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Research Article

Nucleus-Selective Expression of Laccase Genes in the Dikaryotic Strain of *Lentinula edodes*

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Abstract In mating of *Lentinula edodes*, dikaryotic strains generated from certain monokaryotic strains such as the B2 used in this study tend to show better quality of fruiting bodies regardless of the mated monokaryotic strains. Unlike B2, dikaryotic strains generated from B16 generally show low yields, with deformed or underdeveloped fruiting bodies. This indicates that the two nuclei in the cytoplasm do not contribute equally to the physiology of dikaryotic *L. edodes*, suggesting an expression bias in the allelic genes of the two nuclei. To understand the role of each nucleus in dikaryotic strains, we investigated single nucleotide polymorphisms (SNPs) in laccase genes of monokaryotic strains to reveal nuclear origin of the expressed mRNAs in dikaryotic strain. We performed reverse transcription PCR (RT-PCR) analysis using total RNAs extracted from dikaryotic strains (A5B2, A18B2, and A2B16) as well as from compatible monokaryotic strains (A5, A18, and B2 for A5B2 and A18B2; A2 and B16 for A2B16). RT-PCR results revealed that Lcc1, Lcc2, Lcc4, Lcc7, and Lcc10 were the mainly expressed laccase genes in the *L. edodes* genome. To determine the nuclear origin of these laccase genes, the genomic DNA sequences in monokaryotic strains were analyzed, thereby revealing five SNPs in Lcc4 and two in Lcc7. Subsequent sequence analysis of laccase mRNAs expressed in dikaryotic strains revealed that these were almost exclusively expressed from B2-originated nuclei in A5B2 and A18B2 whereas B16 nucleus did not contribute to laccase expression in A2B16 strain. This suggests that B2 nucleus dominates the expression of allelic genes, thereby governing the physiology of dikaryons.

Keywords Dominance, Expression, Laccase, Lentinula, Nucleus

Vegetative cells of basidiomycetes contain two nuclei of different origins in a common cytoplasm. In their natural habitats, hyphae of basidiomycetes grow radially and meet with neighboring hyphae to form a complex network (known as mycelium) through hyphal anastomosis. Dikaryotic cells of vegetative mycelia are generated by mating monokaryotic mycelia derived from haploid basidiospores. Formation of dikaryotic mycelia through mating, which is governed by mating type genes [1, 2], involves multiple complicated processes, including recognition of partner haploid cells, establishment of dikaryotic cells by cell fusion, compatibility check of two nuclei, and nuclear division and transfer through clamp connection [3]. Many of these processes have not been described in detail yet.

Lentinula edodes is a fungal species belonging to Basidiomycota. In nature, it degrades the main components of wood such as cellulose, hemicellulose, and lignin through activities of extracellular enzymes such as cellulase, hemicellulase, lignin peroxidase, and laccase [4-6]. It is one of the representative white-rot fungi. Its fruiting body is a popular edible mushroom grown worldwide. In the life cycle of *L. edodes*, diploid basidia (2n) are formed on the back of pileus as a means of sexual reproduction. Each basidium produces four basidiospores (n) through meiosis. Basidiospores germinate upon environmental stimuli. Germinated haploid cells then undergo a series of mitotic cell division and septum formation that eventually grow into monokaryotic mycelium [3]. In sexual reproduction, monokaryotic mycelia mate with a compatible mating type to form dikaryotic cell (n+n). Subsequently, two nuclei in the common cytoplasm divide independently. The newly formed nuclei then move to neighboring cells through clamp connections to establish dikaryon throughout the

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connected mycelial cells.

An interesting question raised in the process of dikaryogenesis is whether one of the nuclei is dominant in allelic gene expression, since the cytoplasm of mated cell contains both the nuclei independently upon the establishment of dikaryon. Regarding this issue, *Neurospora tetrasperma*, a filamentous ascomycete, has shown biased expression in alleles of genes residing in the two nuclei, with unequal nuclear distribution depending on the developmental stage [7]. This indicates that the nuclei in the common cytoplasm might cooperate in the expression of allelic genes for optimal survival under changing environmental conditions. Moreover, our previous mating experiments have hinted that dikaryotic strains, derived from a certain monokaryotic strain, consistently show better fruiting rate with higher production yield regardless of its partner monokaryotic strains, whereas some monokaryotic strains would yield dikaryotic strains with low fruiting rates, deformed fruiting bodies, and/or low production yields [8]. This suggests that, between the two independent nuclei in the dikaryotic cytoplasm, a nucleus might be dominant over the other, to govern physiological characteristics of the mushroom.

To test this possibility, we determined single nucleotide polymorphisms (SNPs) of allelic genes in monokaryotic strains and investigated the origin of SNPs in mRNA sequences of corresponding genes expressed in dikaryotic strains. Expression of laccase genes was especially of our interest due to their multiple roles in mushroom development and the presence of multiple paralogous genes.

MATERIALS AND METHODS

Strains and culture conditions. Monokaryotic and dikaryotic strains of *L. edodes* 'Chamaram' isolated previously were used for this study [8]. Mushroom strains were cultured in potato dextrose broth (PDB; Vent Tech Bio Co., Eumseong, Korea) or on potato dextrose agar (Oxoid Ltd., Hampshire, UK) at 25°C. Monokaryotic strains A2, A5, A9, A15, and A18 were mated with partner monokaryotic strains B2, B5, and B16 to generate dikaryotic strains A5B2, A15B5, A18B2, A2B16, and A9B16. Monokaryotic B strains were generated by chemical mutagenesis of basidiospores collected from the fruiting body whereas A strains were from the wild type basidiospores of dikaryotic *L. edodes* 'Chamaram' strain [8].

Extracellular enzyme activity assay. Mycelia were cultured in PDB for 2 wk at 25°C. Culture supernatant was collected by centrifugation at 3,000 ×g for 10 min. For laccase activity assay, 20 μL of the culture supernatant was added to 1 mL of a substrate solution containing 2 mM 2′,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS; Sigma-Aldrich, St. Louis, MO, USA) in 50 mM sodium succinate buffer (pH 4.5). The reaction mixture was incubated at 30°C for 5 min. ABTS oxidation was estimated by measuring absorbance at 420 nm. Manganese peroxidase activity was measured using the same substrate solution with additional 0.1 mM $MnSO_4$ and 0.1 mM H_2O_2. One unit of enzyme activity was defined as the amount of enzyme that produces 1 μmol ABTS ($\varepsilon_{420nm} = 3.6 \times 10^4$/M/cm) per minute. Cellulase activity was measured using 5%

Table 1. List of primers used in this study

Gene name	Forward primer (5′→3′)	Reverse primer (5′→3′)
For laccase gene amplification in the genomic DNA		
Lcc1	CAGGAAACAAGCCTTGTG	AGCTCAACTGGATGACCG
Lcc2	CGGTCATCAATGTACAGC	CCAACGCCACTACTAGAC
Lcc3	GTCCTTCGATGCCTTCCA	TGCACCTACGTACCGAAG
Lcc4	CAATGCCGATCCCGTAAC	CCGGAAGAAGGTTCTGTG
Lcc5	GCCTACGGGGTCTGTTTA	AGTGAAGGAACCAAGGAC
Lcc6	GTTATCGAAGTTGACGGT	GACTGGTTTCGAGTAAAG
Lcc7	CTCTCGAACCATTCTTGG	GGTTCCTGTAATAAGTGG
Lcc8	CATGCGTATGAGTACAGC	ACATCATACAAGTCGGCC
Lcc9	GGTATGCCCACATCACCA	CGTTTAACTCCTCATCTGC
Lcc10	CGCAACAGTATGCCGATA	CTTCCGAAATTTGACCACC
Lcc11	CGAGGATTTCAAGTATCCG	TCCAAAGGGTCTATTCCC
Lcc12	CTTACATCTCACCAACCC	GTAGTGTTTCCACCGTTG
Lcc13	GATACAAAGGTGCGCCTG	CGTTGAAGTCGTCAGCAC
Lcc14	GCGAACGGGACATATCCT	GTACCAGAATGTACCGGC
For RT-PCR analysis		
Lcc1	ATGTTTTACTTCTCATCTTTCCTGCT	TTAATTTCCACCAAGTTGTGCAGG
Lcc2	ATGCTTCCCTTCGTTTCTCTTC	TCAAGGTAATTGAGCAGGGGTT
Lcc4	ATGCGTCTACTCTTGACTTC	TCAAAGCTGGTCGGGCT
Lcc7	ATGTTCAAGATCAAATTTGCTCTCG	TCAAGGCAGTTGATCAGGAGT
β-Tubulin	TCAGGTGCCGGTATGGGT	ACTACCAGTACTGTCAGCCAT
GAPDH	CTTCGATTCTAAATACCCTCC	GTAAGTACAACAGGATTTGAGC

RT-PCR, reverse transcription PCR; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

(w/v) cellulose (Sigma-Aldrich) in 50 mL sodium acetate (pH 5.0) as a substrate solution. Culture supernatant (0.15 mL) was mixed with 0.5 mL of the substrate solution and the mixture was incubated at 37°C for 2 hr under agitation at 120 rpm. The reaction mixture was centrifuged at 15,000 ×g for 10 min and the glucose concentration in the supernatant was measured using Glucose Assay Kit (Sigma-Aldrich).

Genomic DNA extraction and PCR conditions. *L. edodes* was grown in PDB for 14 days at 25°C. Mycelia were frozen in liquid nitrogen and ground with a mortar and pestle. Genomic DNA was extracted from the mycelial powder using Genomic DNA Prep Kit (BIOFACT Co., Daejeon, Korea). Genomic DNA was subjected to PCR for the amplification of laccase genes using primer sets shown in Table 1. PCR was performed using a PCR premix (Maxime PCR premix kit, iNtRON Biotechnology, Eumseong, Korea) with following conditions: 94°C for 5 min, followed by 25 cycles of 94°C for 30 sec, 57°C for 30 sec, and 72°C for 1 min, and final 72°C for 10 min. The amplified DNA bands were cloned using a TA cloning kit (pGEM-Teasy vector, Promega, Madison, WI, USA) and their sequences were determined.

For analysis of mRNA sequence variations, total RNA was extracted from the mycelial powder aforementioned using an RNA extraction kit (RNA-spin, iNtRON Biotechnology) Reverse transcription PCR (RT-PCR) was performed using one step RT-PCR kit (Maxime RT-PCR premix kit; iNtRON Biotechnology) with the primer sets listed in Table 1. The amplified cDNAs were cloned into a TA vector (pGEM-T Easy vector, Promega) for sequence analysis.

RESULTS AND DISCUSSION

Extracellular enzyme activities of monokaryotic and dikaryotic strains. *L. edodes* produces various extracellular enzymes including laccase and manganese peroxidase (MnP) for the oxidative degradation of lignin [5, 6] and cellulase for the hydrolysis of cellulose [4]. In this study, we tried to explain the effect of monokaryotic strain on the expression of genes in dikaryotic strain by investigating the wood decomposing enzyme activities of dikaryotic strains and their compatible monokaryotic strains. Since dikaryotic cells have twice as many genes as monokaryotic cells, their enzyme activities are expected to be twice of those of monokaryotic cells, provided that the enzyme genes are co-expressed in both the nuclei of dikaryotic cells. However,

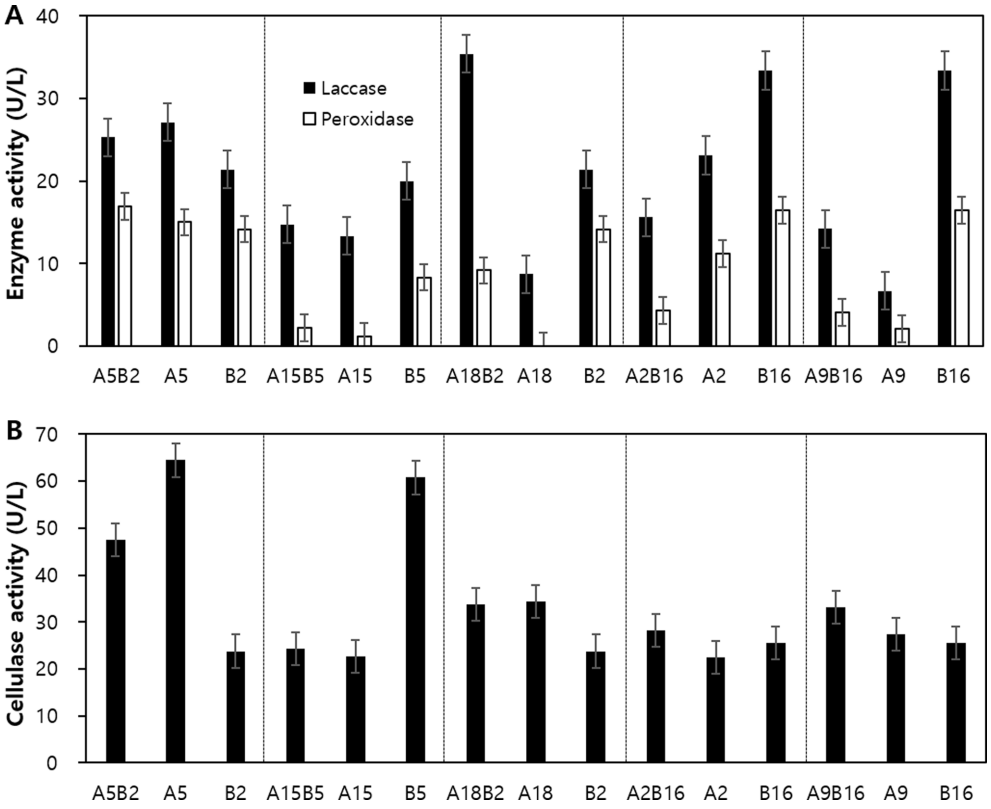

Fig. 1. Extracellular enzyme activities of monokaryotic or dikaryotic strains of *Lentinula edodes*. A, Laccase and peroxidase activities; B, Cellulase activity. Mycelia of monokaryotic (A5, B2, A15, A18, etc.) or dikaryotic (A5B2, A15B5, A18B2, etc.) strains were culture in potato dextrose broth for 2 wk. The enzyme activity in the culture broth was determined using ABTS or cellulose for laccase and peroxidase or cellulase activities, respectively. Each sample was tested in triplicate, and the average was used for comparison.

laccase, peroxidase, or cellulase activities in the culture broth of dikaryotic mycelia were neither synergistic nor additive to those in monokaryotic culture broth (Fig. 1). For example, laccase and peroxidase activities of dikaryotic strain A5B2 were similar to those of the corresponding monokaryotic strains A5 and B2 (Fig. 1A). On the contrary, cellulase activities of monokaryotic strains such as A5 and B5 were higher than those of corresponding dikaryotic strains A5B2 and A15B5, respectively (Fig. 1B). These data suggest the presence of a regulatory mechanism that controls the expression of allelic genes in the two independent nuclei in dikaryotic cytoplasm.

Wood-decaying enzyme activities are important for mushroom development in dikaryotic strains. As shown in Table 2, dikaryotic strains such as A5B2 and A18B2 (containing B2 nucleus in common) showed better production yields with bigger fruiting bodies whereas strains such as A9B16 and A2B16 (containing B16 nucleus in common) produced small underdeveloped fruiting bodies with poor commercial values. The major difference between these two groups of mushrooms was the degree of browning on sawdust substrate during mycelial development. Browning on substrate is an important process in the cultivation of *L. edodes*. It is positively correlated with production yield as well [9]. In a direct correlation with degree of browning, oxidative enzyme activities exhibited by laccase and peroxidase were higher in B2-containing strains than those in B16-containing dikaryotic strains (Table 2).

Laccase genes in the genome of *L. edodes*. White-rot fungi contain more than 10 laccase genes generated by paralogous expansion of ancestral gene [10]. In line with this, *L. edodes* also contains 14 paralogs of laccase genes (Lcc1–Lcc14) based on our recent genome analysis [11]. PCR analyses of these laccase genes in monokaryotic A5, A18, and B2 strains and dikaryotic A5B2 and A18B2 strains detected the presence of all 14 laccase genes regardless of monokaryotic or dikaryotic strains (Fig. 2A). Sequence comparison of these 14 laccase genes with the three most-expressed laccase genes (PO Lacc5, PO Lacc6, and PO

Table 2. Effect of monokaryotic strains on the fruiting body quality and production yield of *Lentinula edodes*

Mono A	Mono B	Total weight (g)[a]	Pileus × Stipe (cm)	Extracellular enzyme activity (U/L)			Degree of browning[b]
				Laccase	Peroxidase	Cellulase	
A5	B2	123	6.0 × 3.5	25.3	16.9	47.5	++++
A18		176	5.0 × 3.7	35.4	9.2	33.8	++++
A9	B16	40	2.0 × 4.9	14.2	4.1	33.2	+
A2		16	2.2 × 5.1	15.6	4.3	28.2	++

[a]Total weight of fruiting bodies after third round of harvest.
[b]Degree of browning was estimated after 90 days of cultivation in sawdust medium.

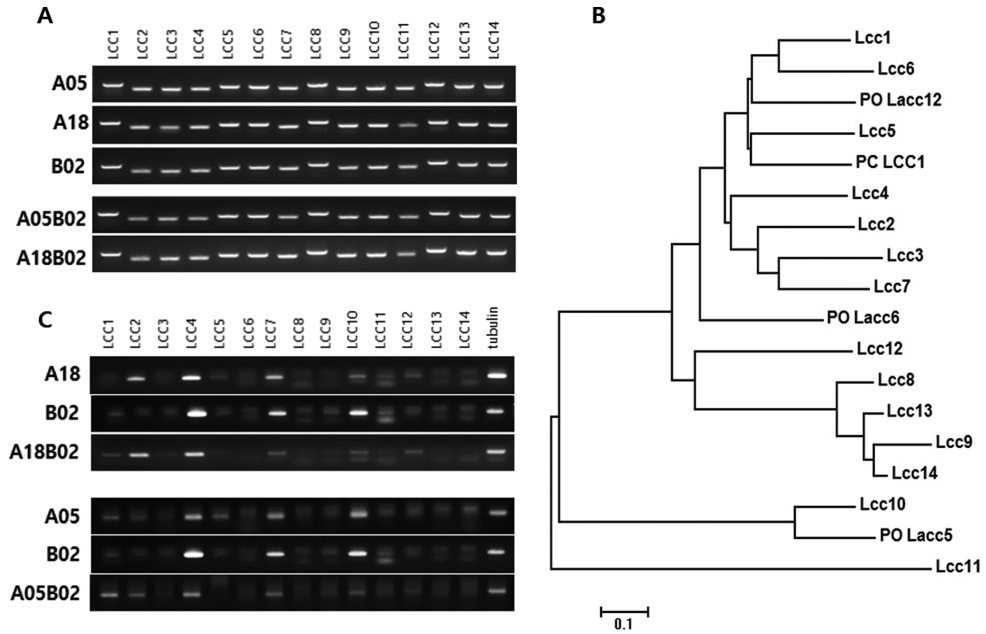

Fig. 2. Analysis of laccase genes in *Lentinula edodes*. A, Laccase genes in the genomes of monokaryotic or dikaryotic strains; B, Analysis of phylogenetic relationship for laccase genes. PO Lacc and PC LCC are some representative laccase genes in *Pleurotus ostreatus* and *Pycnoporus coccineus*, respectively; C, Analysis of laccase mRNA expression.

Lacc12) in *Pleurotus ostreatus* [12] and that expressed mainly in *Pycnoporus coccineus* (PC-LCC1) [13] revealed that laccase genes Lcc1–Lcc7 of *L. edodes* were grouped with PO Lacc6 (a major laccase expressed throughout developmental stages of *Pleurotus ostreatus* [12]) and PC LCC1 (the only secreted laccase in *Pycnoporus coccineus* [13]) (Fig. 2B). By analogy, this group of laccase genes (Lcc1–Lcc7) was expected to be the major expressed laccases in *L. edodes*. Lcc10 of *L. edodes* was similar to PO-Lacc5, a fruiting body-specific laccase in *Pleurotus ostreatus* [12], implying its role in fruiting stage.

Expression of laccase genes. To assess the impact of each monokaryotic strain on the expression of laccase genes in the dikaryotic strain, two dikaryotic strains, A5B2 and A18B2, were cultured along with their monokaryotic strains, A18, A5, and B2 in sawdust-containing liquid medium. RT-PCR analysis, using total RNAs extracted from cultured mycelia, revealed Lcc1, Lcc2, Lcc4, and Lcc7 as the major laccase genes expressed in both dikaryotic strains (Fig. 2C). This was expected, based on their sequence similarities with PO-Lacc6 and PC-LCC1 in *Pleurotus ostreatus* and *Pycnoporus coccineus*, respectively. Lcc12 was additionally expressed in A18B2. In the A18 strain, one of the monokaryotic strains for A18B2, five laccase genes (Lcc2, Lcc4, Lcc7, Lcc10, and Lcc12) were distinctly expressed along with minor expression of Lcc5 and Lcc11. In contrast to A18 strain, B2 monokaryotic strain showed distinct expression of Lcc4, Lcc7, and Lcc10 with minor expression of Lcc1. Lcc12 expressed in A18B2 was conceivably due to A18 strain, because it was not expressed in B2, although B2 strain contributed to the expression of Lcc1 in A18B2. Laccase expression in A5 strain was similar to that of B2 strain, except for minor expression of Lcc2 and Lcc5.

It has been reported that, out of the 12 laccase genes, *Pleurotus ostreatus* can selectively express Lacc5, Lacc6, and Lacc12 depending on the developmental stage [12]. *L. edodes,* in this study, showed that only half of paralogous laccase genes were expressed in the sawdust-containing medium. These observations suggest that the expression of laccase gene is controlled by various physico-chemical signals, as previously shown by the sole expression of LCC1 upon adding copper ion in *Pycnoporus coccineus* in an extreme case [13].

These extracellular enzyme activity data implied a possible interaction between the two nuclei in dikaryotic cell for the production of enzyme. In line with activity data, mRNA expression levels in dikaryotic cell were not the sum of those in monokaryotic strains (Fig. 2C). These results suggest that expression of genes in the two separate nuclei is not independently regulated; rather influenced by each other in dikaryotic cells.

Nucleus-selective expression of laccase genes in dikaryotic cells. One interesting question arising from the above-mentioned extracellular enzyme activity data is whether the homologous gene present in the two independent nuclei has biased expression in one nucleus or common expression in both. To understand gene expression regulation in dikaryotic cells of *L. edodes*, we first analyzed the sequence polymorphisms in the major expressed laccase

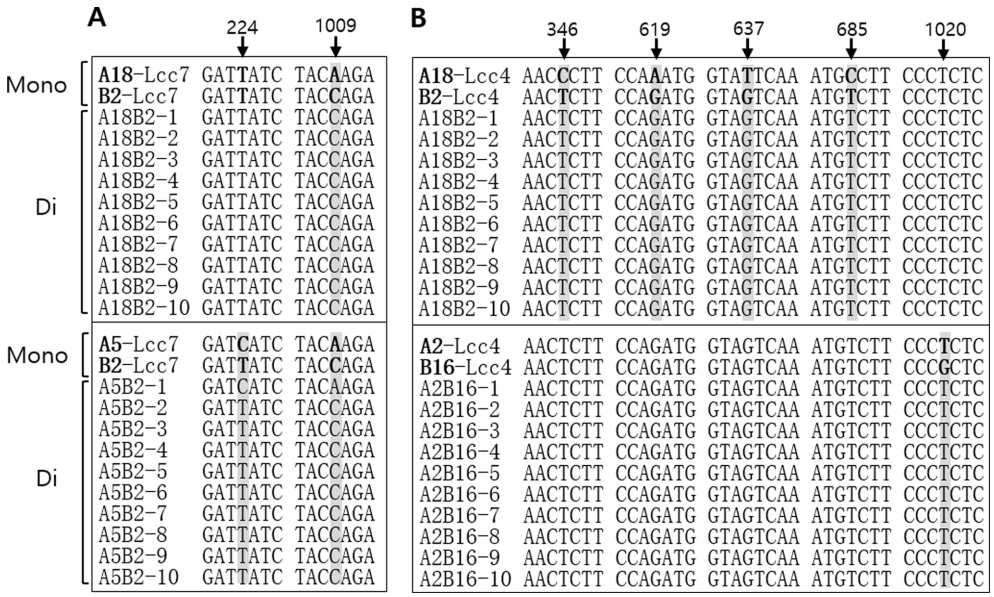

Fig. 3. Nuclear origin of the expressed laccase genes in dikaryotic strains. A, Single nucleotide polymorphism (SNP) analysis of Lcc7 in the mRNA expressed in dikaryotic strains A18B2 and A5B2; B, SNP analysis of Lcc4 in dikaryotic strains A18B2 and A2B16. SNPs in the laccase genes from monokaryotic strains are shown in boldface. Numbers with arrows indicate the position of SNPs. 'Mono' and 'Di' indicate monokaryotic strains and dikaryotic strains, respectively. SNP regions are shaded.

genes such as Lcc1, Lcc4, and Lcc7 in monokaryotic strains A18, A5, A2, B2, and B16. Studies on sequence polymorphisms in mushrooms are rare. However, a recent study on monokaryotic strains of *Tricholoma matsutake* has shown the presence of two SNPs in a 431-bp single copy DNA segment [14]. Through sequence analysis, we found five SNPs in Lcc4 and two SNPs in Lcc7. However, no SNP in Lcc1 was found (Fig. 3). Lcc7 in A18B2 strain can be verified by a single SNP at position 1,009 where the A18 nucleus carries 'A' while the B2 nucleus carries 'C' (Fig. 3A). Similarly, Lcc7 in A5B2 strain can be verified by two SNPs at positions 224 and 1,009. Lcc4 in A18B2 strain carries four SNPs while that in A2B16 strain carries a single SNP at position 1,020 (Fig. 3B). However, no SNP was found in A5B2 strain for Lcc4.

SNPs in each laccase gene in monokaryotic strains were employed to investigate the contribution of each nucleus in dikaryotic strain gene expression. For this, cDNAs of laccase genes were synthesized using mRNAs extracted from A18B2, A5B2, and A2B16 strains. cDNAs were cloned into TA vector. Ten TA clones per gene were randomly selected for sequence determination. cDNA sequences were compared with those from monokaryotic strains. As shown in Fig. 3, cDNA sequences for Lcc7 from A18B2 and A5B2 strains contained SNPs found only in B2 strain (with only one exception for A5B2-1), indicating that Lcc7 was preferentially expressed from the B2 nucleus. This was also true for the expression of Lcc4 in A18B2 strain. Interestingly, Lcc4 in A2B16 strain was exclusively expressed from the A2 nucleus (Fig. 3B). These results reflect a strong preference for the B2 nucleus in laccase gene expression in dikaryotic strains. Expression of these laccases might be related to the degree of browning, thus affecting the quality of fruiting body (Table 2). Dominant expression of laccase genes may explain the effect of B2 monokaryotic strain on phenotypic characteristics of dikaryotic strain, at least partially. In conclusion, we discovered that allelic genes in dikaryotic strains have an expression bias that can govern the phenotype of basidiomycetes. Practically, this paper demonstrates the importance of selecting monokaryotic strain during breeding of mushroom to achieve better quality of fruiting bodies.

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