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RESEARCH ARTICLE

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Discovery and Functional Study of a Novel Genomic Locus Homologous to *Bα*-Mating-Type Sublocus of *Lentinula edodes*

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ABSTRACT

The interaction of mating pheromone and pheromone receptor from the *B* mating-type locus is the first step in the activation of the mushroom mating signal transduction pathway. The *B* mating-type locus of *Lentinula edodes* is composed of *Bα* and *Bβ* subloci, each of which contains genes for mating pheromone and pheromone receptor. Allelic variations in both subloci generate multiple *B* mating-types through which *L. edodes* maintains genetic diversity. In addition to the *B* mating-type locus, our genomic sequence analysis revealed the presence of a novel chromosomal locus 43.3 kb away from the *B* mating-type locus, containing genes for a pair of mating pheromones (*PHBN1* and *PHBN2*) and a pheromone receptor (*RCBN*). The new locus (*Bα-N*) was homologous to the *Bα* sublocus, but unlike the multiallelic *Bα* sublocus, it was highly conserved across the wild and cultivated strains. The interactions of RcbN with various mating pheromones from the *B* and *Bα-N* mating-type loci were investigated using yeast model that replaced endogenous yeast mating pheromone receptor *STE2* with *RCBN*. The yeast mating signal transduction pathway was only activated in the presence of PHBN1 or PHBN2 in the RcbN producing yeast, indicating that RcbN interacts with self-pheromones (*PHBN1* and *PHBN2*), not with pheromones from the *B* mating-type locus. The biological function of the *Bα-N* locus was suggested to control the expression of *A* mating-type genes, as evidenced by the increased expression of two *A*-genes *HD1* and *HD2* upon the treatment of synthetic PHBN1 and PHBN2 peptides to the monokaryotic strain of *L. edodes*.

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1. Introduction

Mating is an important biological process to generate genetic diversity in nature. In fungi, the mating process is initiated by the physical interaction of mating pheromone with pheromone receptors. The pheromone–receptor interaction activates the mating signal transduction pathway that transfers mating signals to the nucleus of each mating partner [1]. Activation of the mating signal transduction pathway in *Saccharomyces cerevisiae* occurs via the mitogen-activated protein kinase (MAPK) cascade upon binding of mating pheromone, a- or α-factor, to membrane-embedded pheromone receptor, Ste2 or Ste3, leading to eventual activation of the transcription factor Ste12 which controls the expression of mating-related genes [2,3].

Fungi belonging to Basidiomycota also require the physical interaction between mating pheromone and membrane receptors to initiate the mating process [1]. However, different from *S. cerevisiae*, the pheromone and receptor genes are genetically linked and are found as pair(s) at a certain chromosomal locus, namely the *B* mating-type locus [4–6]. The *B* mating-type locus is further diversified by allelic variation and paralogous expansion/constriction of the pheromone-receptor gene pair [4,5]. Genome sequence analyses on various mushroom species reveal detailed information on the structures of the *B* mating-type loci. The *B* mating-type locus of *Schizophyllum commune* is composed of linked *Bα* and *Bβ* subloci in 6.1 kb distance, each of which contains a single pheromone receptor gene (*bar3* and *bbr2*, respectively) plus 16 pheromone genes [4]. *S. commune* also has 4 non-mating-type receptor genes (*brl*1–4), *brl*1–3 in the *B* mating-type locus, and *brl*4 in the different scaffold. A recent study has shown their functions in mating (Brl1), hyphal growth (Brl2), and dikaryotic asymmetrical growth (Brl3 and Brl4) [5]. Different from *S. commune*, PR-a and PR-b subloci in the *B* mating-type of *Flammulina velutipes* are located very far from each other (181 kb) [6]. PR-a and PR-b consist of

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STE3-like receptor gene and one and two pheromone genes, respectively. The *B* mating-type locus in *Coprinopsis cinerea* spreads on 35 kb chromosomal region where three subloci, consisting of three variants of *STE3*-like receptor with 2–4 pheromone genes, are present [7–9].

The *B* mating-type locus of *Lentinula edodes*, a popular edible mushroom species in East Asia, is consisted of closely linked *Bα* and *Bβ* subloci, each of which contains a pair of pheromone gene and a *STE3*-type pheromone receptor gene [10]. Our previous investigation on *L. edodes* has revealed that the *B* mating-type diversity was generated by combinations of 5 allelic variants of pheromone receptors (*RCB1*s) from *Bα* sublocus and 3 variants of pheromone receptors (*RCB2*s) from *Bβ* sublocus together with 15 mating pheromones (*PHB*s), resulting in 15 *B* mating-types [11]. Similar *B* mating-type diversities were discovered from *C. cinerea* (79 *B* mating-types) and *S. commune* (81 *B* mating-types) [7,12–15]. Considering the diversity of the pheromones and receptors, the selective recognition of pheromones by receptors is an important process in determining mating compatibility. The pheromone-receptor specificity of *L. edodes* was demonstrated by using a yeast model system using synthetic pheromones [16,17]. It was suggested that a certain PHB recognizes a specific partner Rcb from the same sublocus of different *B* mating-type as a means of non-self recognition in the mating of *L. edodes* [16,17].

In addition to the *B* mating-type locus, a new mating pheromone-receptor gene pair was found in *L. edodes* at a distance of 43.3 kb from the *B* mating-type locus. The presence of this gene pair has drawn our attention because of its uniqueness among mushroom species. Here, we report detailed gene structures in comparison with *PHB*s and *RCB*s from the *B* mating-type locus. We also report the specificity of the new pheromone receptor to the mating pheromones using the yeast model system, and we propose the biological role for this new gene pair in the regulation of *A* mating-type gene expression.

2. Materials and methods

2.1. Strains and culture conditions

A total of 26 strains of *L. edodes* (9 wild and 17 cultivated strains), which were described in our previous study [11], were subjected to the current analysis. *L. edodes* was cultivated in potato dextrose broth (PDB; Oxoid, Hampshire, UK) or on potato dextrose agar (PDA; Oxoid) at 25 °C. *S. cerevisiae* RCY1432 (W303a *sst2Δ::HIS3*, gpa_{Le}*::TRP1*) [17] was a host strain to construct RCBN-expressing model yeast. The yeasts were grown in yeast extract-peptone-dextrose medium (YPD; 1% yeast extract, 2% peptone, 2% glucose) at 30 °C.

2.2. Sequence analysis

The genomic sequence information of *L. edodes* B17 [18] was obtained from MycoCosm site (Joint Genome Institute, US Department of Energy). The *Bα-N* sublocus was identified by BLASTp analysis using *L. edodes* Rcb1 protein as a query sequence. Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) was employed for the multiple sequence comparison. A phylogenetic tree was constructed using MEGA7 (Maximum Likelihood method, 1000 repeats of bootstrapping) [19]. The transmembrane domain in the mating pheromone receptor protein was predicted by the Phobius program (https://phobius.sbc.su.se/).

2.3. Isolation of the RCBN gene and construction of yeast strains

L. edodes was grown in a PDB medium with gentle shaking for 10 d at 25 °C. The mycelia were collected by filtration with Miracloth (Merck, Darmstadt, Germany). The collected mycelia were ground in liquid nitrogen. The mycelial powder was subjected to total RNA extraction using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Reverse transcriptase-PCR (RT-PCR) was performed to generate cDNA for *RCBN* using a HiPi RT-Prime Kit (ELPIS, Daejeon, Korea). The *RCBN* amplicon was obtained by PCR using the cDNA and a specific primer set (Supplementary Table S1). The PCR conditions were as follows: hold for 5 min at 95 °C; 25 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 60 s; and 5 min at 72 °C for a final extension.

For the pheromone-RcbN interaction study, *S. cerevisiae* RCY1467 (*W303a, sst2Δ::HIS3, gpaLe::TRP1, ste2Δ::KanMX6*$^{RCB-N}$) was generated by the integration of *L. edodes RCBN* into the *STE2* site of *S. cerevisiae* RCY1432 (*W303a, sst2Δ::HIS3, gpaLe::TRP1*). The integration procedure is shown in Supplementary Figure S1. Yeast transformation was performed by standard PEG transformation procedure and the transformant was selected on YPD medium containing G-418 (200 μg/ml; Sigma-Aldrich, St. Louis, MO).

2.4. Interaction of PHBN1 or PHBN2 with RcbN

Two pheromone peptides (PHBN1: EHDSEATADTGFC-OMe and PHBN2: EHTDESGSTADTGFC-OMe) with C-terminal methyl esterification were synthesized through a

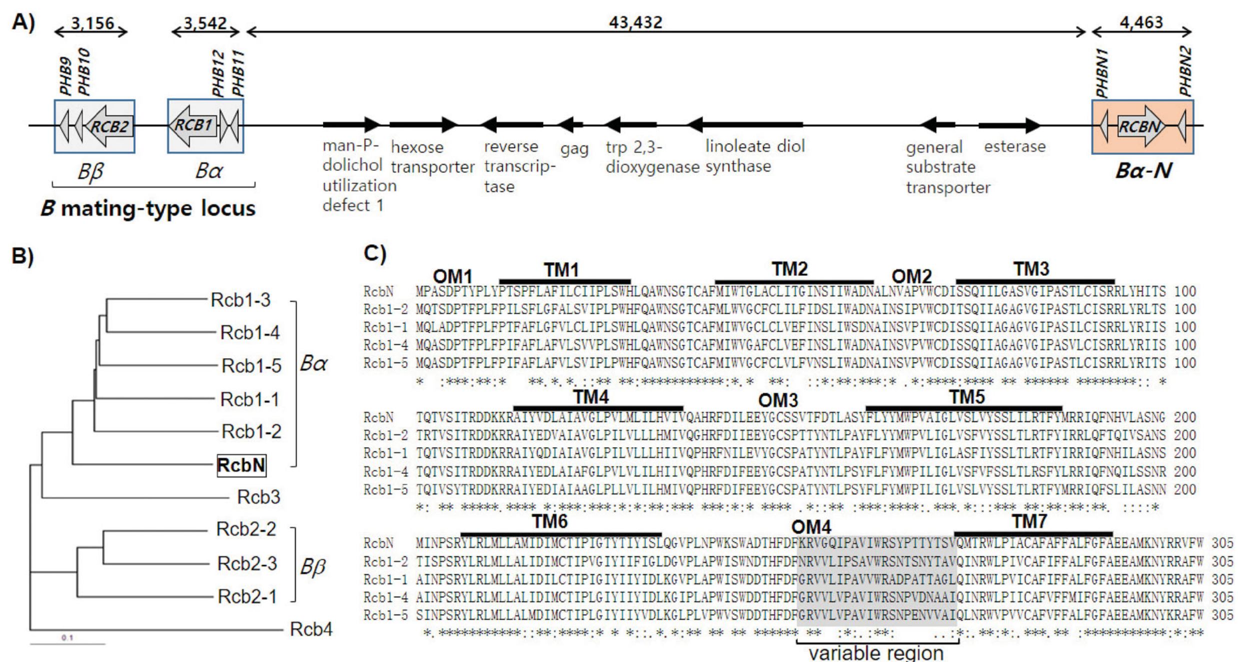

Figure 1. Sequence analysis the *Bα-N* locus in *Lentinula edodes*. (A) Genetic structure of the *B* mating-type locus and the *Bα-N* locus. The sequence was retrieved from the genome sequence of *L. edodes* B17 strains (Scaffold #5: 937,000–997,000). The numbers indicate the DNA length in bps; (B) Phylogenetic analysis of the mating pheromone receptor proteins in *L. edodes*. RcbN in the *Bα-N* locus is belonged to the *Bα* family mating pheromone receptors; (C) Topology of the *Bα* family mating pheromone receptors predicted by Phobius program. Transmembrane domains and outer membrane domains are numbered after TM and OM prefixes, respectively. The variable regions in the OM4 domains are shown in gray box.

commercial service (Genscript, Piscataway, NJ). The physical interaction between pheromones and RcbN was monitored through the level of *FUS1* gene expression in *S. cerevisiae* RCY1467. For this, PHBN1 or PHBN2 was treated for 6 h to yeast cells grown in YPD (OD600 = 0.6) at the concentration of 20 μg/ml. After the treatment, the yeast cells were collected by centrifugation at 3,500 rpm for 10 min and the *FUS1* gene expression was investigated by qRT-PCR using the previously described procedure [17]. The *FUS1* expression was determined using *β-tubulin* as a reference gene. All experiments were triplicated.

The effect of PHBN1 or PHBN2 on the expression of mating-related genes in *L. edodes* was also investigated by the treatment of the pheromones in 20 μg/ml to the *L. edodes* SJ701-M1 strain which was grown in PDB for 10 d. The expression levels of *clp1*, *priA*, *znf2*, *HD1*, and *HD2* were determined as previously described method [17].

3. Results

3.1. Structure analysis of the new mating pheromone and receptor pair

The genome sequence analysis revealed a novel pheromone-receptor gene set 43.3 kb away from the *B* mating-type locus in the genomic scaffold No. 5 of *L. edodes* B17 (Figure 1(A)). The new locus consisted of a *STE3*-type receptor gene (*RCBN*) and two pheromone genes (*PHBN1* and *PHBN2*). Eight genes separated the new locus from the *B* mating-type locus, including mannitol phosphate dolichol utilization defect 1, hexose transporter, and a putative retrotransposon, containing genes for reverse transcriptase (RTase) and gag protein. RcbN, a potential pheromone receptor protein, was homologous to Rcb1 family membrane receptors, which are mating pheromone receptors found from the *Bα* sublocus of *L. edodes* (Figure 1(B)). RcbN was a transmembrane protein containing 7 transmembrane domains (TMs), 4 outer membrane domains (OMs), and 4 cytoplasmic domains (CMs), according to a domain analysis of RcbN and Rcb1 proteins (Figure 1(C)). Because the other OM domains were substantially homologous to all Rcb1 proteins, a variable region discovered in OM4 (Figure 1(C), gray box in OM4) was expected to play a crucial role in the pheromone-specific interaction.

The two pheromones, PHBN1 and PHBN2, belonged to the *L. edodes* mating pheromone Group I and Group II, respectively, which had previously been referred to as the *Bα* mating pheromones (Figure 2(A)) [11]. Both pheromones had a CAAX (C, cysteine; A, aliphatic residue; X, any amino acid) motif at the C-terminus (Figure 2(B)), suggesting that they, like yeast a-factor and *L. edodes* mating pheromones, are membrane-bound pheromones through the C-term farnecylation [20,21]. Because the organization of Group I and/or Group II pheromones with an *RCB1* receptor is a characteristic

Figure 2. Characterization of the mating pheromones (PHBs) of *Lentinula edodes*. (A) Phylogenetic analysis of PHBs from the *B* mating-type and the *Bα-N* loci. PHBN1 and PHBN2 are belonged to the mating pheromone Group I and Group II, respectively; (B) Sequence comparison of PHB polypeptides. Maturation of PHBs occurs by proteolytic cleavage (indicated by arrow) and C-terminal modifications at the CaaX motif (highlighted in red).

genetic structure of the *Bα* sublocus, we named the novel locus as the *Bα-N* locus.

3.2. Analysis of the Bα-N locus in different strains of L. edodes

The *Bα-N* locus in 9 wild strains and 17 cultivated strains of *L. edodes* was examined using the primer set specific for the *Bα-N* locus (Supplementary Table S1). Unlike the *Bα* sublocus, which consists of allelic variants of *RCB1* with different sets of mating pheromone genes depending on the mating-type of the strains [11], the *Bα-N* sequence was nearly identical across all strains, with only a few minor changes (Supplementary Data S1), allowing single primer sets to detect *PHBN1*, *PHBN2*, and *RCBN* (Figure 3(A)). To verify the functionality of the *Bα-N* sublocus, expression of *RCBN* in *L. edodes* SJ701-M1 strain, which contains *RCB1-2* in the *Bα* sublocus and *RCB2-1* in the *Bβ* sublocus [11], was analyzed by quantitative RT-PCR (qRT-PCR). The qRT-PCR analysis revealed that *RCBN* was expressed 1.5 fold higher and 0.5 fold lower than *RCB1-2* and *RCB2-1*, respectively (Figure 3(B)), suggesting that the *Bα-N* sublocus is functional in *L. edodes* although whose detailed function is yet to be verified.

3.3. Analysis of pheromone–receptor interaction in yeast model system

The biological role of *Bα-N* was assessed through the investigation of RcbN-pheromone interaction with the *L. edodes* mating pheromones. To this end, a yeast model system was constructed by replacing the *S. cerevisiae* mating pheromone receptor (*STE2*) with *L. edodes RCBN* (Supplementary Figure S1, strain RCY1467), similar to our previous report [17]. The C-term carboxymethylated synthetic pheromones, including the *Bα*-specific pheromones

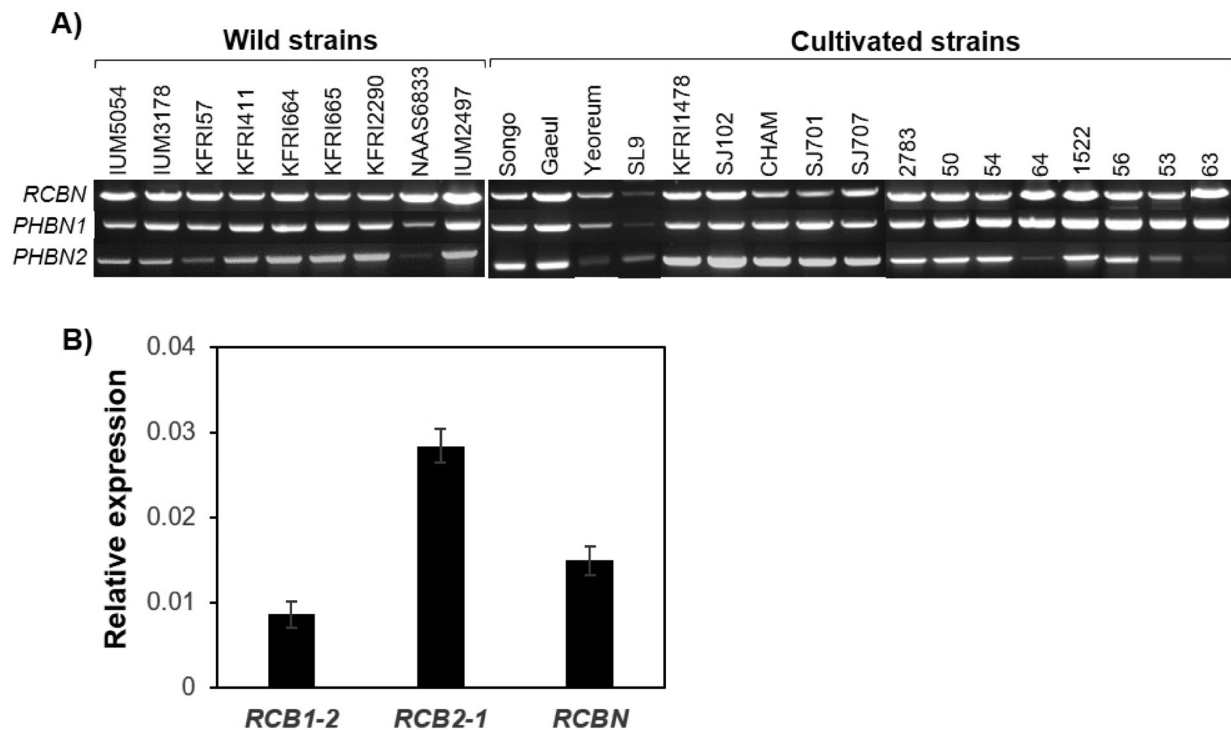

Figure 3. Detection of the *Bα-N* locus in various strains of *Lentinula edodes* and expression analysis of the mating pheromone receptor genes. (A) PCR detection of RCBN, PHBN1, and PHBN2 in the cultivated and wild strains of *L. edodes*; (B) qRT-PCR analysis of *RCB1-2* (*Bα* sublocus), *RCB2-1* (*Bβ* sublocus), and *RCBN* (*Bα-N* locus) in *L. edodes* SJ701-M1. The relative expression levels of the mating pheromone receptor genes were calculated by comparing them to the expression amount of β-tubulin. The error bars are standard errors derived from the triplicated experiments.

(PHB1, PHB2, PHB5, PHB6, PHB8, and PHB11–14), the *Bβ*-specific pheromones (PHB4, PHB7, PHB9, and PHB10), and the *Bα-N* pheromones (PHBN1 and PHBN2), were treated to actively growing cells of RCY1467 (OD600 = 0.5) for 2 h and then the *FUS1* expression was examined as a measure of pheromone-receptor interaction. The yeast cells did not respond to the mating pheromones from the *Bα*- and *Bβ*-specific pheromones, as shown in Figure 4(A). PHBN1 or PHBN2 treatments, on the other hand, resulted in 2.5- and 2.7-fold increased *FUS1* expression, respectively, suggesting the physical interaction between RcbN and PHBN1 or PHBN2 (Figure 4(A)).

3.4. Mating gene expression in L. edodes by Bα-N pheromones

Since the physical interaction between RcbN and PHBN1/PHBN2 was confirmed by the yeast model system, we investigated the expression of genes known to be responsive to the mating pathway activation in *L. edodes*. Synthetic PHBN1 or PHBN2 (20 µg/ml) was treated to the culture medium in which the monokaryotic *L. edodes* SJ701-M1 strain was grown for 3 d. The total RNA extracted from the mycelial cells was subjected to qRT-PCR analysis using specific primer sets targeting *clp1*, *znf2*, *HD1*, *HD2*, and *priA*. The qRT-PCR analysis revealed that the mating-related genes were mostly unresponsive upon the treatment of PHBN1 or PHBN2, except for *HD1* and *HD2* (Figure 4(B)). *HD1* and *HD2* are the two genes constituting the *A* mating-type locus and are known to encode heterodimeric protein complex to control *A*-related mating process as a transcription factor [22].

4. Discussion

The *B* mating-type locus of basidiomycetes consists of mating pheromone(s) and pheromone receptors in pairs. The pheromone and receptor pair can occur at a single locus as shown in *Pleurotus eryngii* [23]. However, the *B* mating-type locus is normally composed of multiple pheromone and receptor pairs, although the numbers are differed depending on fungal species. *S. commune* has two subloci at a close distance, each of which contains a receptor and multiple pheromone genes whereas *C. cinerea* has 3–4 mating-type specific subloci, which are spread on 35 kb chromosomal region [1]. The subloci are not necessarily located at a close distance. The two subloci of *F. velutipes B* mating-type locus are separated by 181 kb distance [6].

L. edodes has been reported to carry two tightly linked mating pheromone (*PHB*)-receptor (*RCB*) gene pairs in the *B* mating-type locus, constituting the *Bα* and *Bβ* subloci [11]. The *B* mating-type diversity is achieved through allelic variations in *RCB1* of *Bα* and *RCB2* of *Bβ*. Reciprocal interaction

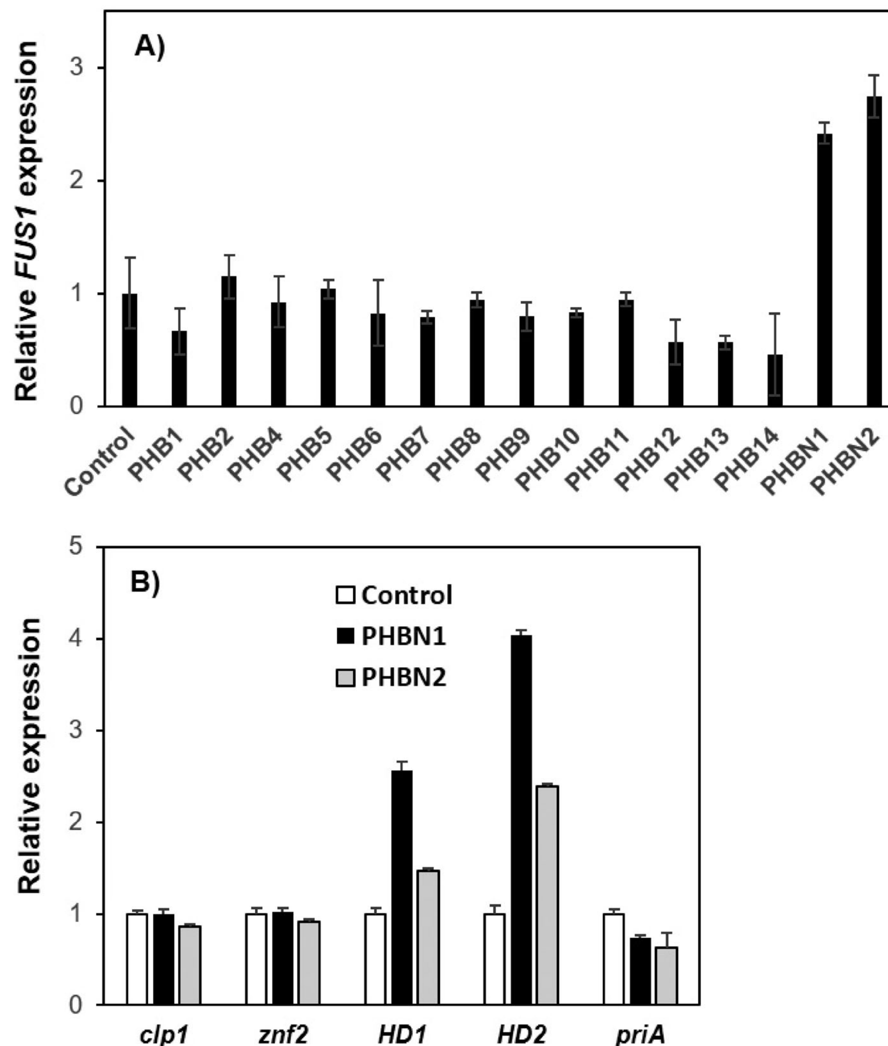

Figure 4. Physical interaction of the mating pheromones with RcbN. (A) PHB-RcbN interaction in yeast model system. *Saccharomyces cerevisiae* RCY1467, replacing *STE2* with *Lentinula edodes RCBN*, was treated with the *B* mating-type pheromones (PHB1–14) and the *Bα-N* pheromones (PHBN1 and PHBN2) in 20 µg/ml. The interaction between PHB and RcbN was determined by the expression of *FUS1* gene as a measure of the activation of the yeast mating signal transduction pathway; (B) Expression of the mating-related genes in *L. edodes* SJ701-M1 upon the treatment of PHBN1 or PHBN2 in 20 µg/ml. The error bars are standard errors derived from the triplicated experiments.

between PHB from a mating partner and Rcb from the other partner of the same sublocus can activate the whole mating process [11]. Besides the *B* mating-type locus, we found a new *PHB-RCB* pair (*PHBN1*, *PHBN2*, and *RCBN*) at a distance of 43 kb in the same genomic scaffold. Different from the *PHB*s and *RCB*s in the *B* mating-type locus, there was no allelic variation in *PHBN1, PHBN2,* and *RCBN* as revealed by sequencing the *Bα-N* sublocus of 26 strains. It appeared that *Bα-N* is an outcome of paralogous expansion of ancestral *Bα* sublocus, since RcbN1 was homologous to Rcb1 and both of the pheromones belonged to the *Bα* mating pheromone family. The presence of RTase and gag genes in between the *B* mating-type locus and the *Bα-N* sublocus implies the possible role of retrotransposon in this gene expansion.

The biological function of the *Bα-N* sublocus was assessed using a yeast model system and synthetic pheromones. RcbN interacts with self-pheromones PHBN1 and PHBN2, rather than pheromones from the *B* mating-type locus (Figure 4). The increased expression of two mating-related genes in *L. edodes* after treatment with PHBN1 and PHBN2 further validated this interaction. Notably, *HD1* and *HD2*, which make up the *A* mating-type locus, were the two genes that responded to PHBN1 and PHBN2. The *A* and *B* mating-type loci are known to be involved in the mating process independently [1], but there are no studies on how the *A* mating-type locus is activated. In this regard, our findings suggest that *Bα-N* activates the *A* mating-type locus independently of the *B* mating pheromone-receptor interaction.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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