Multiple Sex Pheromones and Receptors of a Mushroom-producing Fungus Elicit Mating in Yeast

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The mushroom-producing fungus *Schizophyllum commune* has thousands of mating types defined, in part, by numerous lipopeptide pheromones and their G protein-linked receptors. Compatible combinations of pheromones and receptors encoded by different mating types regulate a pathway of sexual development leading to mushroom formation and meiosis. A complex set of pheromone–receptor interactions maximizes the likelihood of outbreeding; for example, a single pheromone can activate more than one receptor and a single receptor can be activated by more than one pheromone. The current study demonstrates that the sex pheromones and receptors of *Schizophyllum*, when expressed in *Saccharomyces cerevisiae*, can substitute for endogenous pheromone and receptor and induce the yeast pheromone response pathway through the yeast G protein. Secretion of active *Schizophyllum* pheromone requires some, but not all, of the biosynthetic machinery used by the yeast lipopeptide pheromone α-factor. The specificity of interaction among pheromone–receptor pairs in *Schizophyllum* was reproduced in yeast, thus providing a powerful system for exploring molecular aspects of pheromone–receptor interactions for a class of seven-transmembrane-domain receptors common to a wide range of organisms.

INTRODUCTION

*Schizophyllum commune*, a filamentous wood-rotting fungus, belongs to a class of mushroom-producing fungi known as homobasidiomycetes. Such fungi typically have many different mating types in nature – *Schizophyllum* is known to have thousands of “sexes” (Raper, 1966). Mate recognition and sexual development leading to formation of fruiting bodies (mushrooms) and meiosis require the action of two unlinked genetic complexes, called A and B. Each complex is composed of two linked, but genetically separable, loci: Aα and Aβ for the A complex, and Bα and Bβ for the B complex. Each locus exists in multiple versions or specificities within the worldwide population. The Aα locus has 9 different specificities, Aβ has 32, and Bα and Bβ have 9 specificities each (Raper et al., 1960; Koltin et al., 1967; Stamberg and Koltin, 1972). The minimal requirement for a fertile pairing among these numerous, haploid mating types is a difference in specificity at either Aα or Aβ and a difference in specificity at either Bα or Bβ (Raper, 1966).

The genes contained within the B mating-type loci regulate a process of reciprocal fertilization in which nuclei of one mate migrate into and throughout the hyphal cells of the other (Raper, 1966; Koltin and Flexer, 1969; Wessels and Marchant, 1974; reviewed by Raudaskoski, 1998). Characterization of several specificities of the two B mating-type loci indicates that each locus encodes one seven-transmembrane-domain receptor and several putative lipopeptide pheromones (Wendland et al., 1995; Vaillancourt et al., 1997; Fowler et al., 1998). The Bβ1 locus, for example, contains three unique pheromone genes, called bbp1(1), bbp1(2), and bbp1(3), and one unique pheromone receptor gene called bbr1. The protein products of these genes are symbolized Bbp1(1), Bbp1(2), Bbp1(3), and Bbr1 accordingly. Functional analyses of cloned genes revealed the fundamentals of self/non-self-recognition (Figure 1A). No wild-type pheromone–receptor pair encoded within a haploid individual, such as Bbr1 and Bbp1(1) shown in Figure 1A, can activate the B-regulated pathway of development in “self,” where self is defined to include the haploid individual and any other individual with identical B mating-type loci. A single pheromone encoded within a specific Bβ locus can activate a subset of the receptors encoded by all other Bβ specificities. For example, pheromone Bbp1(1) activates the Bβ2 receptor (Figure 1A) as well as four additional Bβ receptors (Vaillancourt et al., 1997). Collectively, the pheromones of any one Bβ specificity can trigger the receptors of all eight non-self Bβ specificities, and similarly, the pheromones of any one Bα specificity can trigger the receptors of all eight non-self Bα
specificities (Wendland et al., 1995; Vaillancourt et al., 1997; Fowler et al., 1998). However, no pheromone encoded by any specificity of the Ba locus can activate receptors encoded within any specificity of the Bb locus and vice versa. This complex set of pheromone–receptor interactions governing mating maximizes the likelihood of outbreeding while minimizing inbreeding.

The numerous variants of pheromones and receptors that naturally exist in Schizophyllum make this organism an attractive system for investigations of the molecular and structural basis for specificity of pheromone–receptor interactions. However, the complexity of this system confounds analysis of its components and the role they play in signal transduction. We therefore attempted to reconstitute Schizophyllum pheromone–receptor interactions in a more genetically tractable system, *Saccharomyces cerevisiae*. Development of a yeast system would allow the examination of individual pheromone–receptor pairs in isolation and facilitate genetic analysis of the specificity determinants of pheromone–receptor interactions.

*S. cerevisiae* has two mating types, MATa and MATα, and mating involves two pheromone–receptor pairs (reviewed by Sprague and Thorner, 1992; Kurjan, 1993). MATa cells express a-factor pheromone and the a-factor receptor Ste2p; MATα cells express a-factor pheromone and the a-factor receptor Ste3p. The two receptors are characterized by seven-transmembrane domains that span the plasma membrane. When bound by pheromone secreted from cells of the opposite mating type, each receptor couples with the same heterotrimeric G protein to initiate a signal-transduction pathway, known as the pheromone-response pathway. Defined effects of pheromone response include transcriptional activation of a large set of genes, cell-cycle arrest, cell fusion, and nuclear fusion (for review, see Sprague and Thorner, 1992; Kurjan, 1993). The mature a-factor pheromone is a simple 13-amino acid peptide. The active form of the a-factor pheromone is a mixture of two farnesylated peptides of 12 amino acids that differ in sequence in one position; these lipopeptides are processed from two similar precursors of 36 and 38 amino acids encoded by the genes *MFA1* and *MFA2* (Michaelis and Herskowitz, 1988).

The presumptive Schizophyllum pheromone precursors appear to be comparable to a-factor in that they are small, ranging in size from 40 to 75 amino acids, and end in a C-terminal signal for farnesylation. This signal is a CaaX motif, where a cysteine residue is followed by two aliphatic residues and ends with any of five specific amino acids (Schafal and Rine, 1992). Processing of the N termini of these pheromone precursors may occur, but has yet to be shown (Casselton and Olesnicky, 1998). A comparison of predicted amino acid sequences from nine Schizophyllum pheromone-precursor genes that have been cloned and tested for func-

![Figure 1](image-url)

**Figure 1.** (A) Interactions of two pheromones and two receptors encoded in the Schizophyllum mating-type loci Bβ1 and Bβ2. The linked Ba and Bb loci contain open reading frames for putative lipopeptide pheromones (circles) and seven-transmembrane domain receptors (rectangles). The distances between and within the loci are not shown to scale. Bβ genes examined in this study, obtained from a Bα1-Bβ1 strain (Mate 1) and a Bα3-Bβ2 strain (Mate 2), are shown as checkered symbols and designated with the gene name (Vaillancourt et al., 1997; Fowler et al., 1998). Bbp1(1) can activate Bbr2 but not Bbr1, and Bbp2(4) can activate Bbr1 but not Bbr2, as indicated by arrows. Additional interactions among gene products encoded in these loci are not indicated. (B) Predicted amino acid sequence of the Bbp2(4) pheromone precursor. The CaaX box recognition motif for protein prenylation is single underlined, and a postulated N-terminal cleavage site for maturation is double underlined (Casselton and Olesnicky, 1998). The predicted amino acid sequence of Bbp1(1) was previously published (Vaillancourt et al., 1997).
tion reveals considerable variation except for the CaaX motif. All five Schizophyllum pheromone-receptor genes analyzed so far are predicted to encode proteins with seven-transmembrane domains. Amino acid sequence comparisons show that these receptors are significantly similar to the pheromone receptors of *S. cerevisiae* (Wendland et al., 1995; Vaillancourt et al., 1997; Fowler, Mitton, and Raper, unpublished).

Previous studies demonstrated that some mammalian G protein-coupled receptors expressed in *S. cerevisiae* showed membrane localization and allowed antagonist and/or agonist binding (King et al., 1990; Price et al., 1995). In one case, a rat somatostatin receptor treated with somatostatin could couple with the yeast G protein to activate the yeast pheromone-response pathway. Here we demonstrate that *Schizophyllum* receptors can be expressed in yeast and can couple with the yeast G protein. In addition, this study presents evidence that *S. cerevisiae* can process and secrete functional pheromones encoded by putative pheromone genes of *Schizophyllum*, thus confirming that these genes encode bona fide sex pheromones. Combinations of pheromones and receptors that are naturally compatible in *Schizophyllum* activate the yeast pheromone-response pathway, while incompatible combinations do not. This system will make the numerous genetic tools applicable to *S. cerevisiae* available for the exploration of interactions among the numerous pheromones and pheromone receptors of *Schizophyllum*.

**Yeast Cultures, Transformations, and Gene Disruptions**

*S. cerevisiae* strains (Table 1) were grown at 30°C on YEPD, synthetic drop-out (SD) media lacking uracil, or SD media lacking both uracil and tryptophan (Treco and Lundblad, 1997). Plasmids were introduced into yeast using the PLAG (polyethylene glycol-lithium acetate-glycerol) method (Chen et al., 1992).

psk-STE3 was constructed by subcloning the *MfeI–SacI* fragment from pSL1 (Hagen et al., 1986) into *EcoRI–Saci*-digested *pBluescriptSK*+ (Stratagene, La Jolla, CA). The 2.3-kilobase (*kb*) *ADE2* *BglII* fragment was subcloned into the *BamHI* site of *pBluescriptSK*+ and the *SpeI–PstI* *ADE2* fragment from this plasmid was subcloned into psk-STE3 to make pSK-STE3:ADE2. The *KpnI–SacI* *ste3:ADE2* fragment from pSK-STE3:ADE2 was used to make STE3 gene replacements by lithium acetate transformation (Chen et al., 1992) in strains *Tn4–1B* and RAK2 (Table 1). The *ram1::URA3* disruption in SM1865 was changed to a *ram1::LEU2* disruption by cleaving pUL9 (Cross, 1997) with *XbaI* to obtain a *ura3::LEU2* fragment and transforming SM1865.

**Schizophyllum Mating, RNA Extraction, and cDNA Synthesis**

The following method for growth and mating of *Schizophyllum* was adapted from Vaillancourt et al. (1997). *S. commune* strains 4–40 (*Aa4-Aβ6/Bα1-Bβ1*) and 4–8 (*Aa4-Aβ6/Bα3-Bβ2*) grown on CYM-agar plates (Raper and Hoffman, 1974) were cut away from the agar and separately macerated in CYM liquid media in a Waring blender to provide inocula for 100-ml liquid cultures. These liquid cultures were grown 24 h at 30°C with shaking at 200 rpm and then macerated again. After this process was repeated, ~1 ml of the final macerates was spread on separate 5-cm squares of semipermeable cellophane membrane (Dupont, Wilmington, DE) placed on CYM agar plates and grown at 30°C for 48 h. The membranes on which strain 4–40 was growing were lifted and placed hyphae-side down on separate 5-cm squares of semipermeable cellophane membrane (Dupont, Wilmington, DE) placed on CYM agar plates and grown at 30°C for 48 h. The membranes on which strain 4–40 was growing were lifted and placed hyphae-side down onto the strain 4–8 cultures. Genes within the B loci are known to be up-regulated after contact between individuals with B loci of different specificities (Vaillancourt et al., 1997). After 8 h of contact, the hyphal mats were stripped from the membranes and flash frozen in liquid nitrogen. Total RNA was isolated by a hot phenol:SDS method as described previously (Devries et al., 1988). Total RNA (10

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**Table 1. S. cerevisiae strains used in this study**

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| SM2331 | MATmfa1Δ1 mfa2Δ1 |           |             |
| SM1188 | MATste4Δ3::TRP1 |           |             |
| SM1865 | MATΔram1::URA3 |           |             |
| LHK1   | MATΔram1::LEU2 |           |             |
| SM2744 | MATΔaxl1::LEU2 Δste23::LEU2 |           |             |
| SM3614 | MATΔste24::LEU2 Δrcel::TRP1 |           |             |
| SM1872 | MATram1Δ1 |           |             |

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Table 2. Oligonucleotides

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The oligonucleotide sequences are shown in the 5’ to 3’ direction. Single underlines indicate EcoRI sites in the upstream primers or BamHI sites in the downstream primers. Double underlines indicate an ATG initiation codon in upstream primers or a termination codon in the reverse strand of downstream primers; 980730-2 binds downstream of the termination codon.

\[ \mu g \] was treated with RNase-free DNase I (Life Technologies, Gaithersburg, MD) and then converted to cDNA with oligo(dT)12-18 primers using the Superscript Preamplification System (Life Technologies) according to the manufacturer’s protocol.

**Construction of Pheromone- and Receptor-Expression Plasmids**

DNA fragments containing *Schizosaccharomyces* pheromone gene- and receptor gene-coding sequences were amplified by PCR, as described below, using oligonucleotides (Genosys, Woodlands, TX) that incorporate EcoRI and BamHI recognition sites at the 5’- and 3’-ends of the open reading frames, respectively. The EcoRI-BamHI fragments were subcloned into pPGK (Kang *et al.*, 1990) to allow expression under the control of the high-expression promoter of the yeast phosphoglycerate kinase gene (PGK).

pPGK-bbr1: oligonucleotides 980420–3 and 980420–4 (Table 2) were used as primers for PCR amplification of the coding region of bbr1 using the pool of cDNAs obtained from the 4-40 × 4-8 mating as template. A standard 50-μl reaction using Taq DNA polymerase (Life Technologies) and a 480 thermocycler (Perkin Elmer-Cetus, Norwalk, CT) was performed.

pPGK-bbr2: Full-length cDNA clones of bbr2 (GenBank Accession AF148501) from a mating of strains 4-40 and 4-39 (Aa4-Aa6/Ba3-Bβ2) were kindly provided by Dr. Marjatta Raudaskoski. The coding region of bbr2 was amplified by PCR from a cDNA template using oligonucleotide primers 980730–1 and 980730–2 (Table 2).

pPGK-bbp2(4) and pPGK-bbp1(1): PCR amplification products containing the bbp1(1) (Vaillancourt *et al.*, 1997) and bbp2(4) (Fowler, Mitton, and Raper, unpublished; GenBank Accession AF148500) coding sequences were generated from genomic clones using oligonucleotide pairs 980730–5 and 980730–6 for bbp1 and 980227–2 and 980227–3 for bbp2(4) (Table 2). No intron interrupts the bbr1 coding region (Raudaskoski *et al.*, 1998) or the bbp2(4) coding region (our unpublished data).

DNA sequences were confirmed using the dyeoxydinucleotide termination sequencing method and the fluorescent label system of the ABI Prism kit (Perkin Elmer-Cetus). Sequencing reactions were run on an ABI 373 DNA Sequencer by the Vermont Cancer Center DNA Analysis Facility.

**Yeast FUS1-lacZ Induction Assays**

Cell culture supernatants were obtained from MATa strains containing pPGK-bbp1(1), pPGK-bbp2(4), or the control vector pPGK in the following manner. Fresh cultures grown to an OD600 of ~1.0, in SD(ura–) medium to maintain plasmids, were diluted to OD600 = 0.1 in SD(ura–) medium and grown for an additional 4 h at 30°C in glass culture tubes. The cultures were centrifuged to obtain the cell culture supernatants. In one set of experiments, secretion of Bbp2(4) by a MATa strain (W303–1B) was tested.

MATa cells containing pPGK-bbr1, pPGK-bbr2, or the control vector pPGK (URA3) as well as the pheromone-inducible reporter construct FUS1-lacZ (pTCFL1, TRP1; Trueheart and Fink, 1989; Chen and Kurjan, 1997) were grown in SD(ura–, trp–) as described for the MATa cells, but after centrifugation the cells were resuspended in 0.5 vol of fresh selective media. MATa cells (1 ml) in fresh medium were mixed with an equal volume of MATa cell supernatant (or fresh SD(ura–) medium for the control) and incubated at 30°C for 2 h in glass culture tubes in a roller drum. The cells were harvested and permeabilized, and β-galactosidase units were calculated as described previously (Reynolds *et al.*, 1997) using o-nitrophenyl β-D-galactopyranoside as the colorimetric substrate. The β-galactosidase data are presented as representative results from experiments repeated two to five times. The activities shown are averages from at least three measurements taken from assays of at least two independent transformants of each strain analyzed. Error bars indicate 1 SD.

**Halo Assays of *S. cerevisiae* Cell Cycle Arrest**

MATa strains containing pPGK or pPGK-bbp2(4) were grown in patches on SD(ura–) master plates 24–48 h. MATa cells (0.3 ml of cells at OD600 = 0.3) were spread on either YEFP plates for the MATa STE3 sst2 strain or SD(ura–) plates for the MATa ste3 sst2 strains containing pPGK or pPGK-bbr1 constructs. The master plates containing the MATa patches were replica plated to the appropriate MATa lawn plates and grown 24–48 h. The plates were analyzed for induction of cell-cycle arrest by secreted pheromone resulting in inhibition of growth of the surrounding lawn (halo formation).

**Mating Assays**

Overlapping perpendicular streaks of MATa strains (his4) and MATa strains (his3) were grown overnight on SD(ura–) medium to maintain pPGK-based plasmids and allow diploids to form. These plates were then replica plated onto SD(his–) medium, which provides a selection for growth of the nutritionally complemented diploid cells produced by mating.
RESULTS

A Schizophyllum Receptor–Pheromone Pair Is Functional in S. cerevisiae

In this study, we asked whether Schizophyllum receptors and pheromones could substitute for their S. cerevisiae counterparts to activate the pheromone-response pathway in S. cerevisiae. The gene encoding the Bb1 receptor Bbr1, called bbr1, and the gene encoding Bp2 pheromone Bbp2(4), called bbp2(4) (Figure 1 and Table 3), were placed under the control of a constitutive promoter derived from the yeast phosphoglycerate kinase (PGK) gene (Kang et al., 1990) to create the plasmids pPGK-bbr1 and pPGK-bbp2(4), respectively. Any possible competition between Bbr1 and the a-factor receptor, Ste3p (Hagen et al., 1986), was avoided by transforming pPGK-bbr1 into a MATa ste3 mutant. Schizophyllum pheromones, including Bbp2(4), are predicted from DNA sequences to be small peptides that are modified with a farnesyl moiety that is attached to the yeast a-factor (Anderegg et al., 1988; Marcus et al., 1991). Expression of Bbp2(4) from pPGK-bbp2(4) was attempted in a MATa strain because these cells modify, process, and secrete the lipopeptide a-factor and thus were thought to provide the best possibility of producing mature Bbp2(4).

The first assay for activation of the pheromone-response pathway by the Bbp2(4)–Bbr1 pheromone–receptor pair utilized the pheromone-inducible FUS1-lacZ reporter gene (Trueheart and Fink, 1989; Chen and Kurjan, 1997). The procedure involved treating cells, containing the receptor and reporter gene constructs, with supernatants from cultures of cells containing the pheromone gene construct. In four paired combinations of MATa culture supernatants with MATa cells, cell culture supernatants of the MATa strains containing pPGK-bbp2(4) or the control plasmid pPGK were mixed with MATa ste3 strains containing pPGK-bbr1 or the control plasmid. The combination of supernatant from the MATa strain containing pPGK-bbp2(4) and MATa cells containing pPGK-bbr1 (and the FUS1-lacZ reporter) showed three- to fourfold higher β-galactosidase levels in comparison with the negative controls (Figure 2). The elevated β-galactosidase activity was ~40% of the activity seen in concurrent a-factor/Ste3p controls, where the genes are expressed from their native chromosomal positions (our unpublished data). The increased β-galactosidase level above background in this test of Schizophyllum gene products required expression of bbp2(4) by the MATa cells and

Table 3. Genes/gene products relevant to this study

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<tr>
<td>S. commune</td>
<td></td>
<td></td>
</tr>
<tr>
<td>bbr1</td>
<td>Pheromone receptor from specificity 1 of the Bβ locus</td>
<td>Vaillancourt et al., 1997</td>
</tr>
<tr>
<td>bbr2</td>
<td>Pheromone receptor from specificity 2 of the Bβ locus</td>
<td>This study</td>
</tr>
<tr>
<td>bbr1(1)</td>
<td>Pheromone 1 from specificity 1 of the Bβ locus</td>
<td>This study</td>
</tr>
<tr>
<td>bbr2(4)</td>
<td>Pheromone 4 from specificity 2 of the Bβ locus</td>
<td></td>
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</table>

from the MATa strain containing pPGK-bbp2(4) and MATα cells containing pPGK-bbr1 (and the FUS1-lacZ reporter) showed three- to fourfold higher β-galactosidase levels in comparison with the negative controls (Figure 2). The elevated β-galactosidase activity was ~40% of the activity seen in concurrent a-factor/Ste3p controls, where the genes are expressed from their native chromosomal positions (our unpublished data). The increased β-galactosidase level above background in this test of Schizophyllum gene products required expression of bbp2(4) by the MATa cells and
Figure 3. Production of Bbp2(4) and response to this pheromone
are not influenced by the presence of α-factor, but Bbp2(4) production
is affected by yeast strain differences. The MATα ste3 strain
(SDK45) tested for pheromone response contained the pheromone-
inducible FUS1-lacZ reporter plasmid and pPGK-bbr1. To test for
any effect on Bbp2(4) signaling by the presence of a-factor, culture
supernatants from isogenic MATα MFA1 MFA2 (SM1058) and
MATα mfa1 mfa2 (SM2331) strains containing either pPGK-bbp2(4)
(black) or pPGK (stripes) were compared. Comparison to a noniso-
genic MATα MFA1 MFA2 strain (W303–1A) showed a strain-related
difference in Bbp2(4) activity. β-Galactosidase assays were done as
in Figure 2.

The Schizophyllum Pheromone–Receptor Pair
Signals through Components of the Yeast
Pheromone-Response Pathway

Three aspects of the pheromone-response pathway were
tested to determine whether the signal generated from the interaction
of the compatible Schizophyllum pheromone–receptor pair Bbp2(4)–Bbr1
follows the same path as a signal generated by a pheromone-activated yeast
receptor. The yeast pheromone receptors interact with a trimeric
G protein composed of Gα1p (α), Ste4p (β), and Ste18p (γ) (Dietzel
and Kurjan, 1987; Miyajima et al., 1987; Jahng et al., 1988; Whiteway
et al., 1989). The Gβγ dimer acts as a positive component to activate
the downstream pathway, whereas the Gα subunit binds to Gβγ to inhibit
signaling by the dimer. Hence, ste4 null mutants, which lack the β subunit,
are defective in response to pheromone

from the nonisogenic MATα MFA1 MFA2 strain W303–1A
(Figure 3). This result suggests that there are strain differ-
ences in production of the secreted, active Schizophyllum
pheromone by S. cerevisiae.

An important response to pheromone in S. cerevisiae is
arrest in the G1 phase of the cell cycle (Kurjan, 1993). In the
“halo” assay (Dietzel and Kurjan, 1987a), pheromone se-
creted from a patch of cells arrests the growth of an under-
lying lawn of cells of the opposite mating type, resulting in
a clear zone immediately surrounding the patch of cells.
Response of the MATα law to α-factor requires the α-factor
receptor, Ste3p (Figure 4A). Expression of Bbr1 in a MATα
ste3 strain resulted in a halo surrounding the MATα cells
expressing Bbp2(4). Controls indicated that Bbp2(4) expres-
sion by the MATα strain and Bbr1 expression by the MATα
strain were necessary for halo formation. Therefore, re-
sponse to the Schizophyllum pheromone Bbp2(4) through the
Schizophyllum Bbr1 receptor was sufficient to promote cell-
cycle arrest.

Another response to pheromone in S. cerevisiae is a mor-
phological change in which the cells produce a mating pro-
jection. No morphological change was seen in MATα cells
(SDK47) expressing Schizophyllum receptor Bbr1 when
treated for ∼12 h with supernatant containing Schizophyllum
pheromone Bbp2(4). In control experiments, MATα STE3
cells (TN44–1B) exposed to culture supernatants containing
a-factor showed morphological changes associated with
pheromone response within 2 h. Previous dose response
analysis for yeast pheromone response indicated that mor-
phological changes require ∼100-fold higher concentrations
of pheromone than are required for cell-cycle arrest or tran-
scriptional induction (Moore, 1983); the absence of mor-
phological changes via the Schizophyllum pheromone–receptor
pair may reflect these differing dosage requirements.

The various responses to pheromone in S. cerevisiae lead
to the mating reaction in which cells, and then nuclei, fuse
to form the MATα/MATα diploid. In mating assays (Figure
4B), production of Bbp2(4) by MATα mfa1 mfa2 cells
(SM2331) allowed mating with MATα ste3 cells (SDK47)
expressing Bbr1, whereas paired combinations that included
control strains that did not express either Bbp2(4) or Bbr1
were defective in mating. Therefore, expression of the
Bbp2(4)–Bbr1 pheromone–receptor pair in S. cerevisiae cells
of opposite mating type can initiate transcriptional induc-
tion, cell-cycle arrest, and mating.
and mating (Whiteway et al., 1989). Culture supernatants containing Bbp2(4) did not induce the FUS1-lacZ reporter gene in a MATa ste4 null mutant expressing Bbr1, but did show about a fourfold induction in the isogenic MATa STE4 strain (Figure 5A), indicating that the Schizophyllum pheromone–receptor pair acts through the G protein of the pheromone-response pathway.

The S. cerevisiae SST2 gene product is involved in recovery from pheromone-induced cell-cycle arrest (Dietzel and Kurjan, 1987a). sst2 Mutants show a greatly increased sensitivity to pheromone and an increase in both the basal and α-factor–induced expression of pheromone-inducible genes (Chan and Otte, 1982). A similar increase in FUS1-lacZ expression was observed in response to Bbp2(4) in the MATa ste3 sst2 mutant expressing Bbr1 (Figure 5B). Cell-cycle arrest in response to secreted yeast pheromones can be detected by the halo assay in sst2 mutants, but not in a wild-type SST2 strain. Whereas Bbp2(4) secretion resulted in halo formation on a lawn of Bbr1-expressing MATa sst2 cells, no halo was observed on a MATa SST2 lawn (our unpublished data). The sst2 mutation therefore increased the sensitivity of cells expressing Schizophyllum receptor Bbr1 to the pheromone Bbp2(4).

Initial assays in this system were done in a ste3 mutant to preclude activation of the pheromone-response pathway through the α-factor receptor, Ste3p, by α-factor present in the culture supernatants (Figure 2). Production of Bbp2(4) in an α-factor–deficient strain (MATa mfa1 mfa2) permitted a test for any effect of coexpression of receptors Ste3p and Bbr1 on signaling. An inhibitory effect of Ste3p upon signaling by α-factor receptor, Ste2p, was previously described for strains that simultaneously express these two receptors (Bender and Sprague, 1989; Hirsch and Cross, 1993). Induction of FUS1-lacZ by Bbp2(4) through Bbr1 was 15-fold over background levels in the ste3 strain as compared with an approximate twofold induction in an isogenic STE3 strain (Figure 6). These results indicated that Ste3p inhibited pheromone-responsive signaling through Bbr1. Signaling by α-
factor through Ste3p, however, was not significantly affected by the expression of Bbr1 (our unpublished data).

**Functional Expression of Bbp2(4) in S. cerevisiae Requires Only Some of the Components Required for a-Factor Processing, Modification, and Secretion**

a-Factor secretion is independent of the classical secretory pathway. The a-factor precursors undergo a series of modification and processing steps, followed by secretion of mature lipopeptide by a transmembrane pump (Michaelis et al., 1992). To determine whether biosynthesis and secretion of active Bbp2(4) involve the same steps required for maturation of a-factor, we used mutant strains defective in several aspects of a-factor production. Each of these mutant strains, containing pPGK-bbp2(4), was used in halo assays to test for secreted Bbp2(4) activity.

The a-factor precursor is farnesylated on a cysteine residue that is part of the C-terminal CaaX sequence (Anderegg et al., 1988; Michaelis and Herskowitz, 1988). The CaaX motif is found at the C termini of all predicted Schizophyllum pheromones, suggesting that prenylation of these molecules is likely. The S. cerevisiae RAM1 and RAM2 genes encode the two subunits of the farnesyltransferase responsible for prenylation of a-factor (He et al., 1991). The ram1 mutant containing pPGK-bbp2(4) did not produce a halo on the lawn expressing Bbr1, whereas the wild-type RAM1 strain containing pPGK-bbp2(4) did produce a halo (Figure 7). A similar defect was seen in a nonisogenic ram2 mutant (our unpublished data). These results suggest that the Bbp2(4) precursor is farnesylated in S. cerevisiae by Ram1p/Ram2p.

After farnesylation of the cysteine residue, the C-terminal aaX residues of the a-factor precursor are removed by the functionally redundant Ste24p and Rce1p proteases (Boyaritchuk et al., 1997; Tam et al., 1998). The ste24 rce1 double mutant expressing Bbp2(4) produced a much smaller halo than the isogenic wild-type strain expressing Bbp2(4) (Figure 7). β-Galactosidase assays with the ste24 rce1 double mutant revealed that the Bbp2(4) activity was <2% of the activity of an isogenic STE24 RCE1 strain (our unpublished data). These results suggest that one or both proteases are nearly essential for production of active Bbp2(4).

After C-terminal proteolysis, the farnesylated cysteine residue of the a-factor precursor is carboxyl methylated by Ste14p (Sapperstein et al., 1994). The ste14 mutant containing Bbp2(4) produced a much smaller halo than the isogenic wild-type STE14 strain expressing Bbp2(4) (Figure 7), and β-galactosidase assays showed that Bbp2(4) activity secreted by the mutant strain was 5–15% of the level obtained from the isogenic STE14 strain (our unpublished data). These
results indicate that Ste14p activity plays an important, but not an essential, role in production of active Bbp2(4). N-terminal processing of a-factor precursor involves two steps. Ste24p acts to carry out the initial N-terminal trimming in addition to its role in C-terminal processing (Fuji-

![Figure 7](image)

In all previous experiments, Bbp2(4) was expressed in MATa cells because it was thought that steps involved in processing, modification, and secretion of Bbp2(4) might be similar to steps used for a-factor production. However, STE6 is the only MATa-specific gene known to play a role in a-factor production (Wilson and Herskowitz, 1984). Because Ste6p was not essential for Bbp2(4) secretion by MATa cells, we tested whether MATa cells could secrete functional Bbp2(4). Levels of induction of the FUS1-lacZ reporter gene were similar using supernatants from either MATa cells containing pPGK-bbp2(4) or MATa cells containing pPGK-

The Specificity of Two Schizophyllum Pheromone–Receptor Pairs Is Reproduced in Yeast

In Schizophyllum, interaction between compatible pheromones and receptors encoded within different B-locus specificities is required for activation of the B-regulated pathway of development. Pheromones and receptors encoded within the same specificity of the B locus are always incompatible and do not activate this pathway (Figure 1A; Vaillancourt et al., 1997; Fowler et al., 1998). We expressed a second pheromone–receptor pair in yeast to determine whether similar compatibility/incompatibility relationships prevail in the reconstituted yeast system. The Bbp1(1) pheromone was expressed in the MATa mfa1 mfa2 strain, and the Bbr2 receptor was expressed in the MATa ste3 strain. This compatible combination induced β-galactosidase activity by approximately threefold (our unpublished data) and also allowed mating (Figure 9). Equally important, pheromone–receptor pairs derived from the same Bβ specificity (e.g., Bbp1(1)/Bbr1 or Bbp2(4)/Bbr2) did not induce the FUS1-lacZ reporter or allow mating. The specificity observed in Schizophyllum was therefore maintained in the reconstituted system in S. cerevisiae.
DISCUSSION

This study demonstrates that interactions of pheromones and receptors encoded by the B mating-type genes of *S. commune* can be reconstituted in *S. cerevisiae* to activate the pheromone-response pathway and mating in yeast. This reconstitution indicates that the seven-transmembrane receptors of *Schizophyllum* can localize appropriately in the plasma membrane in order to allow activation by extracellular pheromone and subsequent coupling to the yeast G protein. The extracellular production of *Schizophyllum* pheromones by yeast indicates that the lipopeptide precursors can be processed, modified, and secreted to produce active pheromones. A previous study in which a rat somatostatin receptor was able to activate the response pathway through the yeast G protein provided a precedent for coupling between the yeast G protein and a heterologous seven-transmembrane receptor (Price et al., 1995). There was no precedent, however, for the secretion of active heterologous lipopeptide pheromones by *S. cerevisiae*. Secretion of the yeast lipopeptide pheromone, a-factor, is independent of the classical secretory pathway and involves a complex set of modification and processing steps. This study indicates that *Schizophyllum* pheromones are modified by farnesylation and demonstrates that active heterologous lipopeptides are secreted by yeast.

**Figure 8.** Bbp2(4) processing and secretion are independent of transporter Ste6p and all other a-specific gene products. The MATa ste3 strain (SDK45) containing the pheromone-inducible FUS1-lacZ reporter plasmid and pPGK-bbp1 was tested for response. Reporter gene assays were done as in Figure 2. (A) The effects of cell culture supernatants from the MATa STE6 strain (W303–1A) and the isogenic ste6 strain (RAK32) containing either pPGK (stripes) or pPGK-bbp2(4) (black) were compared. (B) The effects of cell culture supernatants from MATa (W303–1A) and MATα (W303–1B) cells containing either pPGK (stripes) or pPGK-bbp2(4) (black) were compared.

**Figure 9.** *Schizophyllum* pheromones and receptors maintain their specificity in yeast. Mating was tested as in Figure 4B. Rows: the MATa mfa1 mfa2 strain (SM2331) containing either pPGK (top), pPGK-bbp2(4) (second from top), or pPGK-bbp1(1) (third from top), and the MATa MFA1 MFA2 strain (SM1058) containing pPGK (bottom). Columns: the MATa ste3 sst2 strain (SDK47) containing either pPGK (left), pPGK-bbr1 (second from left), or pPGK-bbr2 (third from left), and the MATα STE3 sst2 strain (Tn44–1B) containing pPGK (right).

**Secretion of Active Schizophyllum Lipopeptide Pheromones by S. cerevisiae**

Similarities of the predicted *Schizophyllum* lipopeptide pheromone precursors to the a-factor precursors suggested the possibility of common processes in the biosyntheses of a-factor and the active forms of *Schizophyllum* pheromones by *S. cerevisiae*. The two a-factor precursors, which differ by a single amino acid within the mature peptide sequence, undergo farnesylation, C-terminal processing, carboxyl methylation, and N-terminal processing. Mature a-factor is then secreted by a specific ATP-binding cassette transporter, Ste6p.

We investigated whether *Schizophyllum* pheromone Bbp2(4) production and secretion in the yeast system used protein.
the same machinery as α-factor production and secretion by analyzing Bbp2(4) expression in mutants defective for each of the steps involved in α-factor production. Interestingly, farnesylation was the only step absolutely required for processing of the Schizophyllum pheromone: mutants defective in α-factor farnesylation (ram1 and ram2) were also defective in production of active Bbp2(4), suggesting that Bbp2(4), secreted by S. cerevisiae, is a lipopeptide and is modified with a farnesyl moiety. Analysis of other mutants showed that two proteases involved in production of the mature α-factor N terminus (Axl1p and Ste23p) are not involved in active Bbp2(4) production. A mutation eliminating the α-factor carboxyl methyltransferase (Ste14p) resulted in ~90% decrease in Bbp2(4) activity, indicating that although the decrease in activity is large, an active form of Bbp2(4) can be produced independently of this protein (Figure 7 and our unpublished data). A double-mutant strain eliminating the C-terminal protease, Rce1p, and the C- and N-terminal protease, Ste24p, showed a small halo (Figure 7) that represents <2% of the activity of a wild-type strain, as quantified by β-galactosidase assays. One or both of these proteases are nearly essential for Bbp2(4) activity. These results suggest either that the Schizophyllum pheromone is processed in a way comparable to α-factor but may be able to utilize other enzymes or that the secretion of active Schizophyllum pheromone does not require all of the processing and modification steps required by α-factor.

Bbp2(4) secretion was independent of the ATP-dependent α-factor transporter, Ste6p, as shown by ste6 mutant studies and by analysis of Bbp2(4) secretion in MATa cells, which do not express STE6 (Figure 8). S. cerevisiae contains ~30 genes predicted to encode other proteins with homology to ATP-binding cassette transporters, and Bbp2(4) export may involve one of these alternative transporters (Taglicht and Michaelis, 1998). Another small lipopeptide product known to be transported in a Ste6p-independent manner is α-factor-related peptide (AFRP), a heptapeptide derived from the C termini of the α-factor precursors (Chen et al., 1997a). Unlike α-factor and Bbp2(4), AFRP does not have pheromone activity. AFRP and Bbp2(4) both require farnesylation for production, but not the N-terminal protease Axl1p. In contrast, methylation by Ste14p is important for robust production of Bbp2(4) activity but appears to be unimportant to AFRP maturation and export. During N-terminal processing of AFRP, the yeast cell appears to use a length-specific protease that measures from the C terminus of the α-factor precursors to the point of N-terminal cleavage, rather than a protease with sequence-specific recognition. It will be interesting to know whether yeast cells use a length-specific mechanism or a site-specific protease(s) to achieve the mature size of Bbp2(4), or whether the N terminus of the Bbp2(4) precursor is processed at all.

**Activation of the Pheromone-Response Pathway by Schizophyllum Receptors**

The activated yeast pheromone receptors signal through the heterotrimeric G protein, composed of Gpa1p (α), Ste4p (β), and Ste18p (γ) (Dietzel and Kurjan, 1987b; Miyajima et al., 1987; Jahng et al., 1988; Whiteway et al., 1989). After G protein activation in S. cerevisiae, the Gβγ dimer (Ste4p/Ste18p) transmits the signal to the downstream pathway. Elimination of Ste4p function therefore abolishes pheromone response and mating (Whiteway et al., 1989). Similarly, signaling by activated Bbr1 was blocked in a ste4 null mutant (Figure 5A), indicating that signaling by the Schizophyllum receptor acts through the yeast G protein. This finding is consistent with the results of two previous investigations in which it was demonstrated that heterologous receptor–G protein couplings could be used to transmit signals in yeast. Expression of the Candida albicans GPA1 homologue in S. cerevisiae allowed mating, indicating that the S. cerevisiae pheromone receptors could interact with C. albicans Gpa1p (Sadhu et al., 1992). A rat somatostatin receptor expressed in S. cerevisiae and activated by exogenous somatostatin induced the pheromone-response pathway through the S. cerevisiae G protein, demonstrating a fruitful interaction between this distantly related receptor and the yeast G protein (Price et al., 1995).

In yeast, Sst2p acts in desensitization to pheromone through Gpa1p (Dohlan and Thorner, 1997); sst2 mutants show greatly increased sensitivity to pheromone and a defect in recovery from pheromone-induced cellcycle arrest (Chan and Otte, 1982; Dietzel and Kurjan, 1987a). The sst2 mutation similarly resulted in increased sensitivity of cells expressing Bbr1 to the secreted Bbp2(4) pheromone. This increased response was evidenced not only by assays of a pheromone-inducible reporter gene (Figure 5B) but in halo assays as well (our unpublished data). These data strengthen the conclusion that the Schizophyllum receptor signals through the S. cerevisiae G protein.

Interestingly, expression of the α-factor receptor, Ste3p, and Bbr1 in the same strain inhibited signaling through Bbr1 (Figure 6). This inhibition was unidirectional: Bbr1 expression did not block signaling through Ste3p (our unpublished data). This result suggests that attempts to couple heterologous receptors to the S. cerevisiae pathway may be more successful if done in a strain lacking endogenous pheromone receptor. The inhibition phenomenon resembles a previous observation that Ste3p inhibits α-factor-induced signaling through Ste2p (Bender and Sprague, 1989; Hirsch and Cross, 1993), but that Ste2p does not inhibit signaling through Ste3p. Recent results indicate that Ste3p inhibition of the Ste2p signal acts at the level of the Gβ subunit, Ste4p, and that a MATa-specific protein is involved in the inhibition process (Kim et al., 1999). A MATa-specific protein cannot be involved in Ste3p inhibition of Bbr1 signaling, however, because our assays tested signaling in a MATa strain.

Overall, the experiments demonstrate that activation of the pheromone-response pathway in yeast by Schizophyllum pheromone–receptor interactions must depend on secreted pheromones, since culture supernatants from pheromone-producing cells induce a response. The probable location of the receptors is within the plasma membrane of the responding cells, since the receptors couple with the plasma membrane-associated yeast G protein.

**Advantages of the Heterologous Expression System for Future Studies of Schizophyllum Pheromone–Receptor Interactions**

The pheromone-stimulated pathway of sexual development in homobasidiomycetes, e.g., Schizophyllum commune and...
Coprinus cinereus, differs in important ways from other fungal mating systems that communicate through secreted pheromones and G protein-linked receptors (reviewed by Vaillancourt and Raper, 1996). In the hemiascomycetes, e.g., S. cerevisiae and Schizosaccharomyces pombe, activation of the pheromone-signaling pathway is necessary for the conjugation of cells of opposite mating type, which is followed by nuclear fusion to establish diploidy. Pheromone signaling is required also for cell conjugation in the dimorphic hemiascomycetes, e.g., Ustilago maydis, Rhodosporidium toruloides, and Tremella sp. In the strictly filamentous homobasidiomycetes, hyphal fusion between two strains is independent of mating type, and self- versus non-self-recognition occurs only after cell fusion. Non-self-recognition (compatibility) is necessary for continued sexual development, and nuclear fusion occurs in specialized cells of the fruiting body well after hyphal fusion. These differences in the pheromone-stimulated pathway of sexual development in homobasidiomycetes suggested no obvious requirement for extracellular secretion of signaling molecules or for mate recognition through cell-surface receptors. The mechanism by which this recognition process acts in the sexual development of this class of fungi is not understood. Production of Schizopyllum pheromones in yeast may allow these pheromones to be concentrated using methods developed for yeast α-factor (Strazdis and Mackay, 1983; Chen et al., 1997a). Isolation and concentration of Schizopyllum pheromone secreted by yeast would facilitate studies on the biochemical nature of these pheromones and the mechanism by which they activate compatible receptors to initiate the B pathway of development in Schizopyllum. As a start toward this goal, we have concentrated Bbp2(4) and have shown that it elicits a dose-dependent halo response in yeast cells that express Bbr1 (our unpublished data).

Functional expression of Schizopyllum pheromones and receptors that can activate the yeast pheromone-response pathway provides a powerful system for addressing the molecular nature of specificity between compatible pheromones and receptors. For example, how do several pheromones of quite different sequences activate the same Schizopyllum receptor, and how can one Schizopyllum pheromone activate several different receptors? To be useful for these studies, the specificity of Schizopyllum pheromone–receptor interactions must be reproducible in this yeast system. We have shown that two pheromone–receptor pairs reproduce their natural specificity in yeast; Bbp1(1) activates Bbr2 but not Bbr1, and Bbp2(4) activates Bbr1 but not Bbr2 (Figure 9). An extrapolation from analyses to date suggest that about half of the estimated 300 or more possible pairings of ββ pheromones and receptors extant in nature activate the B-regulated pathway of development. A comparable number of active and inactive combinations are postulated for the series of βα pheromones and receptors. In addition, a number of mutant variants of both pheromones and receptors are known to alter specificity of interaction (Raper and Raper, 1973; Fowler et al., 1998). Comparisons of predicted amino acid sequences among mutant and natural variants show that both minor and major differences in either type of molecule can result in changes in the spectrum of partners that are used to trigger the identical pathway of sexual development. How is this possible? Exploitation of the heterologous yeast system for extensive and rapid screen-


Sadhu, C., Hoekstra, D., McEachern, M.J., Reed, S.I., and Hicks, J.B. (1992). A G-protein α subunit from asexual Candida albicans func-


