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Molecular Marker Related to Fruitbody Color of *Flammulina velutipes*

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White and brown strains of *Flammulina velutipes* were inter-crossed. All F_1 showed light-brown fruitbody, suggesting that a gene for the brown fruitbody was incompletely dominant against the white one. And backcross experiment showed that more than two genes were involved in color determination. To isolate a molecular marker linked to fruitbody color, a set of primers was designed from a sequence of clones derived by a bulked segregant analysis. These markers showed a specific band which co-segregated with brown fruitbody forming strains.

KEYWORDS: Backcross, Bulked segregant analysis, *Flammulina velutipes*, Molecular marker

Winter mushroom, *Flammulina velutipes*, has been grown commercially by mechanized bottle cultivation system under controlled environment condition since the late 1980's in Korea. Its production has been rapidly increasing, estimated at about 37,955 M/T, followed by oyster mushroom and oak mushroom (Anonymous, 2002). *F. velutipes* belongs to white rot fungi and forms clusters on the dead trunks or stumps of broad-leaved trees such as mulberry, persimmon and poplar trees in early spring or late autumn, in Korea. The fungus shows brown caps and dark brown stipes in wild habitat. Brown fruitbody was not popular in the market as consumers prefer the white ones. Therefore, it is important to develop new variants producing white fruitbody in cultivation. However, basic studies on the inheritance of fruitbody color are lacking, although detailed information on these aspects is crucial to effective mushroom breeding. The isolation of molecular markers linked to genes related to fruitbody color can be achieved by random amplification of polymorphic DNA sequences (Williams *et al.*, 1990) and by using the bulked segregant analysis (Michelmore *et al.*, 1991) to backcross lines of fruitbody color. The objective of this study was to elucidate the genetic and molecular basis for color determination of fruitbody in *F. velutipes*.

Materials and Methods

Strains, growth conditions and mating. Parental strains for the mating and genetic analysis of *F. velutipes* were ASI4019 producing brown fruitbodies in wild flora and a commercial strain ASI4045 producing white fruitbodies. Cultures were grown on potato dextrose agar (PDA) or mushroom complete medium at 25°C. Basidiospores were obtained from spore prints by serial dilution of basidiospores in distilled water. Spore suspension was plated in petri dishes containing approximately 20 *ml* PDA and incubated for three to six days at 25~28°C. Single colony was transferred to a new PDA plate and confirmed as monokaryon by observation of hyphae lacking clamp connection under the microscope. When two monokaryons were combined as a pair, each inoculum was placed about 10 mm apart from its neighbor. After 7~12 days of incubation at 25°C, the clamp connections which appeared in the mycelium from the contact zone between the two paired mycelia were examined to confirm dikaryotization.

Fruitbody formation. Dikaryotic strains obtained by mating between compatible monokaryons were grown on PDA and then the mycelia were transferred to sawdust medium. Mated dikaryon strains were subjected to a standard cultivation method mentioned in previous paper (Byun *et al.*, 1996).

Development isogenic lines. To determine the inheritance of fruitbody color between white commercial strains and brown wild strains, monokaryons were inter-mated. Monokaryons, 4019-15, 18 and 29 of wild brown strain were mated with a monokaryon 4045-9 of commercial white strain. Spores of F_1 were collected and monokaryons were germinated from the spore suspension. Ten monokaryons of each F_1 were isolated and mated with 4045-9 of white strain for making BC_1F_1. The same procedure was applied for BC_2F_1 : two brown isolates in each BC_1F_1 combination were selected and ten monokaryons derived from them were mated with 4045-9 again.

DNA extraction, PCR reaction and sequencing. For DNA isolation each strain was grown in 250 *ml* Erlenm-

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eyer flask containing 50 *ml* of potato dextrose medium for two weeks at 25°C. Mycelia were harvested by filtration through Whatman 3MM filter paper and ground in liquid nitrogen. DNA was purified from 2 g of mycelium employing the protocol described by Zolan *et al.* (1986) with minor modifications. General molecular biology protocols were employed as described by Sambrook *et al.* (1989). Polymerase Chain Reactions (PCRs) for the generation of Randomly Amplified Polymorphic DNA (RAPD) markers were performed as described by Williams *et al.* (1990). The oligonucleotides used as primers for the reaction were 10-mers belonging to the Operon series (Operon Tech. Inc.). Amplification reactions were performed in a GeneAmp 9600 (Perkin Elmer, Norwalk) with the following program : 1 min denaturation at 94°C, 1 min annealing at 37°C, 2 min extention at 72°C, for 40 cycles. Amplification products were analyzed by electrophoresis in 1.8% (w/v) agarose gels in TAE buffer (400 mM Tris, 200 mM sodium acetate, 20 mM EDTA, pH 8.3) and stained with ethidium bromide. As a molecular size marker 1 Kb DNA ladder was used. Appropriate RAPD bands were extracted from the agarose gels and cloned into pGEM-T (Promega, Southhampton, UK). In order to make a specific primer, the plasmid DNAs from these clones were prepared using plasmid purification kit (Promega) and sequenced using the ABI PRISM Big Dye terminator kit (PE Applied Biosystems) by 377 DNA sequencer.

Results and Discussion

Inheritance of fruitbody color and isolation of isogenic lines. To determine the inheritance of fruitbody color between white commercial strains and brown wild strains, monokaryons were inter-mated. Fruitbody color of three lines of F_1 resulted to light brown (Table 1, Fig. 1). This result means that brown is incompletely dominant to white. Ten monokaryons of each F_1 were isolated and

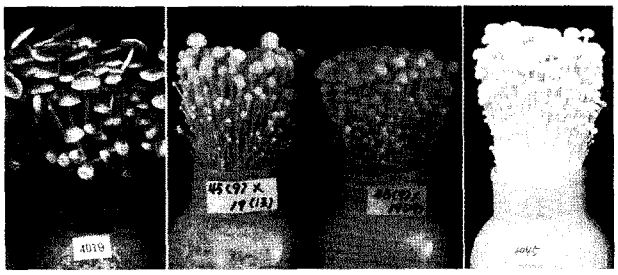

Fig. 1. Fruitbodies in the hybrid between monokaryons isolated from white strain ASI 4045 and brown strain ASI 4019.

mated with 4045-9 of white strain for making BC_1F_1. While F_1 produced light brown fruitbody in all combinations, backcross lines of BC_1F_1 with 4045-9 segregated in color. The ratio of white to brown showed 1:3 and this suggested at least two major genes related to fruitbody color (Table 1). In BC_1F_1, fruitbody production did not differ between white and brown lines. Out of 60 combinations in BC_2F_1, 15 failed to produce a fruitbody. The proportion of white fruitbody formation increased and their yields were lower than those in BC_1F_1 (Table 2), which may be due to heterosis.

White variety of commercial strains in Japan is originated by recessive mutation involving simultaneous inactivation of a series of complementary genes. Kinugawa (1993) suggested that color of mycelium and fruitbody could be controlled by one single major gene with unlinked multiple genes having additive effects. He showed that dark color and pale color gave F_1 progenies producing fruitbodies of intermediate color between two parents. Therefore he suggested a major gene F and recessive allele of f of a commercial vareity, Maruei. The genotype of f/F exhibited features which were intermediate between the f/f and F/F. However, degree of brown color was variable in brown fruitbody in this experiment and it may suggest that several genetic factors are involved. Bro-

Table 1. Genetic analysis of the BC_1F_1 progeny developed from crosses between white strain ASI 4045 and brown strain ASI 4019

Generation	Mating combination	No. of mating	Failure of fruiting	Color	
				White	Brown
Parent	4045			White	
	4019				Brown
F_1	4045-9×4019-15			Light brown	
	4045-9×4019-18			Light brown	
	4045-9×4019-29			Light brown	
BC_1F_1	4045-9×(4045-9×4019-15)	10	3	2	5
	4045-9×(4045-9×4019-18)	10	1	3	6
	4045-9×(4045-9×4019-29)	10	3	1	6
	Total	30	7	6	17
	x^2 = 1.2		P = 0.5~0.25		

Table 2. Distribution of fruitbody yield of different fruitbody color in BC_1F_1 and BC_2F_1 progeny developed from the intercrosses between white strain ASI 4045 and brown strain ASI 4019

Generation	Color	No. of mating	Range of yield (g/850 *ml*)			Mean
			18.5~51.5	51.5~84.5	84.5~117.5	
BC_1F_1	White	6	2	2	2	69±24.4
	Brown	17	2	10	5	70±24.2
	Total	23	4	12	7	69±23.7
Generation	Color	No. of mating	Range of yield (g/850 *ml*)			Mean
			19.5~59.5	59.5~99.5	99.5~139.5	
BC_2F_1	White	16	11	5		54±12.6
	Brown	29	6	14	9	84±30.4
	Total	45	17	19	9	73±29.0

die (1936) stated that the inheritance of the brown color of the mycelium was determined by two pairs of factors (R, r, V and v) which were quite independent of the two pairs of sex factors. The presence of either of the two dominant factors R and V causes a mycelium to possess a brown coloration, but R gives a more intense color than V : RV haplophytes are intense brown, Rv and rV haplophytes are light brown of different intensities, while rv haplophytes are pure white. Our genetic analysis suggests that there may be at least two nuclear genes that control the color of fruitbody. Among the color related genes, the gene on white color seems to be inherited as recessive factor and the gene on brown color is dominant. However, the grade of browning, from grayish white to dark brown in the intra-mating has been seen (Kong *et al.*, 1997b; Byun *et al.*, 1996). We suppose minor genes can affect intensity of fruitbody color. Further genetic analysis is required for the determination of fruitbody color between major and minor gene activity.

Development of molecular marker related to fruitbody color. To find a specific band linked to color, RAPD finger printings were carried out to BC_1F_1 progenies (Fig. 2). Even when they showed different band patterns according to primers, brown progenies had more polymorphic bands than white ones. But there was no distinct bands to related to color. BC_2F_1 progenies (Fig. 3) has a little variation, almost getting homogeneous where no bands related to color appeared. Bulked segregant analysis (Michelmore *et al.*, 1991) involves screening for differences between two pooled DNA samples derived from a segregating population that originated from a single cross. Each pool, or bulk, contains individuals selected to have identical genotypes for a particular genomic region (target locus or region). However, random genotypes at loci were unlinked to the selected region. Larraya *et al.* (1999) identified molecular markers linked to the A incompatibility locus in *Pleurotus ostreatus* by bulked segregant analysis.

Bulked segregant analyses were performed to identify molecular markers linked to fruitbody color in the BC_2F_1 progenies developed from the intercrosses between white strain ASI 4045 and brown strain ASI 4019. Six backcross lines were selected that did not segregate on color in their progenies. DNAs of progenies in a line were ex-

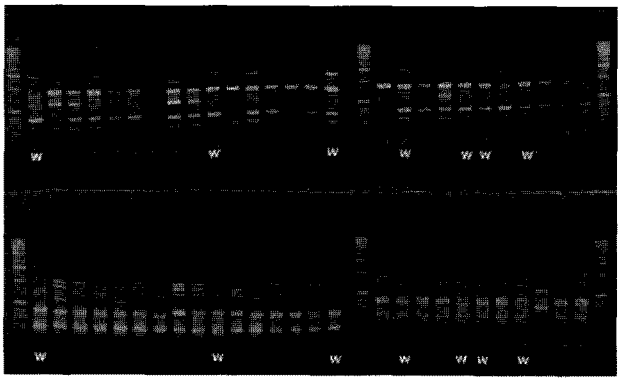

Fig. 2. RAPD fingerprints of BC_1F_1 progenies using OPAX-06 (A) and OPAX-12 (B) primers. W stands for BC_1F_1 progenies which have white fruitbody.

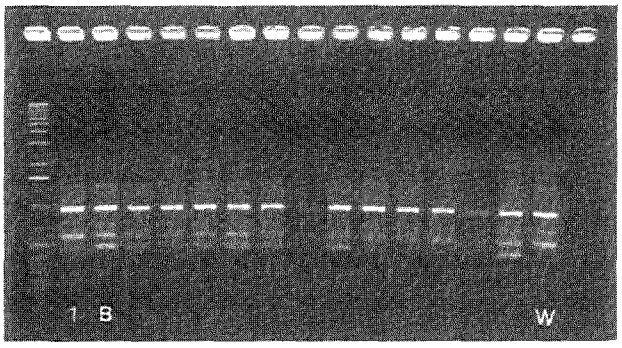

Fig. 3. Comparison of RAPD banding patterns using OPAX-12 primer in BC_2F_1 progeny developed from the intercrosses between white strain ASI 4045 and brown strain ASI 4019. M : 1 kb ladder marker 1 : ASI 4045. B : Brown strains, W : White strain.

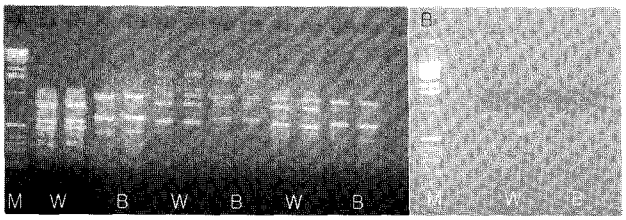

Fig. 4. Isolation of specific marker related to fruitbody color in BC_2F_1 progenies by bulked segregant analysis. A : Comparison of RAPDs from DNA pools of BC_2F_1 progenies which have different fruitbody color. B : Specific markers isolated from different band that afterwards sequenced and analysed.

SEQUENCE SIZE: 634
1 AGGCATCGTGGGGACAAAGTTGATTACTACTAGTAAATGAGGTTCCAGAGGATAGACTCG
61 CCTCAATAGAAAACTGCAGGGTGCTTAGGGTTAGGATTTTATCTAGAACGTGAGCAGGAT
121 TACAGGCGTTCTCAATTTGAGTTTTGGATGAGTTTTGCGGTGTTGTCCGAAAGAGGTGCG
181 CGTTCGACGCGTGGGAGTTTAGATTAGATCTCGCTTGACTTGAACTGTCATGTCTCCTCG
241 TGAGTTCTCCACTTTGGGCGTATTCTCAACTAGTCTGACGCGAATAAGCGTTGCCTCTTT
301 GGAAACAGCCGTCCCCATTTGAACCTGTCCTTCCCCATGGCACGCACCTCGCGCCACAGC
361 ACGTGAAGCGGCACCTACAGGCACTTCGCTCAGAACTCGAAGATGCACGCACGGAAGCCA
421 TTCGTTTACGGAACGCTCTAAAAGTCGCCGAAGCCAACATTGACACGATACAATCTGTCA
481 TCAACGCGCATTGTCGTATTCTTCCCAACATAAACGATCTAATGCCCTCGGAAATACTCG
541 CACACATATTCTCCTTCACATTCACGGCGCCGTTGAATGTGTCTGACTATGAAAGTGGCC
601 CGTGGATGCTTGGAAGGGTCTGTTCACGATGCCT

OLIGO	start	len	tm	gc%	any	3'	seq
LEFT PRIMER	75	20	60.26	55.00	4.00	1.00	TGCAGGGTGCTTAGGGTTAG
RIGHT PRIMER	627	20	59.70	55.00	4.00	2.00	GTGAACAGACCCTTCCAAGC

PRODUCT SIZE: 553, PAIR ANY COMPL: 5.00, PAIR 3' COMPL: 2.00

Fig. 5. Information of the specific primer set LB-2/RB-2 co-segregated with BC_2F_1 progenies that showed brown fruitbody.

tracted and mixed in the same concentration. Each DNA pool was amplified by random primer (Fig. 4A). Four specific bands linked to white and brown fruitbody color were excised and the DNAs were confirmed in gels (Fig. 4B). Each band was extracted from the agarose gels and cloned into pGEM-T.

To make a specific primer, the plasmid DNAs from these clones were sequenced (data not shown). No significant homology to any other sequence in either the GenBank or EMBL data bases was found. Only one primer

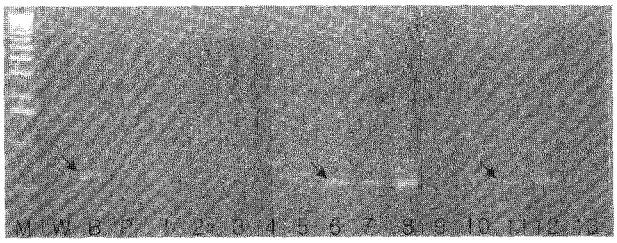

Fig. 6. PCR product obtained by a specific primer set LB-2, RB-2 in BC_2F_1 progenies developed from the intercrosses between white strain ASI 4045 and brown strain ASI 4019. M : 1 Kb DNA ladder, W : White commercial strain ASI 4045, B : Wild brown strain ASI 4019, P1 : Monokaryotic recovery line 45m-9, Lane 1-13 : A part of BC_2F_1 progenies which have white or brown fruitbody. Arrows indicate specific bands amplified by primers.

set (Fig. 5, LB-2 : 5'-TGCAGGGTGC TTAGGGTTAG-3', RB-2 : 5'-GTGAACAGACCCTTCCAAGC-3') among them showed an expected band that is about 600 bp long and was co-segregated with the BC_2F_1 individuals with brown fruitbody (Fig. 6). This primer set derived from BC_2F_1 progeny raised the question of whether this primer set would also react to the other collected dikaryons of a different origin. As such, ten collected dikaryons with white fruitbody and the brown fruitbody were tested.

The primer set responded to the collected dikaryons of different origin and the brown fruitbody (Fig. 7), although it showed different band size according to the strains. This might reflect that the brown strains have different genomic construction. Kong *et al.* (1997a) explained the genetic difference between white and brown strains based on RAPD fingerprinting.

RAPD markers were generated to tag genes of interest (Williams *et al.*, 1990). It is well-established technique widely applied in plant molecular biology since the initial papers of Michelmore's group (Michelmore *et al.*, 1991; Kesseli *et al.*, 1994). This approach has been used to identify the mating genes in other fungi (Judelson *et al.*, 1995;

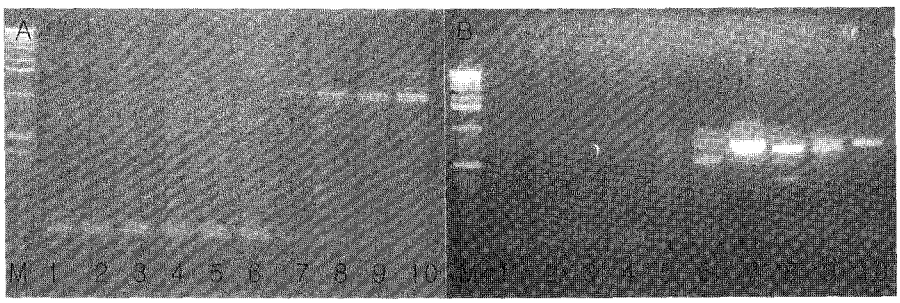

Fig. 7. PCR product obtained by specific primer sets LB-1/RB-1 (A) and LB-2/RB-2 (B) in collected dikaryons of a different origin. M : 1 Kb DNA ladder, Lane order : 1-5 white commercial strains from different source. 1 : Fv4 (New isolate), 2 : ASI 4045, 3 : ASI 4035, 4 : ASI 4036, 5 : ASI 4037, 6-10 : wild brown strain from different origin. 6 : ASI 4001 (Japan), 7 : ASI 4003 (Japan), 8 : ASI 4049 (Suwon, Korea), 9 : ASI 4004 (unknown), 10 : ASI 4057 (China).

Larraya *et al.*, 1999). We developed a set of molecular markers derived from RAPD to apply prescreening for fruitbody color. This is expected to increase the overall efficiency of the screening process.

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