Remarkably Simple Sequence Requirement of the M-Factor Pheromone of *Schizosaccharomyces pombe*

Taisuke Seike,* Yoshikazu Yamagishi,† Hideo Iio,† Taro Nakamura,* and Chikashi Shimoda*,[1]
*Department of Biology and †Department of Material Science and Chemistry, Graduate School of Science, Osaka City University, Sumiyoshi-ku, Osaka 558-8585, Japan

ABSTRACT The mating reaction is triggered by specific pheromones in a wide variety of organisms. Small peptides are used as mating pheromones in yeasts and fungi. In the fission yeast *Schizosaccharomyces pombe*, M-factor is a C terminally farnesylated nonapeptide secreted from M-cells, and its counterpart, P-factor, is a simple peptide composed of 23 amino acids. The primary structure requirements for the biological activity of pheromone peptides remain to be elucidated. Here, we conducted comprehensive substitution of each of the amino acids in M-factor peptide and inspected the mating ability of these missense mutants. Thirty-five sterile mutants were found among an array of 152 mutants with single amino acid substitutions. Mapping of the mutation sites clearly indicated that the sterile mutants were associated exclusively with four amino acid residues (VPYM) in the carboxyl-terminal half. In contrast, the substitution of four amino-terminal residues (YTPK) with any amino acid had no or only a slightly deleterious effect on mating. Furthermore, deletion of the three N-terminal residues caused no sterility, although truncation of a fourth residue had a marked effect. We conclude that a farnesylated hexapeptide (KVPYMC^{Far}–OCH_3) is the minimal M-factor that retains pheromone activity. At least 15 nonfunctional peptides were found to be secreted, suggesting that these mutant M-factor peptides are no longer recognized by the cognate receptor.

SEXUAL reproduction in eukaryotes promotes genome diversity by genetic recombination and thus may accelerate evolution. As a result, mating behavior is important for reproduction. Like many fungi, the fission yeast *Schizosaccharomyces pombe* has two mating types, termed h^+ (P) and h^- (M) (Gutz *et al.* 1974; Egel 1989, 2004). Two haploid cells of opposite mating types mate and form a diploid zygote on starvation of nitrogen (Egel 1971). The diploid zygote undergoes meiosis and forms four haploid spores (Bresch *et al.* 1968; Gutz *et al.* 1974; Egel 1989; Nielsen 2004). Spores germinate in rich nutrient medium and proliferate as haploid vegetative cells.

The specificity of mating types in fission yeast is primarily determined by molecular recognition between a peptide mating pheromone and its cognate receptor. The mating reaction proceeds through several distinguishable steps (Egel 1989; Nielsen 2004). First, cells adhere to each other to form large cell aggregates in a reaction that is termed cell agglutination. Second, cells in aggregates protrude a conjugation tube toward a cell of opposite mating type. M- and P-cells make most contact at the cell tips, where the cell walls separating them are dissolved. Third, the juxtaposed plasma membranes are fused together. Last, after cell fusion, two nuclei derived from each mating type fuse together to form a diploid cell.

A wide variety of organisms use chemical signals to attract the opposite sex and facilitate mating with partners. In *S. pombe*, M-cells secrete the mating pheromone M-factor, which is specifically recognized by its cognate G-protein-coupled receptor (GPCR), Map3, on the surface of P-cells (Davey 1991, 1992; Tanaka *et al.* 1993). On the other hand, P-cells secrete P-factor, which activates the corresponding GPCR Mam2 on M-cells (Kitamura and Shimoda 1991; Imai and Yamamoto 1994). Mating pheromone signals stimulate cells of the opposite mating type to commence the mating reaction. Pheromone signaling evokes several responses in cells of the opposite mating type: G_1 arrest of the cell cycle

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[1]Corresponding author: Department of Biology, Graduate School of Science, Osaka City University, Sugimoto 3-3-138, Sumiyoshi-ku, Osaka 558-8585, Japan. E-mail: shimoda@sci.osaka-cu.ac.jp

(Davey and Nielsen 1994; Imai and Yamamoto 1994), altered patterns of gene transcription (Mata and Bahler 2006; Xue-Franzen *et al.* 2006), sexual cell agglutination (Miyata *et al.* 1997), and polar cell elongation to form characteristic morphology (Fukui *et al.* 1986; Leupold 1987; Davey 1991). The mechanisms leading to cell agglutination followed by cell fusion are not well understood. So far, only Mam3 and Map4 have been proposed to be involved in cell–cell adhesion as putative M-specific and P-specific agglutinins, respectively (Sharifmoghadam *et al.* 2006). These proteins, which are necessary for mating, are thought to be transcriptionally activated downstream of the pheromone-signaling pathway.

M-factor is a nonapeptide whose C-terminal cysteine residue is S-farnesylated and carboxyl-methylated (Davey 1991, 1992; Davey and Nielsen 1994), whereas P-factor is a simple peptide composed of 23 amino acids (Imai and Yamamoto 1994). M-factor is encoded by three redundant genes, *mfm1*$^+$, *mfm2*$^+$, and *mfm3*$^+$ (Davey 1992; Kjaerulff *et al.* 1994). These *mfm*$^+$ genes produce precursor polypeptides composed of 41−44 amino acids, which can produce the same nonapeptide, mature M-factor. Any one of the three genes is sufficient for mating, but M-cells lacking all three genes fail to produce M-factor and are unable to mate, indicating that *mfm1*$^+$, *mfm2*$^+$, and *mfm3*$^+$ are redundant in function but that M-factor is essential for mating (Kjaerulff *et al.* 1994). The biosynthetic pathway of M-factor has not been fully characterized, but is thought to be analogous to the pathway of *Saccharomyces cerevisiae* **a**-factor, which is also S-farnesylated and carboxyl-methylated at the C-terminal Cys residue (Anderegg *et al.* 1988; Davey 1992). The M-factor precursor protein is likely to undergo proteolytic processing and post-translational modifications to produce the mature, active pheromone (Michaelis 1993). Secretion of mature M-factor is mediated by an ATP-binding cassette (ABC) transporter, Mam1 (Christensen *et al.* 1997; Davey *et al.* 1997; Kjaerulff *et al.* 2005; Huyer *et al.* 2006). Importantly, the substrate specificity of the Mam1 transporter is thought to be low (Kjaerulff *et al.* 2005).

The pheromone signaling pathway downstream of the activated receptors is shared by both cell types. Therefore, mating-type specificity in the fission yeast is largely dependent on specific interaction between pheromone peptides and their cognate receptors. In this study, we attempted to generate a comprehensive array of missense M-factor mutants to identify amino acids that are essential for molecular recognition by the Map3 receptor. Except for the C-terminal essential Cys residue, which is chemically modified, we substituted each of the remaining 8 amino acids with the 19 other amino acids by using efficient *in vitro* site-directed mutagenesis with properly designed oligonucleotide primers. We then assayed the mating ability of the array of 152 missense M-factor mutants that were successfully created. As a result, 35 sterile mutants were identified among the 152 missense mutants. On the basis of our results, we discuss the primary structure *vs.* function relationship.

Materials and Methods

Yeast strains, media, and culture conditions

The parent strain of *S. pombe* used for mutagenesis was FS55 (*h*^{90} *mfm1*::*LEU2 mfm2-D4 mfm3*::*ura4*$^+$ *leu1-32 ura4-D18 ade6-M210*), in which the *mfm* deletion alleles were derived from EG773 (Kjaerulff *et al.* 1994). The set of missense *mfm1* mutants created in this study are listed in Supporting Information, Table S1. The *S. pombe* strains that originated differently from the standard laboratory strain (Leupold's strain L972) and other closely related subspecies of the genus *Schizosaccharomyces* are listed in Table S2. Complete medium YE and synthetic media, SD and SSL+N were used for growth. Malt extract medium (ME) and synthetic media (SSL and SSL–N) were used for mating (Egel and Egel-Mitani 1974; Gutz *et al.* 1974; Moreno *et al.* 1991). *S. pombe* cells were grown at 30° and conjugated at 28° unless otherwise stated.

Construction of plasmids

The *mfm1*$^+$ gene was amplified by PCR and cloned into pGEM-T vector (Promega Biotec, Madison, WI). The *Sac*I/*Sal*I fragment containing the *mfm1*$^+$ gene from this vector was then recloned into the integration vector pBS(*ade6*) (K. Tamai, personal communication). The resultant plasmid, referred to as pBS(*ade6–mfm1*$^+$), was used as a template for *in vitro* mutagenesis. After restriction with *Bam*HI near the center of the *ade6*$^+$ gene, pBS(*ade6–mfm1*$^+$) was integrated at the *ade6* locus on chromosome III by transformation of a recipient *S. pombe ade6* auxotrophic strain.

Site-directed mutagenesis by in vitro DNA replication

Site-directed mutagenesis of the M-factor-coding gene, *mfm1*$^+$, was conducted by using *in vitro* mutagenesis reagents (Quik-Change, Stratagene, San Diego, CA). DNA replication using *Pfu* DNA polymerase and pBS(*ade6–mfm1*$^+$) as a template to generate mutated strands *in vitro* was performed as directed by the manufacturer's manual. The degenerate oligonucleotides were designed to introduce various mutated triplet codons at specific sites of the *mfm1*$^+$ ORF. The oligonucleotide primers are listed in Table S3. After replication, the reaction mixture was treated with restriction enzyme *Dpn*I to digest the *mfm1*$^+$ strands used as a template. *Escherichia coli* competent cells (DH5α) were then transformed with the *Dpn*I-treated plasmids. Introduced mutations were confirmed by sequencing the recovered plasmids.

Quantitative assay of zygote formation

Cells were grown to 1×10^7 cells/ml in YE liquid medium and were then resuspended in sterilized water to a cell density of 1×10^8 cells/ml. A 50-μl aliquot of the suspension was spotted onto the ME agar medium, which was incubated for 2 days at 28°. Cells were counted under a differential interference contrast microscope or an ordinary light microscope. Cell types were classified into four groups: vegetative cells (*V*), zygotes (*Z*), asci (*A*), and free spores (*S*). The percentage of zygotes was calculated according to the following equation:

$$\text{Zygotes}\ (\%) = (2Z + 2A + S/2) \times 100 / (V + 2Z + 2A + S/2).$$

Usually, triplicate samples (at least 200 cells each) were counted, and the mean ± SD was calculated.

Quantitative assay of sexual cell agglutination

The intensity of cell agglutination in the *mfm1*$^+$ strain (SS1001) and *mfm1* mutants was estimated according to Shimoda and Yanagishima (1974). Overnight cultures grown in YE liquid medium were washed with sterilized water and resuspended in synthetic growth medium (SSL + N) at a cell density of 2×10^6 cells/ml. After cultivation at 30° for 20 hr with gentle shaking, cells were transferred to synthetic sporulation medium (SSL – N) at a cell density of 4×10^7 cells/ml and cultured at 28° for 4 hr with gentle shaking. An aliquot of the culture was diluted with water, and the optical density at 600 nm was measured (OD_{600} before sonication). Then cell suspension was subjected to sonication (20 kHz) for 10 sec to disperse cell aggregates completely. Immediately after sonication, the OD_{600} was determined (OD_{600} after sonication). The agglutination index was calculated according to the following equation:

$$\text{Agglutination index (AI)} = \text{OD}_{600}\ \text{(after sonication)}/\text{OD}_{600}\ \text{(before sonication)}.$$

This value is a function of an average size of cell aggregates (Yoshida and Yanagishima 1978).

Mass spectrometry

Mass spectrometry was applied to detect mutant M-factor peptides in the culture filtrates. Crude culture supernatants containing M-factor were prepared from homothallic strains as described previously (Davey 1991). Cells were grown in YE liquid medium at 30°, collected by centrifugation, washed three times with SSL, and then resuspended in SSL at a cell density of 1×10^6 cells/ml. Cells were grown for 24 hr at 29° with gentle shaking to the early stationary phase, Amberlite XAD-2 (Stratagene) resin was then added to 10% (v/v), and incubation was continued for another 72 hr. The Amberlite resin was collected by filtration and washed with excess ultrapure water. The absorbed material was eluted with methanol and then subjected to an ordinary desalting procedure suitable for MALDI-TOF samples using a ZipTip (Millipore, Bedford, MA) (Santos *et al.* 2007). A 0.5-μl aliquot was mixed with 0.5 μl of α-cyano-4-hydroxycinnamic acid, spotted on a stainless steel MALDI target plate, and then air-dried. Positive-ion MALDI-TOF spectra were acquired in reflectron mode using an AXIMA-CFR Plus instrument (Shimadzu, Kyoto, Japan).

Results

Comprehensive mutagenesis of *mfm1*$^+$ encoding M-factor peptide

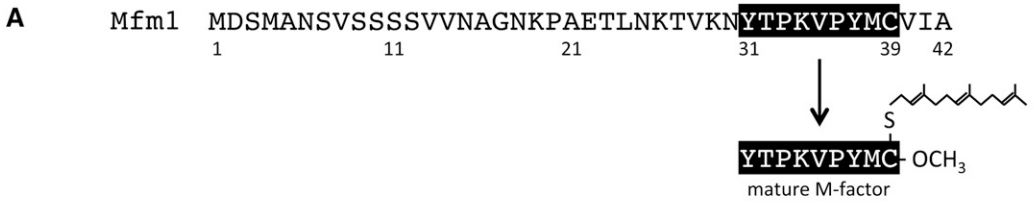
M-factor is a nonapeptide (YTPKVPYMC^{Far}–OCH_3) whose C-terminal Cys residue is *S*-farnesylated and carboxyl-methylated. The M-factor peptide is processed from precursor proteins encoded by *mfm1*$^+$ (Figure 1A), *mfm2*$^+$, and *mfm3*$^+$. We attempted to create a set of single amino acid substitutions throughout the mature peptide. Chemical modifications of Cys are essential for both processing of the precursor protein and the biological activity of the mature peptide (Davey 1992), meaning that the Cys residue is indispensable. Therefore, each of the remaining eight amino acid residues was substituted in turn with the other 19 amino acids by *in vitro* site-directed mutagenesis. We chose the *mfm1*$^+$ gene as a mutagenesis target, because its transcription is the most intense among the three *mfm*$^+$ genes (Kjaerulff *et al.* 1994). We also took codon usage into consideration: for amino acids with multiple codons, the most preferable codon was chosen according to the *S. pombe* codon usage database (Forsburg 1994). The use of degenerate oligonucleotide primers was effective and the strategy was applied successfully; as a result, a full set of 152 missense mutant versions of M-factor were obtained (see Table S1). A homothallic strain lacking all three *mfm*$^+$ genes (called a pheromone-less mutant) fails to produce M-factor and is unable to mate (Kjaerulff *et al.* 1994). Each of 152 mutated *mfm1* genes was integrated at the *ade6* locus of the *h*^{90} pheromone-less strain. Such integrant strains expressed only the mutated *mfm1* gene and thus produced mutant M-factor.

Isolation of 35 sterile mutants carrying missense *mfm1* mutations

Next, the mating efficiency of the 152 missense *mfm1* mutants was inspected by determining the frequency of zygotes. The mutants were incubated on ME solid medium at 28° for 2 days. The SS1001 strain (*mfm1Δ mfm2Δ mfm3Δ ade6-M210* << *mfm1*$^+$) was included as positive control. This integrant strain showed a mating efficiency comparable to that of the wild-type (*mfm1*$^+$ *mfm2*$^+$ *mfm3*$^+$) strain; *i.e.*, the zygote frequency of SS1001 was 73.1 ± 2.2%. The results of 152 missense mutants are summarized in Figure 1B. Repeated microscopic observations revealed that 35 missense mutants failed to mate and were defined as sterile. Figure 1B showed that substitution of amino acid residues in the C-terminal half of M-factor led to a severe defect in mating ability, whereas the substitution of amino acids in the N-terminal half hardly disturbed conjugation. Furthermore, the substitution of amino acids in the C-terminal half of M-factor with basic amino acids led to marked inhibitory effects on mating.

As M-factor induces cell agglutinability in P-cells (Miyata *et al.* 1997), we next examined the sexual agglutinability of all mutants. Cells were first incubated in synthetic medium with a nitrogen source (SSL + N), and were then shifted to medium without nitrogen (SSL − N). The intensity of agglutination (AI) was assayed by photometry (see *Materials and Methods*). In the wild-type culture, strong agglutination was induced. The results of the mutants are presented in Figure 1C. The SS1001 strain (*mfm1*$^+$) showed strong cell agglutination, with an AI of 1.73 ± 0.37. Notably, none of the 35 sterile mutants formed visible cell aggregates (AI < 1.10).

A



B

Substituted amino acids		N ← Amino acid sequence of M-factor → C							
		Y31	T32	P33	K34	V35	P36	Y37	M38
Acidic	D	35.2 ± 7.6	38.4 ± 7.3	28.6 ± 10.3	45.9 ± 13.3	0	20.7±2.3	0	0
	E	46.3 ± 5.7	36.6 ± 7.2	2.7 ± 0.5	3.0 ± 1.8	41.7 ± 12.5	0	0	9.2 ± 5.4
Basic	H	62.4 ± 6.8	47.3 ± 9.4	30.8 ± 3.5	44.9 ± 17.6	0	0	0	0
	K	39.1 ± 7.8	65.4 ± 21.3	21.3 ± 3.9	WT	0	0	0	0
	R	43.7 ± 4.6	58.0 ± 13.8	19.9 ± 2.1	54.9 ± 9.9	0	0	0	0
Neutral Polar	C	36.8 ± 2.5	59.0 ± 3.3	48.2 ± 3.2	26.1 ± 5.9	36.8 ± 8.6	47.7 ± 6.0	8.8 ± 3.2	22.4 ± 4.6
	N	41.1 ± 9.7	56.1 ± 5.5	42.5 ± 4.3	8.1 ± 2.8	0	17.3 ± 12.2	1.8 ± 0.1	0
	Q	51.6 ± 16.9	66.0 ± 3.7	24.8 ± 9.8	44.4 ± 5.1	0	6.5 ± 1.6	0	22.1 ± 3.0
	S	56.9 ± 8.0	37.8 ± 4.9	44.1 ± 12.9	21.4 ± 2.2	1.2 ± 0.5	41.7 ± 4.1	0	0
	T	58.4 ± 4.5	WT	26.6 ± 8.1	26.4 ± 6.8	29.2 ± 15.9	33.8 ± 3.6	0	0
	Y	WT	60.5 ± 4.3	42.7 ± 9.3	51.6 ± 13.2	1.5 ± 0.9	11.9 ± 0.6	WT	10.6 ± 4.7
Neutral Nonpolar	A	40.1 ± 8.9	58.2 ± 5.5	60.6 ± 8.4	20.8 ± 1.7	16.3 ± 7.7	54.0 ± 8.0	0	12.3 ± 6.7
	F	55.8 ± 11.6	40.6 ± 5.0	40.5 ± 6.2	25.5 ± 5.0	2.9 ± 0.8	16.4 ± 2.1	42.1 ± 5.6	20.1 ± 7.8
	G	54.0 ± 9.0	59.9 ± 9.0	46.1 ± 9.6	6.1 ± 1.3	0	47.5 ± 12.3	0	0
	I	29.3 ± 3.0	48.0 ± 5.1	45.9 ± 5.0	21.7 ± 1.9	49.0 ± 7.1	5.6 ± 1.4	20.0 ± 7.3	15.8 ± 6.3
	L	26.3 ± 3.0	57.1 ± 4.1	25.5 ± 5.5	41.8 ± 9.5	10.1 ± 2.1	0	37.6 ± 7.0	45.1 ± 24.2
	M	38.3 ± 5.9	62.0 ± 11.5	25.2 ± 2.4	37.4 ± 6.3	6.5 ± 2.8	13.1 ± 8.1	26.2 ± 5.6	WT
	P	39.0 ± 19.9	36.6 ± 7.3	WT	9.6 ± 3.6	10.7 ± 4.5	WT	0	0
	V	38.9 ± 2.6	54.5 ± 3.8	54.5 ± 2.8	29.8 ± 9.7	WT	29.2 ± 13.4	8.9 ± 1.3	24.6 ± 11.3
	W	43.0 ± 6.7	47.9 ± 7.4	53.2 ± 4.7	24.8 ± 6.1	0	0	26.4 ± 7.8	0

C

Substituted amino acids		N ← Amino acid sequence of M-factor → C							
		Y31	T32	P33	K34	V35	P36	Y37	M38
Acidic	D	1.33 ± 0.13	1.58 ± 0.34	1.24 ± 0.05	1.70 ± 0.08	1.06 ± 0.01	1.16 ± 0.01	1.08 ± 0.01	1.19 ± 0.04
	E	1.47 ± 0.08	1.76 ± 0.25	1.07 ± 0.02	1.11 ± 0.01	1.23 ± 0.03	1.08 ± 0.00	1.06 ± 0.02	1.32 ± 0.03
Basic	H	1.56 ± 0.21	1.67 ± 0.18	1.25 ± 0.02	1.83 ± 0.13	1.05 ± 0.02	1.04 ± 0.01	1.08 ± 0.00	1.10 ± 0.02
	K	1.64 ± 0.04	1.66 ± 0.14	1.19 ± 0.03	WT	1.04 ± 0.00	1.06 ± 0.01	1.05 ± 0.02	1.07 ± 0.08
	R	1.77 ± 0.13	1.71 ± 0.14	1.20 ± 0.01	1.75 ± 0.09	1.05 ± 0.00	1.05 ± 0.01	1.05 ± 0.00	1.16 ± 0.00
Neutral Polar	C	1.52 ± 0.08	1.82 ± 0.27	1.83 ± 0.04	1.33 ± 0.01	1.61 ± 0.09	1.52 ± 0.04	1.09 ± 0.02	1.54 ± 0.08
	N	1.73 ± 0.12	1.84 ± 0.13	1.41 ± 0.09	1.15 ± 0.01	1.09 ± 0.04	1.18 ± 0.02	1.08 ± 0.02	1.11 ± 0.02
	Q	1.72 ± 0.06	1.98 ± 0.00	1.31 ± 0.01	1.46 ± 0.06	1.07 ± 0.05	1.16 ± 0.02	1.08 ± 0.01	1.75 ± 0.07
	S	1.65 ± 0.13	1.65 ± 0.34	1.56 ± 0.06	1.20 ± 0.03	1.17 ± 0.09	1.38 ± 0.03	1.05 ± 0.00	1.21 ± 0.02
	T	1.61 ± 0.09	WT	1.56 ± 0.14	1.22 ± 0.01	1.44 ± 0.04	1.19 ± 0.01	1.06 ± 0.01	1.21 ± 0.02
	Y	WT	1.79 ± 0.24	1.61 ± 0.05	1.85 ± 0.13	1.07 ± 0.02	1.07 ± 0.01	WT	1.02 ± 0.01
Neutral Nonpolar	A	1.61 ± 0.24	1.72 ± 0.22	1.81 ± 0.62	1.36 ± 0.00	1.23 ± 0.00	1.42 ± 0.10	1.07 ± 0.02	1.61 ± 0.05
	F	1.58 ± 0.24	1.80 ± 0.16	1.42 ± 0.03	1.51 ± 0.05	1.12 ± 0.04	1.15 ± 0.01	1.63 ± 0.05	1.46 ± 0.07
	G	1.47 ± 0.11	1.42 ± 0.02	1.71 ± 0.20	1.11 ± 0.02	1.09 ± 0.01	1.58 ± 0.04	1.06 ± 0.02	1.17 ± 0.01
	I	1.67 ± 0.29	1.46 ± 0.46	1.36 ± 0.02	1.30 ± 0.07	1.54 ± 0.05	1.18 ± 0.01	1.40 ± 0.04	1.35 ± 0.02
	L	1.62 ± 0.13	1.65 ± 0.19	1.24 ± 0.02	1.65 ± 0.07	1.11 ± 0.01	1.07 ± 0.00	1.52 ± 0.06	1.66 ± 0.14
	M	1.57 ± 0.21	1.95 ± 0.04	1.25 ± 0.04	1.52 ± 0.06	1.20 ± 0.03	1.12 ± 0.07	1.32 ± 0.01	WT
	P	1.72 ± 0.07	1.22 ± 0.02	WT	1.27 ± 0.01	1.18 ± 0.02	WT	1.07 ± 0.02	1.05 ± 0.02
	V	1.63 ± 0.15	1.51 ± 0.43	1.82 ± 0.16	1.27 ± 0.02	WT	1.16 ± 0.02	1.08 ± 0.01	1.20 ± 0.01
	W	1.81 ± 0.12	1.51 ± 0.42	1.59 ± 0.09	1.37 ± 0.04	1.12 ± 0.07	1.05 ± 0.01	1.26 ± 0.01	1.01 ± 0.01

Figure 1 Mating ability of 152 missense M-factor mutants. (A) Primary structure of the *mfm1*$^+$ gene product. The mature M-factor peptide corresponds to residue Tyr31 to residue Cys39 in the precursor polypeptide. (B) Zygote formation. Frequency of zygotes of the missense mutants of *mfm1* was determined after cultivation of cells on ME agar medium for 2 days. The amino acid sequence of mature M-factor (residues 31–38) is shown at the top and the substituted amino acids grouped according to their chemical nature are shown to the left. Three independent colonies were inspected for zygote formation. The mean zygote percentages with standard deviations are shown. Zygote formation is also indicated as a three-level grading: black, >20%; gray, 0−20%, and white, 0% (sterile). Zygote frequency of the SS1001 strain (*mfm1*$^+$) was 73.1 ± 2.2%. (C) Cell agglutinability. The missense mutants were cultured in SSL−N for 4 hr with gentle shaking. Triplicate samples were taken and the intensity of sexual agglutination was determined. The mean agglutination index (AI) values with standard deviations are presented. The matrix table is constructed as in B. AI values are also indicated as a three-level grading: black, >1.30; gray, 1.10−1.30; and white, ≤1.10 (no aggregate). The AI of the SS1001 strain (*mfm1*$^+$) was 1.73 ± 0.37.

Correlation between zygote formation and agglutination intensity in the mutants

The sterile *mfm1* mutants showed no sexual agglutination. In addition, some leaky mutants with reduced mating ability also showed poor agglutination. As a result, the correlation between efficiency of zygote formation and agglutination intensity was examined in more detail. Macroscopic cell agglutination reflects intense cell-to-cell adhesion between

opposite mating-type cells. Each value of the two parameters in Figures 1, B and C was plotted with zygote frequency on the *x*-axis and AI on the *y*-axis. Figure 2 depicts a clear correlation between the two mating parameters, with a correlation coefficient R^2 of 0.75 ($n = 153$). This linear positive correlation between zygote formation and agglutination intensity suggested that cell-to-cell adhesion is critical for the following cell fusion.

Stepwise deletion of the N-terminal residues of M-factor

As the mating pheromone activity was largely dependent on the primary structure of the C-terminal half of M-factor, we next investigated the effect on mating of deleting residues from the N terminus of the M-factor peptide. We constructed a series of deletion mutants of M-factor, composed of only five to eight amino acids and lacking the N-terminal region of the wild-type peptide (Figure 3A). A mating assay of these deletion mutants revealed that deletion of three amino acid residues from the N terminus of M-factor did not cause a significant reduction in mating ability (Figure 3, B and C). However, removal of one more residue, Lys, dramatically reduced mating capability. Therefore, we conclude that the C-terminal half of M-factor, whose sequence is KVPYMC^{Far}–OCH_3 is sufficient for evoking the mating response. Secretion of this mini M-factor peptide was confirmed as mentioned below (Figure 4D). We also explored the consequence of adding alanine residues to the N terminus of M-factor peptide. An extension of up to two alanine residues affected neither zygote formation nor agglutination (data not shown).

Secretion of sterile-type mutant M-factor peptides

To determine whether the mutant M-factor peptides were secreted by Mam1, we attempted to detect M-factor peptides with altered molecular mass in the culture filtrate by mass spectrometry. As the MALDI–TOF system was not sensitive enough to detect any peaks of M-factor in the culture filtrate, partial purification and concentration of M-factor seemed to be necessary prior to mass spectrometry. Polystyrene resin Amberlite XAD-2 was used to partially purify M-factor according to Davey (1992). As a result, M-factor was detected in the wild-type culture filtrates at a peak in accordance with the expected molecular mass of 1319.6 (Figure 4A). This M-factor peak was absent in the culture filtrate from the *mfm1 mfm2 mfm3* triple deletant, FS55 (Figure 4B).

Next, secretion of the 35 different mutant M-factor peptides in the culture filtrates was examined. Secretion was demonstrated by detecting a peak of expected molecular mass; for example, the M-factor mutant M38N (Met38 substituted with Asn) had a molecular mass of 1302.7 instead of 1319.6 of wild-type M-factor. The appearance of this expected peak coincided with an absence of the wild-type peak (Figure 4C). The above-described mini M-factor peptide composed of six amino acids was also detected (Figure 4D). Likewise, several mutant M-factor peptides were discerned as shown in Figure S1. In summary, among the 35 missense mutants, the following 15 substituted peptides were successfully detected: V35R, V35W, P36E, Y37D, Y37H, Y37Q, Y37R, M38D, M38G, M38H, M38K, M38N, M38R, M38S, and M38W. The other 20 mutant M-factor peptides were not detected, probably owing to an actual reduction in the amount in the culture filtrates or to loss during the purification/concentration procedures. We concluded that at least 15 nonfunctional M-factor peptides are likely to be secreted but not recognized by Map3 receptor proteins.

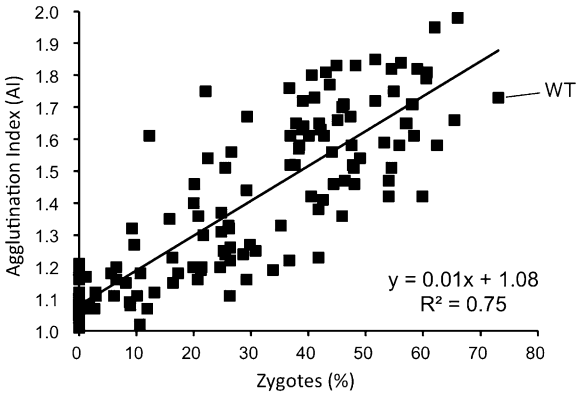


Figure 2 Correlation between zygote frequency and agglutination index of 152 missense M-factor mutants. Frequency of zygotes and AI values of the missense mutants presented in Figure 1 were used. The scatter diagram reveals a clear correlation between percentage of zygotes (*x*) and AI (*y*) of M-factor mutants. The values of the wild-type control were 73.1% (*x*) and 1.73 (*y*). The relationship between the two parameters is described by the linear expression: $y = 0.01x + 1.08$. The coefficient of determination, R^2, is 0.75, indicating a strong correlation between them.

The N-terminal Tyr residue is not essential for processing of M-factor

Processing of the *S. cerevisiae* **a**-factor mating pheromone from a precursor polypeptide has been studied extensively (Marcus *et al.* 1990; Hrycyna *et al.* 1991; Boyartchuk and Rine 1998; Schmidt *et al.* 1998; Tam *et al.* 1998, 2001). By contrast, only limited information is available for *S. pombe* M-factor. The last two sequential steps of **a**-factor processing involve proteolytic cleavage of the N-terminal extension. These steps are catalyzed by different proteases, Ste24p (Fujimura-Kamada *et al.* 1997; Tam *et al.* 2001) and Axl1p (Adames *et al.* 1995) (Figure 5A). In most fungal and yeast pheromones, an N-terminal Tyr residue is highly conserved in the mature peptides (*cf.* Figure 5A). This Tyr residue is thought to be a recognition key for proteolysis. In *S. pombe*, however, our substitution analysis clearly indicated that the N-terminal Tyr31 residue was not required for the biological activity of M-factor (see Figures 1, B and C).

We noted that an Asn residue at the C terminus of the pro-sequence (*i.e.*, Asn30 adjacent to Tyr31) is also conserved among most fungal pheromone peptides (*cf.* Figure 5A) (Martin *et al.* 2011). Interestingly, this residue is Trp

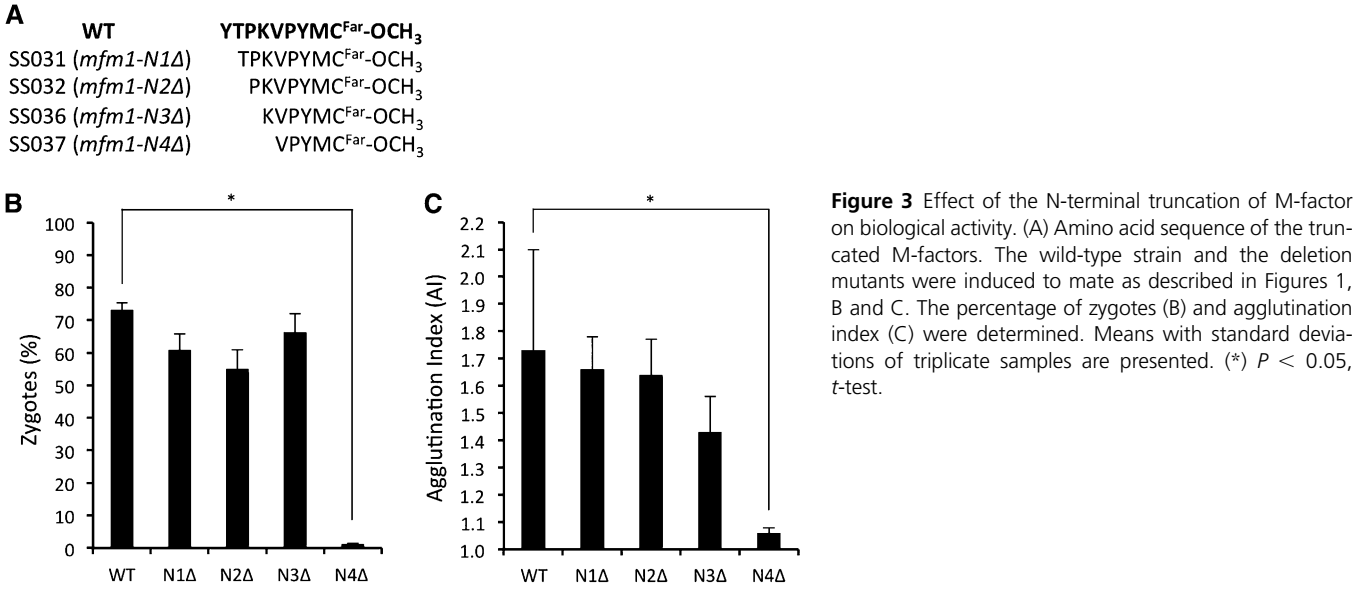


Figure 3 Effect of the N-terminal truncation of M-factor on biological activity. (A) Amino acid sequence of the truncated M-factors. The wild-type strain and the deletion mutants were induced to mate as described in Figures 1, B and C. The percentage of zygotes (B) and agglutination index (C) were determined. Means with standard deviations of triplicate samples are presented. (*) $P < 0.05$, *t*-test.

instead of Asn in 10 putative M-factor precursor proteins of *Schizosaccharomyces octosporus* and *Schizosaccharomyces cryophilus* (Figure 5A). To examine whether this Asn residue is needed for proteolytic cleavage, we introduced missense mutations at this codon (Table S1; SS002−SS019). No M-factor with substitution at the Asn30 residue showed reduced mating efficiency (Figure 5, B and C). In addition, the substitution of Asn30–Tyr31 with Ala30–Ala31 was not inhibitory (SS033). These observations imply that the Asn–Tyr sequence at the putative protease target site of M-factor is not important for its biological activity, and thus processing of M-factor can proceed normally.

Conservation of the primary structure of mature M-factor in S. pombe strains derived from different origins

The primary structure of M-factor is likely to be important for its biological activity (Figures 1, B and C). In fact, substitutions of amino acid residues in the C-terminal half (VPYM) impaired completely or partially the function of M-factor. Surprisingly, amino acid substitutions in the N-terminal half (YTPK) had only a limited effect on mating. Our finding that M-factor is robust to alterations of the primary structure suggests that amino acid substitutions may have occurred quite rapidly during evolution. To test this possibility, we examined 33 strains of *S. pombe* var. *pombe* and close subspecies whose origins differ from that of the standard laboratory strain (L972) first established by U. Leupold (see Table S2). These strains were derived from different sources or geographic locations across the world. All of the *mfm1*, *mfm2*, and *mfm3* genes of these strains were sequenced and compared with the nucleotide sequences of L972 registered at the public database (*Schizosaccharomyces pombe* GeneDB; http://old.genedb.org/genedb/pombe/).

Notably, all of the *mfm* genes of the 34 strains including L972 (*i.e.*, 102 genes in total) were predicted to produce mature M-factor peptides whose primary structure is identical to the known sequence, YTPKVPYMC. Meanwhile, many probable neutral mutations were found in the pro-sequences and intronic sequences (Table 1). One exceptional mutation in the mature M-factor coding region caused no amino acid substitution and thus was a synonymous change. We conclude that the primary structure of mature M-factor is highly conserved in these *S. pombe* strains, although a few neutral mutations have been accumulated in the *mfm* genes.

Discussion

A novel mutational approach to determine the structural requirement of a small peptide pheromone for receptor recognition

Mating-type specificity in yeasts is controlled at several different levels, but the most critical one is the molecular recognition between the peptide-mating pheromone and its cognate receptor protein. To obtain clues to how these molecules specifically interact, mutational analyses have been conducted with budding yeast (Huyer *et al.* 2006). This study took a similar approach, but was more comprehensive in that each of the amino acid residues of this small peptide was substituted by all of the other amino acids. As a result, we succeeded in the creation of 152 such missense mutants. This unprecedented approach enabled us to investigate more thoroughly the structure *vs.* function relationship of M-factor.

Our comprehensive substitution approach clearly indicated that a simple alanine-scanning approach must be applied with care. The results presented here show that the effect of amino acid substitutions at specific residues depends on the amino acid that is substituted. For example, P36A substitution showed no deleterious effect, but P36L caused complete sterility. Conversely, Y37A exhibited a sterile phenotype, but Y37F substitution led to normal mating.

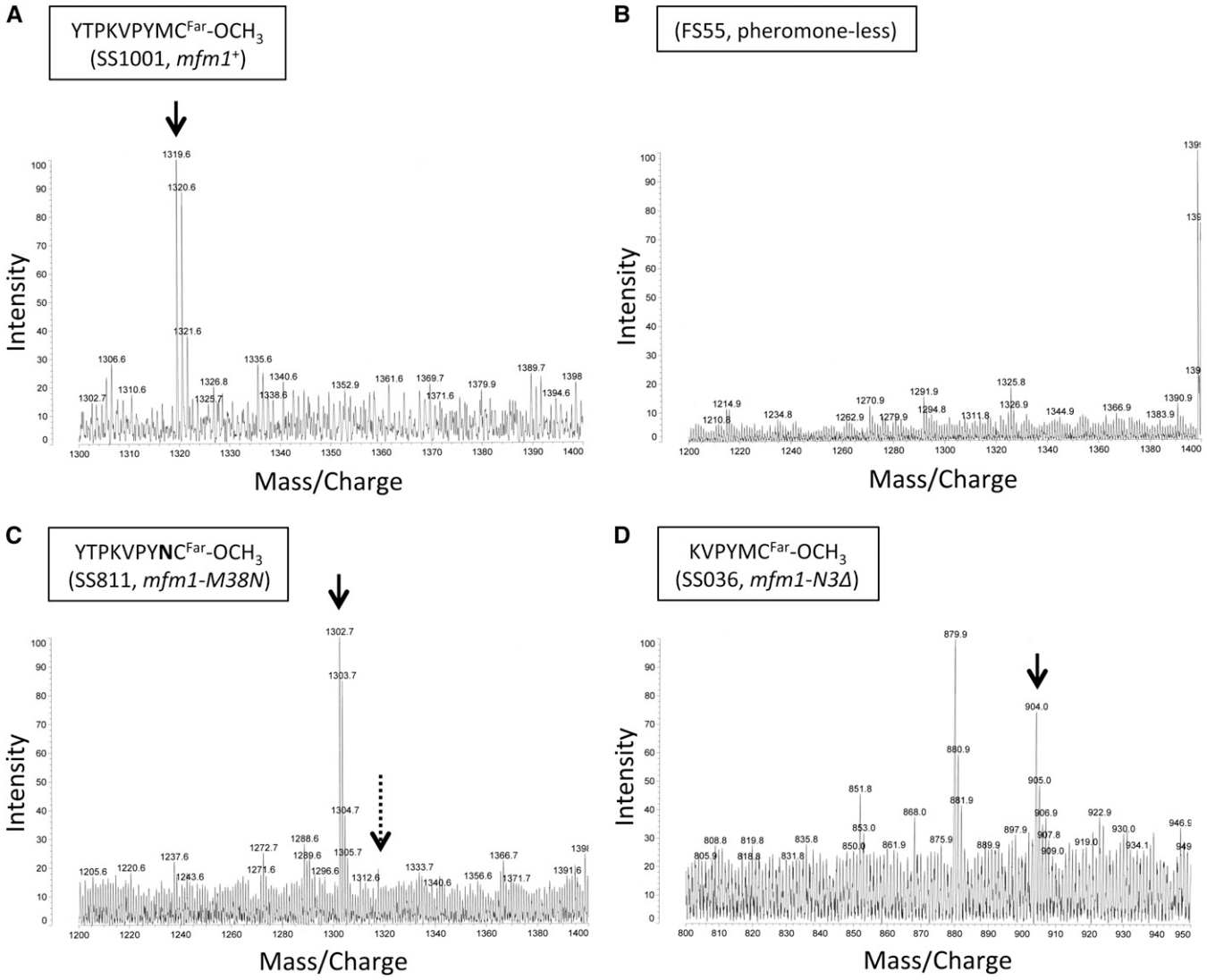


Figure 4 Secretion of mutant M-factors demonstrated by mass spectrometry. Mass spectrometry of M-factor from culture filtrates of a wild-type strain (A; SS1001, *mfm1*$^+$), a pheromone-less mutant (B; FS55, *mfm1Δ*), a missense mutant (C; SS811, *mfm1-M38N*), and a truncated mutant (D; SS036, *mfm1-N3Δ*). The peak corresponding to the mass/charge value expected from the respective amino acid sequence is indicated by the solid downward arrow. The mass/charge position of wild-type M-factor is shown by a dotted arrow in B and C. The expected mass/charge values for M-factor from wild type, *mfm1-M38N* and *mfm1-N3Δ*, are *m/z* 1319.7, 1302.6, and 904.3, respectively. Some other examples of mass spectrometry data are shown in Figure S1.

Similar examples of the different consequences of the substituted amino acids are seen in Figures 1, B and C. In this study, we developed an efficient method applying *in vitro* mutagenesis with degenerate oligonucleotides. This technique will be useful and applicable to biologically active peptides and particular domains of polypeptides to elucidate the function of specific residues or domains reliably.

The N-terminal half of M-factor is dispensable for molecular recognition by its cognate receptor Map3

We isolated 35 sterile mutants among the set of 152 single-substituted missense mutants (Figures 1, B and C). M-factor is secreted by the ABC transporter Mam1, which is highly homologous to *S. cerevisiae* Ste6p, the transporter responsible for the export of **a**-factor mating pheromone (Christensen *et al.* 1997). Mature M-factor peptides, which are formed from the precursor polypeptide by proteolysis, must be recognized by Mam1 transporter on the plasma membrane for secretion. It is possible that some of the nonfunctional mutant M-factor peptides are poorly recognized by Mam1. This seems unlikely, however, because the substrate specificity of the Mam1 transporter is known to be low, as indicated by the following observations: green fluorescent protein is exported by Mam1 when sandwiched between the propeptide sequence and a CAAX motif of M-factor (Kjaerulff *et al.* 2005), and ectopically expressed *S. cerevisiae* Ste6p facilitates the export of M-factor in *S. pombe* cells (Christensen *et al.* 1997). These observations imply that the M-factor-

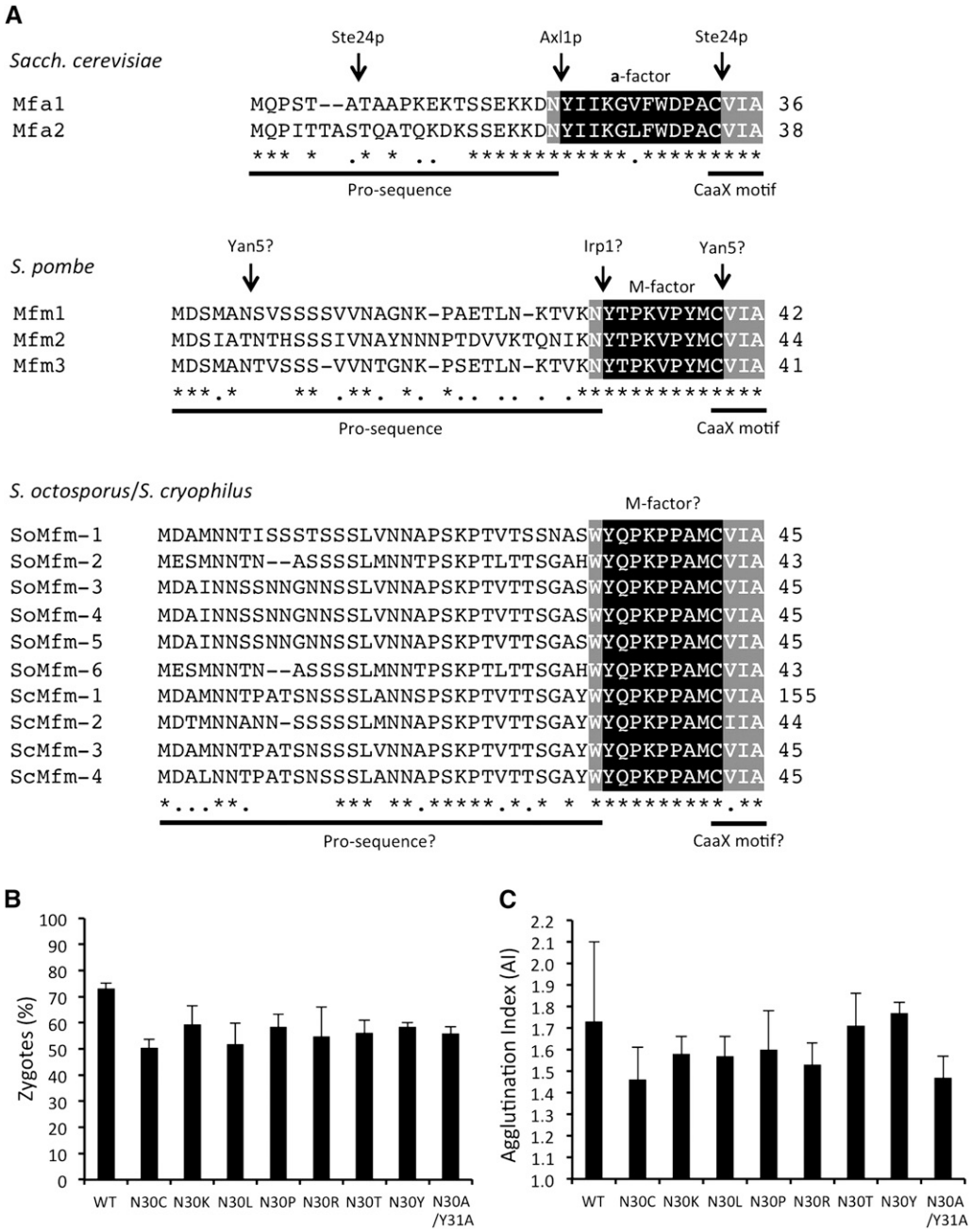


Figure 5 Substitution of amino acid residues at a putative cleavage site of M-factor processing. (A) Sequence alignment of the precursors derived from the different genes of **a**-factor in *S. cerevisiae* (top), of M-factor in *S. pombe* (middle), and of putative M-factor in *S. octosporus* (6 genes) and in *S. cryophilus* (4 genes) (bottom). M-factor-coding genes of *S. octosporus* and *S. cryophilus* were annotated by the *Schizosaccharomyces* group Sequencing Project, Broad Institute of Harvard and MIT (http://www.broadinstitute.org/) (Rhind *et al.* 2011). Putative M-factor precursor proteins of *S. octosporus* are tentatively termed SoMfm-1 to SoMfm-6; similarly, those of *S. cryophilus* are termed ScMfm-1 to ScMfm-4. Numerals to the right of the sequence represent the length of the precursor proteins. Homology of amino acids is shown by asterisks (identical) or dots (similar). Multiple sequence alignment was done by a standard algorithm (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Mature peptide sequences are shown by white letters on black. Flanking conserved residues are indicated by white letters on gray. Experimentally identified proteases involved in the specific cleavage of precursors are shown above the **a**-factor sequence, and the hypothesized proteases for M-factor are also shown. (B and C) Mating ability of the missense mutants was determined as described in Figures 1, B and C. Percentage of zygotes (B) and agglutination index (C) are shown. Means with standard deviations of triplicate samples are presented.

specific transporter Mam1 is probably dependent on the farnesylated Cys residue, but not on other amino acid residues of M-factor. As a result, most of the mutant M-factor peptides should be recognized by the Mam1 transporter and secreted into the culture medium.

In fact, 15 nonfunctional M-factor peptides were successfully detected by mass spectrometry (Figure 4 and Figure S1), indicating that these peptides are indeed secreted by the Mam1 transporter. For the other 20 mutant M-factors, insufficient recognition by the Map3 receptor will need to be confirmed directly by using chemically synthesized peptides. It is probable that most of the 35 sterile M-factors are nonfunctional due to poor recognition by the wild-type receptor Map3, although other possibilities cannot be completely ruled out. We propose the possibility that certain missense mutations of the *map3* gene might enable the receptor to recognize the mutant M-factors. Hopefully, searching for such suppressor *map3* mutants will be feasible, because an efficient screening strategy for isolating mating-sufficient revertants in *S. pombe* is available (Kitamura and Shimoda 1991). Mapping of such suppressor mutations in the *map3* ORF may enable us to identify the ligand-binding sites of the receptor. This kind of genetic analysis of mutant ligand/receptor combinations will hopefully contribute to GPCR biology.

As shown in Figures 1, B and C, the primary structure of M-factor is likely to be associated with its biological activity.

Table 1 Polymorphic alleles in the three M-factor-encoding genes of *S. pombe* strains with different origins from that of the standard laboratory strain L972

Genes	Base substitution and insertion	Amino acid substitution	Regions	No. of strains	Strains
mfm1	T11A	M4K	Propeptide	1	YHL274
	G12A	M4I	Propeptide	3	EF6, NBRC0354, YHL280
	T24C	V8V	Propeptide	1	NBRC0354
	A88G	N30D	Propeptide	3	MS9-1, SP-6, JCM8262
	G147T		Intron	1	YHL271
	G187A		Intron (3′ consensus)	5	JCM8274, YHL264, YHL272, YHL273, YHL274
mfm2	T12C	I4I	Propeptide	5	EF6, NBRC0345, NBRC354, YHL274, YHL280
	A26C	H9P	Propeptide	8	MS9-1, SP-6, JCM8262, JCM1846, YHL267, YHL275, YHL277, YHL282
	T28C	S10P	Propeptide	2	EF6, YHL280
	T39C	I13I	Propeptide	1	EF6
	133 (ins- GCT)		Intron	8	MS9-1, SP-6, JCM8262, JCM1846, YHL267, YHL275, YHL277, YHL282
	T148A		Intron	3	MS9-1, SP-6, JCM8262
	C149T		Intron	3	MS9-1, SP-6, JCM8262
	T160C		Intron	3	MS9-1, SP-6, JCM8262
	A161G		Intron	3	MS9-1, SP-6, JCM8262
mfm3	A9T	S3S	Propeptide	3	JCM8274, JCM1846, YHL282
	G99A	K33K	Mature peptide	3	JCM8274, JCM1846, YHL282
	A121G		Intron	3	JCM8274, JCM1846, YHL282
	T127C		Intron	3	JCM8274, JCM1846, YHL282
	T138C		Intron	3	JCM8274, JCM1846, YHL282
	C140T		Intron	3	JCM8274, JCM1846, YHL282
	G148A		Intron	1	JCM8274
	151 (ins A)		Intron	3	JCM8274, JCM1846, YHL282
	T153C		Intron	3	JCM8274, JCM1846, YHL282
	159 (ins CAAA)		Intron	3	JCM8274, JCM1846, YHL282
	A161G		Intron	3	JCM8274, JCM1846, YHL282
	T162C		Intron	3	JCM8274, JCM1846, YHL282
	T165A		Intron	1	JCM8274

The coding regions of *mfm1*, *mfm2*, and *mfm3* were amplified by PCR. The products were subjected to nucleotide sequencing and compared with the corresponding sequence of L972. Differences from the L972 sequence are listed, and the deduced amino acid substitutions are indicated. The mutation sites are classified into the following three categories: propeptide, intron, and mature peptide. Strains harboring the same alterations are grouped together.

Amino acid substitution of the Y31 and T32 residues with other amino acids did not cause a marked effect on mating efficiency. Amino acid substitution of the P33 and K34 residues led to only a partial mating defect, depending on substituted amino acids (Figure 1B). Interestingly, however, some mutants with substitutions of residues in the C-terminal half (V35, P36, Y37, and M38) showed significantly reduced mating ability. Notably, substitution with basic amino acids caused severe mating defects (Figures 1, B and C), suggesting that the electric charge of amino acids may play a role in the interaction of pheromone peptides with their corresponding receptor Map3. M-factor contains a long hydrophobic farnesyl group at the carboxyl terminus. The importance of the C-terminal half for activity may relate to the hydrophobicity of the farnesyl group. One possible mechanism is that the hydrophobic tail is involved in association of M-factor with the plasma membrane; this association may then facilitate interaction of the C-terminal half of the peptide moiety of M-factor with ligand-binding residues of the Map3 receptor. Mutational analysis of *S. cerevisiae* **a**-factor suggested that the N-terminal region is important for function (Huyer *et al.* 2006). This inconsistency may come from the difference in amino acids recognized by the receptor, although it is also possible that the mutagenesis of **a**-factor was not extensive enough to draw a firm conclusion. Further comprehensive mutagenesis seems necessary to adequately compare the functional sites of these two pheromone peptides.

Our mating assay indicated that amino acid substitutions in the N-terminal half of M-factor can be made without affecting the biological activity. In addition, a mini M-factor composed of 6 amino acid residues without the three N-terminal residues retained activity. Despite these observations, the putative M-factor peptides are fully conserved in 34 strains of *S. pombe* strains collected from different areas. We speculate that the wild-type sequence confers a selective advantage under certain conditions, especially in nature. Mating assays with different experimental conditions may verify such a selective advantage of wild-type M-factor.

Necessity of the conserved dipeptide sequence at a cleavage site of the precursor protein

Prenylation of the C-terminal Cys residue is required for both processing and biological function in *S. pombe* (Davey 1992). Comparison of the primary structure of the pheromone peptides suggests that the two consecutive amino

acids, Asn and Tyr, at the N-terminal cleavage site are well conserved. In fact, the cleavage site of M-factor precursors encoded by the three different genes of *S. pombe* is Asn-Tyr, and this is also the case for two **a**-factor precursor proteins of *S. cerevisiae* (Figure 5A). Despite this conservation, substitutions of these two amino acids with others did not affect mating activity *in S. pombe*, indicating that proteolytic cleavage may occur normally. The cutting site appears to be determined by a broader sequence. In support of this idea, even distant deletions within the N-terminal extension block Axl1p processing in *S. cerevisiae* (Huyer *et al.* 2006). The structure and/or length of the pro-sequence might be important for this cleavage step.

Why are there three redundant genes for M-factor?

The composition of genes encoding pheromone peptides differs between the two mating types in yeasts. In *S. pombe*, P-factor is encoded by a single gene (*map2*$^+$), which contains four tandem copies of mature P-factor-coding sequences (Imai and Yamamoto 1994); by contrast, M-factor is encoded by three unlinked genes, *mfm1*$^+$, *mfm2*$^+$, and *mfm3*$^+$ (Figure 5A). All three genes produce a polypeptide precursor that is then processed to yield a small mature peptide with the same amino acid sequence. Deletion of any one or two of the *mfm* genes causes no remarkable mating defects (Kjaerulff *et al.* 1994). Such multiple structural genes for mating pheromones are often found in other yeasts and fungi (Martin *et al.* 2011): for instance, *S. cerevisiae* contains two redundant pheromone genes, *S. octosporus*, the species most closely related to *S. pombe*, has six genes, and *S. cryophilus* also has four genes (Figure 5A). The redundant genes of *S. octosporus* and *S. cryophilus* also encode M-factor peptides of the same amino acid sequence. Furthermore, the primary structures of the putative M-factors of *S. octosporus* and *S. cryophilus* are the same. Three of the nine amino acid residues (T32, V35, and Y37) of *S. pombe* M-factor are different from those of putative *S. octosporus* and *S. cryophilus* M-factors (Q32, P35, and A37) (Figure 5A). Synthetic M-factor peptides of the latter were found to be ineffective on *S. pombe* P-cells (data not shown). Our assay also showed that a Y37A substitution caused complete sterility (Figure 1B). Therefore, *S. pombe* is reproductively isolated from *S. octosporus* and *S. cryophilus*, at least in terms of the compatibility of M-factor and its cognate receptor.

Why are three redundant genes for M-factor present in the *S. pombe* genome? The identity of amino acid sequences between any pair of the *mfm* gene products is high, ranging from 74 to 94%. All three *mfm* genes contain one intron, and the location of this single intron is also conserved (Kjaerulff *et al.* 1994). Thus, the question is, Why are three redundant genes necessary for the synthesis of M-factor peptides? We can propose the three following possibilities: first, pheromone peptides must be produced quickly in large amounts under environmental conditions favorable for sexual reproduction; second, expression of the respective genes may be differentially controlled, enabling fine-tuning; third, if the receptors are mutated, such redundancy enables the cells to alter one copy to become well adapted to receptor changes, while keeping the others unchanged. In this way, haploid *S. pombe* cells are able to adapt flexibly to various mutational changes in receptor proteins.

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Remarkably Simple Sequence Requirement of the M-Factor Pheromone of *Schizosaccharomyces pombe*

Taisuke Seike, Yoshikazu Yamagishi, Hideo Iio, Taro Nakamura, and Chikashi Shimoda

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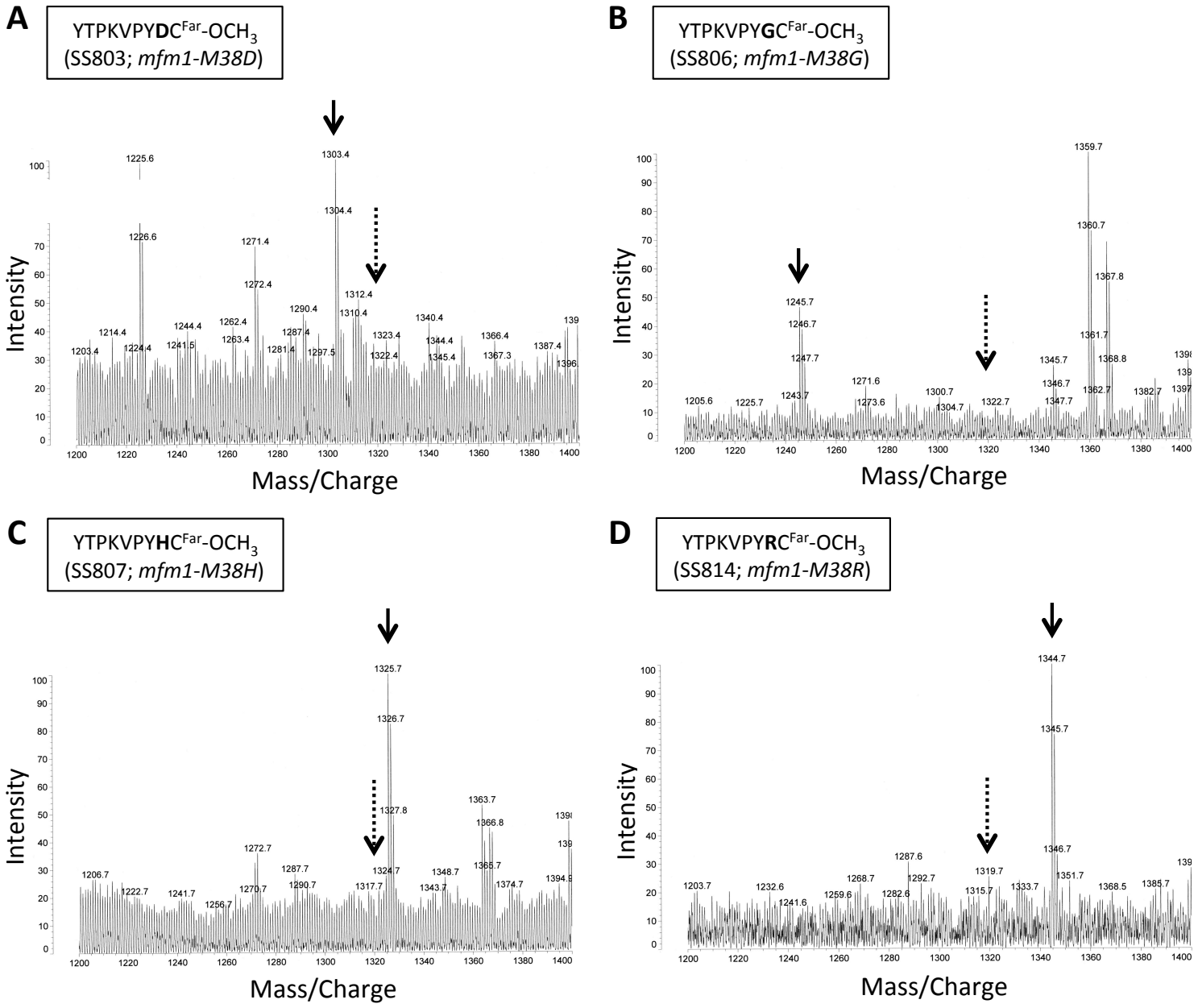

Figure S1 Secretion of mutant M-factors demonstrated by mass spectrometry. Mass spectrometry of partially purified M-factor in the culture filtrates from strains SS803 (A, M38D), SS806 (B, M38G), SS807 (C, M38H) and SS814 (D, M38R). The peak corresponding to the mass/charge value expected from the respective amino acid sequence is indicated by the solid downward arrow. The mass/charge position of wild-type M-factor is shown by a dotted arrow in A, B, C and D. The expected mass/charge values for A, B, C and D are *m/z* 1303.6, 1245.6, 1325.7 and 1344.7, respectively.

Table S1 Strains used in this study

Strains	Genotype	Source
FS55 (FY17222)[a]	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210*	C. Shimoda
SS002	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-N30C*	This study
SS009	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-N30K*	This study
SS010	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-N30L*	This study
SS012	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-N30P*	This study
SS014	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-N30R*	This study
SS016	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-N30T*	This study
SS019	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-N30Y*	This study
SS020	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-N30Stop*	This study
SS031	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-1D*	This study
SS032	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-2D*	This study
SS033	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-N30A/Y31A*	This study
SS036	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-3D*	This study
SS037	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-4D*	This study
SS101	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-Y31A*	This study
SS102	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-Y31C*	This study
SS103	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-Y31D*	This study
SS104	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-Y31E*	This study
SS105	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-Y31F*	This study
SS106	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-Y31G*	This study
SS107	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-Y31H*	This study
SS108	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-Y31I*	This study
SS109	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-Y31K*	This study
SS110	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-Y31L*	This study
SS111	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-Y31M*	This study
SS112	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-Y31N*	This study
SS113	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-Y31P*	This study
SS114	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-Y31Q*	This study
SS115	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-Y31R*	This study
SS116	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-Y31S*	This study
SS117	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-Y31T*	This study
SS118	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-Y31V*	This study
SS119	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-Y31W*	This study
SS120	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-Y31Stop*	This study
SS201	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-T32A*	This study

SS202	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-T32C*	This study
SS203	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-T32D*	This study
SS204	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-T32E*	This study
SS205	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-T32F*	This study
SS206	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-T32G*	This study
SS207	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-T32H*	This study
SS208	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-T32I*	This study
SS209	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-T32K*	This study
SS210	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-T32L*	This study
SS211	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-T32M*	This study
SS212	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-T32N*	This study
SS213	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-T32P*	This study
SS214	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-T32Q*	This study
SS215	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-T32R*	This study
SS216	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-T32S*	This study
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SS317	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-P33V*	This study
SS318	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-P33W*	This study
SS319	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-P33Y*	This study

SS320	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-P33Stop*	This study
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SS406	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-K34G*	This study
SS407	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-K34H*	This study
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SS410	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-K34M*	This study
SS411	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-K34N*	This study
SS412	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-K34P*	This study
SS413	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-K34Q*	This study
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SS419	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-K34Y*	This study
SS420	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-K34Stop*	This study
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SS505	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-V35F*	This study
SS506	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-V35G*	This study
SS507	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-V35H*	This study
SS508	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-V35I*	This study
SS509	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-V35K*	This study
SS510	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-V35L*	This study
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SS512	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-V35N*	This study
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SS514	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-V35Q*	This study
SS515	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-V35R*	This study
SS516	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-V35S*	This study
SS517	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-V35T*	This study

SS518	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-V35W*	This study
SS519	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-V35Y*	This study
SS520	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-V35Stop*	This study
SS601	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-P36A*	This study
SS602	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-P36C*	This study
SS603	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-P36D*	This study
SS604	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-P36E*	This study
SS605	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-P36F*	This study
SS606	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-P36G*	This study
SS607	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-P36H*	This study
SS608	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-P36I*	This study
SS609	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-P36K*	This study
SS610	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-P36L*	This study
SS611	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-P36M*	This study
SS612	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-P36N*	This study
SS613	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-P36Q*	This study
SS614	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-P36R*	This study
SS615	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-P36S*	This study
SS616	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-P36T*	This study
SS617	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-P36V*	This study
SS618	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-P36W*	This study
SS619	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-P36Y*	This study
SS620	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-P36Stop*	This study
SS701	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-Y37A*	This study
SS702	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-Y37C*	This study
SS703	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-Y37D*	This study
SS704	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-Y37E*	This study
SS705	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-Y37F*	This study
SS706	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-Y37G*	This study
SS707	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-Y37H*	This study
SS708	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-Y37I*	This study
SS709	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-Y37K*	This study
SS710	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-Y37L*	This study
SS711	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-Y37M*	This study
SS712	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-Y37N*	This study
SS713	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-Y37P*	This study
SS714	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-Y37Q*	This study
SS715	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-Y37R*	This study

SS716	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-Y37S*	This study
SS717	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-Y37T*	This study
SS718	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-Y37V*	This study
SS719	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-Y37W*	This study
SS720	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-Y37Stop*	This study
SS801	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-M38A*	This study
SS802	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-M38C*	This study
SS803	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-M38D*	This study
SS804	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-M38E*	This study
SS805	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-M38F*	This study
SS806	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-M38G*	This study
SS807	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-M38H*	This study
SS808	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-M38I*	This study
SS809	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-M38K*	This study
SS810	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-M38L*	This study
SS811	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-M38N*	This study
SS812	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-M38P*	This study
SS813	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-M38Q*	This study
SS814	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-M38R*	This study
SS815	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-M38S*	This study
SS816	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-M38T*	This study
SS817	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-M38V*	This study
SS818	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-M38W*	This study
SS819	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-M38Y*	This study
SS820	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1-M38Stop*	This study
SS1001	*h^{90} mfm1::LEU2 mfm2-D4 mfm3::ura4^{+} leu1-32 ura4-D18 ade6-M210<<ade6^{+}:mfm1^{+}*	This study

The *S. pombe* strains constructed in this study will be deposited at the Yeast Genetic Resource Center of Japan, which is supported by the National BioResource Project YGRC/NBRP. [a]This strain was obtained from YGRC/NBRP (http:/yeast.lab.nig.ac.jp/nig/).

Table S2 List of *S. pombe* strains whose origin differs from L972

Strains	Taxonomy	Source	Area	Credit
L972	*Schizosaccharomyces pombe* var. *pombe*	grape juice	Switzerland	A. Osterwalder/U. Leupold
MS5-1	*S. pombe* var. *pombe*			
MS9-1	*S. pombe* var. *malidevorans*			
SP-6	*S. pombe* var. *malidevorans*			
JCM8262	*S. pombe* var. *malidevorans*			Y. Yamada
JCM8274	*S. pombe* var. *pombe*			
JCM21876	*S. pombe* var. *ogasawaraensis*	molasses	Japan	K. Sakaguchi
JCM21877	*S. pombe* var *ioensis*	molasses	Japan	K. Sakaguchi
JCM1846	*S. asporus* (type strain)			
EF6	*S. pombe* var. *pombe*	distillery	Scandinavia	H. Gutz
NBRC0345	*S. pinan* (type strain)	molasses		
NBRC0354	*S. mallacei* (type strain)			
NBRC0365	*S. taito* (type strain)			
YHL264	*S. pombe* var. *pombe*			
YHL265	*S. pombe* var. *pombe*			
YHL266	*S. pombe* var. *pombe*	currant jelly		
YHL267	*S. pombe* var. *pombe*			
YHL268	*S. pombe* var. *pombe*	cane sugar molasses	Japan	R. Nakazawa
YHL269	*S. pombe* var. *pombe*	African millet beer		
YHL270	*S. pombe* var. *pombe*	apple must		
YHL271	*S. pombe* var. *pombe*	young wine	Stellenbosch (South Africa)	van der Walt
YHL272	*S. pombe* var. *pombe*			
YHL273	*S. pombe* var. *pombe*			
YHL274	*S. pombe* var. *pombe*			
YHL275	*S. pombe* var. *pombe*			
YHL276	*S. pombe* var. *pombe*			
YHL277	*S. pombe* var. *pombe*	Soughum beer	Mbabane (Switzerland)	van der Walt
YHL278	*S. pombe* var. *pombe*	maize starch	Johannesburg (South Africa)	van der Walt
YHL279	*S. pombe* var. *pombe*			
YHL280	*S. pombe* var. *pombe*			
YHL281	*S. pombe* var. *pombe*	malt	Holland	
YHL282	*S. pombe* var. *pombe*			
YHL283	*S. pombe* var. *pombe*	cane sugar molasses	Japan	
YHL284	*S. pombe* var. *pombe*			

Table S3 Primers used in this study

Primers	Purpose	Sequence
Mfm1-1	For mutagenesis by Quik-Change	ccgttaagaatNNNacccccaaggttccttacatgtggtaag
Mfm1-2	For mutagenesis by Quik-Change	ccgttaagaattatNNNcccaaggttccttacatgtggtaag
Mfm1-3	For mutagenesis by Quik-Change	ccgttaagaattataccNNNaaggttccttacatgtggtaag
Mfm1-4	For mutagenesis by Quik-Change	ccgttaagaattatacccccNNNgttccttacatgtggtaag
Mfm1-5	For mutagenesis by Quik-Change	ccgttaagaattatacccccaagNNNccttacatgtggtaag
Mfm1-6	For mutagenesis by Quik-Change	ccgttaagaattatacccccaaggttNNNtacatgtggtaag
Mfm1-7	For mutagenesis by Quik-Change	ccgttaagaattatacccccaaggttcctNNNatgtggtaag
Mfm1-8	For mutagenesis by Quik-Change	ccgttaagaattatacccccaaggttccttacNNNtggtaag
Mfm1-9	For mutagenesis by Quik-Change	ccgttaagNNNtatacccccaaggttccttacatgtggtaag
Mfm1(F)	For sequencing	ggaagcttgctgttgggatagctttg
Mfm1(R)	For sequencing	gcaagcttgctctagtaccacagcgtatg
Mfm2(F)	For sequencing	cgtaggtattacaggaagat
Mfm2(R)	For sequencing	cattggaaagttaaggcacg
Mfm3(F)	For sequencing	gtgtccgaatggaataatcc
Mfm3(R)	For sequencing	ggacctaaatctcaatcatc