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# Polyploid engineering by increasing mutant gene dosage in yeasts

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## Summary

**The yeast *Saccharomyces cerevisiae*, widely used for ethanol production, is one of the best-understood biological systems. Diploid strains of *S. cerevisiae* are preferred for industrial use due to the better fermentation efficiency, in terms of vitality and endurance as compared to those of haploid strains. Whole-genome duplications is known to promote adaptive mutations in microorganisms, and allelic variations considerably contribute to the product composition in ethanol fermentation. Although fermentation can be regulated using various strains of yeast, it is quite difficult to make fine adjustment of each component in final products. In this study, we demonstrate the use of polyploids with varying gene dosage (the number of copies of a particular gene present in a genome) in the regulation of ethanol fermentation. Ethyl caproate is one of the major flavouring agents in a Japanese alcoholic beverage called sake. A point mutation in *FAS2* encoding the α subunit of fatty acid synthetase induces an increase in the amount of caproic acid, a precursor of ethyl caproate. Using the *FAS2* as a model, we generated and evaluated yeast strains with varying mutant gene dosage. We demonstrated the possibility to increase mutant gene dosage via loss of heterozygosity in diploid and tetraploid strains. Productivity of ethyl caproate gradually increased with mutant gene dosage among tetraploid strains. This approach can potentially be applied to a variety of yeast strain development via growth-based screening.**

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## Introduction

Yeast has been implemented in the production of various useful compounds. *Saccharomyces cerevisiae,* commonly referred to as budding yeast is employed in the production of ethanol via fermentation (Yamada *et al*., 2010; Fukuda *et al*., 2013). *S. cerevisiae* cells primarily exist in two forms: haploid and diploid (Zhang *et al*., 2017). Although haploid cells cannot endure high-stress conditions such as nutrient starvation, diploid cells survive harsh environmental conditions via sporulation process in sexual reproduction (Fukuda and Honda, 2019). Diploidization has been considered as an evolutionary phenomenon that develops adaptation in response to drastic changes in growth conditions (Mangado *et al*., 2018). Diploid strains of *S. cerevisiae* are commonly used in brewing industries due to the better fermentation efficiency in terms of vitality and endurance as compared to those of haploid strains. However, strenuous efforts have been devoted to develop custom-engineered strains of *S. cerevisiae* to improve the production of ethanol and other compounds such as organic acids.

Mutagenesis is one of the conventional and effective approaches utilized for modifying yeast traits. Ethyl methanesulfonate (EMS) and ultraviolet (UV) light are commonly used mutagens. Mutagenic treatments can induce 100-fold increase in mutations per gene. The mutagenized cells can then be screened for any specific phenotype of interest, such as, auxotrophy and antibiotic sensitivity (Winston, 2008). Due to heterozygosity at one or more loci in diploid cells, genetic alterations that contribute to phenotypic changes as a result of mutagenesis are more complex compared to those in haploid cells. An exception to this would be in the case of recessive mutations, where modified diploid cells carry two alleles of the same gene on homologous chromosomes.

Dominance and recessiveness are fundamental principles of modern genetics. In the case of complete dominance, a phenotype of the heterozygous offspring is identical to one of the homozygous parents. Partial dominance is another type of dominance in which a

heterozygote displays a phenotype that is an intermediate between the phenotypes of both homozygote parents. Cell morphology is one of attractive targets for phenotype analysis because it reflects a wide variety of cellular events (Ohya *et al*., 2005). A recent morphological study on heterozygous diploids of *S. cerevisiae* revealed that majority of essential genes are haploinsufficient (Ohnuki and Ohya, 2018), which indicates the contribution of gene dosage effects on phenotypes. Furthermore, foreign genes were downregulated in diploid cells generated through mating of haploid cells containing various foreign genes (Fukuda and Honda, 2018). Thus, gene dosage (the number of copies of a particular gene present in a genome) is an important factor in yeast trait modification.

*S. cerevisiae* has also been used as an excellent model organism for studying genome evolution (Lu *et al*., 2016). Polyploid (especially tetraploid) yeast cells are employed in several human-activities such as, bread-making (Albertin *et al*., 2009) and lager-brewing (Casaregola *et al*., 2001; Dunn and Sherlock, 2008; Nakao *et al*., 2009; Libkind *et al*., 2011). Due to multiple alleles of the same gene on homologous chromosomes, many polyploid species display higher heterozygosity levels compared to their diploid counterparts as in yeasts (Albertin *et al*., 2009). Although polyploid strains have been extensively used as models for cancer or cell cycle defects (Thorpe *et al*., 2007), there is only few scientific knowledges on polyploid yeasts industrially used in bakery, brewery, etc (Albertin and Marullo, 2012). Additionally, complex heterozygosity associated with diploid yeasts contributes to the difficulty in understanding the gene dosage effect in food industry, where it is quite important to balance out various components to adjust the flavour.

Therefore, in this study, we investigated the dosage effect of the mutant gene of interest using haploids, diploids, triploids and tetraploids with isogenic background (Table 1). *FAS2* encodes α subunit of fatty acid synthetase and the *FAS2-G1250S* mutation leads to an increase in the amount of caproic acid (Ichikawa *et al*., 1991; Aritomo *et al*., 2004), a precursor of ethyl caproate, which is one of the major flavour components in the Japanese alcoholic beverage called sake (Tamura *et al*., 2015). To trace back to its origin, yeast strains with high ethyl caproate-producing ability were isolated based on its resistance to cerulenin (Ichikawa *et al*., 1991), an inhibitor of fatty acid synthesis. We performed quantitative evaluation of the phenotypic change caused due to *FAS2-G1250S* mutation using the cerulenin resistance as an indicator. We also increased the mutant gene dosage within yeast cells via regulating cerulenin concentration in the cultivation media.

**Table 1.** Yeast strain and plasmids used in this study.

| Name | Description | Reference source |
|---|---|---|
| Yeast strain | | |
| BY4741 | *MAT***a** *his3Δ1 ura3Δ0 leu2Δ0 met15Δ0* | Brachmann *et al*. (1998) |
| BY4742 | *MATα his3Δ1 ura3Δ0 leu2Δ0 lys2Δ0* | Brachmann *et al*. (1998) |
| BY4743 | Diploid strain generated by zygosis of BY4741 and BY4742 | Brachmann *et al*. (1998) |
| BY4741C | *MAT***a** *his3Δ1 ura3Δ0 leu2Δ0 met15Δ0 fas2*::*FAS2-G1250S* | Fukuda (2020) |
| BY4742C | *MATα his3Δ1 ura3Δ0 leu2Δ0 lys2Δ0 fas2*::*FAS2-G1250S* | Fukuda (2020) |
| BY4743C | Diploid strain generated by zygosis of BY4741C and BY4742 | Present study |
| BY4743CC | Diploid strain generated by zygosis of BY4741C and BY4742C | Present study |
| BY4743C1 | Diploid strain generated by integration of *FAS2-G1250S* into BY4743 | Present study |
| BY4743C2 | Diploid strain generated from BY4743C through intracellular homologous recombination | Present study |
| K9 | Sake yeast Kyokai No. 9; *MAT***a**/α-type diploid strain | NBRC[a] |
| K9C-S01 | Diploid strain generated by integration of *FAS2-G1250S* into K9; clone No.1 | Present study[b] |
| K9C-S03 | Diploid strain generated by integration of *FAS2-G1250S* into K9; clone No.3 | Present study[b] |
| K9C-S04 | Diploid strain generated by integration of *FAS2-G1250S* into K9; clone No.4 | Present study[b] |
| BY4743AA | *MAT***a**/**a**-type diploid strain derived from BY4743 | Present study |
| BY4743CC-AA | *MAT***a**/**a**-type diploid strain derived from BY4743CC | Present study |
| BY4743-3 | Triploid strain generated by zygosis of BY4743AA and BY4742 | Fukuda (2020) |
| BY4743-3C | Triploid strain generated by zygosis of BY4743AA and BY4742C | Present study |
| BY4743-3CC | Triploid strain generated by zygosis of BY4743CC-AA and BY4742 | Present study |
| BY4743-3CCC | Triploid strain generated by zygosis of BY4743CC-AA and BY4742C | Present study |
| BY4743-3AAA | *MAT***a**/**a**/**a**-type triploid strain derived from BY4743-3 | Fukuda (2020) |
| BY4743-3CC-AAA | *MAT***a**/**a**/**a**-type triploid strain derived from BY4743-3CC | Present study |
| BY4743-3CCC-AAA | *MAT***a**/**a**/**a**-type triploid strain derived from BY4743-3CCC | Present study |
| BY4743-4 | Tetraploid strain generated by zygosis of BY4743-3AAA and BY4742 | Present study |
| BY4743-4C | Tetraploid strain generated by zygosis of BY4743-3AAA and BY4742C | Present study |

**Table 1.** (Continued)

| Name | Description | Reference source |
|---|---|---|
| BY4743-4CC | Tetraploid strain generated by zygosis of BY4743-3CC-AAA and BY4742 | Present study |
| BY4743-4CCC | Tetraploid strain generated by zygosis of BY4743-3CCC-AAA and BY4742 | Present study |
| BY4743-4CCCC | Tetraploid strain generated by zygosis of BY4743-3CCC-AAA and BY4742C | Present study |
| BY4743-4C1 | Tetraploid strain generated by integration of *FAS2-G1250S* into BY4743-4 | Present study |
| BY4743-4CX | Tetraploid strain generated from BY4743-4C through intracellular homologous recombination | Present study |
| BY4743-4C4 | Tetraploid strain generated from BY4743-4CX through intracellular homologous recombination | Present study |
| Plasmid | | |
| pYO323 | yeast expression vector containing $2\mu$ ori and *HIS3* marker | NBRP[c] |
| pUY-Pa1G-HO | $2\mu$ ori, *URA3* marker, $P_{PGK1}$-*a1*-$T_{ADH1}$, and $P_{GAL1}$-*HO*-$T_{ADH1}$ | Fukuda and Honda (2018) |

**a**. Resource was provided by Biological Resource Center (NBRC), NITE, Japan.
**b**. Strain K9C-S02 (clone No.2) was excluded as a false positive clone (without cerulenin resistance).
**c**. Resource was provided by the National BioResource Project (NBRP) of the MEXT (Japan).

## Results and Discussion

### *Confirmation of the FAS2 mutation and cerulenin resistance*

Haploid strains BY4741C (*MAT***a**) and BY4742C (*MAT*$\alpha$) containing the *FAS2-G1250S* mutation (Table 1) were previously generated via homologous recombination (Fukuda, 2020). The difference in nucleotide sequence between the wild-type *FAS2* and *FAS2-G1250S* allele was confirmed using Sanger sequencing (Fig. S1). To easily distinguish the *FAS2-G1250S* mutant from the wild-type gene, we amplified a region of the *FAS2* gene (1424-bp in length) using PCR, cleaved the DNA fragments with the restriction enzyme *Bfa*I (Fig. 1A), and analysed the final products using electrophoresis pattern (Fig. 1B), referring to a past report (Tamura *et al*., 2015). Since haploid strains have a single set of chromosomes, the *FAS2-G1250S* mutant (BY4741C and BY4742C) was distinguished from the *FAS2* gene (BY4741 and BY4742) by the absence of a 645 bp DNA fragment, and the presence of 420 bp and 225bp DNA fragments. In some cases, there was a small amount of undigested DNA fragments were observed even after digestion with *Bfa*I (lanes 2 and 6) as shown in Figure 1B, indicating incomplete digestion. These undigested DNA fragments completely disappeared by modulating the ratio of enzyme to DNA (Fig. S2).

For the quantitative evaluation of the effect of *FAS2-G1250S* mutation, dose responsive curves for cerulenin were compared between *MAT***a** haploid strains, BY4741 and BY4741C. We observed that strain BY4741C was able to grow in cultivation media with approximately 10-fold higher concentration of cerulenin than strain BY4741 (Fig. 1C and Table 2). With $> 50$ $\mu$M cerulenin, there was no significant increase in optical density measured at 620 nm ($OD_{620}$) in case of BY4741C strain. Since fatty acids are essential components of eukaryotic and bacterial cells, a large amount of cerulenin supplemented via extracellular sources would prevent cell growth via inhibition of fatty acid synthesis. The inhibition of fatty acid synthesis using cerulenin is based on its covalent binding to the cysteine residue at the active site of the condensing enzyme component (Kawaguchi *et al*., 1982; Tomoda *et al*., 1987). Although the *FAS2-G1250S* mutation decreases carbon chain elongation during fatty acid synthesis, substitution of the glycine residue at position 1250 causes steric hindrance to cerulenin binding, thereby inducing cerulenin resistance in yeast mutant strains (Aritomo *et al*., 2004).

### *Generation of diploid strains for evaluation of the homozygous or heterozygous mutations*

Subsequently, we generated *MAT***a**/$\alpha$ diploid strains through yeast mating between *MAT***a** and *MAT*$\alpha$ haploid strains (Fig. 2A). We performed DNA fragment analysis using electrophoresis pattern similar to the haploid strains (Fig. 2B). Due to the homozygosity of the *FAS2* gene, diploid strains BY4743 and BY4743CC exhibited the same electrophoresis patterns as haploid strains BY4741 and BY4741C respectively. In the case of the heterozygous diploid strain BY4743C, there we observed four DNA fragments of varying lengths (779, 645, 420 and 225 bp) due to the presence of two alleles, *FAS2* and *FAS2-G1250S*. Next, we compared dose responsive curves for cerulenin among the three diploid strains. Similar to relationship between haploids, diploid strain BY4743CC was able to grow in cultivation media with approximately 10-fold higher concentration of cerulenin than strain BY4743 (Fig. 2C and Table 2). Although homozygous diploid strains BY4743 and BY4743CC exhibited similar dose responsive curves to those of strains BY4741 and BY4741C, respectively, heterozygous diploid strain BY4743C exhibited intermediate behaviour, indicating partial dominance of the *FAS2-G1250S* mutation.

Based on the difference in cerulenin resistance among diploid strains described above, we introduced to a

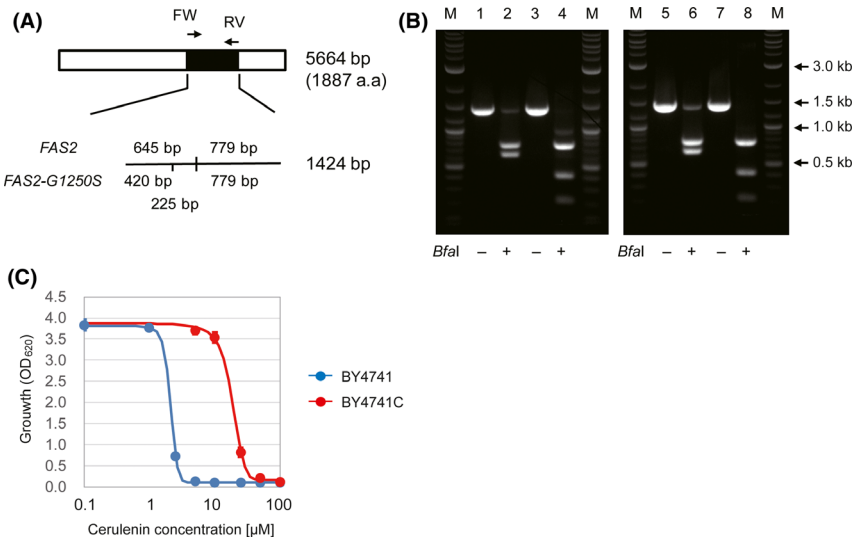



**Fig. 1.** Generation and evaluation of haploid strains containing the *FAS2-G1250S* mutation.A. Confirmation of the *FAS2* mutation site. The *FAS2* genes were amplified using PCR, and the amplified 1424 bp fragment was digested using *Bfa*I. Cleaved DNA fragments of the wild-type *FAS2* and mutated *FAS2-G1250S* are 2 bands (779 and 645 bp) and 3 bands (779, 420 and 225 bp) respectively.B. Cleavage pattern of the PCR-amplified fragment following *Bfa*I digestion. *Bfa*I-digested (+) and non-digested DNA fragments (−) were loaded onto an agarose gel. Lane M indicates a DNA size marker, 1 and 2 indicate strain BY4741, 3 and 4 indicate strain BY4741C, 5 and 6 indicate strain BY4742, and 7 and 8 indicate strain BY4742C.C. Dose-response curves for antibiotic cerulenin. Values are presented as means ± standard deviations from three independent experiments.

**Table 2.** $IC_{50}$ values of cerulenin for growth inhibition of yeast cells.

| Strain | Ploidy | Mutant gene dosage of *FAS2-G1250S* | $IC_{50}$ values of cerulenin [μM] |
|---|---|---|---|
| BY4741 | Haploid | 0 | 2.11 |
| BY4741C | Haploid | 1 | 19.1 |
| BY4743 | Diploid | 0 | 2.11 |
| BY4743C | Diploid | 1 | 4.91 |
| BY4743CC | Diploid | 2 | 21.8 |
| BY4743-3 | Triploid | 0 | 1.83 |
| BY4743-3C | Triploid | 1 | 4.76 |
| BY4743-3CC | Triploid | 2 | 10.7 |
| BY4743-3CCC | Triploid | 3 | 31.4 |
| BY4743-4 | Tetraploid | 0 | 2.04 |
| BY4743-4C | Tetraploid | 1 | 3.58 |
| BY4743-4CC | Tetraploid | 2 | 5.17 |
| BY4743-4CCC | Tetraploid | 3 | 14.0 |
| BY4743-4CCCC | Tetraploid | 4 | 23.5 |

cerulenin-sensitive diploid strain (BY4743) with *FAS2-G1250S* mutation via homologous recombination and increased mutant gene dosage in diploid cells through loss of heterozygosity (LOH). As shown in Fig. 3A, homologous recombination could occur at either of the *FAS2* loci due to its low frequency when strain BY4743 was transformed with PCR fragments with the *FAS2-G1250S* mutation. We then isolated heterozygous cells generated via recombination of the *FAS2* using 10 μM cerulenin, which yielded diploid strain BY4743C1. DNA fragment analysis shows the heterozygosity at the *FAS2* loci in the generated diploid strain (Fig. 3B). LOH is a natural event that generates homozygous loci via chromosomal rearrangement in heterozygous loci (Daigaku *et al*, 2004; Alvaro *et al*, 2006; Andersen *et al*., 2008; Takagi *et al*, 2008). Although the spontaneous frequency of LOH is below $1 \times 10^{-4}$ as reported in previous studies (Hiraoka *et al*, 2000; Fukuda and Honda, 2013; Fukuda *et al*, 2016), it is higher than that of a point mutation, approximately $1 \times 10^{-6}$ (Hayashi and Umezu, 2017). After cultivating strain BY4743C without cerulenin, we isolated homozygous cells generated from heterozygous cells via LOH of the *FAS2* using 25 μM cerulenin (Fig. 3C). In replica plating, five out of eight clones were able to grow in the same condition (25 μM cerulenin). After confirming equivalency, one of the replicated clones was selected as diploid strain BY4743C2. Then, we confirmed the homozygosity at the *FAS2* loci in the generated diploid strain using DNA fragment analysis (Fig. 3D). These results indicated that it is possible to regulate mutant gene dosage of *FAS2-G1250S* in diploid cells using cerulenin resistance as an indicator.

Similarly, we introduced the *FAS2-G1250S* mutation to a sake yeast strain Kyokai No.9 (K9) via homologous recombination. Sake is a traditional Japanese alcoholic

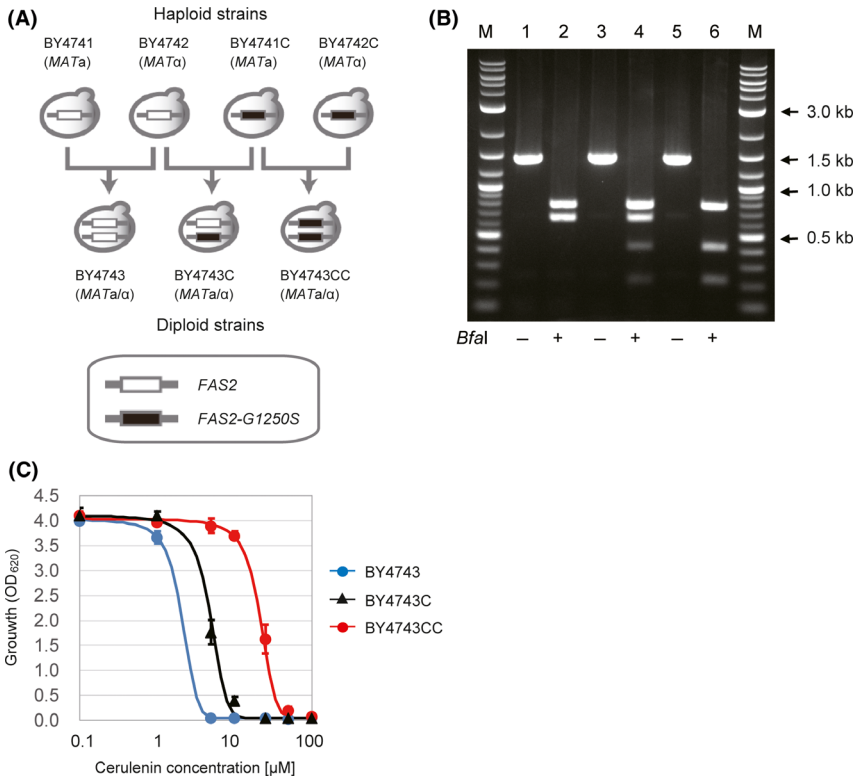



**Fig. 2.** Generation and evaluation of diploid strains with different number of the *FAS2-G1250S* mutation.A. Schematic outline of yeast mating for diploids.B. Cleavage pattern of the DNA fragments. *Bfa*I-digested (+) and non-digested DNA fragments (−) were loaded onto an agarose gel. Lane M indicates a DNA size marker, 1 and 2 indicate strain BY4743, 3 and 4 indicate strain BY4743C, and 5 and 6 indicate strain BY4743CC.C. Dose–response curves for antibiotic cerulenin. Values are presented as means ± standard deviations from three independent experiments.

beverage made from fermented rice, and sake yeast strains have many characteristics suitable for brewing, such as aromatic production and high ethanol tolerance (Katou *et al*, 2008). A previous study (Fukuda *et al*, 2016) has reported that K9 strain maintains a near-diploid DNA content, which was measured using FACS analysis after propidium iodide-staining. Unexpectedly, only homozygous cells were generated via recombination of the *FAS2* gene in K9 strain (Fig. S3A). K9C-S01, K9C-S03 and K9C-S04 strains exhibited reduced cerulenin resistance as compared to the homozygous diploid strain BY4743CC (Fig. 4A). To investigate the copy number of the *FAS2* in K9 strain, real-time PCR was carried out using the haploid strain BY4741 as the reference sample. The relative copy number of the *RAD51* (locating at the right arm of chromosome V) and *PGK1* (locating at the right arm of chromosome III) was calculated against the *FAS2* (locating at the left arm of chromosome XVI). Unlike the *RAD51*, the relative copy number of the *PGK1* gene was approximately double that of the *FAS2* gene in the K9 strain (Fig. 4B). On the other hand, the relative copy number of the *RAD51* and *PGK1* was the identical to *FAS2* in the diploid strain BY4743 (Fig. S3B). These results suggest that K9 strain is an aneuploid and might have lost one of the two *FAS2* loci through spontaneous chromosomal aberration such as mitotic chromosome loss (Fukuda and Honda, 2013). Since chromosomal aneuploidy is frequently seen in other sake brewery yeasts (Kadowaki *et al*, 2017), we further investigated the dosage effect of the *FAS2-G1250S* mutation using laboratory-adapted triploid and tetraploid strains with isogenic background.

### *The dosage effect of the FAS2-G1250S mutation in triploids and tetraploids*

Mating type conversion was carried out (Fig. 5A) according to a previously reported method (Fukuda and Honda, 2018). Triploid and tetraploid strains were generated via yeast mating. Haploid strains BY4741 (*MAT***a**) and BY4742 (*MAT*α) are derived from S288C (Brachmann *et al*, 1998) strain, which is one of the most widely used laboratory-adapted yeast strains, with its genome sequence registered in the *Saccharomyces* Genome Database (http://www.yeastgenome.org/). All S288C-derived strains possess the stuck mutation (Ray *et al*,

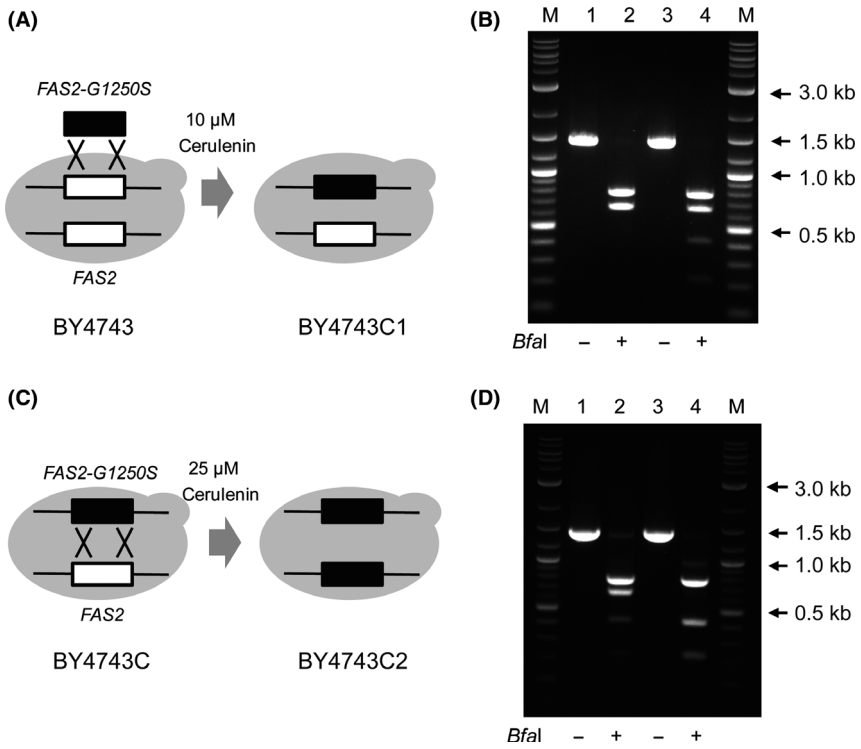



**Fig. 3.** Growth selection of the *FAS2-G1250S* mutant diploid cells.A. Schematic outline of introduction of the *FAS2-G1250S* mutation using homologous recombination. Mutant cells were isolated on a solid media containing 10 μM cerulenin.B. Cleavage pattern of the DNA fragments. *Bfa*I-digested (+) and non-digested DNA fragments (−) were loaded onto an agarose gel. Lane M indicates a DNA size marker, 1 and 2 indicate strain BY4743, and 3 and 4 indicate strain BY4743C1.C. Schematic outline of increase of mutant gene dosage in diploid cells through LOH. Homozygous mutant cells were isolated on a solid media containing 25 μM cerulenin.D. Cleavage pattern of the DNA fragments. *Bfa*I-digested (+) and non-digested DNA fragments (−) were loaded onto an agarose gel. Lane M indicates a DNA size marker, 1 and 2 indicate strain BY4743C, and 3 and 4 indicate strain BY4743C2.

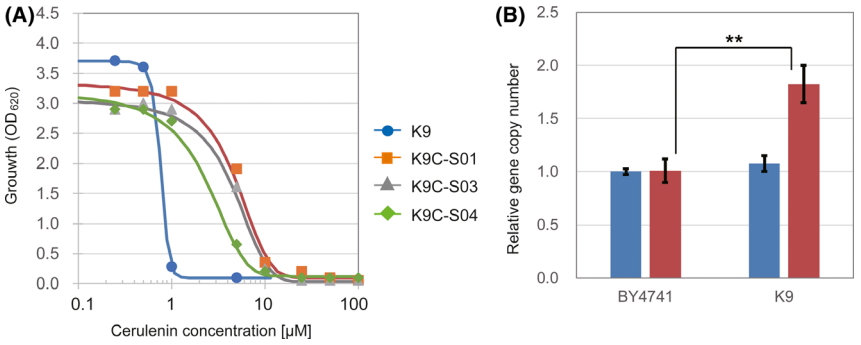



**Fig. 4.** Generation of sake yeast strains with the cerulenin resistance.A. Dose–response curves for antibiotic cerulenin.B. Ploidy analysis using real-time PCR. Blue columns indicate the relative copy number of the *RAD51* against the *FAS2*, and red columns indicate that of the *PGK1*. Values are presented as means ± standard deviations from three replicate experiments (**P* < 0.05, ***P* < 0.01)

1991) at the *HMR***a** locus, which significantly reduces the Ho endonuclease cleavage (Herskowitz and Jensen, 1991) after it is transferred to the *MAT* locus via mating type conversion. As shown in Figure 5A, diploid strains generated in this study maintain both uncleavable *MAT***a** and cleavable *MAT*α genes against the Ho endonuclease. After the introduction of pUY-Pa1G-HO (Table 1), the expression of *HO* in *MAT***a**/α diploid cells could induce unidirectional replacement of DNA sequences at the *MAT* loci from α-type to **a**-type.

*MAT***a**/**a** diploid strains BY4743AA and BY4743CC-AA were generated using *MAT***a**/α diploid strains BY4743 and BY4743CC respectively (Table 1). Further, we generated triploid strains with varying numbers of the *FAS2-G1250S* mutation through yeast mating (Fig. 5B) and compared dose responsive curves for cerulenin among

of the four triploid strains (Fig. 5C). Like diploid strains, homozygous triploid strains BY4743-3 and BY4743-3CCC exhibited similar dose responsive curves to those of haploid strains BY4741 and BY4741C respectively. Furthermore, heterozygous triploid strains BY4743-3C and BY4743-3CC exhibited intermediate behaviours in response to the copy number of the *FAS2-G1250S* mutation (Table 2). These results suggest that phenotypic change in cerulenin resistance is determined by the ratio of partially dominant mutations at the *FAS2* loci.

Triploid strains described above maintain two copies of uncleavable *MAT***a** and one copy of cleavable *MAT*α genes against the Ho endonuclease (Fig. 5A). Using mating type conversion and yeast mating, we generated tetraploid strains with varying numbers of the *FAS2-G1250S* mutation via yeast mating (Table 1) and compared dose responsive curves for cerulenin among the five tetraploid strains (Fig. 5D). Similar to the triploid strains, both homozygous and heterozygous tetraploid strains exhibited gradual phenotypic change in cerulenin resistance according to the ratio of partially dominant mutations at the *FAS2* loci (Table 2). From haploid to tetraploid, homozygous strains (containing either *FAS2* or *FAS2-G1250S*) exhibited similar level of antibiotic resistance regardless of yeast ploidy.

Additionally, we investigated the effect of a single copy of the *FAS2-G1250S* mutation on the antibiotic resistance by comparing the $OD_{620}$ values of yeast cultures with initial cerulenin concentration set at 5 μM (Fig. S4). The antibiotic resistance induced by a single copy of mutation obviously declined as yeast ploidy increased. A similar phenomenon was also observed in the previous report, which used foreign genes encoding fluorescent proteins (Fukuda and Honda, 2018). In this case, the amount of intracellular $Fas2^{G1250S}$ would have declined as yeast ploidy increased, whereas wild-type Fas2 would have increased. Comparing the step size of phenotypic changes, a fine adjustment could be achieved using polyploid strains (polyploid engineering) instead of diploid strains via regulation of cellular functions involved in partially dominant mutations.

### *Polyploid engineering by increasing the dosage of the FAS2-G1250S mutation*

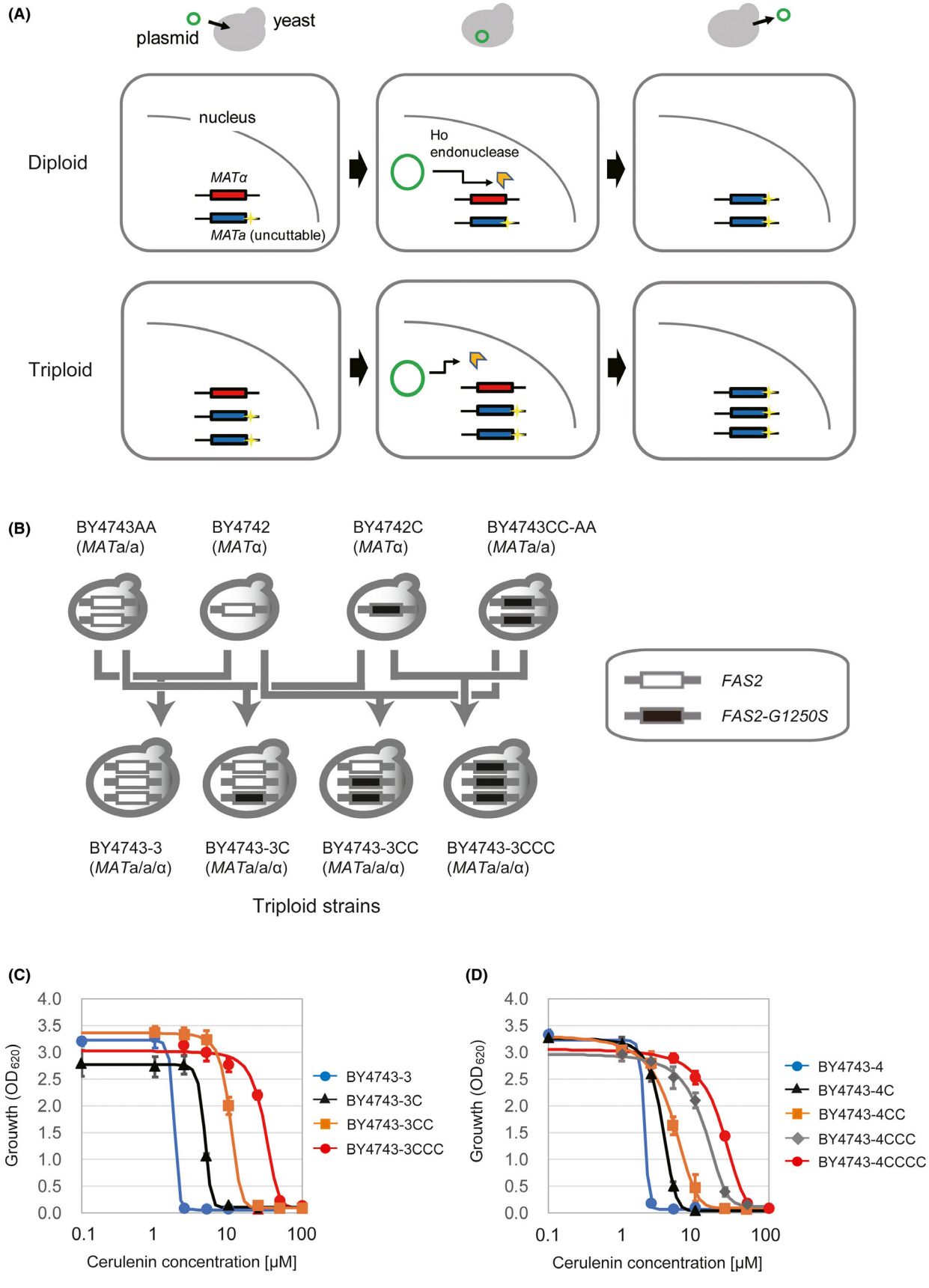
Similar to the diploid strains, we introduced the *FAS2-G1250S* mutation in a cerulenin-sensitive tetraploid strain (BY4743-4) via homologous recombination, and increased mutant gene dosage in tetraploid cells. As shown in Fig. 6A, homologous recombination could occur at one of four *FAS2* loci. Setting the cerulenin concentration at 5 μM, we isolated heterozygous cells generated via recombination of the *FAS2* and yielded tetraploid strain BY4743-4C1. DNA fragment analysis shows the existence of the *FAS2-G1250S* mutation in the generated tetraploid strain (Fig. 6B).

Unlike diploids, multicycle homologous recombination is required for generation of homozygous polyploids containing the same number of mutant alleles as the ploidy from heterozygous polyploids containing one copy of mutant allele. Setting cerulenin concentration at 25 μM, we increased the number of mutant alleles in tetraploid cells via homologous recombination of the *FAS2* (Fig. 6C). In replica plating, two out of four clones were able to grow in the same condition (25 μM cerulenin). After confirming equivalency, one of the replicated clones was selected as tetraploid strain BY4743-4CX. Furthermore, we isolated homozygous cells generated through LOH by increasing cerulenin concentration to 100 μM. In replica plating, three out of three clones were able to grow in the same condition (100 μM cerulenin), and one of them was selected as tetraploid strain BY4743-4C4. Then, we confirmed the homozygosity at the *FAS2* loci in the generated tetraploid strain by DNA fragment analysis (Fig. 6D). These results indicate that it is possible to regulate mutant gene dosage even in polyploid cells using cerulenin resistance as an indicator.

As described above, we revealed the relationship between cerulenin resistance and the *FAS2-G1250S* mutant gene dosage. To confirm whether the yeast cells that survived in cerulenin-rich environments can produce ethyl caproate at high concentration, we cultivated tetraploid strains at low temperature (15°C) appropriate for Japanese sake brewing (Fig. 7). The *FAS2-G1250S* mutation decreases carbon chain elongation activity during fatty acid synthesis, resulting in high productivity of ethyl caproate. Since fatty acids are essential components, an increase in the *FAS2-G1250S* mutant gene dosage caused declined cell growth rates (Fig. 7A). Tetraploid strains BY4743-4CX and BY4743-4C4 were able to grow to the same extent as BY4743-4CC (harbouring 2 copies of the *FAS2-G1250S* mutant allele) and BY4743-4CCCC (harbouring 4 copies of the mutant allele) respectively (Fig. S5A). At the end of cultivation,

**Fig. 5.** Generation and evaluation of triploid and tetraploid strains with different number of the *FAS2-G1250S* mutation.A. Schematic outline of mating type conversion used for polyploid generation following yeast mating. Plasmid pUY-Pa1G-HO was introduced into yeast cells (a/α type). Expression of the *HO* is induced by galactose, resulting in unidirectional conversion of the *MAT* gene from α to a-type. Finally, the plasmid is removed from the yeast cells converted into a-type.B. Schematic outline of yeast mating for triploids.C. Dose–response curves of triploid strains for antibiotic cerulenin.D. Dose–response curves of tetraploid strains for antibiotic cerulenin. Values are presented as means ± standard deviations from three independent experiments.



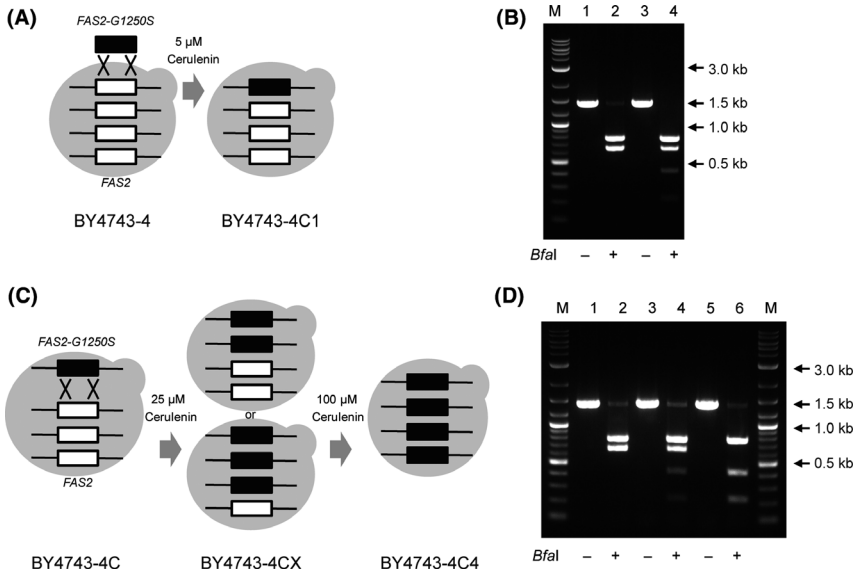



**Fig. 6.** Growth selection of the *FAS2-G1250S* mutant tetraploid cells.A. Schematic outline of introduction of the *FAS2-G1250S* mutation using homologous recombination. Mutant cells were isolated on a solid media containing 5 μM cerulenin.B. Cleavage pattern of the DNA fragments. *Bfa*I-digested (+) and non-digested DNA fragments (−) were loaded onto an agarose gel. Lane M indicates the DNA size marker, 1 and 2 indicate strain BY4743-4, and 3 and 4 indicate strain BY4743-4C1.C. Schematic outline of an increase in mutant gene dosage in tetraploid cells via intracellular homologous recombination. Mutant cells were isolated on a solid media containing 25 μM and 100 μM cerulenin at the first and second step of growth selection respectively.D. Cleavage pattern of the DNA fragments. *Bfa*I-digested (+) and non-digested DNA fragments (−) were loaded onto an agarose gel. Lane M indicates a DNA size marker, 1 and 2 indicate strain BY4743-4C, 3 and 4 indicate strain BY4743-4CX, and 5 and 6 indicate strain BY4743-4C4.

we evaluated and compared the productivity of ethyl caproate among tetraploid strains. Similar to cerulenin resistance, the productivity of ethyl caproate was improved with the *FAS2-G1250S* mutant gene dosage (Fig. 7B). There was no significant difference in the productivity of ethyl caproate between the strains, BY4743-4CCC (harbouring 3 copies of the mutant allele) and BY4743-4CCCC (harbouring 4 copies of the mutant allele). Considering that ethyl caproate concentration continued to increase till the end of cultivation of strain BY4743-4CCCC (Fig. S5B), longer cultivation period might be required to reach a plateau. By evaluating mutant gene dosage effect in tetraploids with isogenic background, it was also found that the trade-off relationship between ethyl caproate productivity and cell growth rate was caused by the *FAS2-G1250S* mutation (Fig. 7C). In addition, we successfully generated yeast strains with high ethyl caproate-producing ability (BY4743-4CX and BY4743-4C4) from heterozygous strain containing one copy of mutant allele (BY4743-4C) via cerulenin screening.

To evaluate the genome stability in the isolated homozygous yeast cells, we carried out passage culture (without cerulenin) of tetraploid strain BY4743-4C4 containing 4 copies of the *FAS2-G1250S* mutation. DNA fragment analysis demonstrated that yeast cells maintained the *FAS2-G1250S* mutation even after 10 passages of culture (Fig. S6A). Mutant gene dosages of yeast cells were estimated by evaluating the cell growth in cultivation medium containing 25 μM cerulenin (Fig. S6B). Compared to the tetraploid strain containing 3 copies of the *FAS2-G1250S* mutation (BY4743-4CCC), passaged cells exhibited higher cerulenin resistance, indicating the existence of 4 copies of the *FAS2-G1250S* mutation. These results indicate that the *FAS2-G1250S* mutation could be stably maintained in yeast cells even in the absence of cerulenin, and the generated yeast cells would exhibit stable performance in the industrial use such as brewing.

### *Challenge in yeast trait modifications using polyploid engineering*

A variety of antibiotics are utilized for selection of yeast cells of interest from mutagenized populations. In this study, we utilized cerulenin to distinguish the activity of the *FAS2-G1250S*. Unfortunately, however, it is not rare to isolate yeast cells having undergone off-target mutations instead of the on-target mutation in antibiotic screening. A previous study (Tamura *et al*, 2015) showed that less than 0.5% of cerulenin resistant mutants exhibited high ethyl caproate-producing ability. Similar to tumour cells, parasites, fungal pathogens or even bacteria, *S cerevisiae* exhibits multidrug resistance

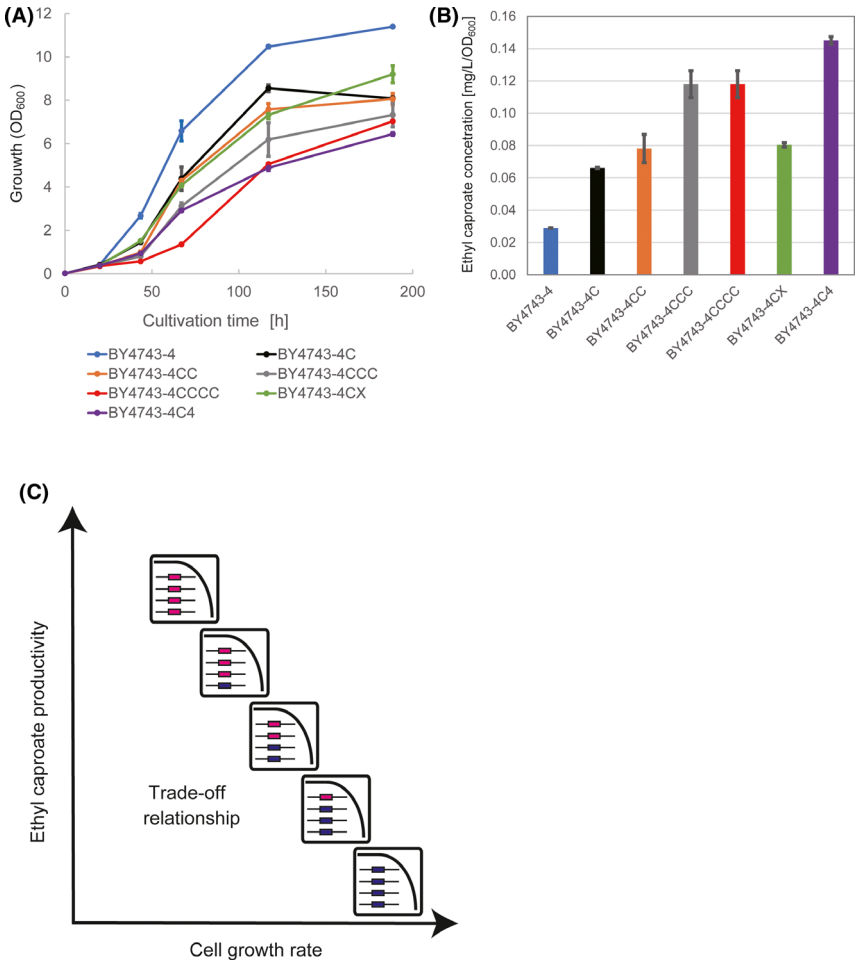



**Fig. 7.** Evaluation for ethyl caproate productivity of tetraploid strains with different number of the *FAS2-G1250S* mutation.A. Growth curve of each strain. The $OD_{600}$ value was monitored during cultivation in YPD10 medium without cerulenin.B. Productivity of ethyl caproate. At the end of cultivation, concentrations of ethyl caproate in supernatants were measured using GC/MS and productivities were calculated by dividing the ethyl caproate concentration with the $OD_{600}$ value. Values are presented as means ± standard deviations from two independent experiments.C. Schematic outline of the trade-off relationship between ethyl caproate productivity and cell growth rate. The *FAS2-G1250S* mutation has given yeast cells high productivity of ethyl caproate at the partial expense of carbon chain elongation activity during fatty acid synthesis.

to unrelated chemicals, as a result of functional enhancement of ATP-binding cassette (ABC) transporters (Jungwirth and Kuchler, 2006). It is known that the genome of *S. cerevisiae* harbours 30 distinct genes encoding ABC transporters, several of which are involved in cellular detoxification (Nourani *et al*, 1997). As described above, there is a possibility of including off-target mutations in case of antibiotic screening.

On the other hand, homologous recombination between intracellular homologous chromosomes occurs with higher frequencies than point mutations in polyploid cells (Hayashi and Umezu, 2017). Therefore, mutant gene dosage in polyploid cells can be increased via antibiotic screening without inducing undesirable off-target mutations. Additionally, excess amount of antibiotics causes annihilation of yeast cells, and shortage of antibiotics makes it difficult to isolate yeast cells with increased mutant gene dosage in polyploid engineering. In this study, we revealed the difference in cerulenin resistance provided by the *FAS2-G1250S* mutant gene dosage in haploids, diploids, triploids, and tetraploids. According to the quantitative analysis of cerulenin resistance, we successfully achieved an increased *FAS2-G1250S* mutant gene dosage via antibiotic screening with appropriate concentration.

## Conclusions

Mutant gene dosage plays a crucial role in yeast trait modification and can be achieved using the principle of partial dominance. In this study, we confirmed the dosage effect of the *FAS2-G1250S* mutation using

haploids, diploids, triploids and tetraploids with isogenic laboratory-adapted yeast strains. In polyploid engineering, homozygous strains generated were successfully isolated at higher concentrations of cerulenin as compared to heterozygous strains. There was an improved productivity of ethyl caproate with increase in mutant gene dosage. Therefore, this approach can be unrestrictedly used for growth-based selections of yeast strains which have been generated through mutagenesis and are currently used in food industries, by taking advantage of partial dominance of mutant genes. It would also facilitate fine adjustments in yeast trait modifications, thereby improving productivity, flavour and taste of compounds processed using industrial yeasts that are used in bakery, brewery, etc.

## Experimental procedures

### *Strains and media*

Detailed information regarding the *S. cerevisiae* laboratory yeast strains and plasmids used is listed in Table 1. Yeast cells were grown in YPD medium (1% yeast extract, 2% peptone, and 2% glucose), YPD10 medium (1% yeast extract, 2% peptone, and 10% glucose), SD medium (0.67% yeast nitrogen base without amino acids [Becton Dickinson and Company, Franklin Lakes, NJ, USA], 2% glucose) or SGR medium (0.67% yeast nitrogen base without amino acids, 2% galactose and 2% raffinose). A final concentration of 2% agar was added to the liquid media to prepare solid media.

### *Construction of yeast strains*

The yeast strains constructed in this study are listed in Tables 1, and Table 2 shows the sequences of oligonucleotides used in this study.

Yeast mating was performed as follows. Two kinds of yeast strains were co-cultivated in 1 mL of YPD medium at 30°C for 1.5 h, with an initial $OD_{600}$ of 0.1 for each. After cocultivation, yeast cells were harvested, washed and resuspended in distilled water. Starting from an initial $OD_{600}$ of 1.0, cell suspensions (100 μl) were spread on SD solid medium with appropriate minimum nutrients for growth selection of zygotes. SD solid medium without methionine and lysine was used for diploid formation. On the other hand, SD solid medium without histidine and lysine was used for triploid and tetraploid formation, following introduction of plasmid pYO323 into haploid strains BY4742 and BY4742C. Plasmid removal after yeast mating was achieved through passage culture in YPD medium (Fukuda, 2020).

To perform chromosomal integration of *FAS2-G1250S* mutation, DNA fragments containing *FAS2-G1250S* were amplified from the genomic DNA of BY4741C strain using oligonucleotides 1: 5′-GTTATTCAATGGTTACAACCC-3′ and 2: 5′-GATGACCAGTCAAGAACTTT-3′. These amplicons were then introduced into yeast cells using the lithium acetate method (Gietz *et al*., 1992). Transformants were selected on solid YPD medium containing cerulenin.

For mating type conversion, pUY-Pa1G-HO was used to transform yeast strains. Transformants were selected on solid SD medium lacking uracil. Mating-type-converted transformants (by the action of the Ho endonuclease) were then grown in SD medium lacking uracil following cultivation in SGR medium. The *URA3* gene encodes orotidine 5'-phosphate decarboxylase (ODCase), which converts 5-FOA into a toxic compound within the cell (Boeke *et al*., 1984). Yeast cells that lacked plasmids containing the *URA3* gene were isolated on solid medium supplemented with 5-fluoroorotic acid (5-FOA). To remove pUYG-HO, transformants were cultured in YPD medium and spread on solid SD medium containing 20 μg $ml^{-1}$ uracil and 0.5 mg $ml^{-1}$ 5-FOA.

### *Investigation of cell growth characteristics*

Each yeast strain was grown in 100 μl of YPD medium containing cerulenin at 30°C, with an initial $OD_{620}$ at 0.01. After 24 h of cultivation, the $OD_{620}$ values of cultures were measured using a photoelectric colorimeter (AE-15F; ERMA Inc., Tokyo, Japan).

### *DNA fragment analysis*

DNA fragments of the partial region (1424 bp) of *FAS2* were amplified using PCR with oligonucleotides 1 and 2 (described above) using genomic DNA as a template. Following DNA cleavage using restriction enzyme *Bfa*I, separation of DNA fragments was carried out using agarose gel electrophoresis. Band patterns were detected using gel imager (FAS-IV; NIPPON Genetic Co, Ltd., Tokyo, Japan).

### *Ploidy analysis using real-time PCR*

Quantitative real-time PCR was performed using Thermal Cycler Dice Real-Time System II (Takara Bio Inc., Shiga, Japan) with Thunderbird SYBR qPCR Mix (Toyobo, Osaka, Japan). The relative gene copy number of the *RAD51* and *PGK1* against the *FAS2* was calculated using the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak, 2008). Haploid strain BY4741 have a single set of chromosomes, which serves as a control strain. Template genomic DNA was isolated from yeast cells cultivated in YPD medium at 30 °C for 24 h. Three sets of PCR primers, oligonucleotides 3: 5′-CGTGGTGAACTAAGCGCAAG-3′ and 4:5′-TAACGACGACTGCAACACCA-3′, 5: 5′-

GCCCCAGGTTCCGTTATTTT-3′ and 6: 5′-ACCTTTT GACCATCGACCTTTC-3′, 7: 5′-GGCAATCAGTGCTC AAGCTG-3′ and 8: 5′-CTCAACATCCATACCGGCCA-3′, were used to evaluate copy number of *RAD51*, *PGK1* and *FAS* respectively.

### *Evaluation for ethyl caproate productivity*

The optical density was measured at 600 nm ($OD_{600}$) using a UV-Visible spectrophotometer (UV-2550; Shimadzu Corporation, Kyoto, Japan). Setting the initial $OD_{600}$ at 0.02, the yeast cells were cultivated in 100 ml of YPD10 medium at 15°C for 8 days without shaking. At the end of cultivation, supernatants were obtained by centrifugation and then injected to gas chromatography mass spectrometer (GC/MS) (7890/5975C; Agilent Technologies, Santa Clara, CA, USA). The amount of ethyl caproate in culture supernatants was evaluated according to the standard method of National Research Institute of Brewing, Japan (Brewing Society of Japan, 2017).

Additionally, daily sampling was carried out, and supernatants obtained by centrifugation were frozen and stored at −20°C. To avoid undesirable chemical reactions (caused by secreted enzymes or uneliminated yeasts) in supernatants used for multi-sample measurement, the frozen-thawed supernatants were incubated at 65°C for 10 min before injection to GC/MS. Although there must be partial loss in the concentrations of ethyl caproate, changing trends during cultivation can be indicated by relative comparison.

## Acknowledgments

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## Funning Information

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## Conflict of interest

The authors declare no competing financial interests.

## Author contributions

N. F. designed the study; N. F., M. F. and Y. Y. conducted experiments; N. F., M. F. and Y. Y. analysed data; and N. F., S. H., M. F., Y. Y. and T. N. wrote the manuscript. All authors have read and approved the final manuscript.

## Ethical approval

This study does not include any experiments with human participants or animals.

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## Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Fig. S1**. Nucleotide sequences of the *FAS2* and *FAS2-G1250S* confirmed by Sanger sequencing. The first line indicates the sequence of the *FAS2* derived from haploid

 

strain BY4742, and the second line indicates that of the *FAS2-G1250S* mutant derived from haploid strain BY4742C.

**Fig. S2**. Cleavage pattern of the DNA fragments of the *FAS2*. *Bfa*I-digested (+) and non-digested DNA fragments (−) were loaded onto an agarose gel. Lane M indicates the DNA size marker 1 and 2 indicate strain BY4741, and 3 and 4 indicate strain BY4742.

**Fig. S3**. Generation and evaluation of sake yeasts with the *FAS2-G1250S* mutation. (A) Cleavage pattern of the DNA fragments of the *FAS2*. *Bfa*I-digested (+) DNA fragments were loaded onto an agarose gel. Lane M indicates a DNA size marker. 1, 2 and 3 indicate the generated sake yeast strains K9C-S01, K9C-S03 and K9C-S04, respectively. (B) Ploidy analysis using real-time PCR. Blue columns indicate the relative copy number of the *RAD51* against the *FAS2*, and red columns indicate that of the *PGK1*.

**Fig. S4**. Comparison of antibiotic resistance among yeast strains containing a single copy of the *FAS2-G1250S* with different ploidy. Yeast cells were cultivated in YPD medium containing 5 μM cerulenin at 30°C for 24 hours, setting initial $OD_{620}$ at 0.01. Black columns indicate homozygous *FAS2-G1250S* mutant strains of haploid (BY4741C), diploid (BY4743CC), triploid (BY4743-3CCC) and tetraploid (BY4743-4CCCC) yeast cells, respectively. Blue columns indicate yeast strains containing a single copy of the *FAS2-G1250S* mutation with varying ploidy (haploid, diploid, triploid, and tetraploid indicate BY4741C, BY4743C, BY4743-3C and BY4743-4C, respectively). Values are presented as means ± standard deviations from three independent experiments.

**Fig. S5**. Evaluation for growth and ethyl caproate producing-ability of tetraploid strains with different number of the *FAS2-G1250S* mutation. (A) The $OD_{600}$ values of yeast cultures (117 hours). (B) Changes in ethyl caproate concentrations. After incubating the collected and frozen-thawed supernatants at 65°C for 10 minutes, ethyl caproate concentrations were measured using GC/MS to trace the time courses (ND: not detected). Values are presented as means ± standard deviations from two independent experiments.

**Fig. S6**. Evaluation for the genome stability of the homozygous tetraploid strain BY4743-4C4 generated through LOH. (A) Cleavage pattern of the DNA fragments of the *FAS2*. Yeast cells were passaged daily with 100-fold dilution using YPD medium without cerulenin for 10 days. *FAS2* was amplified using PCR, and *Bfa*I-digested (+) and non-digested DNA fragments (−) were loaded onto an agarose gel. Lane M indicates a DNA size marker, 1 and 2 indicate passage 1 (P1), 3 and 4 indicate passage 5 (P5), and 5 and 6 indicate strain passage 10 (P10). (B) Comparison of the cerulenin resistance. Yeast cells were cultivated in YPD medium containing 25 μM cerulenin, and the $OD_{620}$ values were measured and compared. Values are presented as means ± standard deviations from three independent experiments. The red dotted line indicates the level of cerulenin resistance of heterozygous tetraploid strain BY4743-4CCC harbouring three copies of the *FAS2-G1250S* mutations.

 