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SHORT COMMUNICATION

Rapid detection for sporeless trait from *Pleurotus pulmonarius* culture extracts by using real-time PCR

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Abstract The release and spread of a large number of basidiospores from the developing fruiting body cause serious problems in the cultivation of edible mushrooms, including *Pleurotus pulmonarius* (Fr.) Quel. The *P. pulmonarius* sporeless mutant, TMIC-30058, has a high potential for breeding sporeless cultivars to reduce these adverse effects. Our previous study (Okuda et al. in *Breeding Science* 59:315–319, 2009) showed two sequence-tagged site (STS) markers for the sporeless trait from this mutant. Here, using these STS markers we present rapid detection of sporeless trait from *P. pulmonarius* culture extracts by real-time PCR. This method enables us to cut down time and labor for screening of the sporeless trait, suggesting its availability for efficient breeding of sporeless cultivars.

Keywords Marker-assisted selection · Mushroom breeding · Oyster mushroom · Sporulation-deficient mutation · STS marker

Pleurotus pulmonarius (Fr.) Quel. (Japanese name: Usuhiratake) is one of the principal commercial species of oyster mushroom (*Pleurotus* spp.) cultivated around the world. The enormous number of spores produced by fruiting bodies can adversely affect the health of mushroom farmers (by respiratory allergic reaction) (Hausen et al. 1974; Sakula 1974; Olsen 1987), the mushroom cultivation facility, and the genetic diversity of natural populations (Hibbett and Donoghue 1996; Obatake et al. 2003). These problems caused by spores are relatively more serious for the cultivation of *Pleurotus* spp. because their fruiting bodies begin to release considerable numbers of spores from a very early stage in development and continue to do so throughout their maturation.

Sporulation-deficient (sporeless) mutants are useful for preventing these problems in mushroom cultivation. To date, sporeless mutants were obtained for various species including model and edible mushrooms. To the best of our knowledge, sporeless strains are currently commercially cultivated for only three species: *Agrocybe cylindracea* (DC.: Fr.) Maire (Murakami 1993), *Pleurotus eryngii* (De Cand.) Gillet (Obatake et al. 2006), and *Pleurotus ostreatus* (Jacq.: Fr.) (Baars et al. 2000). Development of a commercially viable sporeless strain of *P. pulmonarius* is also highly desirable, but no such strain currently exists. In addition, the traditional method of mutation breeding, which uses the artificial or spontaneous mutants as an initial material, requires much time and labor for the test cross and the determination of the sporeless trait of materials through cultivation. Thus, an efficient breeding method using marker-assisted selection (MAS) is expected for detecting the sporeless trait rapidly in the breeding population.

The spontaneous sporeless mutant strain TMIC-30058 of *P. pulmonarius* (Ohira 1979) produces an extremely small number of spores and no unfavorable traits for fruiting body production. As the sporeless mutation is controlled by a single recessive gene (Ohira 1979; Okuda et al. 2009a), this sporeless mutant has high potential for breeding sporeless cultivars. In the previous study, by using

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mapping population including 150 progeny isolates derived from a single cross between two basidiospore isolates from this sporeless and a wild-type isolate, we mapped the sporeless mutation locus with linked markers on the genetic linkage map using the amplified fragment length polymorphism (AFLP) technique (Okuda et al. 2009a). Also, using the technique of bulked segregant analysis-based AFLP (BSA-AFLP), closely linked markers were identified because markers closer to the targeted locus have higher detection efficiency (Okuda et al. 2009b). Of them, two AFLP markers located at a distance of 0 cM (CATC192) and 3.4 cM (CGGG296) from the sporeless mutation locus and were inherited from a sporeless parent (Okuda et al. 2009b). Two AFLP markers, CATC192 and CGGG296, were successfully converted into two STS markers, SD192 and SD296, respectively, for the detection of the sporeless trait reproducibly. Finally, SD192 and SD296 could detect the sporeless trait from TMIC-30058 with 100% and 97.3% efficiency, respectively (Okuda et al. 2009b). However, the practical use of these two markers in breeding with large-scale screening has been limited by the necessity of preparation of purified genomic DNA and electrophoretic analysis.

Real-time polymerase chain reaction (PCR) is a quantitative and precise method with high throughput that can be applied to the analysis of large numbers of samples (Heid et al. 1996). Thus, real-time PCR is widely used in the field of plant pathology, medical science, and environmental studies (Francois et al. 2004; Simon et al. 2005; Mason et al. 2008). In mushrooms, although this method has been used in some reports (Bodles et al. 2006; Parladé et al. 2007; Maeta et al. 2008), no example for useful mutational traits in breeding has been reported. In this study, we describe the real-time PCR-based method with *P. pulmonarius* culture extracts using SD192 and SD296 for the detection of the sporeless trait.

Pleurotus pulmonarius isolates 30058-S1 and 31664-S1 were from basidiospores of two dikaryotic isolates, a sporeless mutant, TMIC-30058, and a wild-type isolate, TMIC-31664, respectively (Okuda et al. 2009a). TMIC-30058 and TMIC-31664 are maintained in the culture collection of the Tottori Mycological Institute (Tottori, Japan).

For PCR amplification, specimens were prepared as follows: cultures were grown at 25°C for 2 weeks on MYG slant (2% malt extract, 2% glucose, 0.2% yeast extract), and then 2-mm mycelial blocks including agar were harvested and incubated at 55°C for 1 h with lysis buffer [20 mM Tris–HCl (pH 8.0), 5 mM ethylenediaminetetraacetic acid (EDTA), 400 mM NaCl, 0.3% sodium dodecyl sulfate (SDS)] and 20 μg Proteinase K, according to instructions from Shimadzu Corporation (Kyoto, Japan). These supernatants were collected with spin-down and used for thermal reaction. Cultures for preparation of purified genomic DNA as control samples were grown at 25°C for 2 weeks on liquid MYG broth, and then mycelia were harvested and freeze-dried. Genomic DNA of each isolate was prepared using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany).

All real-time PCR amplification was performed with iCycler iQ™ (Bio-Rad Laboratories, Hercules, CA, USA) in a 20-μl reaction mixture containing 0.5 μl aforementioned extracted sample or genomic DNA (5 ng/μl), 10 μM each primer, 2× Ampdirect Plus (Shimadzu Corporation), 125× SYBR GreenI Nucleic Acid Gel Stain (Invitrogen, Carlsbad, CA, USA), and 0.5 U Nova*Taq*™ Hot Start DNA polymerase (Novagen, Madison, WI, USA). The PCR procedure was initially started 95°C denaturation for 10 min; followed by 35 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 30 s, and an additional extension for 7 min at 72°C. Each primer pair and that amplification size are listed in Table 1. After amplification was completed, melting curve analysis was performed from 65° to 95°C at 0.5°C/10 s with continuous fluorescence monitoring. The melting temperature (T_m) value was defined as the peak of the curve. T_m value was obtained using iCycler iQ™ Real-Time Detection System software, version 3.0 (Bio-Rad Laboratories). Finally, the sporulation phenotype could be diagnosed by amplification of the PCR products with a desired T_m value.

Real-time PCR using SD192 or SD296 was performed with culture extracts from mycelia or genomic DNAs as template. Culture extracts and genomic DNAs are from 30058-S1 and 31664-S1, respectively. As results, there were no differences between PCR amplification using culture extract and genomic DNA. These data indicate that

Table 1 Characterization of STS primer pairs for the detection of sporeless trait

Marker name	Primer	Primer sequence (5′–3′)	Product size (bp)	Reference
SD192	SD192-F	CAGAATGACGAGGGACTC	144	Okuda et al. (2009b)
	SD192-R	GCGTGCTAATATTATGTCCC		
SD296	SD296-F	CGTTGTCTTCTGAATAGTATTCCT	265	Okuda et al. (2009b)
	SD296-R	GGCGATGCGAGGGGA		

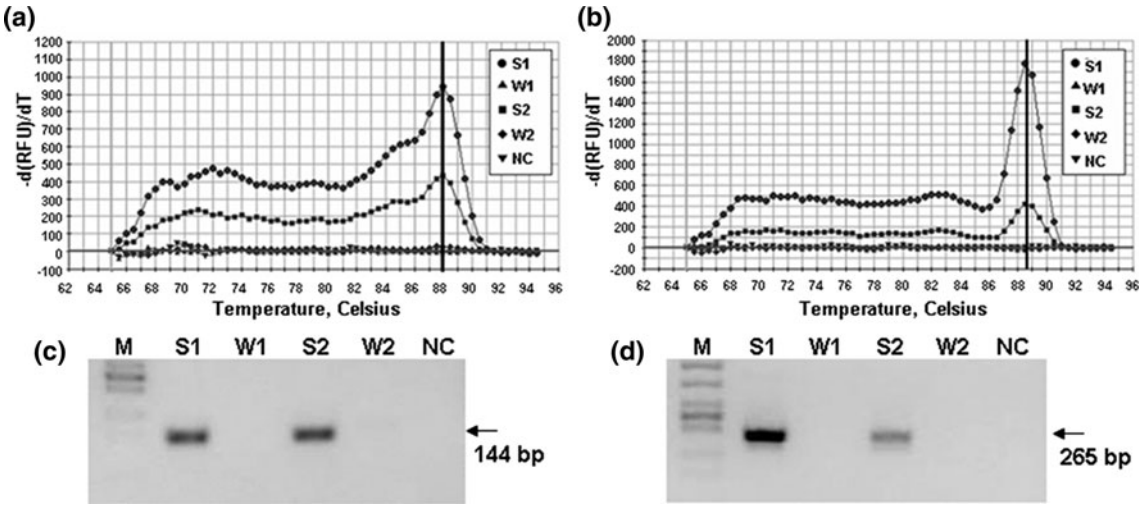

Fig. 1 Analyses during real-time polymerase chain reaction (PCR) with sequence-tagged site (STS) markers for the detection of sporeless trait using culture extract and genomic DNA of sporeless mutant type 30058-S1 and wild type 31664-S1, respectively. **a**, **b** Melting curve analysis using PCR products by SD192 and SD296, respectively. *Heavy vertical line* indicates melting peaks at T_m value. **c**, **d** Electrophoretic analysis of samples from **a** and **b**, respectively. PCR products were fractionated on a 4% agarose gel followed by visualization with ethidium bromide staining. The *columns* represent each template. *S1*, genomic DNA from sporeless mutant; *W1*, genomic DNA from wild type; *S2*, culture extract from sporeless mutant; *W2*, culture extract from wild type; *NC*, negative control; *M*, $\phi \times 174$ *Hin*cII digest

culture extract from mycelium is useful for PCR amplification. On melting analysis, melting peak at T_m values of 88.0° and 88.5°–89.0°C were formed by SD192 and SD296, respectively (Fig. 1a,b). As expected, electrophoretic analysis of these PCR products with 30058-S1 extract showed 144- and 265-bp amplified bands, respectively (Fig. 1c,d). These findings indicate that SD192 and SD296 can be used in the detection of the sporeless trait from TMIC-30058 by using real-time PCR and culture extracts.

Determination of sporulation phenotype in the breeding population by test cross in *P. pulmonarius* generally requires at least 60 days containing mating with tester strains, preparation of the culture seeds, and cultivation to obtain the mature fruiting bodies. Although the two previously developed STS markers, SD192 and SD296, solved the need for these lengthy processes, preparation of purified genomic DNA and electrophoretic analysis were required. Here we showed the method combined these STS markers and real-time PCR without the necessity of purified genomic DNA and electrophoretic analysis. This method makes possible the handling of many specimens in a day by one person. This time- and labor-saving method will be a valuable tool for efficient MAS under large-scale screening in the breeding of sporeless cultivars. To our knowledge, this is the first report of the real-time PCR-based method for useful mutation traits in mushroom breeding.

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References

Baars JJP, Sonnenberg ASM, Mikosch TSP, Van Griensven LJLD (2000) Development of a sporeless strain of oyster mushroom *Pleurotus ostreatus*. In: Van Griensven LJLD (ed) Mushroom science XIV: Proceeding of the 15th international congress on the science and cultivation of edible fungi. Balkema, Rotterdam, pp 317–323

Bodles WJ, Fossdal CG, Woodward S (2006) Multiplex real-time PCR detection of pathogen colonization in the bark and wood of *Picea sitchensis* clones differing in resistance to *Heterobasidion annosum*. Tree Physiol 26:775–782

Francois P, Renzi G, Pittet D, Bento M, Lew D, Harbarth S, Vaudaux P, Scharenzel J (2004) A novel multiplex real-time PCR assay for rapid typing of major staphylococcal cassette chromosome *mec* elements. J Clin Microbiol 42:3309–3312

Hausen BM, Schulz KH, Noster U (1974) Allergic disease caused by the spores of an edible fungus *Pleurotus florida*. Mushroom Sci 9:219–225

Heid CA, Stevens J, Livak KJ, Williams PM (1996) Real time quantitative PCR. Genome Res 6:986–994

Hibbett DS, Donoghue MJ (1996) Implication of phylogenetic studies for conservation of genetic diversity in Shiitake mushrooms. Conserv Biol 10:1321–1327

Maeta K, Ochi T, Tokimoto K, Shimomura N, Maekawa N, Kawaguchi N, Nakaya M, Kitamoto Y, Aimi T (2008) Rapid species identification of cooked poisonous mushrooms by using real-time PCR. Appl Environ Microbiol 74:3306–3309

Mason G, Caciagli P, Accotto GP, Noris E (2008) Real-time PCR for the quantitation of *Tomato yellow leaf curl Sarinia virus* in tomato plants and in *Bemisia tabaci*. J Virol Methods 147: 282–289

Murakami S (1993) Genetic and breeding of spore deficient strains in *Agrocybe cylindracea* and *Lentinus edodes*. In: Chang ST, Buswell JA, Chiu AW (eds) Mushroom biology and mushroom products. The Chinese University Press, Hong Kong, pp 63–69

Obatake Y, Murakami S, Matsumoto T, Fukumasa-Nakai Y (2003) Isolation and characterization of a sporeless mutant in *Pleurotus eryngii*. Mycoscience 44:33–40

Obatake Y, Murakami S, Matsumoto T, Fukumasa Y (2006) Development of a sporeless strain of *Pleurotus eryngii*. Nihon Kingakkai Nishinihon Shibukaiho 15:18–27

Ohira I (1979) Sporulation-deficient mutant in *Pleurotus pulmonarius* Fr. Trans Mycol Soc Jpn 20:107–114

Okuda Y, Murakami S, Matsumoto T (2009a) A genetic linkage map of *Pleurotus pulmonarius* based on AFLP markers, and localization of the gene region for the sporeless mutation. Genome 52:438–446

Okuda Y, Murakami S, Matsumoto T (2009b) Development of STS markers suitable for marker-assisted selection of sporeless trait in oyster mushroom, *Pleurotus pulmonarius*. Breeding Sci 59:315–319

Olsen JA (1987) *Pleurotus* spores as allergens. Mushroom J 172:115–117

Parladé J, Hortal S, Pera J, Galipienso L (2007) Quantitative detection of *Lactarius deliciosus* extraradical soil mycelium by real-time PCR and its application in the study of fungal persistence and interspecific competition. J Biotechnol 128:14–23

Sakula A (1974) Allergy to the spores of *Pleurotus florida*. Lancet 1:137

Simon HM, Jahn CE, Bergerud LT, Sliwinski MK, Weimer PJ, Willis DK, Goodman RM (2005) Cultivation of mesophilic soil crenarchaeotes in enrichment cultures from plant roots. Appl Environ Microbiol 71:4751–4760