In yeast, proline is synthesized from glutamate in the cytosol via the sequential reactions of glutamate phosphorylation to γ-glutamyl phosphate (GP) by γ-glutamyl kinase (GK), reduction to γ-glutamyl semialdehyde (GSA) by γ-glutamyl phosphate reductase (GPR), spontaneous cyclization to Δ^1-pyrroline-5-carboxylate, and finally a second reduction to proline by Δ^1-pyrroline-5-carboxylate reductase (P5CR) (Figure 1). Although yeast has an alternative pathway to produce proline through arginine-ornithine metabolism involving ornithine transaminase and arginase, the pathway via GP is the main contribution to proline in the cell. GK is the rate-limiting enzyme in the yeast proline biosynthesis and its activity is inhibited by proline, which binds to the partially overlapping active/allosteric site. In bacteria and yeast, conversion of glutamate to GP and GP to GSA/P5C are each catalyzed by separate enzymes, whereas in higher eukaroytes these two enzymes are evolved to one bifunctional enzyme with two moieties homologous to GK and GPR.

On the other hand, proline can be utilized as a nitrogen source via the glutamate-glutamine cycle or a carbon source via the TCA cycle. Proline degradation occurs in the mitochondria with the help of the enzymes proline oxidase (POX) and pyrroline-5-caroxylate dehydrogenase (P5CDH).

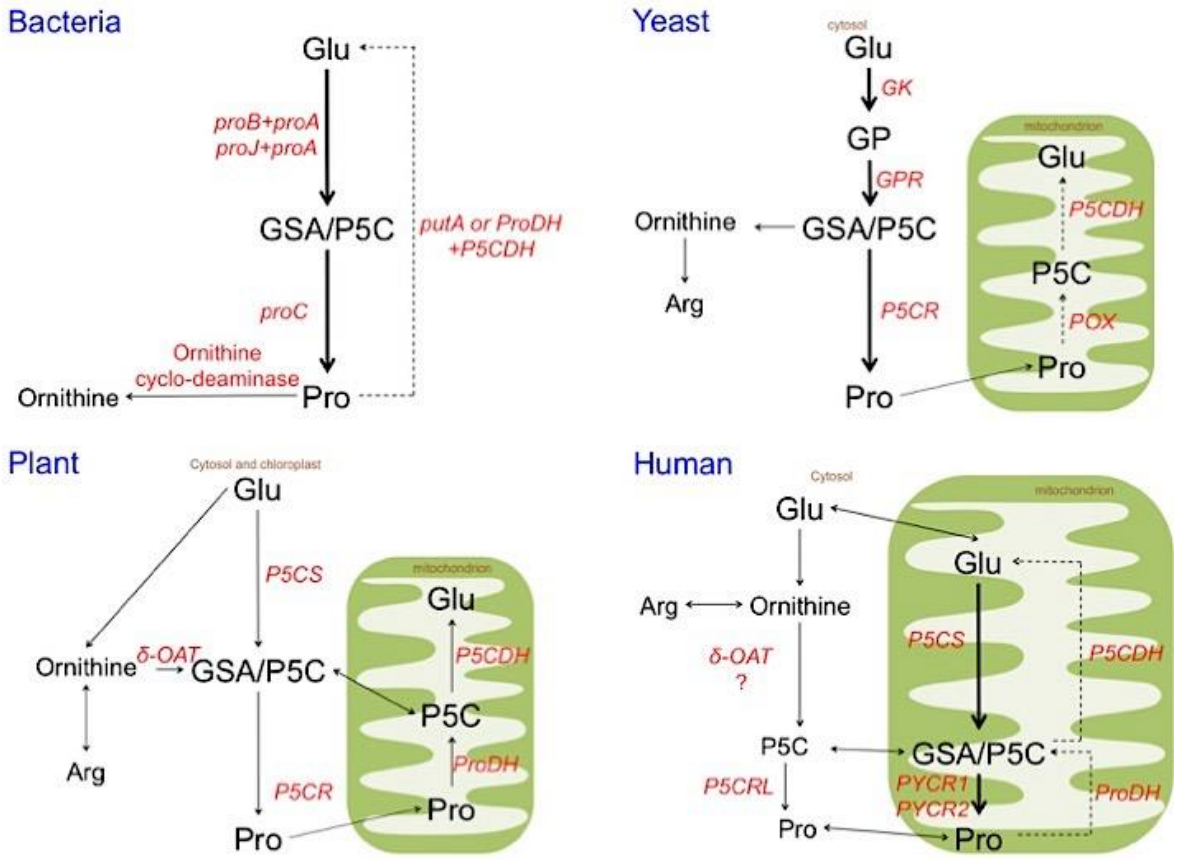

Figure 1. Proline metabolism in bacteria, yeast, plant and human.
GP: *γ-glutamyl phosphate*, GSA: *γ-glutamyl semialdehyde*, P5C: *Δ^1-pyrroline-5-carboxylate*, GK: *γ-glutamyl kinase*, GPR: *γ-glutamyl phosphate reductase*, ProDH: *proline dehydrogenase*, P5CDH: *pyrroline-5-caroxylate dehydrogenase*, POX: *proline oxidase*, δ-OAT: *ornithine-δ-aminotransferase*, P5CS: *Δ^1-pyrroline-5-carboxylate synthetase*, P5CR: *Δ^1-pyrroline-5-carboxylate reductase*, PYCR: *Pyrroline-5-Carboxylate Reductase*

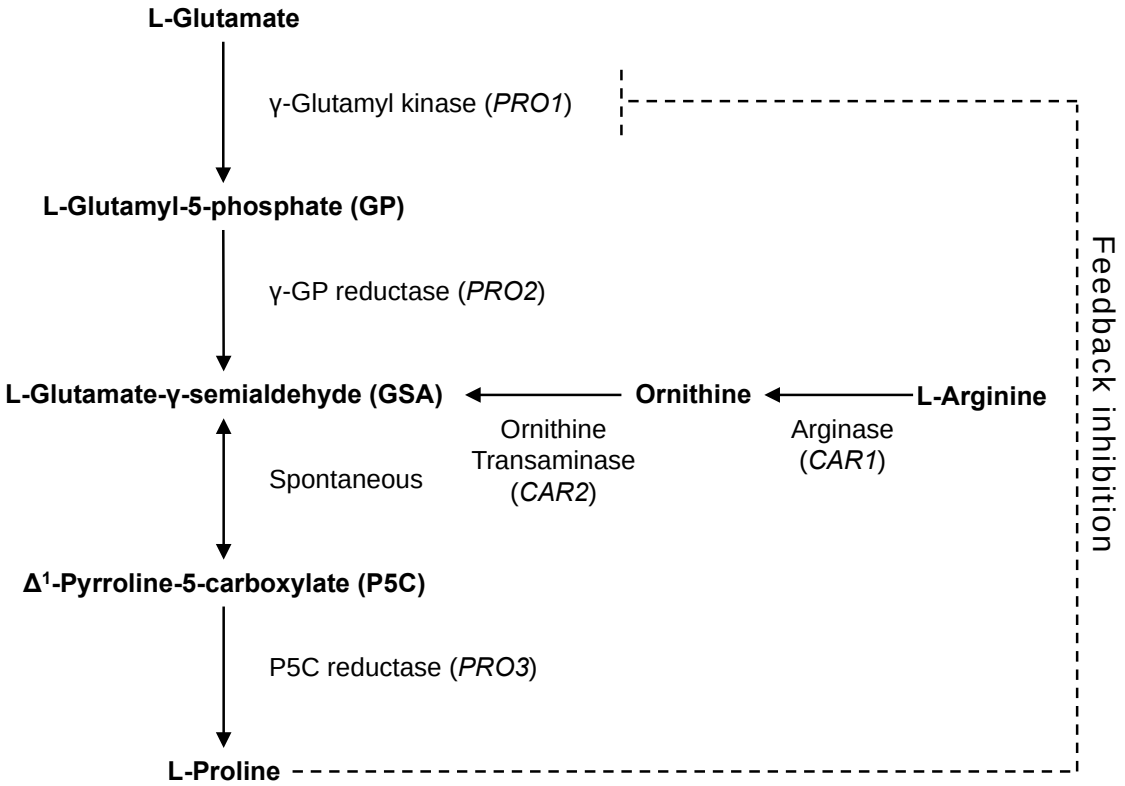

Figure 2. Yeast proline biosynthesis and its regulation.
Proline is synthesized from glutamate. γ-glutamyl kinase (GK), encoded by *PRO1*, is the key enzyme that catalyzes the rate-limiting step of proline biosynthesis in yeast. GK is feedback-inhibited by the end-product proline. Yeast has an alternative pathway for proline synthesis via arginine-ornithine.

1.3. γ-Glutamyl kinase

γ-glutamyl kinase (GK) is the rate-limiting enzyme of proline biosynthesis from glutamate, and its activity is regulated allosterically by the end-product proline (Sekine et al., 2007) (Figure 2). Proline-accumulating strains usually have a mutation on the *PRO1* gene, which encodes GK. Yeast GK consists of an N-terminal kinase domain (KD), a C-terminal PUA (PseudoUridine synthase and Archaeosine transglycosylase) domain and a short linker domain connecting the two (Figure 3). GK contains a highly conserved region in the KD, which is the partially overlapping active and allosteric site of the enzyme (Figure 4). The residues around position 150 in the KD are important for binding with glutamate and proline and are used as a target for mutagenesis to remove the feedback inhibition of the enzyme by proline (Kaino et al., 2008; Takagi et al., 1997). The role

of the PUA domain in yeast GK is not indispensable, but the linker region between the KD and PUA domain is needed for full catalytic activity (Kaino et al., 2012; Tatehashi and Takagi, 2013). In addition, GK is involved in selective autophagy of ribosomes during nitrogen starvation, and the PUA domain might be involved in the interaction with rRNA (Tatehashi et al., 2016).

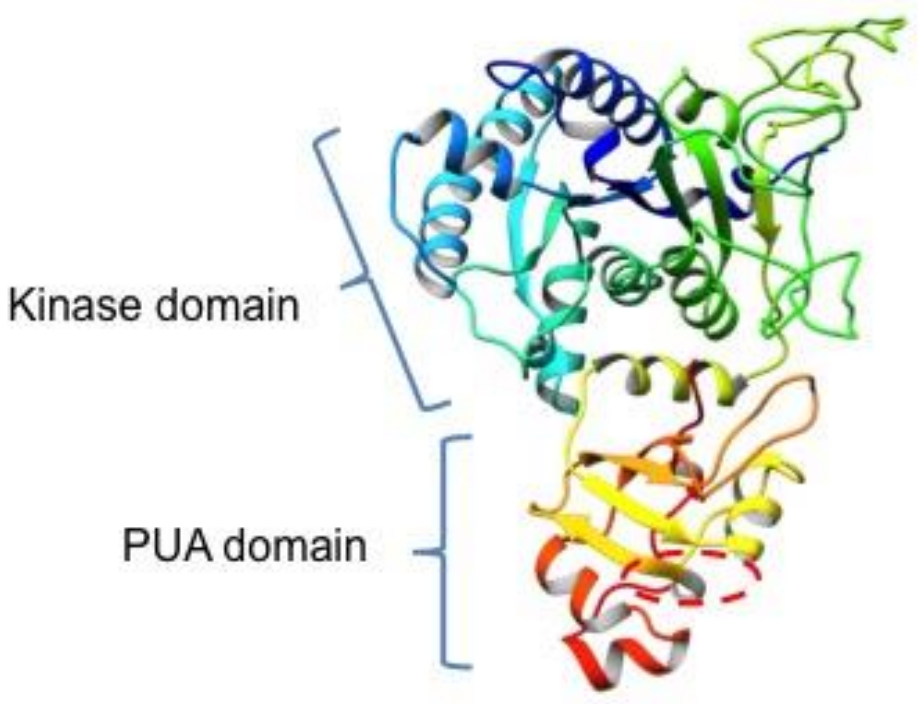

Figure 3. Yeast GK domain structure.
Obtained by homology modeling based on *E.coli* GK (2j5t), yeast GK consists of kinase domain (KD) and pseudouridine synthase and archaeosine transglycosylase (PUA) domain connected by a short linker.

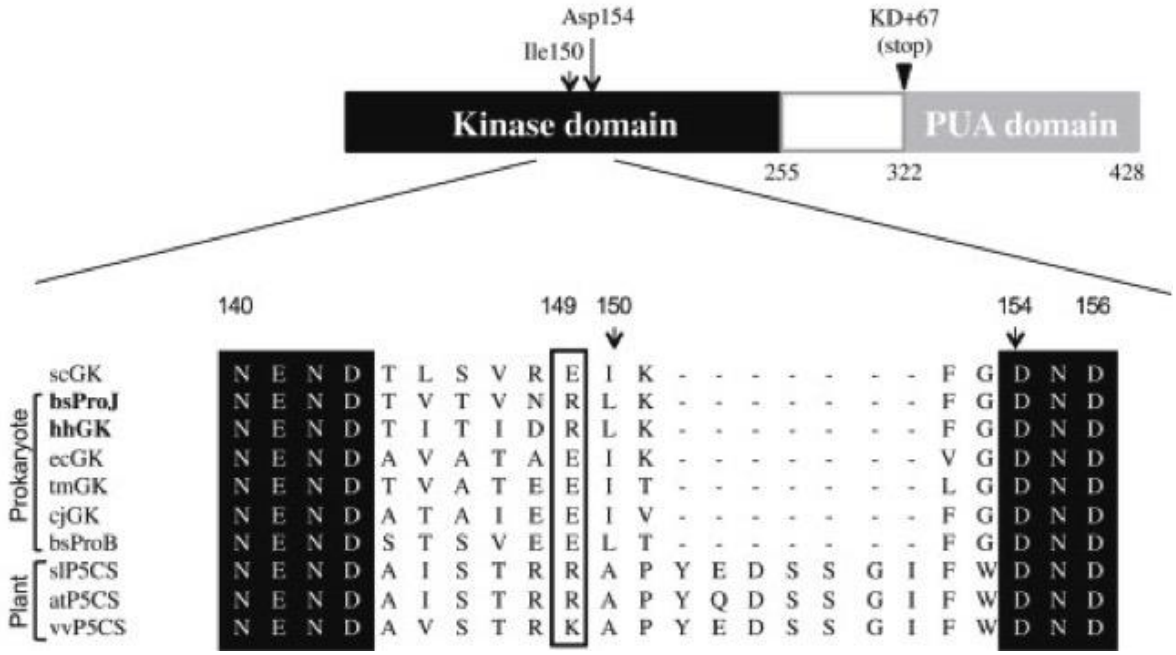

Figure 4. Schematic representation of the S. cerevisiae GK and the alignment of the amino acid sequences around at position 150.
(Tatehashi and Takagi, 2013). Arrows indicate the mutation points. Numbers indicate positions of amino acid residues of the *S. cerevisiae* GK. Identical amino acid residues are shown as black shaded boxes. The residues at position 149 important for feedback inhibition by proline are surrounded by a black box. bsProJ and hhGK shown as bold letters are putative proline-insensitive GK. The sequences used were *Saccharomyces cerevisiae* GK (scGK), *Bacillus subtilis* ProJ and ProB (bsProJ and bsProB), *Halobactillus halophilius* (hhGK), *Escherichia coli* GK (ecGK), *Thermotoga maritime* GK (tmGK), *Campyrobactor jejuni* GK (cjGK), *Solanum lycopersicum* P5CS (slP5CS), *Arabidopsis thaliana* P5CS (atP5CS), and *Vitis vinifera* P5CS (vvP5CS).

1.4. Genetic engineering of proline accumulating yeast

Proline-accumulating yeast strains usually have a mutation in the *PRO1* gene, which encodes γ-glutamyl kinase (GK). GK is the rate-limiting enzyme of proline biosynthesis from glutamate, and its activity is regulated allosterically by the end product proline (Sekine et al., 2007) (Figure 2). The majority of proline-accumulating strains have been obtained by expressing GK variants that are less-sensitive to feedback inhibition by proline (Morita et al., 2003; Sekine et al., 2007). A toxic analogue of proline, azetidine-2-carboxylate (AZC), enters cells via proline permeases and when misincorporated into protein in the place of proline, causes misfolding and thermal instability of the proteins, leading to toxicity in vivo (Trotter et al., 2002). However, by introducing mutation(s) favoring proline accumulation in the corresponding gene(s), the proteins of mutant

cells incorporate proline and the cells become resistant to AZC. In this way, an increased intracellular concentration of proline reduces the toxic effect of AZC.

The first work to obtain AZC-resistant proline-accumulating yeast was done using laboratory yeast by conventional mutagenesis, and the resulting proline-accumulating strains were shown to be tolerant to freeze-thaw stress (Takagi et al., 1997). A subsequent genetic analysis revealed that the D154N mutation in *PRO1* was responsible for the tolerance to AZC (Morita et al., 2003). Next, a PCR random mutagenesis was used to introduce additional beneficial mutations in *PRO1* in order to obtain various mutants with proline accumulation and stress tolerance (Sekine et al., 2007). In addition, baker's yeast mutants with increased freezing stress tolerance were developed by introducing *PRO1* mutations with the help of the self-cloning method (Kaino et al., 2008). In all these attempts to obtain proline accumulating stress-tolerant strains from AZC-resistant mutants, some genetic modification with foreign DNA was involved. Using genetically modified organisms in the food industry is still controversial due to questions about the safety of the products produced. Hence there is a need to isolate baker's yeast strains that will permit safer genetic modifications, such as those using conventional mutagenesis or self-cloning (Steensels et al., 2014). However, consumers are still skeptical about the safety of using self-cloning microorganisms in the production of food for direct and/or indirect consumption (Kayabaşı and Mucan, 2011). Therefore, it might be possible to obtain genetically improved microorganisms by exposing wild-type cells to a conventional mutagen and selecting mutants with improved traits.

1.5. Objectives of my research

As mentioned in the previous section, the use of safer genetic engineering techniques for strain improvement in the microorganisms used for baking and other food-production methods is always preferred. Therefore, building on a previously established technique for conferring proline-accumulation to laboratory yeast, I herein focused on isolating stress-tolerant baker's yeast from proline-accumulating strains that were in turn obtained from AZC-resistant mutants. In addition, in an attempt to increase proline accumulation, I developed recombinant baker's yeast strains with cumulative mutations in *PRO1* and further analyzed the role of excessive intracellular proline in stress tolerance in baker's yeast.

2. Material and methods

2.1. Strains, culture media and plasmids

Japanese diploid baker's yeast strain of *S.cerevisiae* 3346/3347 is used in this study. For plasmid construction, *E.coli* DH5α strain ($F^-\lambda^-$*Φ80lacZΔM15* Δ(*lacZYA argF*)*U169 deoR recA1 endA1 hsdR17*($r_k^-m_k^+$) *supE44 thi-1 gyrA96*) is used. For expression of yeast recombinant protein, Rosetta^{TM}DE3 [F^-*ompT hsdS*$_B$($r_B^-m_B^-$) *gal dcm* (DE3) pRARE (Cam^R)] (Merck) strain is used. *S. cerevisiae* cells were grown in a nutrient rich medium YPD (1% yeast extract, 2% peptone, and 2% glucose), synthetic defined medium SD+Am or SD-N+All (2% glucose, 0.67% yeast nitrogen base without ammonium sulfate and amino acids [Difco Laboratories, Detroit,MI, USA], and 0.5% ammonium sulfate or 0.5% allantoin, respectively), and cane molasses medium (5.88% NEOMOLASSEST [EMlaboratory, Shizuoka, Japan], 0.193% urea, and 0.046% KH2PO4). The composition of the pre-fermentation medium for freezing stress and the liquid fermentation medium for high-sucrose stress was the same as previously described (Sasano et al., 2012a, 2012b), respectively, except that the sucrose concentration was 27.8% and the maltose concentration was 2.78%.. The *S. cerevisiae* recombinant strains were grown in YPD medium or SD-N+All containing 200 μg/ml G418. *Escherichia coli* recombinant strains were grown in Luria-Bertani (LB) complete medium containing 100 μg/ml ampicillin. When necessary, 2% agar was added to solidify the medium.

2.2. Isolation of AZC-resistant mutant

AZC-resistant mutants were obtained by conventional mutagenesis. Random mutations were induced by treatment with 5% ethyl methanesulfonate (EMS) (Rose and Broach, 1991). The mutagenized cells were spread onto SD+Am agar plates containing 2 mg/ml AZC (Bachem, Bubendorf, Switzerland). After incubation at 30 ℃ for 3 days, the resulting colonies were collected and tested for AZC resistance and amino acid production.

2.3. Spot test for AZC resistance

Yeast cells were cultured in 3 ml of YPD medium. After overnight incubation at 30 ℃ with rotary shaking, cells corresponding to an OD_{600} of 1.0 were collected, washed twice, suspended in 1 ml of water, and serially diluted. 5 μl of diluted cell suspensions were spotted on SD-N+All agar

plates containing 2mg/ml or 5mg/ml AZC and incubated at 30 ℃ for 2–7 days. Transcription of the PUT4 gene encoding a proline specific permease is repressed by ammonia (Andréasson et al., 2004) and hence allantoin was used as the nitrogen source to allow uninterrupted influx of AZC.

2.4. Measurement for intracellular amino acid content

Yeast cells were cultured in 3 ml of YPD or cane molasses medium. After overnight cultivation at 30 ℃, 1 ml of the cultured sample was washed and inoculated into 30 ml of the above medium. After cultivation for 48 h at 30 ℃ with rotary shaking, cells corresponding to an OD_{600} of 10 were collected, washed twice and suspended in 0.5 ml of distilled water. Intracellular amino acids in cell suspension were extracted by boiling for 20 min at 100 ℃. After centrifugation (15,000×g, 5 min, 4 ℃), amino acid content in each supernatant was subsequently quantified with amino acid analyzer (AminoTac JLC-500/V; JEOL, Tokyo, Japan). The content of each amino acid was expressed as a percentage of dry cell weight.

2.5. Stress tolerance test

For freeze-thaw stress, the stationary phase cells grown in cane molasses medium for 48 hrs at 30 ℃ with rotary shaking were inoculated in pre-fermentation medium at an initial OD600 of 1.0 and incubated for 4 hrs. Cells corresponding to an OD_{600} of 1.0 were collected, washed twice, and suspended in 1 ml of distilled water. Cell suspension was divided into 100 μl aliquots and frozen at−30 ℃ for 1, 3 and 7 days. After freezing, cell suspension was thawed in at room temperature, then serially diluted and spotfted on YPD agar plates. All the YPD plates were incubated for 2 days at 30 ℃. To determine cell viability, 100 μl of serially diluted suspension was spread on YPD agar plates. After incubation at 30 ℃ for 2 days, the numbers of colonies were counted and the survival cell rates were expressed as percentages, which were calculated as follows: [(number of colonies after freeze-thaw stress) / (number of colonies before freeze-thaw stress)] × 100.

For high-sucrose stress, the stationary phase cells grown in YPD or cane molasses medium for 48 hrs at 30 ℃ with rotary shaking were collected, washed twice, and suspended in 1 ml of distilled water at an OD_{600} of 1.0. Cell suspension was serially diluted and spotted on YPD agar plates containing 40% glucose. All the YPD plates were incubated for 2 days at 30 ℃. To determine cell

viability, the stationary phase cells grown in YPD or cane molasses medium for 48 hrs at 30 °C with rotary shaking were inoculated in 5 ml of liquid fermentation medium at an initial OD_{600} of 1.0 and incubated at 30 °C for 0, 2, 4 and 6 hrs. After incubation, cells were collected, washed twice, and suspended in 1 ml of distilled water. 100 µl of serially diluted suspension was spread on YPD agar plates. After 2 days of incubation at 30 °C, the numbers of colonies were counted and the survival cell rates were expressed as percentages, which were calculated as follows: [(number of colonies after high-sucrose stress) / (number of colonies before high-sucrose stress)] × 100.

2.6. Fermentation test

Fermentation ability was tested in high-sucrose bread dough and frozen dough. For high-sucrose bread dough, 100 g of bread-making flour, 3 g of yeast (66% moisture basis), 30 g of sucrose, 0.5 g of NaCl, and 52 ml of water was mixed thoroughly to make the dough. Total CO_2 gas production was monitored throughout the 2 h incubation at 28 °C. For frozen dough, 100 g of bread-making flour, 3 g of yeast (66% moisture basis), 5 g of sucrose, 0.5 g of NaCl, and 52 ml of water was mixed thoroughly to make the dough. Total CO_2 gas production was monitored throughout the 2 hrs incubation at 30 °C using a Fermograph II (Atto, Tokyo, Japan). The prepared dough was frozen for 7 days at −20 °C and then after thawing for 30 min at 30 °C, total CO_2 gas production was monitored throughout the 1 h incubation at 35 °C.

2.7. Gene cloning and sequencing

The full-length PRO1 gene flanked by 500 bp upstream and downstream of the open reading frame (ORF) was amplified from the genomic DNA of AZC-resistant mutant strains A6 and a22 by high-fidelity PCR using KOD Plus DNA polymerase (Toyobo, Osaka, Japan) using primers PRO1-0 (5′-CAG TGA AGT GTT CAA GGG-3′) and PRO1-5 (5′-CTT CCA AGG GTA GGA AA-3′). To confirm the introduced mutations, the PCR products were sequenced by DNA sequencing. The PRO1 gene DNA sequences of baker's yeast mutants were compared to that of the laboratory strain *S. cerevisiae* S288C using the BLAST search program. The GeneBank accession number for the PRO1 gene is P32264.

2.8. Construction of expression plasmids for the PRO1 genes

For the construction of plasmid pYC130-PRO1 expressing the wild-type PRO1 gene, the P_{ADH1}-PRO1-T_{ADH1} fusion was amplified by high-fidelity PCR using KOD Plus DNA polymerase from pAD-WTPRO1 (Kaino et al., 2012) as the template with primers in-fusion Fw:PRO1 (5′-GCG TAC GCG TCG ACG GTA CCG GGA TCG AAG AAA TGA TGG TAA ATG-3′) and in-fusion Rv:PRO1 (5′-GTT TAA ACG AAT TCG GTA CCG TGT GGA AGA ACG ATT ACA ACA GG-3′) and was cloned into the KpnI site of PYC130 by In-fusion HD Cloning Kit (Takara Bio, Otsu, Japan). The underlined sequences indicate the positions of the KpnI restriction site.

Plasmids pYC130-PRO1-variants, expressing the mutated PRO1 genes were constructed from pYC130-PRO1 using QuickChange Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) with primers Pro1(var)Fw-quickchange and Pro1(var)Rv-quickchange. For the construction of plasmids with multiple substitution, plasmid with single substitution was used as template and primers Pro1(var)Fw-quickchange and Pro1(var)Rv-quickchange was used for QuickChange Site-Directed Mutagenesis to obtain desired plasmids. The nucleotide sequences of the wild-type and mutated PRO1 genes in the newly constructed plasmids were confirmed by DNA sequencing. *S. cerevisiae* strain 3346/3347 was transformed with pYC130-PRO or its variants by lithium acetate method. Transformants were selected on YPD agar plates containing 200 μg/ml G418.

To express and purify the recombinant enzymes from E. coli, plasmids pET53-PRO1(P247S) and pET53-PRO1(E415K) were constructed by Gateway technology (Invitrogen, Carlsbad, CA, USA). A gateway entry vector was generated by BP reaction with pDONR221 using PCR-amplified fragments with forward primer PRO1(attB1)-F (5′-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT AAT GAA GGA TGC TAA TGA GAG TAA ATC G-3′) and reverse primer PRO1(attB2)-R (5′-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTG ACG AGG TGG GAA TGC CAA ATT TTC-3′) (the underlined part of the forward and reverse primer sequences indicates the attB1 and attB2 sequences, respectively) from pQE2-PRO1P247S and pQE2-PRO1E415K. Next, each ORF was cloned into a pET53-DEST vector via an LR reaction. pQE2-PRO1P247S and pQE2-PRO1E415K plasmids were constructed from pQE2-PRO1 (Sekine et al., 2007) using QuickChange Site-Directed Mutagenesis Kit (Agilent Technologies, La Jolla, CA, USA) with primers Pro1(P247S)Fwquickchange (5′-C ATG AAA AGC GAT ACA T*CT GCG AAT ATA GGT AG-3′) and Pro1(P247S)Rv-quickchange (5′-CT ACC TAT ATT CGC AGA*

TGT ATC GCT TTT CAT G-3′); Pro1(E415)Fw-quickchange (5′-GGC TAT AAT GAC AGC A*AA TAT GTC GCT CAT AG-3′) and Pro1(E415)Rv-quickchange (5′-CT ATG AGC GAC ATA TTT* GCT GTC ATT ATA GCC-3′), respectively. The asterisk indicates the position of nucleotide mutations. For the construction of plasmids with multiple substitution, plasmid pDONR221-PRO1 with single substitution was used as template and primers Pro1(var)Fw-quickchange and Pro1(var)Rv-quickchange was used for QuickChange Site-Directed Mutagenesis to obtain desired plasmids. The nucleotide sequences of the wild-type and mutated PRO1 genes in the newly constructed pDONR221 and pQE2 plasmids were confirmed by DNA sequencing.

2.9. Expression and purification of recombinant GK

The recombinant enzyme was expressed and purified as described previously (Tatehashi and Takagi, 2013). The plasmids pET53-PRO1, pET53-PRO1(I150T), pET53-PRO1(P247S), and pET53-PRO1(E415K) were introduced into E. coli Rosetta DE3 [F^- *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3) (CamR)]. Protein concentrations were determined with a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as a standard protein.

2.10. Assay of GK activity

The GK activity was measured at 30 °C by phosphate assay as described previously (Tatehashi and Takagi, 2013). The reaction mixture [final volume, 100 µl (pH 7.4)] contained the following: 400mMglutamate, 20mM ATP, 100mM $MgCl_2$, enzyme, and elution buffer. The glutamate was varied (40–1200 mM) in the presence of fixed concentrations of 20 mM ATP and 100 mM $MgCl_2$. When the proline was varied (0–500 mM), 400 mM glutamate, 20 mM ATP, and 100 mM MgCl2 were used. One unit of activity was defined as the amount of enzyme required to produce 1 µmol of inorganic phosphate (Pi) per min. The program GraphPad Prism (GraphPad Software, San Diego, CA, USA) was used for curve fitting of kinetic data.

Table 1. List of oligonucleotides used in this study

Oligonucleotide	Sequence
in-fusion Fw:PRO1	5’-GCG TAC GCG TCG ACG GTA CCG GGA TCG AAG AAA TGA TGG TAA ATG-3’ underlined part indicates *Kpn*I site
in-fusion Rv:PRO1	5’-GTT TAA ACG AAT TCG GTA CCG TGT GGA AGA ACG ATT ACA ACA GG-3’ underlined part indicates *Kpn*I site
Pro1(I150T)Fw-quickchange	5’-GTT AGA GAA AC*C AAA TTT GGT GAC AAT GAC-3’
Pro1(I150T)Rv-quickchange	5’-ACC AAA TTT GG*T TTC TCT AAC AGA TAG TGT-3’
Pro1(D154N)Fw-quickchange	5’-T AGA GAA ATC AAA TTT GGT A*AC AAT GAC AC-3’
Pro1(D154N)Rv-quickchange	5’-GT GTC ATT GTT* ACC AAA TTT GAT TTC TCT A-3’
Pro1(P247S)Fw-quickchange	5’-C ATG AAA AGC GAT ACA T*CT GCG AAT ATA GGT AG-3’)
Pro1(P247S)Rv-quickchange	5’-CT ACC TAT ATT CGC AGA* TGT ATC GCT TTT CAT G-3’
Pro1(E415)Fw-quickchange	5’-GGC TAT AAT GAC AGC A*AA TAT GTC GCT CAT AG-3’
Pro1(E415)Rv-quickchange	5’-CT ATG AGC GAC ATA TTT* GCT GTC ATT ATA GCC-3’
Pro1(I150T/D154N)Fw-quickchange	5’-TCT GTT AGA GAA AC*C AAA TTT GGT A*AC AAT-3’
Pro1(I150T/D154N)Rv-quickchange	5’-TAA AGT GTC ATT GTT* ACC AAA TTT GG*T TTC-3’

PRO1(attB1)-F	5′-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT AAT GAA GGA TGC TAA TGA GAG TAA ATC G-3′
	underlined part indicates the attB1 sequences
PRO1(attB2)-R	5′-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTG ACG AGG TGG GAA TGC CAA ATT TTC-3′
	underlined part indicates the attB2 sequences

*asterisk indicates the position of nucleotide mutation

Table 2. List of plasmids used in this study

Plasmids	Description	Source
pAD-WTPRO1	P_{ADH1}-$PRO1^{WT}$-T_{ADH1}	(Kaino et al., 2012)
pYC130-PRO1	P_{ADH1}-$PRO1^{WT}$-T_{ADH1}	This study
pYC130-PRO1(P247S)	P_{ADH1}-$PRO1^{P247S}$-T_{ADH1}	This study
pYC130-PRO1(E415K)	P_{ADH1}-$PRO1^{E425K}$-T_{ADH1}	This study
pYC130-PRO1(I150T)	P_{ADH1}-$PRO1^{I150T}$-T_{ADH1}	This study
pYC130-PRO1(D154N)	P_{ADH1}-$PRO1^{D154N}$-T_{ADH1}	This study
pYC130-PRO1(I150T/P247S)	P_{ADH1}-$PRO1^{I150T/P247S}$-T_{ADH1}	This study
pYC130-PRO1-I150T/E415K	P_{ADH1}-$PRO1^{I150T/E415K}$-T_{ADH1}	This study
pYC130-PRO1-D154N/P247S	P_{ADH1}-$PRO1^{D145NT/P247S}$-T_{ADH1}	This study
pYC130-PRO1-D154N/E415K	P_{ADH1}-$PRO1^{D154N/E415K}$-T_{ADH1}	This study
pYC130-PRO1-P247S/E415K	P_{ADH1}-$PRO1^{P247S/E415K}$-T_{ADH1}	This study
pYC130-PRO1-I150T/D154N	P_{ADH1}-$PRO1^{I150T/D154N}$-T_{ADH1}	This study
pYC130-PRO1-I150T/P247S/E415K	P_{ADH1}-$PRO1^{I150T/P247S/E415K}$-T_{ADH1}	This study
pYC130-PRO1-D154N/P247S/E415K	P_{ADH1}-$PRO1^{D154N/P247S/E415K}$-T_{ADH1}	This study
pYC130-PRO1-I150T/D154N/P247S	P_{ADH1}-$PRO1^{I150T/D154N/P247S}$-T_{ADH1}	This study

pYC130-PRO1-I150T/D154N/E415K	P_{ADH1}-$PRO1^{I150T/D154N/E415K}$-T_{ADH1}	This study
pYC130-PRO1-I150T/D154N/P247S/E415K	P_{ADH1}-$PRO1^{I150T/D154N/P247S/E415K}$-$T_{ADH1}$	This study
pQE2-PRO1		(Sekine et al., 2007)
pQE2-PRO1P247S		This study
pQE2-PRO1E415K		This study
pDONR221		
pDONR221-PRO1(WT)		(Tatehashi and Takagi, 2013)
pDONR221-PRO1(P247S)		This study
pDONR221-PRO1(E415K)		This study
pDONR221-PRO1(I150T)		(Tatehashi and Takagi, 2013)
pDONR221-PRO1(D154N)		This study
pDONR221-PRO1-I150T/P247S		This study
pDONR221-PRO1-I150T/E415K		This study
pDONR221-PRO1-D154N/P247S		This study
pDONR221-PRO1-D154N/E415K		This study
pDONR221-PRO1-P247S/E415K		This study
pDONR221-PRO1-I150T/D154N		This study
pET53		
pET53-PRO1(WT)		(Tatehashi and Takagi, 2013)
pET53-PRO1(P247S)		This study
pET53-PRO1(E415K)		This study
pET53-PRO1(P247S)		This study

pET53-PRO1(I150T)	This study
pET53-PRO1(D154N)	This study
pYC130-PRO1-I150T/P247S	This study
pYC130-PRO1-I150T/E415K	This study
pYC130-PRO1-D154N/P247S	This study
pYC130-PRO1-D154N/E415K	This study
pYC130-PRO1-P247S/E415K	This study
pYC130-PRO1-I150T/D154N	This study

3. Results

3.1. Isolation of baker's yeast mutants with increased stress tolerance and identification of favorable mutations

3.1.1. Isolation of baker's yeast mutants with proline accumulation from AZC resistant mutants

Since AZC is known to be a toxic analogue for proline in laboratory and baker's yeast (Kaino et al., 2008; Takagi et al., 1997), I attempted to isolate AZC-resistant mutants from the diploid baker's yeast strain 3346/3347. When cells treated with 5% EMS were directly plated on SD agar plates containing 2 mg/ml AZC, a total of approximately 45 AZC-resistant colonies were collected from two independent experiments. No colonies were grown spontaneously on the AZC-containing agar plates from the cells without mutagenesis treatment (Figure 5).

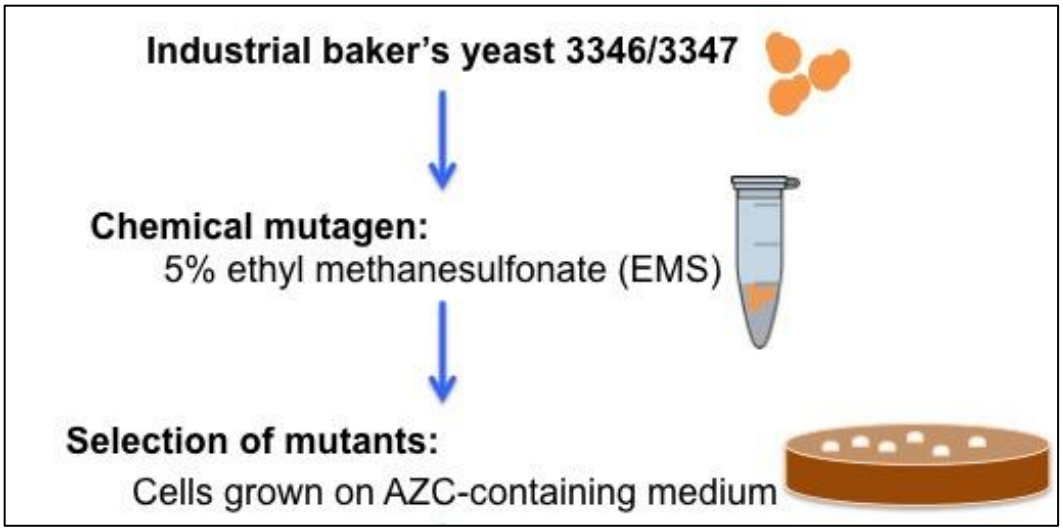

Figure 5. Flowchart of isolating AZC-resistant proline-accumulating baker's yeast mutant by conventional mutagenesis.
Industrial diploid baker's yeast strain 3346/3347 is treated with 5% ethyl methanesulfonate (EMS) and then grown on SD medium containing 2 mg/ml AZC for selection of AZC-resistant proline-accumulating mutants.

The AZC-resistance of the obtained candidates was analyzed by a spot test on SD-N+All medium containing 2 mg/ml AZC. The results confirmed that all the presumed AZC-resistant colonies were indeed resistant to AZC (Figure 6). Next, intracellular amino acids were extracted

from the AZC-resistant mutants and their parental strain, and their contents were quantified with an amino acid analyzer. As expected, many of the mutants had higher levels of intracellular proline than the parent strain (WT), with the difference being as high as 9-fold in mutant A18 (Figure 7).

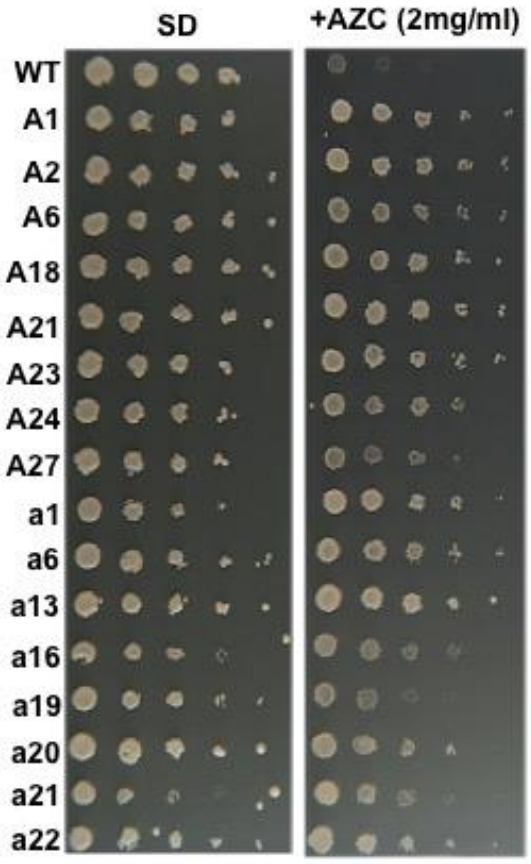

Figure 6. Spot test for AZC-resistance of candidate strains obtained by conventional mutagenesis
Wild-type industrial baker's yeast 3346/3347 (WT) is used as positive control. 1 OD_{600} cells were 10-fold serially diluted to 10^{-4} (from left to right), spotted on SD medium containing 2mg/ml AZC and incubated at 30 ^{O}C for approximately 2 days.

However, the intracellular proline levels in several AZC-resistant mutants were lower than that in the WT. In the mutants with increased levels of intracellular proline, the increase appeared to be mainly due to several mutations occurring during the chemical mutagenesis, particularly in the genes involved in proline metabolism, which were picked out by selection on toxic AZC-containing medium. However, the case of decreased proline levels could be explained by the possibility of mutations in the proline transporter genes, since AZC enters the cell via proline transporters (Andréasson et al., 2004), and hence the reduced uptake and/or increased export of AZC via mutant transporter genes would reduce the toxicity of AZC in the medium.

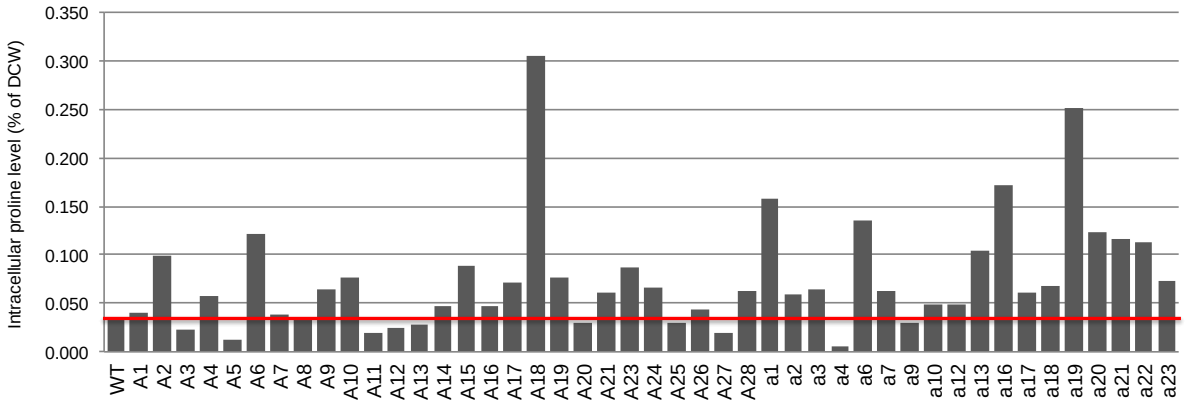

Figure 7. Intracellular proline level of AZC-resistant baker's yeast mutants.
Yeast cells grown in cane molasses medium for 48 h at 30 ^{O}C were collected and intracellular amino acids were extracted by boiling the cell suspension in water. After centrifugation, amino acid content in each supernatant was quantified with amino acid analyzer. Amount of proline is shown as the percentage of dry cell weight (DCW). Red line indicates the level of intracellular proline in the parental strain (WT).

3.1.2. Selection of baker's yeast mutants with enhanced tolerance to baking-associated stress conditions

Based on the profiles of intracellular proline (Figure 7), 16 AZC-resistant mutants were selected for further analysis. These mutants, all of which exhibited increased accumulation of intracellular proline compared to the WT, were subjected to pre-screening by spot test for their freezing and osmotic stress tolerance (Figure 8, Figure 9). As compared to the WT, all the strains except A6, A23, a6, a13, a19, and a22 showed poorer growth in the spot test after freezing stress (Figure 8), and all those except A1, A6, A21, A23, a6, a13, a16, a19, and a22 showed poorer growth in the spot test after osmotic stress (Figure 9). The strains that showed reduced growth in either of the spot tests were then excluded from consideration, and further tests were conducted on strains

A6, A23, a6, a13, a19, and a22, which showed growth equivalent to that of the WT in both the freezing and osmotic-stress spot tests.

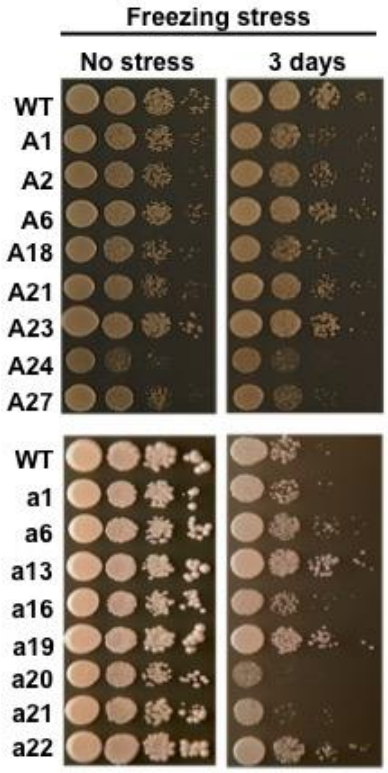

Figure 8. Spot test for freezing-stress tolerance of proline-accumulating baker's yeast mutants.
After cultivation of 48 h at 30 ^{O}C in cane molasses medium, yeast cells were collected, serially diluted, and spotted on YPD agar plates. Also, aliquots of the cells suspension in water was frozen at −30 ^{O}C; then the frozen cell suspension was thawed at 30 ^{O}C for 20min, serially diluted and spotted on YPD agar plates. All spotted plates were incubated for approximately 2 days at 30 ^{O}C.

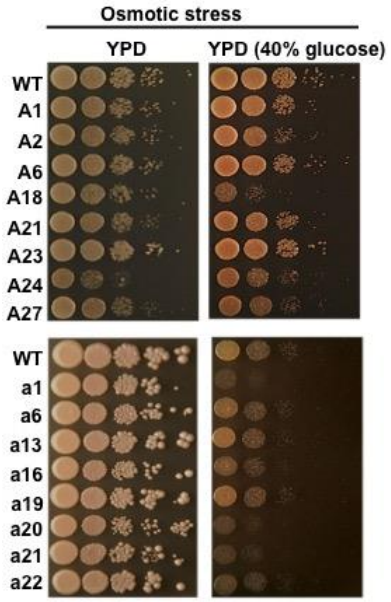

Figure 9. Spot test for high-sugar stress tolerance of proline-accumulating baker's yeast mutants
After cultivation in cane molasses medium for 48 h at 30 ^{O}C, yeast cells were collected, serially diluted, and spotted on YPD agar plates containing 40% glucose. All spotted plates were incubated for several days at 30 ^{O}C.

Next, I performed a cell viability test with the selected strains that accumulated proline after freezing and hyperosmotic stress (Figure 10, Figure 11). Since freeze-thaw and high-sucrose stresses are two major types of baking-associated stress that are experienced by baker's yeast, the relative increase in cell viability was attributed to the stress tolerance of the mutant baker's yeast strains. Viability test results showed that strains A6, a6, a13, and a19 had higher relative cell viability than the WT after freezing stress (Figure 10), whereas the relative survival rates for strains a6, a13, a19, a22 were higher than that of the WT after the high-sucrose stress (Figure 11).

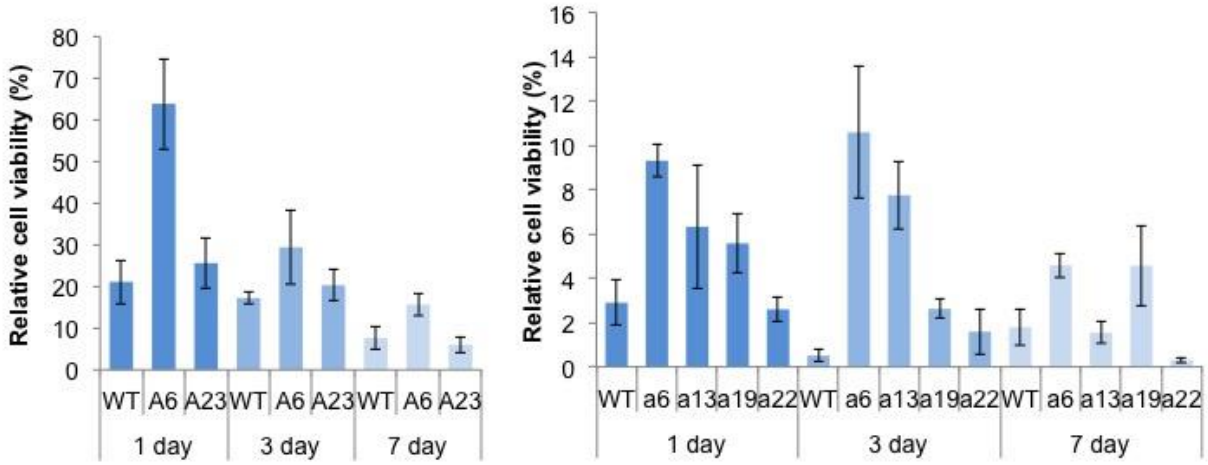

Figure 10. Cell viability test for freezing-stress tolerance of proline-accumulating baker's yeast mutants

Stationary phase cells grown in cane molasses medium for 48 h at 30 ^{O}C were inoculated in pre-fermentation medium and incubated for 4 h. Cells were collected and the cell suspension was divided into 100 µl aliquots and frozen at −30 ^{O}C for 1, 3 and 7 day. The frozen cell suspensions were thawed at room temperature, serially diluted and 100 µl of the diluted suspension was spread on YPD agar plates. After 2 days of incubation at 30 ^{O}C, colonies were counted and number of colonies was expressed in colony forming unit (CFU). CFU without stress was defined as 100%. The values are the means and standard deviations from three independent experiments. Statistically significant differences were determined by Student's t-test (*p< 0.05).

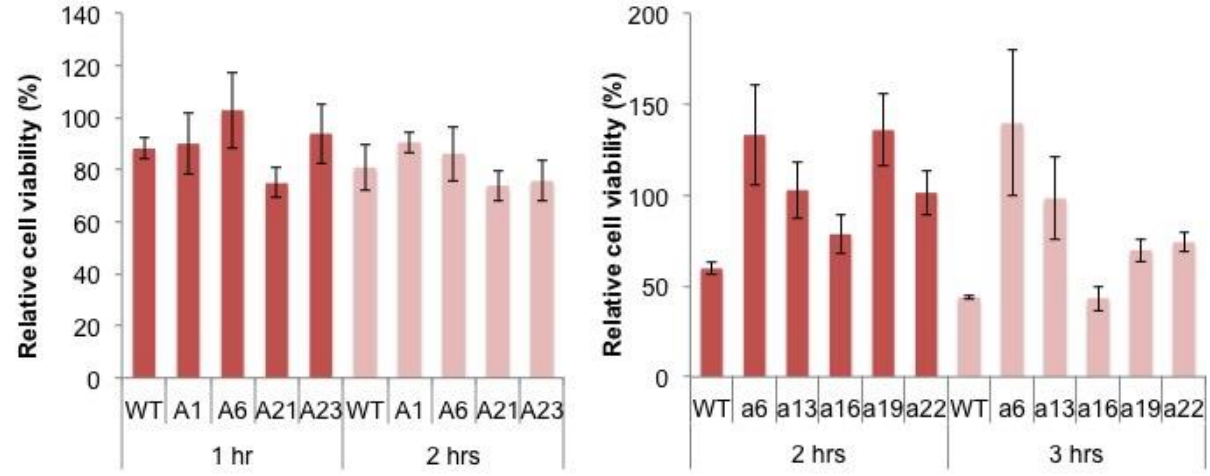

Figure 11. Cell viability test for high-sugar stress tolerance of proline-accumulating baker's yeast mutants (27.8% sucrose).

Yeast cells grown in YPD for 48 h at 30 ^{O}C were inoculated in 5ml of liquid fermentation medium (containing 27.8% sucrose) at an OD_{600} of 1.0 and incubated at 30 ^{O}C for 1, 2 and 3 h. After incubation, cells were collected, diluted and 100 µl of the diluted suspension was spread on YPD agar plates. After 2 days of incubation at 30 ^{O}C, colonies were counted and number of colonies was expressed in colony forming unit (CFU). CFU without stress was defined as 100%. The values are the means and standard deviations from three independent experiments. Statistically significant differences were determined by Student's t-test (*p<0.05).

Also, I made a mixed-dough using the selected strains that accumulate proline and determined the CO_2 gassing power of these strains as an indicator of the fermentation ability in frozen and sweet dough. The relative fermentation ability was expressed as a percentage of total CO_2 gas production for 120 min compared with that in the WT, which was defined as 100% (Figure 12). Strains A6, a19 and a22 exhibited up to an approximately 25% increase in fermentation ability compared with the WT after 7 days of freezing. In the high-sucrose dough, strains A6, a6, a19, ad a22 showed approximately 20% to 60% greater fermentation abilities than the WT. On the other hand, the fermentation ability in strain a13 was almost the same as that in the WT in both frozen and high-sucrose dough. It appears that most of these mutants enhanced the fermentation ability in both frozen dough and sweet dough. I carried out further analysis of the strains A6, a6, a19 and a22, which showed enhanced stress tolerance and improved fermentation in the above tests.

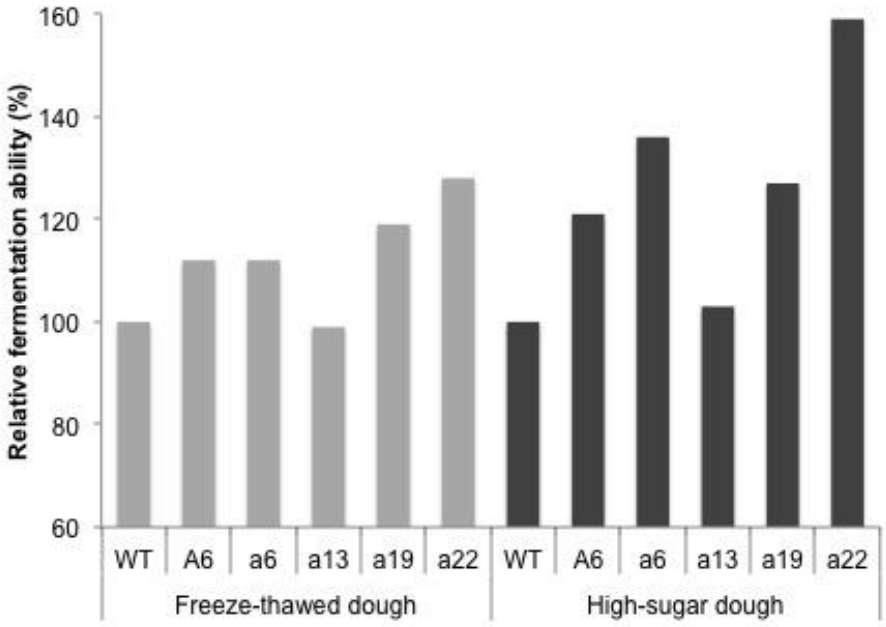

Figure 12. Fermentation ability test using bread dough in freezing or high-sugar stress.
For freeze-thawed dough, 100 g bread-making flour, 3 g yeast (66% moisture basis), 5 g of sucrose, 0.5 g NaCl, and 52 ml water was mixed thoroughly make the dough. The prepared dough is frozen for 7 days at -20 ^{O}C and then after thawing for 30 min at 30 ^{O}C, CO_2 gas production is monitored throughout the 1 h incubation at 35 ^{O}C. For high-sucrose dough, 100 g bread-making flour, 3 g yeast (66% moisture basis), 30 g sucrose, 0.5 g NaCl, and 52 ml water was mixed thoroughly to make the dough. The CO_2 gas production is monitored throughout the 2 h incubation at 28 ^{O}C. The amount of gas produced by parental strain (WT) was defined as 100%.

Chemical mutagenesis induces several mutations throughout the genome, and selection by AZC is likely to pick up strains with mutations in several genes other than those conferring resistance to AZC. Therefore, it is possible that, despite the increase in mutations that cause proline accumulation, the strains may also harbor disadvantageous mutations that give rise to susceptibility to freezing and/or osmotic stress.

3.1.3. Identification of mutations on *PRO1* gene of baker's yeast mutants

In order to identify the mutations that conferred AZC resistance, proline accumulation, and stress tolerance, I analyzed the nucleotide sequences of the *PRO1* genes from the WT strain and the A6, a6, a19, and a22 mutant strains using direct PCR DNA sequencing and compared them to that of the *S. cerevisiae* laboratory strain S288C using the BLAST search program. The results showed that the sequence of the *PRO1* gene in the WT was identical to that in strain S288C. I found that the *PRO1* gene sequence in strain A6 included a mixture of C and T at nucleotide position 739, leading to amino acid replacement of Pro to Ser at position 247 in the amino acid sequence of GK. Another strain, a22, also had a point mutation of G to A at nucleotide position 1243, which led to amino acid replacement of Glu to Lys at position 415 on the GK amino acid sequence. However, neither strain a6 nor strain a19 had any base mutations in its *PRO1* gene sequence.

Here, I focused only on the *PRO1* allele. However, the possibility of mutations in the other genes related to proline metabolism and cellular transport should not be ignored. Hence, it would be worthwhile to determine the whole genome sequence of strains a6 and a19 in order to identify genes that might be responsible for their phenotypes.

3.1.4. Effect of *PRO1* mutations on baker's yeast proline biosynthesis and stress tolerance

To further analyze the *PRO1* mutations found in strains A6 and a22, I constructed two expression plasmids harboring the *PRO1* gene with the same mutations identified in A6 and a22 ($PRO1^{P247S}$ and $PRO1^{E415K}$, respectively) and introduced each plasmid into the parent strain of baker's yeast 3346/3347. Then I examined the AZC resistance, intracellular proline level and baking associated-stress tolerance in the resultant strains, WT, P247S, and E415K, expressing ectopic $PRO1^{WT}$ $PRO1^{P247S}$ and $PRO1^{E415K}$, respectively (Figure 13, Figure). Strains P247S and E415K were resistant to 5 mg/ml AZC and comparable to the previously described strain I150T

expressing *PRO1*^{I150T} (Kaino et al., 2008; Sekine et al., 2007), while the WT showed weaker growth at this concentration of AZC (Figure 13A). In addition, strains P247S and E415K showed intracellular proline levels twice as high as that of WT, although these levels were still lower than that of strain I150T (Figure 13B).

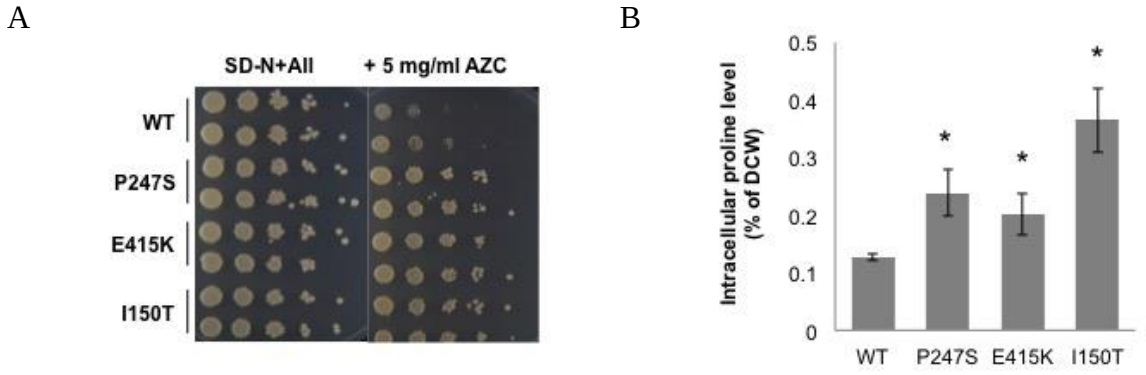

Figure 13. Growth phenotype of recombinant baker's yeast expressing the mutant *PRO1* gene.
(A) AZC-resistance test. Yeast cells cultivated overnight in 2 ml of YPD+G418 at 30 ^{O}C were collected, serially diluted, and spotted on SD-N+All plates containing 5 mg/ml AZC and incubated at 30 ^{O}C for 7 days. (B) Intracellular proline level. Yeast cells cultured in YPD medium for 48 h at 30 ^{O}C were collected, washed twice and then intracellular amino acids were extracted by boiling the cell suspension in water. After centrifugation, amino acid content in each supernatant was quantified with amino acid analyzer. Amount of proline is shown as the percentage of dry cell weight (DCW). The values are the means and standard deviations from three independent experiments. Statistically significant differences were determined by Student's *t* test (*p<0.05).

A freezing-stress test revealed that the cell viability of strains expressing the mutant *PRO1* were two to threefold higher than that of the WT expressing *PRO1*^{WT}, but not as high as that of strain I150T (Figure A). However, no significant differences were observed in the survival rate of the four strains under 2-h exposure to a high-sugar stress condition (Figure B). The AZC-resistance test and amino acid analysis confirmed the phenotypes of strains A6 and a22 that harbored mutations *PRO1*^{P247S} and *PRO1*^{E415K}, respectively.

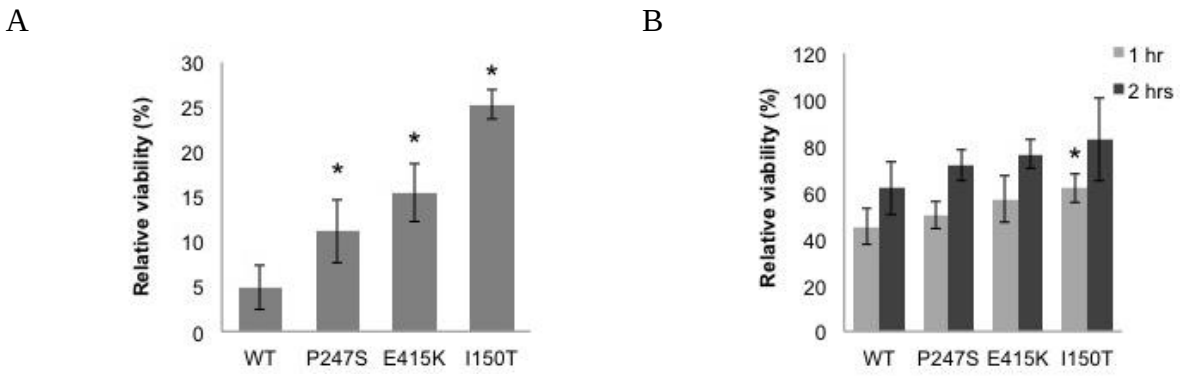

Figure 14. Stress tolerance of recombinant baker's yeast expressing the mutant *PRO1* gene.
(A). Relative viability after 1 day freezing stress. Stationary phase cells grown in YPD medium for 48 h at 30 ^{O}C were inoculated in YPD at an OD_{600} of 1.0 and incubated for 4h. Cells were collected and the cell suspension was divided into 100 µl aliquots and frozen at -30 ^{O}C for 1 day. The frozen cell suspensions were thawed at room temperature, diluted and 100 µl of the diluted suspension was spread on YPD agar plates. After 2 days of incubation at 30 ^{O}C, colonies were counted and number of colonies was expressed in CFU. CFU before freezing was defined as 100%. (B). Relative viability after 1 and 2 hours or exposure to high concentration of sucrose (27.8%). Yeast cells grown in YPD for 48 h at 30 ^{O}C were inoculated in 5 ml of liquid fermentation medium at an OD600 of 1.0 and incubated at 30 ^{O}C for 2 hr. After incubation, cells were collected, diluted and 100 µl of the diluted suspension was spread on YPD agar plates. After 2 days of incubation at 30 ^{O}C, colonies were counted and number of colonies was expressed in CFU. CFU without high-sucrose stress was defined as 100%. The values are the means and standard deviations from three independent experiments. Statistically significant differences were determined by Student's *t* test (*p<0.05).

The results of the stress test supported the idea that the $PRO1P^{247S}$ and $PRO1^{E415K}$ mutations contributed to the freezing stress tolerance of yeast cells. The higher proline level of the strain I150T relative to strains P247S and E415K probably reflects the better freezing stress tolerance of strain I150T. However, a similar phenotype could not be observed for high-sucrose stress. Next, therefore, I considered that it would be worthwhile to identify and analyze mutations in other genes in strains A6 and a22 that confer tolerance to high-sucrose stress on yeast cells.

3.1.5. Enzymatic properties of novel GK variants

The mutations found in the *PRO1* gene of strains A6 and a22 led to some novel amino acid substitutions in GK. First, the substitutions P247S and E415K are distal from the active site, which partially overlaps with the allosteric site of GK. Residues 142 to 154 in the *S. cerevisiae* GK are mapped to a region that was identified as important for allosteric control of the tomato GK (Fujita et al., 2003). Although the sequence similarity in the regions of the two GKs is low, the regions were suggested to constitute part of the proline-binding site. Secondly, the replacement of Glu415 to Lys was found in the PUA domain, which has not been shown to play any role in the catalytic

activity of GK (Kaino et al., 2012). Therefore, I considered that it would be intriguing to see how Pro247 and Glu415 are involved in catalysis of the GK reaction. Hence, I analyzed the kinetic properties of the recombinant GK variants (P247S and E415K). Enzymatic assays of the purified recombinant enzymes revealed that the P247S and E415K variants had higher activity than the WT enzyme with increasing substrate concentration, where the k_{cat} value, which is the number of substrate molecules turned over by an enzyme molecule per second, of variants was double that of the WT enzyme (Figure 15A, Table 3).

Indeed, the activities of the two variants were comparable to that of the I150T variant, which was shown to have increased affinity for substrates and to be insensitive to feedback inhibition by proline (Sekine et al., 2007). When the enzyme assay was done in the presence of proline, the activities of the two GK variants were less sensitive to feedback inhibition at proline concentrations up to 100 mM, while the WT GK was markedly inhibited (Figure 15B). When the IC_{50} value, which is the concentration of proline needed for 50% inhibition, was determined, the E415K and P247S variants showed approximately 7-fold and 30-fold desensitization to proline feedback inhibition, respectively, compared to the WT enzyme (Table 3).

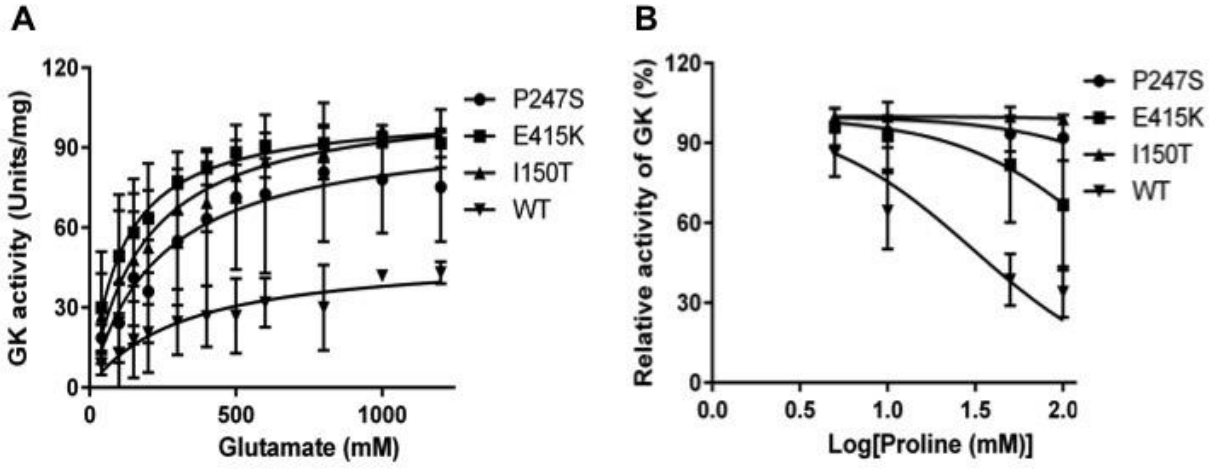

Figure 15. Kinetic properties of the γ-glutamate kinase variants: wild-type (WT), I150T, P247S, and E415K GKs.
(A) Effect of glutamate on the GK activity. The GK activity, which was expressed as unit of enzyme per mg protein, was determined as described in Materials and Methods. (B) Effect of proline on the GK activity. The enzyme activities of each variants of GK in the absence of proline were defined as 100%. The values are the means and standard deviations from three independent experiments.

Table 3. Kinetic parameters of GK enzymatic activity

	Glutamate	Proline
	k_{cat}(s^{-1})	IC_{50} (mM)
P247S	77.4 ± 10.6	931 ± 1.5
E415K	81.6 ± 4.1	204 ± 1.4
I150T	85.6 ± 6.2	12,558 ± 1.2
WT	39.3 ± 6.9	30.8 ± 1.2

The values of k_{cat} and IC_{50} were estimated by GraphPad Prism from Fig. 15A and Fig. 15B, respectively. The data are the mean ± standard deviation of three independent experiments.

Hence, from the above results, I conclude that the decreased sensitivity to proline feedback inhibition in the P247S and E415K variants caused proline accumulation, leading to AZC-resistance in yeast cells expressing $PRO1^{P247S}$ and $PRO1^{E415K}$, respectively.

The sequence alignment showed that proline at position 247 was highly conserved throughout the bacterial and yeast GKs with only kinase activity (Figure 16). However, in the homologous, bi-functional enzymes of Δ1-pyrroline-5-carboxylate synthetase (P5CS) in higher eukaryotes, proline is a non-conserved residue. Based on a preliminary homology modeling study, it appears that Pro247 in the yeast GK lies on the surface of the protein and at some distance from the partially overlapping substrate and inhibitor-binding pocket. Due to the inflexibility of the protein backbone formed around the proline residue, any substitutions at position 247 might affect the folding trajectory and/or dynamics of the protein structure. In addition, the Xaa-Pro bond isomerization occurs frequently in the secondary structure, which may influence the protein stability. In the *E. coli* GK, the interaction between the PUA domains of monomers contributes to the formation of a homodimer of the enzyme (Marco-Marín et al., 2007). Also, removal of the PUA domain from yeast GK dramatically decreased the kinase activity and the enzyme stability (Tatehashi and Takagi, 2013). Although the yeast GK is not shown to form a dimer and Glu415 is not located on the interface of GKs, the substitution at position 415 clearly affected the enzymatic properties.

Therefore, further study is needed to investigate the effect of these novel mutations found in the *PRO1* gene on the structural and functional features of the GK enzyme.

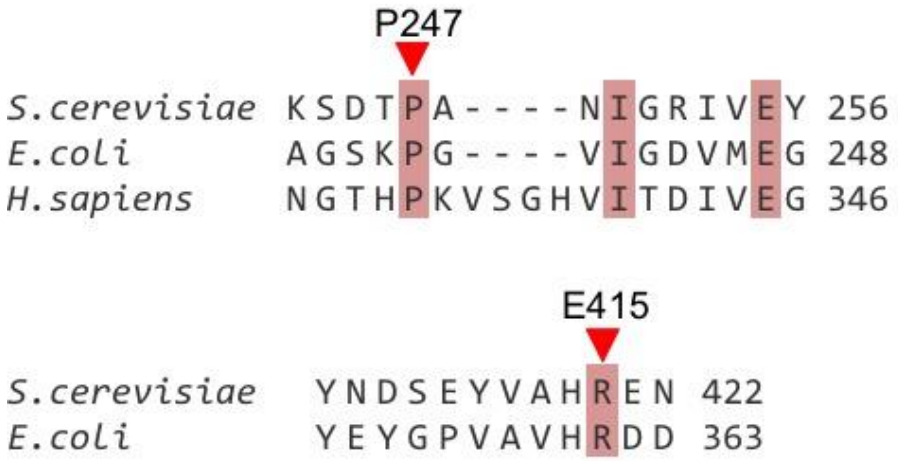

Figure 16. Schematic representation of the *S.cerevisiae* GK and the alignment of the amino acid sequences around at position 247 and 415.
Red triangles indicate the mutation points. P247 residue is in the kinase domain whereas E415 residue is in the PUA domain.

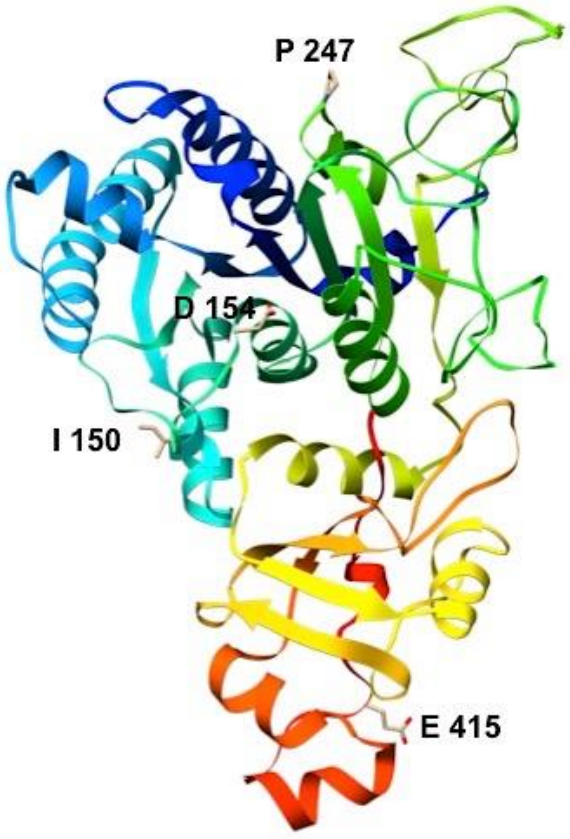

Figure 17. Yeast GK homology modeling.
Wild-type GK protein structure was predicted by homology modelling on I-TASSER based on *E.coli* GK (pdb: 2j5t) as template.

3.2. Cumulative effect of multiple *PRO1* mutations

Proline accumulation and/or active proline biosynthesis is known to confer stress tolerance. Proline accumulation and/or active proline biosynthesis is known to confer stress tolerance. However, the questions of how much proline is sufficient to confer stress tolerance and whether or not the proline concentration is correlated with the degree of stress tolerance have not been adequately addressed. I therefore investigated the possibility of excessive proline accumulation in the yeast cells and its effect on stress tolerance. For this purpose, I developed plasmids harboring *PRO1* with multiple substitution mutations, where the double, triple and quadruple substitution mutations are combinations of the previously reported *PRO1* mutations (I150T and D154N) and the two novel *PRO1* mutations (P247S and E415K) obtained in this study.

The AZC-resistance and intracellular proline levels were analyzed, and stress tolerance was tested in the baker's yeast strains 3346/3347 expressing these newly designed plasmids individually. In addition, plasmids with multiple amino acid substitutions in *PRO1* were constructed for recombinant enzyme expression in *E. coli* in order to study the effects of cumulative mutations on the activity of GK.

3.2.1. AZC-resistance and proline accumulation

First, I tested the AZC-resistance of each multiple mutation variant (Figure 18). All recombinant yeasts expressing the *PRO1* variants showed resistance to 5 mg/ml AZC, while the WT variant was susceptible to the same dosage of AZC. This result suggested that an excessive amount of proline in the cell confers resistance to AZC; however, in order to elucidate the correlation between AZC-dosage and the number of point mutations in the *PRO1*, the strains should be analyzed using a higher concentration of AZC (>5 mg/ml) in the medium. In conclusion, all proline-accumulating strains showed AZC-resistance.

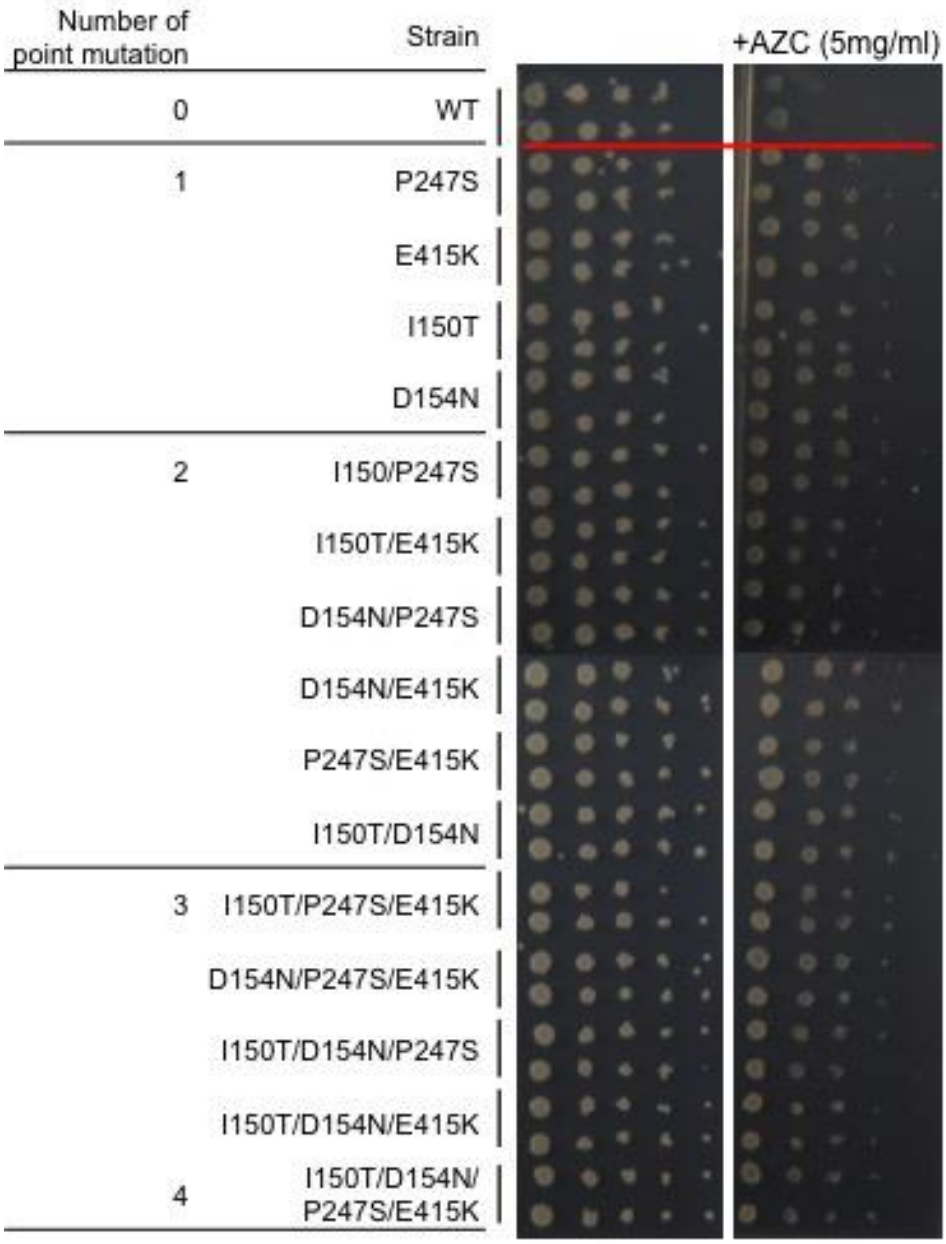

Figure 18 Spot test of recombinant baker's yeasts expressing variants of GK for their AZC-resistance.
Yeast cells cultivated overnight in 2 ml of YPD+G418 at 30 ^{O}C were collected, serially diluted, and spotted on SD-N+All plates containing 5 mg/ml AZC and incubated at 30 ^{O}C for 7 days.

Next, I measured the intracellular proline content in the recombinant yeast expressing *PRO1* variants (Figure 19). In the double substitution mutants, the additional point mutation in *PRO1* resulted in a further increase in proline accumulation compared to that by the single point mutation variants. However, no further improvements in intracellular proline level were seen in quadruple or triple mutation variants compared to double mutation variants. This result shows that each

combination of point mutations in all double mutant variants at least doubled the proline level compared to the single mutation variants, which suggests that every combination was cumulative. In addition, this result suggests that the residues P247 and E415 each make a significant contribution to the catalytic activity of GK. However, the possibility of excess proline export, in order to maintain the amino acid homeostasis in the cell, may not be ignored in triple and quadruple mutation variants.

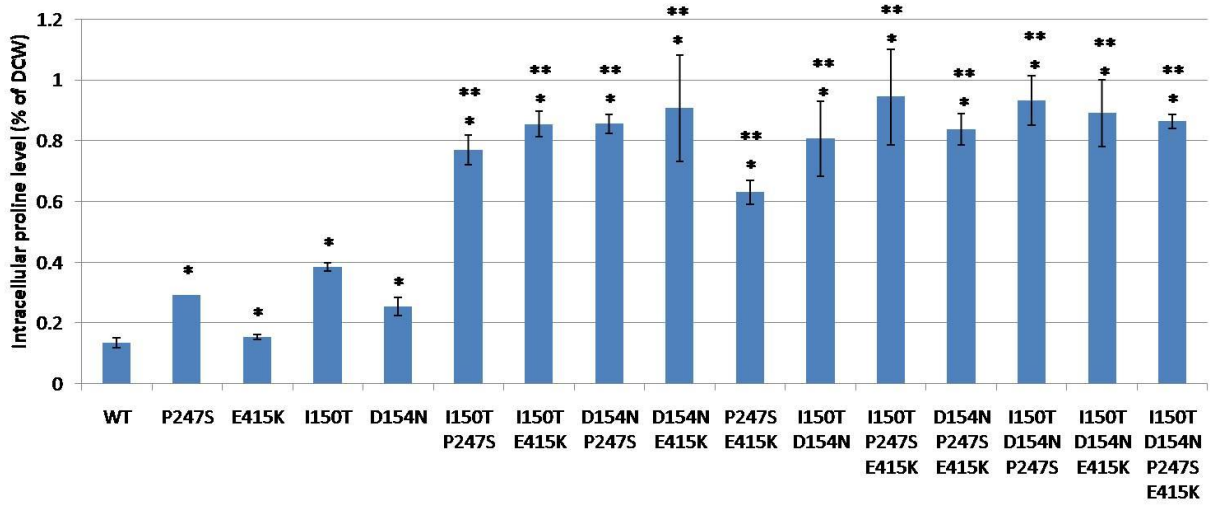

Figure 19. Intracellular proline level of recombinant baker's yeast expressing variants of GK. Intracellular proline level. Yeast cells cultured in YPD medium for 48 h at 30 ^{O}C were collected, washed twice and then intracellular amino acids were extracted by boiling the cell suspension in water. After centrifugation, amino acid content in each supernatant was quantified with amino acid analyzer. Amount of proline is shown as the percentage of dry cell weight (DCW). The values are the means and standard deviations from three independent experiments. Statistically significant differences were determined by Student's *t* test (p<0.05, * significant difference compared to WT, ** significant difference compared to single substitution mutant).

3.2.2. Enzymatic properties of GK with multiple substitutions

To study the effect of cumulative mutations in *PRO1*, recombinant GK enzyme variants were developed and purified for enzyme assay. The results (Figure 20) showed that the WT GK was sensitive to proline and lost all activity at 100 mM of proline, while the positive control GK^{I150T} was insensitive to proline and its relative activity stayed strong even at very high concentrations of proline. Also, with the exceptions of GK^{E415K} and $GK^{D154N/E415K}$, all mutant variants of GK were desensitized towards proline, and they retained as much as 70% of their relative activity (relative

to 0 mM proline) at a higher concentration of proline. In addition, when I calculated the IC_{50}, or the concentration of proline required to inhibit 50% of the enzyme activity, I found that the IC_{50} was increased 2-15 times in the GK mutants (Table 4). The result I obtained from an enzymatic assay with increasing proline concentrations in the reaction mixture showed that each combination of amino acid substitutions contributed to desensitization to the inhibitory effect of proline, resulting in higher proline synthesis. Some variants, such as $GK^{P247S/E415K}$, initially showed an increase in relative activity at a lower concentration of proline, which could indicate that proline stabilizes the protein, and that, as the proline concentration in the reaction mixture increases, the protective effect of proline is overcome by the inhibitory effect of proline on the GK enzyme. The higher proline levels in the double-mutants compared to I150T when expressed in yeast cells, despite some double mutant enzymes exhibiting higher sensitivity to proline, was likely to be due to increased turnover for glutamate by these enzymes, and hence the specific activity of these enzymes in the presence of varying concentrations of substrate.

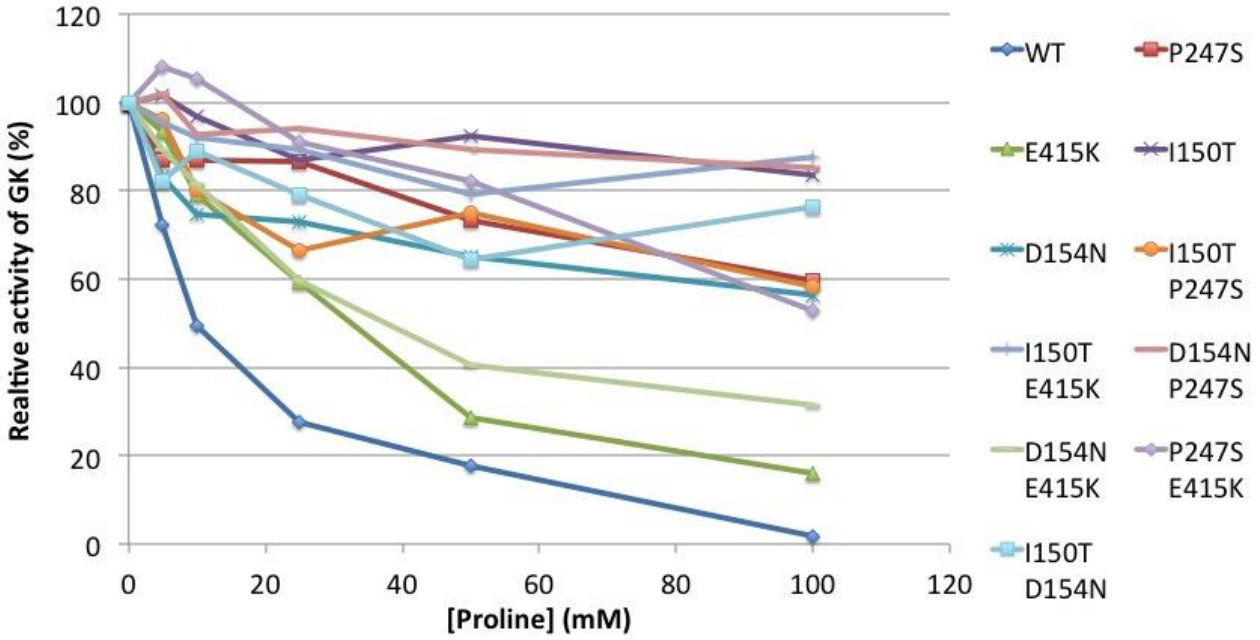

Figure 20. Inhibition of enzymatic activity of GK variants by proline.

The enzyme activities of each variants of GK in the absence of proline were defined as 100%.

Table 4. Kinetic parameters of GK variants

	IC_{50} ([Proline]mM)
WT	25.3
P247S	124.0
E415K	46.6
I150T	298.0
D154N	107.0
I150T/P247S	114.7
I150T/E415K	374.3
D154N/P247S	322.2
D154N/E415K	57.3
P247S/E415K	99.7
I150T/D154N	193.7

The IC_{50} values were estimated from Fig. 20.

3.2.3. Stress tolerance

Here, in order to see whether excess proline in the cell may give rise to increased stress tolerance, I analyzed the stress tolerance of recombinant yeasts that can accumulate excessive proline in their cells.

3.2.3.1. Freezing stress

First, I performed a spot test in which cells were grown in rich medium for 48 hrs to allow them to accumulate proline, and then untreated cells and cells exposed to 1, 3 or 7 days of freezing were spotted on YPD medium to check the extent of growth deterioration after freezing stress. After 1 day of freezing, all mutant variants grew as well as the WT variant. Even after 3 to 7 days of freezing, no significant growth difference between the WT and mutant variants was observed (Figure 21). Next, I carried out a viability test. None of the multiple mutation variants showed increased freezing stress tolerance compared to the WT and single mutation variants. Although the intracellular proline levels were higher in these mutants, the GK enzyme stability could be affected

by various combinations of mutations, and proteins in an unnatural conformation may cause ER stress, leading to an overall stagnation in stress tolerance.

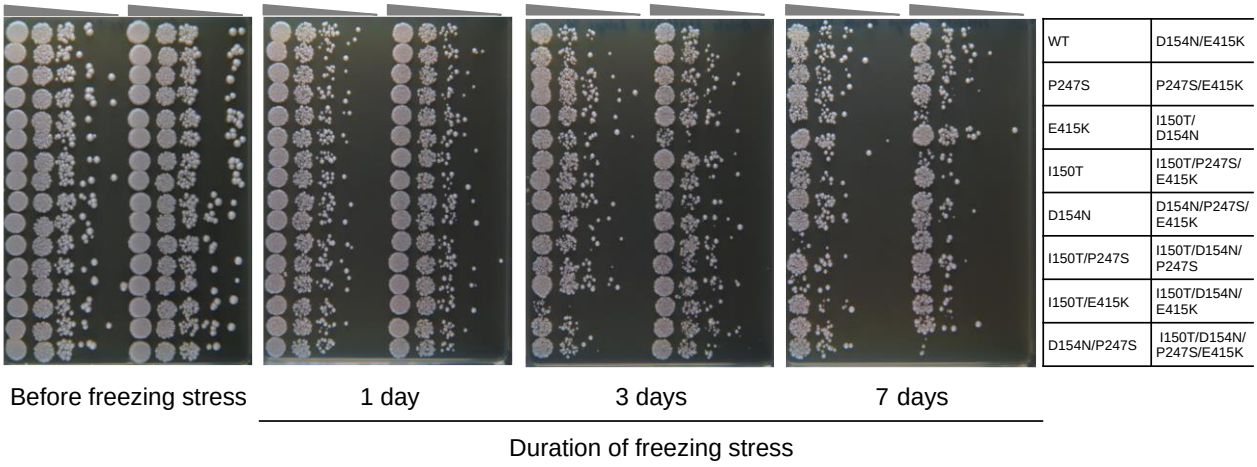

Figure 21. Freezing stress tolerance test recombinant baker's yeast expressing variants of GK. After cultivation of 48 h at 30 ^{O}C in YPD, yeast cells were collected, serially diluted, and spotted on YPD agar plates. Also, aliquots of the cells suspension in water was frozen at −30 ^{O}C; then the frozen cell suspension was thawed at 30 ^{O}C for 20min, serially diluted and spotted on YPD agar plates. All spotted plates were incubated for approximately 2 days at 30 ^{O}C.

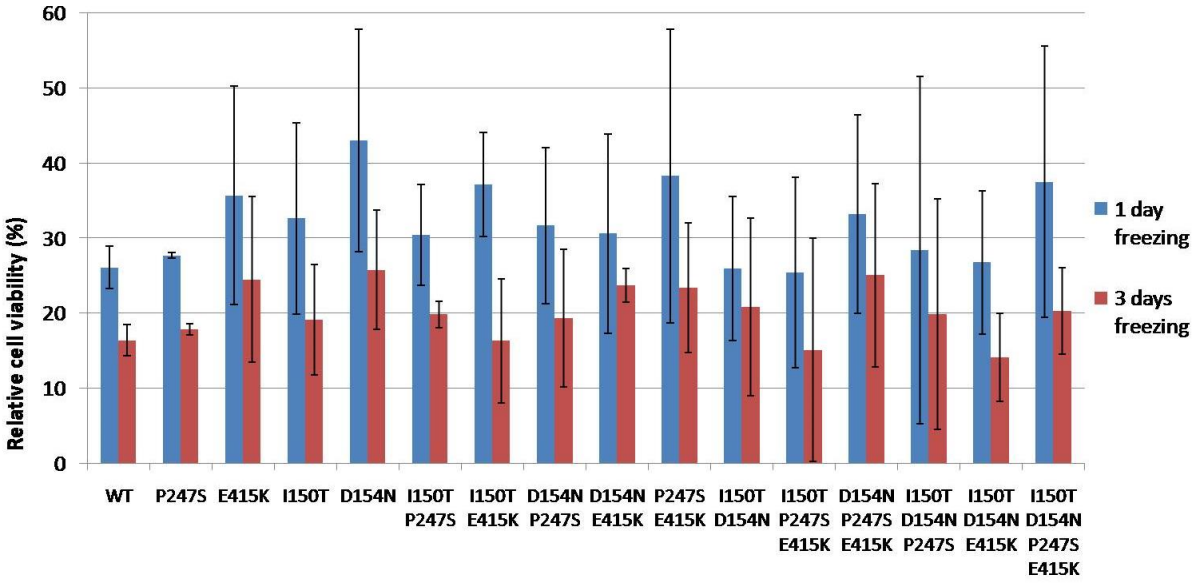

Figure 22. Viability test for freezing stress of recombinant baker's yeast expressing variants of GK Stationary phase cells grown in cane molasses medium for 48 h at 30 ^{O}C were inoculated in YPD at an OD_{600} of 1.0 and incubated for 4h. Cells were collected and the cell suspension was divided into 100 µl aliquots and frozen at -30 ^{O}C for 1 and 3 days. The frozen cell suspensions were thawed at room temperature, diluted and 100 µl of the diluted suspension was spread on YPD agar plates. After 2 days of incubation at 30 ^{O}C, colonies were counted and number of colonies was expressed in CFU. CFU before freezing was defined as 100%.

3.2.3.2. High-sugar stress

First, I performed a spot test in YPD containing 40% glucose as high-sugar stress using WT as a negative control and P247S, E415K, I150T and D154N as positive controls. The phenotypes in the spot test showed that P247S, I150T and D154N exhibited higher growth compared to the negative control WT, confirming the findings of previous reports (Figure 23). However, some of the strains accumulating excessive proline in the cell were susceptible to 40% glucose in the spot test. These were D154N/P247S and I150T/D154N/P247S/E415K.

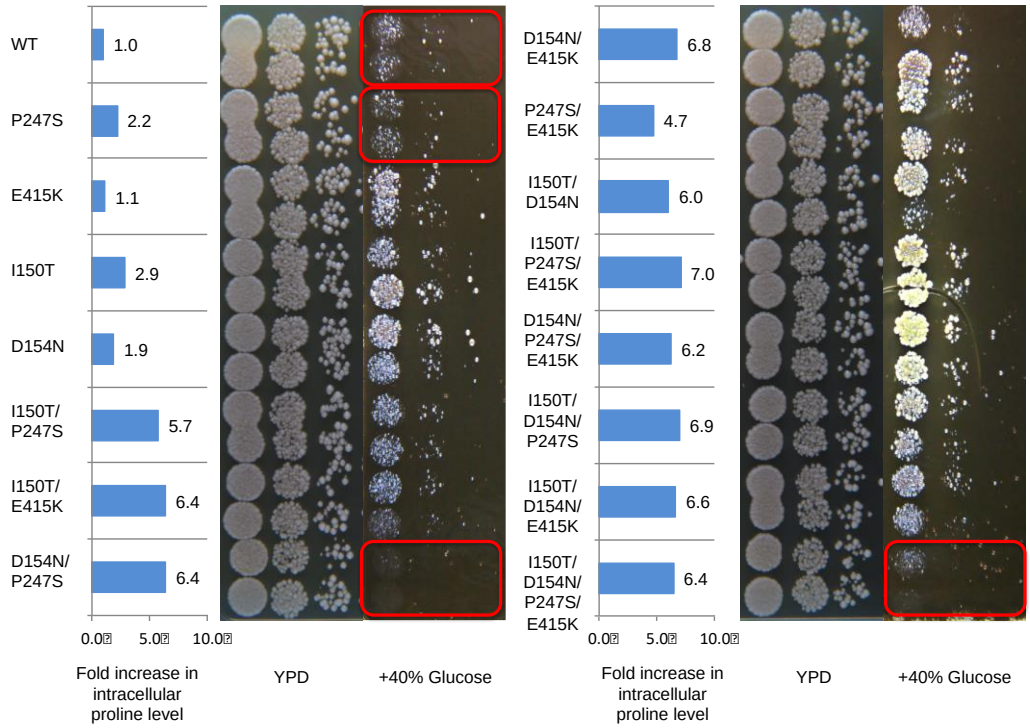

Figure 23. Spot test for High-sugar stress recombinant baker's yeast expressing variants of GK. After cultivation in cane YPD for 48 h at 30 ^{O}C, yeast cells were collected, serially diluted, and spotted on YPD agar plates containing 40% glucose. All spotted plates were incubated for several days at 30 ^{O}C. Blue bars are fold increase in proline intracellular proline level relative to wild-type calculated from Fig. 19.

In the cell viability test (Figure 24), all *PRO1* mutant variants exhibited decreased growth after 2 hrs of high-sucrose stress; however, after 4 hrs of incubation in the high-sucrose medium, the cells began to recover, as evidenced by the greater cell viability at 4 hrs relative to that at 2 hrs. Hence, at 4 hrs, the relative cell viability was improved in all mutant variants. However, despite the expectation that excessive proline accumulation might confer higher stress tolerance, no further improvement was observed in the multiple mutation variants with increased proline accumulation

after 4 hrs of incubation in medium containing a high concentration of glucose. This might be attributable not only to a high concentration of proline but also to fine tuning of the proline-glutamate cycle, which in turn regulates the NADP-NAD cycle, which is important in stress tolerance. In addition, the protein stability of the particular variant of the mutant GK enzyme in the presence of high-sugar stress is an important factor in the stress tolerance.

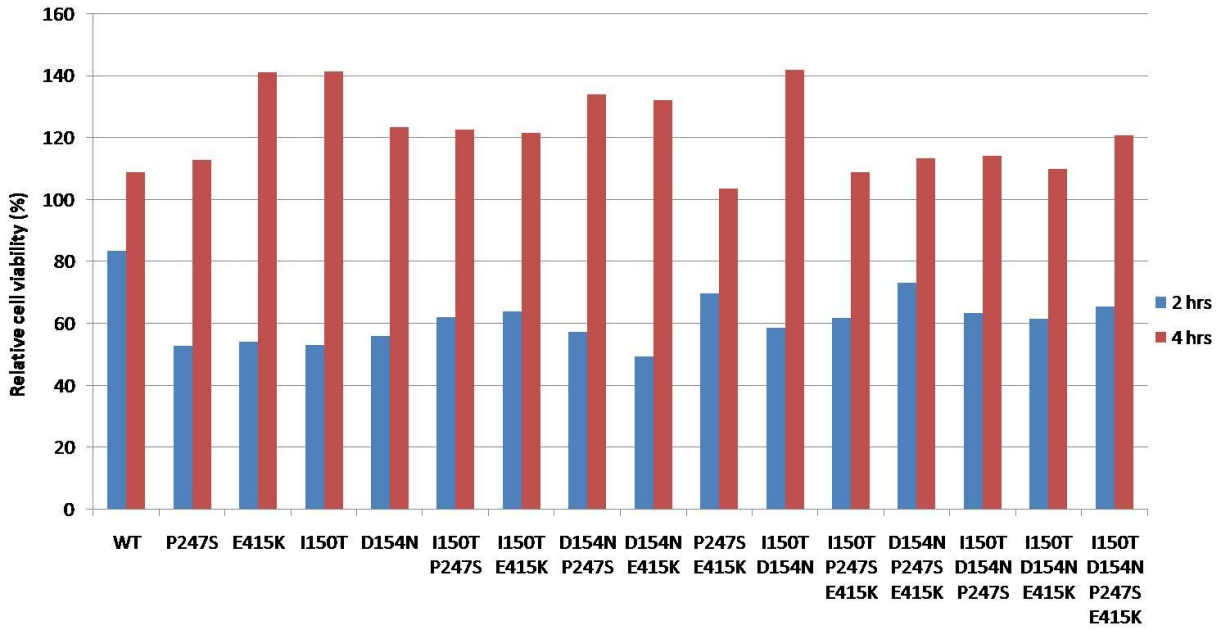

Figure 24. Viability test for high-sugar stress of recombinant baker's yeast expressing variants of GK. Yeast cells grown in cane molasses for 48 h at 30 ^{O}C were inoculated in 5 ml of liquid fermentation medium (containing 27.8% sucrose) at OD_{600} of 1.0 and incubated at 30 ^{O}C for 2 and 4 hrs. After incubation, cells were collected, diluted and 100 µl of the diluted suspension was spread on YPD agar plates. After 2 days of incubation at 30 ^{O}C, colonies were counted and number of colonies was expressed in CFU. CFU without high-sucrose stress was defined as 100%.

4. Discussion

4.1. Stress tolerant baker's yeast isolated by conventional mutagenesis

In this chapter, I isolated stress-tolerant baker's yeast by conventional mutagenesis and identified two novel *PRO1* mutations, P247S and E415K, which confer AZC-resistance and proline accumulation in the cell.

Freezing stress and high-sugar stress are two important forms of baking-associated stress. Although these stresses propagate distinctive cellular experiences, they both lead to ROS generation. During freezing stress, ice crystals are formed in the cellular compartments and the freeze-thaw cycle may give rise to changes in the structure and conformation of biomolecules, resulting in a decrease in antioxidant enzyme activity in some systems (Baek and Skinner, 2012; Hermes-Lima and Storey, 1993) and an increase in ROS (Park et al., 1998). On the other hand, high-sugar stress brings about osmotic pressure in the cell and an increase in ROS levels resulting from active glycoxidation (Landolfo et al., 2008; Valishkevych et al., 2016). Proline is one of the amino acids shown to confer resistance to various stresses, including freeze-thaw, osmotic and oxidative stress, and proline accumulation is one of the stress responses observed in plants and bacteria. In addition, the ROS-scavenging properties of proline have been well documented (Takagi, 2008), and several studies have investigated hydroxyl radical-scavenging mechanism (Signorelli et al., 2015). As a natural response to stress, yeasts accumulate glutathione, trehalose or glycerol, but not proline. Therefore, attempts have been made to genetically manipulate yeast cells so that they accumulate proline (Morita et al., 2003; Takagi et al., 1997), using the property of feedback-inhibition of *PRO1* by proline as a target for mutations. In this study, I used a similar approach while taking into consideration the safety concerns related to GMO products. Hence, the Japanese industrial diploid baker's yeast 3346/3347 was used in the conventional mutagenesis, and AZC-resistant mutants were isolated (Figure 5, Figure 6). Through a two-step screening that consisted of measuring the proline levels of AZC-resistant mutants (Figure 7) and analyzing the stress tolerance of proline-accumulating mutants (Figure 8, Figure 9, Figure 10, Figure 11, Figure 12), I obtained four strains, A6, a6, a19, a22, which had increased stress tolerance compared to the parental strain. Among the four strains obtained, I found that the *PRO1* gene sequence in strain A6 included a mixture of C and T at nucleotide position 739, leading to amino acid replacement of Pro to Ser at position 247 in the amino acid sequence of GK. The strain a22 had a point mutation of G to A at nucleotide position 1243, which led to an amino acid replacement of Glu to Lys at position

415 on the primary amino acid sequence of GK. However, neither strain a6 nor strain a19 had any base mutations in its *PRO1* gene sequence. In addition, the mutation P247S in A6 is a dominant as it resulted from nucleotide substitution only on one allele. Here, I focused only on the *PRO1* allele, and thus the possibility of mutations in the other genes related to proline metabolism and cellular transport should not be ignored. Hence, it would be worthwhile to determine the whole genome sequence of strains a6 and a19 in order to identify mutations contributing to their observed phenotypes of AZC-resistance, proline accumulation and stress tolerance. Next, I focused on characterization of the novel mutations P247S and E415K in *PRO1*. For this purpose, I developed four yeast expression plasmids pYC130-PRO1-variant with the respective mutations WT, P247S, E415K and I150T (positive control). The recombinant diploid baker's yeast 3346/3347 expressing each of the above-mentioned plasmids was tested for their AZC-resistance, proline content and stress tolerance. The results showed that the mutations P247S and E425K were indeed responsible for the AZC-resistance and increased proline levels in the cells (Figure 13), suggesting that the strains A6 and a22 owe their AZC-resistance and proline accumulation to the P247S and E415K mutations in *PRO1*, respectively. In the stress-tolerance test, recombinant yeasts expressing each novel GK mutant showed increased cell viability under freezing stress (Figure), while no significant improvement was observed under high-sugar stress (Figure). This unexpected result of the high-sugar stress test may have been attributable to the incubation time of 1 or 2 hrs being insufficient for cells to develop adaptation or accumulate enough proline to show a positive response to the stress. I thus suggest increasing the incubation time in the medium containing a high concentration of sucrose.

Finally, I analyzed the enzymatic properties of mutant GK by purifying recombinant enzymes and testing their enzymatic activity in the presence of increasing concentrations of glutamate and proline. Both P247S and E415K of GK showed increased activity with increasing substrate concentration, and both levels of activity were comparable to that of the positive control I150T (Figure 15A). On the other hand, the sensitivity to feedback inhibition was slightly different between the two novel mutants, with the P247S variant showing less sensitivity to the increasing proline concentrations, maintaining almost 90% of full acidity at proline concentrations as high as 100 mM (Figure 15B). Based on the results from the analysis of enzymatic properties (Figure 15, Table 3), I conclude that P247S is a more favorable mutation for increased proline synthesis in the cell. This was also supported by the observations from the homology modeling of yeast GK, which

showed that the proline residue at 247 was located at a bend, and that its replacement with serine might have resulted in a more flexible conformation of the active site pocket.

In the above experiments, the feature of intracellular proline accumulation conferring AZC-resistance was used successfully to obtain stress resistant baker's yeast strains A6 and a22, and their phenotypes, which resulted from the two novel mutations P247S and E425K, respectively, were confirmed by characterizing the recombinant yeast and studying the kinetic properties of the recombinant enzyme.

4.2. Cumulative effect of multiple *PRO1* mutation

In this chapter, I studied the cumulative effect of the *PRO1* mutations I150T and D154N, which were characterized previously (Morita et al., 2003; Sekine et al., 2007), and P247S and E4125K, which are novel substitutions obtained in this study. For this purpose, I constructed 15 pYC130 plasmids harboring variants of *PRO1* with various combinations of the amino acid substitutions described above (Table 1). These plasmids were expressed individually in the baker's yeast 3346/3347 to analyze how the variants differed from each other and from the WT in terms of AZC-resistance, intracellular proline content and stress tolerance. In the AZC-resistance test (Figure 6), all variants showed growth better than the WT on 5 mg/ml AZC. However, no differences in AZC-resistance were observed between mutant variants. If I presume that the degree of AZC-resistance should differ among the mutant variants, the same spot test could be carried out under different concentrations of AZC >5 mg/ml. Such analysis could be key to identifying mutations in *ALDH18A1* (homologous to *PRO1* in humans), since it has been proposed that there may be a link between AZC toxicity and the etiology of multiple sclerosis (Rubenstein et al., 2009), although detoxification of AZC in humans has not been well studied and the involvement of ALDH18A1 or the cellular proline levels is still unknown.

Next, I measured the intracellular proline level in yeasts expressing each of the variants of *PRO1*. Here, the combination of two substitutions showed a synergetic effect, since the intracellular proline level was two-fold higher in the double substitution mutants compared to the single mutation variants. However, there was no further increase in proline level in triple substitution mutants as against double substitution mutants and it was the same with quadruple substitution variants. This was the opposite of my expectation, and measuring the extent of leakage of excess amino acid to maintain proline homeostasis in the cell should be considered.

Since there was no significant difference between the double and triple/quadruple substitution mutants, I focused on the enzymatic properties of double substitution mutants in the next analysis. Using purified recombinant GK variants harboring single and double substitution, I analyzed the sensitivity of feedback inhibition in these variants (Figure 20, Table 4). Here, the activity of the WT enzyme was drastically decreased with increasing proline concentration, confirming the high sensitivity of the WT to feedback inhibition, while the relative enzyme activity remained strong for the positive controls I150T, D154N in the presence of a high concentration of proline, confirming the removal of feedback inhibition in these variants. With the exception of

D154N/E145K, all variants harboring double substitutions showed removal of feedback inhibition to nearly the same degree as the positive control. This observation shows that the removal of feedback inhibition was attributable to the increase in proline in double substitution mutants.

Finally, I examined the stress tolerance of multiple substitution mutants to observe whether excess proline results in increased stress tolerance in yeast. Recombinant baker's yeasts 3346/3347 expressing pYC130-*PRO1*-variant plasmids were tested for their freezing stress and high-sugar stress tolerance (Figure 22, Figure 24). In contrast to my expectation, there was no significant increase in stress tolerance in the multiple substitution mutants compared to the single substitution mutants. This result suggested that balanced proline recycle with active proline degradation (Sasano et al., 2012b) rather than very high intracellular proline concentration is more important in stress tolerance, which is in agreement with a previous study (Kavi Kishor and Sreenivasulu, 2014). In addition, the proper localization of proline may play an important role in stress tolerance, since excess proline is accumulated in the vacuoles (Matsuura and Takagi, 2005).

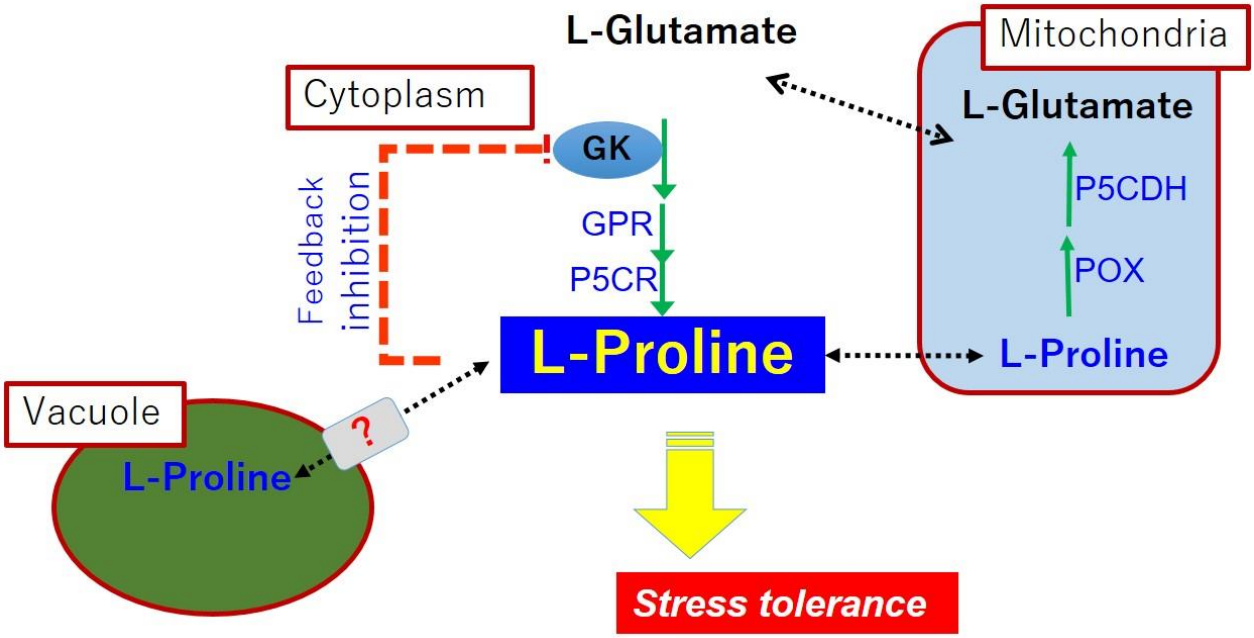

Figure 25. Proline as stress protectant in baker's yeast.
Cellular proline homeostasis including right concentration and localization of proline in the cell and proline recycle is important in overall action of proline as stress protectant. GK: *γ-glutamyl kinase*, GPR: *γ-glutamyl phosphate reductase*, P5CR: *Δ^1-pyrroline-5-carboxylate reductase*, POX: *proline oxidase*, P5CDH: *pyrroline-5-caroxylate dehydrogenase*

5. Conclusions

This study is the first to report the isolation of diploid baker's yeast mutants with proline accumulation by conventional mutagenesis and to confirm the resulting increase in tolerance to freeze-thaw and high-sucrose stresses. Here, I found that the novel *PRO1* mutations (*PRO1*^{P247S}, *PRO1*^{E415K}) in baker's yeast caused desensitization to the proline feedback inhibition of GK, leading to intracellular proline accumulation. Although self-cloning yeasts, which do not contain any foreign genes or DNA sequences except for yeast DNA, do not have to be treated as genetically modified yeasts, the conventional methods for breeding baker's yeasts are more acceptable to consumers than the use of self-cloning yeasts. Thus, the approach described here could be a practical method for breeding novel baker's yeasts and brewer's yeasts. Moreover, the novel mutations of *PRO1* identified in this study can be applied to strain improvement in other industrial strains, such as recombinant bioethanol yeast. To understand the role of P247 and E425K in yeast GK in more detail, it would be advantageous to know the crystal structure of yeast GK and thus to carry out molecular dynamics analysis of the yeast GK protein structure rather than studying the already defined crystal structure of *E. coli* GK.

Also in this study, I analyzed the cumulative effect of multiple amino acid substitutions in *PRO1*. When various combinations of the active site mutations (I150T and D154N) and the novel mutations (P247S and E415K) were used, the AZC-resistance and proline accumulation increased, but no significant improvement of stress tolerance was observed in the yeasts expressing variants of *PRO1* with multiple mutations. In consideration of all the above results, the method described herein is a promising approach for improving yeast strains by introducing novel mutations in *PRO1* to increase the compatible solute proline in cells.

6. Future perspectives

Based on this study, it is worth to carry out whole genome sequence analysis for the strains a6 and a19, in order to identify the mutation(s) that contribute to their proline accumulation and stress tolerance phenotypes.

Moreover, possibility of excess proline export from the cell should be considered and, it is suggested to determine the concentration of proline in the culture medium growing cells with high proline accumulation.

Because yeast GK crystal structure is not defined, *E.coli* GK, which has highest similarity to yeast GK in terms of domain structure and primary amino acid sequence, can be used for molecular dynamics analysis in order to determine detailed contribution of the novel mutations P247S and E425K in change in enzyme kinetics.

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8. References

Andréasson, C., Neve, E.P.A., Ljungdahl, P.O., 2004. Four permeases import proline and the toxic proline analogue azetidine-2-carboxylate into yeast. Yeast 21, 193–199. https://doi.org/10.1002/yea.1052

Baek, K.-H., Skinner, D.Z., 2012. Production of reactive oxygen species by freezing stress and the protective roles of antioxidant enzymes in plants. J. Agric. Chem. Environ. 1, 34–40. https://doi.org/10.4236/jacen.2012.11006

Fichman, Y., Gerdes, S.Y., Kovács, H., Szabados, L., Zilberstein, A., Csonka, L.N., 2015. Evolution of proline biosynthesis: enzymology, bioinformatics, genetics, and transcriptional regulation. Biol. Rev. 90, 1065–1099. https://doi.org/10.1111/brv.12146

Fujita, T., Maggio, A., García-Roís, M., Stauffacher, C., Bressan, R.A., Csonka, L.N., 2003. Identification of regions of the tomato γ-glutamyl kinase that are involved in allosteric regulation by proline. J. Biol. Chem. 278, 14203–14210. https://doi.org/10.1074/jbc.M212177200

Hayat, S., Hayat, Q., Alyemeni, M.N., Wani, A.S., Pichtel, J., Ahmad, A., 2012. Role of proline under changing environments: A review. Plant Signal. Behav. 7, 1–11. https://doi.org/10.4161/psb.21949

Hermes-Lima, M., Storey, K.B., 1993. Antioxidant defenses in the tolerance of freezing and anoxia by garter snakes. Am. J. Physiol. 265, R646-52. https://doi.org/10.1152/ajpregu.1993.265.3.R646

Kaino, T., Tasaka, Y., Tatehashi, Y., Takagi, H., 2012. Functional analysis of the C-terminal region of γ-glutamyl kinase of Saccharomyces cerevisiae. Biosci. Biotechnol. Biochem. 76, 454–61.

Kaino, T., Tateiwa, T., Mizukami-Murata, S., Shima, J., Takagi, H., 2008. Self-Cloning Baker's Yeasts That Accumulate Proline Enhance Freeze Tolerance in Doughs. Appl. Environ. Microbiol. 74, 5845–5849. https://doi.org/10.1128/AEM.00998-08

Kaul, S., Sharma, S.S., Mehta, I.K., 2008. Free radical scavenging potential of L-proline: evidence from in vitro assays. Amino Acids 34, 315–20. https://doi.org/10.1007/s00726-006-0407-x

Kavi Kishor, P.B., Sreenivasulu, N., 2014. Is proline accumulation per se correlated with stress tolerance or is proline homeostasis a more critical issue? Plant. Cell Environ. 37, 300–311. https://doi.org/10.1111/pce.12157

Kayabaşı, A., Mucan, B., 2011. An Empirical Study of Consumer Attitudes and Perceptions Toward Genetically Modified Foods (GMF). Eur. J. Soc. Sci. Akıncılar Caddesi 25, 52–65.

Landolfo, S., Politi, H., Angelozzi, D., Mannazzu, I., 2008. ROS accumulation and oxidative damage to cell structures in Saccharomyces cerevisiae wine strains during fermentation of high-sugar-containing medium. Biochim. Biophys. Acta - Gen. Subj. 1780, 892–898. https://doi.org/10.1016/J.BBAGEN.2008.03.008

Liang, X., Dickman, M.B., Becker, D.F., 2014. Proline biosynthesis is required for endoplasmic reticulum stress tolerance in Saccharomyces cerevisiae. J. Biol. Chem. 289, 27794–806. https://doi.org/10.1074/jbc.M114.562827

Marco-Marín, C., Gil-Ortiz, F., Pérez-Arellano, I., Cervera, J., Fita, I., Rubio, V., 2007. A novel two-domain architecture within the amino acid kinase enzyme family revealed by the crystal structure of Escherichia coli glutamate 5-kinase. J. Mol. Biol. 367, 1431–46. https://doi.org/10.1016/j.jmb.2007.01.073

Matsuura, K., Takagi, H., 2005. Vacuolar Functions Are Involved in Stress-Protective Effect of Intracellular Proline in Saccharomyces cerevisiae 100, 538–544. https://doi.org/10.1263/jbb.100.538

Morita, Y., Nakamori, S., Takagi, H., 2003. L-proline accumulation and freeze tolerance of Saccharomyces cerevisiae are caused by a mutation in the PRO1 gene encoding gamma-glutamyl kinase. Appl. Environ. Microbiol. 69, 212–9. https://doi.org/10.1128/AEM.69.1.212-219.2003

Park, J.-I., Grant, C.M., Davies, M.J., Dawes, I.W., 1998. The Cytoplasmic Cu,Zn Superoxide Dismutase of Saccharomyces cerevisiae Is Required for Resistance to Freeze-Thaw Stress GENERATION OF FREE RADICALS DURING FREEZING AND THAWING. J. Biol. Chem. 273, 22921–22928.

Pérez-Torrado, R., Panadero, J., Hernández-López, M., Prieto, J., Randez-Gil, F., 2010. Global expression studies in baker's yeast reveal target genes for the improvement of industrially-relevant traits: the cases of CAF16 and ORC2. Microb. Cell Fact. 9, 56. https://doi.org/10.1186/1475-2859-9-56

Rajendrakumar, C.S., Suryanarayana, T., Reddy, A.R., 1997. DNA helix destabilization by proline and betaine: possible role in the salinity tolerance process. FEBS Lett. 410, 201–5.

Ribotta, P.D., León, A.E., Añón, M.C., 2001. Effect of Freezing and Frozen Storage of Doughs on Bread Quality. J. Agric. Food Chem. 49, 913–918. https://doi.org/10.1021/jf000905w

Rose, M.D., Broach, J.R., 1991. Cloning genes by complementation in yeast. Methods Enzymol. 194, 195–230.

Rubenstein, E., McLaughlin, T., Winant, R.C., Sanchez, A., Eckart, M., Krasinska, K.M., Chien, A., 2009. Azetidine-2-carboxylic acid in the food chain. Phytochemistry 70, 100–104. https://doi.org/10.1016/j.phytochem.2008.11.007

Sasano, Y., Haitani, Y., Hashida, K., Ohtsu, I., Shima, J., Takagi, H., 2012a. Enhancement of the proline and nitric oxide synthetic pathway improves fermentation ability under multiple

baking-associated stress conditions in industrial baker's yeast. Microb. Cell Fact. 11, 40. https://doi.org/10.1186/1475-2859-11-40

Sasano, Y., Haitani, Y., Ohtsu, I., Shima, J., Takagi, H., 2012b. Proline accumulation in baker's yeast enhances high-sucrose stress tolerance and fermentation ability in sweet dough. Int. J. Food Microbiol. 152, 40–43. https://doi.org/10.1016/j.ijfoodmicro.2011.10.004

Sekine, T., Kawaguchi, A., Hamano, Y., Takagi, H., 2007. Desensitization of feedback inhibition of the Saccharomyces cerevisiae gamma-glutamyl kinase enhances proline accumulation and freezing tolerance. Appl. Environ. Microbiol. 73, 4011–9. https://doi.org/10.1128/AEM.00730-07

Shima, J., Takagi, H., 2009. Stress-tolerance of baker's-yeast (Saccharomyces cerevisiae) cells: stress-protective molecules and genes involved in stress tolerance. Biotechnol. Appl. Biochem. 53, 155–164. https://doi.org/10.1042/BA20090029

Signorelli, S., Dans, P.D., Coitiño, E.L., Borsani, O., Monza, J., 2015. Connecting Proline and γ-Aminobutyric Acid in Stressed Plants through Non-Enzymatic Reactions. PLoS One 10, e0115349. https://doi.org/10.1371/journal.pone.0115349

Smirnoff, N., Cumbes, Q.J., 1989. Hydroxyl radical scavenging activity of compatible solutes. Phytochemistry 28, 1057–1060. https://doi.org/10.1016/0031-9422(89)80182-7

Steensels, J., Snoek, T., Meersman, E., Nicolino, M.P., Voordeckers, K., Verstrepen, K.J., 2014. Improving industrial yeast strains: exploiting natural and artificial diversity. FEMS Microbiol. Rev. 38, 947–995. https://doi.org/10.1111/1574-6976.12073

Takagi, H., 2008. Proline as a stress protectant in yeast: physiological functions, metabolic regulations, and biotechnological applications. Appl. Microbiol. Biotechnol. 81, 211–223. https://doi.org/10.1007/s00253-008-1698-5

Takagi, H., Iwamoto, F., Nakamori, S., 1997. Isolation of freeze-tolerant laboratory strains of Saccharomyces cerevisiae from proline-analogue-resistant mutants. Appl. Microbiol. Biotechnol. 47, 405–11.

Takagi, H., Taguchi, J., Kaino, T., 2016. Proline accumulation protects *Saccharomyces cerevisiae* cells in stationary phase from ethanol stress by reducing reactive oxygen species levels. Yeast 33, 355–363. https://doi.org/10.1002/yea.3154

Tatehashi, Y., Takagi, H., 2013. Characterization of γ-glutamyl kinase mutants from Saccharomyces cerevisiae. J. Biosci. Bioeng. 116, 576–579. https://doi.org/10.1016/j.jbiosc.2013.05.018

Tatehashi, Y., Watanabe, D., Takagi, H., 2016. γ-Glutamyl kinase is involved in selective autophagy of ribosomes in *Saccharomyces cerevisiae*. FEBS Lett. 590, 2906–2914. https://doi.org/10.1002/1873-3468.12318

Trotter, E.W., Kao, C.M.-F., Berenfeld, L., Botstein, D., Petsko, G.A., Gray, J. V, 2002. Misfolded proteins are competent to mediate a subset of the responses to heat shock in Saccharomyces cerevisiae. J. Biol. Chem. 277, 44817–25. https://doi.org/10.1074/jbc.M204686200

Valishkevych, B. V., Vasylkovska, R.A., Lozinska, L.M., Semchyshyn, H.M., 2016. Fructose-Induced Carbonyl/Oxidative Stress in *S. cerevisiae* : Involvement of TOR. Biochem. Res. Int. 2016, 1–10. https://doi.org/10.1155/2016/8917270

Wang, F., Zeng, B., Sun, Z., Zhu, C., 2009. Relationship Between Proline and Hg2+-Induced Oxidative Stress in a Tolerant Rice Mutant. Arch. Environ. Contam. Toxicol. 56, 723–731. https://doi.org/10.1007/s00244-008-9226-2