FEMS Yeast Research, 17, 2017, fox045

doi: 10.1093/femsyr/fox045
Advance Access Publication Date: 24 June 2017
Research Article

RESEARCH ARTICLE

Mating response and construction of heterothallic strains of the fission yeast *Schizosaccharomyces octosporus*

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One sentence summary: We have characterized the mating response of *Schizosaccharomyces octosporus*, and successfully constructed heterothallic strains by genetic manipulation.

Editor: Hyun Kang

ABSTRACT

The fission yeast *Schizosaccharomyces octosporus* is one of four species in the genus *Schizosaccharomyces*. Recently released genome sequence data provide useful information for comparative studies. However, *Sz. octosporus* has not yet been genetically characterized because there have been no heterothallic strains of this species. Here we report the construction of stable heterothallic strains of *Sz. octosporus* for genetic crosses. First, we continuously observed the mating process of a homothallic strain, yFS286, and determined the mating frequency of *Sz. octosporus* on various sporulation media. It showed, on average, 30% zygote formation on mating, and a higher frequency of zygotes (43.8 ± 4.7%) on PMG medium. Regardless of sporulation, the number of spores within an ascus was variable. *Schizosaccharomyces octosporus* forms eight-spored asci, but preferentially produced four-spored asci on MEA or YMoA medium. To obtain heterothallic strains essential for genetic analyses, we isolated spontaneous mutants showing heterothallic-like phenotypes. We also constructed stable heterothallic strains by deleting the silent *mat* region. As a result, we established the following heterothallic strains, TS162 as h^+ and TS150/TS161 as h^-, which successfully mated with each other. These genetic tools will be useful for yeast genetics such as molecular cloning, gene complementation tests and tetrad (octad) analysis.

Keywords: fission yeast; gene manipulation; heterothallic strain; mating; mating type; *Schizosaccharomyces octosporus*

INTRODUCTION

Schizosaccharomyces is a genus of fission yeasts, which currently comprises four species: *Sz. pombe* (Leupold 1950), *Sz. octosporus* (Beijerinck 1894), *Sz. cryophilus* (Helston *et al.* 2010) and *Sz. japonicus* (Yukawa and Maki 1931). Recently, whole-genome sequences of these four species have been released by the Broad Institute (Rhind *et al.* 2011). These sequence data are expected to provide new insights into evolution of the genus *Schizosaccharomyces*. In fact, comparative genomic analyses have revealed that ~4000 genes are shared among these four species, although they also have hundreds of unique genes (Rhind *et al.* 2011). Such differences in gene content might confer potentially distinct cellular properties on the four yeast species.

On the one hand, *Sz. pombe* is a well-studied model organism. Over the past 70 years, genetic studies based on *Sz. pombe* have contributed to basic biology in many fields, especially the cell cycle (Beach, Durkacz and Nurse 1982; Lee and Nurse 1987), chromosome dynamics (Hirano *et al.* 1986; Samejima *et al.* 1993)

Received: 13 February 2017; **Accepted:** 20 June 2017

Table 1. Strains used in this study.

Strains	NIG ID	Species	Genotype	Source
L968		*Sz. pombe*	h^{90}	Our stock
yFS286		*Sz. octosporus*	h^{90}	NBRP yeast[a]
OY14		*Sz. cryophilus*	h^{90}	NBRP yeast[a]
NIG2008	NIG2008	*Sz. japonicus*	h^{90}	Our stock
TS147	NIG10001	*Sz. octosporus*	h^{+}-like	This study
TS148	NIG10002	*Sz. octosporus*	h^{+}-like	This study
TS149	NIG10003	*Sz. octosporus*	h^{90} sterile mutants	This study
TS150	NIG10004	*Sz. octosporus*	h^{-}	This study
TS161	NIG10005	*Sz. octosporus*	h^{-S} mat2-P/mat3-M::bleMX6	This study
TS162	NIG10006	*Sz. octosporus*	h^{+S} mat2-P/mat3-M::hphMX6	This study

[a] http://yeast.lab.nig.ac.jp/yeast/

and gene transcription (Jenuwein and Allis 2001; Nakayama *et al.* 2001; Volpe *et al.* 2002). *Schizosaccharomyces pombe* genes have prospective homologs in higher organisms, including human; thus, understanding the biological functions of *Sz. pombe* is a matter of great importance (Wood *et al.* 2002). On the other hand, *Sz. japonicus* shows unique physiological features that are not seen in other fission yeasts, including semi-open mitosis (Aoki *et al.* 2011), hyphal growth (WICKERHAM and DUPRAT 1945; Niki 2014) and light response (Okamoto *et al.* 2013). In addition, the larger cell size of *Sz. japonicus* has enabled us to observe its chromosome behavior more easily (Aoki *et al.* 2011). In contrast, *Sz. octosporus* and *Sz. cryophilus* have been scarcely used in research so far.

Beijerinck first described the species of *Sz. octosporus*, which he isolated from dried currants and figs (Beijerinck 1894). Although *Sz. octosporus* and *Sz. japonicus* produce an ascus containing eight spores, *Sz. octosporus* is more closely related to *Sz. pombe* than to *Sz. japonicus*. Indeed, *Sz. octosporus* has a very similar life cycle to *Sz. pombe* except for the number of spores within its asci. Previously, genetic hybridization between *Sz. octosporus* and *Sz. pombe* was achieved by using protoplast fusion (Sipiczki 1979; Sipiczki *et al.* 1982), but usually these species do not interbreed, probably because the primary structures of their mating pheromones are clearly different (Seike *et al.* 2012). Recently, the very closely related species *Sz. cryophilus* was found in *Sz. octosporus* culture (Helston *et al.* 2010). Cells of *Sz. cryophilus* are visually indistinguishable from those of *Sz. octosporus*. The principal difference between the species is optimal temperature for cell growth: 25°C leads to optimal growth of *Sz. cryophilus* but 30°C is lethal, whereas *Sz. octosporus* can grow at 30°C (Helston *et al.* 2010). *Schizosaccharomyces cryophilus* also produces an eight-spored ascus, but its ability to sporulate is extremely low (Helston *et al.* 2010). In contrast, these two species show strong non-sexual flocculation, probably enabling them to prevent various stresses.

Usually, fission yeasts proliferate as haploid cells under vegetative growth. In *Sz. pombe*, on starvation of nitrogen, two haploid cells of opposite mating types, termed h^{+} (P) and h^{-} (M), mate and form a diploid zygote (Egel and Egel-Mitani 1974). The diploid zygote immediately undergoes meiosis and forms four haploid spores within an ascus (Bresch, Müller and Egel 1968; Gutz *et al.* 1974; Nielsen 2004). *Schizosaccharomyces pombe* L968 is a homothallic (namely h^{90}) strain. It possesses one expression locus (*mat1*) and two silent loci (*mat2* and *mat3*) at the *mat* region (over 15 kb) on chromosome II, and transfers genetic information from each of silent loci to the expression locus, resulting in mating-type switching. h^{+} cells express two *mat-P* genes (*mat-Pc* and *mat-Pi*), whereas h^{-} cells express two *mat-M* genes (*mat-Mc* and *mat-Mi*) (Kelly *et al.* 1988). The *mat-Pc* and *mat-Mc* genes initially regulate the expression of genes involved in mating such as mating pheromones, whereas the *mat-Pi* and *mat-Mi* genes are required to initiate meiosis after mating (Willer *et al.* 1995). Our previous study revealed that *Sz. japonicus* also possesses the *mat* locus (Furuya and Niki 2009); however, only the *mat-Pc* and *mat-Mc* genes exist at the *mat* locus of *Sz. japonicus*, and no genes equivalent to *mat-Pi* and *mat-Mi* have been found. Therefore, each gene is likely to regulate a series of other genes during the mating process.

Heterothallic haploid strains are useful for selecting desired phenotypes by tetrad (octad) dissection, for investigating the function of mating-type-specific genes, and for mating tests of reproductive isolation between closely related species. Strains L975 (h^{+}) and L972 (h^{-}) are available in *Sz. pombe* (Leupold 1958) and NIG2017/NIG2025 (h^{+}) and NIG2028 (h^{-}) are available in *Sz. japonicus* (Furuya and Niki 2009), but heterothallic strains of the other two species have not been reported. Despite their potential as novel model organisms, *Sz. octosporus* and *Sz. cryophilus* are limited by a lack of genetic tools. Here we report the successful construction of stable heterothallic strains of *Sz. octosporus* that possess sporulating capability. By using a modified method of DNA transformation, we deleted the *mat-2P/mat3-M* silent region in order to prevent mating-type switching. The resulting strains could not self-mate because their homothallism was completely loss. Thus, we established TS162 (h^{+}) and TS161 (h^{-}) as stable heterothallic strains. In addition, we characterized the mating response of *Sz. octosporus*. Remarkably, *Sz. octosporus* produced a variable number of spores within an ascus depending on nutrient conditions. Such a feature has not been seen in the other three fission yeast species. Flowering studies with *Sz. octosporus* might shed light on evolution of the mechanism that regulates spore production in ascomycetes.

MATERIALS AND METHODS

Strains, media and culture conditions

The strains used in this study are listed in Table 1. All constructed strains were derived from yFS286, which is the 'type' (wild-type) strain of the *Schizosaccharomyces octosporus* species. The draft genome of this strain has been published by the Broad Institute (Rhind *et al.* 2011).

The media used in this study were made mainly in accordance with the recipe for *Sz. pombe* (Moreno, Klar and Nurse 1991). Yeast cells were grown in yeast extract (YE) medium (5 g/l of Bacto yeast extract [BD Bioscience, Sparks, MD, USA] and 30 g/l

Table 2. List of media used in this study.

Medium	State	Agar (g/l)
Complete medium[a]		
YE	Liquid	–
YEA	Solid	15.0
Mating and sporulation media[b]		
ME	Liquid	–
MEA	Solid	15.0
EMM2A	Solid	20.0
EMM2−N	Liquid	–
YMoA	Solid	18.0
PMG	Solid	20.0
SPA	Solid	30.0
MYA (ME+YMoA)[c]	Solid	18.0

[a]Yeast extract-based rich medium.
[b]Nitrogen-limited medium.
[c]Blend medium: YMoA mixed with ME (defined in this study).

of D-glucose [Sigma-Aldrich, St. Louis, MO, USA]), supplemented with adenine (75 mg/l), uracil (50 mg/l) and leucine (50 mg/l). For solid medium, 15 g/l of Bacto agar [BD Bioscience, Sparks, MD, USA] was added to YE medium to form yeast extract agar (YEA). Antibiotics (hygromycin B [Wako Pure Chemical Industries, Ltd., Osaka, Japan] and zeocin [Invivogen, San Diego, CA, USA]) were added to YEA medium at a final concentration of 100 μg/ml.

The following media were used for mating and sporulation (Table 2): malt extract (ME) (30 g/l of Bacto Malt Extract [BD Bioscience, Sparks, MD, USA]), malt extract agar (MEA) (addition of 15 g/l of Bacto agar to ME, pH 5.5), Edinburgh minimal medium 2 (EMM2) (2.2 g/l of Na_2HPO_4, 3 g/l of potassium hydrogen phthalate, 5 g/l of NH_4Cl, 20 g/l of D-glucose, 1 × salts [50 × salts: 53.5 g of $MgCl_2{\cdot}6H_20$, 0.74 g of $CaCl_2{\cdot}2H_2O$, 50 g of KCl and 2 g of Na_2SO_4], 1 × vitamins [1000 × vitamins: 1 g of sodium pantothenate, 10 g of nicotinic acid, 1 g of inositol and 10 mg of biotin] and 1 × minerals [10 000 × minerals: 5 g of H_3BO_3, 4 g of $MnSO_4$, 4 g of $ZnSO_4{\cdot}7H_2O$, 2 g of $FeCl_3{\cdot}6H_2O$, 0.4 g of $H_2MoO_4{\cdot}H_2O$, 1.0 g of KI, 0.4 g of $CuSO_4{\cdot}5H_2O$ and 10 g of citric acid]), EMM2A (addition of 20 g/l of Bacto agar to EMM2), EMM2−N (EMM2 medium lacking NH_4Cl), yeast morphology agar (YMoA) (35 g/l of Difco yeast morphology agar [BD Bioscience, Sparks, MD, USA]), pombe minimal glutamate (PMG) (NH_4Cl in EMM2A is replaced with 3.75 g/l of L-glutamic acid) and sporulation agar (SPA) (10 g/l of D-glucose, 1 g/l of KH_2PO_4, 1 × vitamins and 30 g/l of Bacto agar). Screening for heterothallic strains was performed on ME+YMoA blend medium (named 'MYA' medium: 30 g/l of Bacto malt extract and 35 g/l of Difco yeast morphology agar), on which yFS286 sporulated after forming single colonies. Cells were incubated at 30°C for growth and mating, unless stated otherwise.

Time-lapse imaging

To observe the mating process of *Sz. octosporus*, living cells (yFS286) scraped from a YEA plate were mounted onto a glass-bottom Petri Dish (Matsunami Glass Industries, Ltd., Osaka, Japan), and a thin film of PMG medium (~120 μl) was laid over the cells. Time-lapse images were taken by a disk scanning confocal system (Cell Voyager CV1000; Yokogawa, Tokyo, Japan) using a microscope equipped with an oil immersion objective lens (×60) and a back-illuminated EMCCD camera in a stage incubator maintained at 30°C by a temperature controller. Frames were taken every 10 min (those taken every 20 min are shown in Fig. 2A and C) overnight. Digital images were analyzed with Image-J software (http://rsbweb.nih.gov/ij/features.html).

NeurotransRed C2 staining

Cells grown on YEA medium were transferred to PMG medium for 12 h, and then a few cells scraped from the PMG plate were washed once with sterilized water. They were stained for 30 min at room temperature with 10 μg/ml of NeurotransRed C2 (also known as FM4–64, from Setareh Biotech, Eugene, OR, USA) in water, and the vacuolar membrane was observed by microscopy.

Quantitation of mating frequency and number of spores

Cells grown on YEA medium were resuspended in sterilized water to a density of 1×10^8 cells/ml. A 30-μl aliquot of the suspension was spotted onto various solid media for mating and sporulation, and the inoculated petri dish was incubated for an appropriate period to induce mating (2 days for *Sz. pombe*, and 3 days for *Sz. octosporus*) at 30°C. The number of cells was counted under a differential interference contrast (DIC) microscope. Cell types were classified into four groups: vegetative cells (V), zygotes (Z), asci (A) and free spores (S). Mating frequency was calculated by the following equation:

$$\text{Mating}\,(\%) = 100 \times (2Z + 2A + S/2)/(V + 2Z + 2A + S/2) \quad \text{[for } \textit{Sz.pombe}\text{]}$$

$$\text{Mating}\,(\%) = 100 \times (2Z + 2A + S/4)/(V + 2Z + 2A + S/4) \quad \text{[for } \textit{Sz.octosporus}\text{]}$$

In all cases, triplicate samples (with at least 300 cells each) were counted, and the mean ± standard deviation (SD) was calculated. At the same time, the number of spores within asci (>100 asci) was counted as tetrads, hexads and octads.

Cell proliferation assay under various culture conditions

Cells were pre-cultured in YE medium overnight. The pre-cultured cells were harvested, and washed with sterilized water once prior to inoculation into the main culture. The cell proliferation assay used ME liquid medium supplemented with nutrient mixtures as follows: ammonium sulfate (3.5 g/l), L-asparagine (1.5 g/l), D-glucose (10 g/l), L-histidine (10 mg/l), L-methionine (20 mg/l), L-tryptophan (20 mg/l), biotin (2 μg/l), calcium pantothenate (400 μg/l), folic acid (2 μg/l), inositol (2 mg/l), nicotinic acid (400 μg/l), aminobenzoic acid (200 μg/l), pyridoxine hydrochloride (400 μg/l), riboflavin (200 μg/l), thiamine hydrochloride (400 μg/l), boric acid (500 μg/l), copper sulfate (40 μg/l), potassium iodide (100 μg/l), ferric chloride (200 μg/l), manganese sulfate (400 μg/l), molybdic acid (200 μ/l), zinc sulfate (400 μg/l), potassium phosphate monobasic (1 g/l), magnesium sulfate (500 mg/l), sodium chloride (100 mg/l) and calcium chloride (100 mg/l). The added nutrient mixtures were made in accordance with the Difco manual.

Cells were inoculated at a density of 1×10^6 cells/ml into 5 ml of medium in an L-shaped test tube. The optical density (OD) of culture tubes was automatically recorded by using a biophotorecorder (TVS062CA; Advantec Toyo Co. Ltd., Tokyo, Japan). During 2 days of cultivation with continuous shaking at 30°C, the

OD_{600} of each tube containing *Sz. octosporus* cultures was measured every 10 min. The cell proliferation assay was performed in triplicate per samples. The mean ± SD growth rate μ (h^{-1}) was calculated as the slope of a line fitted to the exponential growth curve (Table 5).

Determination of mating type by PCR

PCR with primers oTS103 (*mat-Pi* specific primer: 5′-CTTGACACACTCTGCACAGGTATCT-3′) and oTS110 (*mat-Mc* specific primer: 5′-TCCTCTTTCCAAAGTTGACCAACCAG-3′) was used to determine the mating type of *Sz. octosporus* strains (e.g. Fig. 6A). Primer oTS103 with oTS13 (the common primer: 5′-TTTGGAAATGTTTAAGTGGGATTTGATTCTGTCA-3′) produces a PCR product of 1.8 kb, whereas primer oTS110 with oTS13 produces a PCR product of 1.7 kb. In the case of a homothallic strain (h^{90}), both bands were obtained (Fig. 7E). The PCR was performed by using KOD FX Neo DNA polymerase (TOYOBO, Osaka, Japan) with the following heating program: 94°C for 2 min, and 35 cycles of 98°C for 10 s, 58°C for 30 s and 68°C for 1 min.

RNA extraction followed by real-time PCR

Schizosaccharomyces octosporus cells were pre-cultured in YE medium overnight, and used at a density of 4×10^7 cells/ml to inoculate a 5-ml batch of EMM2−N liquid medium, which was continuously shaken at 30°C for 12 h. Next, 1 ml of this culture was harvested for RNA extraction by using an RNeasy® Mini Kit (Qiagen, Hilden, Germany). Complete digestion of DNA was carried out to remove any contaminating DNA in the RNA solution before clean-up for isolation of RNA. For real-time PCR (RT-PCR), cDNA was synthesized from 500 ng of RNA by using a SuperScript® VILO cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The program for RT-PCR was as follows: 94°C for 2 min, and 30 cycles of 98°C for 10 s, 58°C for 30 s and 68°C for 30 s. The primers used to amplify the DNA segment containing *mat-Pc*, *mat-Mc* and *ura4* (control) were oTS359 (5′-ATCCAGTGTTCTTACCGGTTCTAAC-3′) and oTS360 (5′-GTGTTCTTTATGCCAGAGGGTAAGG-3′), oTS363 (5′-GCTGCATATTTTGTTCCAGAAGGC-3′) and oTS110, and oTS364 (5′-TGTCATTAAGACGCATGTCGATGTC-3′) and oTS365 (5′-AGTGGCCAGACTTCCTTTAGAAGAC-3′), respectively.

Plasmid construction for targeted gene deletion in *Schizosaccharomyces octosporus*

To delete the *mat2-P/mat3-M* region (5.9 kb containing the silent *mat2-P* and *mat3-M* cassettes) in *Sz. octosporus* cells, first, the 5′-upstream sequence (~1 kb) was amplified from yFS286 genomic DNA by using the primers oTS153 (5′-TTCGTAC GCTGCAGGATTCCGCTTAATTTTTGATGGATCTTCATGACA-3′) and oTS154 (5′-GCTAAACAGATCTGGCTCATCGATGTTCTTTAGA AATAAGTAAACCCGT-3′). The DNA fragment was fused to a linearized vector derived from pFA6a-bleMX6 or pFA6a-hphMX6, prepared by inverse PCR using KOD FX Neo DNA polymerase with the primers oTS35 (5′-CCAGATCTGTTTAGCTTGCCTCGTC-3′) and oTS36 (5′-CCTGCAGCGTACGAAGCTTCA-3′) by an In-Fusion® HD Cloning Kit (Clontech, Mountain View, CA, USA). The resultant plasmids were pTS38 [pFA6a-bleMX6(SomatUP)] and pTS39 [pFA6a-hphMX6(SomatUP)]. Next, the 3′-downstream sequence (~1 kb) of the *mat2-P/mat3-M* region was amplified from yFS286 genomic DNA by using the primers oTS155 (5′-GCTGTCGATTCGATAGACGCGCTAAAGCTTTGATACAAAAGT-3′) and oTS156 (5′-GAGACCGGCAGATCCCGACCATATGTTTAAGTCG TTTATTTAGGTTTGC-3′). The DNA fragment was fused to a linearized vector derived from pTS38 or pTS39, prepared by inverse PCR with the primers oTS37 (5′-GGATCTGCCGGTCTCCCTATAGT-3′) and oTS38 (5′-TATCGAATCGACAGCAGTATAGCGACC-3′) as mentioned above. Lastly, pTS40 [pFA6a-bleMX6(SomatUPDOWN)] and pTS41 [pFA6a-hphMX6(SomatUPDOWN)] (Fig. 7A) were constructed. DNA fragments from each plasmid, namely, a 3.1-kb fragment amplified from pTS40, and a 3.8-kb fragment amplified from pTS41 using oTS153 and oTS156 (Fig. 7A), were purified through a Wizard® PCR cleaning system (Promega, Madison, WI, USA), and 1 μg of the DNA was used for transformation.

Transformation of *Schizosaccharomyces octosporus* cells

Schizosaccharomyces octosporus cells were grown in YE medium supplemented with adenine, uracil and leucine. Cultures in log phase (~2×10^7 cells/ml) were harvested by spinning 50 ml of culture at 10 000 rpm for 3 min at 4°C. Cell pellets were washed with 20 ml of ice-cold sterilized water, followed by 10 ml of ice-cold sterilized water twice. Cells were suspended in 5 ml of 1 M sorbitol and 125 μl of 1 M dithiothreitol (DTT). They were incubated for 15 min at 30°C and then spun down at 10 000 rpm for 3 min at 4°C. The pellet was suspended in 1 ml of 1 M sorbitol, transferred to a 1.5-ml tube and spun at 12 000 rpm for 3 min at 4°C. Finally, cells were suspended in 100 μl of 1 M sorbitol, to which transforming DNA (1 μg of a linearized DNA fragment for transformation) was added. The mixture was mixed gently by pipetting. After incubation on ice for 5 min, the cells were electroporated by a Bio-Rad Gene Pulser Xcell electroporator (cuvette, 2 mm; settings, 3.0 kV, 25 μF, 200 ohms). Immediately after electroporation, 1 ml of 1 M sorbitol was transferred to a test tube containing 5 ml of YE medium, and then incubated for at least 16 h at 30°C with shaking. Lastly, cells from the diluted recovery medium were plated onto selective agar medium. Transformants appeared in 5–6 days at 30°C.

Assay for sexual agglutination

To induce sexual agglutination, cells were transferred from YE medium to EMM2−N medium after washing three times. Cells in 2-ml batches of EMM2−N medium were continuously shaken at 30°C for 12 h at a density of 4×10^7 cells/ml. After incubation, the plates were photographed.

Octad analysis of heterothallic strains

A mixture of heterothallic strains at a 1:1 ratio was spotted on PMG medium and incubated at 30°C for 3 days. Growing cells on the PMG medium were suspended in sterilized water containing 1 mg/ml of Zymolyase®-20T (Nacalai Tesque Inc., Kyoto, Japan), and then incubated at 37°C for 5 min. They were then transferred onto YEA medium for spore dissection. After 1 h, spore dissection was performed by using a yeast dissection microscope (MSM300; Singer Instruments, Somerset, UK). Dissected spores were incubated for 3–4 days at 30°C to obtain progenies. The mating types of the progenies were determined by using PCR.

Flow cytometry

Cells were prepared from YE overnight culture and washed once with sterilized water before 70% EtOH was added at 4°C. Aliquots of fixed cells in 50 mM sodium citrate buffer were incubated for 2 h at 37°C with 1 mg/ml of RNase A (Wako, Tokyo,

Table 3. Doubling time of four fission yeasts in YE medium.

Temp, °C	*Sz. pombe*	*Sz. octosporus*	*Sz. cryophilus*	*Sz. japonicus*
30	143.05 ± 8.37	200.69 ± 7.01	n.d.	115.73 ± 6.66
25	193.54 ± 12.09	240.06 ± 4.61	400.07 ± 6.40	171.49 ± 10.86

Doubling time (min) was calculated as the mean ± standard deviation of biological triplicates.
n.d.: not detected

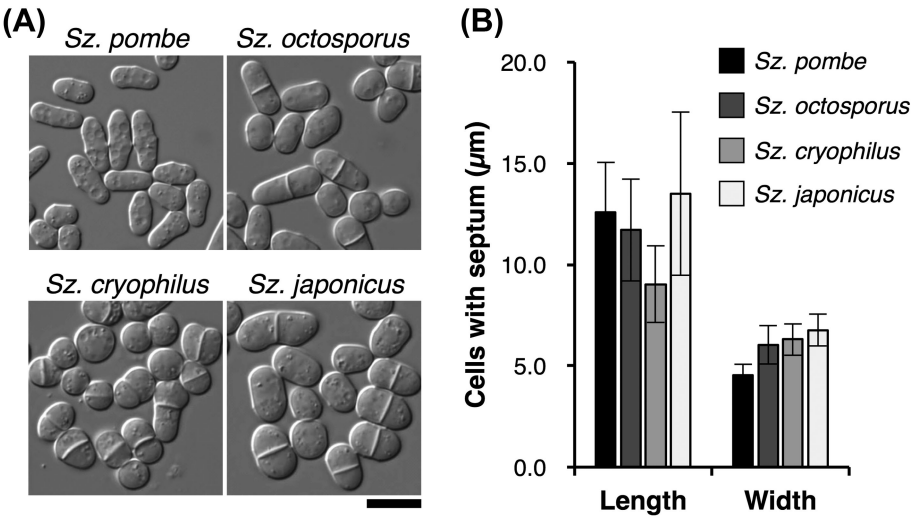

Figure 1. Vegetative growth of four fission yeast species: (**A**) Morphology of vegetative cells of *Sz. pombe* (L968), *Sz. octosporus* (yFS286), *Sz. cryophilus* (OY14) and *Sz. japonicus* (NIG2008) in YE medium. Scale bar, 10 μm. (**B**) The length and width of cells with septa were measured to compare cell size among the four species (100 cells each).

Japan), and then treated with 10 μg/ml of propidium iodide (PI; Thermo Fisher Scientific, Waltham, MA, USA) to determine the quantity of DNA in cells. Uptake of PI was measured using a FACS Calibur instrument (Becton Dickinson Biosciences, Mississauga, Canada). Samples containing 20 000 cells were analyzed using FL2-A detection line (filter FL2; emission, 560–600 nm), as shown in Fig. S2 (Supporting Information).

RESULTS

Comparison of four fission yeast species under vegetative growth

Similar to other fission yeasts, *Schizosaccharomyces octosporus* cells show vegetative growth under nitrogen-rich conditions such as YE medium. We determined the doubling time of the four fission yeast species in YE medium (Table 3). At 30°C, the doubling time of *Sz. octosporus* was 200.69 ± 7.01 min (vs143.05 ± 8.37 min for *Sz. pombe*; both n = 3), while at 25°C, the doubling time of *Sz. octosporus* was 240.06 ± 4.61 min (vs 193.54 ± 12.09 min for *Sz. pombe*) (Table 3). As previously reported (Helston *et al.* 2010; Niki 2014), *Sz. japonicus* grew the fastest (115.73 ± 6.66 min) and *Sz. cryophilus* did not grow at 30°C (Table 3).

We also compared cell size among the four species. Each h^{90} wild-type strain was grown in YE medium to the log phase, and images were obtained by a DIC microscope (Fig. 1A). To make direct comparisons, we measured both the length and the width of cells with septa. *Schizosaccharomyces octosporus* cells were a little shorter than *Sz. pombe* cells (11.71 ± 2.19 vs 12.60 ± 2.13 μm), but were much wider (6.04 ± 0.82 vs 4.55 ± 0.46 μm) (Fig. 1B); as a result, *Sz. octosporus* cells look a little bigger than *Sz. pombe* cells. In addition, *Sz. japonicus* cells were the longest (13.51 ± 3.51 μm) and widest (6.77 ± 0.68 μm) among the four species, and *Sz. cryophilus* were the shortest (9.04 ± 1.65 μm) in YE medium (Fig. 1B).

Mating process of *Schizosaccharomyces octosporus*

Next, we observed the mating process of *Sz. octosporus*. Time-lapse images of mating cells of the h^{90} wild-type strain were continuously taken. When plated on PMG medium, haploid cells of opposite mating type began to fuse within 6–8 h to form diploid zygotes, which had spherical structures that could be visually observed (e.g. Fig. 2A; arrowheads). To examine whether these structures were vacuoles, we stained the zygotes with NeurotransRed C2 (FM4–64; a selective stain for yeast vacuolar membranes). As shown in Fig. 2B, the periphery of the spherical structures was clearly stained by NeurotransRed C2, indicating that these structures were vacuoles. The *Sz. octosporus* zygotes were very different from the *Sz. pombe* ones. Each diploid zygote sporulated after a few hours, and eventually formed eight mature spores within an ascus in 5–6 h (Fig. 2C).

Induction of mating of *Schizosaccharomyces octosporus* on various media

The mating response of fission yeast depends on the composition of the medium. We examined the mating efficiency of *Sz. octosporus* (yFS286) on various solid media (MEA, EMM2A, YMoA, PMG and SPA). For comparison, the mating response of a homothallic *Sz. pombe* strain (L968) was also examined. To observe the mating response, we spotted cells of homothallic *Sz. octosporus* (yFS286) onto MEA plates. After 3 days of incubation at 30°C, *Sz. octosporus* mated and formed asci. The diploid formed

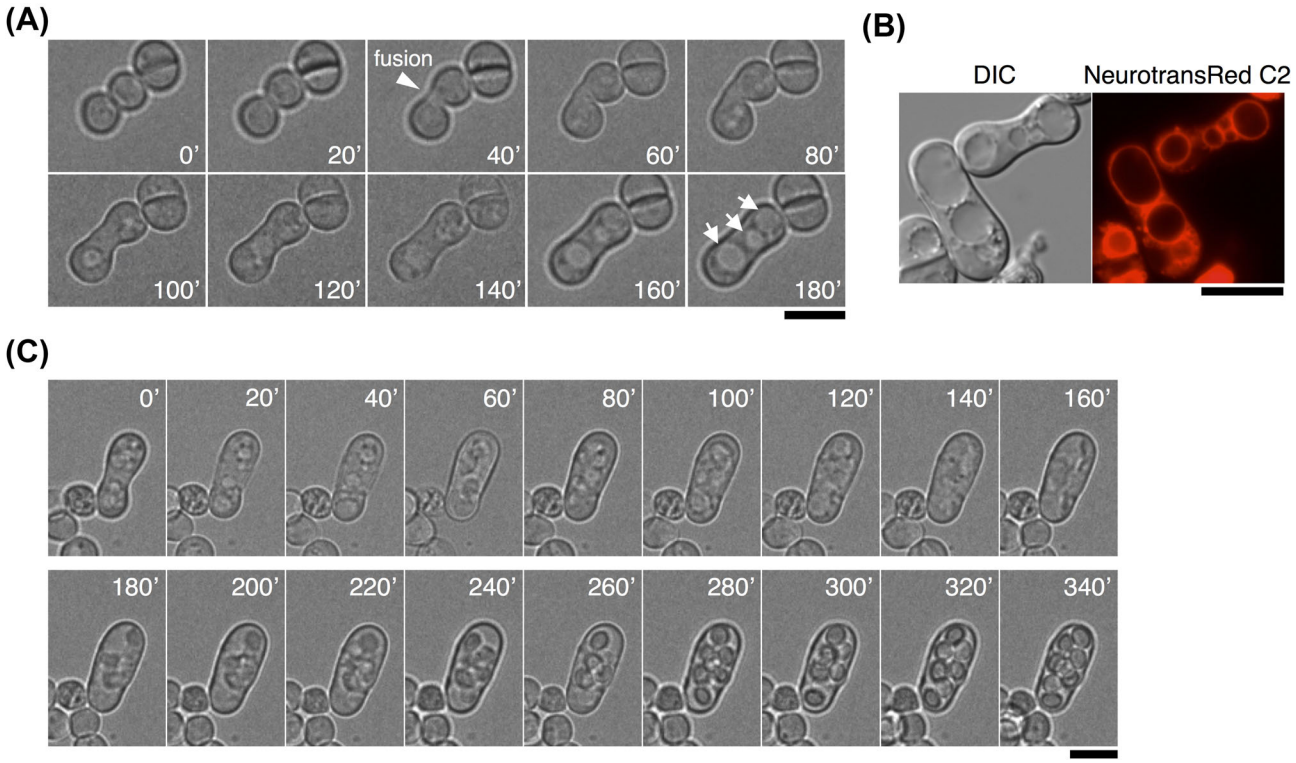

Figure 2. Continuous observation of *Sz. octosporus* mating cells. (**A**) Time-lapse images of *Sz. octosporus* (yFS286) mating cells on PMG medium. Two haploid cells fused and became a diploid zygote, which had spherical structures (arrowheads). Frames were taken every 20 min. (**B**) NeurotransRed C2 fluorescence of representative zygotes. Most *Sz. octosporus* zygotes had two or more vacuole structures. (**C**) Time-lapse images of *Sz. octosporus* (yFS286) sporulating cells on PMG medium. A diploid zygote formed eight haploid spores within an ascus. Frames were taken every 20 min. Scale bar, 10 μm.

asci with a maximum of eight spores. In addition, asci containing tetrads and hexads were formed, as seen in Fig. 3A. As shown in Fig. 3B, the frequency of zygotes of *Sz. octosporus* was lower than that of *Sz. pombe*. Mating of *Sz. pombe* occurred efficiently with an average of more than 50% on every medium, whereas *Sz. octosporus* showed a relatively lower mating efficiency (30% on average); however, *Sz. octosporus* showed the highest mating frequency on PMG medium (43.8 ± 4.7%). Unexpectedly, the percentage of tetrads, hexads and octads asci depended on the type of medium (Fig. 3C). In fact, the diploid zygote formed a majority of four-spored asci (tetrads) on MEA or YMoA medium, with a few eight-sored asci (octads) (Fig. 3C). In contrast, more than half of the asci contained eight haploid spores on EMM2A or PMG medium. Thus, the number of spores of *Sz. octosporus* varied greatly according to the medium. These results clearly indicate that, in *Sz. octosporus*, meiosis followed by sporulation is likely to be affected by certain external factors (e.g. nutrients).

Essential nutrients for sufficient growth

Schizosaccharomyces octosporus cells (yFS286) grew poorly on MEA, whereas *Sz. pombe* cells (L968) and *Sz. japonicus* (NIG2008; homothallic strain) grew well on this medium (Fig. 4A). This result suggests that the nutrient composition of ME alone is insufficient for growth of *Sz. octosporus*. We therefore investigated the effect of various solid media selected for mating and sporulation (EMM2A, YMoA, PMG and SPA) on cell growth. *Schizosaccharomyces octosporus* modestly proliferated on PMG medium, and formed single colonies with sufficient growth on EMM2A or YMoA medium (Fig. 4A). In fact, on SPA (nitrogen-free) medium, fission yeast underwent sporulation with almost no vegetative growth (Fig. 4A). Therefore, the availability of nitrogen might affect cell growth.

To exclude the possibility that ME medium contains a growth inhibitor specific for *Sz. octosporus*, but not for *Sz. pombe* or *Sz. japonicus*, the growth test was repeated using a blended medium made by mixing ME and YMoA (named 'MYA' medium). We considered that, if such an inhibitor is present in ME medium, cell growth would also be inhibited on MYA medium. On the contrary, however, *Sz. octosporus* proliferated much faster on MYA medium than on YMoA medium, with the result that bigger single colonies were formed owing to synergy effects (Fig. 4A). In addition, MYA medium enabled the cells to mate favorably (see Fig. 3B). These tests indicated that the deficiency in growth of *Sz. octosporus* in ME medium was certainly due to a lack of nutrition.

Because the deficiency in *Sz. octosporus* growth in ME medium was alleviated by mixing ME with YMoA, we focused on the constituents of YMoA to determine the growth requirements of *Sz. octosporus*. YMoA is a rich defined solid medium, providing six categories of nutrients: nitrogen sources (N), carbon source (C) in sufficient concentration, a few amino acids (A), vitamins (V), minerals (M) and salts (S) in addition to agar (according to the Difco manual). As indicated in the Materials and methods section, various mixtures of these nutrients were therefore formulated.

First, we excluded one of six categorized constituents (Table 4) from MY liquid medium (i.e. MYA lacking agar). *Schizosaccharomyces octosporus* cells were incubated in each liquid medium at 30°C, and OD_{600} was measured with a photorecorder. In MY medium, cells grew faster with a growth rate of 0.151 ± 0.004 h^{-1} (Fig. 4B and Table 5), which was ~10 times faster than the growth rate in ME medium (0.014 ± 0.003 h^{-1}).

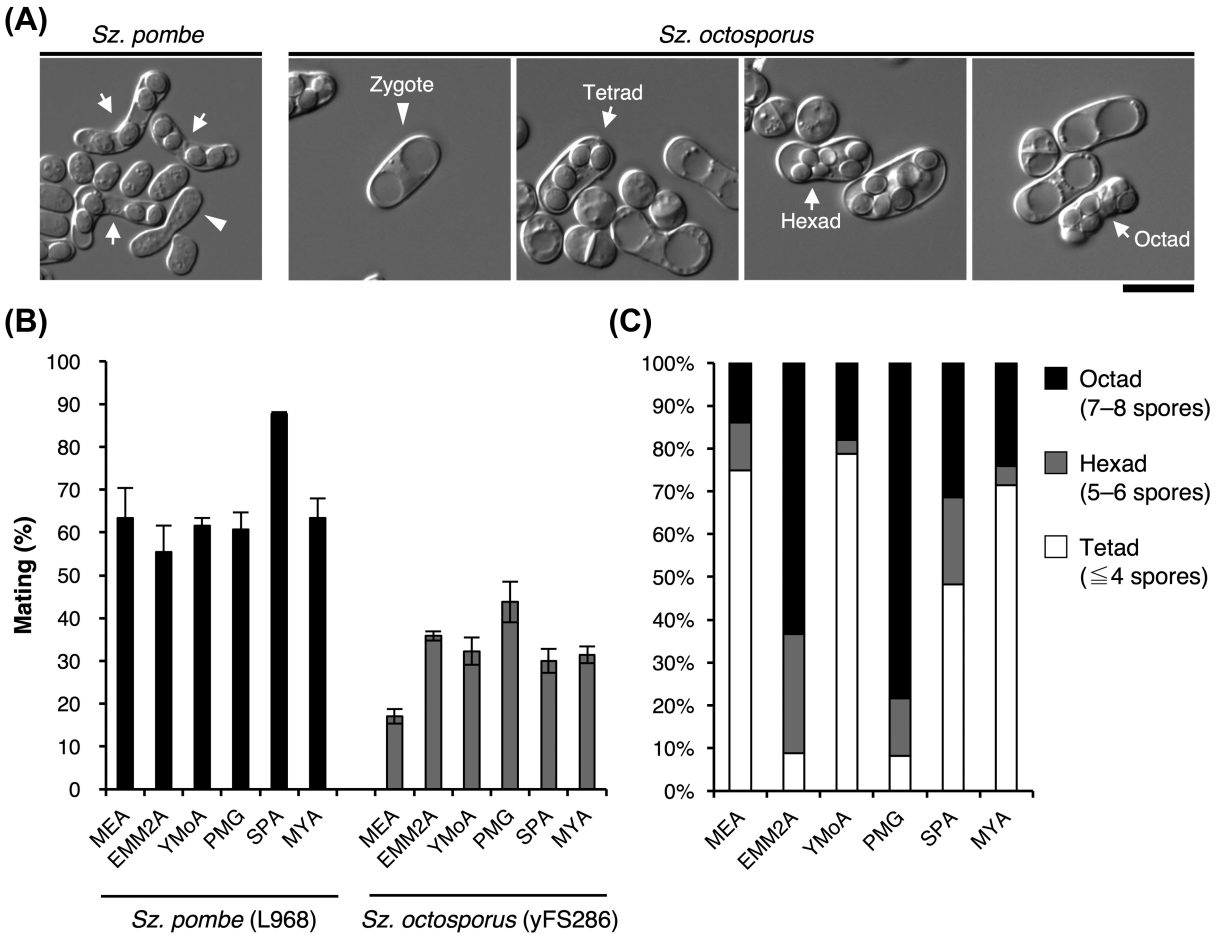

Figure 3. Mating response of *Sz. pombe* and *Sz. octosporus*. (**A**) Morphology of zygotes and asci in *Sz. pombe* (L968) and *Sz. octosporus* (yFS286). When nitrogen starved, cells of opposite mating type fuse to form diploid zygotes (arrowheads) that undergo meiosis to generate haploid spores. Schizosaccharomyces *pombe* asci (arrows) contain exactly four spores, but *Sz. octosporus* asci (arrows) contain up to eight spores. Typical tetrads (four-spored ascus), hexads (six-spored ascus) and octads (eight-spored ascus) are shown. Scale bar, 10 μm. (**B**) Mating ability of *Sz. pombe* and *Sz. octosporus* on various sporulation solid media. Mating efficiency on six different sporulation media (MEA, EMM2A, YMoA, PMG, SPA and MYA) was determined by measuring the frequency of zygotes. Data are the mean ± standard deviation of triplicate samples. At least 300 cells were examined for each sample. (**C**) Quantification of spore number within asci. Data are the calculated results based on (**B**) (at least 100 asci were examined).

In particular, nitrogen sources and salts were needed for growth (Fig. 4B).

Next, we examined the effects of growth in ME medium supplemented with only one of categorized constituents (Table 4). As shown in Fig. 4C, the addition of nitrogen sources and/or salts improved growth of *Sz. octosporus* (also see Table 5). When supplemented with both nitrogen and salts, *Sz. octosporus* showed significantly improved growth and formed single colonies on MEA plates (Fig. 4A). Taken together, *Sz. octosporus* needs nitrogen (e.g. ammonium acetate and asparagine) and salts (e.g. potassium phosphate monobasic, magnesium sulfate, sodium chloride and calcium chloride) for satisfactory growth, and it is desirable to include a carbon source, vitamins and minerals. ME medium is overwhelmingly lacking in nitrogen for growth of *Sz. octosporus* species, suggesting that efficient utilization of nitrogen is crucial for cell growth.

Isolation of heterothallic strains by screening of spontaneous mutants

Stable heterothallic strains of *Sz. octosporus* had not been isolated at the start of this study. Therefore, to isolate heterothallic strains of *Sz. octosporus*, colonies derived from cells of the homothallic strain (yFS286) were spread on MYA plates, where they underwent sporulation after a short period of growth. After 4 days of incubation at 30°C, single colonies on MYA plates were observed. Approximately 5.4×10^4 colonies were inspected for their sporulation capability by using efficient colony staining with iodine vapor (Gutz *et al.* 1974) (Fig. 5A). In this assay, non-sporulating colonies are not stained darkly by iodine vapor, but remain white or yellowish. This screening revealed 89 putative iodine-negative colonies (Fig. 5A), which were microscopically inspected to confirm a sterile phenotype. Among 45 colonies inspected, there were a few false-positives, but 16 colonies showed sterility or extremely low mating proficiency as expected from the iodine staining. Furthermore, we identified four colonies (named TS147–TS150) as truly sterile mutants produced by spontaneous mutation (Fig. 5B).

Next, the identified four strains were crossed with each other. We considered that if a set of heterothallic strains were included among them, then some combinations would have significant mating ability. In fact, a mating assay showed that the sterility of three of these strains was recovered by mixing with the others. Two strains (TS147 and TS148) mated with the strain TS150 (Fig. 5C). The only exception was TS149, which did not mate with any strain (Fig. 5C). These results suggested that three of the

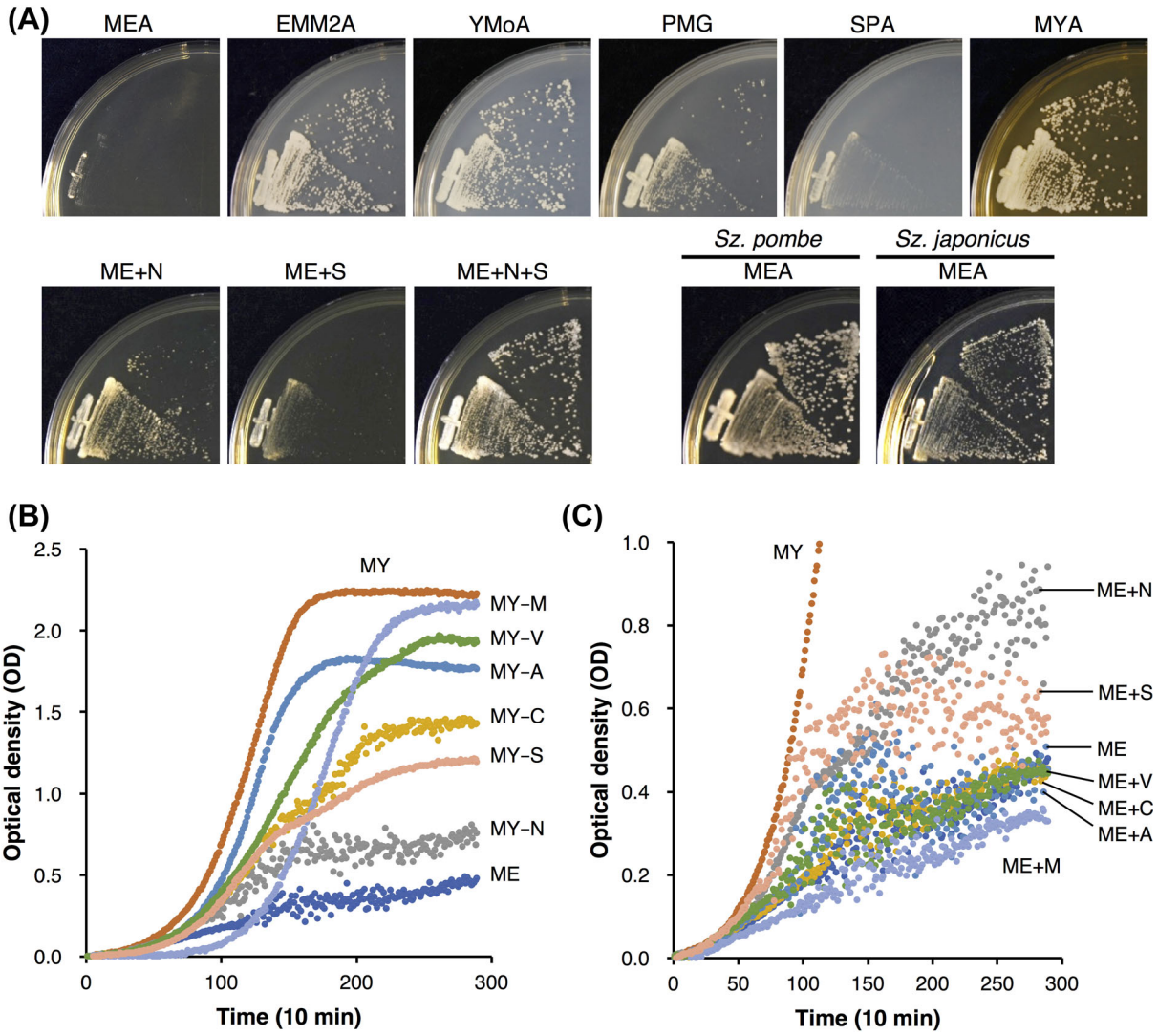

Figure 4. Growth kinetics on/in sporulation media under various conditions. (**A**) Growth of *Sz. octosporus* cells on various sporulation media. As a control, *Sz. pombe* and *Sz. japonicus* cells were grown on MEA medium. (**B**) To investigate the growth requirements of *Sz. octosporus*, yFS286 was cultured in ME liquid medium supplemented with various nutrients. A series of test tubes were prepared with MY liquid medium excluding one by one: N, C, A, V, M and S (Table 4). (**C**) A series of test tubes were prepared with ME liquid medium adding one by one: N, C, A, V, M and S (Table 4). Cultures were inoculated at an initial OD_{600} of 0.05 and incubated at 30°C with shaking. Cell density was measured every 10 min for 2 days by using a biophotorecorder. Data are the means of biological triplicates.

strains (TS147, TS148 and TS150) were likely to be phenotypes of a heterothallic strain.

Mating-type specific sterility of the isolated colonies

A stable heterothallic strain should express only intact *mat-P* or intact *mat-M* genes. The *Schizosaccharomyces* genome database of the Broad Institute indicates that *Sz. octosporus* possesses a *mat*-cassette. This *mat*-cassette contains three genes: *mat-Pc* (354 bp), *mat-Pi* (477 bp) and *mat-Mc* (540 bp) (Fig. 6A). Based on the sequence database, the homologous genes of *Sz. pombe* share 63% identity with *mat-Pc*, 39% identity with *mat-Pi* and 58% identity with *mat-Mc*; in addition, *Sz. pombe* possesses a *mat-Mi* gene, which has not been annotated in *Sz. octosporus*. In fission yeast cells, the mating type is determined by the configuration at the *mat1* locus. In an h^+ cell, P type is specified by the *mat1-P* allele, whereas in an h^- cell, M type is specified by the *mat1-M* allele. To determine the mating type of the four strains (TS147–TS150), we carried out a PCR assay. The primers for PCR assay were designed based on the sequence of the mating-type region. The common primer oTS13 was located at the proximal 5′-side of the *mat1* locus (Fig. 6A), whereas the specific primers oTS103 and oTS110 corresponded to sequences contained in the *mat-Pi* gene and the *mat-Mc* gene, respectively (Fig. 6A). Thus, if P-type information were present in the *mat1* locus, oTS13 and oTS103 primers would yield a PCR product of 1.8 kb, whereas for M-type information, oTS13 and oTS110 primers would give rise to a PCR product of 1.7 kb. In the wild-type homothallic strain (yFS286), the primer set oTS13 and oTS103 for P type produced the 1.8-kb DNA fragment, and the primer set oTS13 and oTS110 for M type produced the 1.7-kb DNA fragment (Fig. 6B) because the homothallic strain is a mixed population of P-type and M-type cells. As shown in Fig. 6B, both DNA fragments were detected in all sterile strains, indicating that the exchange of the configuration at the *mat1* locus is not completely loss.

We further tested the expression of mating-type specific mRNAs in the strains (TS147–TS150). RT-PCR was performed on total

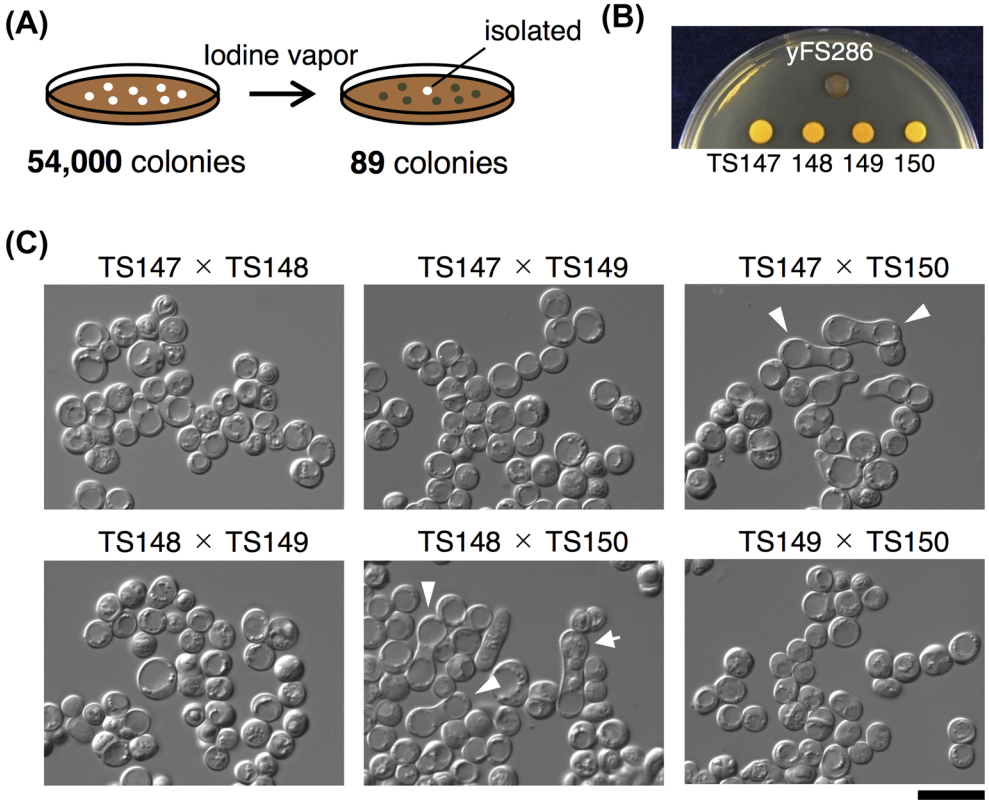

Figure 5. Isolation of non-sporulating colonies by spontaneous screening. (**A**) Screening for heterothallic strains. Cells of the homothallic strain (yFS286) were allowed to form single colonies on MYA plates for 4 days at 30°C. (**B**) Cells grown on YEA were resuspended in sterilized water to a cell density of 1×10^8 cells/ml. A 5-μl aliquot of the suspension was spotted onto MYA plates, and incubated for 4 days at 30°C. The cells were exposed to iodine vapor for 1 min. (**C**) Four non-sporulating colonies were crossed with each other on MYA plates for 3 days at 30°C. Microphotographs show that the mating inability of two colonies (TS147 and TS148) was rescued by mixing with another colony (TS150). Arrowheads, diploid zygotes; arrows, asci. Scale bar, 10 μm.

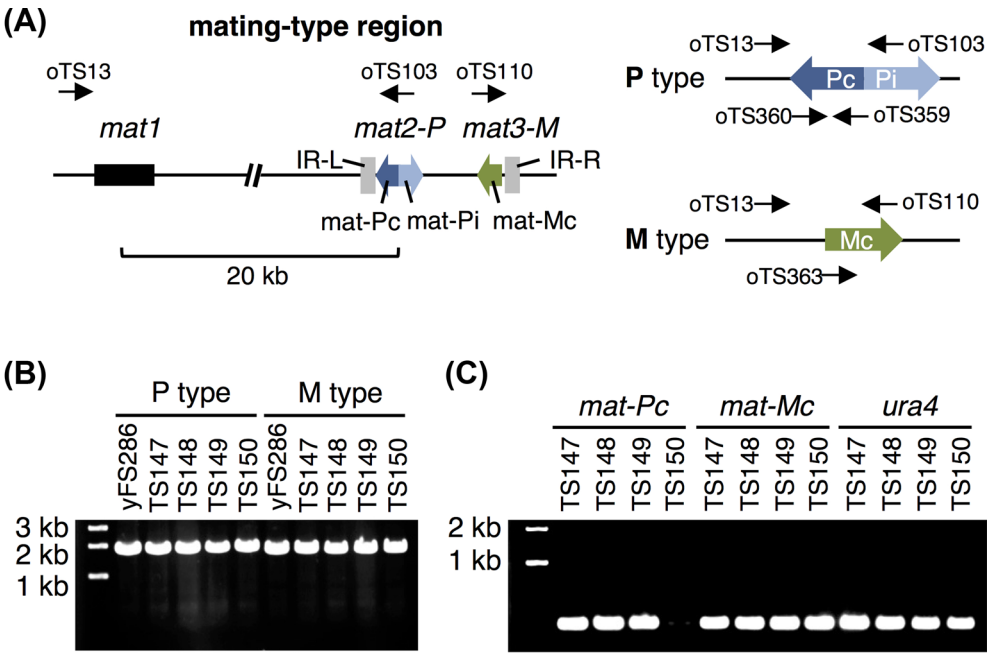

Figure 6. TS147 and TS148 are h^+-like strains and TS150 is h^- strain. (**A**) Schematic representation of the mating-type region located on the (presumed) chromosome II in *Sz. octosporus*. The mating-type region consists of three linked loci: *mat1* (black box), *mat2-P* and *mat3-M*. The *mat2-P*/*mat3-M* region is surrounded by two inverted repeats, *IR-L* and *IR-R*, which have highly conserved sequences (gray arrows). Mating type is specified by the different genes in the so-called *mat*-cassette: P type by the *mat-Pc* and *mat-Pi* genes, and M type by the *mat-Mc* gene. The position of each primer (oTS13, oTS103, oTS110, oTS359, oTS360 and oTS363) in the mating-type regions is shown. (**B**) Genomic DNA was extracted from YE cultures, and mating type was determined by PCR. For all strains, both bands were detected. (**C**) Total RNA was collected from EMM2–N cultures, and RT-PCR was performed to detect the designated mRNAs.

Table 4. Composition of the blend medium, MYA.

Constituent	Amount (g/l)
Nitrogen sources (N)	
Ammonium sulfate	3.5
L-Asparagine	1.5
Carbon source (C)	
D-Glucose	10
Amino acids (A)	
L-Histidine	0.01
L-Methionine	0.02
L-Tryptophan	0.02
Vitamins (V)	
Biotin	0.000002
Calcium pantothenate	0.0004
Folic acid	0.000002
Inositol	0.002
Nicotinic acid	0.0004
Aminobenzoic acid	0.0002
Pyridoxine hydrochloride	0.0004
Riboflavin	0.0002
Thiamine hydrochloride	0.0004
Minerals (M)	
Boric acid	0.0005
Copper sulfate	0.00004
Potassium iodide	0.0001
Ferric chloride	0.0002
Manganese sulfate	0.0004
Molybdic acid	0.0002
Zinc sulfate	0.0004
Salts (S)	
Potassium phosphate monobasic	1
Magnesium sulfate	0.5
Sodium chloride	0.1
Calcium chloride	0.1
Bacto Malt Extract	30
Bacto Agar (for solid)	18

Table 5. Growth rate of yFS286 in ME liquid media supplemented with various nutrients.

Medium	Growth rate (μ)(h^{-1})
ME	0.014 ± 0.003
MY	0.151 ± 0.004
MY−N	0.023 ± 0.003
MY−C	0.079 ± 0.004
MY−A	0.140 ± 0.035
MY−V	0.106 ± 0.016
MY−M	0.121 ± 0.016
MY−S	0.084 ± 0.009
ME+N	0.050 ± 0.016
ME+C	0.013 ± 0.007
ME+A	0.017 ± 0.005
ME+V	0.015 ± 0.009
ME+M	0.011 ± 0.004
ME+S	0.029 ± 0.006

Growth rate was calculated as the mean ± standard deviation of biological triplicates.

RNA from cultures of all four strains. The *Sz. octosporus* cells were cultured in EMM2−N medium for 12 h at 30°C (see Materials and Methods section). The primer sets were designed to detect one of two mRNAs (*mat-Pc* and *mat-Mc*). Figure 6C shows that only *mat-Mc* mRNA and not *mat-Pc* mRNA was expressed in TS150; therefore, we concluded that TS150 must be an h^- cell. Curiously, both *mat-Pc* and *mat-Mc* mRNAs were detected from TS147 and TS148; nevertheless, these strains could mate only with TS150. Because TS147 and TS148 behaved as h^+-like strains, we hypothesized that they might produce a non-functional *mat-Mc* mRNA. To examine this possibility, we attempted to sequence the *mat-Mc* gene of TS147 and TS148. The *mat-Mc* genes were amplified by the primer set oTS253 and oTS254) and were sequenced. In comparison to the genomic DNA of the homothallic yFS286 strain, however, no mutations in the *mat-Mc* gene in either strain were found (data not shown). Thus, the h^+-like phenotypes of both TS147 and TS148 are possibly due to a defect in M-specific genes, but the details remain unclear.

Gene targeting to construct heterothallic strains

The above data showed that the TS150 strain was the same phenotype as an h^- heterothallic strain, which cannot express the *mat-Pc* gene. In contrast, TS147 and TS148 seemed to behave like an h^+ heterothallic strain, but they still expressed both the *mat-Pc* and the *mat-Mc* genes. To obtain truly heterothallic strains, we therefore deleted the silent *mat-2P/mat-3M* region by gene manipulation.

Although manipulation of an endogenous gene locus in *Sz. octosporus* has been reported previously (Gu, Yam and Oliferenko 2012), a successful method for routine gene manipulation has not been described. We therefore tried to target exogenous DNA according to methods used for other fission yeast species (Prentice 1992; Furuya and Niki 2009). Two circular DNAs (pTS40 and pTS41; Fig. 7A) with two homology arms (1 kb) flanking each side of the *mat-2P/mat-3M* region were used. Each fragment of DNA was amplified using a primer set (oTS153 and oTS156) from pTS40 and pTS41 and introduced into the yFS286 strain by electroporation. Cells were pulsed at 3.0 kV (15.0 kV/cm), 25 μF and 200 ohms. Through the process of homologous recombination, the linearized DNA should be recombined into the genome to disrupt the *mat-2P/mat-3M* region. We screened for colonies that grew on zeocin (Ble)-containing (for pTS40) or hygromycin B (Hyg)-containing (for pTS41) plates, and obtained successful transformants from each plate. If homologous recombination had occurred at the correct place, then the mating type of the isolated transformant should be fixed to either P or M (Fig. 7B), and the transformant should show a sterile phenotype. We therefore inspected the mating capability of transformants to identify sterile colonies under a microscope. As expected, Ble- and Hyg-resistant colonies that could not mate by themselves were found.

The cells of these colonies were crossed with each other, and cultured on PMG plates to induce mating. As a result, we identified two strains, termed TS161 (Ble-resistance) and TS162 (Hyg-resistance), each of which successfully mated with the other (Fig. 7C). We also tested the sexual agglutination that occurs between opposite mating-type cells prior to cell fusion. Each strain was cultured in nitrogen-free EMM2−N medium for 12 h. Whereas the homothallic strain yFS286 exhibited significant sexual agglutination, neither TS161 nor TS162 visually aggregated (Fig. 7D). Only the mating response between TS161 and TS162 progressed as well as that of the homothallic cells. To determine the mating type of these strains, the PCR assay was

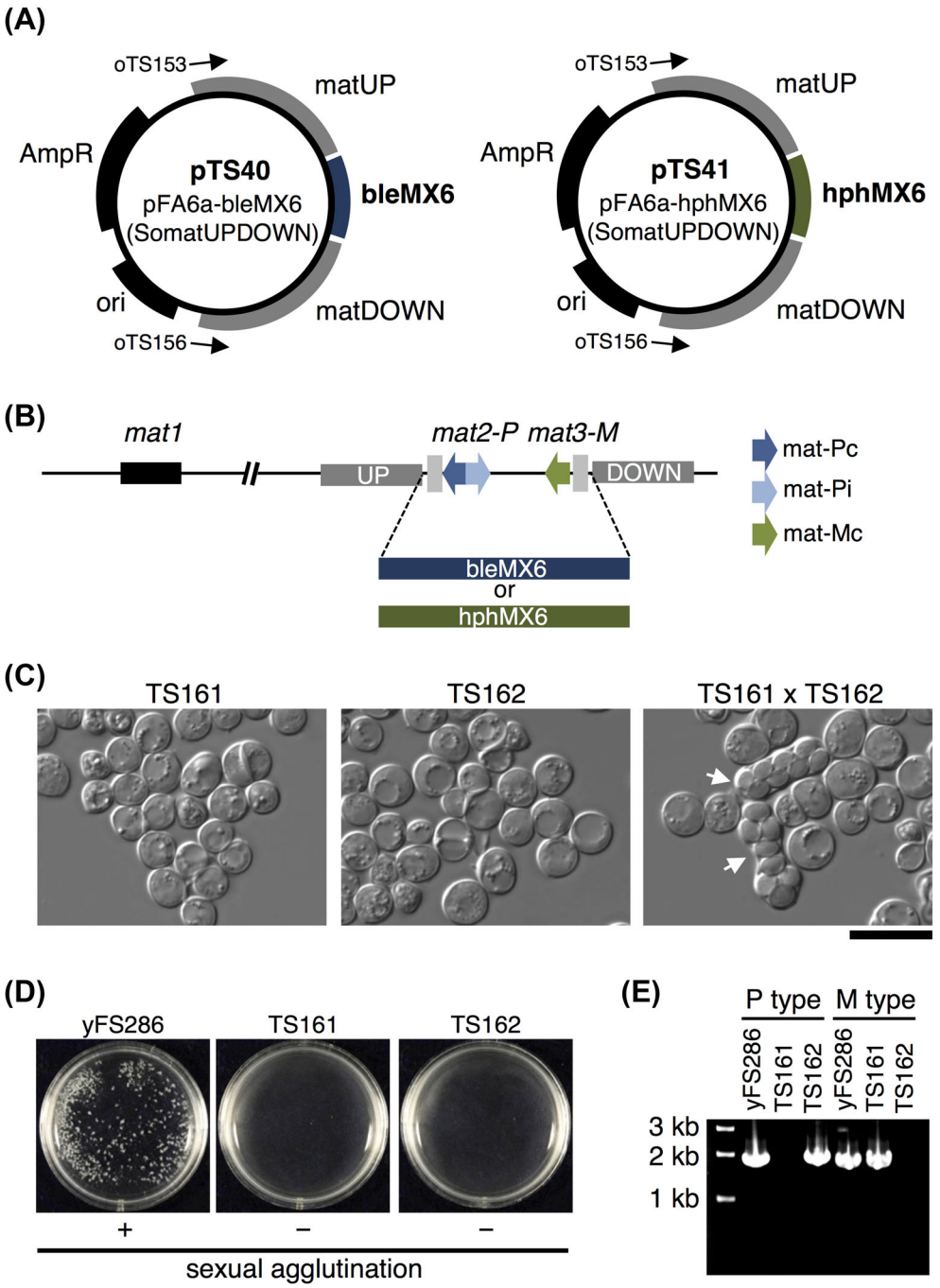

Figure 7. Construction of heterothallic strains by gene manipulation. (**A**) Maps of the two plasmids used to delete the *mat2-P/mat3-M* silent region. bleMX6 and hphMX6 are drug-resistance genes used for selection in *Sz. octosporus*. matUP and matDOWN, located at either end of the *mat2-P/mat3-M* region, were derived from the *Sz. octosporus* genomic sequences for integration by homologous recombination. DNA fragments amplified from the plasmids by the primer set oTS153 and oTS156 were used for transformation. (**B**) *mat2-P/mat3-M* disruption strategy. If recombination takes place at this locus, the cell will have a *mat2-P/mat3-M* disruption and show resistance to zeocin or hygromycin B, and thus the mating type of the cell will be fixed to either P or M. (**C**) Morphology of zygotes and asci in mating cells of TS161 and TS162. Arrows, asci. Scale bar, 10 μm. (**D**) Sexual agglutination of yFS286 (homothallic strain), TS161 and TS162. Each strain was precultured in YE medium overnight, and then cultured in EMM2−N medium for 12 h. During incubation, cultures in 3.5-cm petri dishes (Thermo Fisher Scientific, Waltham, MA, USA) were gently shaken. Visible agglutination is seen only in the wild-type strain yFS286. (**E**) Genomic DNA was extracted from YE cultures, and mating type was determined by PCR.

carried as described above. Figure 7E shows clearly that TS162 produced the 1.8-kb DNA fragment after PCR with the primers oTS13 and oTS103 for P type; in contrast, TS161 produced the 1.7-kb DNA fragment with the primers oTS13 and oTS110 for M type. Thus, in both strains, homothallism was lost as expected. Therefore, TS162 was designed as h^+ and TS161 was designed as h^-.

Next, we investigated whether the progenies showed 4:4 mating types in the eight spores from the crosses between the heterothallic strains of opposite mating types. Spores were produced by crossing TS161 and TS162, and analysis of octad dissection was carried out. As result of the octad analysis of three asci, eight progenies were grown from each ascus. Furthermore,

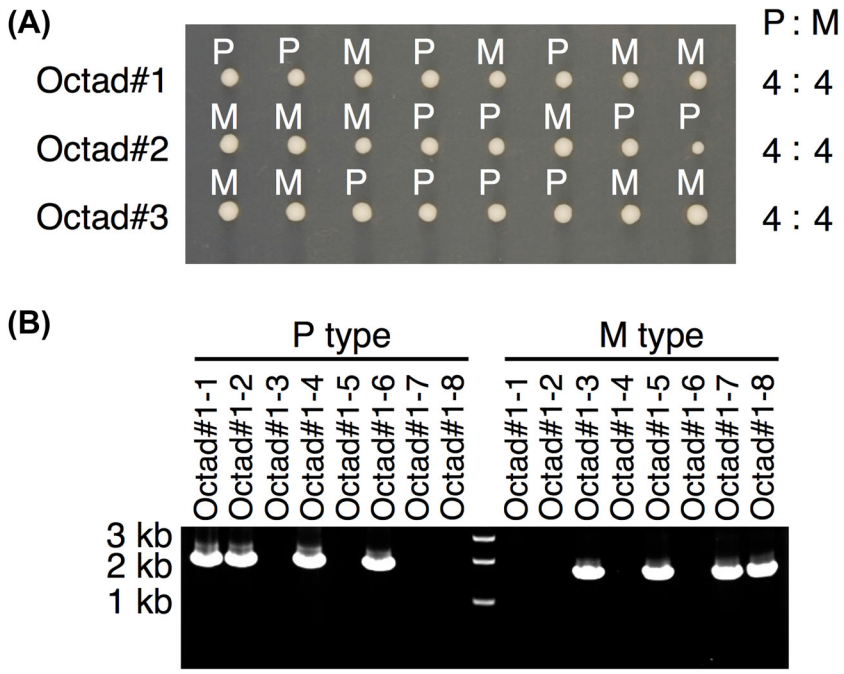

Figure 8. Octad analysis of the constructed heterothallic strains, TS161 and TS162. (**A**) Analysis of 24 spores from three octads. The mating types of these progenies were determined by both crossing with each other and mat-PCR. The mating types all showed the expected 4:4 ratio. (**B**) Results of PCR determination of mating type in Octad no.1 is shown as an example.

the mating types of these progenies were determined by crossing with TS161 or TS162. Half of the progenies could mate with the other half (Fig. 8A), whereas none of the progenies formed by themselves. We further determined the mating types of eight progenies from a representative octad (Octad no. 1). As shown in Fig. 8B, the mating types showed a 4:4 ratio as we expected. In addition, drug resistance test of the progenies from the octad also strongly indicated that TS161 and TS162 must be heterothallic strains (Fig. S1, Supporting Information). Even in the tetrads, Ble-resistant and Hyg-resistant progenies appeared in a 2:2 ratio (Fig. S1); however, we could not analyze the progenies from hexads because of the low frequency of spore germination. Ultimately, these findings show that we succeeded in constructing stable heterothallic strains of *Sz. octosporus* by gene manipulation.

DISCUSSION

Variable numbers of spores in *Schizosaccharomyces octosporus*

In ascomycetes, a zygote develops into an ascus. Meiosis then leads to four haploid nuclei, which is usually followed by a further mitotic division that results in eight nuclei within each ascus (Chiu and Moore 1996). The nuclei become enclosed within prospore membranes to form spores that are aligned inside the ascus. Here, we have shown that the number of spores that form within the asci of *Sz. octosporus* depends on the nutrient conditions (Fig. 3C) and that *Sz. octosporus* does not always form eight haploid spores.

Given that eight spores are formed within asci in some fission yeast species due to an extra post-meiotic mitosis, similarly to many filamentous ascomycetes, the regulation of spore number probably occurs at the level of commitment to post-meiotic mitosis. This is consistent with our finding that spores in *Sz. octosporus* tetrads have normal haploid DNA content, as shown in Fig. S2 (Supporting Information). Our study clearly indicated that *Sz. octosporus* frequently formed four spores within asci on MEA, YMoA and SPA (D-glucose 10 g/l), as compared with either EMM2A or PMG (D-glucose 20 g/l) (Fig. 3C). Thus, carbon-limited conditions might be related to the regulation of spore number in *Sz. octosporus*.

Requirements for satisfactory growth

We found that *Sz. octosporus* (yFS286) could not proliferate on MEA plates (Fig. 4A). MEA is a standard solid medium that has been used to screen for mating-defective mutants in both *Sz. pombe* and *Sz. japonicus* (Furuya and Niki 2009; Seike, Nakamura and Shimoda 2015). ME medium is overwhelmingly lacking in nitrogen for satisfactory growth of *Sz. octosporus*. In a previous investigation of the growth requirements of *Sz. octosporus* (Northam and Norris 1951), the yeast grew satisfactorily on medium containing vitamins (inositol, pantothenic acid, nicotinic acid and biotin), minerals and amino acids (histidine, methionine and adenine), in addition to glucose and nitrogen (asparagine and ammonium sulphate). In fact, in this study, the growth defect of *Sz. octosporus* in ME medium was recovered by adding the constituents of YMoA (Fig. 4B and C). In terms of sporulation media for *Sz. octosporus*, however, MEA is not suitable, whereas PMG medium was optimal among those that we investigated (Fig. 3B).

Successful construction of heterothallic strains of *Schizosaccharomyces octosporus*

To obtain heterothallic strains in this study, we first searched for non-sporulating colonies by random screening. Among ~5.4 × 10^4 colonies, we identified 16 sterile colonies. In *Sz. octosporus* cells, the frequency of the appearance of sterile mutants by

spontaneous mutations was thus 2.96×10^{-4} (16/54,000), which is almost the same as the value of 3.00×10^{-4} (6/20 000) reported in *Sz. japonicus* cells (Furuya and Niki 2009). Ultimately, we identified three mutants showing phenotypes of a heterothallic strain (TS147, TS148 and TS150) (Fig. 5B and C). TS150 was confirmed as an h^- heterothallic strain because it produces *mat-Mc* mRNA but not *mat-Pc* mRNA. In contrast, TS147 and TS148 are likely to be pseudo-h^+ heterothallic strains (Egel 1973) because they express intact *mat-Pc* and *mat-Mc* mRNAs, but could not mate with h^+ cells. Probably, the M-specific mating system is aberrant in these strains.

To construct stable heterothallic strains by deleting *mat-2P/mat-3M* silent region, we first tried to transform the relevant PCR fragment into yFS286 by the lithium acetate methods used for *Sz. pombe* (Ito *et al.* 1983); however, no transformants were obtained. We therefore referred to the transformation method of electroporation used for *Sz. japonicus* (Furuya and Niki 2009), and modified it for *Sz. octosporus*. Our findings indicated that, for *Sz. octosporus*, an electric pulse condition of 3.0 kV, 25 μF, 200 ohms led to more efficient transformation. The transformation efficiency was ca. 1.57×10^{-7} cells when 1 μg of DNA was transferred. By using modified gene manipulation, we established two stable heterothallic strains of *Sz. octosporus*, TS161 and TS162, which are h^-- and h^+-heterothallic strains, respectively (Fig. 7E). We verified that TS161 mated with TS147 and TS148, and TS162 mated with TS150, while mat-PCR revealed the fixation of *mat* locus in the two strains. Although the TS161 and TS162 strains have Ble and Hyg resistance, respectively, the expression level of each was very low due to silencing by heterochromatin at the *mat-2P/mat-3M* region; thus, for selection of one or the other, it will be necessary to use plates containing lower drug concentrations (e.g. 50 μg/ml).

Lastly, we attempted to construct stable heterothallic strains of *Sz. cryophilus* in the same manner. Although some transformants were successfully isolated (data not shown), the homothallic strain (OY14) of *Sz. cryophilus* showed a sterile phenotype on every sporulation media that we tested; therefore, we could not judge whether the isolated transformants were heterothallic strains (data not shown). Further consideration of the mating conditions for *Sz. cryophilus* is required. In conclusion, our constructed heterothallic strains of *Sz. octosporus* mate only with opposite mating-type cells, and therefore will be powerful tools in yeast genetics to accelerate comparative studies across the genus *Schizosaccharomyces*.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSYR* online.

ACKNOWLEDGEMENTS

We thank National BioResource Project Japan (NBRP) for strains. We also thank Chikashi Shimoda and Taro Nakamura at Osaka City University for helpful comments, Mitsuko Endo for technical assistance, and all members of our laboratory for beneficial suggestions.

FUNDING

This study was supported by Grant-in-Aid for JSPS Research Fellow (Research Project number: JP15J03416).

Conflict of interest. None declared.

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