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## Functional Characterization of an $\alpha$ -Factor-Like *Sordaria macrospora* Peptide Pheromone and Analysis of Its Interaction with Its Cognate Receptor in *Saccharomyces cerevisiae*

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The homothallic filamentous ascomycete *Sordaria macrospora* possesses genes which are thought to encode two pheromone precursors and two seven-transmembrane pheromone receptors. The pheromone precursor genes are termed *ppg1* and *ppg2*. The putative products derived from the gene sequence show structural similarity to the  $\alpha$ -factor precursors and a-factor precursors of the yeast *Saccharomyces cerevisiae*. Likewise, sequence similarity has been found between the putative products of the pheromone receptor genes *pre2* and *pre1* and the *S. cerevisiae* Ste2p  $\alpha$ -factor receptor and Ste3p a-factor receptor, respectively. To investigate whether the  $\alpha$ -factor-like pheromone-receptor pair of *S. macrospora* is functional, a heterologous yeast assay was used. Our results show that the *S. macrospora*  $\alpha$ -factor-like pheromone precursor PPG1 is processed into an active pheromone by yeast *MAT $\alpha$*  cells. The *S. macrospora* PRE2 protein was demonstrated to be a peptide pheromone receptor. In yeast *MAT $\alpha$*  cells lacking the endogenous Ste2p receptor, the *S. macrospora* PRE2 receptor facilitated all aspects of the pheromone response. Using a synthetic peptide, we can now predict the sequence of one active form of the *S. macrospora* peptide pheromone. We proved that *S. macrospora* wild-type strains secrete an active pheromone into the culture medium and that disruption of the *ppg1* gene in *S. macrospora* prevents pheromone production. However, loss of the *ppg1* gene does not affect vegetative growth or fertility. Finally, we established the yeast assay as an easy and useful system for analyzing pheromone production in developmental mutants of *S. macrospora*.

The life cycle of ascomycetes can be either homothallic or heterothallic. Homothallic species are self-fertile and are able to complete the sexual cycle without a mating partner. In heterothallic ascomycetes, mating occurs only between cells of opposite mating type, which attract each other by secreting pheromones (for reviews see reference 9, 46, and 47). Each mating type produces its own specific pheromone. These can be divided into two groups depending on the pathway of synthesis and secretion.

The pheromone response system of the heterothallic ascomycetous yeast *Saccharomyces cerevisiae* is a well-studied model of pheromone and pheromone receptor interaction. Binding of pheromones to their specific receptors triggers a G-protein-linked signal transduction pathway that induces the expression of several genes, facilitating the fusion of *MAT $\alpha$*  and *MAT $\alpha$*  cells to form diploid *MAT $\alpha$* / $\alpha$  cells (44). The peptide synthesized by *MAT $\alpha$*  cells is derived from a precursor with a C-terminal CaaX (where C is cysteine, a is an aliphatic residue, and X is any amino acid residue) motif, a signal for carboxymethylation and farnesylation. The mature lipopeptide pheromone, called a-factor, is secreted via an ATP-binding cassette transporter (7). *MAT $\alpha$*  cells produce  $\alpha$ -factor, which is secreted by the classical yeast secretory pathway (9, 35, 73). The  $\alpha$ -factor is a 13-amino-acid peptide derived from a precursor containing several copies of the 13-amino-acid sequence in tandem repeats. The first step of precursor processing is the removal of

the signal sequence of the prepro- $\alpha$ -factor (79). Further processing includes the Kex2 endoprotease, which cleaves the pro-protein at characteristic KR dipeptides (36), the dipeptidyl-aminopeptidase Ste13p, which recognizes the dipeptide XA or XP (35), and the carboxypeptidase Kex1p, which removes C-terminal extensions (16).

The *S. cerevisiae* pheromone receptors for a-factor (Ste3p) and  $\alpha$ -factor (Ste2p) are members of the large family of G-protein-coupled receptors, which contain seven-transmembrane domains. In contrast to ascomycetes, only lipopeptide pheromones of the *S. cerevisiae* a-factor group and Ste3p-like lipopeptide receptors have been found in basidiomycetes (4, 12, 20, 43, 51, 70, 78). Interestingly, putative pheromone precursor genes encoding two different mating pheromones have been identified not only in heterothallic filamentous ascomycetes, such as *Neurospora crassa*, *Cryphonectria parasitica*, *Magnaporthe grisea*, and *Podospira anserina*, but also in the homothallic filamentous ascomycete *Sordaria macrospora* (3, 58, 69, 81).

The first step in the sexual reproduction of mycelial ascomycetes is to bring together two compatible nuclei in the same cell. In heterothallic species, a specialized hypha termed trichogyne is sent out from a female prefruiting body to grow towards a fertilizing male cell of the opposite mating type. A functional male cell may be a uninucleate spermatium or microconidium or a multinucleate macroconidium. Recently, it was demonstrated that male and female fertility of heterothallic mycelial ascomycetes depends on the interactions of pheromones with their specific receptors (17, 39, 40, 77). In the homothallic ascomycete *S. macrospora*, conidiospores, spermatia, and trichogynes are absent, suggesting that phero-

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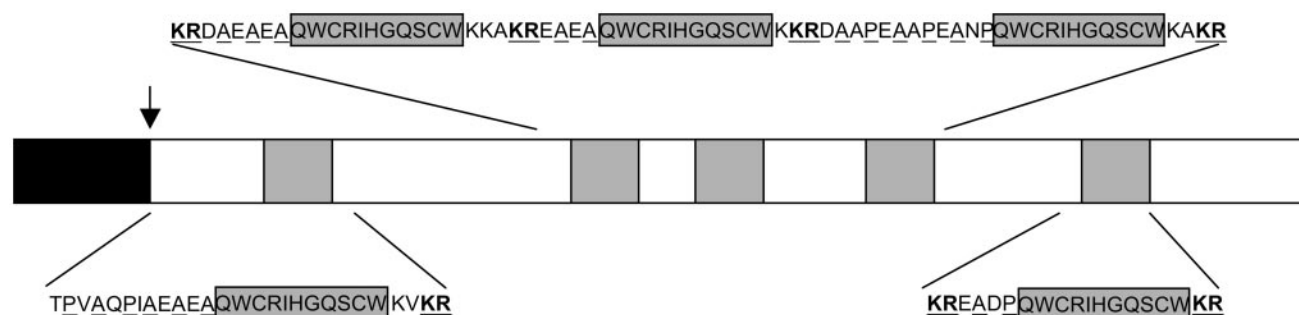


FIG. 1. Schematic structure of the *S. macrospora* pheromone precursor PPG1. The predicted PPG1 pheromone precursor consists of a hydrophobic signal sequence (black box) and five repeats of the undeca-peptide QWCRIHGQSCW (grey boxes). The putative cleavage site of the signal sequence is marked by a vertical arrow, KR dipeptides are indicated in bold letters and underlined, and XA/XP repeats, putative cleavage sites for a dipeptidyl-aminopeptidase, are underlined.

mones are not needed either for sensing a mating partner or for initializing fertilization events.

Nevertheless, two putative pheromone precursor genes, named *ppg1* and *ppg2*, are transcriptionally expressed in *S. macrospora*. The *ppg2* gene encodes a lipopeptide with a putative farnesylated and carboxymethylated C-terminal cysteine residue. The PPG1 precursor consists of a putative hydrophobic signal sequence and five repeats of the undeca-peptide QWCRIHGQSCW. Each of the five repeated sequences is surrounded by maturation signals similar to those of the  $\alpha$ -factor pheromone precursors of *S. cerevisiae* (58) (Fig. 1). The presence of these maturation motifs suggests that the *S. macrospora* PPG1 precursor is processed to an undeca-peptide pheromone, hereafter referred to as pheromone PHE1. In addition to pheromone genes, two pheromone receptor genes, termed *pre1* and *pre2*, have also been identified and shown to be transcriptionally expressed in *S. macrospora* (59). The predicted products of both genes are proteins with seven transmembrane domains. The *pre1* gene product shows extensive amino acid similarity to the  $\alpha$ -factor receptor Ste3p of *S. cerevisiae* and to lipopeptide pheromone receptors of basidiomycetes. The *S. macrospora* pheromone receptor PRE2 exhibits significant sequence similarity to the *S. cerevisiae* Ste2p  $\alpha$ -factor receptor (59).

In filamentous ascomycetes, two functions of pheromones have been suggested. One is the regulation of initial recognition between trichogynes and spermatia of opposite mating types, which is essential for sexual reproduction in heterothallic filamentous ascomycetes. Second, pheromones are thought to play a role in postfertilization events (18). This may be equally important for heterothallic and homothallic fungi. In mycelial ascomycetes, an essential postfertilization event takes place during the development of dikaryotic hyphae, enabling karyogamy and meiosis. It has therefore been suggested that recognition between nuclei is mediated by the nucleus-limited expression of mating type-specific pheromones and receptors. In addition, these pheromones and receptors are thought to be limited to the plasma membrane region close to the individual nucleus. The spatial restriction of signaling components is proposed to facilitate recognition between two nuclei (21, 67, 72).

The fact that pheromone and pheromone receptor genes are transcribed in the homothallic ascomycete *S. macrospora* supports the idea that pheromones and receptors are functional in this fungus.

In order to examine the functionality of *S. macrospora* pheromone precursor PPG1 and pheromone receptor PRE2, the yeast *S. cerevisiae* was used as a heterologous assay system. A synthetic peptide was designed to analyze whether the undeca-peptide predicted from the PPG1 sequence can act as an active peptide pheromone and trigger the pheromone response in the heterologous yeast system. Furthermore, we disrupted the pheromone precursor gene *ppg1* of *S. macrospora* to investigate its involvement in fruiting body and ascospore development.

#### MATERIALS AND METHODS

**Strains, culture conditions, and transformation.** *Escherichia coli* strain SURE was used as the host for plasmid amplification (30). Cloning and propagation of recombinant plasmids were done with standard protocols (65). All fungal strains used in this work are summarized in Table 1. *Saccharomyces cerevisiae* strain 7416-12-3 was used as a host for plasmids containing *S. macrospora* pheromone receptor gene *pre2* or control plasmid pPGK in halo assays (42). Supernatants used in assays for pheromone activity were taken from cultures of strain W303-1B carrying either the *S. macrospora ppg1* gene or the empty vector pPGK (22). To ensure that the synthetic yeast  $\alpha$ -mating pheromone was functioning as intended in the halo assay set-up, strain 786-11-1 was used as a control (32). Strains 7416-12-3 and 786-11-1 are both derived from yeast strain 381G (31). As appropriate, yeast strains were grown in YEPD or SD minimal medium lacking uracil or uracil and tryptophan (71). Transformation of *S. cerevisiae* was done by electroporation according to Becker and Lundblad (2) in a Multiporator (Eppendorf) at 1.5 kV. For each transformation, 100 ng of plasmid DNA was used.

The *Sordaria macrospora* wild-type strain K (S48977) was available from our laboratory collection (Department of General and Molecular Botany, Bochum, Germany). The *S. macrospora* developmental mutant strains used for screening of halo formation are impaired in forming female gametangia (asc mutants),

TABLE 1. Strains used in this study

Organism	Strain	Relevant genotype	Reference or source
<i>Sordaria macrospora</i>	S48977	Wild type, homothallic	This study
	per44 (S48143)	Sterile, no ascospores	This study
	per46 (S43021)	Sterile, no ascospores	This study
	S52063	<i>ppg1::hyg<sup>R</sup></i> , fertile	This study
<i>Saccharomyces cerevisiae</i>	W303-1B	<i>MAT<math>\alpha</math></i>	22
	7416-12-3	<i>MAT<math>\alpha</math> cry1 ade2-1 his4-580 lys2 trp1 tyr1 SUP4-3 leu2 ura3 TYR1<sup>+</sup> sst2-1 ste2-10::LEU2</i>	42
	786-11-1	<i>MAT<math>\alpha</math> cry1 ade2-1 his4-580 lys2 trp1 tyr1 SUP4-3 ade3 leu2 ura3 TYR1<sup>+</sup> sst2-1</i>	32

TABLE 2. Oligonucleotides used in this work

Oligonucleotide	Sequence (5'-3')
ppg1S1	CATGAAGTTCACCTCCCTCTTGTTCATCTTCGCCGCGTGGCTCCGCCACCCCGGC
ppg1S2	CATGGCCGGGGTGGCGGAGGCCACGGCGCGAAGATGACAAGAGGGGAGGGTGAACCT
ETP3	CATGGTAGCCCTACCAACCTCCTCTACTACCGTCTCGCTCTGCGGCCTCGGTTCCGCCCTGCCAGC
ETP4	CATGGTGGCAGGCGGAACCGAGGCGCGACAGGCGGAGGACCGGTAGTGAGGAGGAGGTTGGTGAGGGCTAC
pre2-1	GGATCCATGGCTTCATGCGCTTCCCAACA
pre2-2	GGATCCCTACTCAAAAAGACCTCGGCTTCTG
phe1-1	GAATTCATGAAGTTCACCTCCCTCTT
phe1-2	GAATTCCTAGTTCTCATCAGAGTGAGCAG
pb41	GATCTCAATAACGACACCATCAC
pb42	GAATTCGAGATACTGAAAGGGTC
pb8	GAGAATGTGGCTCCATCTCCA
tC1	GATCCGCCTGGACGACTAAACC
h3	ACTCGTCCGAGGGCAAAGGAATAG
pb6	TGTCAAGGAAAATACCAAGGCCG

perithecia (pro mutants), or ascospores (per mutants) or produce unshaped, piled perithecia (pile mutants). They were generated by UV or ethyl methane-sulfonate mutagenesis from the wild-type strain (49, 50, 54, 60). Transformation of *S. macrospora* was performed according to Nowrousian et al. (54). All *S. macrospora* strains were cultivated on corn meal medium (27). For the halo assays analyzing PPG1 pheromone secretion and for the Western analysis of enhanced green fluorescent protein (EGFP) secretion, *S. macrospora* wild-type and mutant strains and transformants were grown for 3 to 4 days in liquid CM medium (54).

**Construction of plasmids.** In order to determine whether a peptide pheromone encoded by the *ppg1* gene is secreted by *S. macrospora*, we fused the signal sequence (SS) of the *ppg1* gene in-frame to the *egfp* gene of plasmid p82.9 (61). The SS region of the *ppg1* gene was reconstituted with two annealed complementary oligonucleotides (ppg1S1 and ppg1S2). The sequences of all oligonucleotides used in this work are listed in Table 2. The signal sequence was inserted into the NcoI site of p82.9 in the sense (pSppg1-1) and in the antisense (pSppg1-2) orientations. As a control, the signal sequence of an aspartic proteinase gene (*etp*) was fused either in sense in-frame with the *egfp* gene (pSetp-1) or antisense (pSetp-2) (61). The 1.4-kb EcoRI *hph* cassette of pCB1003 was inserted into all constructs to provide a selectable marker (8).

The yeast expression vector pPGK contains unique EcoRI, HindIII, and BamHI cloning sites between the phosphoglycerate kinase (PGK) promoter and the transcription terminator sequences (37). The open reading frames (ORFs) of the genes encoding PPG1 and PRE2 were inserted into pPGK to facilitate expression of the *S. macrospora* genes and to analyze the interaction of the two gene products. The genes were amplified from wild-type DNA by PCR with oligonucleotides pre2-1/pre2-2 (*pre2*) and phe1-1/phe1-2 (*ppg1*) as primers. The PCR products containing the *ppg1* and *pre2* coding sequence were inserted into the PCR cloning vector pDrive (Qiagen, Hilden, Germany). Sequence analysis of the PCR fragments (MWG-Biotech Customer Service, Ebersberg, Germany) confirmed the successful amplification and cloning of the desired fragments. Afterwards plasmids were digested with restriction enzyme BamHI or EcoRI. The resulting 1.7-kb *pre2* BamHI and 835-bp *ppg1* EcoRI fragments were cloned into the single BamHI and EcoRI restriction sites of the vector pPGK, respectively. An overview of all plasmids used in this work is given in Table 3.

**Pheromones.** The synthetic *S. macrospora* PHE1 pheromone (Gln-Trp-Cys-Arg-Ile-His-Gly-Gln-Ser-Cys-Trp) was synthesized by Eurogentec (Seraing, Belgium). Synthetic *S. cerevisiae*  $\alpha$ -factor (Trp-His-Trp-Leu-Gln-Leu-Lys-Pro-Gly-Gln-Pro-Met-Tyr) was obtained from Sigma-Aldrich (Munich, Germany; T6901). Pheromones were diluted in 2% dimethyl sulfoxide, and 1.5 nmol was used for halo and *lacZ* induction assays.

**Determining the effects of *S. macrospora* pheromones on *S. cerevisiae*.** For halo assays, strain 7416-12-3 (*MATa*  $\Delta$ *ste2 sst2-1*) containing plasmid pPre2 or control plasmid pPGK was grown in SD medium lacking uracil to an optical density at 600 nm ( $OD_{600}$ ) of 1.0 to 1.3. One milliliter of cells was embedded in 5 ml of soft agar. To obtain a lawn, the cell-containing soft agar was plated onto SD plates lacking uracil and containing 0.1 M citric acid (pH 4.5). To test the interaction between the PRE2 receptor produced by the cells in the lawn and certain pheromones, 20  $\mu$ l of the following solutions was applied to filter disks (6 mm diameter; Schleicher & Schuell) and placed onto the lawn, either yeast culture supernatants (*MATa* strain W303-1B containing pPpg1 or control plasmid pPGK) or culture medium of *S. macrospora* strains grown for 3 to 4 days in CM medium or 1.5 nmol of synthetic pheromones. The plates with the yeast lawns were grown for 24 to 48 h and analyzed for pheromone-induced cell cycle arrest

by the halo assay. Measuring the distance from the center of the filter disks to the edge of the zone of growth inhibition determined the size of the halo. Plates were scanned electronically and recorded images were edited with Adobe Photoshop 6.0.

Analysis of shmoo formation was done according to Sprague (74). Yeast *MATa* cells were grown to a density of  $5 \times 10^6$  to  $10^7$  cells/ml and were treated either with pheromone at a concentration of 5.0  $\mu$ M or with dimethyl sulfoxide. Samples were removed at 0 h, 2 h, and 4 h and examined by differential interference contrast light microscopy on a Zeiss Axiophot microscope. Images were recorded with an MC80DX camera, and the Adobe Photoshop 6.0 software was used for further processing.

**Yeast *FUS1* and *lacZ* induction assays.** Yeast cell cultures of *MATa* transformants carrying plasmid pPpg1 or plasmid pPGK as a control were grown in SD medium lacking uracil. Supernatants were obtained by centrifugation when growth had reached an  $OD_{600}$  of  $\approx 1.0$ . Similarly, *S. macrospora* wild-type and mutant strains were grown in liquid CM medium, and cell-free supernatants were harvested after 3 to 4 days of growth.

Yeast *MATa* cells (7416-12-3) carrying pPre2 or the control vector pPGK, together with the pheromone-inducible reporter construct pTCFL1 (*FUS1::lacZ*) (11), were grown overnight to an  $OD_{600}$  of  $\approx 1.0$  in SD medium lacking uracil and tryptophan and harvested by centrifugation. Cell pellets were resuspended in 0.5 volume of fresh selective medium.

Equal volumes (10 ml) of receptor-containing *MATa* cells and supernatants of *MATa* *S. cerevisiae* or *S. macrospora* cultures containing the pheromones of interest were mixed and incubated in glass flasks at 30°C and 220 rpm for 2 h. In *lacZ* assays with synthetic pheromone, 1.5 nmol of synthetic PHE1 pheromone was added to 10 ml of *MATa* transformants expressing the *S. macrospora* pheromone receptor gene *pre2*. After the incubation period, the activity of  $\beta$ -galactosidase was measured. This was done by determining *o*-nitrophenyl- $\beta$ -D-galactopyranoside cleavage by crude protein extracts (64). Crude protein extracts were obtained by destroying the yeast cells with glass beads (0.5-mm diameter) in the presence of the proteinase inhibitor phenylmethylsulfonyl fluoride. The method

TABLE 3. Plasmids used in this work

Plasmid	Vector	Insert	Reference
pSM1		<i>gpd</i> promoter and <i>egfp</i> gene	61
pSppg1-1	p82.9	<i>ppg1</i> signal sequence fused sense to <i>egfp</i> gene	This study
pSppg1-2	p82.9	<i>ppg1</i> signal sequence fused antisense to <i>egfp</i> gene	This study
pSetp-1	p82.9	<i>etp</i> signal sequence fused sense to <i>egfp</i> gene	61
pSetp-2	p82.9	<i>etp</i> signal sequence fused antisense to <i>egfp</i> gene	61
pPGK		PGK expression cassette	37
pPre2	pPGK	<i>S. macrospora pre2</i> ORF	This study
pPpg1	pPGK	<i>S. macrospora ppg1</i> ORF	This study
pTCFL1		<i>FUS1</i> -inducible <i>lacZ</i> reporter gene	11
p204-20	pBCKS(+)	1,622-bp EcoRI fragment of cosmid clone H4	This study
p210-1	p204-20	<i>hph</i> cassette from pCB1003	This study

of Bradford (5) was used to determine the protein concentration. Enzyme activity was normalized to the protein concentration in the samples. Six to seven independent measurements of activity were taken from assays of two different transformants, and the mean activities were calculated.

**Generation of *ppg1*-deficient *S. macrospora* mutants.** A 1,622-bp EcoRI fragment containing the *ppg1* coding sequence and its flanking regions was subcloned from cosmid H4 (58) into pBCKS(+) (Stratagene) to obtain plasmid p204-20. The hygromycin phosphotransferase gene (*hph*) within pCB1003 (8) was isolated as a 1.4-kb SalI fragment and cloned into the SalI cleavage site of p204-20 (see Table 3). This caused the desired disruption of the ORF of the *ppg1* gene. The resulting plasmid construct was referred to as p210-1.

To insert the generated *ppg1-hph* construct into *S. macrospora*, a 3.4-kb fragment from p210-1 was amplified by PCR with primers pb41 and pb42 (Table 2). Transformation of *S. macrospora* was done according to Nowrousian et al. (54). *ppg1* mutants were identified by Southern blot analysis. Genomic DNA was isolated as described previously (62). Successful homologous recombination was confirmed by PCR amplification with primers pb8 and tC1 for the 5'-flanking region and primers h3 and pb6 for the 3'-flanking region (see Table 2). Fungal transformants are often heterokaryotic, and mycelia carry transformed and non-transformed nuclei. A single spore isolate of a primary transformant carrying the disrupted *ppg1* gene was therefore investigated further.

**Immunodetection of secreted EGFP.** EGFP secretion in *S. macrospora* was put under the control of the signal sequences from *S. macrospora* genes *ppg1* and *etp*. To achieve this, the signal sequences were fused in the sense and antisense orientations in frame with the *egfp* gene. Immunodetection of secreted EGFP was performed as previously described (61). As a control, a pSM1 transformant expressing the *egfp* gene at high levels was used. In the pSM1 transformant, the EGFP protein is not secreted and is therefore located in the cytoplasm. After *S. macrospora* transformants had grown for 3 days in liquid CM medium, 5 ml of the cell-free medium was removed from the culture, and proteins secreted into the medium were precipitated by adding 10% trichloroacetic acid. Subsequently, precipitated proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to standard protocols (45). The proteins were transferred to a polyvinylidene difluoride Western blotting membrane (Biometra), using a semidry blotting system (Biometra). A polyclonal anti-GFP living color peptide antibody (BD Bioscience) (diluted 1:500) was used for the detection of EGFP. The chemiluminescence Western blotting kit (Roche) was used for visualization as described by the manufacturer.

**Confocal laser microscopy.** Confocal laser microscopy was carried out on a Zeiss LSM 510 META confocal system version 3.0 (excitation/emission 488 nm blue, 10% argon laser power, Ch2-1, BP505-550 filter, Ch2/72  $\mu$ m pinhole, Zeiss Axiovert 100 M, Plan Apo 63/1.4 oil lens). Preparation of *S. macrospora* strains was done as described previously (61).

## RESULTS

**Functional expression of *S. macrospora* pheromones and pheromone receptors in *S. cerevisiae*.** In order to analyze the functionality of the *S. macrospora* peptide pheromone and the PRE2 pheromone receptor, we heterologously expressed the *S. macrospora ppg1* and *pre2* genes in *S. cerevisiae* and tested them for interaction. The coding sequences of the *ppg1* and *pre2* gene were amplified and put under the control of a constitutive promoter derived from the *S. cerevisiae* phosphoglycerate kinase (*PGK*) gene (37), resulting in plasmids pPpg1 and pPre2, respectively. To avoid competition between the *S. cerevisiae*  $\alpha$ -factor receptor Ste2p and the *S. macrospora* PRE2 receptor, we used strain 7416-12-3 (*MATa*  $\Delta$ *ste2 sst2-1*), lacking the *S. cerevisiae* *STE2* gene, as a host for expression of the *S. macrospora pre2* gene.

In an attempt to optimize the conditions for *ppg1* expression and pheromone synthesis in an *S. cerevisiae* host, the *MATa* (W303-1B) strain was chosen because its MF $\alpha$ 1p and MF $\alpha$ 2p precursor proteins are structurally similar to the PPG1 preproprotein of *S. macrospora* (58). To measure the activation of the heterologously expressed *S. macrospora* PRE2 receptor in *MATa* cells, an agar diffusion bioassay (halo assay) was employed. The assay is based on the fact that pheromone expo-

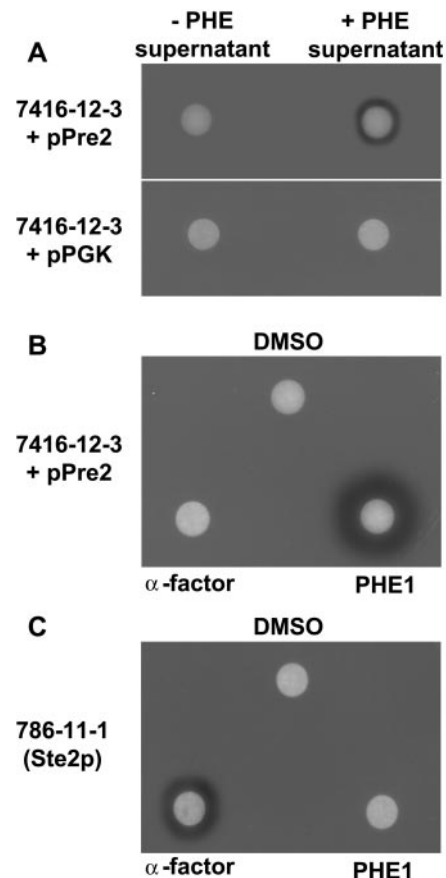


FIG. 2. Pheromone-induced growth arrest of *S. cerevisiae* transformants expressing the *S. macrospora pre2* pheromone receptor gene. (A) Quantitative halo test of *MATa* lawns (7416-12-3;  $\Delta$ *ste2 sst2-1*) either expressing the *S. macrospora pre2* pheromone receptor gene (+PRE2) or carrying the mock vector pPGK (+pPGK). Sterile filter disks contained 20  $\mu$ l of cell-free culture supernatant of *MATa* cells expressing (+PHE) or not expressing (-PHE) the *S. macrospora ppg1* gene. (B) Response of *MATa* lawn (strain 7416-12-3) expressing the *S. macrospora* PRE2 receptor to a single dose of synthetic pheromone PHE1 (1.5 nmol/disk),  $\alpha$ -factor (1.5 nmol/disk), and 2% dimethyl sulfoxide (DMSO; 10  $\mu$ l/disk). (C) Functionality of  $\alpha$ -mating factor was proved on a *MATa sst2-1* lawn (strain 786-11-1) as described for B.

sure triggers a cell division arrest in *S. cerevisiae* cells. In *S. cerevisiae*, binding of a pheromone to its receptor leads to the release of GDP from the G-protein  $\alpha$ -subunit Gpa1p, followed by the binding of GTP and the liberation of the G-protein  $\beta\gamma$ -subunits (Ste4p/Ste18p). The  $\beta\gamma$ -dimer initiates the mitogen-activated protein kinase cascade, resulting in gene transcription, morphological changes, and a G<sub>1</sub> cell cycle arrest (23, 25).

In addition to the *ste2* mutation, the yeast *MATa* strain used in the halo assay also carried the *sst2-1* mutation. The *S. cerevisiae* *SST2* gene encodes a regulator of G-protein signaling (RGS) protein, which is crucial for recovery from pheromone-induced cell cycle arrest (22). Mutants lacking *SST2* are approximately 100-fold more sensitive to pheromones (24). Supernatants of *MATa* transformants secreting an *S. macrospora* peptide pheromone were applied to filter disks and placed on a lawn of *MATa* cells expressing the *S. macrospora* pheromone receptor gene *pre2* (Fig. 2A). As a control, the supernatant of *ppg1-MATa* transformants was applied to a *MATa* lawn con-

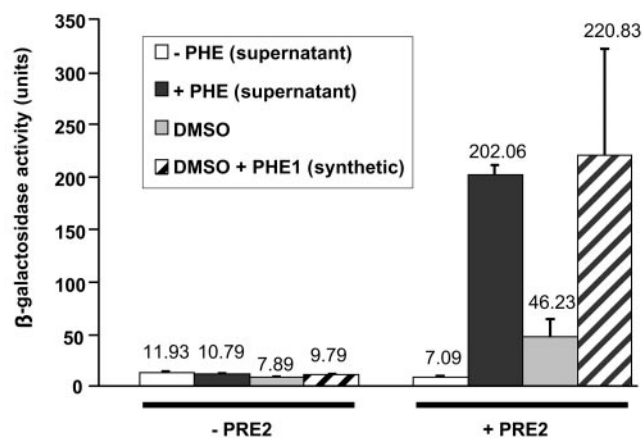


FIG. 3. Activation of an inducible *S. cerevisiae* gene by the *S. macrospora* pheromone-receptor pair. Response to culture supernatants or synthetic pheromone was tested with a *MATa Δste2 sst2-1 S. cerevisiae* strain containing the pheromone-inducible *FUS1-lacZ* reporter plasmid and either pPre2 (+PRE2) or pPGK (–PRE2). Cell-free supernatants were collected from *MATa* transformants (W303-1B) secreting the *S. macrospora* peptide pheromone (black bar) or from *MATa* control transformants carrying pPGK (white bar). In addition, 1.5 nmol of synthetic PHE1 pheromone (striped bar) or an equal volume of the solvent dimethyl sulfoxide (DMSO) was used (grey bar).  $\beta$ -Galactosidase activity was assayed to assess pheromone response. Activities shown are averages from seven independent measurements taken from assays of two different transformants. Error bars are given as indicated.

taining only the mock vector pPGK. Alternatively, the supernatant of a *MATa* culture carrying pPGK was applied to a *MATa pre2* lawn. Application of supernatants of *S. cerevisiae* cells secreting an *S. macrospora* peptide pheromone resulted in a bare zone immediately surrounding the filter disk. In the controls, no halo formation was observed (Fig. 2A). Hence, *S. cerevisiae MATa* cells expressing the *S. macrospora ppg1* pheromone precursor gene must have been able to secrete an active pheromone which promotes cell cycle arrest in *S. cerevisiae MATa* cells expressing the *S. macrospora pre2* gene.

Although *S. cerevisiae MATa* cells seemed capable of processing the *S. macrospora* PPG1 precursor, the precise nature of the active pheromone remained unknown. So far, no pheromones have been purified from filamentous ascomycetes. However, since the overall structure of the PPG1 protein from *S. macrospora* resembles that of *S. cerevisiae* MF $\alpha$ 1p and MF $\alpha$ 2p with respect to signal sequence, cleavage sites for endopeptidases, dipeptidyl-aminopeptidases, and carboxypeptidases, we predicted that the active *S. macrospora* pheromone was an undecapeptide, QWCRIHGQSCW (Fig. 1).

To experimentally test the hypothesis that the undecapeptide PHE1 is one active form of an *S. macrospora* pheromone, the peptide QWCRIHGQSCW was synthesized *in vitro*; 1.5 nmol was added to an *S. cerevisiae MATa Δste2 sst2-1* lawn producing the *S. macrospora* receptor PRE2 (Fig. 2B). Halo formation occurred in response to the synthetic undecapeptide pheromone. However, no halo formation was seen when synthetic *S. cerevisiae*  $\alpha$ -factor or dimethyl sulfoxide was added in control experiments. As a positive control, we were able to induce halo formation when we applied the synthetic *S. cerevisiae* mating  $\alpha$ -factor to strain 786-11-1 (*MATa sst2-1*), which produces the *S. cerevisiae*  $\alpha$ -factor receptor Ste2p. Conversely,

application of the synthetic PHE1 pheromone to *MATa Ste2p* cells resulted in no detectable halo (Fig. 2C). Thus, we were able to demonstrate that in *S. cerevisiae* cells expressing the *S. macrospora pre2* gene, specific interaction occurs between the QWCRIHGQSCW undecapeptide PHE1 and the *S. macrospora* PRE2 receptor.

To quantify the extent of pheromone activation on gene expression, we used the pheromone-inducible *FUS1-lacZ* reporter gene (11, 76). *S. cerevisiae* cells expressing the *S. macrospora* receptor gene *pre2* and the *FUS1-lacZ* reporter gene construct were treated with either culture supernatants of *MATa* cells containing pPpg1 or the control plasmid pPGK, synthetic pheromone, or 2% dimethyl sulfoxide (Fig. 3). The sixfold increase in  $\beta$ -galactosidase activity in the reporter strains after addition of 2% dimethyl sulfoxide might be due to a nonspecific activation of the PRE2 receptor by the solvent. However, a 20-fold increase in  $\beta$ -galactosidase activation in comparison to the negative controls was observed when the supernatant of *MATa* cells expressing the *ppg1*-encoded pheromone or the synthetic peptide QWCRIHGQSCW was added to the cells. This result suggests that the heterologous *S. macrospora* receptor PRE2 is capable of triggering the *S. cerevisiae* pheromone response pathway when activated by the synthetic peptide or the *S. cerevisiae* supernatant.

As a response to pheromone interaction, *S. cerevisiae* shows cell cycle arrest, pheromone-induced gene expression, and morphological changes. Cells exhibit polarized growth towards the mating partner, leading to pear-shaped forms (shmoos) of unconjugated haploid cells (19). Treatment of *MATa Δste2 sst2-1* cells expressing the *S. macrospora* receptor PRE2 with synthetic pheromone for 2 h produced the characteristic morphological changes. The same extent of morphological changes was observed in the control experiments, where *MATa sst2-1* cells (strain 786-11-1) carrying the endogenous Ste2p receptor were exposed to synthetic  $\alpha$ -factor (Fig. 4). In summary, we have shown that the *S. macrospora pre2* gene, when expressed in an *S. cerevisiae MATa Δste2* mutant, can initiate the phero-

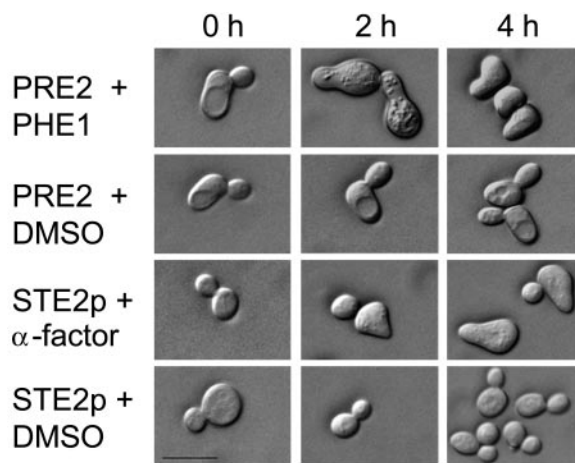


FIG. 4. Shmoosing of *S. cerevisiae* in response to the synthetic pheromone PHE1 and to *S. cerevisiae*  $\alpha$ -factor. *MATa Δste2 sst2-1* cells expressing the *S. macrospora pre2* gene were treated for 0, 2, or 4 h with either synthetic pheromone PHE1 at 5  $\mu$ M or dimethyl sulfoxide. As a control, a *MATa sst2-1* strain was treated with synthetic  $\alpha$ -factor or with dimethyl sulfoxide. Bar, 10  $\mu$ m.

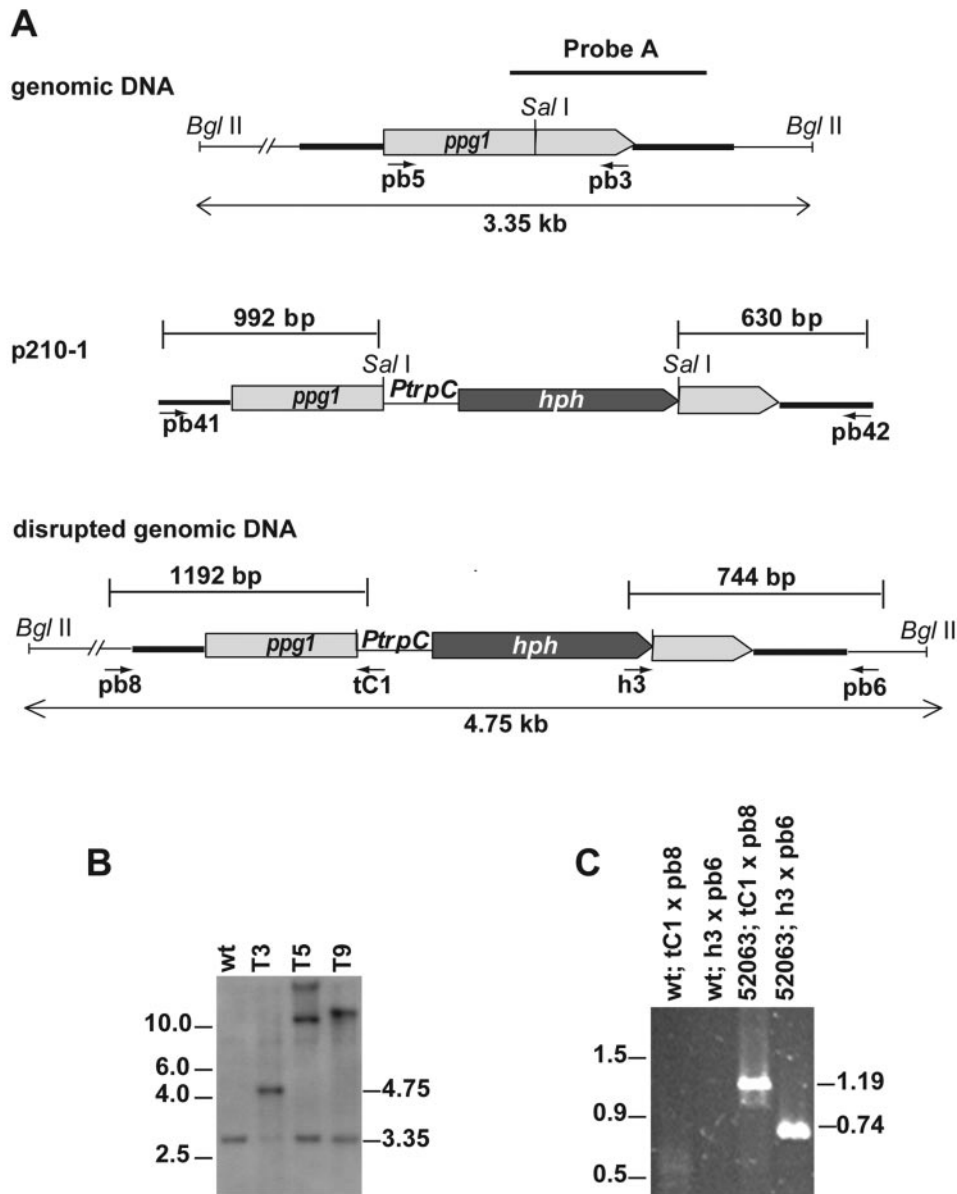


FIG. 5. Disruption of the *S. macrospora ppg1* gene. (A) Structure of the *ppg1* genomic region and construction of the *ppg1::hph* disruption strain. The positions of primers used to amplify the disruption construct from plasmid p210-1 and verify homologous recombination at the *ppg1* locus are indicated. *PtrpC*, *Aspergillus nidulans trpC* promoter. (B) Southern analysis. Genomic DNA from wild-type *S. macrospora* (wt) and three transformants (T3, T5, and T9) was digested with *Bgl*II, separated on a 1% agarose gel, blotted, and hybridized with the 1.2-kb  $^{32}$ P-labeled probe indicated in A. (C) PCR analysis for verification of homologous recombination from a single spore isolate (strain S52063) of transformant T3 and the wild type. The positions of primers are indicated in A.

mone response pathway leading to cell cycle arrest, transcriptional activation of genes, and shmoo formation in the presence of *S. macrospora* peptide pheromone.

**An  $\alpha$ -factor-like pheromone is secreted by *S. macrospora*.** The secretion of diffusible peptide pheromones, which are recognized by pheromone receptors on the cell surface of a mating partner with an opposite mating type, initiates mating in heterothallic ascomycetes. In order to assess whether the homothallic fungus *S. macrospora* secretes a peptide pheromone into the extracellular medium and whether the *ppg1*-encoded pheromone was responsible for pheromone secretion, we disrupted the coding region of the *ppg1* gene of *S. macro-*

*spora* by introducing a hygromycin resistance cassette (*hph*) by homologous recombination. The homologous recombination was confirmed by Southern blot and PCR analysis (Fig. 5). Phenotypic inspection of independent single-spore isolates revealed no differences from the wild type with respect to the timing of sexual development or the morphology and number of perithecia, asci, or ascospores.

Application of the cell-free culture supernatant of an *S. macrospora* wild-type strain onto the *S. cerevisiae MATa* lawn expressing PRE2 resulted in cell cycle arrest, proving that the culture medium of the wild-type *S. macrospora* was able to induce the pheromone response pathway in *S. cerevisiae*. How-

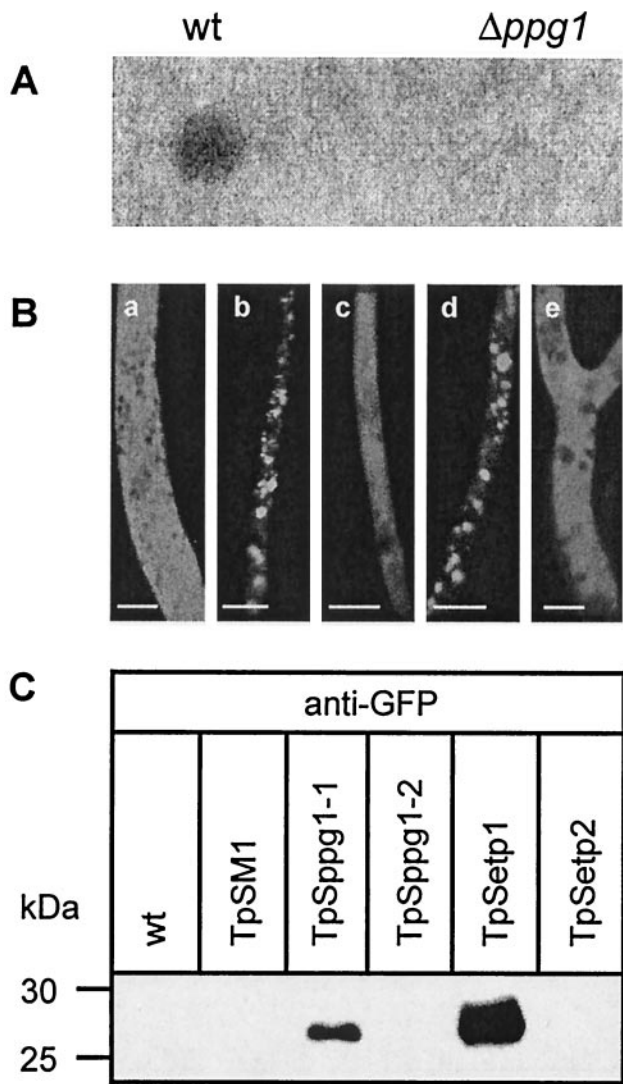


FIG. 6. *ppg1*-encoded peptide pheromone of *S. macrospora* is secreted into the culture medium. (A) Halo assays with fungal culture supernatants. To measure the peptide pheromone which is secreted into the culture medium, 20  $\mu$ l of culture medium of wild-type *S. macrospora* and an *S. macrospora ppg1::hph* disruption mutant (strain S52063) was spotted on an *S. cerevisiae* tester lawn (*MATa  $\Delta$ ste2 sst2-1*) expressing the *S. macrospora* pheromone receptor PRE2. Analysis of halo formation was performed after 24 to 48 h of incubation of plates at 30°C. (B) Analysis of EGFP secretion mediated by N-terminal signal sequences of PPG1. Fluorescence microscopy. Hyphae of *S. macrospora* transformants carrying either pSM1 (a), pSppg1-1 (b), pSppg1-2 (c), pSetp1 (d), or pSetp2 (e) were analyzed by fluorescence microscopy. Scale bars represent 5  $\mu$ m. (C) Western blot. Equal amounts of extracellular cell-free culture medium were analyzed from wild-type *S. macrospora* (wt) and transformants containing either pSM1 (TpSM1), pSppg1-1 (TpSppg1-1), pSppg1-2 (TpSppg1-2), pSetp1 (TpSetp1), or pSetp2 (TpSetp2). Culture medium was fractionated on a denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted on a polyvinylidene difluoride membrane. Western blot detection of EGFP protein was carried out with a polyclonal GFP antibody. Size markers were low-range polypeptides (Bio-Rad), and their sizes are indicated at the left.

ever, when we applied the culture medium of the *ppg1* disruption mutant onto the *S. cerevisiae* lawn expressing the *pre2* gene, no halo was formed (Fig. 6A). Thus, the halo formation observed with the culture medium of the *S. macrospora* wild-type

strain was due to the expression of the *ppg1* gene and secretion of the encoded pheromone.

The 16 N-terminal residues of the pheromone precursor PPG1 of *S. macrospora* constitute a hydrophobic region that may act as a signal sequence during translocation of the precursor across the membrane of the endoplasmic reticulum (58). This is the critical initial step of protein secretion, which is then followed by sorting into the Golgi network (15). To analyze whether the predicted signal sequence alone is able to mediate secretion, we fused the coding sequence of the PPG1 signal sequence in frame to an *egfp* reporter gene.

On the basis of plasmid p82.9, we constructed plasmids pSppg1-1 and pSppg1-2. Plasmid pSppg1-1 contains the signal sequence in the sense orientation. As a control, it was introduced in the antisense orientation into pSppg1-2. Both plasmids were transformed into wild-type *S. macrospora*, and transformants were analyzed for EGFP fluorescence (Fig. 6B). Microscopic investigation revealed a different localization of EGFP in transformants containing pSppg1-1 than in transformants containing pSppg1-2. In transformants expressing the PPG1 signal sequence in the sense orientation, the GFP fluorescence appeared to be distributed in distinct patches in the fungal hyphae, suggesting localization within cytoplasmic vesicles. Transformants carrying pSetp1 were analyzed as a control. The plasmid pSetp1 contains the signal sequence of the *S. macrospora etp* gene fused to the *egfp* gene. Previously it has been shown that the signal sequence of the *etp* gene can mediate the secretion of EGFP in *S. macrospora* (61). As can be seen from Fig. 6B, transformants containing pSetp1 show the same localization of EGFP within cytoplasmic vesicles as pSppg1-1 transformants. In contrast, EGFP was distributed throughout the cytoplasm in transformants containing the PPG1 signal sequence in the antisense orientation (TpSppg1-2). A similar pattern was observed for pSetp2 transformants, which carry an antisense ETP signal sequence fused to EGFP and for pSM1 transformants expressing the *egfp* gene.

Extracellular culture broth of *S. macrospora* transformants was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting with a polyclonal antibody against GFP in order to prove the presence of EGFP in the culture medium (Fig. 6C). The 27-kDa EGFP polypeptide was detected in the culture medium of pSppg1-1 and pSetp1 transformants. No signal was detected in culture supernatants of wild-type, pSM1, pSppg1-2, and pSetp2 transformants. Thus, the signal sequence derived from PPG1 was shown to be sufficient to mediate secretion of EGFP.

***S. cerevisiae* as a powerful screening system for the identification of pheromone mutants of *S. macrospora*.** In heterothallic species, mutations conferring male and/or female sterility can be detected directly because of their sterility effects in heterozygous crosses. However, recessive mutations that effect postfertilization fruiting-body development will remain undetected in heterothallic species until the mutant allele is available in both mating types, thus allowing homozygous crosses. In the homothallic *S. macrospora*, recessive mutations can be tested directly for defects in fruiting-body development. Therefore, *S. macrospora* was used to generate numerous mutants which are blocked at various stages of sexual development (28, 49, 50). These mutants were divided into four main groups: mutants displaying defects in ascogonium development, called



TABLE 4. Halo screening of developmental mutants<sup>a</sup>

Mutant type	No. screened	Halo size		
		Wild type	> Wild type	< Wild type
asc	3	2		1
pro	37	25	5	7
per	36	22	7	7
pile	5	5		

<sup>a</sup> Halo sizes were larger than, smaller than, or comparable to the halo size caused by wild-type *S. macrospora*.

asc mutants; mutants affected in early fruiting-body development, named pro (protoperithecia) mutants; mutants that form perithecia but no ascospores, termed per (perithecia) mutants; and mutants generating malformed and piled fruiting bodies, termed pile mutants (49). Using the halo assay, we have been able to demonstrate that the wild-type strain of *S. macrospora* does secrete a peptide pheromone.

Since pheromones are supposed to be involved in postfertilization events and fruiting-body development (18), we wanted to test if this assay may be suitable for analyzing pheromone production in the *S. macrospora* fruiting-body mutants. Three *S. macrospora* asc mutants, 37 pro mutants, 36 per mutants, and 5 pile mutants were screened by the halo assay. A lawn of *S. cerevisiae* strain 7416-12-3 (*MATa Δste2 sst2-1*) expressing the *S. macrospora pre2* pheromone receptor gene was used. For each of the 81 *S. macrospora* mutants tested, 20 μl of culture medium was applied to an individual *S. cerevisiae* lawn. The culture medium of 54 mutants caused halo formation, which was approximately the same size as the halo seen with the culture medium of wild-type *S. macrospora* (Table 4). However, a change in production or secretion of the peptide pheromone was seen in several mutants. Compared to the halo formed with culture medium from wild-type *S. macrospora*, the culture medium of 12 mutants caused larger halos. In 15 cases, smaller halos were observed (Table 4).

As an example, mutant per44, showing a broader halo, and

mutant per46, showing no halo formation, were analyzed in more detail (Fig. 7A). The culture supernatants of both *S. macrospora* mutants were subsequently tested for their ability to activate the pheromone-inducible *FUS1-lacZ* reporter gene. In comparison to the wild-type culture medium, only a slight decrease in the level of reporter gene induction was seen with the supernatant of mutant per46. The application of supernatant of mutant per44 caused a twofold increase in *FUS1-lacZ* activation levels compared to the wild-type level. Activities were reduced to background level if the same supernatants were added to *S. cerevisiae* control strains lacking the *S. macrospora* PRE2 receptor (Fig. 7B). Thus, the heterologous *S. cerevisiae* system proved useful in identifying *S. macrospora* mutants affected in *ppg1* gene expression.

## DISCUSSION

**Interactions of pheromones and receptors of the homothallic filamentous ascomycete *S. macrospora* can be reconstituted in the yeast *S. cerevisiae*.** Despite being self-fertile, the filamentous ascomycete *S. macrospora* does carry and express two pheromone precursor genes and two pheromone receptor genes (58, 59). We expressed the α-factor-like pheromone precursor PPG1 and its cognate receptor PRE2 in the yeast *S. cerevisiae* to provide experimental evidence that the pheromones and receptors of *S. macrospora* are truly functional.

This study demonstrated that the *S. macrospora* pheromone precursor gene *ppg1* and the pheromone receptor gene *pre2* are functionally homologous to the *S. cerevisiae* *MFα1* and *MFα2* pheromone genes and the *STE2* receptor gene, respectively. Despite the fact that the amino acid sequence of the *S. macrospora* PRE2 receptor shows limited similarity to the *S. cerevisiae* Ste2p α-factor receptor sequence (23.0% identity over 274 amino acids), PRE2 does act as a pheromone receptor in an *ste2*-deficient *S. cerevisiae* mutant (*MATa Δste2 sst2-1*). Activation of the *S. cerevisiae* pheromone response pathway through heterologously expressed receptors has so far been

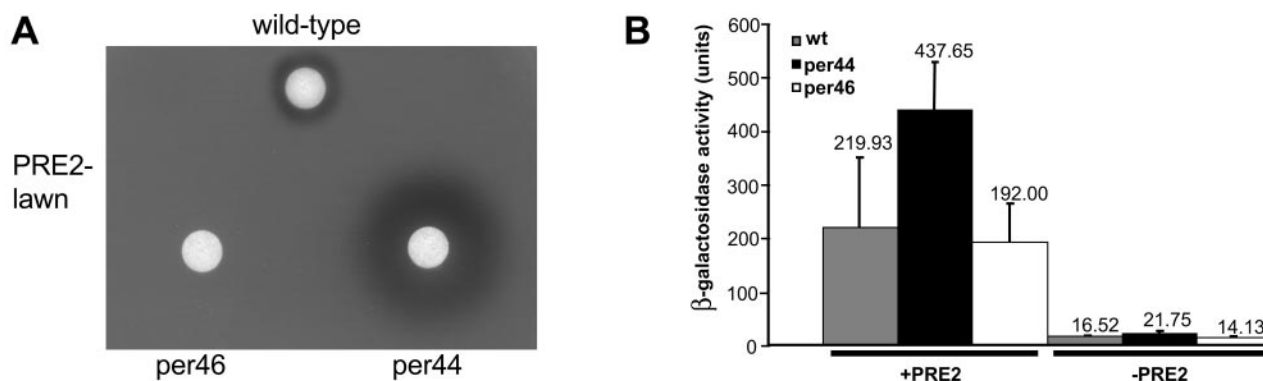


FIG. 7. Halo assays and *FUS1-lacZ* induction assays with cell-free culture medium of wild-type *S. macrospora* and developmental mutants per44 and per46. (A) Culture medium (20 μl) of wild-type *S. macrospora* and mutant strains per44 and per46 were applied on an *S. cerevisiae* tester lawn (*MATa Δste2 sst2-1*) expressing the *S. macrospora* pheromone receptor PRE2. (B) For *S. cerevisiae* *FUS1-lacZ* induction assays, culture medium of wild-type *S. macrospora* (grey bar) and mutant strains per44 (black bar) and per46 (white bar) was taken after growth of strains in liquid CM medium. Pheromone response to the culture medium was tested with a *MATa Δste2 sst2-1* *S. cerevisiae* strain carrying the pheromone-inducible *FUS1-lacZ* reporter plasmid and either pPre2 (+PRE2), encoding the *S. macrospora* PRE2 receptor, or the mock vector pPGK (-PRE2); 10 ml of culture medium of *S. macrospora* strains was mixed with an equal volume of *pre2*-expressing *S. cerevisiae* cells. β-Galactosidase activity was assayed, and activities shown are averages of six independent measurements taken from assays of two different transformants. Error bars are given as indicated.

demonstrated for a number of mammalian G-protein-coupled receptors (26, 41, 63, 66, 75) and for a-factor-like pheromone receptors from basidiomycetes (29, 33, 57).

$\alpha$ -factor, which activates the Ste2p receptor in *S. cerevisiae*, is a 13-amino-acid peptide. It is processed from larger polypeptide precursors containing several copies of the active tridecapeptide. Similarly, an active *S. macrospora* peptide pheromone is thought to be an undecapeptide derived from the PPG1 precursor. This precursor contains five copies of the amino acid sequence QWCRIHGQSCW (58). In this work we demonstrated that a peptide pheromone secreted by *S. cerevisiae* MAT $\alpha$  cells expressing the *ppg1* gene interacts with the PRE2 receptor. So far, only a-factor-like pheromones of the basidiomycetes *Coprinus cinereus* and *Schizophyllum commune* have been heterologously expressed in *S. cerevisiae* (29, 57). Furthermore, we were able to show that the synthetic undecapeptide PHE1 (QWCRIHGQSCW) was biologically active when tested in the *S. cerevisiae* system, suggesting that the synthetic peptide might represent one active form of the *S. macrospora* peptide pheromone. The lowest concentration of synthetic PHE1 to cause visible halo formation was found to be 1.5 pmol.

The size of the halo formed when synthetic PHE1 was added to a MAT $\alpha$   $\Delta$ ste2-PRE2 lawn was compared to the halo seen in a MAT $\alpha$  lawn treated with synthetic  $\alpha$ -factor. PHE1 seemed to have a more profound effect than the  $\alpha$ -factor (Fig. 2B and C). One possible explanation for this is the presence of the extracellular protease Bar1p in *S. cerevisiae* cells. The enzyme encoded by the *BARI* gene cleaves *S. cerevisiae*  $\alpha$ -factor between leucine 6 and lysine 7, rendering it nonfunctional. The enzymatic degradation of the  $\alpha$ -factor enables the target cells to recover from arrest in the G<sub>1</sub> phase of the cell cycle (13, 48). The PHE1 pheromone does not contain leucine or lysine residues. This may protect it from enzymatic cleavage, making it more stable than *S. cerevisiae*  $\alpha$ -factor.

Activation of the heterologously expressed PRE2 receptor by synthetic pheromone PHE1 or an *S. macrospora* peptide pheromone secreted from MAT $\alpha$  cells initiates the *S. cerevisiae* pheromone response pathway, resulting in characteristic changes. These include cell cycle arrest, gene activation, and the formation of one or more projections on the cell surface, commonly termed shmoo morphology (Fig. 2 to 4). It has previously been shown that pheromone receptors of basidiomycetes heterologously expressed in an *S. cerevisiae* host can initiate gene expression and cell cycle arrest in response to their cognate pheromone (29, 33, 57). However, formation of the shmoo morphology upon pheromone application has not been observed so far in experiments expressing basidiomycete receptors in *S. cerevisiae* (29). Dose-response curves have been determined for each of the three responses as a function of  $\alpha$ -factor concentration in *S. cerevisiae*. The analysis revealed that approximately 100-fold-higher concentrations of pheromones are required for the induction of shmoo formation than for transcriptional activation or cell cycle arrest (52). In contrast to the *S. commune* receptor, the *S. macrospora* PRE2 receptor is able to mediate all pheromone responses when expressed in *S. cerevisiae* (29).

Since, in *S. cerevisiae*, PRE2 was activated by extracellular pheromones and was able to trigger the pheromone response, it seems likely that PRE2 has been incorporated into the

plasma membrane and might interact with the Gpa1p protein of *S. cerevisiae*. This hypothesis is supported by the fact that the MAT $\alpha$   $\Delta$ ste2 strain used in our analysis carried a mutation in the *SST2* gene. Sst2p acts in desensitization to pheromone through negatively regulating the *S. cerevisiae* G $\alpha$ -subunit Gpa1p. *S. cerevisiae* *sst2-1* mutants are therefore at least 100-fold more sensitive to pheromones and fail to recover from pheromone-induced cell cycle arrest (10, 22, 24). Accordingly, increased sensitivity to pheromone was present in Sst2p-deficient MAT $\alpha$   $\Delta$ ste2 cells expressing PRE2, but PHE1 never caused cell cycle arrest in wild-type *SST2* strains (our unpublished results). The third intracellular loop of the *S. cerevisiae* Ste2p receptor is important for G-protein activation (14). However, no homologies in the amino acid sequences of Ste2p and PRE2 were found in the region predicted to form the third intracellular loop (59). Thus, properties other than sequence homology have to account for the ability of PRE2 and the *S. cerevisiae* G-protein to interact.

The importance of heterotrimeric G-proteins during fertilization and sexual development has been demonstrated for the heterothallic fungus *Neurospora crassa*, which is closely related to *S. macrospora* (1, 34, 38, 80). The *N. crassa* a-factor-like pheromone receptor PRE1 is thought to couple to the G-protein  $\alpha$ -subunit GNA-1 of the heterotrimeric G-protein, thereby transferring the pheromone signal (39). A gene encoding a protein similar to the GNA-1 subunit of *N. crassa* has been identified in *S. macrospora*, and the PRE2 receptor may interact with this protein in a similar way as hypothesized for *N. crassa*. In addition to a *gna-1* homologue, several genes encoding putative downstream components of the pheromone signal transduction cascade, including genes coding for subunits of G-proteins, RGS proteins, mitogen-activated protein kinase cascade components, and transcription factors have been isolated from *S. macrospora* (Table 5) (56). These findings suggest not only that pheromones and pheromone receptors are similar in structure and functionality, but also that the signal transduction pathways in *S. macrospora*, *N. crassa*, and *S. cerevisiae* are highly conserved.

***S. macrospora* secretes a peptide pheromone that activates the pheromone response pathway of MAT $\alpha$  cells expressing the pre2 receptor gene.** Halo assays revealed that cell-free culture medium of *S. macrospora* wild-type strains contained components which can induce cell cycle arrest in MAT $\alpha$  cells expressing the *S. macrospora* PRE2 receptor. It was previously demonstrated that the growth medium of the basidiomycete *Schizophyllum commune* applied to *S. cerevisiae* cells expressing a *Schizophyllum commune* receptor can activate expression of a *FUS1-lacZ* reporter gene (33). This suggests that filamentous fungi secrete pheromones into the culture medium.

The SignalP version 1.1 program (53) predicted a hydrophobic secretion signal in the N terminus of the *S. macrospora* PPG1 protein, which was expected to be cleaved between amino acids 16 and 17 (Fig. 1). The signal sequence of the *S. cerevisiae*  $\alpha$ -factor precursor MF $\alpha$ 1p has been used to manipulate secretion of heterologous proteins in *S. cerevisiae* for several years (6). The GFP fluorescence of transformants carrying the PPG1 signal sequence-*egfp* sense construct appeared as distinct patches distributed along the fungal hyphae, suggesting localization within cytoplasmic vesicles. Fluorescence signals in PPG1 signal sequence-*egfp* antisense transfor-

TABLE 5. Putative *S. macrospora* homologues of *S. cerevisiae* genes that encode components of the pheromone response pathway and processing of  $\alpha$ -factor<sup>a</sup>

Function <sup>b</sup>	<i>S. cerevisiae</i> gene; ORF name	Putative <i>S. macrospora</i> homologue (accession no. of partial gene sequence); <i>E</i> value
G-protein $\alpha$ subunit	<i>GPA1</i> ; YHR005C	<i>gna-1</i> (AJ879481); 5.2e-38
G-protein $\beta$ subunit	<i>STE4</i> ; YOR212W	<i>gnb-1</i> (AJ879478); 2.8e-33
Serine threonine kinase	<i>STE20</i> ; YHL007C	<i>ste20</i> (AJ879480); 3.2e-26
MAPKKK	<i>STE11</i> ; YLR362W	<i>nrc-1</i> (AJ879482); 1.3e-19
MAPKK	<i>STE7</i> ; YDL159W	<i>ste7</i> (AJ879473); 4.1e-33
MAPK	<i>FUS3</i> ; YBL016W	<i>fus3</i> (AJ879483); 3.4e-62
Transcription factor	<i>STE12</i> ; YHR08W	<i>ste12</i> (AJ879472); 1.3e-43
Regulator of G-protein signaling	<i>SST2</i> ; YLR452C	<i>rgs1</i> (AJ879475); 2.8e-17
Protein tyrosine phosphatase; adaptation to pheromone	<i>PTP3</i> ; YER075C	<i>ptp3</i> (AJ879476); 2e-6
Protein tyrosine phosphatase; adaptation to pheromone	<i>MSG5</i> ; YNL053W	<i>msg5</i> (AJ879477); 3.7e-15
Carboxypeptidase; $\alpha$ -factor processing	<i>KEX1</i> ; YGL203C	<i>kex1</i> (AJ879474); 2.2e-59
Subtilisin-like protease; $\alpha$ -factor processing	<i>KEX2</i> ; YNL238W	<i>kex2</i> (AJ879479); 9.3e-81

<sup>a</sup> Partial gene sequences from *S. macrospora* genomic DNA were compared with *S. cerevisiae* ORF proteins (using BLASTX) available through the *Saccharomyces* genome database (<http://www.yeastgenome.org/>). *E* (expected) values of *S. macrospora* partial ORFs are indicated with respect to the corresponding *S. cerevisiae* protein.

<sup>b</sup> MAPK(KK), mitogen-activated protein kinase (kinase kinase).

ants were distributed evenly throughout the cytoplasm (Fig. 6B). By means of Western blot analysis, we demonstrated that the PPG1 signal peptide carries sufficient information to enable EGFP to pass through the secretory pathway and to be released into the culture medium.

Using PCR, we identified the sequences of putative homologues of *S. cerevisiae* genes encoding the  $\alpha$ -factor precursor processing enzymes Kex2p and Kex1p in the *S. macrospora* genome (Table 5). Reverse transcription-PCR and Northern blot analysis confirmed that these genes are transcriptionally expressed in *S. macrospora* (data not shown). Thus, processing of the PPG1 precursor in *S. macrospora* may follow the same pathway as described in *S. cerevisiae*. According to our findings, we postulate that the undecapeptide QWCRIHQSCW most likely represents one active form of an  $\alpha$ -factor-like pheromone in *S. macrospora* because it can activate the PRE2 receptor in the heterologous *S. cerevisiae* system. However, based on our data, we cannot exclude the possibility that longer or shorter forms of the peptide pheromone exert a pheromone function in *S. macrospora* or that the active peptide in the *S. cerevisiae* assay may have a different structure than the native, mature pheromone produced in *S. macrospora*.

**Disruption of the *S. macrospora ppg1* gene prevents secretion of the peptide pheromone but does not affect sexual development.** After disrupting the *ppg1* gene, we were no longer able to induce halo formation in an *S. cerevisiae* *MATa* lawn expressing the *pre2* gene when we added culture medium from the *S. macrospora ppg1* mutant. Thus, we proved that disruption of the *ppg1* gene resulted in a defect of peptide pheromone production and secretion. However, no other phenotypic changes have been observed in the *S. macrospora ppg1* mutant. Despite lacking a functional *ppg1* gene, the *S. macrospora ppg1* mutant was able to develop fruiting bodies, asci, and ascospores, and its vegetative growth was not affected.

The homothallic ascomycete *S. macrospora* expresses both pheromone genes throughout its development, whereas heterothallic ascomycetes express pheromone genes predominantly in conidia, and expression occurs in a mating type-specific manner (3, 17, 58, 69, 81). Deletion of pheromone genes in the heterothallic ascomycetes *Podospira anserina* and *Cryphonectria parasitica* revealed that pheromones play an es-

ential role in promoting fertilization. Strains carrying these mutations are male sterile because their conidia are unable to attract and mate with cells of the opposite mating type. In *P. anserina*, deletion of pheromone genes affects male fertility without impairing vegetative growth or postfertilization events (17). The same observation was made in *C. parasitica*, where the *Mf1-1* gene encoding the  $\alpha$ -factor-like pheromone was deleted by gene replacement (77). Our observations in *S. macrospora* were very similar, even though defects in male sterility could not be demonstrated because *S. macrospora* does not produce conidia. On the contrary, deletion of one of the two copies of the *C. parasitica* gene encoding the  $\alpha$ -factor-like pheromone resulted in a pleiotropic phenotype. *Mf2-2* mutants display reduced sexual reproduction. A cross between an *Mf2-2* mutant (as the female) and a wild-type strain (as the male) produced only barren perithecia (82). It was therefore assumed that *Mf2-2* of *C. parasitica* is required during a developmental phase after fertilization and that the CaaX-type pheromone acts in a dosage-specific manner in post-fertilization events (77). Similarly, *N. crassa mfa-1* mutants in which the critical cysteine residue of the open reading frame had been changed to the nonprenylatable residue tyrosine (YAAX mutants), as well as mutants with intact open reading frames but multiple mutations in the 3' non-coding region (CAAX mutants), were shown to display delayed and reduced vegetative growth and aberrant sexual development. Surprisingly, this pleiotropic phenotype was observed in both mating types. It was therefore postulated that the lipopeptide pheromone of *N. crassa* may have an additional role in cementing hyphae together to stabilize the structure of the perithecium (40).

Northern blotting and reverse transcription-PCR analysis indicated that, in *N. crassa*, in contrast to pheromone precursor genes, expression of the receptor genes does not occur in a mating type-specific manner (39, 59). Deletion of the *N. crassa pre-1* gene encoding the  $\alpha$ -factor-like receptor does not affect vegetative growth or male fertility. On the other hand,  $\Delta$ *pre1* *matA* mutants are sterile females, because their trichogynes are unable to recognize and fuse with *matA* conidia (39). Deletion of two putative pheromone receptor genes, *gprA* and *gprB*, in the homothallic filamentous ascomycete *Aspergillus nidulans*

resulted in the production of fewer and smaller fruiting bodies carrying a reduced number of ascospores. Fruiting-body formation in homothallic conditions was absent in the  $\Delta$ *gprA*  $\Delta$ *gprB* double mutant (68). This result implies that a pheromone-receptor system is essential for postfertilization events during the sexual development of homothallic ascomycetes. It has to be expected that the second active pheromone-receptor pair (PPG2 and PRE1) of *S. macrospora* is able to functionally complement the disruption of the *ppg1* gene in *S. macrospora*. This would explain why no defects in sexual development were detected in the *S. macrospora ppg1* mutant.

**The *S. cerevisiae* system provides a tool to measure pheromone secretion in developmental mutants of *S. macrospora*.** Expressing the functional *S. macrospora* receptor PRE2 in *S. cerevisiae*, we developed a powerful tool for studying pheromone secretion in developmental mutants of *S. macrospora*. Of 81 developmental mutants tested in this work, 27 secreted a different amount of peptide pheromone than the wild type (Table 4). The culture medium of these 27 strains led to the formation of either smaller or larger halos. The halo assay results for two mutants, per44 (larger halo) and per46 (no halo), were confirmed by testing their culture medium for activation of the pheromone-inducible *FUS1-lacZ* reporter (Fig. 7). Induction of the reporter gene was only slightly decreased with the supernatant of mutant per46. We detected a twofold increase in the induction of *FUS1-lacZ* with the supernatant of mutant per44. The *FUS1-lacZ* reporter assay was therefore more sensitive than the halo assay. Northern blot analysis showed that transcriptional expression of *ppg1* was elevated in mutant per44, while in per46 the transcript level was only slightly decreased (our unpublished results). Currently we do not know which genes are defective in mutants per44 and per46.

However, four of the mutants screened (pro1, pro1, pro22, and per5) have previously been complemented by transformation with an indexed cosmid library. The *pro1* gene encodes a transcription factor, and the *pro22* gene codes for a putative membrane protein (50, 55). The PRO11 protein is a membrane-associated protein and thought to be a scaffolding protein in several signal transduction pathways (60). The defect in mutant per5 lies in the *acl1* gene, encoding a subunit of an ATP-citrate lyase (54). The culture medium of per5 led to wild-type-like halo formation in the *S. cerevisiae* assay, whereas the culture medium of pro1, pro11, and pro22 caused increased halo sizes. In this context, it is of interest that interspecies microarray hybridization of *N. crassa* with targets of *S. macrospora* mutants pro1, pro11, and pro22 revealed that the pheromone precursor gene *ppg1* is among the genes which are upregulated more than twofold in these mutants (55). These findings indicate that alterations in pheromone gene expression may be involved in the sexual development of homothallic ascomycetes.

The heterologous *S. cerevisiae* system offers an easy and valuable tool to identify mutants carrying a defect in *ppg1* gene expression and pheromone secretion pathways. Further analysis of these mutants will help us to establish the way in which pheromones are processed in filamentous ascomycetes and how pheromones are involved in postfertilization events of filamentous ascomycetes.

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