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FULL PAPER

Supatra Ratanatragooldacha · Cao Hui · Atsushi Kumata
Yutaka Kitamoto

The constitution of incompatibility factor and mating characteristics of spore isolates in a bipolar mushroom, *Pholiota nameko*

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Abstract *Pholiota nameko* is a wood-rotting edible mushroom that carries a bipolar *A* incompatibility factor gene. The linkage analysis of the multiple allelomorphic *A* factor gene demonstrated that sexual reproduction produced a monospore isolate carrying a new *A* factor gene in addition to two parental mating types of isolates. However, 10%–30% of the modified monospore isolates could not produce a dikaryon with both of the parental monokaryons by crossing. It is concluded that the bipolar *A* incompatibility factor gene of *P. nameko* is constituted of two functional subunits, *A*α and *A*β, which might be successively located beside each other with an apparent genetic distance of 0.3 centi-Morgan between them on the same chromosome. Further, some monospore isolates that did not conjugate with both parental monokaryons could produce dikaryons with different monokaryotic stocks with either one of the parental mating types. This result suggests that the crossing capability of these isolates were essentially those for one of the mating types of the parental monokaryons, but that their function for mating activity was made partially by unequal crossing-over in the process of sexual recombination.

Key words Basidiomycete · Bipolar mating system · Incompatibility factor · Linkage analysis · *Pholiota nameko*

S. Ratanatragooldacha · C. Hui[1] · Y. Kitamoto (✉)
Department of Biochemistry and Biotechnology, Faculty of Agriculture, Tottori University, 4-101 Koyamacho Minami, Tottori 690-0945, Japan
Tel. +81-857-31-5371; Fax +81-857-31-5371
e-mail: kitamoto@muses.tottori-u.ac.jp

A. Kumata
Fukushima Prefecture, Forest Research Center, Koriyama, Japan

[1]*Present address*:
Edible Fungi Institute, Shanghai Academy of Agricultural Sciences, China

Introduction

The mating system of basidiomycetes such as basidiomycetous yeasts and mushrooms is controlled by incompatibility factor genes that regulate the formation of the dikaryon between complementary pairs of monokaryons carrying different incompatibility factors. The incompatibility factors of basidiomycetous yeasts function as allelomorphic genes in their sexual reproduction (Rowell 1955; Banuett and Herskowitz 1989). In contrast, the incompatibility factors of basidiomycetous mushrooms are constituted of one or two sets of multiple allelomorphic genes called bipolar and tetrapolar incompatibility factors, respectively (Whitehouse 1949). Mushrooms such as *Coprinus cinereus* (Schaeff.: Fr.) S.F. Gray (Casselton and Kues 1994), *Flammulina velutipes* (Curt.: Fr.) Sing. (Ashan-Aberg 1960), *Lentinula edodes* (Berk.) Pegler (Takemaru 1961), and *Schizophyllum commune* Fr. (Frankel and Ellingboe 1977) were demonstrated to carry tetrapolar incompatibility factors. In the tetrapolar mushrooms, both *A* and *B* factors are constructed of two subunits, α and β subunits, that are located on the different chromosomes, respectively (Day 1960; Frankel and Ellingboe 1977; Takemaru et al. 1995; Ratanatragooldacha et al. 2001). However, little is known about the constitution and function of incompatibility factors in bipolar basidiomycetous mushrooms.

Pholiota nameko (T. Ito) S. Ito and Imai in Imai is a wood-rotting edible mushroom that carries a bipolar *A* incompatibility factor (Arita and Takemaru 1962). The present study is conducted to reveal the genetic constitution of the bipolar *A* incompatibility factor gene in *P. nameko* by linkage analysis. The crossing characteristic of the monospore isolates produced as recombination products in the sexual reproduction is also discussed.

Table 1. Monokaryotic stocks of *Pholiota nameko*

Stock no.	Incompatibility factor
NX-1	*A1*
NX-15	*A2*
NX-20	*A2*
NX-2	*A2*
NGW-12	*A3*
NX-3	*A3*
NGW-19	*A4*
NGW-20	*A4*
NX-4	*A4*
NF-7	*A5*
NF-8	*A5*
NX-5	*A5*
NX-6	*A6*

Materials and methods

Test organisms

Monokaryotic lines of *Pholiota nameko* were obtained by monospore isolation from the fruit-bodies of various wild strains, as detailed in our previous report (Masuda et al. 1995). According to the method applied for the determination of nuclear DNA content of the hyphal cells of *P. nameko* with propidium iodide (Cao et al. 1999), these stocks were confirmed to have haploid DNA content. The incompatibility factors of these monokaryotic stocks were determined by crossing them with tester stocks. Thirteen monokaryons with six types of bipolar incompatibility factors were assigned as *A1*, *A2*, *A3*, *A4*, *A5*, and *A6*, respectively (Table 1).

Method for producing dikaryotic hybrid stocks

The dikaryotic stocks used for linkage analysis of the incompatibility factor of this mushroom were prepared by reciprocal crossing of two compatible monokaryotic stocks. The inoculation for crossing was done by planting two different monokaryotic stocks 4 mm apart in the center of a potato dextrose agar (PDA) plate (Nissui Seiyaku, Tokyo, Japan). After incubation for 7–10 days at 25°C, the colony on the PDA plate at the contact zone between two parental monokaryons was inspected under a microscope for the formation of clamp connections as evidence for dikaryotization. The hybrid dikaryotic mycelium thus confirmed was transplanted onto a PDA slant for the preparation of hybrid stocks.

The cultivation of fruit-bodies of dikaryotic hybrid stocks

The cultivation of fruit-bodies in the test mushroom was carried out by using a sawdust substrate. The substrate was prepared by mixing beech sawdust (*Fagus sieboldi*) and rice bran at a volumetric ratio of 5:1 and adjusting the moisture content to about 65%. About 40 ml of the substrate was placed into a 100-ml Erlenmeyer flask, plugged with silicon-polarized rubber (Shinétsu Chemical, Tokyo, Japan), and autoclaved at 121°–123°C for 15 min. After cooling the flask in air, the mycelium fragment from the stock culture was inoculated and incubated for about 30 days at 25°C in the dark. After completing spawn running, the culture was then incubated at 10°C under continuous light at 200 lux with fluorescent light tubes for the formation of fruit-bodies. The mature pilei of the fruit-bodies were cut off with a knife and put into a Petri dish to obtain spore prints.

Isolation of basidiospore-derived monokaryotic stocks

Five ml of sterilized water was pipetted onto the spore prints in the petri dishes, which were vigorously shaken to prepare the spore suspension. The densities of spores in the suspensions were determined by counting with a hemocytometer under a microscope and the suspension was then diluted to about $1 \times 10^4 - 1 \times 10^6$ cells/ml. Then, 0.1 ml of the suspension was mixed with 2 ml of melted PDA soft agar medium at 50°C in the test tube and poured onto the PDA plate to prepare the double-layer agar culture. After incubating the culture at 25°C for 3–4 days, the colonies that appeared on the plate were isolated and transferred onto PDA slants. These slants were incubated for about 7 days at 25°C before using in the crossing experiments.

Determination of the incompatibility factor of monospore isolates

The mycelium of the monospore isolate was identified by crossing them with the tester monokaryotic stocks of known mating types. The isolates were crossed with tester strains that had the same incompatibility factor composition as each of the two parental monokaryotic stocks. The isolate showing incompatibility with a parental monokaryon tester but compatible with either of the tester monokaryons was considered to indicate that the test isolate had the same nuclear type as the former tester. If the test monokaryotic isolates could not produce clamp connections with parental monokaryons, the isolates were further applied for the crossing tests by using different monokaryotic tester stocks carrying the same incompatibility factors (see Table 1). If the test monokaryons showed incompatibility characteristics different from those of the parental monokaryons, it was identified to be the recombinant carrying the new *A* incompatibility factor. The new incompatibility factor produced by recombination was designated *Ar* with a numerical suffix.

Results and discussion

Linkage analysis of the *A* incompatibility factor constitution

To examine whether the bipolar *A* incompatibility factor gene of *P. nameko* was constituted of subunits similar to

those of the tetrapolar mushrooms, linkage analysis of monospore isolates from three different hybrid dikaryotic stocks was conducted. Two monokaryons carrying different mating factors were crossed. The resulting basidiospore-derived monokaryotic isolates were backcrossed with both the parental monokaryons to determine the genotypes of their incompatibility factors.

The results of the linkage analysis of the incompatibility factor in a hybrid stock, NX-2(*A2*) × NGW-20(*A4*), are shown in Table 2a. Among 300 monospore isolates, it was found that 1 isolate was carrying *Ar1* in addition to two parental mating types of isolates: 121 of *A2* and 152 of *A4*. However, it was also found that 26 modified isolates could not produce a dikaryon against both parental monokaryons. We assume that these modified isolates were the products of unequal crossing-over in the process of sexual reproduction. Further, an isolate carrying the new *A* incompatibility factor (I-8: *Ar1*) was the product of the successful meiotic recombination in sexual reproduction. In the case of the NGW-12(*A3*) × NGW-19(*A4*) hybrid, 223 of 300 isolates produced dikaryons against either one of the parental monokaryons, and 1 isolate (II-127: *Ar2*) produced dikaryons against both parental monokaryons.

However, 76 isolates did not produce dikaryons against two parental monokaryons. This result contrasts with the apparently small number of isolates carrying the *A3* factor as compared with the expected segregation ratio (1:1) between two types of parental mating factors, *A3* and *A4* (Table 2b). Similarly, we found an unusually small number of *A2* factored monospore isolates among 300 monospore isolates from the NA-15(*A2*) × NGW-19(*A4*) hybrid and also detected an isolate (III-17) carrying *Ar3* (Table 2c). The formation of *Ar* monokaryons by sexual reproduction suggests that the bipolar *A* incompatibility factor gene of *P. nameko* is constituted of two functional subunits, for example, *Aα2* and *Aβ2* in the parental monokaryon NX-2 (*A2*), which might be located successively beside each other without any actual genetic distance on the same chromosome. The apparent genetic distance between two subunits of the *A* incompatibility factor on the first chromosome was averaged to be 0.3 centi-Morgan (cM). Giasson et al. (1989) estimated that 0.6 cM corresponded to about 100 kbp of DNA in *Schizophyllum commune*.

Table 2. The results of backcrossing test of monospore isolates from three different hybrids against the corresponding parental monokaryons

a. NX-2(*A2*) × NGW-20(*A4*)

Tester monokaryon		Number of spore isolates
NX-2(*A2*)	NGW-20(*A4*)	
+	+	1
+	−	152
−	+	121
−	−	26

b. NGW-12(*A3*) × NGW-19(*A4*)

Tester monokaryon		Number of spore isolates
NGW-12(*A3*)	NGW-19(*A4*)	
+	+	1
+	−	142
−	+	81
−	−	76

c. NA-15(*A2*) × NGW-19(*A4*)

Tester monokaryon		Number of spore isolates
NA-15(*A2*)	NGW-19(*A4*)	
+	+	1
+	−	154
−	+	46
−	−	99

Crossing characteristic of three monospore isolates carrying different *Ar* incompatibility factors

The crossing characteristics between either one of the three *Ar* isolates with several different monokaryotic tester stocks carrying *A1–A6* were examined. As shown in Table 3, two recombinant isolates, I-8 and II-127, produced dikaryons against all tester stocks. The isolate, III-17, showed mating capability against the testers except for NX-2 and NA-20, both of which had the *A2* factor. However, the isolate could produce a dikaryon against NA-15 with the *A2* factor, suggesting that the mating characteristic of this isolate was essentially a new type, but its incompatibility factor gene was partially defective in the process of recombination. Consequently, these three isolates were the products of the recombination between the two subunits of the *A* incompatibility factor gene in this bipolar mushroom.

Table 4 shows the reciprocal crossing among three *Ar* isolates. All these isolates produced dikaryons with the other isolates. Therefore, I-8 may have the subunit constitution in combination with *A2* and *A4* factors, i.e., of either *Aα2Aβ4* or *Aα4Aβ2*. Similarly, II-127 and III-17 might have the *A* incompatibility factor constitutions of *Aα3Aβ4* or *Aα4Aβ3* and *Aα4Aβ2* or *Aα2Aβ4*, respectively.

Crossing characteristic of the monospore isolates carrying the partially defective incompatibility factor gene produced by sexual recombination

There are three possible cases of recombination for the incompatibility factor *A* gene in the meiosis of *P. nameko*. If recombination occurs outside the region for the incompatibility gene on the chromosome, it is expected to produce two types of isolates carrying either one of the parental monokaryon incompatibility factors. However, if the recombination occurs just between the *Aα* and *Aβ* region of the incompatibility factor gene, the recombinant isolate thus produced may carry a new *A* incompatibility factor (*Ar*) that is active against both the parental monokaryons. On the other hand, if the recombination occurs in the intra-*Aα* or-*Aβ* region of the incompatibility subunit, the subunit is divided into two substructures on the resulting

Table 3. Crossing of the incompatility factor gene recombinant isolates from three different hybrids against the tester monokaryons with different incompatibility factors

Tester stock	Recombinant stock		
	I-8 (NX-2 × NGW-20)	II-127 (NGW-12 × NGW-19)	III-17 (NA-15 × NGW-19)
NX-1(*A1*)	+	+	+
NX-2(*A2*)	+	+	−
NA-20(*A2*)	+	+	−
NA-15(*A2*)	+	+	+
NX-3(*A3*)	+	+	+
NX-4(*A4*)	+	+	+
NX-5(*A5*)	+	+	+
NF-7(*A5*)	+	+	+
NF-8(*A5*)	+	+	+
NX-6(*A6*)	+	+	+

Table 4. Crossing of the incompatility factor gene recombinant isolates from three different hybrids against the tester monokaryons with different incompatibility factors

Tester stock	Recombinant stock		
	I-8 (NX-2 × NGW-20)	II-127 (NGW-12 × NGW-19)	III-17 (NA-15 × NGW-19)
I-8(*Ar1*)	/	+	+
II-127(*Ar2*)	+	/	+
III-17(*Ar3*)	+	+	/

Table 5. Crossing characteristic of the monospore isolates carrying partially defected incompatibility factor gene produced by sexual reconbination

a. Monospore isolates derived from NX-2(*A2*) × NGW-20(*A4*)

Tester monokaryon				Number of spore isolates
NA-15(*A2*)	NA-20(*A2*)	NGW-19(*A4*)	NX-4(*A4*)	
+	−	−	−	3
+	+	−	−	2
−	+	−	−	3
−	−	+	−	1
−	−	−	−	17

b. Monospore isolates derived from NGW-12(*A3*) × NGW-19(*A4*)

Tester monokaryon				Number of spore isolates
NX-3(*A3*)	NGW-9(*A3*)	NGW-20(*A4*)	NX-4(*A4*)	
−	+	−	−	2
−	−	−	−	74

c. Monospore isolates from NA-15(*A2*) × NGW-19(*A4*)

Tester monokaryon				Number of spore isolates
NX-2(*A2*)	NA-20(*A2*)	NGW-20(*A4*)	NX-4(*A4*)	
+	−	−	−	2
−	−	+	+	1
−	−	−	−	94

chromosome. The last case suggests that the fragmentation of the *A* incompatibility genes may cause reduction or defects of mating capability against complementary monokaryons. Actually, among each 300 monospore isolates from either one of the three hybrids tested, 19–94 isolates had defective capability to produce dikaryons against the parental monokaryons (see Table 2). To examine the production of partially defective incompatibility factors in the sexual recombination of *P. nameko*, crossing tests were carried out by using other tester stocks expressing the same mating types as those of the parental monokaryons.

In the case of 26 defective isolates from the NX-2(*A2*) × NGW-20(*A4*) hybrid, 8 isolates produced dikaryons with one or two of the tester stocks carrying the *A2* factor, and 1 isolate produced dikaryons against one tester stock carrying the *A4* factor (Table 5a). Therefore, we assumed that these 9 isolates might carry partially defective incompatibility factor genes that could be only mated against superior monokaryons above the complemented parental monokaryon. However, the remaining 17 isolates could not produce dikaryons with any *A2* or *A4* stocks tested. In the case of 76 defective isolates from the NGW-12(*A3*) × NGW-19(*A4*) hybrid, 2 isolates produced dikaryons against one tester stock carrying the *A3* factor, but the others could not produce dikaryons with any *A3* or *A4* tester stocks (Table 5b). Similarly, 3 isolates from the NA-15(*A2*) × NGW-19(*A4*) hybrid showed partially defective mating capability against different tester monokaryons to produce dikaryons, and others completely lost their mating capability against the test monokaryons (Table 5c). These facts suggest that the defect or partial defect of mating capability against the complementary monokaryons was the result of modifying the function of the incompatibility genes by unequal crossing-over in the meiotic recombination. We estimated that the productivity of the monospore isolates carrying partially defective incompatibility factors might be about 10% from several commercial strains of *P. nameko* (unpublished data). From this criterion, the high productivities of defected monospore isolates from NGW-12 × NGW-19 and NA-15 × NGW-19 were unexpected results. If one of the parental monokaryons, NGW-12 or NA-15, carries the defective incompatibility gene, it might be expected that the crossing of NGW-12(*A3*) × NGW-19(*A4*) or NA-15(*A2*) × NGW-19(*A4*) produces more defective monospore isolates than those from dikaryotic hybrids produced with two parental monokaryons carrying normal incompatibility factor genes against each other.

Further research is in progress to analyze the details of the mating characteristics of partially defective monospore isolates by using parental monokaryons carrying the auxotrophic marker that is very close to the locus of the *A* incompatibility factor on the first chromosome of this mushroom.

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