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Molecular analysis of *B* mating type diversity in *Lentinula edodes*

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ABSTRACT

Pheromone and pheromone receptor genes in *B* mating type locus constitute *B* mating type of fungi belonging to Basidiomycota. In *Lentinula edodes*, multiple *B* mating types have been suggested based on genome sequence analysis and mating assay. Here we report finding of new alleles of pheromones (*phbs*) and pheromone receptors (*rcbs*) to constitute five alleles of *rcb1* (*rcb1-1*∼*rcb1-5*) with nine associated *phbs* in *Bα* sublocus and three alleles of *rcb2* (*rcb2-1*∼*rcb2-3*) with five associated *phbs* in *Bβ* sublocus. Each *rcb* was primarily associated with two *phbs*. Each *phb-rcb* pair was recurrently discovered as a distinct unit in various monokaryotic and dikaryotic strains, regardless of subloci. This allowed us to propose 15 *B* mating types through combinations of five *Bα* and three *Bβ* subloci. PCR analyses with primer sets probing alleles of *rcb1* and *rcb2* demonstrated that all monokaryotic strains contained one of the five *rcb1*s and one of the three *rcb2*s representing *Bα* and *Bβ* subloci, respectively, thereby enabling designation of their *B* mating types via combinations of *Bα* and *Bβ*. PCR analyses also revealed the presence of one or two alleles of *rcb1* and *rcb2* representing *B* mating type of each nuclei in dikaryotic cytoplasm. Further investigation of 111 dikaryotic strains, including 83 cultivated and 28 wild strains collected from East Asia, revealed that *B8* and *B11* mating types constructed by *rcb1-3* + *rcb2-2* and *rcb1-4* + *rcb2-2*, respectively, were more prevalent in cultivated strains. In *B* mating type pairs representing each nucleus in dikaryons, *B2B12*, *B4B8*, and *B8B11* were frequently occurring pairs in cultivated strains while none of them was found in wild strains. Such prevalence indicates that certain nuclei have been preferentially selected in the generation of cultivated strains. Our findings shed new light on the construction of *B* mating type in *L. edodes* and provide practical tools in the breeding of new cultivars.

1. Introduction

Filamentous fungi belonging to Basidiomycota spend most of their life cycle as mycelia with one (monokaryon) or two nuclei (dikaryon) in the cytoplasm. For sexual reproduction, monokaryotic mycelia will mate with compatible partners to form fused mycelia which maintain two compatible nuclei inside cells. In fused mycelial cells, these two nuclei undergo mitotic division. Divided nuclei then move to next cells through clamp connections to establish dikaryons throughout connected mycelial cells (Kües, 2000). Dikaryotic mycelia can occasionally form fruiting bodies with basidia as sexual organs upon physico-chemical signals. In the basidium of heterothallic basidiomycetes, nuclear fusion (karyogamy) followed by meiotic division produces four sexual spores (basidiospores), resulting in monokaryotic mycelia upon germination (Kües, 2000).

Self or non-self recognition in sexual reproduction is enabled by two unlinked mating type loci, *A* and *B* (Bakkeren et al., 2008; Casselton and Olesnicky, 1998). The *A* mating type locus encodes two homeodomain proteins (HDs). Each HD proteins forms heterodimeric transcription factor with HD protein from compatible partner to control mating events, including nuclear pairing, clamp cell formation, and coordination of nuclear division (Casselton and Olesnicky, 1998). The *B* mating type locus is consisted of pheromone and pheromone receptor genes (Kües, 2015). Mating pheromones in basidiomycetes contain CaaX motif, implying their localizations to cell membrane through acylation at the cysteine residue (Huyer et al., 2006; Michaelis and Barrowman, 2012), similar to a-factor of ascomycete fungi (Caldwell et al., 1995). Pheromone receptors in basidiomycetes are homologous to Ste3p (a-factor receptor) of yeasts (Kües, 2015). Pheromone and receptor interaction is thought to activate the mating pathway involving MAP kinase-dependent signal transduction (Casselton and Olesnicky, 1998; Raudaskoski et al., 2012).

Both mating types can be multiple to increase chances of inter-strain mating in nature. Multiplicity of mating types arises from allelic

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variations of genes residing in multiple subloci constituting both *A* and *B* mating type loci. *Coprinopsis cinerea* has *a*, *b*, and *d* subloci in the archetypal *A* mating type locus and Groups *1*, *2*, and *3* subloci in the *B* mating type locus consisted of *HD1-HD2* gene pairs and pheromone-receptor pairs, respectively (Riquelme et al., 2005; Kües, 2015). Allelic variations in genes at each sublocus together with combinations of subloci have generated more than 240 *A* and 70 or 79 *B* mating types (Riquelme et al., 2005; O'Shea et al., 1998). Similarly, it has been reported that *Schizophyllum commune* contains 288 *A* and 81 *B* mating types (Raper et al., 1958; Kües, 2015).

Mating behavior of *Lentinula edodes* has been of our interest because of its practical importance in the mushroom industry (Ha et al., 2015). Similar to *C. cinerea* and *S. commune*, *L. edodes* carries multiallelic *A* and *B* mating types. Through mating analyses of wild populations, Tokimoto et al. (1973) have suggested 40-65 *A*s and 63–100 *B*s from 33 Japanese wild strains. Later, Lin et al. (2003) have reported 66 *A*s and 72 *B*s (predicted to be 121 *A* and 151 *B*) from 53 Chinese wild strains. However, these numbers have inherent uncertainty because they have been mainly estimated by mating analyses that rely on empirical observations such as the presence of clamp connections and the formation of barrage lines as measures of successful mating. To establish more reliable method for estimating mating types, we have focused on allelic variations in pheromone and receptor to see if we can employ them to verify *B* mating types. Using similar approach, we have recently reported variable sequence regions in *A* mating type loci of *L. edodes* that represent different mating type alleles (Ha et al., 2018).

Genome analyses of three monokaryotic strains (939 P26, 939 P42, and SUP2) of *L. edodes* have revealed that the genetic structure of *B* mating type locus is more complex than that of *A* mating type locus (Wu et al., 2013). Each *B* mating type locus consists of two mating type-specific receptors (*rcb*s), one or two pheromone genes (*phb*s), and two non-mating type *rcb*s. The whole gene-set in this locus lies in a single chromosome with an approximate length of 37 Kbp. Mating type-specific receptors *rcb1* and *rcb2* are found together with their associated *phb*s whereas non-mating type receptors (*rcb3* and *rcb4*) are devoid of associated *phb* gene. *rcb1* and *rcb2* with their coupled *phb*s reside in subloci *Bα* and *Bβ*, respectively, to constitute the *B* mating type locus. Recent genome analysis of *L. edodes* strain W1-26 has revealed the same *Bα* and *Bβ* subloci as strain 939 P42, with an additional sublocus at approximately 30 Kbp downstream from the *Bα* sublocus that contains two *phb*s (*le_pp4* and *le_pp5*) and an *rcb* (*le_pr5*) (Chen et al., 2016).

rcb1 and *rcb2* contain significant amounts of allelic variations, suggesting a multiallelic nature of the *B* mating type. In strain 939 P26, the *Bα* sublocus is comprised of *phb5*, *phb6*, and *rcb1*-939 P26 (*rcb1-2*), while the *Bβ* sublocus contains *phb7*, *phb4*, and *rcb2*-939 P26 (*rcb2-1*). Similarly, strain SUP2 carries *phb1*, *phb2*, and *rcb1*-SUP2 (*rcb1-1*) in the *Bα* sublocus and *phb3*, *phb4*, and *rcb2*-SUP2 (*rcb2-2*) in the *Bβ* sublocus. Each mating type-specific receptor pairs with two pheromone genes except *rcb1*-939 P42 (*rcb1-3*) of strain 939 P42 which has only one pheromone gene *phb8* in the *Bα* sublocus whereas *rcb2*-939 P42 (*rcb2-2*) retains *phb3* and *phb4* in the *Bβ* sublocus (Wu et al., 2013). The pheromone gene names published as "*Lephbx*" (Wu et al., 2013) are simplified to *phbx* in this study. The pheromone receptor genes published as "*Lercb1* or *2*-strain name" are also renamed to *rcb1-x* or *rcb2-x* to better represent the allelic variations.

Based on these previous findings, we explored the diversity of *L. edodes B* mating type at molecular level using sequence information of *rcb*s and *phb*s from 111 strains, including 83 cultivated and 28 wild strains, collected from East Asia.

2. Materials and methods

2.1. Strains, culture conditions, and mating assay

A total of 111 strains of *L. edodes* were obtained from various collections, including Culture Collection and DNA Bank of Mushroom (CCDBM, University of Incheon, Korea), Korea Forest Research Institute (KFRI, Korea), National Institute of Agricultural Sciences (NAAS, Korea), and Forest Mushroom Research Center (FMRC, Korea) (Supplementary Table S1). Of these 111 strains, 83 were cultivated strains collected from East Asian countries, including 18, 20, and 45 strains from China, Japan, and Korea, respectively, and 28 were wild strains collected from mountainous areas in southern part of Korean peninsula. Mycelia of *L. edodes* were grown on potato dextrose agar (PDA, Oxoid, UK) or in potato dextrose broth (PDB, Difco, USA) at 25 °C. Mating analysis and genomic DNA extraction were performed as described previously (Ha et al., 2018).

2.2. PCR analysis

For sequence determination of pheromone and receptor genes, primer sets specific for *phb*s and *rcb*s were designed using consensus sequence regions (Supplementary Table S2). PCR was performed with the following conditions: 94 °C for 5 min; 30 cycles of amplification reaction at 94 °C for 45 s 45 s, 60 °C for 30 s, and 72 °C for 30 ~ 120 s; and 72 °C for 5 min. PCR products were analyzed by agarose gel electrophoresis. Target DNA was extracted from agarose gel and then cloned into a TA vector as described previously (Ha et al., 2018). Sequences of *phb*s and *rcb*s were determined by sequencing 1st *phb* and 2nd *phb-rcb1* for *Bα* sublocus and *rcb2*-3rd *phb* and 4th *phb* for *Bβ* sublocus. Results are summarized in Supplementary Fig. S1. All sequences discovered in this study were deposited at Genbank (Supplementary Table S3).

2.3. Generation of monokaryon by de-dikaryotization

When necessary, monokaryotic strain was generated directly from a dikaryotic strain without making fruiting body using modified protocol of Miyazaki et al. (2000). In brief, mycelia cultured in mushroom complete medium (MCM, MB cell, USA) for 10 days were isolated using a filter paper (Grade 41, Whatman, UK) and subsequently washed twice with distilled water. Cell walls were disrupted in 0.6 M sucrose solution by treatment with a 2.5% a lysing enzyme solution (Sigma, USA) for 3 h at 25℃. Resulting protoplasts were filtered through Miracloth (Calbiochem, USA). The filtrate was then centrifuged at 1000 rpm for 1 min. Protoplasts were washed twice with 0.6 M sucrose solution and suspended in washing solution followed by spreading onto MCM agar containing 0.6 M sucrose. Agar plate was incubated for one week. Monokaryotic mycelia devoid of clamp connections were isolated under a light microscope.

2.4. Gene and protein analyses

Exon structures of *rcb* genes were analyzed using FGENESH with gene-finding parameters specific for *Moniliophthora* (Salamov and Solovyev, 2000). Transmembrane domain in RCB was predicted with Phobius software (http://phobius.sbc.su.se/, Käll et al., 2004). Phylogenetic trees for RCBs and PHBs were constructed by Maximum Likelihood method with 1000 repeats of bootstrapping using MEGA7 (Kumar et al., 2016).

3. Results

3.1. Identification of alleles of phb and rcb in B mating type locus

Analysis of recently published genome sequence of CHAM-B17 strain (Shim et al., 2016), a monokaryotic strain derived from basidiospore of *L. edodes* CHAM strain (Ha et al., 2015), revealed that the *B* mating type locus consisted of *phb11*, *phb12*, and *rcb1-4* in the *Bα* sublocus and *phb9*, *phb10*, and *rcb2-3* in the *Bβ* sublocus (Fig. 1A). Comparison of this new *B* mating type with other three known *B* mating types (Wu et al., 2013) showed that each mating type-specific *rcb* paired