Mating Signals Control Expression of both Starch Fermentation Genes and a Novel Flocculation Gene *FLO8* in the Yeast *Saccharomyces*

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In the yeast *Saccharomyces diastaticus*, expression of both glucoamylase-producing (*STA*) genes and a novel flocculation gene *FLO8* was greatly diminished by the mating-type locus *MATa*/*MATα*.

In 1978, H. Tamaki isolated three polymorphic genes (*STA1*, *STA2*, and *STA3*) for starch fermentation in the yeast *Saccharomyces diastaticus*¹⁾ and demonstrated that haploid strains of *S. diastaticus* carrying any one of the unlinked *STA* genes produce one of the extracellular glucoamylase isozymes I, II, and III.²⁾ Recently we observed that the glucoamylase production was greatly diminished in diploid strains that are homozygous for the *STA* genes but are heterozygous for the co-dominant mating-type alleles *MATa* and *MATα*. However, full expression of the producing ability was recovered in diploid strains homozygous for both *STA* and *MAT*.

Flocculation genes are widely distributed in the yeast *Saccharomyces cerevisiae*, which is very closely allied to *S. diastaticus*.³⁾ Flocculation has been studied mostly with brewer's strains of yeasts because of its importance to the brewing industry.^{4,5)} We isolated a novel flocculation gene, *FLO8*, from *S. diastaticus* strain 5106-32D, provided by H. Tamaki. The *FLO8* gene is loosely linked to *arg4* on chromosome VIII, and details about this gene will be published elsewhere (manuscript submitted). Diploid strains heterozygous for *FLO8* but homozygous for *MAT* were flocculent, indicating a dominant phenotype of the *FLO8* gene, but diploid strains heterozygous for both *FLO8* and *MAT* were non-

flocculent.

In this communication, we describe how the expression of two *STA* genes (*STA1* and *STA3*) and the flocculation gene *FLO8* is controlled by the constitution of the mating-type locus. A preliminary report of this work was presented at the Annual Meeting of the Agricultural Chemical Society of Japan, 1983.⁶⁾

MATERIALS AND METHODS

Strains. Yeast strains used and their relevant genotypes are listed in Table I.

Culture media. YPS medium contained 1% yeast extract, 2% peptone, and 3% soluble starch. YPSB medium was YPS medium with the addition of $3.3 \times 10^{-3}\%$ bromocresol purple. YEPD medium contained 1% yeast extract, 2% peptone, and 2% dextrose. If necessary, the media were solidified by 2% agar. Sporulation plates contained 1% potassium acetate, 0.1% yeast extract, 0.05% dextrose, and 2% agar.

Fermentation test for starch. Fermentation tests were carried out by the classical Durham-tube method¹⁾ as follows: a loopful of cells was inoculated into 5 ml of YPS medium in a test tube (1.8 × 18 cm) with a Durham tube, and the test tube stood at 28°C for up to 3 weeks.

Flocculation test. Cells were cultured with shaking in a test tube (1.5 × 10.5 cm) containing 1 ml of YEPD medium at 28°C for 1~2 days. The degree of flocculation was estimated first by the appearance of the sedimented cells.

TABLE I. STRAINS

Strain	Genotype ^a	Source
5104-9C	α <i>his6 ura3 STA1</i>	H. Tamaki
5106-32D	α <i>arg4 his2 lys2 FLO8 STA1</i>	H. Tamaki
YIY2-11A	<i>a leu2-3,112 his2 STA1</i>	This work
YIY2-12D	<i>a leu2-3,112 his2 lys2 STA1</i>	This work
YIY4-6B	<i>a arg4 lys2 STA1</i>	This work
YIY11-1B	<i>a leu2-3,112 STA1</i>	This work
YIY4-3A	α <i>arg4 his4 STA1</i>	This work
YIY12-2D	α <i>leu2-3,112 his2 his4 STA1</i>	This work
YIY247	<i>a his4 STA1</i>	This work
YIYD11-7D	α <i>arg4 lys2 FLO8 STA1</i>	This work
YIYD11-9D	α <i>his2 FLO8 STA1</i>	This work
YIY1-6A	<i>a leu2-3,112 his4 flo^o</i>	This work
YIY256	<i>a arg4 FLO8 STA1</i>	This work
YIY257	<i>a leu2-3,112 his2 his4 FLO8 STA1</i>	This work
YIY258	<i>a his2 his4 lys2 FLO8 STA1</i>	This work
YIY260	<i>a leu2-3,112 his2 his4 lys2 FLO8 STA1</i>	This work
YIY262	<i>a his4 lys2 FLO8 STA1</i>	This work
YIY268	α <i>arg4 FLO8 STA1</i>	This work
YIY269	α <i>leu2-3,112 arg4 his2 his4 lys2 FLO8 STA1</i>	This work
YIY271	α <i>leu2-3,112 his2 his4 FLO8 STA1</i>	This work
YIY277	α <i>his4 FLO8 STA1</i>	This work
YIY280	<i>a leu2-3,112 his2 flo^o</i>	This work
YIY281	<i>a his4 lys2 flo^o</i>	This work
YIY284	<i>a leu2-3,112 his4 flo^o</i>	This work
YIY285	<i>a leu2-3,112 his4 lys2 flo^o</i>	This work
YIY292	α <i>lys2 flo^o</i>	This work
YIY294	α <i>his2 his4 lys2 flo^o</i>	This work
YIY297	α <i>leu2-3,112 his2 his4 flo^o</i>	This work
YIY299	α <i>his4 flo^o</i>	This work
YIY223	<i>a cdc6 ade1 ade2 his2 his7 leu2 met2 STA1 flo^o</i>	This work
YIY204	α <i>cdc6 his4 STA1 FLO8</i>	This work
YIY259	<i>a leu2-3,112 arg4 STA1</i>	This work
YIY261	<i>a arg4 his2 lys2 STA1</i>	This work
5301-17B	α <i>lys7 STA3</i>	H. Tamaki
YIYSTA3-2	<i>a leu2-3,112 his2 his4 STA3</i>	This work
YIYSTA3-3	<i>a leu2-3,112 his4 STA3</i>	This work
YIYSTA3-5	<i>a his2 his4 STA3</i>	This work

^a *flo^o* indicates the absence of a flocculation gene in cells; in the asci prepared from the cross *FLO8* $\times$ *flo^o*, the flocculating ability was segregated as 2 (flocculating): 2 (non-flocculating).

and second by the nature of the floc after degradation of the sediment with a Vortex mixer. This method was routinely used for characterization of the flocculating ability in genetic studies.

Protoplast fusion. Diploid strains homozygous for *MAT* were constructed by the standard protoplast-fusion method⁷⁾ with selection for prototrophy. Most of the fusants are expected to be diploids, since all of the six fusants examined were estimated to be diploids by tetrad analysis of the hybrids constructed by the crosses between each of the fusants and a standard diploid strain homozygous for *MAT*.

Assay for glucoamylase activity. Cells were cultured in YPS medium at 28°C for 3 days. The culture supernatant was obtained by centrifugation, dialyzed extensively against deionized water, and assayed for glucoamylase activity. The reaction mixture (final volume, 0.5 ml) for the assay contained 0.4% soluble starch, 80 mM sodium acetate buffer (pH 5.0) and the dialyzed enzyme solution. The reaction mixture was incubated at 50°C for 20 min, and the reducing sugar (glucose) formed was determined by the standard Somogyi-Nelson method.²⁾ Under the conditions used here, the activity proceeded linearly at least for 30 min. The activity was presented as increased absorbance at 660 nm for 20 min per 0.1 ml of the enzyme

TABLE II. MATING-TYPE REGULATION OF THE *STA* GENES

Genotype		Strain ^a	Fermentation ^b	Halo ^c	Glucoamylase activity
<i>STA1</i>	<i>MATa</i>	YIY2-11A	3	+	21.5
		YIY2-12D	3	+	13.0
		YIY4-6B	3	+	13.5
		YIY11-1B	3	+	22.0
<i>STA1</i>	<i>MATα</i>	YIY4-3A	3	+	18.5
		YIY12-2D	3	+	17.0
		5104-9C	3	+	11.0
<i>STA1/STA1</i>	<i>MATa/MATα</i>	YIY2-11A × YIY4-3A	13	—	0.2
		YIY2-12D × YIY4-3A	14	—	0.6
		YIY11-1B × YIY4-3A	13	—	0.5
		YIY4-6B × YIY12-2D	20	—	0.2
<i>STA1/STA1</i>	<i>MATa/MATa</i>	YIY2-11A × YIY4-6B	3	+	12.0
		YIY11-1B × YIY4-6B	3	+	17.5
		YIY247 × YIY2-11A	3	+	13.5
		YIY247 × YIY2-12D	3	+	14.0
<i>STA1/STA1</i>	<i>MATα/MATα</i>	YIY4-3A × 5104-9C	3	+	16.0
		YIY4-3A × YIYD11-9D	3	+	9.5
		YIYD11-7D × YIYD11-9D	3	+	8.5
		YIYD11-7D × YIY12-2D	3	+	17.5
<i>STA3</i>	<i>MATa</i>	YIYSTA3-2	4	+	2.0
		YIYSTA3-3	3	+	2.4
		YIYSTA3-5	4	+	1.7
<i>STA3</i>	<i>MATα</i>	5301-17B	3	+	1.8
<i>STA3/STA3</i>	<i>MATa/MATα</i>	YIYSTA3-2 × 5301-17B	11	—	0.2
		YIYSTA3-3 × 5301-17B	7	—	0.3
		YIYSTA3-5 × 5301-17B	8	—	0.2

^a Diploid strains heterozygous for *MAT* were constructed by sexual mating of the two haploid strains indicated by the cross. Diploid strains homozygous for *MAT* were constructed by the protoplast-fusion method using the two haploid strains indicated by the cross.

^b Numbers presented are culture days required for visible gas formation in a Durham tube.

^c +, halo-forming; and —, non-halo-forming.

solution.

Genetic analysis. Genetic techniques including tetrad analysis were described previously.⁷⁾

RESULTS

Mating-type regulation of the STA genes

We used three independent diagnoses for glucoamylase production, 1) direct measurement of glucoamylase activity secreted in the culture medium, 2) fermentation test for starch, and 3) halo formation for a simple estimation of the enzyme activity on a plate. For the halo formation test, a small amount of

cells were smeared on a YPSB plate and the plate was incubated at 28°C for 4~6 days and at 4°C for 2 days. Glucoamylase-producing cells formed halos around their colonies, while non-producing cells did not. This method has allowed us to determine glucoamylase-producing abilities of the large number of cultures that are necessary in genetic studies. Table II summarizes the glucoamylase production in haploid strains carrying either *MATa* or *MATα* and diploid strains either heterozygous or homozygous for *MAT*. In diploid strains homozygous for *STA1* or *STA3* but heterozygous for *MAT*, the fermentation

TABLE III. MATING-TYPE REGULATION OF THE *FLO8* GENE

Genotype		Strain ^a	Flocculation ^b
<i>FLO8</i>	<i>MATa</i>	YIY256	+++
<i>FLO8</i>	<i>MATα</i>	5106-32D	+++
<i>flo°</i>	<i>MATa</i>	YIY1-6A	—
<i>flo°</i>	<i>MATα</i>	YIY292	—
<i>FLO8/FLO8</i>	<i>MATa/MATα</i>	YIY257 × YIY268	+++
		YIY258 × YIY268	+++
		YIY260 × YIY268	+++
		YIY262 × YIY268	+++
<i>FLO8/FLO8</i>	<i>MATa/MATa</i>	YIY256 × YIY257	+++
		YIY256 × YIY258	+++
		YIY256 × YIY260	+++
		YIY256 × YIY262	+++
<i>FLO8/FLO8</i>	<i>MATα/MATα</i>	YIY268 × YIY271	+++
		YIY268 × YIY277	+++
<i>FLO8/flo°</i>	<i>MATa/MATα</i>	YIY280 × YIY268	—
		YIY281 × YIY268	—
		YIY285 × YIY268	—
<i>FLO8/flo°</i>	<i>MATa/MATa</i>	YIY256 × YIY280	+
		YIY256 × YIY281	+
		YIY256 × YIY284	++
		YIY256 × YIY285	+
<i>FLO8/flo°</i>	<i>MATα/MATα</i>	YIY268 × YIY292	+++
		YIY268 × YIY294	++
		YIY268 × YIY297	+++
		YIY268 × YIY299	++

^a Diploid strains heterozygous for *MAT* and fusant strains homozygous for *MAT* were constructed as described in Table II (footnote a). All strains except 5106-32D and YIY1-6A were derived from the cross 5106-32D × YIY1-6A which showed 2 (flocculating) : 2 (non-flocculating) segregation.

^b + + +, highly flocculent; + +, moderately flocculent; +, weakly flocculent; and —, non-flocculent.

ability and the glucoamylase activity were greatly diminished, and halo formation could not be detected. However, in fusant strains homozygous for both *STA1* and *MAT*, the glucoamylase production recovered to the level of haploid strains. These results indicate that *MATa/MATα* represses glucoamylase production.

Mating-type regulation of the *FLO8* gene

Table III summarizes the flocculating abilities of haploid strains carrying either *MATa* or *MATα* and of diploid or fusant strains either homozygous or heterozygous for *FLO8* and *MAT*. In the background of *FLO8/FLO8*, constitution of the mating type allele did not affect the flocculating ability, however, in the background of *FLO8/flo°*, diploid strains heterozygous for *MAT* were non-flocculent, al-

though fusant strains homozygous for *MAT* were flocculent. These results indicate that *MATa/MATα* represses the expression of the *FLO8* gene in diploid strains heterozygous for *FLO8*.

cdc6 Chromosome loss

To confirm that *MATa/MATα* represses the expression of both *STA1* and *FLO8*, we constructed, on the basis of mitotic chromosome loss caused by the *cdc6* mutation as described by Kawasaki,⁸⁾ a diploid strain YIY237 homozygous for *cdc6* and *STA1* but heterozygous for *MAT* and *FLO8* by sexual mating of haploid strains YIY223 and YIY204. The diploid strain YIY237 shows neither glucoamylase-producing nor flocculating abilities. Cells of the strain YIY237 were cultured in YEPD medium to log phase at 28°C and

TABLE IV. HEAT TREATMENT OF THE DIPLOID STRAIN YIY237 HOMOZYGOUS FOR *cdc6* AND *STA1* BUT HETEROZYGOUS FOR *MAT* AND *FLO8*

Strain	Mating type	<i>cdc6</i> ^a	Auxotrophic markers ^b							Flocculation ^c	Halo ^d	Glucoamylase activity	Genotype of <i>MAT</i>
			<i>ade1</i>	<i>ade2</i>	<i>his2</i>	<i>his4</i>	<i>his7</i>	<i>leu2</i>	<i>met2</i>				
YIY223	<i>a</i>	—	—	—	—	+	—	—	—	—	+	7.0	<i>MATa</i>
YIY204	α	—	+	+	+	—	+	+	+	++	+	4.0	<i>MATα</i>
YIY237	Non-mater	—	+	+	+	+	+	+	+	—	—	0.1	<i>MATa/MATα</i>
YIY250	<i>a</i>	—	+	+	+	+	+	—	+	++	+	7.0	<i>MATa</i>
YIY251	<i>a</i>	—	+	+	+	+	+	—	+	++	+	4.3	<i>MATa</i>
YIY248	α	—	+	+	+	—	+	+	+	++	+	4.0	<i>MATα</i>
YIY249	α	—	+	+	+	—	+	+	+	++	+	4.5	<i>MATα</i>
YIY237-5	α	—	+	+	+	—	+	+	+	++	+	5.0	<i>MATα</i>
YIY237-7	α	—	+	+	+	—	+	+	+	++	+	6.2	<i>MATα</i>

^a (—) indicates temperature-sensitive phenotype.^b (+) indicates prototroph and (—) indicates auxotroph.^c See footnotes in Table III.^d See footnotes in Table II.

TABLE V. ULTRAVIOLET TREATMENT OF THE DIPLOID STRAINS HOMOZYGOUS FOR *STA1* BUT HETEROZYGOUS FOR *MAT*

Strain	Mating type	Auxotrophic markers ^a					Halo ^b	Glucoamylase activity	Spore formation ^c	Genotype of <i>MAT</i>
		<i>his2</i>	<i>his4</i>	<i>leu2</i>	<i>lys2</i>	<i>arg4</i>				
YIY4-3A	α	+	—	+	+	—	+	6.4	—	<i>MAT</i> α
YIY259	<i>a</i>	+	+	—	+	—	+	5.2	—	<i>MAT</i> <i>a</i>
YIY261	<i>a</i>	—	+	+	—	—	+	6.2	—	<i>MAT</i> <i>a</i>
YIY4-3A × YIY259	Non-mater	+	+	+	+	—	—	0.1	+	<i>MAT</i> <i>a</i> / <i>MAT</i> α
YIY4-3A × YIY261	Non-mater	+	+	+	+	—	—	0.2	+	<i>MAT</i> <i>a</i> / <i>MAT</i> α
UV1	<i>a</i>	+	+	+	+	—	+	5.6	—	<i>MAT</i> <i>a</i> / <i>MAT</i> <i>a</i>
UV2	<i>a</i>	+	+	+	+	—	+	5.0	—	<i>MAT</i> <i>a</i> / <i>MAT</i> <i>a</i>
UV7	<i>a</i>	+	+	+	+	—	+	6.0	—	<i>MAT</i> <i>a</i> / <i>MAT</i> <i>a</i>
UV8	<i>a</i>	+	+	+	+	—	+	4.0	—	<i>MAT</i> <i>a</i> / <i>MAT</i> <i>a</i>
UV3	α	+	+	+	+	—	+	4.4	—	<i>MAT</i> α / <i>MAT</i> α
UV4	α	+	+	+	+	—	+	5.6	—	<i>MAT</i> α / <i>MAT</i> α
UV10	α	+	+	+	+	—	+	5.6	—	<i>MAT</i> α / <i>MAT</i> α
UV11	α	+	+	+	+	—	+	5.6	—	<i>MAT</i> α / <i>MAT</i> α
UV13	α	+	+	+	+	—	+	3.5	—	<i>MAT</i> α / <i>MAT</i> α
UV6	α	+	—	+	+	—	+	6.0	—	<i>MAT</i> α
UV9	α	+	—	+	+	—	+	7.4	—	<i>MAT</i> α
UV12	α	+	—	+	+	—	+	5.4	—	<i>MAT</i> α
UV5	Non-mater	+	+	+	+	—	+	6.2	+	<i>MAT</i> <i>a</i> / <i>MAT</i> α

^a See footnotes in Table IV.^b See footnotes in Table II.^c (+) indicates spore-forming phenotype and (—) indicates non-spore-forming phenotype.

plated on the YPSB plates. The plates were incubated as 35°C (a restrictive temperature for the *cde6* mutation) for 6 hr (survival, 10%) and then incubated at 28°C for 6 days and at 4°C for 2 days. Halo-forming colonies were picked up and subcultured to single colonies on the YPSB plate. The clones were examined for mating type, auxotrophic markers, flocculating ability and glucoamylase-producing ability. The results are presented in Table IV. All halo-forming clones produced glucoamylase activity and showed mating ability (either *a* or α) and flocculating ability. The clones showing mating type *a* were *leu2* and the clones showing mating type α were *his4*. These results indicate that one of two chromosomes (III) in the diploid strain YIY237 was lost by the heat treatment (35°C) and the resulting diploids monosomic for chromosome III showed mating type and thus showed glucoamylase-producing and flocculating

abilities.

Treatment with ultra-violet light

Another approach to confirm the mating-type regulation of the *STA1* gene was carried out on the basis of mitotic recombination or gene conversion, chromosome loss, and mutation caused by ultra-violet (UV) light. Diploid strains homozygous for *STA1* but heterozygous for *MAT* were constructed by the crosses YIY4-3A × YIY259 and YIY4-3A × YIY261. The diploid strains showed no glucoamylase-producing ability. The diploid strains were cultured in YEPD medium to log phase, plated on the YPSB plates and treated with UV light for 30 sec (survival, 10%). The plates were incubated at 28°C for 6 days and at 4°C for 2 days. Halo-forming clones were picked up and subcultured to single colonies on the YPSB plate. The clones UV1 to UV8 were obtained from the strain YIY4-3A ×

YIY259, and the clones UV9 to UV13 were obtained from the strain YIY4-3A × YIY261. Mating ability, auxotrophic markers, glucoamylase production, and spore-forming ability were examined with these clones. The results are presented in Table V. All clones except UV5 showed mating ability. The auxotrophic markers of the clones indicate the genotypes for *MAT*; *MATa/MATa* and *MATα/MATα* may be due to mitotic recombination or gene conversion, and *MATα* (monosomic for chromosome III) may be due to chromosome loss. The strain UV5 was a non-mater and showed spore-forming ability, suggesting that the mutation caused by UV light derepressed the *MATa/MATα* repression against *STA1*. The strain UV5-3D (*MATa*) was one of the glucoamylase-producing segregants from the asci prepared from the strain UV5. In the asci prepared from the hybrid constructed by the cross between the strain UV5-3D and a wild-type strain YIY269 (*MATα STA1*), the glucoamylase-producing ability was segregated as 4 (producing):0 (non-producing). Diploid strains heterozygous for *MAT*, constructed by the crosses between the strain UV5-3D and any one of the wild-type strains carrying *STA1*, showed glucoamylase-producing ability, suggesting that a dominant mutation against *MATa/MATα* repression is present in the strain UV5-3D. Further experiments to characterize this mutation are now in progress.

DISCUSSION

Our results show that *MATa/MATα* represses the expression of two of the *STA* genes (*STA1* and *STA3*) and the flocculation gene *FLO8*. It would be interesting to isolate and characterize the DNA sequences of the *STA* and *FLO* genes, since it is speculated that regulatory regions of the genes may contain a common DNA sequence which accepts mating signals.

Regulatory mutations occurring adjacent to structural genes and causing overproduction of associated gene products have been found in

S. cerevisiae. In a class of regulatory mutations at the *CYC7*, *CAR1*, *CAR2*, *DUR1*,²⁹⁾ and *ADR3*¹⁰⁾ loci, the overproduction is greatly diminished in diploids that are heterozygous at *MAT*. Moreover, there are several *his4* mutations which are subject to *MAT* control.¹¹⁾ In every case, the mutations are caused by insertion of Ty (transposable yeast) elements at the 5' non-coding region of the structural genes. It is known that transcription of the Ty elements themselves is repressed in diploid cells heterozygous at *MAT*.¹²⁾ However, it is obscure how Ty elements subject genes to *MAT* control and whether other mechanisms for *MAT* control are present or not.

Recently, we cloned the *STA1* gene (manuscript No. 3-307, accepted by *Agric. Biol. Chem.*) and the study using the cloned gene will provide some information on the *MAT* control, while it is not known what the *MAT* control against *STA* and *FLO* genes means for the growth of yeast cells and in the evolution of *S. diastaticus* and *S. cerevisiae* (manuscript No. 3-333, accepted by *Agric. Biol. Chem.*).

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