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Artificial Conversion of the Mating-Type of *Saccharomyces cerevisiae* without Autopolyploidization

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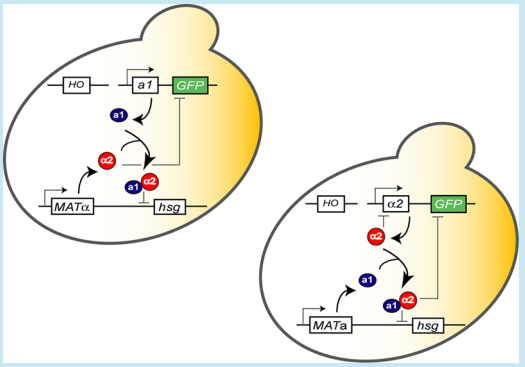
Supporting Information

ABSTRACT: Crossbreeding is a classical yeast hybridization procedure, where the mating of haploid cells of opposite mating-type, *MAT*a and *MATα* cells, produces a new heterozygous diploid. Here, we describe a method to generate haploid *MAT*a and *MATα* cells using mating-type conversion caused by expression of the *HO* gene, which encodes an endonuclease. Importantly, our method prevents the autopolyploidization that typically arises during artificial mating-type conversion. This facilitates isolation of the desired mating-type of yeast cells with simple and easy procedure. In the current study, we designed a suitable genetic circuit for each haploid cell and converted *MATα* haploid cells into *MAT*a haploid cells and vice versa, demonstrating the utility of constructed artificial regulation network to prevent autopolyploidization. Via forced expression of the a1 gene in *MATα* haploid cells or of α2 in *MAT*a haploid cells, the undesirable mating ability of yeast cells was completely suppressed. We confirmed the success in prevention of autopolyploidization by ploidy analysis. This new approach provides a reliable and versatile tool for yeast crossbreeding, so that it will be useful for scientific research and industrial applications of yeast.



KEYWORDS: *mating-type switching, autopolyploidization, yeast, crossbreeding*

Yeast has been widely studied and successfully utilized to produce numerous useful compounds. Fermentative production such as ethanol production using the yeast *Saccharomyces cerevisiae* is a promising technology, as is protein production, the production of some types of organic acids, *etc.*[1] In order to increase the productivity and expand in application, however, strenuous efforts have been devoted to the production of custom-engineered strains.

Crossbreeding is a classical hybridization approach used to improve and combine traits of yeast strains in which mating of haploid cells of opposite mating-type results in a new, heterozygous, diploid strain.[2−7] Because parental diploid and polyploid strains are never able to mate directly, isolation of mating strains (typically, haploid strains) via sporulation is a prerequisite for crossbreeding. Unfortunately, the resulting hybrids do not always inherit all of the desirable properties of the parental strains due to machinery of sexual reproduction, which is accompanied by homologous recombination. Thus, to generate and isolate hybrids suitable for industrial purposes, it is imperative to screen a large number of hybrid constructs.[2,7] Furthermore, the requirement to start with a pair of haploid strains, *MAT*a and *MATα* mating-types, restricts possibilities for combining previously isolated and engineered strains. Therefore, a powerful approach that can be used to generate mating strains with the specific desired properties and mating-type is required in order to accelerate progress in yeast breeding technology and strain engineering.

The HO endonuclease is expressed in homothallic yeast and mediates mating-type switching. This process involves replacement of a or α DNA sequences at the *MAT* locus by opposite mating-type sequences derived from one of two silent donor loci, *HMR*a and *HMLα*.[8−10] Applied to yeast breeding, homozygous diploid strains are constructed via autodiploidization, which comes about during conversion of the mating-type in the presence of expression of the *HO* gene.[11] Conversion of mating-type has the potential to generate a desired strain that can inherit all of the genetic properties of the parental strains except for the *MAT* locus. However, autopolyploidization impedes isolation of the desired mating strains and subsequent crossbreeding.

Here we designed a genetic circuit to prevent autopolyploidization arising during mating-type conversion using the mating-type genes a1 and α2. As shown in Figure 1A, *MAT*a haploids express the a1 gene, whereas *MATα* haploids express α2 from their respective *MAT* loci. In both haploids, haploid-specific genes (*hsg*) are expressed and induce mating responses. By contrast, *MAT*a/α diploids express both a1 and α2 genes from heterologous *MAT* loci, resulting in the formation of the a1-α2 complex, which represses expression of *hsg*.[12] Therefore, introduction of a1 into *MATα* haploid cells or α2 into *MAT*a haploid cells would be expected to lead to the formation of the

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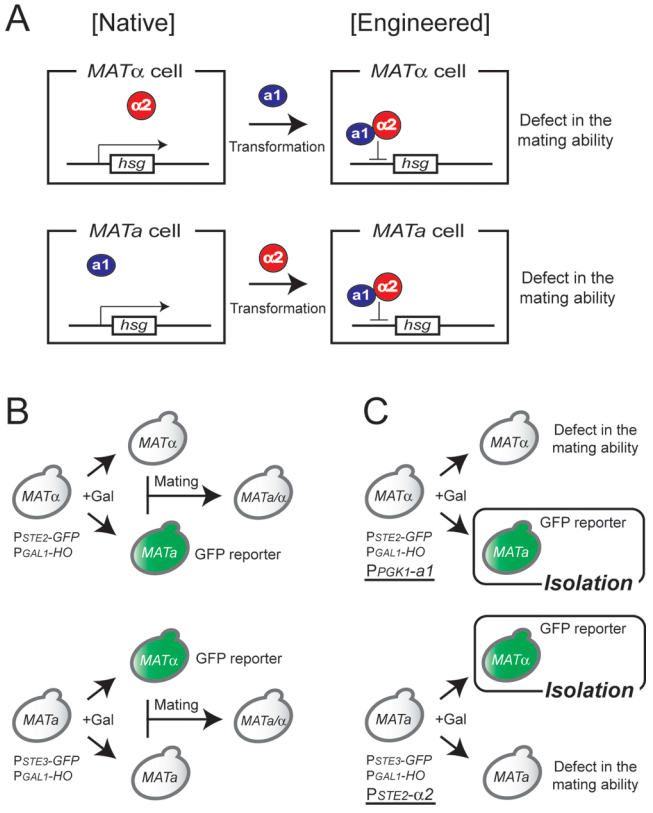

Figure 1. Schematic outline of experimental design. (A) Artificial formation of the a1-α2 complex in haploid cells to repress their mating abilities. The introduction of the a1 gene into *MAT*α haploids or of the α2 gene into *MAT*a haploids was predicted to lead to formation of the a1-α2 complex. This would repress expression of haploid-specific genes (*hsg*) required for mating responses, similar to *MAT*a/α diploids, which normally express both a1 and α2 from heterologous *MAT* loci. (B) The conventional method of converting mating-type is accompanied by autoploidization. Expression of the *HO* gene is induced via galactose, which triggers mating-type conversion. Although the desired mating-type of yeast cells (highlighted in green) expressing the *GFP* reporter gene is present in the cell mixture, these cells would frequently form diploid zygotes by mating with parental mating-type of yeast cells, thus depleting them from the cell population. (C) New approach using ectopic formation of the a1-α2 complex to avoid autopolyploidization during mating-type conversion. The a1-α2 complex suppresses mating ability. As a consequence, yeast cells with the desired mating-type are expected to be stable and not subject to autopolyploidization. Isolation of these cells can be achieved using fluorescence intensities as an indicator.

a1-α2 complex and a subsequent defect in mating ability via repression of *hsg* (Figure 1A). In this work, we show the feasibility of this approach and its powerful ability to provide mating strains for yeast crossbreeding without autopolyploidization.

RESULTS AND DISSCUSSION

General Strategy. An outline of experimental design to establish a rapid and reliable method for production of *MAT*a or *MAT*α cells using artificial mating-type conversion without autopolyploidization is shown in Figure 1. Conversion of mating-type has previously been applied to production of mating strains;[13] however, this approach faces the problem of autopolyploidization, which results in undesirable non-hybrid cells. Here we used the mating-type genes a1 and α2 to suppress the undesirable mating abilities of yeast cells that would normally induce autopolyploidization (see Figure 1) In our system, undesirable mating abilities of yeast cells were suppressed utilizing the knowledge that the a1-α2 complex represses expression of *hsg*, which should be naturally seen in diploid cells (Figure 1A).

In the current study, we utilized *MAT*a and *MAT*α haploids as the parental strains to receive conversion of the mating-type. These strains are normally subject to a high frequency of autopolyploidization during mating-type conversion similar to natural homothallic yeasts possessing a mating-type switching mechanism.[14] We examined whether our approach was useful to avoid autopolyploidization even when applied to mating-type conversion of these strains.

To recognize the desired mating-type among a population of cells subjected to mating-type conversion, we introduced a *GFP* reporter gene under the control of the a- or α-type-specific promoter (Figure 1B and C). Expression of the *HO* gene, controlled by the *GAL1* promoter, was induced by addition of galactose. As shown in Figure 1B, haploids of the opposite mating-type, generated by *HO* gene expression, can mate with the parental haploids. In contrast, because our method suppresses the mating ability of parental haploids, thus preventing autopolyploidization, the haploids generated using our approach should be stable following termination of expression of the *HO* gene. They could then be isolated using GFP-fluorescence as the indicator (Figure 1C).

GFP Reporter Expression As an Indicator of *MAT*a or *MAT*α Mating-Type. Our strategy to generate the desired mating strains via conversion of mating-type is outlined in Figure 1C. To discriminate mating-type-converted cells from others, a *GFP* reporter gene was expressed under the control of an a-type-specific promoter, *STE2p* (P_{STE2}-*GFP*), or an α-type-specific promoter, *STE3p* (P_{STE3}-*GFP*). As shown in Figure 2, expression of the *GFP* reporter gene successfully resulted in an increase in the fluorescence intensities of *MAT*a (Figure 2A) and *MAT*α cells (Figure 2B). These results suggest that it is possible to identify *MAT*a or *MAT*α cells in the resulting cell populations using fluorescence intensity as an indicator.

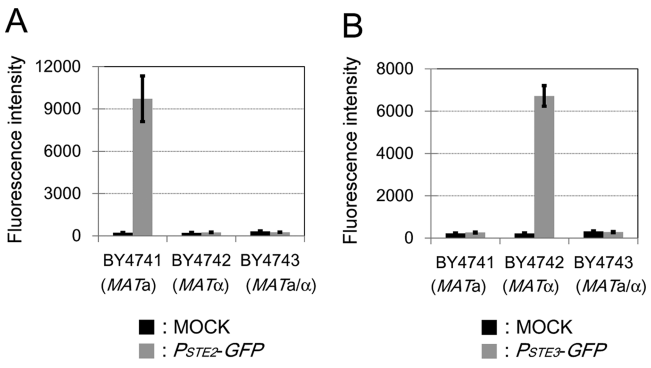

Figure 2. Fluorescent reporter assay to evaluate P_{STE2}-*GFP* and P_{STE2}-*GFP* as indicators of yeast mating-type. (A) The expression level of the *GFP* reporter gene under the control of the *STE2p* (P_{STE2}-*GFP*) was quantified and compared among BY4741 (*MAT*a), BY4742 (*MAT*α), and BY4743 (*MAT*a/α) strains. (B) The expression level of the *GFP* reporter gene under the control of the *STE3p* (P_{STE3}-*GFP*) was quantified and compared among BY4741 (*MAT*a), BY4742 (*MAT*α), and BY4743 (*MAT*a/α) strains. Standard deviations of three independent experiments are presented.

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Blocking the Mating Abilities of Haploids via Constitutive Expression of the a1 or α2 Gene. Forced expression of the α2 gene in *MAT*a haploids, which express the a1 gene or, conversely, forced expression of a1 gene in the α2-expressing *MAT*α haploids should lead to formation of the a1-α2 complex (Figure 1A). To inhibit mating responses that would induce autopolyploidization, the a1 or α2 gene was expressed under the control of the *PGK1* promoter,[15] which functions independently of mating-type (P_{PGK1}-a1 or P_{PGK1}-α2).

The a1-α2 complex can repress the expression of a-type-specific genes (*asg*) and α-type-specific genes (*αsg*) in addition to *hsg*.[16] Thus, we expected that expression of the *GFP* reporter under the control of *STE2p* (active in *MAT*a cells) or *STE3p* (active in *MAT*α cells) would be repressed when the a1-α2 complex is formed in a haploid cell. As shown in Figure 3A, neither BY4741 (*MAT*a) with P_{PGK1}-α2 nor BY4742 (*MAT*α) with P_{PGK1}-a1 exhibited GFP fluorescence, suggesting that the a1-α2 complex successfully formed and repressed transcription in these cells. Surprisingly, whereas overexpression of the a1 gene did not have a detectable effect on the function of *STE2p* in *MAT*a cells, overexpression of α2 significantly decreased expression of the *GFP* reporter controlled by *STE3p* in *MAT*α cells (see Figure 2).

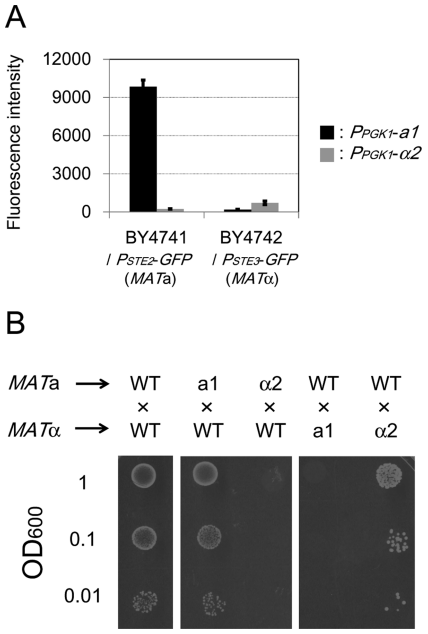

Figure 3. Evaluation of the performance of the artificially formed a1-α2 complex using P_{PGK1}-a1 or P_{PGK1}-α2. (A) Fluorescent reporter assay to quantify the expression level of the *GFP* reporter gene. Standard deviations of three independent experiments are presented. (B) Diploid growth assay to investigate the mating abilities of yeast cells. BY4741 was used as the *MAT*a parental strain and BY4742 as the *MAT*α parental strain. At the top of the image, WT indicates BY4741 or BY4742 without plasmids, a1 indicates yeast transformants containing P_{PGK1}-a1, and α2 indicates yeast transformants containing P_{PGK1}-α2.

We next investigated the mating abilities of haploid cells using a growth assay on diploid-selective solid media[17,18] without methionine and lysine (Figure 3B, left image), without methionine, lysine, and histidine (center image), or without methionine, lysine, and leucine (right image). The lack of histidine or leucine was required to retain plasmids containing either the a1 or α2 gene. Diploid cells are normally generated between wild-type (WT) *MAT*a and *MAT*α cells. We found that *MAT*a cells expressing the α2 gene (α2 in the *MAT*a lane of Figure 3B) and *MAT*α cells expressing the a1 gene (a1 in the *MAT*α lane) never mated with their mating partners. Unfortunately, a slight decrease in the number of generated diploid cells was also observed for *MAT*α cells expressing the α2 gene (α2 in the *MAT*α-lane of Figure 3B).

According to previous reports, α2 functions as the repressor of *asg*[19−21] and has a low affinity for the a1-α2 complex-binding sites, which represses expression of *αsg* and *hsg*.[22] Presumably, overexpression via P_{PGK1}-α2 might have induced accumulation of α2 on the a1-α2 complex-binding site, followed by recruitment of repressor complex, resulting in repression of P_{STE3}-*GFP* and *hsg*. The results of other studies have shown that overexpression of the α2 gene can lead to morphological changes in yeast cells (e.g., hyperpolarized cells and bipolar budding)[23] as a result of changes in gene expression. Thus, we concluded that for the purposes of our study, *PGK1p* is a suitable promoter for expression of a1 but not for expression of α2 due to difficulty in isolation of target cells generated via conversion of the mating-type, i.e., low fluorescence signal of the reporter (Figure 1C).

Specific Expression of the α2 Gene in *MAT*a Cells To Avoid Undesirable Repression of Reporter Gene Expression. An improved strategy regarding the α2 gene is shown in Figure 4A. The plasmid pL3G-2α contains *STE2p*-α2 (active in *MAT*a cells) and *STE3p*-*GFP* (active in *MAT*α cells). In *MAT*a cells with pL3G-2α, α2 should form the a1-α2 complex by binding to a1, resulting in repression of *hsg*. However, as overexpression of α2 is not induced in *MAT*α

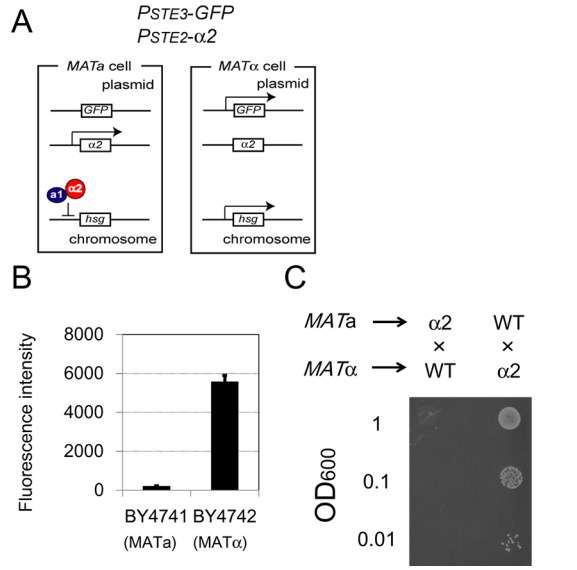

Figure 4. Design and evaluation of improved expression of the α2 gene. (A) Schematic outlines of gene expression in *MAT*a and *MAT*α cells using the plasmid pL3G-2a, which contains P_{STE3}-*GFP* and P_{STE2}-α2. (B) Fluorescent reporter assay to quantify the expression level of the *GFP* reporter gene. Standard deviations of three independent experiments are presented. (C) Diploid growth assay to investigate the mating abilities of yeast cells. BY4741 was utilized as the *MAT*a parental strain, and BY4742 was utilized as the *MAT*α parental strain. At the top of the image, WT indicates BY4741 or BY4742 without plasmids, and α2 indicates yeast transformants containing P_{STE2}-α2.

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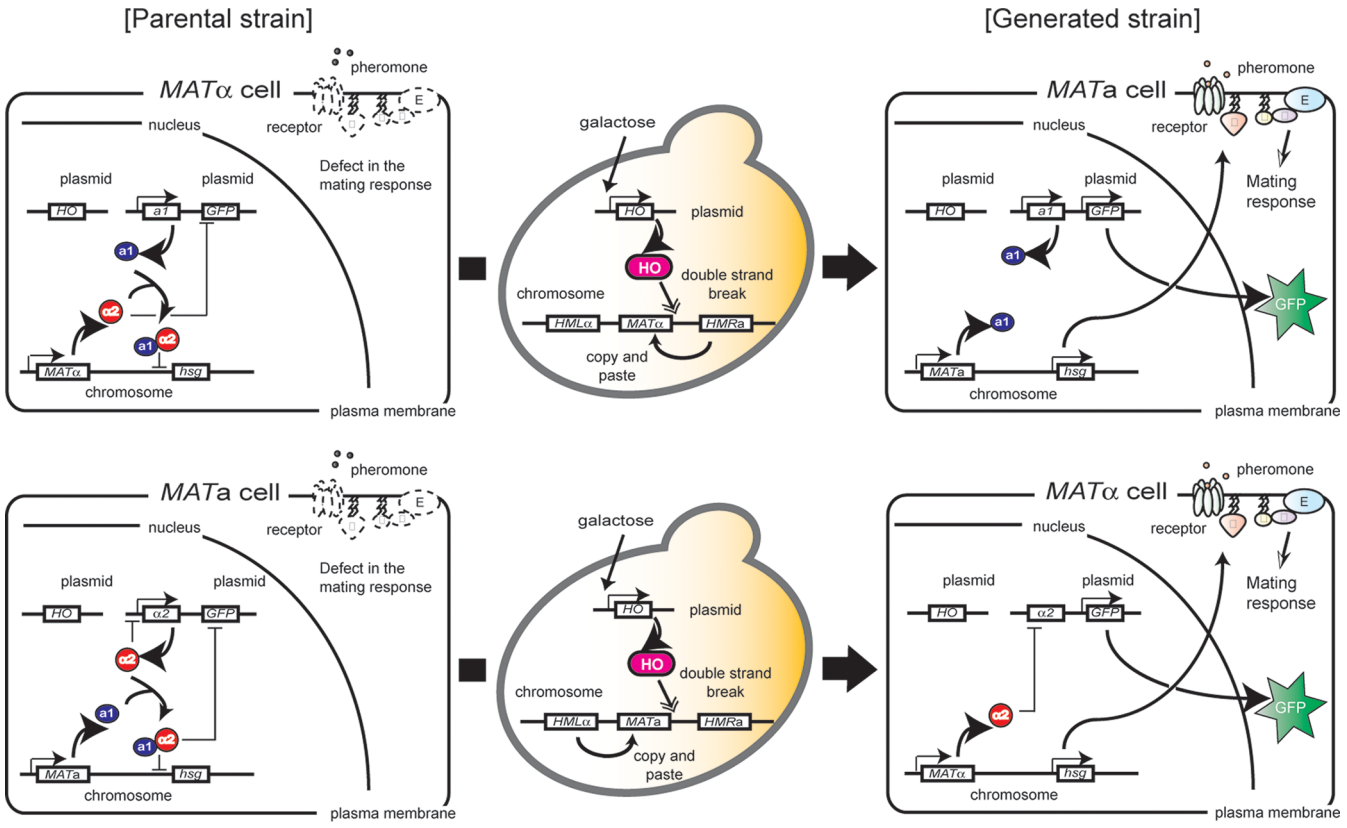

Figure 5. Schematic outline of yeast mating-type regulation and our engineered approach. Artificial formation of the a1-α2 complex in parental strains suppresses the mating response by repressing expression of haploid-specific genes (*hsg*), which encode components of the mating signaling pathway. The *MAT*α parental strain indicates HR42-K with pH2G-Pa1 and pUYG-HO, and the *MAT*a parental strain indicates HR41-K with pL3G-α2 and pUYG-HO (see Table 1). The addition of galactose induces expression of the *HO* gene, triggering mating-type conversion. Next, the generated strains expressing the *GFP* reporter gene can be isolated in the absence of complications that would normally arise via autopolyploidization. Note that the designed circuits for *MAT*a and *MAT*α cells are asymmetric.

cells, expression of *hsg* and the *GFP* reporter would be maintained.

Figure 4B shows the fluorescence intensity of *MAT*a or *MAT*α cells with pL3G-2α. As expected, *STE2p*-α2 did not have a detectable negative effect on *STE3p-GFP* in *MAT*α. We next tested the mating abilities of these two transformants (α2 in the *MAT*a and *MAT*α lanes) using wild-type cells of opposite mating-type as the mating partners. The results of a growth assay carried out using diploid-selective solid media without methionine, lysine, and leucine are shown in Figure 4C. As expected for this improved strategy, *STE2p*-α2 successfully suppressed the undesirable mating ability of *MAT*a cells without disrupting the mating response of the *MAT*α cells (Figure 4C). We concluded that *STE2p* is a suitable promoter for expression of the α2 gene.

Artificial Regulation Network Designed for Conversion of Mating-Type without Autopolyploidization. A model that describes how our artificial gene network can be used for mating-type conversion is shown in Figure 5. As described above, P_{PGK1}-a1 successfully suppresses the mating ability of *MAT*α cells, and *STE2p*-α2 successfully suppresses that of the *MAT*a cells. To perform conversion of mating-type, we needed to additionally introduce a source of the *HO* gene (pUYG-HO; Table 1). In parental strains, the a1-α2 complex represses expression of *hsg*, which is required for mating signal transduction. In these strains, addition of galactose induces expression of the *HO* gene. The HO protein is known to make a double-stranded break at the *MAT* locus, and from one of two silent donor loci (*HMR*a and *HML*α), a or α DNA sequences are then copied and pasted into the *MAT* locus. Because with our system the resulting strain should have the opposite mating-type, it should be positive for green fluorescence, attributable to expression of the *GFP* reporter gene, and thus can be isolated.

Conversion of Mating-Type from *MAT*α into *MAT*a. As shown in Figure 6A, all strains, including BY4741 and BY4742, were derived from a representative yeast lab strain S288C.[24] This strain contains a mutation (*inc*) at the *HMR*a locus ($HMRa_{inc}$) that severely reduces HO endonuclease cleavage when it is at the *MAT* locus.[9] In addition, BY4741 contains the *inc* mutation at the *MAT*a locus ($MATa_{inc}$), making it insensitive to HO endonuclease cleavage. Because these strains cannot be used directly as the hosts for mating-type conversion, an improved strain without the *inc* mutation, HR42-K, was prepared from BY4742 using homologous recombination to replace $HMRa_{inc}$ with intact *HMR*a ($HMRa_{cut}$; Table 1). The new HR42-K strain was utilized as the host for conversion of mating-type from *MAT*α into *MAT*a as described in Figure 1C.

Yeast cells were cultivated in SGal media to induce mating-type conversion and then spread on solid media without galactose (to suppress expression of the *HO* gene) and containing uracil (to promote dropout of the plasmid containing the *HO* gene). Subsequently, the colonies generated were individually grown in SD media, and then their fluorescence intensities, an indicator of *MAT*a mating-type, were quantified. Approximately 30% of the examined clones

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Table 1. Yeast Strains and Plasmids Used in This Study

name	description	ref source
	Yeast Strains	
BY4741	*MAT*a$_{inc}$[a] *his3Δ1 ura3Δ0 leu2Δ0 met15Δ0*	24
MCF4741	BY4741 *Figure 1:: Figure 1-EGFP-loxP-kanMX4-loxP*	present study
HR41-K	*mat*a$_{inc}$::*MAT*a$_{cut}$[b] *his3Δ1 ura3Δ0 leu2Δ0 lys2Δ0 hmr*a$_{inc}$::*HMR*a$_{cut}$*-loxP*	present study
BY4742	*MATα his3Δ1 ura3Δ0 leu2Δ0 lys2Δ0*	24
HR42-11T	BY4742 *hmr*a$_{inc}$::*HMR*a$_{cut}$*-loxP-kanMX4-loxP*	present study
HR42-K	BY4742 *hmr*a$_{inc}$::*HMR*a$_{cut}$*-loxP*	present study
BY4743	BY4741/BY4742 (*MAT*a$_{inc}$/*α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 met15Δ0/MET15 ura3Δ0/ura3Δ0*)	24
BY43-kan	BY4741/HR42-11T	present study
HR43-kan	HR41-K/HR42-11T	present study
MCF43-kan	MCF4741/HR42-K	present study
	Plasmids	
pYO323	yeast expression vector containing *2μ* ori and *HIS3* marker	NBRP[c]
pYO325	yeast expression vector containing *2μ* ori and *LEU2* marker	NBRP
pYO326	yeast expression vector containing *2μ* ori and *URA3* marker	NBRP
pHY-2GA	P_{STE2}*-EGFP* in pYO323	present study
pLY-3GC	P_{STE3}*-EGFP* in pYO325	present study
pH2G-Pa1	P_{PGK1}-a*1* in pHY-2GA	present study
pH2G-P*α*2	P_{PGK1}-*α2* in pHY-2GA	present study
pL3G-Pa1	P_{PGK1}-a*1* in pLY-3GC	present study
pL3G-P*α*2	P_{PGK1}-*α2* in pLY-3GC	present study
pL3G-2*α*	P_{STE2}-*α2* in pLY-3GC	present study
pUYG-HO	P_{GAL1}*-HO* in pYO326	present study
pUC57-LKL	containing *loxP-kanMX4-loxP*	Genscript Inc.
pUC57-Cre	containing *Cre* gene encoding DNA recombinase	Genscript Inc.
pNHK12	containing P_{ADH1}*-EGFP*	NBRP
pAT471	yeast expression vector containing *CEN4/ARS1* ori, *URA3* marker, and *HO*	NBRP

[a]*MAT*a$_{inc}$ or *HMR*a$_{inc}$ contains the stuck mutation that severely reduces *HO* endonuclease cleavage. [b]*MAT*a$_{cut}$ or *HMR*a$_{cut}$ contains the normal, cuttable sequence for *HO* endonuclease cleavage. [c]Resources were provided by the National Bio-Resource Project (NBRP) of the MEXT, Japan.

exhibited GFP fluorescence, suggesting the successful production of *MAT*a cells (Figure 6B). Some colonies exhibited obviously lower fluorescence intensity as compared with BY4741 containing *STE2p-GFP* (see Figure 2A), suggesting that *MATα* cells were also present in the colony. These mixed colonies consisting of *MAT*a and *MATα* cells might be produced by the partial and redundant conversion of mating-type during colony formation despite the absence of galactose (to suppress expression of the *HO* gene) and presence of uracil (to promote dropout of the plasmid containing the *HO* gene).

Among colonies containing *MAT*a cells, three colonies displayed high-intensity green fluorescence signal, equivalent to what is seen with BY4741 containing *STE2p-GFP*. We selected the colony (No. 1) with the highest fluorescence intensity and observed fluorescence intensity after passage of the culture (see Supplementary Figure S1). Even after two passages of the culture, the cells continued to exhibit green fluorescence at levels almost equivalent to what we see for BY4741 containing *STE2p-GFP*. This suggests that the colonies with high-intensity green fluorescence comprise *MAT*a cells in which HO endonuclease activity was successfully suppressed. Thus, we concluded that colony No.1 represents the target cells we had aimed to produce and performed another round of colony isolation to exclude all plasmids. The resulting strain was termed HR41-K.

To verify the mating ability of HR41-K, a diploid growth assay described above was carried out using HR42-11T (Table 1) as the mating partner. Because HR41-K and BY4742 have the same auxotrophy, pYO323 (containing *HIS3* marker) was introduced into HR41-K and BY4741 (control strain), and pYO325 (containing *LEU2* marker) was introduced into BY4742. This allowed us to select diploid cells using solid media without histidine and leucine. As a result of diploid growth selection, HR41-K successfully mated with the *MATα* cells, as well as the existing *MAT*a strain, BY4741 (Figure 6C). The zygote formed from the mating of BY4741 and HR42-11T was termed the BY43-kan strain, and that of HR41-K and HR42-11T was termed the HR43-kan strain (Table 1). These strains were used for the following analysis.

To confirm the absence of autopolyploidization within the generated HR41-K strain, ploidy analysis was performed using real-time PCR. In Figure 6D, the normalized copy number of the *PGK1* gene using the *kanMX4* gene as the reference reveals the ploidy of each strain (see Methods). HR43-kan was determined to be diploid, possessing 2 sets of chromosome DNA, as was BY43-kan. Thus, HR41-K was assumed to be haploid, suggesting it avoided autopolyploidization as expected using our engineered strategy. These results show that autopolyploidization was successfully avoided in the process of converting the mating-type of these cells from *MATα* to *MAT*a.

Conversion of Mating-Type from *MAT*a into *MATα*. The HR41-K strain was next utilized as the host receiving conversion of mating-type from *MAT*a into *MATα* as described in Figure 1C. The steps we used to convert mating-type were the same as described in the previous section. Figure 7A shows the fluorescence intensities of the isolated colonies, which in this context indicate a *MATα* mating-type. Approximately 25%

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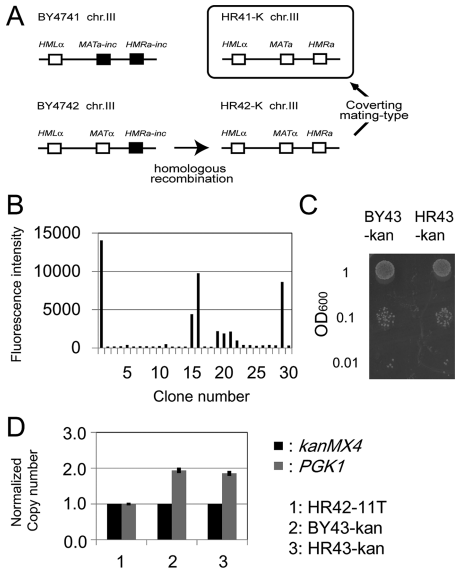

Figure 6. Conversion of mating-type from *MATα* to *MAT*a without autopolyploidization of the resulting *MAT*a cells. (A) Schematic outline of the approach used to build the host strain HR42-K for mating-type conversion. *MAT* and *HM* loci are in chromosome III. The blank boxes indicate that the locus contains the normal and endonuclease-sensitive sequence for the HO endonuclease cleavage, and filled boxes indicate the *inc* mutation (insensitive to cleavage when the sequence is at the *MAT* locus). (B) Fluorescent reporter assay to quantify expression of the *GFP* reporter gene (P_{STE2}-*GFP*). Standard deviations of three independent experiments are presented. (C) Diploid growth assay to investigate the mating abilities of yeast cells. (D) Ploidy analysis using real-time PCR. The normalized copy number of the *PGK1* gene is an indicator of ploidy. Standard deviations of three replicate experiments are presented.

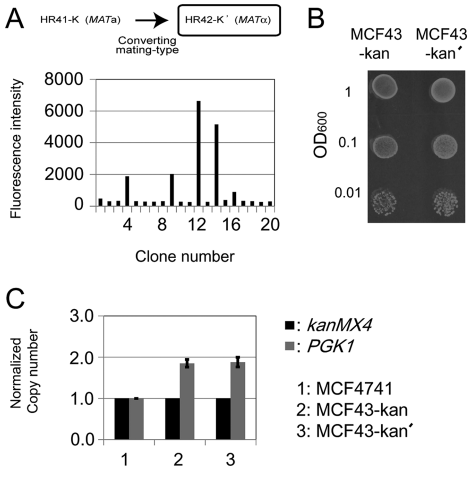

Figure 7. Conversion of mating-type from *MAT*a to *MATα* without autopolyploidization of the resulting *MATα* cells. (A) Fluorescent reporter assay to quantify expression of the *GFP* reporter (P_{STE3}-*GFP*). Standard deviations of three independent experiments are presented. (B) Diploid growth assay to investigate the mating abilities of yeast cells. (C) Ploidy analysis using real-time PCR. The normalized copy number of the *PGK1* gene is an indicator of ploidy. Standard deviations of three replicates are presented.

of the examined clones exhibited GFP fluorescence, suggesting the successful production of *MATα* cells. Several colonies showed a low level of fluorescence, suggesting these colonies consist of a mixture of *MAT*a and *MATα* cells. In addition, two colonies showed a high level of green fluorescence, equivalent to what we observe for BY4742 containing *STE3p-GFP* (Figure 2B). We selected the colony (No. 12) with the highest fluorescence intensity and performed another round of colony isolation to exclude all plasmids. The resulting strain was tentatively termed HR42-K′ to distinguish it from HR42-K (Figure 7A).

The HR42-K′ strain should be identical to HR42-K if it did indeed avoid autopolyploidization during mating-type conversion as expected for our strategy. To compare the mating abilities of HR42-K′ and HR42-K, a diploid growth assay was carried out using solid media without methionine and lysine. MCF4741 (Table 1) was utilized as the mating partner. HR42-K′ successfully mated with the *MAT*a cells as well as HR42-K (Figure 7B). The zygote formed from the mating of MCF4741 and HR42-K was termed the MCF43-kan strain (Table 1), and that of MCF4741 and HR42-K′ was termed the MCF43-kan′ strain.

Ploidy analysis was also performed using real-time PCR as described above. As shown in Figure 7C, MCF43-kan′ has the same ploidy as MCF43-kan (diploid strain), suggesting that HR42-K′ is identical to the haploid strain HR42-K. These results show the absence of autopolyploidization during conversion of mating-type from *MAT*a into *MATα*, as expected using our strategy.

Conclusion. We have established a new approach for the production of *MAT*a and *MATα* cells using an artificial mating-type conversion strategy that avoids the problem of autopolyploidization. Using haploid strains as model cases, we showed that artificial formation of the a1-α2 complex can be used to avoid autopolyploidization and furthermore that it is possible to isolate *MAT*a and *MATα* cells generated via mating-type conversion using reporter gene expression as a marker for mating-type. On the basis of the results shown here, we expect our method to provide a reliable and versatile tool for yeast crossbreeding, such that it will have a significant impact on the advancement of yeast-based biosynthesis approaches and in other experimental areas of research.

METHODS

Strains and Media. The *Saccharomyces cerevisiae* genotypes used in this study are summarized in Table 1. For detailed information about plasmid and yeast construction, see Supporting Information Materials and Methods. Each plasmid was introduced into yeast cells using the lithium acetate method.[25] YPD media contained 1% yeast extract, 2% peptone, and 2% glucose. SD media contained 0.67% yeast nitrogen base without amino acids (YNB; Becton Dickinson and Company, Franklin Lakes, NJ, USA) and 2% glucose. SGal media contained 0.67% YNB and 2% galactose. A final concentration of 2% agar was added to make solid media.

Fluorescent Reporter Assay. The *EGFP* gene was used as a fluorescent reporter to indicate mating-type. Reporter-containing cells were incubated at 30 °C for 18 h, harvested, and washed with distilled water. The cells were then resuspended in 100 μL of distilled water to an optical density of 5.0 at 600 nm (OD_{600} = 5.0). GFP fluorescence intensities were measured using the Infinite 200 fluorescence microplate reader (Tecan Japan Co., Ltd., Kawasaki, Japan). For detection of the GFP signal, the excitation wavelength was set at 485 nm with a bandwidth of 20 nm, and the emission wavelength was set at 535 nm with a bandwidth of 25 nm. The gain was set at 50.

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Diploid Growth Assay. Evaluation of mating abilities was performed as follows. Each engineered yeast strain was cultivated with the mating partner in 1 mL of YPD medium at 30 °C for 1.5 h, with an initial OD_{600} of each haploid cell type of 0.1. After cultivation, yeast cells were harvested, washed, and resuspended in distilled water. Starting from an initial OD_{600} of 1, 0.1, or 0.01, 10 μL of cell suspensions were spotted on SD solid medium without the appropriate amino acids for growth selection of diploid cells. After incubation at 30 °C for 2 days, the image data were recorded for diploid colonies on solid medium.

Conversion of Mating-Type. Yeast cells were grown in 1 mL of SD media, then transferred to 1 mL of SGal media (initial OD_{600} of 0.3) to induce expression of the *HO* gene. The cells were subsequently cultivated for 18 h, harvested and washed with distilled water. Cell suspensions of the appropriate dilutions were spread on SD solid medium without the appropriate amino acids and uracil and incubated at 30 °C for 2 days. The resulting colonies were individually cultivated and their fluorescence intensities were measured as described above.

Ploidy Analysis Using Real-Time PCR. Yeast haploid strains (control strains) and the mating-type-converted strains were mated with the opposite mating-type strains, MCF4741 (*MAT*a) or HR42-11T (*MATα*), shown in Table 1. These strains have one copy of the *kanMX4* gene integrated into the yeast chromosome DNA, which serves as a reference for ploidy analysis. Template genomic DNA was isolated from yeast cells cultivated in YPD medium at 30 °C for 18 h. Quantitative real-time PCR was performed using an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) with Thunderbird SYBR qPCR Mix (Toyobo, Osaka, Japan). Two sets of PCR primers, kanMX 98F: 5′-AATC-AGGTGCGACAATCTATCGA-3′ and kanMX 178R: 5′-CAACGCTACCTTTGCCATGTT-3′, and PGK1 331F: 5′-GCCCCAGGTTCCGTTATTTT-3′ and PGK1 413R: 5′-ACCTTTTGACCATCGACCTTTC-3′, were used to detect the *kanMX4* and *PGK1* genes respectively. Because one copy of the *PGK1* gene exists in one set of chromosomal DNA, the normalized copy number of *PGK1* can be calculated using the standard curve method together with *kanMX4* as the reference for 1 copy/cell, such that the *PGK1/kanMX4* ratio is an indicator of ploidy. The chromosome ploidy of the mating-type-converted strains was estimated by subtracting 1 (corresponding to the chromosome ploidy of the mating partners) from that of the zygotes.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Details on the construction of strains and plasmids and sequence of oligonucleotides used for construction of plasmids and yeast strains. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

N.F. designed the study, conducted experiments, analyzed data and cowrote the manuscript. S.M. conducted experiments and analyzed data. S.H. analyzed data and co-wrote the manuscript.

Notes

The authors declare the following competing financial interest(s):Regarding conflict of interest, we declare that all authors are inventors on a pending patent using aspects of this system.

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■ ABBREVIATIONS

EGFP, enhanced green fluorescent protein; *MAT* locus, mating-type locus; *HM* loci, hidden mating-type loci; *hsg*, haploid-specific genes; *asg*, a-type-specific genes; *αsg*, *α*-type-specific genes

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