Functional Characterization of an α-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 α-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 α-factor receptor and Ste3p a-factor receptor, respectively. To investigate whether the α-factor-like pheromone-receptor pair of S. macrospora is functional, a heterologous yeast assay was used. Our results show that the S. macrospora α-factor-like pheromone precursor PPG1 is processed into an active pheromone by yeast MATα cells. The S. macrospora PRE2 protein was demonstrated to be a peptide pheromone receptor. In yeast MATα 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. These can be divided into two groups depending on the pathway of synthesis and secretion. The pheromone receptor system of the heterothallic ascomycete 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 MATα and MATα cells to form diploid MATαα cells. The peptide synthesized by MATα cells is derived from a precursor with a C-terminal CaaX motif, and X is any amino acid residue, a signal for carboxymethylation and farnesylation. The mature lipopeptide pheromone, called α-factor, is secreted via an ATP-binding cassette transporter. MATα cells produce α-factor, which is secreted by the classical yeast secretory pathway. The α-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-α-factor. Further processing includes the Kex2 endoprotease, which cleaves the pro-protein at characteristic KR dipeptides, the dipetidyl-aminopeptidase Ste13p, which recognizes the dipeptide XA or XP, and the carboxypeptidase Kex1p, which removes C-terminal extensions.

The S. cerevisiae pheromone receptors for α-factor (Ste3p) and α-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 α-factor group and Ste3p-like lipopeptide receptors have been found in basidiomycetes. 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 Podospora anserina, but also in the homothallic filamentous ascomycete Sordaria macrospora. 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. In the homothallic ascomycete S. macrospora, conidiospores, spermatia, and trichogynes are absent, suggesting that phero-
mones are not needed either for sensing a mating partner or for initializing fertilization events.

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

In order to examine the functionality of \textit{S. macrospora} pheromone precursor \textit{PPG1} and pheromone receptor \textit{PRE2}, the yeast \textit{S. cerevisiae} was used as a heterologous assay system. A synthetic peptide was designed to analyze whether the undecapeptide predicted from the \textit{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 \textit{ppg1} of \textit{S. macrospora} to investigate its involvement in fruiting body and ascospore development.

**MATERIALS AND METHODS**

**Strains, culture conditions, and transformation.** \textit{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. \textit{Saccharomyces cerevisiae} strain 7416-12-3 was used as a host for plasmids containing \textit{S. macrospora} pheromone receptor gene \textit{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 \textit{S. macrospora} \textit{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 \textit{S. macrospora} to investi-

![Fig. 1. Schematic structure of the \textit{S. macrospora} pheromone precursor PPG1. The predicted PPG1 pheromone precursor consists of a hydrophobic signal sequence (black box) and five repeats of the undecapeptide 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.](image-url)

**TABLE 1. Strains used in this study**

| Organism       | Strain   | Relevant genotype    | Reference or source |
|----------------|----------|----------------------|---------------------|
| \textit{Sordaria macrospora} | S48977               | Wild type, homothallic | This study          |
| per44 (S48143) | sterile, no ascospores | \(ppg1::Jog^R\), sterile, no ascospores | This study          |
| per66 (S43021) | sterile, no ascospores | \(ppg1::Jog^R\), sterile, no ascospores | This study          |
| S52063         | sterile, no ascospores | \(ppg1::Jog^R\), sterile, no ascospores | This study          |
| \textit{Saccharomyces cerevisiae} | W303-1B         | MAT\(\alpha\)         | This study          |
| 7416-12-3      | MAT\(\alpha\) cry1 ade2-1 his4-580 | MAT\(\alpha\) cry1 ade2-1 his4-580 | 22                  |
|                | hs2 trp1 trp1 SUP4-3 leu2 | hs2 trp1 trp1 SUP4-3 leu2 | 42                  |
|                | ura3 TYRI\(\star\) sst2-1 ste2-10: :: LEU2 | ura3 TYRI\(\star\) sst2-1 ste2-10: :: LEU2 |                       |
| 786-11-1       | MAT\(\alpha\) cry1 ade2-1 his4-580 | MAT\(\alpha\) cry1 ade2-1 his4-580 | 32                  |
|                | hs2 trp1 trp1 SUP4-3 leu2 | hs2 trp1 trp1 SUP4-3 leu2 | 32                  |
|                | ura3 TYRI\(\star\) sst2-1 | ura3 TYRI\(\star\) sst2-1 | 32                  |
were grown for 24 to 48 h and analyzed for pheromone-induced cell cycle arrest

medium or 1.5 nmol of synthetic pheromones. The plates with the yeast lawns

S. macrospora

supernatants (pPGK) or culture medium of

S. macrospora

Oligonucleotide

Sequence (5'-3')

ppg1S1 .......................... CATGAAGTTCACCCTCCCTCTGCTCCTCGG

ppg1S2 .......................... CATTGCCGGGGTTGCGGAGCCAGGCGCCG

ETP3 .......................... CATGGCTGGCAGGGCGGACCCGAGGCCG

ETP4 .......................... CATGGCTGGCAGGGCGGACCCGAGGCCG

pre2-1 .......................... GGTACCCTGGCCTTCCTCCCAACA

pre2-2 .......................... GATCTCCGCTATGCTTCCTCTG

phe1-1 .......................... GAATTCTAGATGGTACACTCCCAACCTAT

phe1-2 .......................... GAATTCTAGATGGTACACTCCCAACCTAT

pfdS ........................... GGATCCCTACTCAAAAGACCTCGGCTTCTG

pb41 ........................... GGATCCATGGCTTCATGCGCTTCCCCAACA

pb8 ........................... GAGAATTTGCCTGATCCTTCC

tC1 ............................. GATCCGCCTGGACGACTAAACC

h3 .............................. ACTCGTCGGGAGGGCCAGGATAAG

pbs .............................. TGTCAGAAGAAATACCAAGGGC

TABLE 2. Oligonucleotides used in this work

ppg1 and ppg2 genes were inserted into pPGK to facilitate

expression of the

the genes encoding Ppg1 and Pre2 were inserted into pPGK to facilitate

expression of the

The SS region of the

sequence (SS) of the

pheromone encoded by the

ppg1 gene is secreted by S. macrospora, we fused the signal

sequence and to analyze the interaction of the two

pre2 genes. The genes were amplified from wild-type DNA by PCR with

PCR products containing the

and mutant strains and transformants were grown for 3 to 4 days in liquid CM

medium (54).

mutant strains and transformants were grown for 3 to 4 days in liquid CM

medium lacking uracil to an optical density at

OD600 of 1.0 to 1.3. One milliliter of cells was embedded in 5 ml of soft

plasmid pPGK was grown in SD medium lacking uracil to an optical density at

OD600 of 1.0 in SD medium lacking uracil and

Scanned electronically and recorded images were edited with Adobe Photoshop 6.0.

Analysis of shmoo formation was done according to Sprague (74). Yeast

MAT+/H9251

or

MAT-/H11003

S. macrospora cultures containing the pheromones of

S. macrospora

in the liquid CM medium for 3 to 4 days.

Yeast

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 OD600 of ~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

etp gene (pSetp-1) or antisense (pSetp-2) (61). The 1.4-kb EcoRI

hphp 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

perihelia (pro mutants), or ascospores (per mutants) or produce unshaped,
piled perihelia (pilate mutants). They were generated by UV or ethyl

methylene-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

Determination of

the effects of S. macrospora

pheromones on S. cerevisiae. For halo

assays, strain 7416-12-3 (MATa leu2 tsst2/1) containing plasmid Prsv2 or control

plasmid pPGK was grown in SD medium lacking uracil to an optical density at

600 nm (OD600) of 1.0 to 1.3. One milliliter of cells was embedded in 5 ml of soft

agar. To obtain a cell, the containing-soft cell was plated onto SD plates

lack uracil and containing 0.1 M citric acid (pH 4.5). To test the interaction

between the PR2 receptor produced by the cells in the lawn and certain

pheromones, 20 µl 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 pPg1 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.

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 inoculated 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 β-galac-

tosidase was measured. This was done by determining o-nitrophenyl-β-d-galac-

topyranoside cleavage by a crude protein extract (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     | pPGK   | gpd promoter and egfp gene | 61        |
| pSppg1-1 | p82.9  | ppg1 signal sequence fused to egfp gene | This study |
| pSppg1-2 | p82.9  | ppg1 signal sequence fused to egfp gene | This study |
| pSetp-1  | p82.9  | etp signal sequence fused to egfp gene | 61        |
| pSetp-2  | p82.9  | etp signal sequence fused to egfp gene | 61        |
| pPGK     | pPGK   | S. macrospora pre2 ORF | 37        |
| pPpg1    | pPGK   | S. macrospora ppg1 ORF | This study |
| pTCFL1   | pPGK   | Fus1-inducible lacz reporter gene | 11        |
| p204-20  | pBCKS(+) | 1.622-bp EcoRI fragment of cloned lacz H4 | This study |
| p210-1   | p204-20 | hphp 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 PC1 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 (Biome-trax), 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/515 nm) using 10% argon laser power, Ch2-1, BP505-550 filter, Ch2/72 filter (Zeiss LSM 510 META confocal system version 3.0). Immunofluorescence images were acquired with the Zeiss LSM 510 META confocal system camera (Zeiss). The EGFP fluorescence signal was corrected against a fluorescently labeled 4′,6′-diamidino-2-phenylindole (DAPI) control image. Image processing was performed using the Adobe Photoshop program.

**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 constitutive promoters derived from the *S. cerevisiae* *STE2* and *S. macrospora* *STE2* genes, respectively. To avoid competition between the heterologously expressed *S. macrospora* *ppg1* and *S. cerevisiae* *PRE2* receptor to a single dose of synthetic pheromone, binding of a pheromone to its receptor leads to the release of GDP from the G-protein subunits (Ste4p/Ste18p). The Ste4p/Ste18p is crucial for recovery from pheromone-induced cell cycle arrest (23, 25).

In addition to the ste2 mutation, the yeast *MATα* 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 *MATα* transformants secreting an *S. macrospora* peptide pheromone were applied to filter disks and placed on a lawn of *MATα* cells expressing the *S. macrospora* pheromone receptor gene *pre2* (Fig. 2A). As a control, the supernatant of *ppg1-MATα* transformants was applied to a *MATα* lawn con-
containing 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 MFα1p and MFα2p 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 ss2-1 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 α-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 α-factor to strain 786-11-1 (MATa ss2-1), which produces the S. cerevisiae α-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 pPG1 or the control plasmid pPGK, synthetic pheromone, or 2% dimethyl sulfoxide (Fig. 3). The sixfold increase in β-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 β-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 (shmooos) of unconjugated haploid cells (19). Treatment of MATa Δste2 ss2-1 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 ss2-1 cells (strain 786-11-1) carrying the endogenous Ste2p receptor were exposed to synthetic α-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. 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 ss2-1 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). β-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.](image-url)

![FIG. 4. Shmooing of S. cerevisiae in response to the synthetic pheromone PHE1 and to S. cerevisiae α-factor. MATa Δste2 ss2-1 cells expressing the S. macrospora pre2 gene were treated for 0, 2, or 4 h with either synthetic pheromone PHE1 at 5 μM or dimethyl sulfoxide. As a control, a MATa ss2-1 strain was treated with synthetic α-factor or with dimethyl sulfoxide. Bar, 10 μm.](image-url)
mone response pathway leading to cell cycle arrest, transcriptional activation of genes, and shmoo formation in the presence of *S. macrospora* peptide pheromone.

An α-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. macrospora* 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* MATα 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-
ever, when we applied the culture medium of the \textit{ppg1} disruption mutant onto the \textit{S. cerevisiae} lawn expressing the \textit{pre2} gene, no halo was formed (Fig. 6A). Thus, the halo formation observed with the culture medium of the \textit{S. macrospora} wild-type strain was due to the expression of the \textit{ppg1} gene and secretion of the encoded pheromone.

The 16 N-terminal residues of the pheromone precursor PPG1 of \textit{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 \textit{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 \textit{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 \textit{S. macrospora} etp gene fused to the \textit{egfp} gene. Previously it has been shown that the signal sequence of the \textit{etp} gene can mediate the secretion of EGFP in \textit{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 \textit{egfp} gene.

Extracellular culture broth of \textit{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.

\textit{S. cerevisiae} as a powerful screening system for the identification of pheromone mutants of \textit{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 \textit{S. macrospora}, recessive mutations can be tested directly for defects in fruiting-body development. Therefore, \textit{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
smaller halos were observed (Table 4). Formed with culture medium from wild-type omon was seen in several mutants. Compared to the halo ever, a change in production or secretion of the peptide pheromone was observed in mutants.

The culture medium of 54 mutants caused halo formation, of wild-type S. cerevisiae culture medium was applied to an individual lawn of S. macrospora. 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 (MAT∼a 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.

**TABLE 4. Halo screening of developmental mutants**

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

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

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**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 secrete a peptide pheromone. Since pheromones are supposed to be involved in postfer-tilization events and fruiting-body development, 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 (MAT∼a 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).

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**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 (MAT∼a 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 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).

α-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 MATα 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 PRE1 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 MATα Δste2-PRE2 lawn was compared to the halo seen in a MATα lawn treated with synthetic α-factor. PHE1 seemed to have a more profound effect than the α-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 α-factor between leucine 6 and lysine 7, rendering it nonfunctional. The enzymatic degradation of the α-factor enables the target cells to recover from arrest in the G1 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 α-factor.

Activation of the heterologously expressed PRE2 receptor by synthetic pheromone PHE1 or an S. macrospora peptide pheromone secreted from MATα 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 α-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 MATα Δste2 strain used in our analysis carried a mutation in the SST2 gene. Sst2p acts in desensitization to pheromone through negatively regulating the S. cerevisiae Gα-subunit Gpa1p. S. cerevisiaesst2-1 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 MATα Δste2 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 α-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 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 MATα 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 MATα 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 α-factor precursor MFα1p 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-
mants 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 α-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 QWCRIHGQSCW most likely represents one active form of an α-factor-like pheromone in S. macrospora because it can activate the PPRE2 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.

Table 5. Putative S. macrospora homologues of S. cerevisiae genes that encode components of the pheromone response pathway and processing of α-factor*

| Function                          | S. cerevisiae gene; ORF name | Putative S. macrospora homologue (accession no. of partial gene sequence); E value |
|-----------------------------------|------------------------------|----------------------------------------------------------------------------------|
| G-protein α subunit               | GPA1; YHR056C                | gna-1 (AJ879481); 5.2e-38                                                        |
| G-protein β subunit               | STE4; YOR212W                | gnb-1 (AJ879478); 2.8e-33                                                        |
| Serine threonine kinase           | STE20; YHL067C               | ste20 (AJ879480); 3.2e-26                                                       |
| MAPKK                             | STE11; YLR362W               | nrc-1 (AJ879482); 1.3e-19                                                        |
| MAPKK                             | STE7; YDL159W                | ste7 (AJ879473); 4.1e-33                                                        |
| MAPK                              | Fus3; YBL0162                | fus3 (AJ879483); 3.4e-6                                                         |
| Transcription factor              | STE12; YHR08W                | ste12 (AJ879472); 1.3e-4                                                        |
| Regulator of G-protein signaling  | Sst2; YLR452C                | rgl1 (AJ879475); 2.8e-17                                                         |
| Protein tyrosine phosphatase; adaption to pheromone | PTPP; YER075C | ptp3 (AJ879476); 2e-6                                                           |
| Protein tyrosine phosphatase; adaption to pheromone | MSG5; YNL053W | msg5 (AJ879477); 3.7e-15                                                          |
| Carboxypeptidase; α-factor processing | KEX1; YGL203C | kex1 (AJ879474); 2.2e-59                                                          |
| Subtilisin-like protease; α-factor processing | KEX2; YNL238W | kex2 (AJ879479); 9.3e-81                                                          |

* 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.

A functional 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 N. crassa, 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 α-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 α-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 postfertilization 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 C’ 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 peritheicum (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 α-factor-like receptor does not affect vegetative growth or male fertility. On the other hand, N. crassa pre1 mataA 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 Δper4 Δαshr 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 PRO1 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 act1 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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