TRANSFER OF MITOCHONDRIA OF *HANSENULA WINGEI* INTO PROTOPLASTS OF *SACCHAROMYCES CEREVISIAE* BY MINI-PROTOPLAST FUSION

Kenji YAMASHITA, Hirosuke FUKUDA, Kosaku MURATA and Akira KIMURA
Research Institute for Food Science, Kyoto University, Uji, Kyoto 611, Japan

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1. Introduction

Intraspecific transfer of mitochondria has been reported with isolated mitochondria, although the efficiency of the transformation was very low ($\sim 10^{-8}$) [1]. Using mini-protoplasts containing mitochondria, but not nuclei, intraspecific transfer of mitochondria was also achieved in [2].

This study presents data on the intergeneric transfer of mitochondria from *Hansenula wingei* into protoplasts of *Saccharomyces cerevisiae* using the mini-protoplast method. Mini-protoplasts were prepared by treating log-phase cells of *H. wingei* with a lytic enzyme (zymolyase). In these dividing cells, migration of mitochondria into buds or daughter cells precedes that of the nuclei. After separation, the mini-protoplasts were fused with the normal protoplasts prepared from a respiration-deficient (ρ^0) strain of *S. cerevisiae*. Seven fusants were isolated and two of them were shown to be haploid cells of *S. cerevisiae* to which only mitochondria, but not nuclei had been transferred from *H. wingei*.

2. Materials and methods

2.1. *Strains used*

A cytoplasmic petite mutant BO60AF-1 (*a ade2 arg4 leu2 trp CAP° ERY° OLI° ρ°*) [1] was used as the mitochondrial recipient and a respiration-sufficient haploid strain of *H. wingei* (21 *ade1 his1* ρ^+) was used as mitochondrial donor.

2.2. *Preparation of protoplasts*

Protoplasts were prepared by the method in [2] with a slight modification. Cells of BO60AF-1 were cultured in YPD medium (1.0% yeast extract, 2.0% peptone, 2.0% dextrose) to log phase. The cells were harvested, washed once with distilled water and then suspended in protoplast buffer (pH 7.5). To 10 ml cell suspension (0.1 g wet cells/ml), 2-mercaptoethanol and zymolyase-60 000 were added successively to final concentration of 7.0 μM and 10 μg/ml, respectively. The mixture was incubated with gentle shaking for 30 min at 30°C. Protoplasts were collected by centrifugation at 1000 × *g* for 5 min, washed twice and resuspended in 10 ml protoplast buffer. Protoplasts were prepared by the same procedure from the respiratory-sufficient (ρ^+) haploid of *H. wingei*, aerobically grown in YPG medium (1.0% yeast extract, 2.0% peptone, 3.0% glycerol) to mid-log phase. This procedure gave a mixture of the usual large nucleated protoplasts and small anucleated ones (mini-protoplasts) containing mitochondria but not nuclei. The large protoplasts were removed by centrifugation at 2000 × *g* for 5 min, and the mini-protoplasts were collected by centrifugation at 3500 × *g* for 15 min and washed twice with protoplast buffer.

2.3. *Protoplast fusion*

Mini-protoplasts prepared from 6.9 g wet cells of *H. wingei* (mitochondrial donor) and protoplasts prepared from 1.0 g wet cells of BO60AF-1 (mitochondrial recipient) were mixed and carefully suspended in 5.0 ml 35% (w/v) polyethylene glycol(PEG)-4000 containing 50 mM $CaCl_2$ and incubated for 15 min at 30°C. Following this, the fusion mixture was centrifuged for 15 min at 3500 × *g*, and the precipitate was resuspended in 1.0 ml protoplast buffer. To regenerate the fused protoplasts, 0.1 ml aliquots of the suspension were spread on the selection medium and the plates were incubated at 30°C. The selection

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medium contained 0.67% yeast nitrogen base, 40 μg/ml each of adenine sulfate, arginine, leucine, tryptophan, 3.0% glycerol, 0.1% glucose, 1.0 M sorbitol (as stabilizer of protoplasts) and 2.5% agar.

2.4. *Determination of DNA content*

DNA extraction and determination were carried out by the modified methods in [3,4].

3. Results and discussion

The petite mutant BO60AF-1 could not grow on selective medium, since this strain could not assimilate glycerol for growth. The mitochondrial donor cells of *H. wingei* also could not grow on the selection medium, because this strain requires histidine for growth. When mini-protoplasts from *H. wingei* were fused with protoplasts from BO60AF-1 in the presence of polyethylene glycol and $CaCl_2$, several respiratory-sufficient colonies appeared on the selection medium after incubation for 5–6 days at 30°C. The fusants were divided into two groups as shown in fig.1. The first example (I) represents fusants formed from protoplasts (recipient) and anucleate mini-protoplasts (mitochondrial donor). These fusants requite adenine, arginine, leucine and tryptophan for growth. The second example (II) represents fusants formed from protoplasts of recipient and donor cell contaminating the mini-protoplasts preparation. Two of the fusants isolated (strain 3,4 or F-1,F-2 in table 1) required adenine, arginine, leucine and tryptophan for growth, showing the same nutritional requirements as the recipient strain BO60AF-1. These two strains were, therefore, concluded to be transformants made by the transfer of mitochondria in the mini-protoplasts. The efficiency of transformation was 10^{-6}. Two other fusants (strain 5,6 or F-3,F-4 in table 1) required none of the above-mentioned additives for growth. Their DNA content was twice that of F-1 and F-2, indicating that they were the fused products of the two strains used. Strains 7 or F-5 in table 1 exhibited

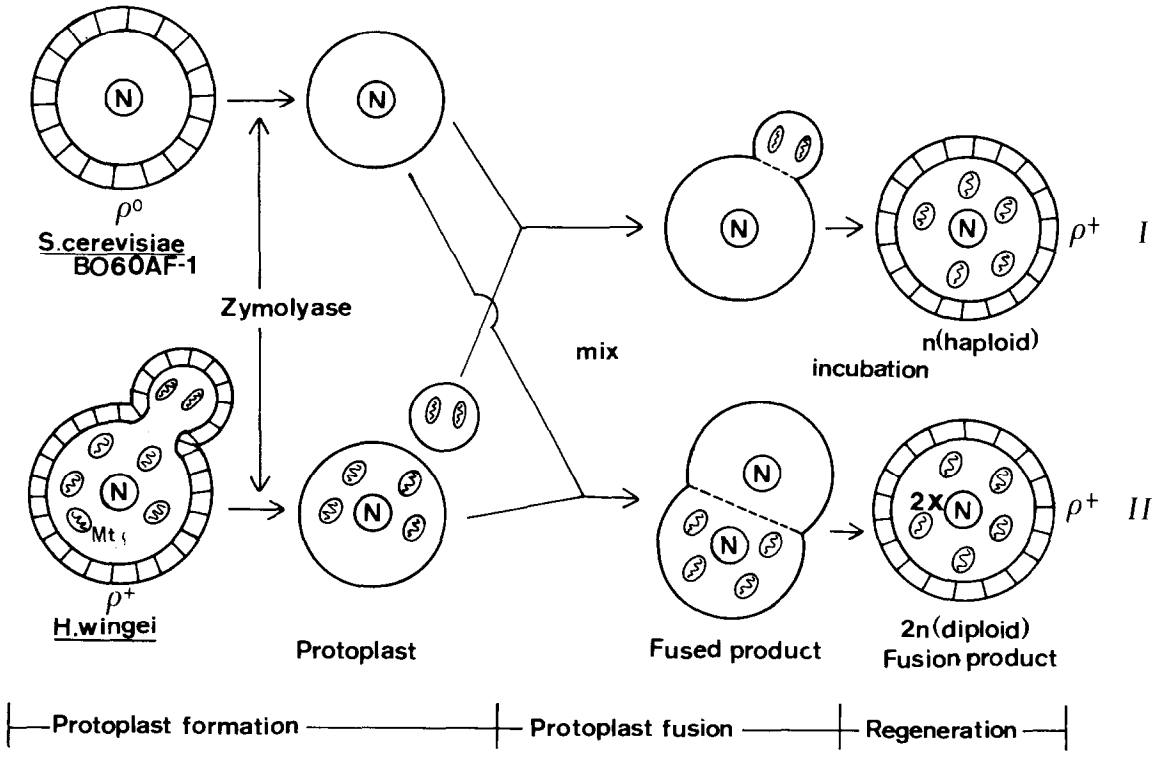

Fig.1. Design of protoplast fusion between *H. wingei* and *S. cerevisiae* BO60AF-1. This process consists of three steps of protoplast formation, fusion and regeneration. *Abbreviations:* N, nuclei; Mt, mitochondria.

Table 1

Strains	ρ	Auxotrophic markers						DNA content ($\mu g/10^8$ cells)	Ploidy
		ade2	*arg4*	*leu2*	*trp*	*ade1*	*his1*		
(1) *H. wingei*	+	+	+	+	+	–	–	1.65	*n*
(2) *S. cerevisiae* BO60AF-1	°	–	–	–	–	+	+	1.55	*n*
(3) F-1	+	–	–	–	–	+	+	1.62	*n*
(4) F-2	+	–	–	–	–	+	+	1.61	*n*
(5) F-3	+	+	+	+	+	+	+	3.17	2*n*
(6) F-4	+	+	+	+	+	+	+	3.22	2*n*
(7) F-5	+	+	+	+	+	+	+	2.18	aneuploid
(8) F-6	+	–	–	–	–	– (+)[a]	– (+)[a]	1.67	*n*

[a] Glycerol was used as a carbon source; other auxotrophic requirements were determined by using glucose as a carbon source

no auxotrophic markers, but the DNA content was only 2.18 $\mu g/10^8$ cells; therefore, they may be considered to be aneuploid. We isolated another interesting fusant (F-6), which showed auxotrophic requirements for adenine, arginine, leucine, tryptophan and histidine only when glucose was used as the carbon source. Further study of this strain is under way.

The mini-protoplasts method was shown to be feasible for the intergeneric transfer of mitochondria. By using this method, we are planning to transfer the mitochondria of *Hansenula jadinii* into protoplasts of *Saccharomyces cerevisiae* as the next step in analyzing the fermentative mechanism of CDP-choline production [5–7].

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