Mycobiology 32(1): 11-16 (2004)

Breeding of *Flammulina velutipes* Strains Adaptable to Elevated-temperature

Won-Sik Kong*, Yong-Hyun Cho, Chang-Sung Jhune, Young-Bok Yoo and Kwang-Ho Kim[1]

Applied Microbiology Division, National Institute of Agricultural Science and Technology, Rural Development Administration, Suwon 441-707, Korea

[1]College of Agriculture and Life Science, Konkuk University, Seoul 143-701, Korea

(Received February 13, 2003)

Winter mushroom, *Flammulina velutipes*, needs low temperature during its cultivation. To save on farm costs, especially during summer, a strain adaptable to a higher or elevated-temperature must be developed. At the start of breeding program, parental strains which could endure high temperature were obtained. Seuenty four dikaryotic strains were collected and divided into four groups according to the nature of temperature. They also had different fruiting temperature. Finally we selected three brown strains ASI 4048, 4057 and 4072, and collected their spores. These selected strains can germinate even at a high temperature of 32°C, which were dramatically higher than the other strains. Based on these results, the new white strain adapted to mid-temperature by backcross mating was developed. Molecular markers were applied to select white fruitbody producing strains without cultivation. They showed a specific band which co-segregated with brown fruitbody forming strains in BC_1F_1 progenies. Selected white strains were tested under several elevated temperature conditions.

KEYWORDS: Backcross mating, Breeding, Elevated-temperature adaptable strains, *Flammulina velutipes*, Molecular marker

Winter mushroom, *Flammulina velutipes*, belongs to white rot fungi. Their fruits become exposed on branches and trunks of hardwood trees in early spring or late autumn in Korea. Wild types of the fungus show brown caps and dark brown stipes. Low temperature is one of the most important environmental factors that affect the fruitbody formation. Brown fruitbody is not popular in the market as consumers prefer white ones. Therefore it is important to develop new varieties producing white fruitbody. This mushroom also needs a low temperature during its cultivation. To save on farm costs, especially during summer, a strain adaptable to elevated temperature must be developed. The objective of this study was to develop a new commercial strain of white fruitbody which can endure relatively high temperature. At the start of the breeding program, parental strains which could endure high temperature were obtained. Tests on 74 dikaryotic strains were conducted to determine their temperature requirements. Molecular markers were applied to select the white strains with no need for cultivation.

Materials and Methods

Strains, growth conditions and mating. Strains of *Flammulina velutipes* were collected from wild flora in Korea and some strains were commercial. Cultures were grown on potato dextrose agar (PDA) or mushroom complete medium at 25°C. Basidiospores were obtained from spore prints. Monokaryons were isolated 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 25~35°C. Single colony was transferred to new PDA plate and confirmed as monokaryon by observing hyphae lacking clamp connection under the microscope. Dikaryons were developed by mating between monokaryons. 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, clamp connections in the mycelium taken from the contact zone between two paired mycelia and from either side of the zone were examined. A mycelium was considered monokaryotic when its hyphae had simple septa, and dikaryotic when clamp connections were observed.

Development of a new mid-temperature strain by backcrossing. Seventy-four collected strains were inoculated on PDA, subjected to five different temperatures and measured after seven days. They were kept in 25°C for 60 days to determine if they develop fruits in high temperature. Spores from selected strains were collected and germinated in four different temperatures. Randomly, ten monokaryons germinated at high temperature (32°C) were selected. They were mated with ten monokaryons from ASI 4074 spores in the same condition. ASI4074 is a commercial strain which has a white fruitbody. Hybrid dikaryons were cultivated and three strains from each combination were selected. They were designated as B48, B57 and B72, respectively. Their monokaryons were isolated by the same procedures and mated again with the

*Corresponding author <E-mail: wskong@rda.go.kr>

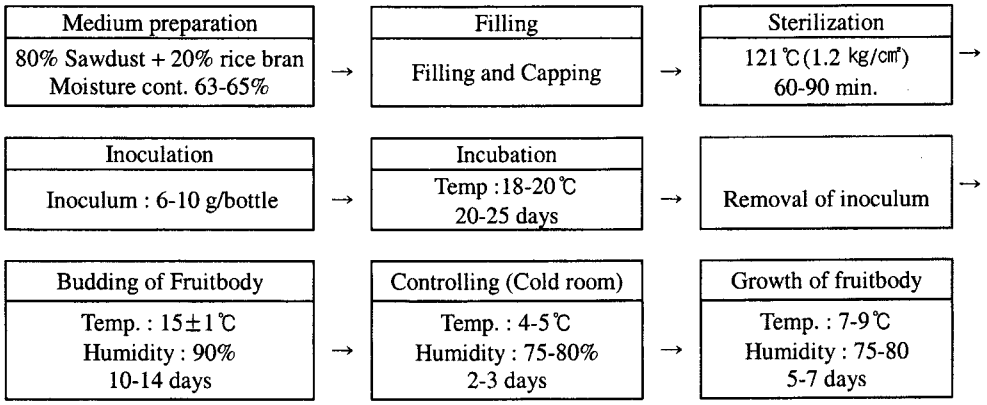

Fig. 1. The standard process of the bottle cultivation of winter mushroom, *F. velutipes*.

same parental strains from ASI4074. Dikaryons were confirmed as having clamp connections by microscope. DNA of the progenies in each combination was amplified by the specific primer set LB-2/RB-2 (Kong *et al.*, 2004) to select the white strains. Strains with no band in gel were cultivated in different conditions to determine whether they adapt to mid-temperature.

Fruitbody formation. Dikaryotic strains obtained by mating between compatible monokaryons were grown on PDA. Then, the mycelia were transferred to sawdust medium. Figure 1 shows the standard cultivation process of the mated dikaryon strains. Elevated temperature conditions for selection were as follows; Conditions of high temperature fruiting were 18°C in the budding stage of fruitbody, 4°C at controlling and 7°C in the growing stage. Conditions of no cold room treatment were 14°C in the budding stage and 7°C in the growing stage without the controlling stage of 4°C. High temperature growth conditions were 14°C in the budding stage, 4°C at controlling and 10°C in the growing stage.

DNA extraction and PCR reaction. For DNA isolation each strain was inoculated at four spots on PDA covered with cellophane to facilitate harvesting the mycelia. After four days, they were harvested and pulverized in 1.5 *ml* tube with liquid nitrogen. DNA was extracted in a tube employing the protocol described by Zolan *et al.* (1986) with minor modifications. General molecular biology protocols described by Sambrook *et al.* (1989) were employed. Williams *et al.* (1990) procedure for Polymerase Chain Reactions (PCRs) to apply molecular marker was followed. The oligonucleotides used as primers for the reaction were LB-2 (5'-TGCAGGGTGCTTAGGGTTAG-3') and RB-2 (5'-GTGAACAGACCCTTCCAAGC-3') (Kong *et al.*, 2004). Amplification reactions were performed in a GeneAmp 9600 (Perkin Elmer, Norwalk) with the following program : one cycle of four minutes at 94°C; 35 cycles of one minute denaturation at 94°C, one minute annealing at 55°C, two minute extention at 72°C; one cycle of a final extension for ten minutes at 72°C. 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 1Kb DNA ladder was employed.

Results and Discussion

Selection of parental strains adapted for mid-temperature. Seventy-four strains were collected to determine which of them can endure relatively high temperature. These were inoculated on PDA in five different temperatures and were measured after seven days (Table 1). Most strains grew best at 25°C. These were divided into four groups according to temperature responses. Strains in the high temperature group grew better at 30~35°C than those in the other groups. In the broad temperature response group, the strains grow well at all temperatures. These results suggest that there may be variations of temperature responses in genetic resources. Masuda *et al.* (1995) proposed that monokaryons which grew fast at high temperature could be used for breeding high-optimum temperature strains.

All 74 strains were kept for 60 days in 25°C to determine if they could fruit in high temperature (Table 2). Within 30 days, only three strains formed fruitbodies which were all brown. After 60 days, 30 strains out of 74 strains formed fruitbodies. When cultivated in a bottle filled with sawdust, 13 brown strains and only one white strain produced fruitbodies. Hence, we selected three brown strains and one white strain as mating parents: ASI 4048, 4057 and 4072 as brown strains and ASI 4074 as a white strain.

Spore germination at high temperatures. Spores from selected strains and several other strains were collected and germinated in 4 different temperatures (Table 3).

Table 1. Grouping of collected strains on the basis of mycelium growth at different temperatures

Group	Strain No. (ASI)	Mycelium growth at temperatures (mm/7 days)					
		10°C	15°C	20°C	25°C	30°C	35°C
Low temp. (10~15°C)	4002(B)	28	45	56	73	59	8
	4046(B)	21	49	58	75	64	8
	4065(B)	22	50	59	75	52	7
Medium temp. (20~25°C)	4025(B)	12	40	55	78	65	10
	4064(B)	15	51	58	78	65	16
	4031(W)	16	47	54	78	66	11
High temp. (30~35°C)	4057(B)	15	32	49	74	80	22
	4070(B)	17	44	59	73	76	13
	4072(B)	20	47	56	78	79	11
Broad temperature response	4048(B)	23	52	57	80	79	18
	4059(B)	20	51	55	82	77	13
	4069(B)	21	52	62	80	80	24
Mean	74 strains	164.0	417.8	508.2	6614.5	5517.3	93.5

Table 2. Selection of high temperature fruiting strains

Trial	Treatment for fruiting	Selected strains (ASI)	
		Brown strains	White strains
1st.	PDA plate (30 days, 25°C)	4065, 4070, 4072	
2nd.	PDA plate (60 days, 25°C)	4002, 4008, 4022, 4028, 4048, 4059, 4064, 4069, 4070, 4072,	4015, 4026, 4027, 4031, 4034, 4037, 4040, 4041, 4043, 4046, 4050, 4053, 4055, 4056, 4066, 4071, 4074
3rd.	Bottle cultivation (60 days, 25°C)	4002, 4003, 4022, 4025, 4048, 4058, 4059, 4062, 4064, 4068, 4070, 4072, 4073,	4074

Table 3. Germination rate of spores according to temperature

Grouping	Strain No. (ASI)	Germination rate of spores according to temperature (%)			
		25°C	30°C	32°C	35°C
Low temp. type	4023	1.8	0.63	–	0
"	4045	6.93	1.29	–	0
"	4047	3.85	1.51	–	0
Wide reponse type	4074	1.84	1.13	0.07	0
"	4048	8.25	4.8	3.8	0
High temp. type	4057	66.88	38.4	17.2	0
"	4072	30.4	23.9	27.7	0

Spores germinated at 25°C with the highest rate from 1.8% to 66.88%. These results show that germination rates of spores were affected by strain itself. The rates were dramatically decreased according to temperature. Spores from the strains which belong to low temperature group did not germinate over 30°C. Spores from selected strains germinated at rate from 3.8% to 27.7% at 32°C. Table 2 shows that spores from ASI4074 which produce white fruitbody at second and third trials germinated at a rate of 0.07%.

To compare culture characteristics, colonies were transferred to a new PDA plate and investigated at 25°C after seven days (Table 4). Monokaryons from white fruitbody producing strains showed almost all white colonies. Colonies at 32°C formed into fluffy and dense types. Monokaryotic fruiting was a typical trait of this mushroom (Kong *et al.*, 1997b). A single recessive gene (Takemaru, 1961) or more than two recessive genes (Kinugawa, 1993) was thought to be responsible for monokaryotic fruiting. This was checked at 10°C after one month. Monokaryons from high temperature were not able to fruit well (Table 4). Fruiting varies with different genotypes and is related to temperature response. Randomly, ten monokaryons which germinated at high temperature (32°C) were selected and mated with ten monokaryons from ASI 4074 spores in the same condition. ASI 4074 is a commercial strain which has white fruitbody.

A new strain developed by back-cross and molecular marker assisted selection. Hybrid dikaryons were cultivated and three strains were selected from each combination. All F_1 hybrids showed colored fruitbodies although the intensities were different (Table 5). A high productive

Table 4. Distribution of monokaryons according to culture characteristics

Strain No. (ASI)	No. of monokaryons	Color distribution (%)			Mycelial density distribution (%)			Aerial hyphae distribution (%)			Mono-karyotic fruiting rate (%)
		White	Light brown	Brown	High	Medium	Low	Fluffy	Medium	Strandy	
4023	30	57	40	3	20	37	43		20	80	73
4045	30	100			26	37	37	20	17	63	43
4047	37	95	5		5	22	73	8	14	78	11
4074	38	100			16	63	21	21	37	42	13
4048	29	41	48	11	79	17	4	38	38	24	41
4048(H)	19	11	89		100			68	32		5
4057	24	29	54	17	58	8	34	21	33	46	21
4057(H)	13	23	38	38	69	23	8	54	31	15	15
4072	11	36	64		100			45	45	9	91
4072(H)	15	73	27		80	20		93	7		–

H : High temperature germination.

Table 5. Characteristics of fruit bodies on the dikaryons derived from back-crossing (F_1) for the development of a new mid-temperature strain

Generation	Strain No. & Combination	Color of fruitbody	Shape of pilus	Length of stipe (mm)	Thickness of stipe (mm)	Yield (g/850 cc)	Remarks
P	4074	W/W	A	127	3	125	Parents
	4048	LY/DB	B	90	3	120	
	4057	LY/DB	B	70	4	100	
	4072	LY/B	B	65	3	95	
F_1	74-1x48-9	LB/DB	B	110	2	166	B48
	74-1x57-9	LY/LB	A	85	3	153	B57
	74-8x72-1	LB/DB	C	120	3	155	B72
BC_1F_1	74-2(B72-5)	LB/DB	B	120	2.5	160	Discard
	74-6(B72-4)	W/W	C	120	3	144	
	74-6(B72-9)	W/W	B	125	2	140	
	B72-5(74-6)	W/W	C	140	2.5	130	
	B72-8(74-6)	W/W	B	140	2.5	153	
	74-6(B57-3)	W/W	B	100	3	122	
	B57-2(74-6)	W/W	C	130	3	132	
	74-2(B57-2)	W/W	C	140	3	130	
	B48-3(74-4)	W/W	B	110	3	132	
	B48-3(74-6)	W/W	C	100	2.5	127	
	B48-3(74-3)	W/W	C	120	3.5	110	

*Combination : A(B) was isolated at A side, A × B at contact area, and B(A) at B side.
*Color of fruitbody : W. white, B. brown, L. light, D. dark, pilus/stipe.
*Shape of pilus : A. Plano-convex B. Convex C. Hemispherical D. Paraboloid.

strain from each combination was chosen. They were designated as B48, B57 and B72. Their monokaryons were isolated by the same procedures and mated again with the same parental strains from ASI 4074. Dikaryons were confirmed as clamp connection by microscope and designated as BC_1F_1 (Table 5).

Fruitbody color is one of the most important considerations in the breeding program (Kong *et al.*, 1997a). Standard cultivation of developed hybrids requires the installation of a large scale pilot plant. Thus, to increase the overall efficiency of the screening process, pre-screening methods to vegetative mycelium was recommended (Kitamoto *et al.*, 1993). RAPD (Random Amplified Polymorphic DNA) marker is well-established technique widely applied in plant molecular biology since the initial papers of Michelmores group (Michelmore *et al.*, 1991; Kesseli *et al.*, 1994). This was used to tag genes of interest and to identify mating genes in other fungi (Judelson *et al.*, 1995; Larraya *et al.*, 1999). We developed a set of molecular markers derived from RAPD to apply pre-screening for fruitbody color (Kong *et al.*, 2004). DNA of the progenies in each BC_1F_1 combination were amplified

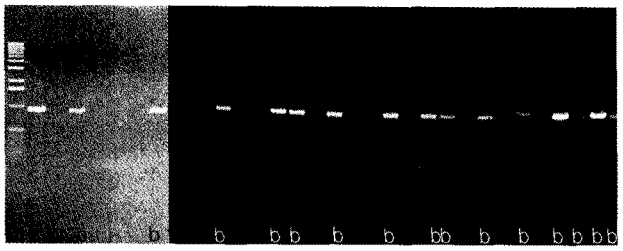

Fig. 2. PCR product obtained by a specific primer set LB-2, RB-2 in BC_1F_1 progenies developed from the intercrosses between brown strain ASI 4072 and white strain ASI 4074. M : 1 kb DNA ladder, B : Wild brown strain ASI 4072 , W : White commercial strain ASI 4074. The other lane : A part of BC_1F_1 strains composed of 3 mating combination. b : BC_1F_1 isolates which have brown fruitbody.

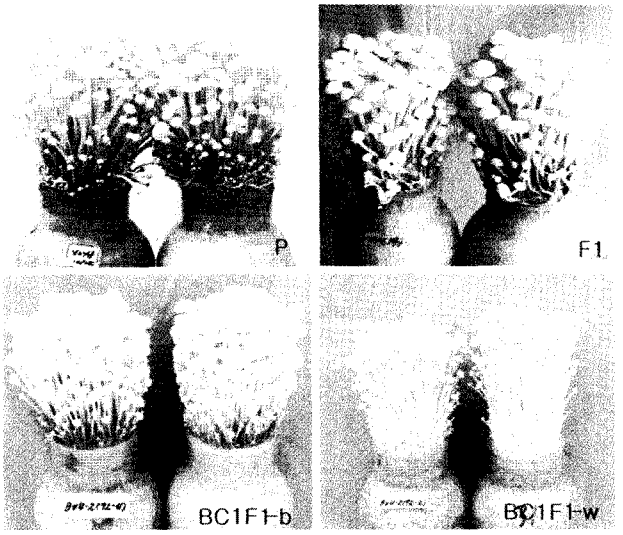

Fig. 3. Fruitbodies on the dikaryons represented in the P, F_1 and BC_1F_1 for the development of a new strain. P is ASI4048, one of parental strain which produce brown fruitbody. F_1 is a hybrid mated between monokaryons from white strain ASI4074 and brown strain ASI4048. BC_1F_1-b is a progeny in BC_1F_1 which showed a specific band at molecular marker assisted selection. BC_1F_1-w is a progeny in BC_1F_1 which showed no band at molecular marker assisted selection.

by the specific primer set LB-2/RB-2 to select white strains (Fig. 2). The band size was about 1 kb. This was due to the parents with different origins (Kong *et al.*, 2004). The strains that have no band in gel were cultivated and the characteristics of fruitbodies were confirmed (Table 5, Fig. 2). Most of them produced white fruitbodies. Some BC_1F_1 progenies which showed specific band produced brown fruitbody (Fig. 3).

The white strains with no band in gel were cultivated in different cultivation conditions to determine if they adapt to mid-temperature (Table 6). Conditions for high temperature fruiting were changed from 14°C to 18°C in the budding stage of fruitbody. Conditions of no cold room treatment were absent of the controlling stage of 4°C. High temperature growth conditions were changed 7°C to 10°C in the development stage. All tested strains decreased their productivity on set conditions, especially severe in excluded low temperature treatment. But strain B72-8(74-6) produced good yields both in standard and high temperature growth conditions.

In these experiments, an effective breeding system with pre-screening was applied. Selecting parental strains that possess desirable traits is the most important. It is reasonable to expect wide variations in morphological and culture characteristics among the mating hybrids. To recover practical traits including color, backcross method was the most powerful tool. Molecular markers were applied to vegetative mycelium to increase the efficiency of the screening. However, the use of self mating on selected strain seems to be effective to achieve minute improvements as a next step.

Table 6. Productivity of *Flammulina velutipes* according to the cultivation method

Strain No. (ASI)	Yield according to the cultivation method[a] (g/850 *ml*)			
	Standard (1)	High temperature fruiting (2)	No cold room treatment (3)	High temperature growth (4)
74-6(B72-4)	144	115	88	96
74-6(B72-9)	140	117	93	79
B72-8(74-6)	153	114	118	138
74-6(B57-3)	122	114	75	113
B57-2(74-6)	132	106	77	100
74-2(B57-2)	130	87	79	108
B48-3(74-4)	132	117	75	100
B48-3(74-6)	127	99	88	122
B48-3(74-3)	110	109	106	102
4072	95	90	88	83
4074	150	125	105	114

[a]Cultivation method. 1. Standard temperature: Fruiting 14°C, Cold room 4°C, Growth 7°C. 2. High temperature fruiting : Fruiting 18°C, Cold room 4°C, Growth 7°C. 3. No cold room treatment : Fruiting 14°C, Growth 7°C. 4. High temperature growth : Fruiting 14°C, Cold room 4°C, Growth 10°C.

Reference

Judelson, H. S., Spielman, L. J. and Shattock, R. C. 1995. Genetic mapping and non-Mendelian segregation of mating type loci in the oomycete, *Phytophthora infestans*. *Genetics* **141**(2): 503-512.

Kesseli, R. V., Paran, I. and Michelmore, R. W. 1994. Analysis of a detailed genetic linkage map of *Lactuca sativa* (lettuce) constructed from RFLP and RAPD markers. *Genetics* **136**(4): 1435-1446.

Kinugawa, K. 1993. Physiology and the breeding of *Flammulina velutipes*. Pp 87-109. *In:* Genetics and Breeding of Edible Mushrooms. Gordon and Breach Science Publishers.

Kitamoto, Y., Nakamata, M. and Masuda, P. 1993. Production of a novel white *Flammulina velutipes* by breeding. Pp 65-86. *In:* Genetics and Breeding of Edible Mushrooms. Gordon and Breach Science Publishers.

Kong, W. S., Kim, D. H., You, C. H., Cha, D. Y. and Kim, K. H. 1997a. Genetic relationships of *Flammulina velutipes* isolates based on ribosomal DNA and RAPD analysis. *RDA. J. Indus. Crop Sci.* **39**(1): 28-40.

_____, _____, Kim, Y. H., Kim, K. S., You, C. H., Byun, M. O. and Kim, K. H. 1997b. Genetic variability of *Flammulina velutipes* monosporous isolates. *Kor. J. Mycol.* **25**(2): 111-120.

Kong, W. S., You, C. H., Yoo, Y. B., Kim, G. H. and Kim, K. H. 2004. Molecular marker related to fruitbody color in *Flammulina velutipes*. *Kor. J. Mycol.* **31** (in press).

Larraya, L., Penas, M. M., Cruz Santos, G. P., Ritter, E., Pisabarro, A. G. and Ramirez, L. 1999. Identification of incompatibility alleles and characterisation of molecular markers genetically linked to the A incompatibility locus in the white rot fungus *Pleurotus ostreatus*. *Curr. Genet.* **34**: 486-493.

Masuda, P., Nogami, T., Mori, N. and Kitamoto, Y. 1995. An empirical rule for breeding high-optimum-temperature strains of *Flammulina velutipes*. *Trans. Mycol. Soc. Japan* **36**: 158-163.

Michelmore, R. W., Paran, I. and Kesseli, R. V. 1991. Identification of markers linked to disease-resistance genes by bulked segregant analysis: A rapid method to detect markers in specific genomic regions by using segregating populations. *Proc. Natl. Acd. Sci.* **88**: 9828-9832.

Sambrook, J, Fritsch, E. F. and Maniatis, T. 1989. Molecular cloning : a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Takemaru, T. 1961. Genetical studies on fungi, . The mating system in Hymenomycetes and its genetical mechanism. *Biol. J. Okayama Univ.* **7**: 133-211.

Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalski, J. A. and Tingey, S. V. 1990. DNA polymorphism amplified by arbitary primer are useful as genetic markers. *Nucleic Acids Research* **18**: 6513-6535.

Zolan, M. E. and Pukkila, P. J. 1986. Inheritance of DNA methylation in *Coprinus cinerius*. *Mol. Cell. Biol.* **6**: 195-200.