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FULL PAPER

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Isolation of the *B* incompatibility factor mutants in *Pleurotus ostreatus*

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Abstract *B* incompatibility factor mutants (*Bmut*) in *Pleurotus ostreatus* were recovered from common-*B* mating heterokaryons resulted from matings between wild-type monokaryons with different *A* but the same *B* factors (*A1B2* and *A2B2*) after NTG mutagenesis. The mutant monokaryons such as *A1B2mut* and *A2B2mut* were observed to have regularly uninucleated hyphal cells and to be compatible with each other. Matings between *A1B2mut* and *A2B2mut* monokaryons produced stable heterokaryons (*A1B2mut* + *A2B2mut*) that had binucleated hyphal cells with true clamp connections and formed normal fruit-bodies. Mating tests using basidiospore progeny from each of these heterokaryons revealed the bipolar mating pattern. Genetic analysis suggested that the mutation of *B* factor in *P. ostreatus* might occur in the *B* incompatibility factor genes.

Key words *B* incompatibility factor · Mutagenesis · *Pleurotus ostreatus*

Introduction

Pleurotus ostreatus (Jacq.: Fr.) Kummer is an excellent edible mushroom widely cultivated in many countries of the world. As in most hymenomycetes, the mating system of this fungus, leading to the formation of a dikaryon that can differentiate to produce fruiting bodies, is known to be bifactorial heterothallism (tetrapolality) controlled by two unlinked multialletic factors, *A* and *B* (Terakawa 1960; Eguenio and Anderson 1968; Anderson et al. 1991).

In tetrapolar hymenomycetes, the regulatory role of the *A* and *B* incompatibility factors in developmental processes during dikaryotization is evident from genetic studies on mutation in either or both of the two factors (Raper 1966; Casselton 1978). Mutants in *A* and *B* factor genes (*Amut* and *Bmut*) have been isolated so far in *Shizophyllum commune* Fr. (Raper et al. 1965; Koltin and Raper 1966; Koltin and Flexer 1969; Koltin 1970), *Coprinus cinereus* (Schaeff.: Fr.) S.F. Gray (Haylock et al. 1980; Swamy et al. 1984), *C. lagopus* (Fr.) Fr. (Parag 1962; Day 1963), and *Lentinula edodes* (Berk.) Pegler (Murakami and Hasebe 2000). *A* factor mutations result in constitutive expression of *A* factor-regulated development in monokaryons, and their phenotype is similar to that of common-*B* heterokaryons showing conjugate nuclear division and pseudoclamp formation (Day 1963). On the other hand, monokaryons with mutated *B* factor genes exhibit behavior similar to common-*A* heterokaryons showing septum breakdown and nuclear migration (Parag 1962; Koltin and Flexer 1969). Monokaryons mutated in both *A* and *B* factors (*Amut Bmut*) convert to sexually fertile homodikaryons in terms of function and morphology resembling dikaryons (Raper et al. 1965; Koltin 1970; Swamy et al. 1984).

These incompatibility factor mutants are also very useful materials for elucidating the construction and function of mating type genes. Recent molecular studies on the loci of *A* and *B* factors in *S. commune* and *C. cinereus* have revealed that *A* factor genes code for homeodomain proteins functioning as transcription factors and *B* factor genes code pheromone and pheromone receptors (Casselton and Kues 1994; Kothe 1999). Particularly, the *Amut Bmut* double mutant strain has been utilized to generate various recessive developmental mutants available for analysis of the mechanism of fruit-body formation in *C. cinereus* (Kanda and Ishikawa 1986; Kanda et al. 1989). The present article describes the isolation of the *B* incompatibility factor mutants in *P. ostreatus* and their genetic and cytological properties.

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Materials and methods

A wild-type dikaryon TMIC-4127 (*A1B1* + *A2B2*) was used in this study. Many single spore monokaryons were isolated from this dikaryon and maintained on a malt extract agar medium [MA medium, composed of 2% malt extract (Difco, Detroit, MI, USA) and 2% agar (Wako, Tokyo, Japan)]. Mycelial culture was carried out in plastic Petri dishes (Ina Optika, Ina, Japan; 90 × 15 mm) containing 24 ml MA. Mating types of these monokaryons were analyzed, and each four monokaryons with *A1B2* (No. 8, No. 24, No. 59, No. 63) or *A2B2* (No. 5, No. 9, No. 27, No. 39) were used to construct common-*B* mating cultures. Mating of monokaryons was performed by placing two small plugs of mycelia carring *A1B2* and *A2B2*, 10 mm apart, on the periphery of the MA plates. Four matings with the same combination were carried out on one plate. Ten plate cultures were performed for each of 16 cross-combinations. After 10 days of incubation, 0.25 ml *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG) solution (4.8 mg/ml) was poured into a hole (diameter, 10 mm) that had been opened in the center of the MA plates using a cork borer. The plate cultures were incubated at 25°C in the dark. After 2 months incubation, 12 small mycelial plugs were randomly transferred from each of the mating colonies to new MA plates. After several days, these colonies were examined under a light microscope for the presence of clamp connections. For microscopy of nuclei in isolates, slide cultures were performed as follows. A small piece of mycelium was inoculated on a thin film of MA medium on a sterilized glass slide. The glass slides were placed in a sterilized Petri dish containing wet filter paper that then was sealed with Parafilm (American National Can, Neenah, WI, USA). The cultures were incubated at 25°C in the dark for 3–7 days. The mycelia growing on the slides were fixed with an ethanol : acetic acid (3 : 1) solution for 30 min at 4°C. The nuclei of hyphal cells fixed were stained with 4′,6-diamidino-2-phenylindole (DAPI) dissolved in NS buffer (Kuroiwa and Suzuki 1980), and observed under an Olympus BH-2 fluorescence microscope with a UV excitation apparatus.

Results and discussion

Of a total of 640 common-*B* mating cultures thus treated, 21 of isolates were found to produce dikaryons having apparently true clamp connections (Table 1), indicating the presence of the mutated *B2* factor. All these dikaryons was similar in mycelial growth to the parental dikaryon on MA medium and produced normal fruit-bodies on a sawdust medium (SD medium; sawdust of *Fagus crenata* Blume : rice bran, 3 : 1 in volume, moisture content 65%). To further examine the possibility of the occurrence of *B2* factor mutation in these dikaryons, basidiospore progeny (*Sp.*) from respective fruit-bodies of three randomly selected dikaryons [(24 × 5) D10-4, (24 × 9) D7-8, and (59 × 5) D7-3] were subjected to mating type analysis. As shown in Table 2, the analysis of *B* factor specificity in all progenies revealed that there was a new *B* factor, namely, mutated *B2* factor (*B2mut*), which was compatible with the original *B2* factor. Also, because four mating types (*A1B2mut*, *A2B2mut*, *A1B2*, and *A2B2*) appeared in every progeny analyzed, the *B2* factor mutation was considered to occur in one of the two nuclei of the monokaryons used for mating. It can be concluded that, for *P. ostreatus*, mutation of the *B* incompatibility factor was efficiently induced from common-*B* heterokaryons by mutagenesis treatment, as reported for other tetrapolar hymenomycetes (Parag 1962; Koltin and Raper 1966; Haylock et al. 1980; Swamy et al. 1984; Murakami and Hasebe 2000).

Table 1. Recovery from common-*B* heterokaryons treated with NTG

Stock no. of monokaryon, *A2B2*	Stock no. of monokaryon, *A1B2*			
	No. 8	No. 24	No. 59	No. 63
No. 5	+ (3)	+ (3)	+ (4)	+ (1)
No. 9	−	+ (3)	−	+ (2)
No. 27	+ (1)	+ (1)	+ (1)	+ (1)
No. 39	−	+ (1)	−	−

+ and − indicate presence and absence of clamps, respectively; number of isolations is in parentheses
NTG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine

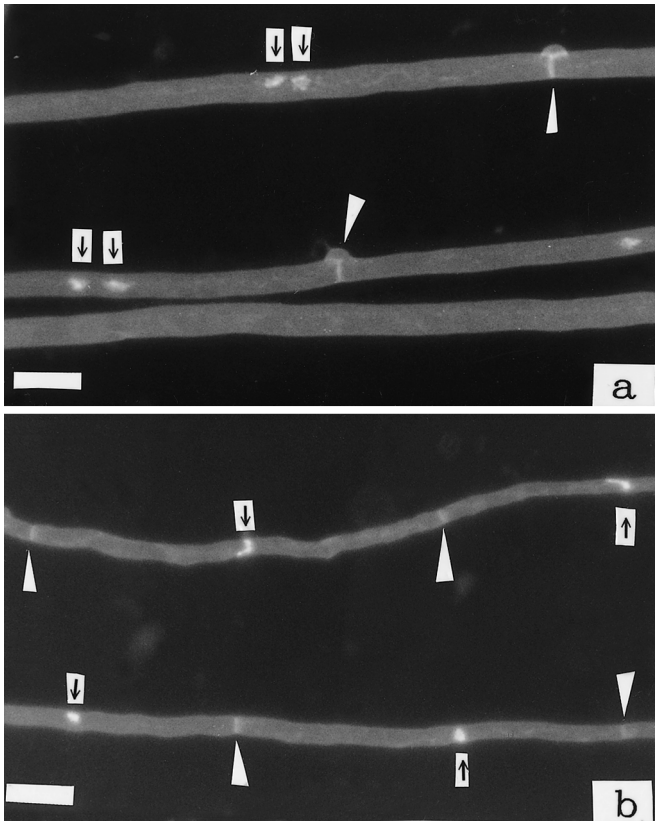

Fig. 1. Fluorescent photo micrographs of mycelia of *Sp*. 18 × *Sp*. 16; *A1B2mut* + *A2B2mut* dikaryon (**a**) and (24×5) D10-4*Sp*. 18; *A1B2mut-1* monokaryon (**b**). *Arrows*, nuclei; *arrowheads*, clamp (**a**) and septum (**b**). *Bar* 10 μm

Table 2. Mating type analysis of basidiospore progenies of three mutated dikaryons resulting from common-*B* heterokaryons after NTG mutagenesis

Mutated dikaryons[a]	Total no. of single spore isolates analyzed	Segregation in progeny					
		A1Bmut	*A1B2*	*A2Bmut*	*A2B2*	χ^2 (1:1:1:1)	*P*
(24 × 5) D10-4	84	25	26	14	19	4.48	0.30–0.20
(24 × 9) D7-8	90	14	28	23	25	7.78	0.10–0.05
(59 × 5) D7-3	93	14	28	21	30	6.83	0.10–0.05

[a] The parentheses and following numbers show parental monokaryons (*A1B2* × *A2B2*) and isolates, respectively

Table 3. Segregation of mating types of basidiospore progenies derived from the crosses between *A1B2mut-1*, *A2B2mut-1*, and wild-type monokaryons

Crosses[a]	Segregation in progeny					
	A1B1	*A1Bmut*	*A2B1*	*A2Bmut*		*P*
Sp. 18 × *Sp*. 16; *A1B2mut-1* × *A2B2mut-1*		96		104	χ^2 (1:1) = 0.32	0.70–0.50
Sp. 18 × No. 38; *A1B2mut-1* × *A2B1*	52	47	51	37	χ^2 (1:1:1:1) = 3.01	0.50–0.30
Sp. 16 × No. 16; *A2B2mut-1* × *A1B1*	37	47	49	40	χ^2 (1:1:1:1) = 2.24	0.70–0.50

[a] *Sp*. 18 and *Sp*. 16 are spore isolates derived from a mutated dikaryon, (24 × 5) D10-4
No. 38 and No. 16 are incompatibility factor tester strains derived fom a wild-type strain, TMIC-4127

Table 4. Mating pattern analysis of 20 basidiospore progeny from the cross between *A1B2mut-1* and *A2B2mut-1* monokaryons from a mutated dikaryon, (24 × 5) D10-4

		A1B2mut-1								*A2B2mut-1*											
		2	3	4	6	13	14	17	18	1	5	7	8	9	10	11	12	15	16	19	20
A1B2mut-1	2	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+
	3	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+
	4	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+
	6	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+
	13	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+
	14	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+
	17	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+
	18	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+
A2B2mut-1	1	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
	5	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
	7	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
	8	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
	9	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
	10	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
	11	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
	12	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
	15	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
	16	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
	19	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
	20	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−

+ and − indicate presence and absence of clamp connections, respectively

To examine the stability of the *B2* factor mutation, monokaryons with *A1B2mut* and *A2B2mut* mating types were serially transferred at 1-month intervals on MA medium. It was recognized that the monokaryons could maintain their mutation characteristic over 1 year. Newly established dikaryons between (24×5) D10-4*Sp*.16 (*A1B2mut-1*) and (24×5) D10-4*Sp*.18 (*A2B2mut-1*) monokaryons derived from a mutated dikaryon, (24×5) D10-4, had true clamp connections and binucleated hyphal cells (Fig. 1a) and formed normal fruit-bodies on SD medium. Using 20 basidiospores randomly isolated from the *A1B2mut-1* + *A2B2mut-1* dikaryon, their mating patterns were analyzed. As a result, their mating types were found to be grouped only into two types, *A1B2mut-1* and *A2B2mut-1*, indicating a bipolar mating a pattern (Tables 3,4). In addition, when the *Bmut* monokaryons were crossed to compatible monokaryons with different *B* factors, the *A* and *B* factors segregated into four mating types in approximately equal frequency, as expected from a normal meiosis (Table 3). Also, in every progeny tested, the *B* factors seg-

regated at a 1:1 ratio for *B2mut-1* and *B1*, and the original *B2* did not appear, suggesting that the mutation had likely occurred in *B* factor genes.

To evaluate the effect of *B* factor mutation on morphogenesis, the mutants were microscopically examined with respect to nuclear distribution and septum structure. The hyphal cells of *B2mut* monokaryons were predominately uninucleate and had an intact septum structure (Fig. 1b). This observation was similar to the findings in *C. cinereus* (Haylock et al. 1980; Swamy et al. 1984) and *L. edodes* (Murakami and Hasebe 2000) in which *Bmut* mycelia were uninucleate and exhibited no septal disruption. The observations in these fungi were apparently different from those in *S. commune*, where hyphal cells of the *Bmut* showed septal disruption and were predominantly multinucleate (Koltin and Flexer 1969; Raper 1966; Raudaskoski 1976, 1998). Moreover, upon mating to other compatible monokaryons, the *B* factor mutants of *P. ostreatus* were shown to be able to behave as both nuclear donor and recipient.

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