

RESEARCH ARTICLE

Identification and characterization of the *Komagataella phaffii* mating pheromone genes

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One sentence summary: The mating pheromone genes of the yeast *Komagataella phaffii* were identified and characterized.

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ABSTRACT

The methylotrophic yeast *Komagataella phaffii* (*Pichia pastoris*) is a haploid yeast that is able to form diploid cells by mating once nitrogen becomes limiting. Activation of the mating response requires the secretion of a- and α -factor pheromones, which bind to G-protein coupled receptors on cells of opposite mating type. In *K. phaffii*, the genes coding for the α -factor (*MFA*), the pheromone surface receptors and the conserved a-factor biogenesis pathway have been annotated previously. Initial homology-based search failed to identify potential a-factor genes (*MFA*). By using transcriptome data of heterothallic strains under mating conditions, we found two *K. phaffii* a-factor genes. Deletion of both *MFA* genes prevented mating of a-type cells. *MFA* single mutants were still able to mate and activate the mating response pathway in α -type cells. A reporter assay was used to confirm the biological activity of synthetic a- and α -factor peptides. The identification of the a-factor genes enabled the first characterization of the role and regulation of the mating pheromone genes and the response of *K. phaffii* to synthetic pheromones and will help to gain a better understanding of the mating behavior of *K. phaffii*.

Keywords: *Komagataella phaffii*; *Pichia pastoris*; yeast; mating; mating factor; pheromone

INTRODUCTION

Yeast mating is mediated by secreted peptide pheromones binding to surface receptors on cells of the opposite mating type. In ascomycetes, one cell type secretes unmodified hydrophilic peptides (known as α -factor), whereas cells of the other mating type produce hydrophobic peptides with a characteristic C-terminal farnesyl and carboxymethyl modification (a-factor). Secretion of these small peptides is essential for mating partner recogni-

tion and activation of the genes required for diploid cell formation.

The different aspects of the mating process are well understood in the model yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* but are much less characterized in other relevant yeast species. The *S. cerevisiae* α -factor pheromone is an unmodified hydrophilic peptide of 13 amino acids secreted exclusively by haploid cells of the α -mating type. It is encoded by *MFA1* and *MFA2* and translated as precursor containing four

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and two copies of the mature α -factor, respectively (Kurjan and Herskowitz 1982; Singh et al. 1983). In *S. cerevisiae* a-type cells, the MFA1 and MFA2 genes are constitutively transcribed and translated into precursor proteins of 36 and 38 amino acids, respectively (Michaelis and Herskowitz 1988). While the α -factor is processed and secreted via the classical secretory pathway, the a-factor is modified in the cytosol. Following translation, the C-terminal CAAX motif (C is cysteine, A is usually aliphatic, X can be any amino acid) is recognized by a cytosolic farnesyl transferase consisting of two subunits (Ram1 and Ram2) resulting in farnesylation of the C-terminal cysteine residue. Subsequently, the three C-terminal amino acids (AAX) are cleaved by the proteases Rce1 or Ste24 and a carboxylmethyl group is added to the farnesylcysteine by the isoprenyl cysteine carboxyl methyltransferase Ste14. The N-terminus of a-factor is processed in two consecutive proteolytic cleavage steps by zinc proteases Ste24 and Axl1, resulting in mature a-factor peptides with a length of only 12 amino acids differing solely in a single residue (Betz et al. 1987; Michaelis and Barrowman 2012). Mature a-factor is exported from the cytosol by Ste6, an ATP-binding cassette (ABC) transporter located in the plasma membrane (Kuchler, Sterne and Thorne 1989). Once secreted, a-factor binds to the G-protein coupled receptor Ste3 on the surface of α -type cells, activating a mitogen-activated protein kinase (MAPK) signaling cascade resulting in the activation of genes required for mating, growth of mating projections (shmoos) and arrest in the G1 phase of the cell cycle (Jones and Bennett 2011; Merlini, Dudin and Martin 2013).

The enzymes of the a-factor biogenesis pathway are conserved in yeasts and higher eukaryotes. However, due to their short length and poor sequence conservation, the genes encoding a-factor pheromones are hard to identify. Besides the well-characterized *S. cerevisiae* mating factors, a-factor pheromones have been investigated in a number of other yeast species like *Sc. pombe* (M-factor), *Kluyveromyces lactis* and *Candida albicans* (Davey 1992; Kjaerulff, Davey and Nielsen 1994; Dignard et al. 2007; Ongay-Larios et al. 2007). All the identified proteins contain a C-terminal CAAX motif for farnesylation and were found to be essential for mating of a-type cells. The number of genes encoding the a-factor varies in different species, from only one gene copy in *C. albicans* or *Eremothecium gossypii* to up to 12 predicted MFA genes in *C. tropicalis* (Dignard et al. 2007; Wendland, Dunkler and Walther 2011). It has been hypothesized that the redundancy in the MFA genes might be necessary to allow evolution of pheromones required for the development of new reproductive groups while ensuring mating within a species (Seike, Nakamura and Shimoda 2015).

The methylotrophic yeast *Komagataella phaffii* (also referred to as *Pichia pastoris*) is a haploid yeast that is able to form diploids by mating if nitrogen becomes limiting. Wild-type strains are homothallic and can undergo mating-type switching by inversion of the chromosomal region between two mating-type (MAT) loci located on chromosome 4. While the expression of the MAT genes at MAT locus 1 is downregulated by telomere position effects, the mating type of a cell is determined by the MAT genes actively transcribed from locus 2. Both MAT loci are flanked by large inverted repeat regions which have been proposed as homologous regions involved in mating-type switching (Hanson, Byrne and Wolfe 2014). Deletion of the 'outer' repeat region at MAT locus 2 (including DIC1-2) prevents mating-type switching and allowed the generation of *K. phaffii* strains with stable mating types (Heisting, Gasser and Mattanovich 2018).

The *K. phaffii* α -factor is encoded by a single MFA gene (CBS7435_Chr2-0522) and is translated as a precursor peptide

consisting of a pre-pro leader sequence and nine copies of the mature α -factor flanked by Kex2 and Ste13 processing sites (Kuberl et al. 2011). Experiments using the pre-pro leader sequence have shown that it can be used for efficient secretion of recombinant proteins (Kuberl et al. 2011) but no further studies characterizing the *K. phaffii* α -factor peptide itself have been conducted. Orthologs of α - and a-factor receptor genes (STE2 and STE3, respectively) as well as all the genes essential for a-factor processing and secretion in other yeasts can be found in the *K. phaffii* genome using homology-based search (Kuberl et al. 2011; Valli et al. 2016). However, so far no gene coding for the a-factor pheromone has been identified.

Within this study we could identify two putative MFA genes in the genomes of *K. phaffii* and *K. pastoris* by using a combination of transcriptomics data analysis and homology search. The role of the newly identified peptides in *K. phaffii* mating was confirmed using MFA deletion mutants and fully modified synthetic pheromones. Furthermore, we analyzed the mating phenotype of *mfa* Δ mutants and the response of *K. phaffii* cultures to synthetic α -factor.

MATERIALS AND METHODS

Yeast strains and vectors

All *K. phaffii* strains described in this study (Table 1) are based on the homothallic CBS2612 wild-type strain. Generation of the *dic1-2* Δ strains with stable mating types was described in detail in Heisting, Gasser and Mattanovich (2018). To obtain the mating-factor deletion strains, the whole coding sequence of the target gene was replaced by an antibiotic resistance marker using the split-marker technique (Fairhead et al. 1996; Gasser et al. 2013). Homologous flanking regions of around 1000 bp for integration were PCR amplified from *K. phaffii* genomic DNA with primers containing *Bsa*I restriction sites and fusion sites (FS) for Golden Gate cloning (FS AB and CD for the 5' and 3' region, respectively) using the previously published GoldenPiCS system (Weber et al. 2011; Prielhofer et al. 2017; Sarkari et al. 2017). Marker sequences were amplified from available plasmid vectors to introduce FS BC. In the next step, a vector (BB3) containing the marker gene flanked by the two homologous regions was assembled by Golden Gate cloning using *Bsa*I. This vector was used as template for PCR-based amplification of the two knockout cassettes overlapping in the marker gene sequence. For the construction of the P_{FUS1}-eGFP reporter construct, the sequence upstream of the FUS1 start codon (Chr4:560823..560339 [FR839631.1]) was amplified from genomic DNA of *K. phaffii* CBS2612 using primers containing FS AB and *Bsa*I restriction sites for cloning into a BB1 GoldenPiCS vector. In the next step, the expression cassette consisting of the enhanced green fluorescent protein (eGFP) coding sequence under control of the FUS1 promoter and *S. cerevisiae* CYC1 terminator was assembled from the available BB1 constructs into a BB3 vector using *Bpi*I. The final plasmid also contained a NatMX resistance cassette for selection with nourseothricin and the AOX1 terminator sequence as homologous region for genomic integration after linearization with *Asc*I. Empty pPUZ-ZLE vectors were used for the integration of additional marker genes required for the selection of diploid cells into the TDH3 promoter or AOX1 terminator region, respectively (Stadlmayr et al. 2010). All primers used in this study are listed in Table S1 (Supporting Information).

Table 1. List of strains used in this study.

Strain name	Genotype	Mating type	Source
CBS2612	Wild type	mixed	
CBS2612 <i>dic1-2Δ</i> (α)	<i>dic1-2Δ::loxP</i>	MAT α	Heistingering, Gasser and Mattanovich (2018)
CBS2612 <i>dic1-2Δ</i> (a)	<i>dic1-2Δ::loxP</i> , MAT α - <i>loxP</i> ^a	MAT α	Heistingering, Gasser and Mattanovich (2018)
CBS2612 <i>mfa1Δ</i>	<i>mfa1Δ::loxP-natMX-loxP</i>	mixed	This study
CBS2612 <i>mfa2Δ</i>	<i>mfa2Δ::loxP-ZeoR-loxP</i>	mixed	This study
CBS2612 <i>mfa1Δ mfa2Δ</i>	<i>mfa1Δ::loxP-natMX-loxP</i> <i>mfa2Δ::loxP-ZeoR-loxP</i>	mixed	This study
CBS2612 <i>mfaΔ</i>	<i>mfaΔ::loxP-kanMX-loxP</i>	mixed	This study
CBS2612 <i>ste6-1Δ</i>	<i>ste6-1Δ::loxP-natMX-loxP</i>	mixed	This study
CBS2612 <i>mfa1Δ</i> (a)	<i>dic1-2Δ mfa1Δ::loxP-natMX-loxP</i>	MAT α	This study
CBS2612 <i>mfa2Δ</i> (a)	<i>dic1-2Δ mfa2Δ::loxP-ZeoR-loxP</i>	MAT α	This study
CBS2612 <i>mfa1Δ mfa2Δ</i> (a)	<i>dic1-2Δ mfa1Δ::loxP-natMX-loxP</i> <i>mfa2Δ::loxP-ZeoR-loxP</i>	MAT α	This study
CBS2612 <i>ste6-1Δ</i> (a)	<i>ste6-1Δ::loxP-natMX-loxP</i>	MAT α	This study
CBS2612 <i>dic1-2Δ</i> (α) P _{FUS1} -eGFP	<i>dic1-2Δ 5'-AOX1tt-P_{FUS1}-eGFP-ScCYC1tt-natMX-AOX1tt-3'</i>	MAT α	This study
CBS2612 <i>dic1-2Δ</i> (a) P _{FUS1} -eGFP	<i>dic1-2Δ 5'-AOX1tt-P_{FUS1}-eGFP-ScCYC1tt-natMX-AOX1tt-3'</i>	MAT α	This study

^aThe *dic1-2Δ*(a) strain contains one *loxP* sequence between *SLA2-2* and *MAT α 1* in *MAT* locus 1 and another *loxP* sequence in *MAT* locus 2 between and *SLA2-1* and *MAT α 1*.

Cultivation conditions

Yeast cells were grown in standard YP medium (yeast extract 10 g/l, soy peptone 20 g/l) containing 2% glucose as carbon source. All liquid cultures were grown at 25°C. After transformation, cells were plated on YPD agar (2% agar-agar) supplemented with the appropriate antibiotics (zeocin 50 μ g/ml, geneticin 500 μ g/ml, nourseothricin 100 μ g/ml). Increased antibiotic concentrations of up to 100 μ g/ml zeocin and 1 mg/ml geneticin were used for the selection of diploid cells on mating plates. Mating conditions were defined as cultivation in nitrogen-free acetate medium (mating medium, 0.5% sodium acetate, 1% potassium chloride, 1% glucose).

RNA extraction

Cultivations and sampling for RNA extractions were performed as described in Heistingering, Gasser and Mattanovich (2018). Three colonies of each strain were cultivated in parallel. RNA was isolated according to the TRI reagent (Sigma-Aldrich) protocol. Residual genomic DNA was digested using the Ambion DNA-free kit (Invitrogen), and RNA was precipitated overnight (1/10 volume 3M sodium acetate, 2.5 volumes absolute ethanol, 1 μ l glycogen) at -20°C to remove traces of phenol. RNA concentrations and integrity were analyzed using a Nanodrop spectrophotometer and a Bioanalyzer (Agilent) using the RNA 6000 Nano Kit.

RNA sequencing data analysis

Solexa Sequencing of poly(A)-enriched cDNA libraries was performed at the VBCF NGS Unit (www.vbcf.ac.at). The unsorted, unaligned BAM files were converted and split into paired-end FASTQ files with the TopHat2 tool *bam2fastx* v2.1.0 (Kim et al. 2013) and subsequently trimmed and quality checked using *Trim-Galore* v0.4.2 (Babraham Bioinformatics 2016) using default parameters. As preparation for the count quantification, a transcript index was created using the latest *K. phaffii* CBS7435 annotation (FR839628.1, FR839629.1, FR839630.1, FR839631.1,

FR839632.1; Valli et al. 2016) and the kallisto v0.43.0 subprogram index (Bray et al. 2016). Subsequently, the read count for each sample was determined using kallisto subprogram *quant* (Bray et al. 2015). For differential expression analysis (Love et al. 2015) with R (R Development Core Team 2016), the packages *tximport* and *tximportData* (Soneson, Love and Robinson 2015), *readr* (Wickham et al. 2017) and *DESeq2* (Gentleman et al. 2004; Huber et al. 2015; Love, Anders and Huber 2017) were used for each comparison. Visualization of the expression analysis results was also carried out with R. Transcripts with a log₂ fold-change of below -1 and above 1 combined with an adjusted *p*-value below 0.05 were considered to be significantly regulated. For the detection of new, so far unannotated transcripts, the RNA sequencing data were analyzed with the HISAT-StringTie-Balgon suite (Pertea et al. 2016). Coverage histograms were visualized with *JBrowse* (Skinner et al. 2009). The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar, Domrachev and Lash 2002) and are accessible through GEO Series accession number GSE111973.

Quantitative PCR

For quantitative PCR, RNA samples were treated with the Ambion DNA-free kit (Invitrogen) and RNA integrity was analyzed by agarose gel electrophoresis. All cDNA was synthesized using oligo(dT)₂₃ primers (NEB) and the Biozym cDNA synthesis kit. PCR reactions were performed using the Sensi Mix SYBR Hi-Rox kit (Bioline) on a Rotor-Gene Q instrument (Qiagen). Purified PCR products of the analyzed genes were used to generate standard curves for quantification. Transcript levels were normalized to *ACT1* (PP7435_Chr3-0993) expression.

Mating protocols

Qualitative mating experiments were performed similarly to Tolstorukov and Cregg (2007). Strains to be mated were streaked out in parallel on YPD agar plates directly from glycerol stocks and incubated at 30°C. After approximately 24 h, cells were replicated twice at an angle of 90°C onto mating agar (0.5% sodium

acetate, 1% potassium chloride, 1% glucose, 2% agar) and incubated at 25°C for 3 days. Diploid cells were selected by replica plating onto YPD agar containing the appropriate antibiotics. Cell growth at crossing points was evaluated after incubation at 30°C for 3 days.

For the semiquantitative mating assay, fresh colonies grown on YPD agar were used to inoculate non-selective YPD medium. After 18–20 h, approximately 6.5×10^7 cells of each strain were mixed, plated on mating agar plates and incubated at 25°C for 3 days. Cell numbers were estimated by measuring the optical density at 600 nm (OD_{600}), with an OD_{600} of 1 corresponding to 1×10^7 cells. Mated cells were washed from the plates with phosphate buffered saline (PBS) (KH_2PO_4 0.24 g/l, $Na_2HPO_4 \cdot 2 H_2O$ 1.8 g/l, KCl 0.2 g/l, NaCl 8 g/l) and appropriate dilutions were plated on YPD agar plates containing either one or both of the antibiotics used for the selection of diploid cells. Colonies were counted after incubation at 30°C for 3 days. The mating efficiency was calculated as the percentage of cells growing on the plates containing both antibiotics in relation to the number of cells growing in the presence of one of the antibiotics (using the cell count on the antibiotic plates with the lower number of cells).

Synthetic pheromones

The *K. phaffii* a- and α -factor peptides were synthesized by Thermo Fisher Scientific with a purity >95%. Both a-factor peptides were obtained with the C-terminal farnesyl and carboxymethyl modifications. Aliquots were dissolved in dimethyl sulfoxide and diluted to working concentrations with sterile H_2O + 0.5 mg/ml BSA. The α -factor peptide was dissolved directly in sterile H_2O + 0.5 mg/ml BSA. *Saccharomyces cerevisiae* a-factor was purchased from ZYMO Research. All synthetic peptides were added to the cultures at final concentrations of 10–20 μ g/ml.

Enhanced GFP reporter assays

For assays with the P_{FUS1} -eGFP reporter strains, precultures were grown in 24-well deep well plates in YP medium without glucose. After 24 h, cells were harvested and resuspended in yeast nitrogen base (YNB) medium (YNB without amino acids and ammonium sulfate 3.4 g/l, potassium phosphate buffer 0.1M, pH 6). For the main cultures, 2 ml YNB medium supplemented with two glucose release feed beads (6 mm diameter, Kuhner AG, glucose release rate: $0.79 \times \text{time}^{0.66} = \text{mg glucose released per feed bead}$, theoretical glucose concentration without consumption after 40 h: 9 g/l) were inoculated at a starting OD_{600} of 2. To test the induction of the *FUS1* promoter by a second strain of opposite mating type, equal numbers of cells from the separate precultures were mixed to reach the same starting OD_{600} . Synthetic pheromones and solvent controls were added around 18 h after the start of the main culture. After 40 h in YNB medium, cells were analyzed by flow cytometry and microscopy.

Flow cytometry

For flow cytometry cells were diluted in PBS (KH_2PO_4 0.24 g/l, $Na_2HPO_4 \cdot 2 H_2O$ 1.8 g/l, KCl 0.2 g/l, NaCl 8 g/l) and green fluorescence (FL1 channel, 505–545 nm), forward and side scatter of 20 000 cells per sample were measured on a Gallios Flow Cytometer (Beckman Coulter). Data were analyzed with the Kaluza analysis software (Beckman Coulter). GFP-positive cells were

gated using the $dic1-2\Delta P_{FUS1}$ -eGFP α -type strain in the absence of pheromone as reference.

Microscopy

Microscopy was performed with a Zeiss Axio Observer.Z1/7 microscope using a LCI Plan-Neofluar 63x/1.3 water immersion objective. Cell morphology was analyzed in bright-field mode. For fluorescence microscopy of eGFP-expressing cells, the 38 HE eGFP shift free (E) filter set (Zeiss) was used. Pictures were processed with the Zen 2.3 lite (blue edition) software (Carl Zeiss Microscopy GmbH).

RESULTS

Identification of two putative MFA sequences

Due to their short coding sequences and poor sequence conservation, genes encoding a-factor mating pheromones are hard to identify by homology search (Dignard et al. 2007; OhEigearthaigh et al. 2011). To annotate possible MFA genes in the genome of *K. phaffii*, we used RNA sequencing data of *K. phaffii* CBS2612 (the homothallic wild-type strain) as well as of stable a- and α -type strains derived from it (Heisting, Gasser and Mattanovich 2018). Sequencing reads of all strains grown in rich medium and under mating conditions were mapped to the available genome sequence of the CBS7435 strain (Kuberl et al. 2011; Valli et al. 2016). Note that no full genome sequence of the *K. phaffii* CBS2612 type strain is available at the moment. However, the sequences of all relevant regions investigated in this study were verified to be identical to the CBS7435 sequence. All so far not annotated open reading frames (ORFs) shorter than 200 bp that were expressed only by a-type or wild-type cells under mating conditions were analyzed. By this method, one candidate gene (MFA1) with an ORF of 123 bp (Chr1:1587540..1587662 in CBS7435 [FR839628.1]), encoding a 40 amino acid protein containing the conserved C-terminal CAAX motif, was identified. Using this sequence, a second potential a-factor gene was identified by homology search (tBLASTn). Like MFA1, the MFA2 ORF (Chr2:1840284..1840406 in CBS7435 [FR839629.1]) encodes a 40 amino acid protein containing a C-terminal CAAX motif. The ORF is part of an unknown gene annotated as CBS7435.Chr2-1002, which, according to the RNA sequencing data analysis, was upregulated to similar levels in all strains under mating conditions. However, a mating-type-specific expression pattern could be observed in the enriched coverage histograms when only the relevant sequence region was considered (Figs S1 and S2, Supporting Information).

An alignment of the *K. phaffii* a-factor sequences is shown in Fig. 1A. The two identified proteins have an overall sequence identity of 63%. Before secretion of the mature mating factors via the Ste6 transporter, yeast a-factor peptides are modified by a number of conserved enzymes. The genome of *K. phaffii* encodes orthologs of all proteins known to be required for a-factor maturation in *S. cerevisiae*. Therefore, the N-terminal cleavage site on the *K. phaffii* a-factor sequences was predicted by comparison of the amino acid sequences to sequences of known a-factor peptides from other yeasts (Fig. 1B). In *S. cerevisiae*, the N-terminus is processed in two consecutive steps carried out by Ste24 and Axl1, with Axl1 cleaving after KDN in the sequence (Michaelis and Barrowman 2012). Cleavage site sequences vary between different yeast species; however, sequence analysis showed that many of the described sites contain lysine residues and have a conserved asparagine at the final position. In *K. phaffii*, cleavage

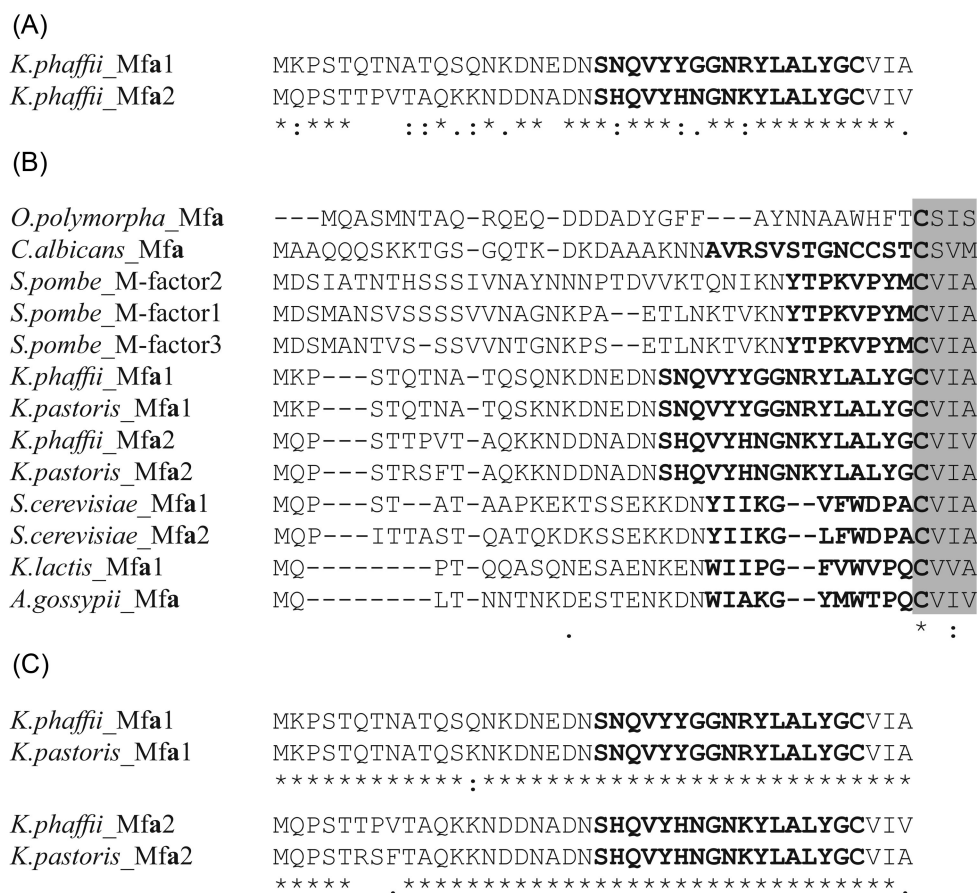


Figure 1. Alignments of yeast a-factor sequences. Protein sequences were aligned using Clustal Omega (Sievers et al. 2011). Identical amino acids are marked with an asterisk, a colon indicates residues with highly similar properties and residues with weakly similar properties are indicated by a point. Sequences of the mature peptides are shown in bold. (A) Alignment of the newly identified *K. phaffii* a-factor sequences. (B) Alignment of a-factor peptides from different yeast species. All sequences contain the conserved C-terminal CAAX motif (highlighted in gray). (C) Sequences of the *K. pastoris* a-factor peptides. The predicted mature peptides are identical to the *K. phaffii* a-factor peptide sequences.

of the N-termini after EDN and ADN, respectively, would result in mature peptides of 17 amino acids with four residues difference between MFA1 and MFA2. Of the genes required for mating-factor cleavage, farnesylation and export, only transcript levels of RAM1 (PP7435_Chr3-0565), encoding the β -subunit of the CAAX farnesyltransferase, and STE6-1 (PP7435_Chr3-0320) were significantly upregulated under mating conditions. For RAM1, transcript levels were higher in the wild type than in pure cultures of a- or α -type cells, which points to an additional induction of gene expression by pheromone (log₂ fold-changes: wild type 3.56, a-type 1.82, α -type 2.05). The transcription of the STE6-1 gene was found to be mating type specifically upregulated only in a-type and wild-type cells (log₂ fold-changes: wild type 1.62, a-type 1.66).

Using the *K. phaffii* sequences to perform homology-based search also allowed the identification of two MFA genes in *K. pastoris*. As in *K. phaffii*, the two genes are located on two different chromosomes (MFA1 Chr1:1743827..1743708 [CP014584.1], MFA2 Chr4:570171..570290 [CP014587.1], ATCC 28485 (Love et al. 2016)) and encode 40 amino acid proteins, differing from their *K. phaffii* orthologs only in 1 or 4 amino acids, respectively (Fig. 1C). No further a-factor sequences of other closely related yeast species with available genome sequences could be identified.

Only simultaneous deletion of both MFA genes prevents mating of a-type cells

To confirm the role of the newly identified sequences and to analyze the effect of MFA deletions on the mating behavior of *K. phaffii*, different knockout strains were generated. Genes were deleted in the homothallic CBS2612 wild-type strain as well as the previously published *dic1-2 Δ (a)* or *dic1-2 Δ (α)* strains with stable mating types (Heistingering, Gasser and Matanovich 2018). In all cases, the whole gene was replaced by an antibiotic resistance marker gene. The *mfa1 Δ mfa2 Δ* double mutants were generated by consecutive transformation of two deletion cassettes. Furthermore, strains with deletion of MFA (CBS7435_Chr2-0522) or the a-factor transporter STE6-1 (PP7435_Chr3-0320) were generated. To analyze the influence of the mating-factor gene deletions on the mating behavior, all strains were mated against the unmodified a- and α -type strains using a standard mating protocol.

Both of the MFA single mutants (*mfa1 Δ (a)* and *mfa2 Δ (a)*) were still able to mate with an α -type mating partner. Only deletion of both MFA genes from the a-type strain resulted in a complete loss of mating ability, indicating that the two candidate genes encode functionally redundant proteins (Fig. 2A). Mating experiments with the homothallic wild-type strain

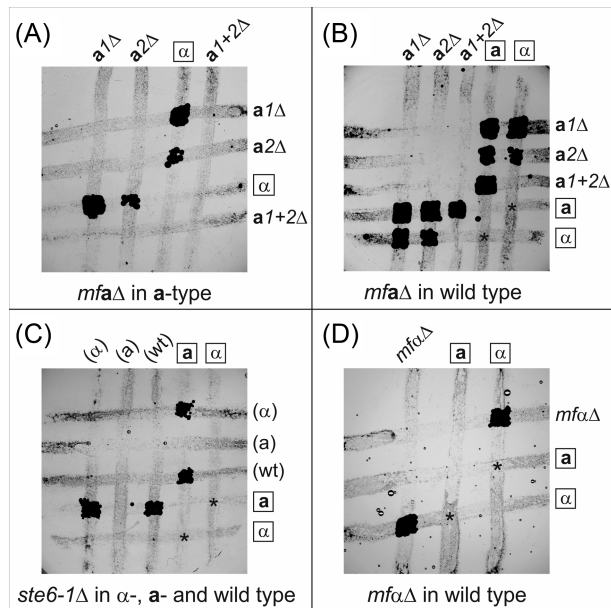


Figure 2. Mating phenotype of mating-factor deletion strains. Diploid cells were selected on YPD agar containing the appropriate antibiotics. All different mutants were mated against the *dic1-2Δ* a- and α -type strain indicated by a framed a and α . Note that the two tester strains carried the same resistance gene, preventing the selection of diploid cells formed by these strains in the positions indicated by an asterisk. (A) Diploid cell formation by *mfaΔ* a-type strains. Deletion of both MFA genes abolished mating with α -type cells. (B) Diploid cell formation by homothallic *mfaΔ* strains. Deletion of both MFA genes prevented mating of a-type cells, but did not affect α -cell mating. (C) Deletion of the a-factor transporter gene *STE6-1* abolished mating of a-type cells. The cell type of the *ste6-1Δ* strains is given in parentheses. (D) Diploid cell formation by homothallic *mfaΔ* cells. Only a-type cells were able to mate and form diploids with the *dic1-2Δ*(α) strain.

consisting of mutants of both mating types gave similar results (Fig. 2B). While the single *mfaΔ* mutants were able to mate with a- and α -type cells, MFA double mutants and the *ste6-1Δ* strain (Fig. 2C) were unable to mate with α -type cells. However, mating with the *dic1-2Δ*(a) strain was still possible for both the *mfa1Δ* *mfa2Δ* and the *ste6-1Δ* strain, showing that the effect of the mutations is mating type specific. The same behavior was also observed for the *mfaΔ* strain, where mating was only possible with the *dic1-2Δ*(α) strain (Fig. 2D).

MFA single mutants mate with reduced efficiency

To assess the contribution of the individual a-factor peptides to the overall mating efficiency of the *K. phaffii* a-type strain, the mating efficiency of the different mutants was analyzed by a semiquantitative mating assay. In this assay, MFA single and double mutants were mated against the unmodified *dic1-2Δ*(α) strain.

The mating efficiency of the unmodified *dic1-2Δ* strains was found to be around 10% (Fig. 3). Mating with strains deleted in MFA1 and MFA2 led to a dramatic decrease in efficiency to 0.9% and 0.1%, respectively. This indicated a synergistic effect of the two a-factor pheromones in *K. phaffii* and showed that the expression of both MFA genes is required for efficient mating. A few diploid cells could also be obtained when crossing the *mfa1Δ* *mfa2Δ*(a) double mutant with the *dic1-2Δ*(α) strain, resulting in a mating efficiency of 0.01%.

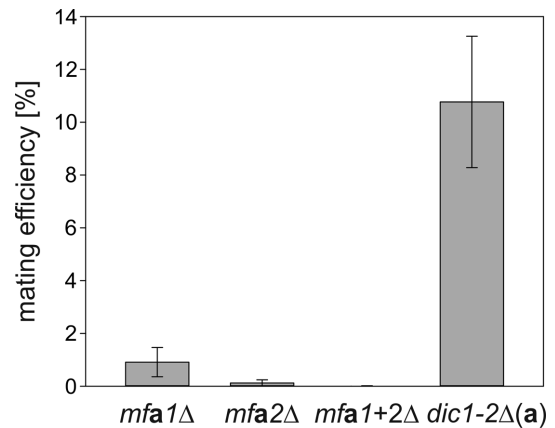


Figure 3. Mating efficiency of *mfaΔ* a-type strains. Single and double mutants as well as the *dic1-2Δ*(a) strain were mated with the *dic1-2Δ*(α) strain. Error bars represent the standard deviation of four independent mating experiments. A Student's t-test showed significant differences between all strains (p -value < 0.02).

Expression of one MFA gene is sufficient for mating pathway activation

Binding of mating pheromones to the pheromone surface receptors Ste2 and Ste3 (for α - and a-factor, respectively) activates a MAPK signaling cascade resulting in the activation of the genes necessary for shmoo formation and cell fusion (Jones and Bennett 2011; Merlini, Dudin and Martin 2013). The membrane protein Fus1 localizes at the shmoo tip and is required for efficient fusion of two mating cells. In *S. cerevisiae*, *FUS1* expression is strongly induced by mating pheromones (McCaffrey et al. 1987; Trueheart, Boeke and Fink 1987). A similar pattern of regulation could be found in *K. phaffii*, where *FUS1* (PP7435_Chr4-0336) was only expressed under mating conditions and significantly upregulated in the wild-type cultures containing cells of both mating types compared to the a- and α -type samples (log2 fold-changes under mating conditions: a-type/wild type -2.65, α -type/wild type -2.39). Fusion of the *FUS1* promoter to the β -galactosidase or GFP gene has previously been used as a tool to study *FUS1* regulation and the induction of the pheromone response pathway in other yeasts (Trueheart, Boeke and Fink 1987; Marcus et al. 1991; Fujimura 1992; Houser et al. 2012). To investigate the induction of mating in response to the different mating factors, the *K. phaffii* *FUS1* promoter (Chr4:560823..560339 [FR839631.1]) was used to generate an eGFP reporter construct. This construct was integrated into the *K. phaffii* genome at the *AOX1* terminator locus of the unmodified a- and α -type strain, resulting in the reporter strains *dic1-2Δ*(a) P_{FUS1} -eGFP and *dic1-2Δ*(α) P_{FUS1} -eGFP.

To analyze the induction of the mating response in the reporter strains in the presence of the different mating-factor deletion strains, equal numbers of cells were mixed and cultivated under nitrogen and glucose limiting conditions, as shmoo formation and *FUS1* expression were strongest under these conditions. After around 40 h, the fraction of fluorescent cells was measured by flow cytometry. Changes in cell morphology were assessed by microscopy.

Flow cytometry analysis (Fig. 4A) showed no eGFP expression in the cultures of the reporter strains alone, confirming that nitrogen and glucose limitation alone is not sufficient for induction of *FUS1* expression in haploid *K. phaffii* cells. Only after cocultivation with cells of opposite mating type, an eGFP-positive population was observed. Mixing the a-type reporter strain with

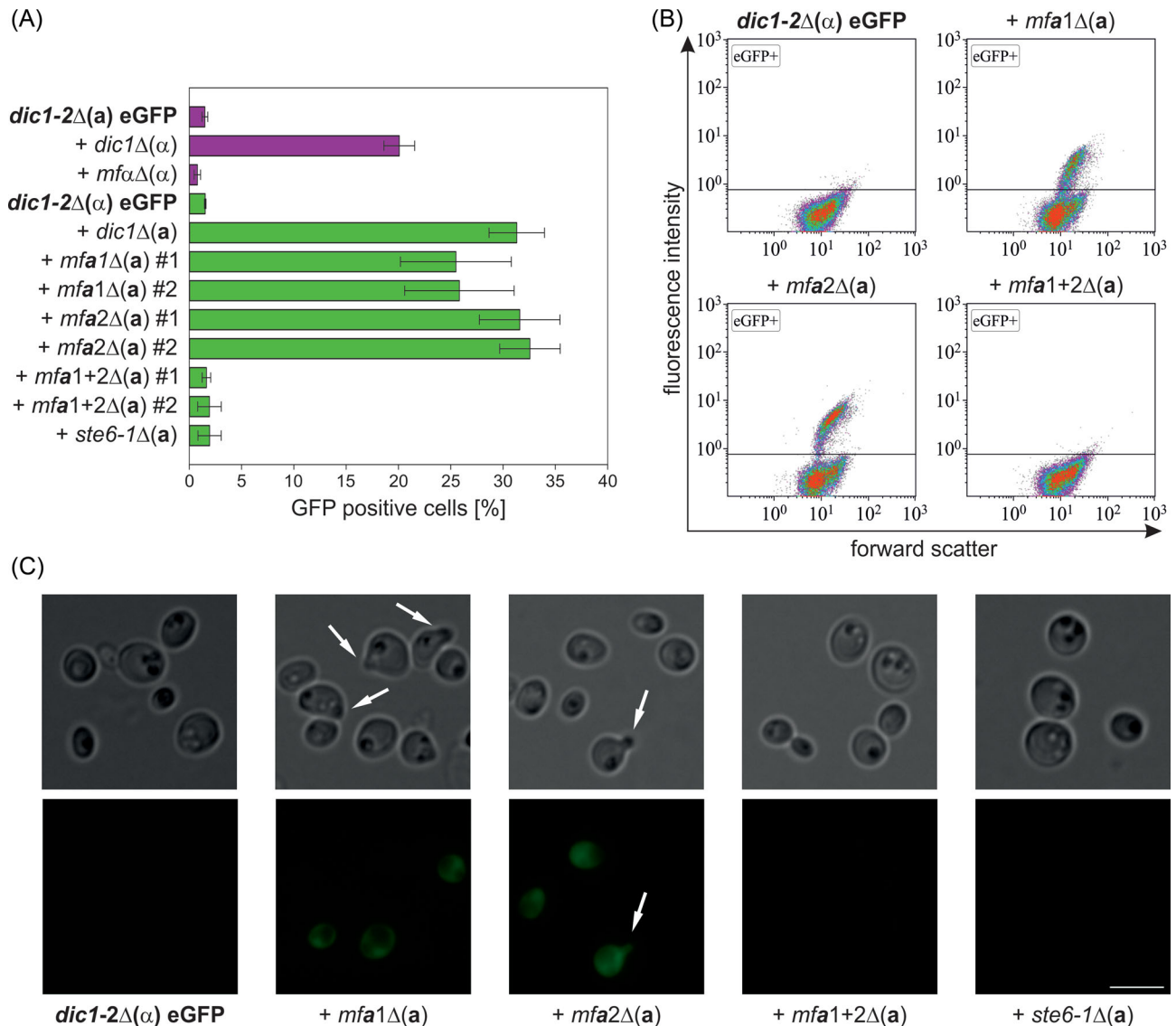


Figure 4. Induction of the mating response by mating factor deletion strains. The different *a*- and α -factor deletion strains were co-cultivated with the respective *dic1-2Δ* P_{FUS1} -eGFP reporter strain of the opposite mating type under nitrogen and glucose limiting conditions. (A) The proportion of eGFP-positive cells in a culture was analyzed by flow cytometry. Error bars represent the standard deviation of three independent cultivations. (B) Representative flow cytometry plots of the α -type reporter strain alone and after co-cultivation with different *mfaΔ* strains. A distinct population of fluorescent cells was observed in all cultures with the *mfaΔ* single mutants, while the *mfa1Δ mfa2Δ* double mutant did not induce eGFP expression. (C) Microscopy pictures of the α -type reporter strain alone and after co-cultivation with the *mfaΔ* and *ste6-1Δ* strains. Only the *mfaΔ* single mutants were able to induce eGFP expression and a clear shmoo phenotype. Arrows indicate shmoo formation. Bar, 5 μ m.

mfaΔ cells failed to induce eGFP expression, showing that the generated reporter strains are sensitive to the presence of mating pheromone. As expected from the mating experiments, the *mfaΔ* single mutants, secreting only one of the *a*-factor peptides, could still induce eGFP expression in the α -type reporter strain. No eGFP expression was observed when α -type reporter cultures were mixed with either the *mfa1Δ mfa2Δ* double mutant or the *ste6-1Δ* strain. Representative flow cytometry plots of cultures containing the *mfaΔ* strains are shown in Fig. 4B. Note that in the mixed cultures the theoretical maximum of eGFP-positive cells is 50%. Shmoo formation could only be observed in cultures containing the *dic1-2Δ(a)* P_{FUS1} -eGFP reporter strain together with one of the *mfaΔ(a)* single mutants (Fig. 4C). Interestingly, no green cells forming shmoo could be observed with the

mfa1Δ(a) strain, while *mfa2Δ(a)* cells were able to induce shmoo formation in *dic1-2Δ(a)* P_{FUS1} -eGFP cells.

Activation of the mating response by synthetic pheromones

The same P_{FUS1} -eGFP reporter assay was used to test the activity of synthetic mating pheromones. To ensure full activity, the proposed mature *a*-factor peptides were synthesized including the C-terminal methylation and farnesylation. The effect of synthetic *K. phaffii* α -factor was also investigated. The peptides were added to cultures of the *dic1-2Δ* P_{FUS1} -eGFP *a*- or α -type strains after around 18 h in nitrogen and glucose limiting conditions. After another 24 h, the percentage of eGFP-positive cells was

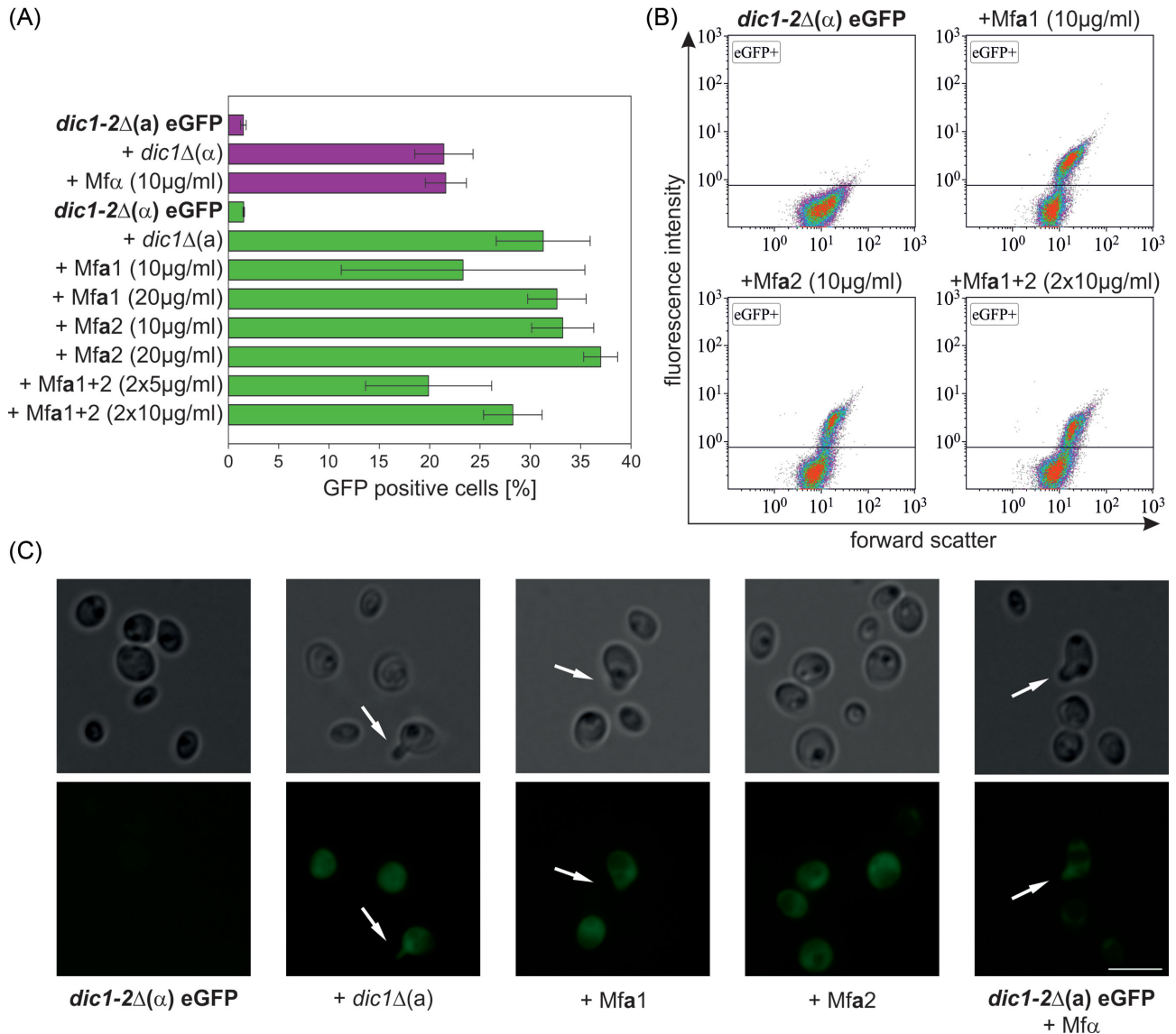


Figure 5. Induction of the mating response by synthetic a- and α -factor (Mfa and Mfa α). The synthetic peptides were added to cultures of the *dic1-2Δ* P_{FUS1} -eGFP reporter strains cultivated under nitrogen and glucose limiting conditions. (A) The portion of eGFP-positive cells was analyzed by flow cytometry. Error bars represent the standard deviation of three independent cultivations. (B) Representative flow cytometry plots of the α -type reporter strain alone and after addition of the a-factor peptides. Addition of the two peptides alone and in combination led to the formation of a distinct population of eGFP-positive cells. (C) Bright-field and fluorescence microscopy pictures of the α -type reporter strain alone and after co-cultivation with *dic1-2Δ(a)* cells or addition of synthetic a-factor. Clear shmoo formation was only observed in mixed cultures with *dic1-2Δ(a)* cells and after addition of a-factor 1. Synthetic α -factor induced shmoo formation in *dic1-2Δ(a)* P_{FUS1} -eGFP cells. Bar, 5 μ m.

determined by flow cytometry. Changes in cell morphology were assessed by microscopy.

Addition of either a-factor peptide to a final concentration of 10 μ g/ml led to a clear induction of eGFP expression in around 30% of the cells of the P_{FUS1} -eGFP reporter strain (Fig. 5A). Representative flow cytometry plots of cultures after a-factor addition are shown in Fig. 5B. The proportion of eGFP-positive cells increased only slightly when the peptide concentration was increased to 20 μ g/ml and the addition of both a-factor peptides to the same culture did not result in a higher number of induced cells. At the same concentration, synthetic *K. phaffii* α -factor was able to induce the mating response in around 20% of the cells (Fig. 5A). No response could be observed when the *S. cerevisiae* a-factor peptide was used. Overall, the absolute portion of induced

reporter cells after addition of the pheromones was about half compared to induction by pheromones secreted by cells of opposite mating type in the mixed cultures, which contained only 50% reporter cells. Synthetic a-factor 1 at a concentration of 10 μ g/ml led to shmoo formation to a similar extent as in the control (Fig. 5C). This was also observed for a-type cells after the addition of α -factor. Addition of the same amount of a-factor 2 did not induce shmoo formation.

Expression of MFA genes is induced by pheromone

To confirm the transcriptional regulation of the mating-factor genes observed in the RNA sequencing data, samples from cultures during exponential growth in rich medium and after

24 h in mating medium were analyzed by quantitative PCR. To obtain mixed mating-type cultures, equal numbers of a- and α -type cells from the precultures were inoculated into mating medium.

As expected, the transcript levels of *MFA1*, *MFA2*, *MF α* and the surface receptor genes *STE2* and *STE3* were very low in rich medium (YPD), with expression levels below 1% of the reference gene *ACT1* (Fig. 6). *MFA1* and *MFA2* were both upregulated in a-type cells under mating conditions (Fig. 6A and B). The highest a-factor transcript levels were detected in the mixed cultures containing a- and α -type cells, indicating induction of *MFA* gene expression by a-factor. However, even in the mixed mating-type cultures, *MFA1* transcript levels were around eight times higher than levels of *MFA2*. The expression of *MF α* was upregulated only in α -type cells under mating conditions (Fig. 6C). Interestingly, no further induction of gene expression was observed in the presence of a-type cells and transcript levels were around five times lower than the *MFA1* levels in the same cultures. To emphasize the strong differences in expression levels between the three genes, a direct comparison of the mating-factor transcript levels in the different strains under mating conditions is shown in Fig. 6D. Transcript levels of the α -factor receptor *STE2* were strongly upregulated in a-type cells under mating conditions (Fig. 6E). The absolute levels detected in the mixed cultures were similar to the a-type samples. However, as these cultures contained only 50% of a-type cells, this likely resulted from an additional 2-fold increase in transcript level per cell in the presence of a-factor. The same expression pattern was also observed for the a-factor receptor *STE3* (Fig. 6F).

DISCUSSION

The preferentially haploid yeast *K. phaffii* can undergo mating and meiosis under nitrogen limitation conditions, and orthologs of many of the genes involved in the MAPK pathway leading to activation of the mating response in other yeasts are found in the genome. This includes the pheromone surface receptor genes *STE2* and *STE3* as well as the genes coding for a-factor and the conserved biogenesis pathway of the a-factor pheromone. However, no gene coding for the *K. phaffii* a-factor itself has been identified so far. Annotation of yeast a-factor genes by standard homology-based search is difficult because of their short sequence length and relatively poor sequence conservation. Furthermore, as the mating genes of *K. phaffii* are only expressed under nitrogen limitation conditions, relevant transcripts would not be detected in transcriptome analyses of strains under standard growth conditions (Valli et al. 2016). Starting from RNA sequencing data of previously generated heterothallic *K. phaffii* strains (Heistingering, Gasser and Mattanovich 2018) under mating conditions, we were able to identify two independent *MFA* genes. The systematic search for short ORFs expressed in a mating-type-specific manner and containing a C-terminal CAAX motif yielded *MFA1*. *MFA2* was subsequently identified using *MFA1* homology search. The *MFA2* ORF is part of a predicted gene (CBS7435.Chr2-1002), probably wrongly annotated by automated search to code for a non-conserved hypothetical protein. It is likely that *MFA2* was not considered during the first analysis because the average expression over the whole annotated gene was similar in both mating types. However, mating-type-specific expression could be observed in the short *MFA2* region and was also confirmed by quantitative PCR.

Both identified *K. phaffii* *MFA* sequences code for 40 amino acid long proteins with an overall sequence identity of 63%

and contain the conserved C-terminal CAAX motif required for a-factor modification. The proposed N-terminal AxII cleavage site after the asparagine at position 20 was predicted by comparison to other known a-factor sequences. The resulting mature peptides of 17 amino acids are slightly longer than the well-studied a-factor peptides of *S. cerevisiae* and the *Sc. pombe* M-factor, which have a confirmed length of 12 and 9 amino acids, respectively (Betz et al. 1987; Davey 1992). In our experiments, synthetic a-factor peptides with the predicted N-termini were able to activate the mating response pathway and to induce shmoo formation in α -type cells. The sequences of the N-terminal processing sites need to be confirmed by future experiments.

Analysis of the mating phenotype of *MFA* mutants showed that only *mfa1 Δ mfa2 Δ* double deletion strains were unable to mate or induce the mating response pathway in α -type cells. *MFA* single mutants were still able to induce the mating response in a similar number of α -type cells as the unmodified a-type strain. They were also able to mate with α -type cells, although with lower efficiency. These results indicated that the two newly identified genes both encode a variant of the a-factor and that there are no further a-factor genes in the genome of *K. phaffii*. For both of the single mutants, the mating efficiency was reduced to below 10% of the mating efficiency of the unmodified a-type strain. This strong reduction in efficiency might be due to a synergistic effect of the two peptide variants. However, no such effect could be observed after simultaneous addition of both pheromones in comparison to addition of one of the synthetic peptides alone. Another possibility is that with only one of the genes being expressed, the total a-factor concentration might simply be too low for an efficient induction of the mating response. *Saccharomyces cerevisiae* *mfa Δ* single mutants were shown to mate with a similar efficiency as wild-type cells while secreting only about half the amount of a-factor (Michaelis and Herskowitz 1988). Similarly, the deletion of one of the three M-factor gene copies of *Sc. pombe* did not affect mating, even though pheromone production was found to be reduced. In *MFA* double mutants, the mating efficiency was reduced to 25–30% compared to the wild type (Kjaerulff, Davey and Nielsen 1994).

Deletion of the gene encoding the a-factor transporter *Ste6* has been shown to prevent a-factor secretion and mating of *S. cerevisiae* a-type cells (Kuchler, Sterne and Thorner 1989). The same mating-type-specific non-mating phenotype was observed for *K. phaffii* *ste6-1 Δ* strains, indicating that *STE6-1* is the only gene coding for the a-factor transporter. The additionally annotated *STE6-2* (PP7435.Chr1-1574) and *STE6-3* (PP7435.Chr2-1257) genes most likely encode other closely related ABC transporters. As expected, the deletion of the single *MF α* gene abolished mating of α -type cells.

In *K. phaffii*, the expression levels of mating specific genes are very low during growth in rich medium and increase only under nitrogen starvation conditions (Heistingering, Gasser and Mattanovich 2018). The same pattern of gene expression was also observed for the mating-factor genes, with transcript levels below 1% of the *ACT1* control in rich medium and strong induction to up to 2500% of *ACT1* expression for *MFA1* under mating conditions. Furthermore, gene expression was found to be mating type specific, with the *MFA* genes being expressed only in a-type and *MF α* being expressed only in α -type cells. It is likely that the expression of the *MFA* and *MF α* genes is activated by *Mata2* and *Mat α 1*, respectively, as it has been observed for the corresponding surface receptor genes, although this remains to be shown in further experiments (Heistingering, Gasser and

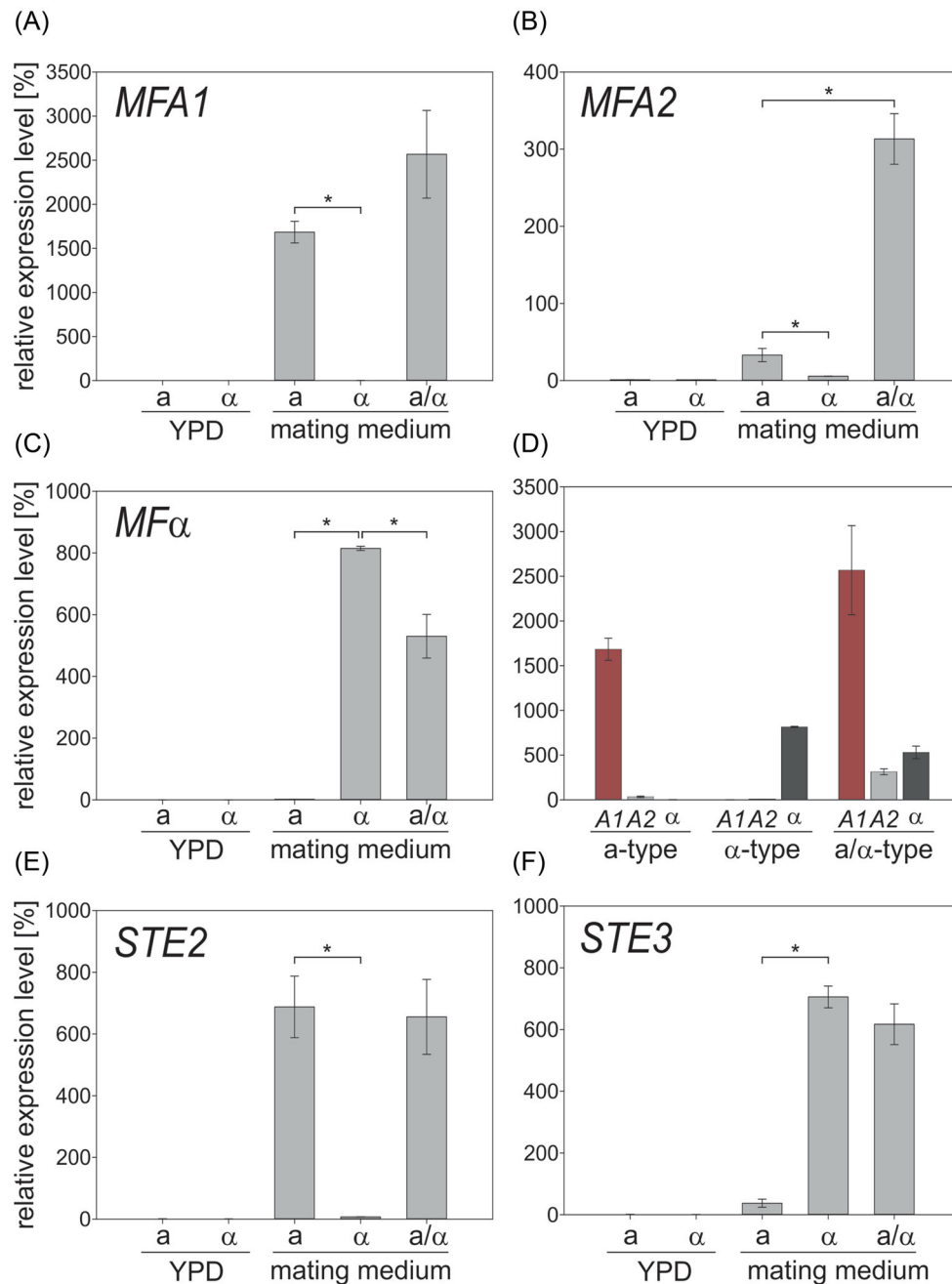


Figure 6. Transcript levels of mating factor and pheromone surface receptor genes. Cultures of *dic1-2Δ* a- and α -type cells were analyzed during exponential growth in rich medium (YPD) and under mating condition. Mixed mating-type cultures consisted of an equal number of cells of both mating types grown under mating conditions. Transcript levels were normalized to *ACT1* expression. Error bars represent the standard deviation of three biological replicates. Differences in gene expression between the cultures in mating medium were analyzed using a Student's t-test. Significant differences with a *p*-value < 0.05 are indicated by an asterisk. (A) *MFA1* was highly expressed in a-type cells under mating conditions. An additional increase in transcript level was measured in the mixed mating-type cultures. (B) Transcription of *MFA2* was induced in a-type cells under mating conditions and further increased significantly in the mixed mating-type cultures. (C) Mating-type-specific expression of *MF α* under mating conditions. (D) Direct comparison of the mating-factor transcript levels in the different cultures in mating medium. (E) Expression levels of *STE2* under mating conditions were similar in pure a-type and mixed cultures. (F) *STE3* transcript levels are upregulated in α -type cells and mixed cultures under mating conditions.

Mattanovich 2018). Under mating conditions, transcript levels of *MFA1* were up to 50 times higher than levels of *MFA2*. This difference in transcript levels was less pronounced in the mixed cultures containing both mating types where *MFA1* levels were only around eight times higher. In these cultures, the presence of α -type cells led to an additional increase in the *MFA1* and

MFA2 transcript levels. An additional induction of pheromone expression in the presence of a pheromone of the opposite mating type has been described for several other yeasts like *S. cerevisiae*, *Sc. pombe* and *C. albicans* (Strazdis and MacKay 1983; Achstetter 1989; Imai and Yamamoto 1994; Kjaerulff, Davey and Nielsen 1994; Zhao et al. 2005; Dignard et al. 2007). It likely

serves as a feedback mechanism to ensure high enough local pheromone levels to induce the mating process. Interestingly, in our experiments no additional induction of $MF\alpha$ expression was observed in mixed mating-type cultures. The fact that $MF\alpha$ levels are already strongly induced by nitrogen limitation and one transcript encodes nine copies of the mature α -factor could be a possible reason why further induction of α -factor signal might not be necessary for efficient mating. The measured expression levels of the corresponding pheromone receptor genes in the mixed cultures were equal to the levels obtained for samples containing only one mating type, indicating a 2-fold increase in receptor expression per cell in the presence of pheromone of the opposite mating type. In a homothallic *K. phaffii* wild-type culture containing a higher portion of α -type cells, a-factor receptor *STE2* was expressed at lower levels than in a pure a-type culture while the transcript levels of the α -factor receptor *STE3* were highest in the mixed cultures (Heistingering, Gasser and Mattanovich 2018 and RNA sequencing data).

Addition of synthetic mating pheromones to cultures under mating conditions resulted in an induction of the mating response pathway in about 20–30% of the population, confirming that the peptides are able to act as mating pheromones. Interestingly, even after addition of cells of opposite mating type only around 60% of the reporter cells expressed eGFP. The missing induction of the mating response in a rather large portion of the population might be one of the reasons for the low mating efficiency of *K. phaffii*. Other indicators for the biological activity of mating pheromones are arrest of the cells in the G1 phase of the cell cycle and changes in cell morphology to the so-called shmoo phenotype (Merlini, Dudin and Martin 2013). Many of the *K. phaffii* mating genes are only activated under nitrogen starvation conditions in which hardly any mitotic growth is observed. Therefore, it is difficult to use cell cycle arrest to assess the response to pheromone. In *Sc. pombe*, deletion of the adenylate cyclase (*CYR1*) to mimic nitrogen starvation was used to induce mating gene expression in growing cultures, enabling the demonstration of G1 arrest in response to mating pheromone (Maeda, Mochizuki and Yamamoto 1990; Imai and Yamamoto 1994). It remains to be tested if this strategy could also be applied in *K. phaffii*. Under the conditions used, shmoo formation could always be observed in mixed mating-type cultures of the unmodified strains. It was also induced by synthetic α -factor and, to a lesser extent, by a-factor 1. However, no shmoo formation could be observed after the addition of a-factor 2 or in fluorescent cells of the α -type reporter strain in cultures with the *mfa1* Δ strain. It is unclear what causes this difference in the response to the a-factor peptides. Actual shmoo formation probably requires higher concentrations of a-factor than the activation of the *FUS1* promoter, which was similar for both a-factor peptides and only slightly higher with the *mfa2* Δ strain than in the cultures with the *mfa1* Δ strain. As *MFA2* expression levels are lower, the amounts of active a-factor secreted by *mfa1* Δ cells could be just too low to induce the shmoo phenotype. It is possible that local areas of increased pheromone concentration close to actively secreting cells are responsible for the more efficient induction in mixed mating-type cultures, although cultures were well mixed and no cell aggregation was observed. It has been described for *S. cerevisiae* that shmoo formation after addition of a-factor was less pronounced than for α -factor (Betz, MacKay and Duntze 1977). Another study with *S. cerevisiae* found that although lower concentrations of synthetic a-factor than α -factor were necessary to induce G1 arrest, only little shmoo formation was observed at concentrations where α -factor could efficiently

induce shmoo formation (O'Reilly et al. 2012). Generally, concentrations of pheromones required to induce a response in *K. phaffii* were a little higher than described for *S. cerevisiae*. At least 10 $\mu\text{g/ml}$ synthetic pheromone was necessary to achieve mating pathway activation in more than 10% of the cells. In comparison, similar concentrations of 5–10 $\mu\text{g/ml}$ synthetic α -factor can be used to induce G1 arrest in *S. cerevisiae* cultures, leading to a shmoo phenotype in >95% of the cells (Breedon 1997; Wu, Liu and Huang 2011). For *S. cerevisiae* a-factor, growth inhibition and shmoo formation were observed with as little as 40–200 ng/ml of purified or synthetic pheromone (Anderegg et al. 1988; Xue et al. 1989; Marcus et al. 1991; O'Reilly et al. 2012). The need for high local pheromone concentrations might be a reason why *K. phaffii* requires rather high cell densities for efficient mating. In *S. cerevisiae*, the barrier proteases Bar1 and Afb1 produced by a- and α -type cells, respectively, are required for fast recovery from the response to pheromone (Sprague and Herskowitz 1981; MacKay et al. 1988; Huberman and Murray 2013). In *K. phaffii*, a potential protease showing a-type specific expression under mating conditions is the aspartyl protease Yps1–5 (PP7435.Chr3–0313). Interestingly, no α -specific genes coding for a potential a-factor degrading enzyme could be found in our dataset.

Within this study we identified the two *MFA* genes of *K. phaffii*. Both a-factor variants contribute to efficient mating of a-type cells and show a mating-type-specific expression pattern under nitrogen-limiting conditions. Furthermore, we could confirm that the α -factor is encoded by a single *MF\alpha* gene which is essential for α -cell mating. Expression level analysis indicated an additional induction of a-factor and surface receptor gene expression upon pheromone binding. It remains to be investigated why this additional layer of activation could not be detected for the *MF\alpha* gene. Using synthetic peptides we could demonstrate the biological role of the identified sequences in mating pathway activation.

SUPPLEMENTARY DATA

Supplementary data are available at [FEMSYR](https://www.femsy.com) online.

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Conflicts of interest. None declared.

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