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Tetraploid Formation through the Conversion of the Mating-type Alleles by the Action of Homothallic Genes in the Diploid Cells of *Saccharomyces* Yeasts

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Mating type conversion by the homothallic genes in the diploid cells occurred subsequent to the spontaneous or ultraviolet-light induced appearance of homozygosity of the mating-type alleles, **a**/**a** and α/α, from the **a**/α configuration. When homothallic, semi-homothallic or heterothallic **a**/α diploid cells were incubated with an excess population of **a** or α haploid cells having complementary nutritional markers in nutrient medium, prototrophic colonies appeared with a frequency of approximately 10^{-5} of the diploid cells on subsequent plating of the mating mixture on minimum medium. However, when **a**/α cells (irradiated with low dose of ultraviolet-light to accelerate the mitotic recombination) were directly plated on nutrient agar, and each colony appearing on the plate was tested for its mating response with the standard haploid cells, striking differences were observed depending on the genotypes for homothallism of the diploid cells. None of the 1,000 colonies so far tested of a perfect homothallic strain (the Ho type) showed mating reaction, while some isolated colonies of a heterothallic strain showed either **a** or α mating type activity. In the isolates showing mating potency from an Hp type semi-homothallic strain, solely α mating-type was observed, whereas in those from an Hq type semi-homothallic diploid showed solely **a** mating-type. These findings suggest that switching of the **a**/α heterozygous configuration to **a**/**a** and α/α occurs in both heterothallic and homothallic cells. And the conversion of the **a**/**a** alleles to α/α or vice versa, or from **a**/**a** and α/α to **a**/α depends on the genotype for homothallism of the cell. The former conversion is followed by cell fusion between the converted and the unconverted cells to produce tetraploid cells. This inference was supported by tetrad analyses and determination of cell size and deoxyribonucleic acid contents of the cells of supposed tetraploid clones. Photomicrographic traces of cell fusion to produce tetraploid cells by the cultivation of a diploid ascospore derived from the supposed tetraploid clones also supported the above view.

Fertile *Saccharomyces* yeasts are classified into heterothallic and homothallic strains. In a heterothallic strain, cultivation of an ascospore gives rise to a haploid clone having either **a** or α mating-type, and diploidization occurs by fusion of two haploid cells of opposite mating types. In a homothallic strain, conversion of one mating-type allele to the other occurs efficiently by the mutagenic action of the homothallic genes,[1,2] and diploidization occurs by the fusion of the converted and unconverted cells in a single haploid ascospore-culture.[3] Conversion of one mating-type allele to the other is controlled by three kinds of homothallic genes, each consisting of a single pair of alleles, *HO*/*ho*, *HMa*/*hma*, or *HM***a**/*hm***a**.[4] Depending on the genotype of the three loci and the mating-type alleles in an ascospore, three different types of diploid homothallic strain arise on the

cultivation of the ascospore. One shows a perfectly homothallic life cycle with the **a** (or *α*) *HO hma hm***a** or **a** (or *α*) *HO HMα HM***a** genotype (the Ho type); the other two are semi-homothallic in the **a** *HO hma HM***a** spore culture (the Hp type) and the *α HO HMα hm***a** spore culture (the Hq type). Any spore having a genotype other than those described above gives rise to stable heterothallic haploid clones.[4] From the relationships between the genotypes and the phenotypes in homothallism, it was indicated that the combination of the *HMα* and/or *hm***a** alleles with the *HO* allele is essential for the *α* to **a** conversion while for the **a** to *α* conversion, the *HM***a** and/or *hmα* alleles should be combined with the *HO* allele. Conversion of one mating-type allele to the other occurs within a few generations of spore germination and the activity of the homothallic genes is blocked as soon as heterozygosity of the mating-type alleles is established by zygote formation between cells with complementary mating types.[3]

On the other hand, it has been reported that industrial yeasts are commonly polyploid: most brewer's yeasts are tetraploid, some are triploid,[5~7] and tetraploid strains of baker's yeasts have also been reported.[8] These observations suggest polyploid cells may have advantages in industrial uses. In fact, we previously tried to breed a yeast strain suitable for alcoholic fermentation of a mash prepared by hydrolysis of starch, but resultant diploid strains never gave more potent fermentation than the authentic polyploid strains (unpublished data).

This paper describes evidences which support the view that the activities of the homothallic genes are also effective during vegetative growth of diploid cells homozygous for the mating-type alleles. Conversions occurred in the **a**/**a** and *α*/*α* cells derived from an **a**/*α* cell by mitotic recombination and **a**/**a**/*α*/*α* tetraploid cells were produced through cell fusion between the converted and unconverted cells. It was also suggested that single allele conversion occurs in **a**/**a** and *α*/*α* diploid cells and produces **a**/*α* cells.

Materials and Methods

Strains The strains used are listed in Table 1. All strains were selected from our stock cultures for Yeast Genetics, and were purified by single cell isolation with the aid of a micromanipulator. Two heterothallic haploid strains, A-22-16B (**a**) and A-23-5B (*α*), were used as standards for the determination of mating type.

Media The nutrient medium contained 10 g of yeast extract (Daigo Eiyo Chemicals and Co. Ltd.), 20 g of polypeptone (Daigo Eiyo Chemicals and Co. Ltd.) and 20 g of glucose per liter. For testing auxotrophic markers, minimum medium (0.67% of Difco Yeast Nitrogen Base without amino acids and 2% of glucose in distilled water, pH 5.0) with appropriate supplement of nutrients was used. Sporulation was performed by smearing cells on an agar medium containing 0.5% anhydrous potassium acetate and incubation for 2 days at 30°C. All solid media were prepared by addition of 20 g of agar per liter of the media.

Genetic methods Mating types were determined by the mass mating method[10] or by the appearance of prototrophs from the combination with standard strains marked with the complementary auxotrophic markers on minimum medium. Tetrad dissection was carried out by the method of Johnston and Mortimer.[11] Occurrence of cells showing **a** or *α* mating type from **a**/*α* diploid cells was detected by the procedure of synchronized zygote formation[12,13] with slight modification. Diploid **a**/*α* cells to be tested were cultivated in nutrient medium with shaking for 24 hr at 30°C. The cells were harvested and resuspended in sterile distilled water to give cell density of approximately 2×10^7 cells per ml. A portion (0.5 ml) of the cell suspension was mixed with the same volume of cell suspension of strain A-22-16B (**a**) or A-23-5B (*α*) in nutrient medium at approximately 1×10^8 cells per ml. The suspension was shaken (40 mm amplitude at 120 rpm) at 30°C for 2 hr after addition of 5 ml of fresh nutrient medium. The mixed culture was centrifuged at 3,000 rpm for 5 min and the cell pellet was allowed to stand for 1 hr at 30°C without decantation of the supernatant. The pellet was then resuspended in the same supernatant and shaken for 1 hr at 30°C. Finally, the cells were plated on minimum medium after appropriate dilution with sterile water and the plates were incubated at 30°C for 2–3 days. The colonies appearing on the plate were scored assuming they were derived from the

Table 1. List of strains used.

Strain	Genotype*			Remarks
	mating type	homothallic gene	genetic markers	
T-1068	**a** / *α*	*ho* *HMα* *hma* / *ho* *HMα* *hma*	*ade* 1 / + ; + / *arg* 4 ; + / *his* 4 ; + / *leu* 2 ; *lys* 2 / *lys* 2 ; + / *thr* 4 ; + / *trp* 1 ; + / *ura* 3	Heterothallism
T-1071-8	**a** / *α*	*ho* *HMα* *hma* / *ho* *HMα* *hma*	*ade* 1 / + ; + / *arg* 4 ; + / *his* 4 ; + / *leu* 2 ; *lys* 2 / *lys* 2 ; + / *thr* 4 ; + / *trp* 1 ; + / *ura* 3	Heterothallism
T-1269-38C	**a** / *α*	*HO* *HMα* *HMa* / *HO* *HMα* *HMa*	*lys* 2 / *lys* 2 ; *met* 4 / *met* 4	Homothallism (Ho)
S-14-9C	**a** / *α*	*HO* *hma* *HMa* / *HO* *hma* *HMa*	*his* 4 / *his* 4 ; *leu* 2 / *leu* 2 ; *lys* 2 / *lys* 2	Homothallism (Hp)
T-1023-23B	**a** / *α*	*HO* *HMα* *hma* / *HO* *HMα* *hma*	*ade* 1 / *ade* 1 ; *arg* 4 / *arg* 4 ; *his* 4 / *his* 4 ; *leu* 2 / *leu* 2 ; *lys* 2 / *lys* 2 ; *trp* 1 / *trp* 1	Homothallism (Hq)
T-891-13B	**a** / *α*	*HO* *HMα* *hma* / *HO* *HMα* *hma*	*ade* 1 / *ade* 1 ; *his* 4 / *his* 4 ; *lys* 2 / *lys* 2	Homothallism (Hq)
A-22-16B	**a**		*ade* 5 *aro* 1 *hom* 2 *leu* 1 *lys* 7	Heterothallism
A-23-5B	*α*		*hom* 2 *leu* 1	Heterothallism

* The terminology of genetic symbols follows that proposed by Plischke *et al.*[9]

zygotes between a cell having mating ability and a cell of the standard haploid.

Determination of cell volumes and deoxyribonucleic acid (DNA) contents Cells to be tested were shaken in nutrient medium for 36 hr at 30°C. To determine cell volume, photomicrographs of cells were taken at a fixed magnification approximately 900, and more than 50 cells were measured along their short and long axes. Each cell volume was calculated assuming that it was ellipsoidal. DNA was extracted by the method of Schneider[14] from approximately 10^9 cells. DNA contents of the extract were determined by measuring absorbance at 600 nm using a diphenylamine reagent.[15] Calf thymus DNA (Seravac Laboratory Ltd.) was employed as standard. For estimation of cellular DNA contents, cell numbers in each sample were counted by Coulter Counter (Model ZBI, Coulter Electronics, Inc.) after slight sonication (Sonic 300, Artex System Corporation; 20 k cycle/sec, 60 w, for 15 sec).

Results

Appearance of mating types among a/α diploid cells Cells showing **a** or *α* mating type appear spontaneously among **a**/*α* diploid cells during the vegetative growth by mitotic crossing over between the mating-type locus and the centromere on chromosome III, by mitotic gene conversion or by mutation. These events are enhanced by treatment with various recombinogens or mutagens.[16,17] Cells showing mating potency can be detected by scoring the appearance of prototrophic zygotes with the standard haploid cells having appropriate complementary genetic markers. Here their occurrence was detected by the technique of synchronized zygote formation. Results clearly indicated that conversions in both directions, *i.e.*, from the **a**/*α* non-mater cells to the cells showing **a** or *α* mating type, occurred with the frequency of approximately 10^{-5} in all the strains tested, although characteristic biases in the frequencies were suggested in some strains, particularly in the Hp and Hq homothallic strains (Table 2).

Isolation of clones showing mating response from a/α diploids To see

Table 2. Spontaneous occurrence of cells showing mating potency in heterothallic and homothallic **a**/*α* diploids.

Strain	Thallism	Occurrence of cells showing mating potency (prototrophic colonies per 10^5 **a**/*α* diploid cells)							
		Exp. 1		Exp. 2		Exp. 3		Exp. 4	
		a*	*α***	**a**	*α*	**a**	*α*	**a**	*α*
T–1068	Heterothallism	0.30	0.98	—	—	—	—	—	—
T–1071–8	Heterothallism	—	—	0.46	0.42	0.48	0.34	0.43	0.22
T–1269–38C	Homothallism (Ho)	0.35	0.28	0.74	0.30	1.06	0.35	0.35	0.24
S–14–9C	Homothallism (Hp)	0.17	2.37	0.14	0.55	0.17	0.89	0.15	0.34
T–1023–23B	Homothallism (Hq)	—	—	0.97	0.15	1.22	0.24	0.68	0.05
T–891–13B	Homothallism (Hq)	0.67	0.35	—	—	—	—	—	—

* Number of switches from **a**/*α* to **a** scored by occurrence of prototrophic colonies with the *α* haploid cells having complementary auxotrophic markers.
** Number of switches from **a**/*α* to *α* scored with the **a** haploid cells as described above.

whether the homothallic genes are effective in diploid cells showing mating type during their vegetative growth, cells of **a**/*α* diploids were irradiated with a low dose (approximately 20 percent survival) of ultraviolet light to accelerate mitotic recombination. The irradiated cells were plated on nutrient agar and the plates were incubated at 30°C for 3 days. Several colonies appearing on the plates were picked up and the isolates were tested for their mating reaction with the standard haploid strain having **a** or *α* mating type. In heterothallic strains, T–1068 and T–1071–8, both **a** and *α* mating-type clones were obtained with frequencies of 0.3% to 3.8%, while in the Ho type homothallic strain, T–1269–38C, neither of **a** and *α* clones could be isolated from 1,000 colonies examined. In the Hp and Hq type homothallic strains, on the other hand, clones showing solely *α* or **a** mating type were obtained (Table 3).

The observations listed in Tables 2 and 3 might be interpreted as indicating that homozygosity at the mating-type locus, *i.e.*, **a**/**a** and *α*/*α*, occurred in the original **a**/*α* diploid cells irrespective of the thallism. If an excess population of haploid *α* or **a** cells was present in the culture, as in the experiments listed in Table 2, the **a**/**a** and *α*/*α* cells could immediately mate with the haploid cells and the zygotes would give rise to prototrophic colonies by the complementary combination of the auxotrophic markers. Even if homozygosity at the mating-type locus occurred in a pure culture, the probability of cell fusion with the opposite

Table 3. Occurrence of colonies showing mating potency from **a**/*α* diploids after irradiation of low dose of ultraviolet-light.

Strain	Thallism	Number of colonies examined	Number of colonies showing mating potency	
			a	*α*
T–1068	Heterothallism	425	16	5
T–1071–8	Heterothallism	1,000	3	4
T–1269–38C	Homothallism (Ho)	1,000	0	0
S–14–9C	Homothallism (Hp)	1,550	0	25
T–1023–23B	Homothallism (Hq)	780	15	0
T–891–13B	Homothallism (Hq)	1,100	27	0

mating-type cells in the culture before vegetative growth begins would be low because of the rare occurrence of homozygosity. Thus the conversion of a mating-type allele must occur during vegetative multiplication in the homothallic **a**/**a** or α/α cells. It is possible to speculate two alternative types of conversion of a mating-type allele in **a**/**a** and α/α diploid cells. One is from the **a**/**a** and α/α homozygous alleles to the **a**/α configuration and the other from **a**/**a** to α/α or vice versa. The former conversion will give rise to a non-mater **a**/α diploid from both **a**/**a** and α/α cells. In the latter conversion, cell fusion will occur between the converted and unconverted cells and give rise to **a**/**a**/α/α tetraploid cells. Since both directions of the mating-type conversion, **a** to α and α to **a**, are possible in the Ho type homothallic cells, transiently appearing **a**/**a** and α/α cells would be quickly converted to the **a**/α or **a**/**a**/α/α cells as described above Thus, it will be expected that all the colonies in the Ho strain show no mating reaction with the standard as observed in Table 3. In the Hp strain, however, α to **a** conversion is blocked, whereas in the Hq strain **a** to α conversion is not possible. Hence the α/α cells in the Hp strain and the **a**/**a** cells in the Hq strain could maintain the homozygosity at the mating-type locus through their vegetative growth.

Occurrence of tetraploid clones from various a/α diploid strains To confirm the above possibilities, colonial clones which did not show mating reaction with either of the standard haploid cells in Table 3 were inspected under a microscope. Since cells of higher ploidy are expected to have larger size,[7,18~20] clones showing larger cell size than the original diploid cells were selected and purified by the single cell isolation with the aid of a micromanipulator. Cell volumes and DNA contents per cell were determined. From the plates inoculated with the ultraviolet-light irradiated **a**/α cells of the Ho, Hp, or Hq type homothallic strains, we were able to isolate several colonial clones showing larger cell size more easily than from those of a heterothallic strain, although their frequencies of occurrence were not scored. The supposed tetraploid clones (larger cell clones) were tested for their ability to sporulate and compared in cell size and DNA content with their parental diploid strains (Table 4). The strains showing larger cell size, T–1071–8–U33, T–1269–38C–U1, S–14–9C–U37, and T–1023–23B–U16, were thought to be tetraploid, since their cell volumes and DNA contents per cell were almost twice those of their parental diploid strains. The result of similar determination of two heterothallic **a** (T–1071–8–U1) and α (T–1071–8–U2) clones derived from the heterothallic strain T–1071–8 and the hybrid between them supports that those two clones showing mating types are diploid. However, a tetraploid strain, T–1071–8–U33, obtained from the same strain, T–1071–8, might be caused by an event other than the process speculated above, because this strain does not carry the *HO* allele which is indispensable for the mating-type conversion. A possible mechanism for the tetraploid formation in the heterothallic strain will be that 50 percent of cases of mitotic crossing over between the mating-type locus and the centromere will produce the reciprocal products, **a**/**a** and α/α, from an **a**/α diploid cell. Then these **a**/**a** and α/α cells will form a zygote, being adjacent sister cells.

It is possible to expect three types of segregation with respect to the mating types in asci from an **a**/**a**/α/α tetraploid (Table 5) as described by Roman *et al.*[21] In a heterothallic strain, 4 non-mater (**a**/α): 0 mater, 2 non-mater (**a**/α): 1 **a**(**a**/**a**): 1α(α/α), and 2 **a**(**a**/**a**): 2 α(α/α) segregations will be expected in asci. If the *HO hma hm***a** and *HO HMα HM***a** genotypes (the Ho strains) were effective for mating-type conversion in both directions in the **a**/**a** and α/α diploid cells as well as in the **a** and α haploid cells, all asci from the Ho type homothallic tetraploid should show a 4 non-mater: 0 mater segregation, while three types of segregation, 4 : 0, 2 : 2, and 0 : 4, would be expected with respect to the diploid: tetraploid ratios in each tetrad culture assuming that the **a**/**a** and α/α cells were converted

Table 4. Cell volume and DNA content of the clones showing mating potency or showing larger cell size obtained from various **a**/*α* diploids.

Strain	Type of homothallism	Mating type	Spore formation	Cell volume (μm^3)	DNA contents (mg/10^{11} cells)	Expected	
						ploidy	genotype of mating-type alleles
T-1071-8	Heterothallism	non*	+	115.1	6.26	Diploid	**a**/*α*
T-1071-8-U1	Heterothallism	**a**	−	116.9	6.82	Diploid	**a**/**a**
T-1071-8-U2	Heterothallism	*α*	−	126.1	7.65	Diploid	*α*/*α*
T-1071-8-U33	Heterothallism	non	+	200.6	13.68	Tetraploid	**a**/**a**/*α*/*α*
U1 × U2**	Heterothallism	non	+	214.1	14.56	Tetraploid	**a**/**a**/*α*/*α*
T-1269-38C	Homothallism (Ho)	non	+	102.6	4.98	Diploid	**a**/*α*
T-1269-38C-U1	Homothallism (Ho)	non	+	218.7	7.98	Tetraploid	**a**/**a**/*α*/*α*
S-14-9C	Homothallism (Hp)	non	+	93.3	4.31	Diploid	**a**/*α*
S-14-9C-U6	Homothallism (Hp)	*α*	−	92.4	5.21	Diploid	*α*/*α*
S-14-9C-U37	Homothallism (Hp)	non	+	170.0	8.72	Tetraploid	**a**/**a**/*α*/*α*
T-1023-23B	Homothallism (Hq)	non	+	120.5	6.12	Diploid	**a**/*α*
T-1023-23B-U6	Homothallism (Hq)	**a**	−	132.4	5.04	Diploid	**a**/**a**
T-1023-23B-U16	Homothallism (Hq)	non	+	251.9	12.28	Tetraploid	**a**/**a**/*α*/*α*

* Non-mater.
** Obtained from the cross between T-1071-8-U1 and T-1071-8-U2 strains.

to **a**/**a**/*α*/*α* tetraploid. Since *α* to **a** conversion in the Hp strain and **a** to *α* conversion in the Hq strain are not expected, a tetraploid originating from an Hp type homothallic strain will show the 4 non-mater: 0 mater, 3 non-mater: 1 *α*(*α*/*α*) and 2 non-mater: 2 *α*(*α*/*α*) segregations in asci, and that from an Hq type will give the 4 non-mater: 0 mater, 3 non-mater: 1 **a**(**a**/**a**), and 2 non-mater: 2 **a**(**a**/**a**) segregations. All non-mater clones in the asci showing a 4 non-mater: 0 mater segregation should be diploids and two clones of three non-maters in the asci showing a 3 non-mater: 1 mater segregation should be diploids and the remaining one should be tetraploid. All the non-maters in the asci showing a 2 non-mater: 2 mater segregation should be tetraploids.

To confirm the above possibilities, tetrad analysis of the supposed tetraploid strains was performed (Table 6). The clones showing large cell size, *i.e.*, supposed tetraploids, sporulated well and the ascospores showed good viability by tetrad dissection. The original

Table 5. Three principal types of asci expected from three types of homothallic **a**/**a**/*α*/*α* tetraploid.

Types of homothallism	Type of segregation											
	I				II				III			
	A	B	C	D	A	B	C	D	A	B	C	D
	a/*α*	**a**/*α*	**a**/*α*	**a**/*α*	**a**/*α*	**a**/*α*	**a**/**a**	*α*/*α*	**a**/**a**	**a**/**a**	*α*/*α*	*α*/*α*
Ho	**a**/*α**	**a**/*α*	**a**/*α*	**a**/*α*	**a**/*α*	**a**/*α*	**a**/**a**/*α*/*α**	**a**/**a**/*α*/*α*	**a**/**a**/*α*/*α*	**a**/**a**/*α*/*α*	**a**/**a**/*α*/*α*	**a**/**a**/*α*/*α*
Hp	**a**/*α*	**a**/*α*	**a**/*α*	**a**/*α*	**a**/*α*	**a**/*α*	**a**/**a**/*α*/*α*	*α*/*α***	**a**/**a**/*α*/*α*	**a**/**a**/*α*/*α*	*α*/*α*	*α*/*α*
Hq	**a**/*α*	**a**/*α*	**a**/*α*	**a**/*α*	**a**/*α*	**a**/*α*	**a**/**a****	**a**/**a**/*α*/*α*	**a**/**a**	**a**/**a**	**a**/**a**/*α*/*α*	**a**/**a**/*α*/*α*

* **a**/*α* and **a**/**a**/*α*/*α* have no mating response.
** **a**/**a** and *α*/*α* have mating response of **a** and *α* respectively.

Table 6. Tetrad segregation in asci from supposed tetraploids.

Strain	Thallism	Segregation of mating type (non*: **a** : *α*)							Expected genotype of mating-type alleles
		4:0:0	3:1:0	3:0:1	2:1:1	2:2:0	2:0:2	0:2:2	
T-1071-8	Heterothallism	0	0	0	0	0	0	13	**a**/*α*
T-1071-8-U33	Heterothallism	9	0	0	1	0	0	4	**a**/**a**/*α*/*α*
U1×U2**	Heterothallism	10	0	0	4	0	0	4	**a**/**a**/*α*/*α*
	Homothallism(Ho):								
T-1269-38C	original diploid	14	0	0	0	0	0	0	**a**/*α*
T-1269-38C-U1	tetraploid	28	0	0	0	0	0	0	**a**/**a**/*α*/*α*
	Homothallism(Hp):								
S-14-9C	original diploid	0	0	0	0	0	6	0	**a**/*α*
S-14-9C-U37	tetraploid	17	0	8	0	0	3	0	**a**/**a**/*α*/*α*
	Homothallism(Hq):								
T-1023-23B	original diploid	0	0	0	0	20	0	0	**a**/*α*
T-1023-23B-U16	tetraploid	14	11	0	0	4	0	0	**a**/**a**/*α*/*α*

* Non-mater
** Obtained from the cross between T-1071-8-U1 and T-1071-8-U2 strains.

heterothallic diploid strain, T-1071-8, showed a 2**a**: 2*α* segregation in all asci so far tested. Strain T-1071-8-U33, which is a colonial isolate of strain T-1071-8 having larger cell size, showed the segregation pattern in asci expected from a heterothallic tetraploid strain with respect to the mating types. A hybrid clone prepared by the cross between two derivatives from T-1071-8 showing **a** (T-1071-8-U1) and *α* mating types (T-1071-8-U2) showed the same segregation pattern as that of strain T-1071-8-U33 by tetrad analysis. In tetrads of the supposed tetraploids obtained from the homothallic **a**/*α* strains, the phenotypic segregations in asci agree with those expectations, as described in Table 5. All the tetrad segregants from the supposed tetraploid of the Ho type, T-1269-38C-U1, were non-mater, and most of the asci were classified into three types of segregation, *i.e.*, 4 diploid: 0 tetraploid, 2 diploid : 2 tetraploid, and 0 diploid: 4 tetraploid. Segregants from any type of asci again showed a 4 non-mater: 0 mater segregation in each ascus on further tetrad dissection.

The three typical types of asci from strain T-1269-38C-U1 are shown in Table 7. Further tetrad analysis of the segregants and the cell volume determination accord well with the expectation that strain T-1269-38C-U1 is tetraploid, while the DNA contents of the supposed tetraploids in the segregants showed lower levels than those of the other supposed tetraploids (Tables 4, 7, 8 and 9). In the tetrads from the tetraploid having the Hp (Tables 6 and 8) and Hq (Tables 6 and 9) genotypes for homothallism, we observed the three types of asci with respect to the diploid: tetraploid ratio. The diploid non-mater segregants from them always showed 2 non-mater : 2 *α* (the Hp clones) or 2 non-mater : 2**a** (the Hq clones) and the supposed tetraploid segregants showed the characteristic segregation pattern depending on the genotype for homothallism. All these results accord with those expected, as described in Table 5, and strongly suggest that the clones showing large cells are tetraploid having the **a**/**a**/*α*/*α* genotype, and that the homothallic genes are effective in the **a**/**a** and *α*/*α* diploid cells with the same specificity for the mating-type alleles as observed in the haploid cells.

During the course of the above experiment, however, some asci of the supposed tetraploid clones showed the 1 diploid: 3 tetraploid and 3 diploid : 1 tetraploid segregations

Table 7. Further analysis of three asci showing typical types of segregation from the supposed homothallic (Ho) tetraploid strain, T-1269-38C-U1.

Ascus	Mating type	Spore formation	Cell volume (μm^3)	DNA contents (mg/10^{11} cells)	Tetrad analysis		Expected	
					No. of asci tested	4non:0 mater	ploidy	genotype of mating-type alleles
1 A	non*	+	93.4	4.55	20	20	Diploid	**a**/*α*
1 B	non	+	107.9	4.92	12	12	Diploid	**a**/*α*
1 C	non	+	103.7	5.48	18	18	Diploid	**a**/*α*
1 D	non	+	97.6	5.08	20	20	Diploid	**a**/*α*
3 A	non	+	219.8	8.16	16	16	Tetraploid	**a**/**a**/*α*/*α*
3 B	non	+	201.5	7.43	12	12	Tetraploid	**a**/**a**/*α*/*α*
3 C	non	+	107.6	5.09	15	15	Diploid	**a**/*α*
3 D	non	+	101.5	5.41	15	15	Diploid	**a**/*α*
14 A	non	+	209.2	7.82	17	17	Tetraploid	**a**/**a**/*α*/*α*
14 B	non	+	190.1	7.20	14	14	Tetraploid	**a**/**a**/*α*/*α*
14 C	non	+	192.3	7.99	17	17	Tetraploid	**a**/**a**/*α*/*α*
14 D	non	+	196.2	8.23	15	15	Tetraploid	**a**/**a**/*α*/*α*

* Non-mater.

according to the estimation of ploidy by cell size. For example, one ascus showed 1 diploid : 3 tetraploid and 4 asci gave 3 diploid: 1 tetraploid segregations out of 28 asci so far tested in T-1269-38C-U1. If the mating-type alleles of the **a**/**a** or *α*/*α* diploids were converted to *α*/*α* or **a**/**a**, respectively, in a homothallic strain, the above segregations would not be expected (Table 5). This fact suggests that another possibility, *i.e.*, the **a**/**a** or *α*/*α* to **a**/*α* conversion, might be occurred in some spore-cultures of those asci. Occurrence

Table 8. Further analysis of three asci showing typical types of segregation from the supposed homothallic (Hp) tetraploid strain, S-14-9C-U37.

Ascus	Mating type	Spore formation	Cell volume (μm^3)	DNA contents (mg/10^{11} cells)	Tetrad analysis				Expected	
					No. of asci tested	4non:0 mater	3non:1*α*	2non:2*α*	ploidy	genotype of mating-type alleles
1 A	non*	+	87.5	4.30	12	0	0	12	Diploid	**a**/*α*
1 B	non	+	92.7	4.67	12	0	0	12	Diploid	**a**/*α*
1 C	non	+	99.2	4.36	12	0	0	12	Diploid	**a**/*α*
1 D	non	+	88.7	4.78	19	0	0	19	Diploid	**a**/*α*
7 A	non	+	87.2	4.29	20	0	0	20	Diploid	**a**/*α*
7 B	non	+	161.5	8.33	15	4	6	5	Tetraploid	**a**/**a**/*α*/*α*
7 C	non	+	86.1	4.27	17	0	0	17	Diploid	**a**/*α*
7 D	*α*	−	93.7	4.91	—	—	—	—	Diploid	*α*/*α*
6 A	*α*	−	94.7	5.10	—	—	—	—	Diploid	*α*/*α*
6 B	non	+	182.2	8.38	16	7	3	6	Tetraploid	**a**/**a**/*α*/*α*
6 C	*α*	−	91.3	4.95	—	—	—	—	Diploid	*α*/*α*
6 D	non	+	168.6	8.17	14	5	7	2	Tetraploid	**a**/**a**/*α*/*α*

* Non-mater.

Table 9. Further analysis of three asci showing typical types of segregation from the supposed homothallic (Hq) tetraploid strain, T-1023-23B-U16.

Ascus		Mating type	Spore formation	Cell volume (μm^3)	DNA contents (mg/10^{11} cells)	Tetrad analysis				Expected	
						No. of asci tested	4non:0 mater	3non:1a	2non:2a	ploidy	genotype of mating-type alleles
5	A	non*	+	100.7	6.06	19	0	0	19	Diploid	a/α
	B	non	+	118.2	5.37	18	0	0	18	Diploid	a/α
	C	non	+	122.3	6.94	20	0	0	20	Diploid	a/α
	D	non	+	110.1	6.19	19	0	0	19	Diploid	a/α
2	A	a	−	110.9	6.21	—	—	—	—	Diploid	a/a
	B	non	+	230.8	11.42	11	6	5	0	Tetraploid	a/a/α/α
	C	non	+	103.6	6.42	18	0	0	18	Diploid	a/α
	D	non	+	120.8	6.02	19	0	0	19	Diploid	a/α
8	A	non	+	188.6	10.62	10	7	1	2	Tetraploid	a/a/α/α
	B	non	+	236.6	10.11	13	5	6	2	Tetraploid	a/a/α/α
	C	a	−	130.7	5.56	—	—	—	—	Diploid	a/a
	D	a	−	130.5	5.97	—	—	—	—	Diploid	a/a

* Non-mater.

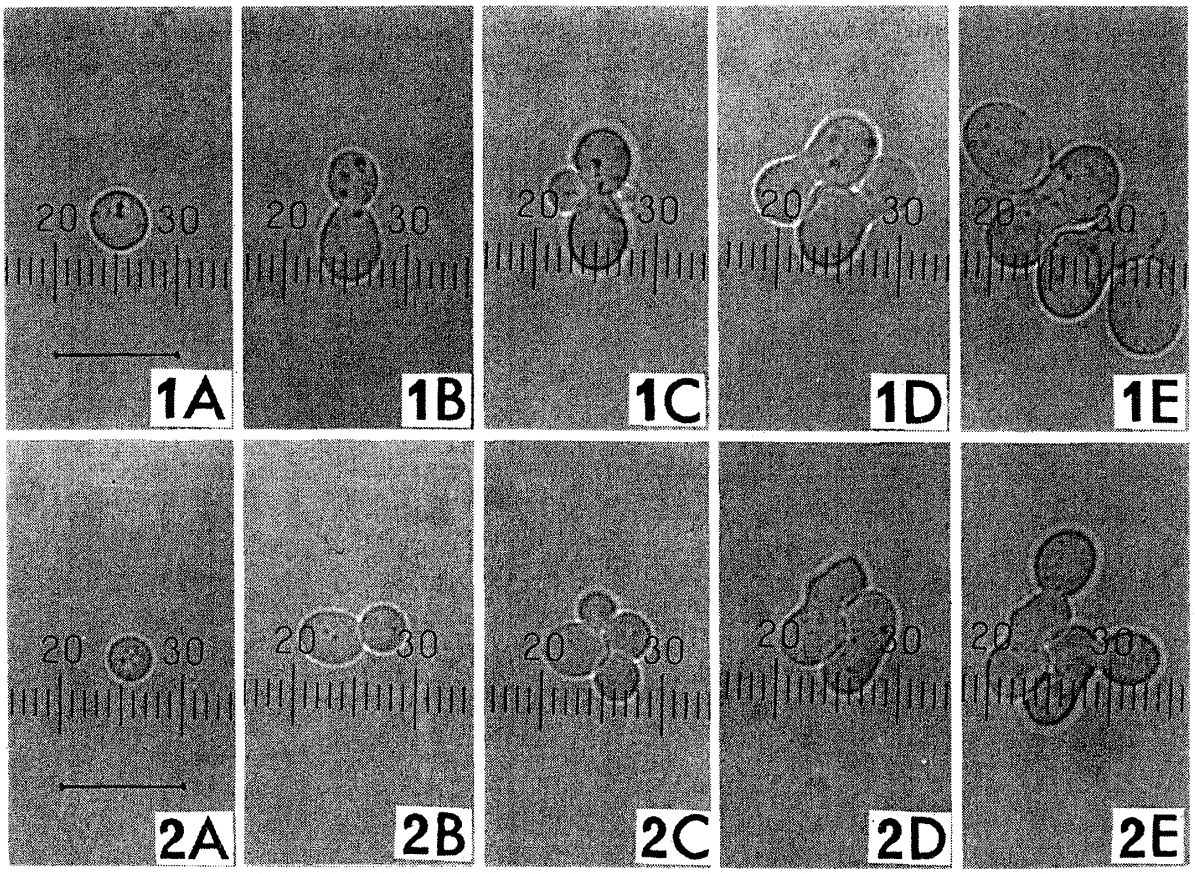

Fig. 1. Twin zygote formation during the incubation of diploid (1A–1E) and haploid (2A–2E) ascospores from the Ho type homothallic tetraploid and diploid strains, respectively. Asci were dissected and several single ascospore cultures were made on thin agar film of nutrient medium. Photomicrographs were taken at 1 (A), 4.5 (B), 6 (C), 7 (D) and 8 (E) hr after incubation at 30°C. The bar denotes 10 μm.

of this type of conversion in the tetrad segregants of the supposed tetraploids having the Hp or Hq genotype was also suggested by tetrad data (not shown).

Tetraploidization by cell fusion during outgrowth of diploid spores It was strongly suggested by the above genetic studies that diploid cells or diploid ascospores having the **a**/**a** or *α*/*α* mating-type alleles are changed to either sporogenous non-mater tetraploid or diploid cells by the effects of homothallic genes during their vegetative growth. In a haploid homothallic ascospore, it has been observed that the diploidization occurs mostly at the four-cell stage after the second division of cells immediately following spore germination.[3,22] To determine the manner of tetraploidization in a culture of a homothallic diploid ascospore, several ascospores from strain T-1269-38C-U1 carrying a homozygous genotype for *HO*, *HMα* and *HM***a** alleles were placed on a piece of nutrient agar film separately. The agar films were sealed and incubated at 30°C. It was observed that approximately 30% of the ascospores from 15 asci so far tested could form twin zygotes (Fig. 1, 1A to 1E). This observation was quite similar to that observed during the outgrowth of a homothallic haploid spore (Fig. 1, 2A to 2E), and suggests that both the mating-type alleles in the **a**/**a** and *α*/*α* genotypes were effectively converted to the opposite alleles within a few generations of spore germination as well as in a haploid cell. Then tetraploidization occurred through the zygote formation between the converted and unconverted cells, although it is uncertain which cells at the four-cell stage are converted. Germination sites of the large cells from a zygote were variable in each case.

Cell fusion between two cells in a spore culture could double the ploidy. However, some spore cultures (2 of 60 ascospores) showed only one zygote formation during the mitotic growth of the cells. These cultures produced a mixed population of large and small cells, both of which are non-mater and sporogenous. The large cells were derived

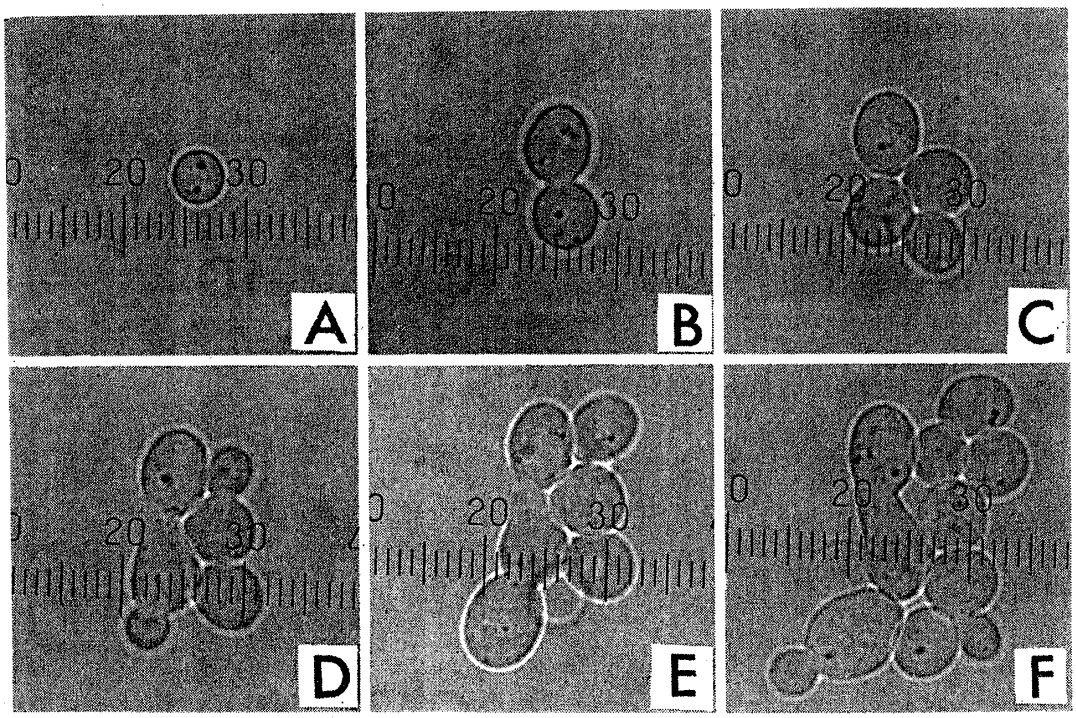

Fig. 2. Single zygote formation by the Ho type homothallic diploid ascospore during incubation on nutrient agar film at 30°C. Photomicrographs were taken at 1 (A), 4 (B), 6 (C), 7 (D), 8 (E) and 9 (F) hr after incubation at 30°C. Only one zygote was formed at the four-cell stage and the further cultivation gave rise to a culture with mixed population of large and small cells. The smallest unit of scale is approximately 1 μm.

from the zygote, and small ones might have originated from unfused cells (Fig. 2). These observations, along with the exceptional asci observed in the foregoing tetrad analysis, further strengthen the possibility that some of the **a**/**a** or α/α genotype would be converted to **a**/α.

Discussion

The process for breeding of tetraploid clones most probably occurs in two main steps. 1) First, there is a genetical event to produce the **a**/**a** and α/α configuration at the mating-type locus from the **a**/α diploid cells. This might be either mitotic recombination or mutation at the mating-type locus, though we have not determined which events occurred in the present cases. However, it has been proposed that mitotic recombination, most possibly by mitotic crossing over between the mating-type locus and the centromere on chromosome III, plays a major role in the occurrence of homozygosity of the mating-type alleles.[19] We detected this event with a frequency of one in 10^5 cells spontaneously by the modified synchronized mating method (Table 2). Frequency of the **a**/α to **a**/**a** or α/α switches was significantly increased to approximately 0.3–3.8% of cell population by low dose of ultraviolet-light irradiation (20 percent survival) (Table 3). Furthermore, it might be expected that the frequencies of occurrence of **a**/**a** cells and α/α cells in the **a**/α diploid culture are the same in any **a**/α diploid culture, irrespective of the thallism of the cells (Tables 2 and 3). 2) Then, mating-type conversion occurs effectively in some fraction of the **a**/**a** and α/α diploid cells by the action of homothallic genes as well as in haploid cells. This is followed by cell fusion between the converted and unconverted cells. The resultant zygote produces a new tetraploid bud, as observed in a diploid ascospore which presumably has the **a**/**a** or α/α configuration (Fig. 1). These facts suggest that it is possible to select **a**/**a**/α/α tetraploid clones by microscopic inspection of individual colonies as tetraploid colonies, consisting of cells showing larger cell size than diploid cells, will be expected with the frequency of $10^{-2}\sim10^{-3}$ (Table 3). Furthermore, genetic and cytological observations (Fig. 2) strongly suggested that there will also occur another possibility, *i.e.*, conversion of the **a**/**a** and α/α configuration to **a**/α.

There is also a possible mechanism to produce tetraploid cells in heterothallic strains by direct fusion of the **a**/**a** and α/α cells which might be derived from an **a**/α diploid cell as the reciplocal products of a mitotic crossing over. We were able to isolate such a strain, T-1071-8-U33 (Tables 4 and 6), although its frequency of occurrence is thought very low in comparison with that in a homothallic strain.

Though several models have been proposed to explain the interconversion of mating types[2,4,23] (J. B. Hicks and I. Herskowitz, personal communication), the exact mechanism of the homothallic genes has not been established, nor is the dominance-recessiveness between each pair of alleles of the *HM*α and *HM***a** genes understood yet, while the evidence for the dominance of the *HO* allele over the *ho* allele has been presented by Hopper and Hall.[24] The observations described in the present communication demonstrate a new method for breeding tetraploid strains, though several questions remain to be solved for full understanding of the phenomena as described above. The procedure does not require auxotrophic markers necessary for selection due to the low frequency of zygote formation in a forced mating mixture by the conventional polyploid breeding techniques.[19,25] Most of the tetraploid strains obtained by this procedure sporulate well and segregate **a**/**a**/α/α tetraploids, **a**/**a** and α/α diploids along with **a**/α diploids depending on the genotype for homothallism. Those segregants will be usefull for further construction of polyploid cells.

On the other hand, it has been reported that a number of the yeast strains used in

the brewing industry and bakery are polyploid.[5~8] In previous papers,[20,26] we described a novel type of semi-homothallic strain, the *das* (diploid **a** sporogenous) strain, which is of special interest in its application to the breeding of triploid cells. The procedure described in the present communication is more widely applicable to breeding polyploid strains than that for the *das* strain, because it does not require such a special genetic trait.

Though all the test strains used in the present study were homozygous for the homothallic genes, our results clearly demonstrate that the homothallic genes are effective in the diploid cells. This finding suggests a possible approach to the dominance-recessiveness of the alleles at *HMα* and *HM***a** loci, although there remains the difficulty due to the direct linkage between these two loci and the mating-type gene on chromosome III.[27]

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