Activation of the Mating Pheromone Response Pathway of *Lentinula edodes* by Synthetic Pheromones

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**ABSTRACT**

Pheromone (PHB)-receptor (RCB) interaction in the mating pheromone response pathway of *Lentinula edodes* was investigated using synthetic PHBs. Functionality of the C-terminally carboxymethylated synthetic PHBs was demonstrated by concentration-dependent induction of a mating-related gene (*znf2*) expression and by pseudoclamp formation in a monokaryotic strain S1-11 of *L. edodes*. Treatment with synthetic PHBs activated the expression of homeodomain genes (HDs) residing in the A mating type locus, and of A-regulated genes, including *znf2, clp1*, and *priA*, as well as genes in the B mating type locus, including pheromone (*phb*) and receptor (*rcb*) genes. The synthetic PHBs failed to discriminate self from non-self RCBs. PHBs of the B4 mating type (B4 PHBs) were able to activate the mating pheromone response pathway in both monokaryotic S1-11 and S1-13 strains, whose B mating types were B4 (self) and B12 (non-self), respectively. The same was true for B12 PHBs in the B4 (non-self) and B12 (self) mating types. The synthetic PHBs also promoted the mating of two monokaryotic strains carrying B4-common incompatible mating types (A5B4 × A1B4). However, the dikaryon generated by this process exhibited abnormally high content of hyphal branching and frequent clamp connections and, more importantly, was found to be genetically unstable due to overexpression of mating-related genes such as *clp1*. Although synthetic PHBs were unable to discriminate self from non-self RCBs, they showed a higher affinity for non-self RCBs, through which the mating pheromone response pathway in non-self cells may be preferentially activated.

**1. Introduction**

In filamentous basidiomycetes, mating occurs through hyphal fusion of two compatible monokaryotic strains. Mating compatibility is determined by mating type-specific genes in genetic loci A and B. Particularly, pheromone and pheromone receptor at the B locus are involved in the initial stage of mating through the activation of the pheromone response pathway. The pheromone response pathway of basidiomycetes has been suggested to share strong genetic similarities with the most well-studied model fungus, *Saccharomyces cerevisiae* [1,2]. In *S. cerevisiae*, pheromone-receptor interaction activates the transcription of genes related to the mating process through the MAP kinase-mediated signaling cascade [3]. The pheromone response pathway of *MATa* haploid cells is activated by the binding of α-factor from *MATa* cells to pheromone receptor Ste2, whereas that of *MATα* cells is activated by the binding of a-factor from *MATa* cells to receptor Ste3 [3,4]. Unlike the freely diffusible α-factor, a-factor is a membrane-incorporated pheromone. The precursor of α-factor (36 residues) from *MATα* cells is converted into a 12-amino acid mature peptide through proteolytic cleavage catalyzed by membrane-bound metalloprotease Ste24 [5–7]. The C-terminus of α-factor is prenylated and carboxymethylated at the cysteine residue in a CAAX (C, cysteine; A, aliphatic residue; X, any amino acid) motif by the activities of Ram1/Ram2 and Ste14, respectively [8,9]. Mature α-factor is translocated across the membrane by exporter protein Ste6 [10].

Filamentous basidiomycetes have only Ste3-type pheromone receptors, which are activated by a-factor-like pheromones [11]. Similar to α-factor, pheromones in this class are incorporated into cell membrane through acylation at the cysteine residue in the C-terminal CAAX motif [11]. Therefore, it is highly possible that activation of the mating pathway occurs only after membrane fusion between two compatible cells, since there are no diffusible pheromones in basidiomycetes. The B mating locus of basidiomycetes is much more complex than the single receptor-pheromone pair in haploid cells of *S. cerevisiae*. 

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In *Schizophyllum commune*, 81 B mating types have been suggested to occur through recombination between nine allelic $B_x$ and nine allelic $B_b$ subloci [2,12]. The $B$ locus of *Coprinopsis cinerea* consists of three subloci, with two alleles in sublocus 1, five alleles in sublocus 2, and seven alleles in sublocus 3, constituting a total of 70 theoretical $B$ mating types [13]. In these basidiomycetes, the pheromone response pathway is activated only when non-self pheromones are bound to compatible pheromone receptors at the same sublocus [14–16]. The specificity of a pheromone to a pheromone receptor relies on subtle variations in their amino acid sequences [2,13,17]. One example of this was shown in *Ustilago maydis* using synthetic peptides that mimic mature pheromones. Synthetic pheromone a1 was able to activate only a2 receptor, while synthetic pheromone a2 was found to activate a1 receptor [18]. Alanine scanning of a1 pheromone showed that Gly5, Gly9, and Tyr10 are highly important amino acid residues in the activation of pheromone pathways, and these residues have been suggested to play a role in the binding of pheromone to target receptor.

*Lentinula edodes* is one of the most cultivated mushrooms worldwide, and particularly in East Asia. Much effort has been made to develop new strains suitable for changing climates and alternative substrates. One such example is our classical mating experiment in monokaryotic strains from various origins [19]. During this experiment, we got to know that the mating type of *L. edodes* is multiallelic both in the $A$ and $B$ loci [20,21]. The $B$ mating locus has been shown to consist of two subloci, $B_x$ and $B_b$, similar to *S. commune* [22]. Moreover, we have found 15 $B$ mating types through a combinatorial assortment of five $B_x$ and three $B_b$ subloci [21]. The $B_x$ and $B_b$ subloci are respectively composed of five alleles of pheromone receptors ($rcb$s) with nine associated pheromones ($phb$s), and of three alleles of $rcb$s with five $phb$s [21]. Each sublocus contains an $rcb$ and two $phb$ genes. For example, the $B_x$ subloci of the $B4$ and $B12$ mating types, two frequently occurring $B$ mating types, are constituted by $phb5-phb6-rcb1-2$ and $phb11- phb12-rcb1-4$, respectively, while the $B_b$ subloci of the $B4$ and $B12$ mating types are consisted of $rcb2-1-phb7-phb4$ and $rcb2-3-phb9-phb10$, respectively (Figure 1(A)). The complexity of the $B$ mating type in *L. edodes* has led us to investigate the pheromone-receptor interactions. Hence, we investigated the effect of synthetic pheromones on the expression of mating genes as a measure of the specificity of pheromone-receptor interactions. In the present study, eight synthetic peptides corresponding to the mature forms of $B4$ and $B12$ PHBs were employed to determine the specificity of RCB proteins using two monokaryotic strains, S1-11 and S1-13, which have the $B4$ and $B12$ mating types, respectively.

![Figure 1. Pheromone and pheromone receptor structures in the $B$ mating type locus of *L. edodes*. (A) Structures of the $B4$ and $B12$ mating types. The $B$ mating type loci consist of distinct $B_x$ and $B_b$ subloci which contain different pheromones ($phb$s) and receptor genes ($rcb$s). Arrow boxes indicate the direction of transcription. (B) Amino acid sequence of mature pheromones. (C) Amino acid sequence comparison of pheromone receptors. Loop regions are shown within boxes and the amino acids predicted to be located in transmembrane domains are colored in red. RCB1-2 and RCB1-4 represent pheromone receptors found in the $B_x$ sublocus of $B4$ and $B12$ mating types, respectively, whereas RCB2-1 and RCB2-3 are the receptors found in the $B_b$ sublocus of $B4$ and $B12$, respectively.](image-url)
2. Materials and methods

2.1. Strains and growth conditions

The monokaryotic strains S1-11 and S1-13 of *L. edodes*, whose *B* mating types were determined to be *B4* and *B12*, respectively [19], were employed to investigate the self- and nonself-PHB responses. Their *A* mating types were *A5* and *A1* for S1-11 and S1-13, respectively [19]. The monokaryotic strain S1-10, whose mating types were *A1* in the *A* mating type locus and *B4* in the *B* mating type locus, was used to induce forced mating between *B*-common monokaryotic strains. These strains had been generated from basidiospores of the dikaryotic SJ701 strain [19]. All strains were grown on potato dextrose agar (PDA; Oxoid, Hampshire, England) at 25°C.

2.2. Pheromone response assay using synthetic PHBs

PHB peptides were commercially synthesized with carboxymethylation at the C-terminal cysteine residue (Genscript, Piscataway, New Jersey). The C-terminal carboxymethyl group is suggested to be important for the binding of pheromone to receptor [23,24]. In order to investigate the pheromone response in *L. edodes*, mycelia grown for two weeks in potato dextrose broth (PDB; Oxoid, Hampshire, England) at 25°C were harvested and then washed twice with distilled water. Collected mycelia were suspended in fresh PDB containing synthetic PHBs (20 μg/ml) for 12 h at 25°C. Pheromone response was determined by measuring the expression of downstream genes in the mating pheromone pathway. For this, total RNA from PHB-treated mycelia was extracted using RNeasy Plant Mini Kit (Qiagen, Hinden, Germany) and was then transcribed into cDNA using a cDNA synthesis kit (TOPscript; Enzynomics, Daejeon, Korea) with an oligo-dT primer. Real-time qRT-RCR was performed using Lightcycler Nano system (Roche; Manheim, Germany) with the synthesized cDNA (100 ng/μl) and FastStart Universal SYBR Green Master (Roche; Manheim, Germany). Primer sets used for this analysis are described in Supplementary Table S1. PCR was carried out under the following conditions: hold for 10 min at 95°C; 45 cycles of 95°C for 15 s and 60°C for 60 s. The relative expression of the target gene was calibrated on the basis of the cycle threshold value of the *L. edodes* β-tubulin gene (GenBank accession number AF106239). All assays were performed in triplicate.

2.3. Mating assay and microscopic examination

For mating, two monokaryotic mycelia were placed on PDA at a distance of 0.5 cm from each other. The generation of the dikaryon was determined by observation of clamp connections under the light microscope after two weeks of incubation at 25°C. Mating between common *B* strains was induced by treatment with 10 μl of a mixture of synthetic PHBs (1 mg/ml) in the middle of two monokaryotic strains. Mycelia were stained with calcifluor white (Sigma-Aldrich, St. Louis, MO) so as to enhance their visualization. The stained mycelia were examined under a fluorescence microscope (AX-80; Olympus, Tokyo, Japan) at 400× magnification with excitation at 355 nm.

3. Results

3.1. Structures of pheromones and pheromone receptors

Mature PHBs were predicted to be polypeptides with a length of 11–16 amino acids (Figure 1(B)), similar to a-factor of *S. cerevisiae*. PHB5 and PHB11 at the *Bz* sublocus of *B4* and *B12*, respectively, shared high sequence identity. Only two amino acids were different at the positions 2 (H/R) and 5 (D/N), whereas PHB6 in *B4* was remotely related to these two. PHB12 was different in length and sequence composition to the three PHBs. PHBs at the *Bf* sublocus were shorter and contained different sequence motifs, such as EA and A(G)FC. Similar to PHB5 and PHB11, PHB7 in *B4* and PHB10 in *B12* shared high sequence homology.

Pheromone receptor (RCB) was predicted to be a membrane protein with seven transmembrane domains. RCBs were highly homologous regardless of the subloci to which they belong. However, even in this case, RCBs at *Bz* differed from those at *Bf* in sequence compositions. The loop domain L7, which conceivably allows RCBs to recognize their specific ligands (PHBs), was the most variable sequence region predicted to be located in the outer membrane (Figure 1(C)).

3.2. Synthetic pheromones are functional in the activation of the mating pathway

In order to better understand the pheromone-receptor interaction, we synthesized PHB polypeptides with a C-terminal carboxymethylation that can mimic mature PHBs in *L. edodes*, as it also does in *C. cinerea* [25]. Since the mature PHB in basidiomycetes is a polypeptide known to bind to the membrane through farnesylation at the C-terminus [10], we tested the functionality of synthetic pheromones on the expression of genes related to the mating pathway of *L. edodes*. First, the expression of *znf2*, a downstream transcription factor of the mating pathway [26,27], was investigated through RT-PCR.
analysis, upon addition of a mixture of Bz pheromones from either the B4 or the B12 mating type to the actively growing monokaryotic strain S1-11, whose B mating type is B4. As shown in Figure 2(A), Znf2 expression was induced by both pheromone mixtures in a concentration-dependent manner. However, the B12-Ba pheromones, PHB11 and PHB12, were more effective at inducing znf2 than the B4-Ba pheromones, PHB5 and PHB6. The expression levels were saturated at concentrations of 2.5 μg/ml and 5 μg/ml for B12-Ba pheromones and B4-Ba pheromones, respectively.

Next, we examined the microscopic structure of mycelia using calcoflour staining after treatment with a mixture of B12 PHBs (PHB11, PHB12, PHB9, and PHB10) to the actively growing mycelia of the S1-11 strain. The dikaryotic strain generated through the mating of S1-11 and S1-13 showed typical clamp connections with septal structure (Figure 2(B)). Interestingly, the monokaryotic S1-11 strain also formed a clamp-like structure (pseudoclamp) without septum upon treatment with B12 PHBs (Figure 2(C)). These data indicate that (1) the synthetic PHBs in soluble forms are functionally active in the activation of the mating pathway even without C-terminal farnesylation; (2) B4 pheromones can activate their own mating pheromone pathway (self-activation); and (3) cross-activation by B12-Bz pheromones appeared to be favored over self-activation by B4-Bz pheromones in the B4 mating pheromone pathway (Figure 2).

### 3.3. Synthetic PHBs induce genes in the mating pathway

We next explored the expression of genes in the mating pheromone pathway, including A mating type locus genes (HD1 and HD2), clp1, znf2, and pri-A, upon treatment with mixtures of B4 or B12 pheromones at a fixed total concentration of 20 μg/ml. Treatment with PHBs from individual subloci, i.e. B4-Bz PHBs (PHB5 + PHB6, 10 μg/ml each), B4-Bβ PHBs (PHB4 + PHB7, 10 μg/ml each), B12-Bz PHBs (PHB11 + PHB12, 10 μg/ml each), or B12-Bβ PHBs (PHB9 + PHB10, 10 μg/ml each), was able to induce the expression of the five mating genes in the S1-11 strain (Supplementary Figure S1). The combined treatment with B4 pheromones (PHB5 + PHB6 + PHB4 + PHB7, 5 μg/ml each) or B12 pheromones (PHB11 + PHB12 + PHB9 + PHB10, 5 μg/ml each) was also able to induce the mating genes (Figure 3). In the S1-11 strain (B4 mating type), both B4 and B12 pheromone mixtures induced the expression of mating genes with different degrees of activation (Figure 3). Similarly, both pheromone mixtures were able to activate mating genes in the S1-13 strain (B12 mating type), except for HD1 and HD2 (Figure 3). There is currently no explanation for the unresponsiveness of these two genes in the A mating type locus to the mating pheromones in the S1-13 strain. However, these data suggest that PHBs can induce self-activation as well as cross-activation of the mating pheromone pathway.

### 3.4. PHBs selectively induce genes in the B mating type locus

We further investigated the effect of PHBs on the expression of genes in the B mating type locus by treating the S1-11 strain with self PHBs (B4-Bz PHBs, PHB5 and PHB6; B4-Bβ PHBs, PHB4 and
PHB7) and with non-self PHBs (B12-Bα PHBs, PHB11 and PHB12; B12-Bβ PHBs, PHB9 and PHB10). Unlike the genes of the mating pheromone pathway, PHBs from a single sublocus, regardless of whether self or non-self PHBs were used for treatment, failed to activate any phb or rcb genes in the B4 mating type locus (Figure 4). However, a combination of Bα PHBs and Bβ PHBs from B4 (self) or B12 (non-self) was able to selectively activate the expression of two mating type genes (phb5 and rcb1-2) and a non-mating type rcb gene (rcb3) (Figures 4 and 5). Knowing that the genes in the B mating type locus are induced only in the presence of a mixture of Bα and Bβ PHBs, we also examined the expression of B mating type genes in the monokaryotic S1-13 strain after treatment with B4 PHBs (non-self) or B12 PHBs (self). Unlike S1-11, the three phbs (i.e. phb11 and phb12 at the Bα sublocus and phb9 at the Bβ sublocus) were induced by treatment with either self or non-self PHBs (Figure 5(C)), whereas rcbs, including rcb1-4 from the Bα sublocus and a nonmating type rcb3, were overexpressed, similar to observations in the S1-11 strain (Figure 5(D)).

3.5. Synthetic PHBs can induce mating in an incompatible mating pair

Mating between two monokaryotic strains occurs only when the two strains have different A and B mating types. Since expression of genes in the A mating type locus is also controlled by the mating pheromone pathway as described above, the mating process in L. edodes is essentially governed by pheromone-receptor interactions. This suggests that a monokaryon can mate with a monokaryon of an incompatible mating type, provided that the mating pheromone pathway is activated. To prove this hypothesis, we employed two monokaryotic strains, S1-11 and S1-10, whose mating types are A5B4 and A1B4, respectively. These two strains are basically incompatible for mating because of the B mating type they have in common. However, we were able to generate a dikaryotic strain (S1-11 × S1-10) through a classical mating experiment on PDA medium containing a mixture of B12 PHBs. The strain S1-11 × S1-10 exhibited a slower growth rate than its monokaryotic strains, whereas the dikaryotic
strain S1-11 × S1-13, which was generated by compatible mating between S1-11 and S1-13 (A1B12), exhibited a better growth than the monokaryotic strains (Figure 6(A)). Mycelia of S1-11 × S1-10 showed more frequent and irregular branching than mycelia of S1-11 × S1-13 (Figure 6(B) and Supplementary Figure S2). S1-11 × S1-10 mycelia also presented frequent septations and multiple clamp connections that resulted in short cells without nuclei (Figure 6(B)).

To account for this irregular development, we examined the expression of genes in the mating pheromone pathway. Most notably, clp1, which has been known to be involved in clamp formation [28], was found to be highly expressed in S1-11 × S1-10 (Figure 6(C)). Expression of the HD2 gene, unresponsive to PHBs in monokaryotic strains (Figure 3), was 4.5-fold higher in S1-11 × S1-10 than in S1-13 × S1-10 (Figure 6(C)). The nonmating type receptor rcb3 was also 4-fold overexpressed in S1-11 × S1-10. Detailed functions of the latter two are yet to be clarified. The dikaryon generated by this process was very poor in growth on PDA.

4. Discussion

Self/non-self recognition in the mating process of basidiomycetes is governed by interactions between mating pheromones and pheromone receptors. Furthermore, pheromones and pheromone receptors are essentially multiallelic, and therefore, the molecular mechanism underlying the correct matching of pheromone and receptor is one of the main aspects in the mating process of basidiomycete. C. cinerea is known to have more than 79 B mating types [12,13,29]. Selective and non-self interaction of pheromone and pheromone receptor, and thus the selective activation of the mating pathway, in this mushroom has been shown by mating analysis of tester strains and transformants, harboring plasmids expressing pheromones and receptors [29–31]. Strains of S. cerevisiae that express mushroom pheromone receptor proteins also exhibit a selective interaction with plasmid-borne pheromones or synthetic peptides of C. cinerea [17,25] and S. commune [14,15].

L. edodes, a popular edible mushroom of industrial importance, has been shown to contain more than five alleles of rcb5 with nine associated phb5, and three alleles of rcb5 with five associated phb5, in the Bx and Bβ subloci (of B mating type locus), respectively [21]. The complexity of phb5 and rcb5 has prompted us to investigate self/non-self recognition in the pheromone interaction with its receptor. In the present study, we demonstrated that PHB peptides can activate the mating pathway in monokaryotic strains of L. edodes in a concentration-dependent manner, regardless of their B mating
types (Figure 2), and even if the activation efficiency of non-self PHBs is higher than that of self PHBs (Figure (2A)). Activation of the pheromone response pathway appears to require PHB-RCB interactions from both \( B_a \) and \( B_b \) subloci (Figures 3–5).

Both synthetic PHBs (\( B_4 \) and \( B_12 \)) were able to induce the expression of \( znf2 \), a gene known to be controlled by \( HD \) genes from the \( A \) mating type locus [26], in the S-11 strain (\( B_4 \) mating type) in a concentration-dependent manner (Figure 2(A)). Addition of synthetic PHBs induced pseudoclamps in monokaryotic mycelia (Figure 2(C)), indicating that PHBs can activate the mating pathway in monokaryons even without mating. Clamp cell formation is a hallmark of successful mating in most basidiomycetes, and is controlled by the \( A \) mating type locus-regulated \( clp1 \) gene [28,32]. Activation of \( A \) genes and \( A \)-regulated genes by mating pheromones was further demonstrated by the treatment with a mixture of self or non-self PHBs (Figure 3). \( priA \), a gene involved in primordia formation [33], was also expressed in the PHB-treated monokaryotic strains (Figure 3). PHBs were also able to induce the expression of genes in the \( B \) mating type locus, but only upon treatment with both \( B_x \) and \( B_\beta \) PHBs in the monokaryotic strains (Figures 4 and 5). Particularly, \( phb5 \) and \( rcb1-2 \) in the \( B_x \) sublocus of \( B_4 \) and \( phb11, phb12 \) and \( rcb1-4 \) in the \( B_\beta \) sublocus of \( B_12 \) were highly expressed upon treatment with a mixture of synthetic PHBs. The biological meaning of the asymmetric expression of \( B \) genes, as well as the notably high expression of the non-mating type receptor \( rcb3 \), demands further investigation.

As described above, activation of the pheromone response pathway through a compatible PHB-RCB interaction is essential at the initial stage of the mating process. In basidiomycetes, this appears to occur during the hyphal fusion between two monokaryotic mycelia, because all pheromones found in basidiomycetes are a-factor-like membrane-bound peptides. Our present study demonstrates that RCB can bind to pheromones without discriminating between self and non-self PHBs, while studies in \( C. \) cinerea and \( S. \) commune have shown specific non-self interactions [29–31]. One possible explanation of this phenomenon is that the genetically encoded pheromone is localized to the cell membrane through C-terminal farnesylation, and therefore it is not in a correct orientation to interact with the RCB in the same membrane. Provision of soluble synthetic PHBs in the present study may help overcome the orientation problem. However, if both self and non-self PHB-RCB interactions occur simultaneously in nature, how does the RCB discriminate.
between self and non-self PHBs? A higher binding affinity of RCB for non-self PHBs over self PHBs can be one explanation. Preferred binding of non-self PHBs to RCB may outcompete the binding of self PHBs as shown in Figure 2(A). Additionally, even if the self PHB-RCB interaction occurs and thus results in a successful mating, the dikaryon generated from incompatible partners appears to be eliminated during adaptation due to the decreased fitness. This was demonstrated by the decreased growth rate with the irregular branching and septation in the mycelia of the dikaryotic strain generated by forced mating between monokaryotic S1-11 and S1-10 strains (both of them have the same B mating type) (Figure 6).

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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