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 **a**-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

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A
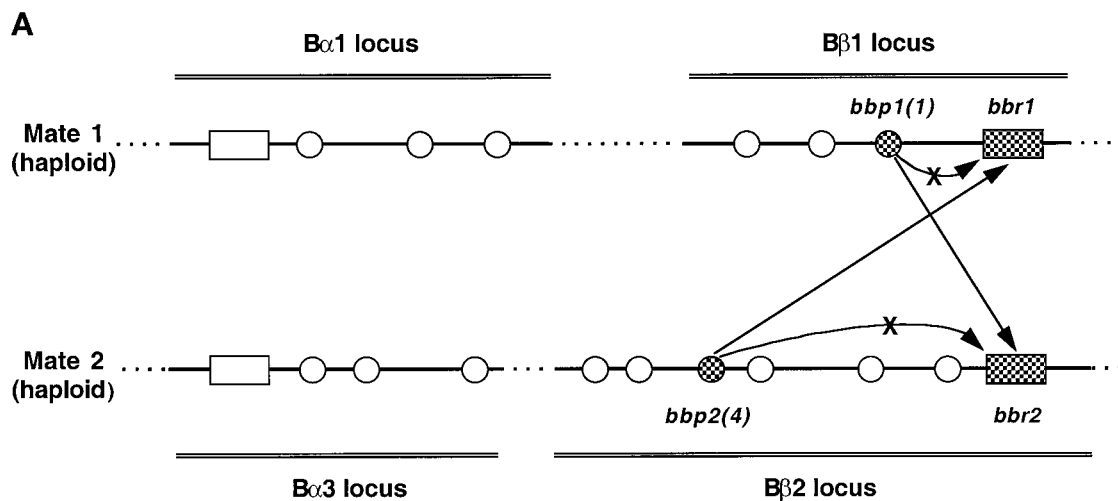
B

MDDFITLDFLEDTTPVFDFAPPTPNELTPEGYDEF<u>MR</u>MVANSDSPDGYFGGY<u>CVVA</u>

Figure 1. (A) Interactions of two pheromones and two receptors encoded in the *Schizophyllum* mating-type loci Bβ1 and Bβ2. The linked Bα and Bβ 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).

specificities (Wendland *et al.*, 1995; Vaillancourt *et al.*, 1997; Fowler *et al.*, 1998). However, no pheromone encoded by any specificity of the Bα locus can activate receptors encoded within any specificity of the Bβ 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, *MAT**a*** and *MATα*, and mating involves two pheromone–receptor pairs (reviewed by Sprague and Thorner, 1992; Kurjan, 1993). *MAT**a*** cells express **a**-factor pheromone and the α-factor receptor Ste2p; *MATα* cells express α-factor pheromone and the **a**-factor receptor Ste3p. The two receptors are characterized by sev-

en-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 α-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 (Schafer 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-

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

Isogenic to:	Strain	Genotype[a]	Reference/source
W303-1A	W303-1A	*MAT***a**	Kurjan and Dietzel, 1987
	W303-1B	*MAT*α	Kurjan and Dietzel, 1987
	RAK2	*MAT*α*ste4::HIS3*	R. Akada
	RAK32	*MAT***a***ste6::HIS3*	R. Akada
	Tn44-1B	*MAT*α*sst2::LEU2*	Kurjan and Dietzel, 1987
	SDK45	*MAT*α*ste3::ADE2*	This study
	SDK47	*MAT*α*ste3::ADE2 sst2::LEU2*	This study
	SDK48	*MAT*α*ste3::ADE2 ste4::HIS3*	This study
SM1058	SM1058	*MAT***a**	Michaelis and Herskowitz, 1988
	SM2331	*MAT***a***mfa1-Δ1 mfa2-Δ1*	Chen *et al.*, 1997b
	SM1188	*MAT***a**Δ*ste14-3::TRP1*	Hrycyna *et al.*, 1991
	SM1865	*MAT***a**Δ*ram1::URA3*	S. Michaelis
	LHK1	*MAT***a**Δ*ram1::LEU2*	This study
	SM2744	*MAT***a**Δ*axl1::LEU2* Δ*ste23::LEU2*	S. Michaelis
	SM3614	*MAT***a** Δ*ste24::LEU2* Δ*rce1::TRP1*	Tam *et al.*, 1998
None	SM1872	*MAT***a***ram2-1*	S. Michaelis

[a] Strains isogenic to W303-1A contain the markers *ade2-1 his3-11,15 leu2-3, 112 trp-1 ura3-1 can1-100*; strains isogenic to SM1058 contain the markers *trp1 leu2 ura3 his3 can1*; and SM1872 contains the markers *leu2 ura3 his3 ade8 lys2 can1 cyh4* in addition to the markers shown.

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*.

MATERIALS AND METHODS

Plasmid Isolation

Escherichia coli strains TG1, HB101, and DH5α were used for plasmid production. *E. coli* transformations were done by electroporation using the Gene Pulser (Bio-Rad, Hercules, CA), and plasmids were isolated with the Qiaprep kit (Qiagen, Valencia, CA).

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 *Mfe*I–*Sac*I fragment from pSL1 (Hagen *et al.*, 1986) into *Eco*RI-*Sac*I–digested pBluescriptSK+ (Stratagene, La Jolla, CA). The 2.3-kilobase (kb) *ADE2 Bgl*II fragment was subcloned into the *Bam*HI site of pBluescriptSK+, and the *Spe*I–*Pst*I *ADE2* fragment from this plasmid was subcloned into pSK-*STE3* to make pSK-*ste3::ADE2.* The *Kpn*I–*Sac*I *ste3::ADE2* fragment from pSK-*ste3::ADE2* was used to make *STE3* gene replacements by lithium acetate transformation (Chen *et al.*, 1992) in strains Tn44–1B and RAK2 (Table 1). The *ram1::URA3* disruption in SM1865 was changed to a *ram1::LEU2* disruption by cleaving pUL9 (Cross, 1997) with *Xba*I 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 (Aα4-Aβ6/Bα1-Bβ1) and 4–8 (Aα4-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 blendor 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 onto the strain 4–8 cultures. Genes within the B loci are known to be up-regulated after contact between individuals 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

Table 2. Oligonucleotides

Gene	Oligonucleotide	Position	Sequence[a]
bbr1	980420-3	Upstream	TTC<u>GAATTC</u><u>ATG</u>CACCCCGAGTTTGCCCCCGTAG
	980420-4	Downstream	TCC<u>GGATCC</u><u>CTA</u>CCTGCGCCCACCGGGGAACAC
bbr2	980730-1	Upstream	TTC<u>GAATTC</u><u>ATG</u>TACTCCAACGACCCGACG
	980730-2	Downstream	TCC<u>GGATCC</u>TTAAAGGACGACGCGGTGTG
bbp1(1)	980730-5	Upstream	TTC<u>GAATTC</u><u>ATG</u>GACGCCTTCACCGCC
	980730-6	Downstream	TCC<u>GGATCC</u><u>CTA</u>CGCAACCACGCACCAC
bbp2(4)	980227-2	Upstream	CG<u>GAATTC</u><u>ATG</u>GACGACTTCATCACC
	980227-3	Downstream	CG<u>GGATCC</u>GGCTACGCCGGG<u>TCA</u>CGCC

[a] The oligonucleotide sequences are shown in the 5′ to 3′ direction. Single underlines indicate *Eco*RI sites in the upstream primers or *Bam*HI 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.

μg) 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 *Schizophyllum* pheromone gene- and receptor gene-coding sequences were amplified by PCR, as described below, using oligonucleotides (Genosys, Woodlands, TX) that incorporate *Eco*RI and *Bam*HI recognition sites at the 5′- and 3′-ends of the open reading frames, respectively. The *Eco*RI–*Bam*HI 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 (Aα4-Aβ6/Bα3-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 dideoxynucleotide 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 *MAT***a** strains containing pPGK-*bbp1(1)*, pPGK-*bbp2(4)*, or the control vector pPGK in the following manner. Fresh cultures grown to an OD$_{600}$ of ~1.0, in SD(ura−) medium to maintain plasmids, were diluted to OD$_{600}$ = 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 *MAT*α strain (W303–1B) was tested.

MATα 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 *MAT***a** cells, but after centrifugation the cells were resuspended in 0.5 vol of fresh selective media. *MAT*α cells (1 ml) in fresh medium were mixed with an equal volume of *MAT***a** 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

*MAT***a** strains containing pPGK or pPGK-*bbp2(4)* were grown in patches on SD(ura−) master plates 24–48 h. *MAT*α cells (0.3 ml of cells at OD$_{600}$ = 0.3) were spread on either YEPD plates for the *MAT*α *STE3 sst2* strain or SD(ura−) plates for the *MAT*α *ste3 sst2* strains containing pPGK or pPGK-*bbr1* constructs. The master plates containing the *MAT***a** patches were replica plated to the appropriate *MAT*α 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 *MAT***a** strains (*his4*) and *MAT*α 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.

Table 3. Genes/gene products relevant to this study

Gene(s)	Protein product(s)	Reference
S. cerevisiae		
STE4	Gβ subunit	Whiteway *et al.*, 1989
STE6	**a**-factor transporter	McGrath and Varshavsky, 1989; Kuchler *et al.*, 1989
SST2	Regulator of G protein signaling	Dietzel and Kurjan, 1987
STE3	**a**-factor receptor	Hagen *et al.*, 1986
MFA1, MFA2	**a**-factor peptide precursors	Michaelis and Herskowitz, 1988
RAM1, RAM2	α- and β-subunits of farnesyl transferase	He *et al.*, 1991
STE14	Carboxyl methylase	Sapperstein *et al.*, 1994
STE24	N- and C-terminal protease of **a**-factor precursors	Boyartchuk *et al.*, 1997; Tam *et al.*, 1998; Fujimura-Kamada *et al.*, 1997
RCE1	C-terminal protease of **a**-factor precursors	Boyartchuk *et al.*, 1997
STE23, AXL1	N-terminal proteases of **a**-factor precursors	Adames *et al.*, 1995; Chen *et al.*, 1997b
S. commune		
bbr1	Pheromone receptor from specificity 1 of the Bβ locus	Vaillancourt *et al.*, 1997
bbr2	Pheromone receptor from specificity 2 of the Bβ locus	This study
bbp1(1)	Pheromone 1 from specificity 1 of the Bβ locus	Vaillancourt *et al.*, 1997
bbp2(4)	Pheromone 4 from specificity 2 of the Bβ locus	This study

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 Bβ1 receptor Bbr1, called *bbr1*, and the gene encoding Bβ2 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 *MATα ste3* mutant. *Schizophyllum* pheromones, including Bbp2(4), are predicted from DNA sequences to be small peptides that are modified with a farnesyl group (Wendland *et al.*, 1995; Vaillancourt *et al.*, 1997; Fowler, Mitton, and Raper, unpublished), the same lipid 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 *MAT**a*** 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 *MAT**a*** culture supernatants with *MATα* cells, cell culture supernatants of the *MAT**a*** strains containing pPGK-*bbp2(4)* or the control plasmid pPGK were mixed with *MATα ste3* strains containing pPGK-*bbr1* or the control plasmid. The combination of supernatant

from the *MAT**a*** 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 *MAT**a*** cells and
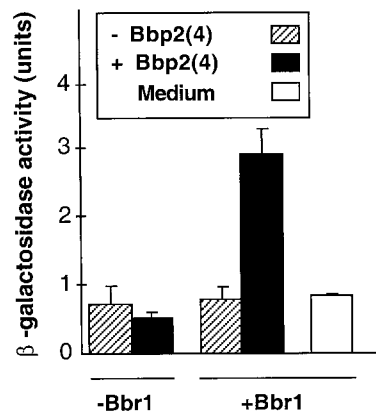

Figure 2. Activation of a yeast pheromone-inducible gene by a *Schizophyllum* pheromone–receptor pair. Cell culture supernatants were collected from *MAT**a*** strains (W303–1A) containing pPGK-*bbp2(4)*, which encodes the *Schizophyllum* pheromone Bbp2(4) (black), or the control vector pPGK (stripes). Response to culture supernatants or a fresh medium control (white) was tested using a *MATα ste3* strain (SDK45) containing the pheromone-inducible *FUS1-lacZ* reporter plasmid and either pPGK-*bbr1*, which encodes *Schizophyllum* receptor Bbr1, or pPGK. β-Galactosidase activity was assayed to assess pheromone response. Error bars here and in subsequent figures represent 1 SD.
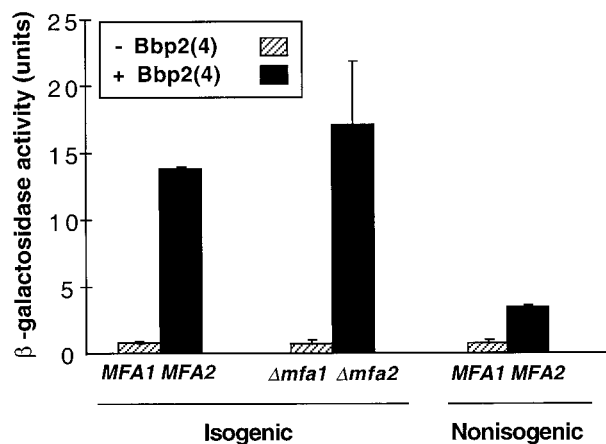
Figure 3. Production of Bbp2(4) and response to this pheromone are not influenced by the presence of **a**-factor, but Bbp2(4) production is affected by yeast strain differences. The *MATα ste3* strain (SDK45) tested for pheromone response contained the pheromone-inducible *FUS1-lacZ* reporter plasmid and pPGK-*bbr1.* To test for any effect on Bbp2(4) signaling by the presence of **a**-factor, culture supernatants from isogenic *MATa MFA1 MFA2* (SM1058) and *MATa mfa1 mfa2* (SM2331) strains containing either pPGK-*bbp2(4)* (black) or pPGK (stripes) were compared. Comparison to a nonisogenic *MATa MFA1 MFA2* strain (W303–1A) showed a strain-related difference in Bbp2(4) activity. β-Galactosidase assays were done as in Figure 2.

expression of *bbr1* by the *MATα* cells. These results indicate that an active *Schizophyllum* pheromone Bbp2(4) is secreted by *S. cerevisiae* and that the *Schizophyllum* receptor Bbr1 is also functionally expressed. Furthermore, Bbr1 is inactive until stimulated by Bbp2(4), at which time the receptor transduces a signal that elicits a response from the *FUS1-lacZ* reporter gene, indicating that the receptor is capable of coupling with the *S. cerevisiae* pheromone-response pathway (see below).

In the previously described experiment, the *MATa* strain used to secrete Bbp2(4) also secreted **a**-factor, but the **a**-factor could not induce signaling in the *MATα ste3* strain due to the absence of the **a**-factor receptor, Ste3p. It was possible, however, that the simultaneous expression of the **a**-factor and Bbp2(4) precursors might influence Bbp2(4) maturation or secretion or that secreted **a**-factor could interfere with the interaction between Bbp2(4) and the Bbr1 receptor. We therefore compared Bbp2(4) activity in culture supernatants from an **a**-factor-deficient strain (SM2331, *MATa mfa1 mfa2*; Michaelis and Herskowitz, 1988) with that from an isogenic wild-type strain (Figure 3, isogenic strains; SM1058, *MATa MFA1 MFA2*). Activation of the *FUS1-lacZ* reporter gene through Bbr1 was comparable for these culture supernatants, indicating that **a**-factor production does not interfere with Bbp2(4) production or affect interaction of Bbp2(4) with its receptor.

The two isogenic strains (SM2331, SM1058) tested above for any interference between **a**-factor and Bbp2(4) are not isogenic with the strain (W303–1A) used in the initial assay. Culture supernatants containing Bbp2(4) obtained from the *MATa MFA1 MFA2* strain SM1058 induced four- to sixfold higher β-galactosidase levels than did culture supernatants

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

An important response to pheromone in *S. cerevisiae* is arrest in the G_1 phase of the cell cycle (Kurjan, 1993). In the "halo" assay (Dietzel and Kurjan, 1987a), pheromone secreted from a patch of cells arrests the growth of an underlying lawn of cells of the opposite mating type, resulting in a clear zone immediately surrounding the patch of cells. Response of the *MATα* lawn to **a**-factor requires the **a**-factor receptor, Ste3p (Figure 4A). Expression of Bbr1 in a *MATα ste3* strain resulted in a halo surrounding the *MATa* cells expressing Bbp2(4). Controls indicated that Bbp2(4) expression by the *MATa* strain and Bbr1 expression by the *MATα* strain were necessary for halo formation. Therefore, response 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 morphological change in which the cells produce a mating projection. 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 morphological changes require ~100-fold higher concentrations of pheromone than are required for cell-cycle arrest or transcriptional induction (Moore, 1983); the absence of morphological 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 *MATa/MATα* diploid. In mating assays (Figure 4B), production of Bbp2(4) by *MATa 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 induction, cell-cycle arrest, and mating.

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 Gpa1p (α), Ste4p (β), and Ste18p (γ) (Dietzel and Kurjan, 1987b; 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
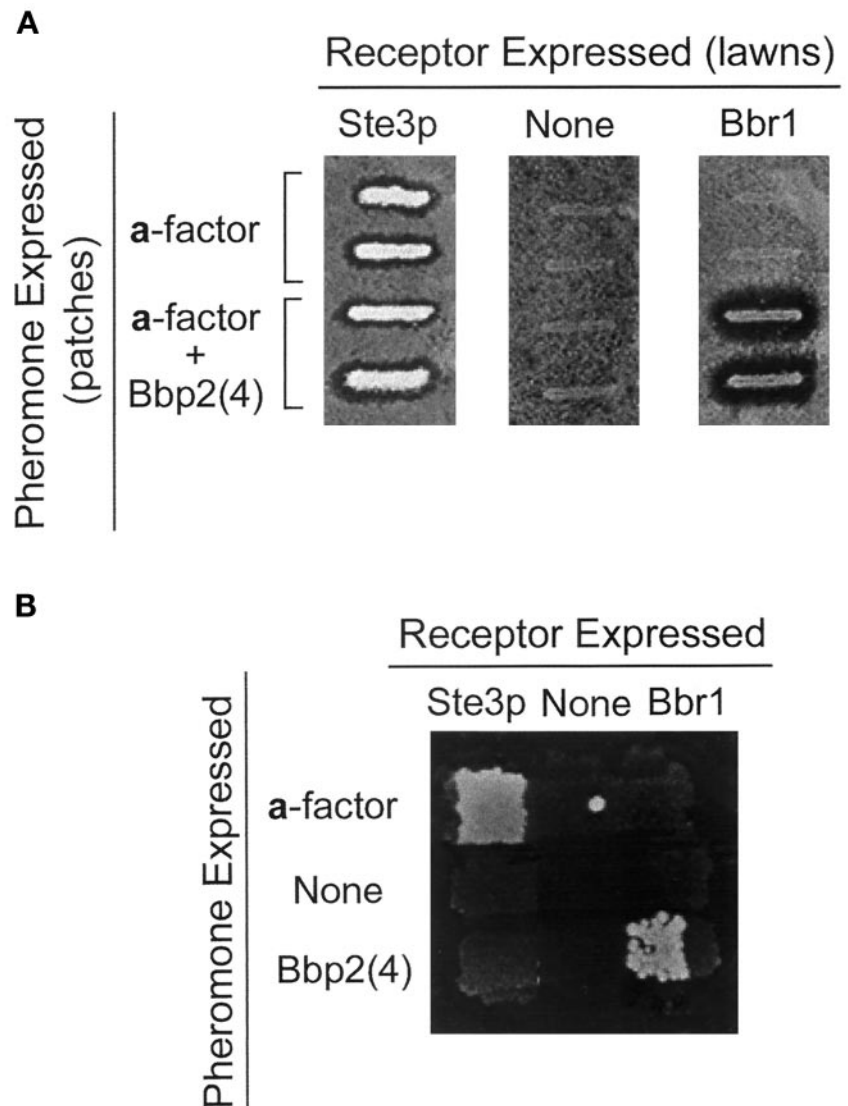
A

Receptor Expressed (lawns)

B

Receptor Expressed

Figure 4. A compatible pheromone–receptor pair from *Schizophyllum* elicits cell-cycle arrest and mating. (A) Cell-cycle arrest in response to pheromone was tested by the halo assay. Master plates with duplicate patches of *MAT**a*** cells (W303–1A) containing either the control vector pPGK (upper two patches in each panel) or pPGK-*bbp2(4)* (lower two patches) were replica plated on *MATα* lawns to test growth arrest by secreted pheromone. The lawns used to test response were *STE3 sst2* (Tn44–1B) containing pPGK (left panel), *ste3 sst2* (SDK47) containing pPGK (middle panel), and *ste3 sst2* (SDK47) containing pPGK-*bbr1*. (B) Mating tests were performed between *MAT**a*** and *MATα* cells by pairing complementing auxotrophs (*his4* versus *his3*) and assaying for growth of diploids on SD(his−) medium. Rows: the *MAT**a*** *MFA1 MFA2* strain (SM1058) containing pPGK (top), the *MAT**a*** *mfa1 mfa2* strain (SM2331) containing pPGK (middle), and the *MAT**a*** *mfa1 mfa2* strain containing pPGK-*bbp2(4)*. Columns: The *MATα STE3* strain (Tn44–1B) containing pPGK (left), the *MATα ste3* strain (SDK47) containing pPGK (middle), the *MATα ste3* strain (SDK47) containing pPGK-*bbr1* (right).

and mating (Whiteway *et al.*, 1989). Culture supernatants containing Bbp2(4) did not induce the *FUS1-lacZ* reporter gene in a *MATα ste4* null mutant expressing Bbr1, but did show about a fourfold induction in the isogenic *MATα 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 *MATα 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 *MATα sst2* cells, no

halo was observed on a *MATα 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 **a**-factor receptor, Ste3p, by **a**-factor present in the culture supernatants (Figure 2). Production of Bbp2(4) in an **a**-factor–deficient strain (*MAT**a*** *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 **a-**
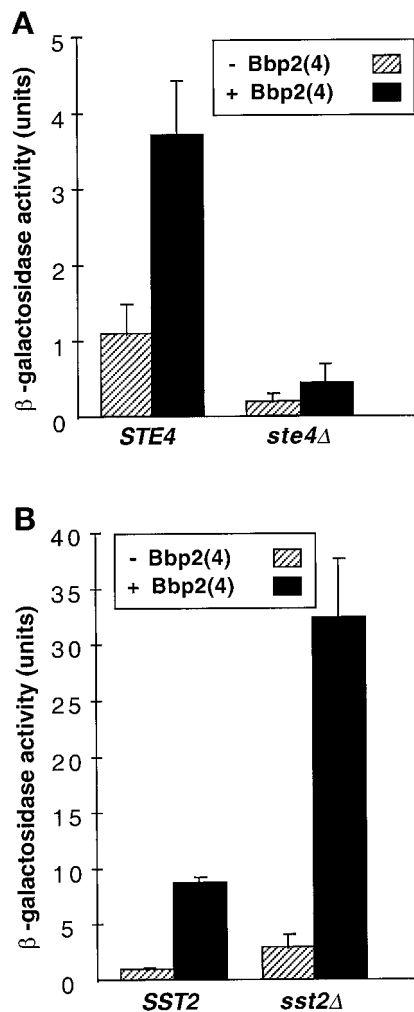
A

B

Figure 5. Components of the pheromone-response pathway govern signaling from the *Schizophyllum* pheromone–receptor pair. The *MATα* strains tested for response contained the pheromone-inducible *FUS1-lacZ* reporter plasmid and pPGK-*bbr1*. Cell culture supernatants were from a *MATa* strain (W303–1A) that contained either pPGK (stripes) or pPGK-*bbr2(4)* (black). Reporter gene assays were done as in Figure 2. (A) Comparison of responses by *MATα ste3 STE4* (SDK45) and *MATα ste3 ste4* (SDK48) strains indicate that Ste4p, the β-subunit of the yeast G protein, is required for signaling via activated Bbr1. (B) Comparison of responses by *MATα ste3 SST2* (SDK45) and *MATα ste3 sst2* (SDK47) strains indicate that *sst2*, a mutation that enhances sensitivity to yeast pheromone, also increases sensitivity to *Schizophyllum* pheromone.

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 ma-
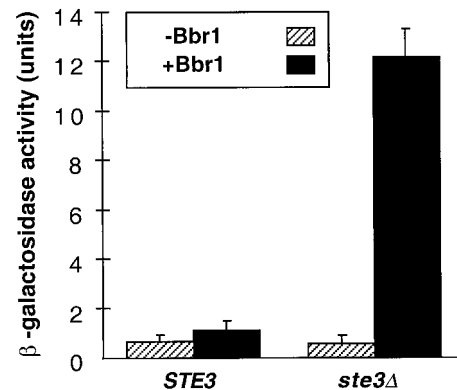

Figure 6. The yeast Ste3p receptor inhibits response to activated *Schizophyllum* Bbr1. Cell culture supernatants of the *MATa* strain (W303–1A) containing pPGK-*bbp2(4)* were used for all treatments. Responses by the *MATα STE3* (W303–1B) and *MATα ste3* (SDK45) strains containing either pPGK (striped) or pPGK-*bbr1* (black) were compared. Reporter gene assays were done as in Figure 2.

ture 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 (Boyartchuk *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 expressing 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
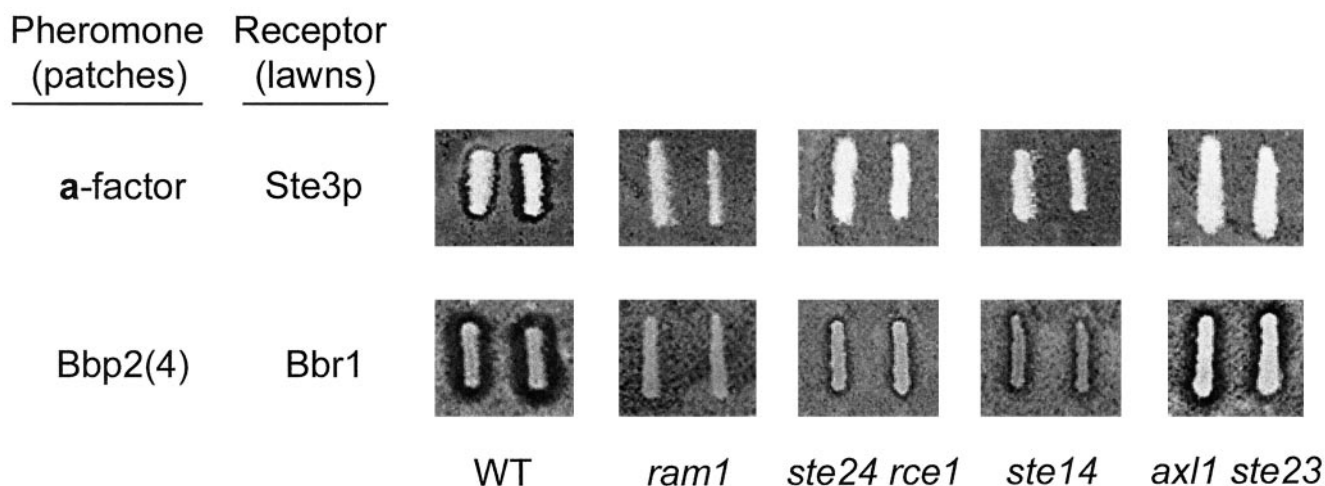
Figure 7. Active, secreted Bbp2(4) requires only some of the **a**-factor processing and modification machinery. Halo assays, in duplicate, were done as in Figure 4A. Top row: Secreted **a**-factor was monitored using halo assays on *MATα sst2 STE3* (Tn44–1B) lawns. *MAT***a** cell patches tested for **a**-factor production were the wild-type parental strain ("WT", SM1058) and isogenic mutants defective in farnesylation (*ram1*, LHK1), C-terminal processing (*ste24 rce1*, SM3614), carboxyl methylation (*ste14*, SM1188), and N-terminal processing (*axl1 ste23*, SM2744), all containing pPGK. Bottom row**:** A *MAT***a** *mfa1 mfa2* mutant strain (SM2331) containing pPGK-*bbp2(4)* ("WT") served as the control for Bbp2(4) production. The *ram1*, *ste24 rce1*, *ste14*, and *axl1 ste23* mutant strains described above, but containing pPGK-*bbp2(4)*, were tested for Bbp2(4) production. Halo assays were done on *MATα sst2 ste3* (SDK47) lawns containing pPGK-*bbr1*. Quantifications of Bbp2(4) activity in the *ste24 rce1* and *ste14* mutants were done by *FUS1-lacZ* reporter gene assays (see text for details). The same mutant strains containing pPGK, tested on *MATα sst2 ste3* (SDK47) lawns containing pPGK-*bbr1,* showed no halo effect (our unpublished data).

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 (Fujimura-Kamada *et al.*, 1997; Tam *et al.*, 1998). The reduced halo size (Figure 7) and the extremely low levels of β-galactosidase produced by the *ste24 rce1* mutant suggest that Ste24p may be required for Bbp2(4) activity. Processing of the mature N terminus of **a**-factor involves the functionally redundant Axl1p and Ste23p proteases (Adames *et al.*, 1995; Chen *et al.*, 1997b). The *axl1 ste23* mutant expressing Bbp2(4) showed halos of similar size to those of the wild-type control (Figure 7), indicating that Axl1p and Ste23p are not required for production of active Bbp2(4).

Secretion of the mature **a**-factor lipopeptide occurs through the transporter Ste6p, which is a family member of the ATP-binding cassette transporters (Kuchler *et al.*, 1989; McGrath and Varshavsky, 1989). The level of active Bbp2(4) produced by *ste6* mutants was about half of the level produced by the wild-type *STE6* strain, as assayed by β-galactosidase activity (Figure 8A). Halo assays also indicated that the *ste6* mutation did not significantly affect Bbp2(4) secretion (our unpublished data). Bbp2(4) can therefore be secreted independently of Ste6p.

In all previous experiments, Bbp2(4) was expressed in *MAT***a** 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 *MAT***a**-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 *MAT***a** cells,

we tested whether *MATα* cells could secrete functional Bbp2(4). Levels of induction of the *FUS1-lacZ* reporter gene were similar using supernatants from either *MATα* cells containing pPGK-*bbp2(4)* or *MAT***a** cells containing pPGK-*bbp2(4)* (Figure 8B). This result is consistent with a Ste6p-independent transport mechanism and indicated that no *MAT***a**-specific gene products are required for Bbp2(4) production.

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 *MAT***a** *mfa1 mfa2* strain, and the Bbr2 receptor was expressed in the *MATα 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*.
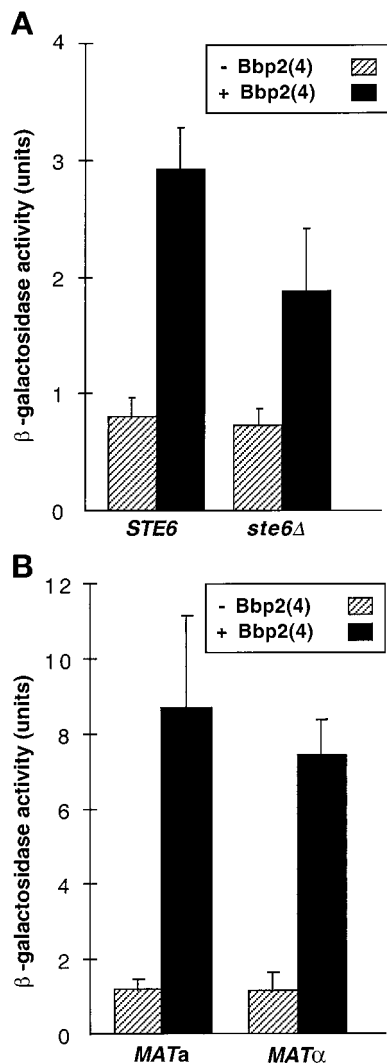
A

B

Figure 8. Bbp2(4) processing and secretion are independent of transporter Ste6p and all other **a**-specific gene products. The *MAT*α *ste3* strain (SDK45) containing the pheromone-inducible *FUS1-lacZ* reporter plasmid and pPGK-*bbr1* was tested for response. Reporter gene assays were done as in Figure 2. (A) The effects of cell culture supernatants from the *MAT***a** *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 *MAT***a** (W303–1A) and *MAT*α (W303–1B) cells containing either pPGK (stripes) or pPGK-*bbp2(4)* (black) were compared.

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
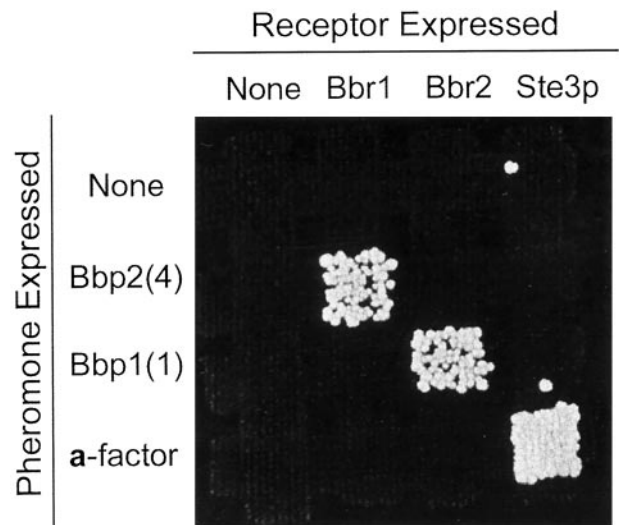

Figure 9. *Schizophyllum* pheromones and receptors maintain their specificity in yeast. Mating was tested as in Figure 4B. Rows: the *MAT***a** *mfa1 mfa2* strain (SM2331) containing either pPGK (top), pPGK-*bbp2(4)* (second from top), or pPGK-*bbp1(1)* (third from top), and the *MAT***a** *MFA1 MFA2*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

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.

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

the same machinery as **a**-factor production and secretion by analyzing Bbp2(4) expression in mutants defective for each of the steps involved in **a**-factor production. Interestingly, farnesylation was the only step absolutely required for processing of the *Schizophyllum* pheromone: mutants defective in **a**-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 **a**-factor N terminus (Axl1p and Ste23p) are not involved in active Bbp2(4) production. A mutation eliminating the **a**-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 **a**-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 **a**-factor.

Bbp2(4) secretion was independent of the ATP-dependent **a**-factor transporter, Ste6p, as shown by *ste6* mutant studies and by analysis of Bbp2(4) secretion in *MATα* 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 **a**-factor-related peptide (AFRP), a heptapeptide derived from the C termini of the **a**-factor precursors (Chen *et al.*, 1997a). Unlike **a**-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 **a**-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 G$\beta\gamma$ 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 (Dohlman and Thorner, 1997); *sst2* mutants show greatly increased sensitivity to pheromone and a defect in recovery from pheromone-induced cell-cycle 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 **a**-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 *MAT***a**-specific protein is involved in the inhibition process (Kim *et al.*, 1999). A *MAT***a**-specific protein cannot be involved in Ste3p inhibition of Bbr1 signaling, however, because our assays tested signaling in a *MATα* 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 hemibasidiomycetes, 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 *Schizophyllum* pheromones in yeast may allow these pheromones to be concentrated using methods developed for yeast **a**-factor (Strazdis and MacKay, 1983; Chen *et al.*, 1997a). Isolation and concentration of *Schizophyllum* 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 *Schizophyllum*. 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 *Schizophyllum* 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 *Schizophyllum* receptor, and how can one *Schizophyllum* pheromone activate several different receptors? To be useful for these studies, the specificity of *Schizophyllum* 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 Bβ 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 Bα 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 sytem for extensive and rapid screening of the effects of amino acid substitutions on *Schizophyllum* pheromone–receptor interactions will help us to answer this question. The information obtained from such studies should complement studies of other complex systems of ligand–seven-transmembrane receptor interactions such as those involved in mammalian olfaction, pheromone sensing, and taste (Buck and Axel, 1991; Dulac and Axel, 1995; Belluscio *et al.*, 1999; Hoon *et al.*, 1999; Rodriguez *et al.*, 1999). This body of information may lead to a general understanding of the basic structure–function relationships between ligands and receptors.

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