J. Microbiol. Biotechnol. (2007), **17**(6), 1041–1044

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Genetic Variability and Phylogenetic Relationship Among Proton-Beam-Irradiated Strains of *Pleurotus ostreatus*

KWON, HYE JIN[1], YONG JIN PARK[2], YOUNG BOK YOO[3], SOON YOUNG PARK[3], AND WON SIK KONG[3]*

[1]*Doorae Institute of Environment-Friendly Agriculture, Seoul 137-850, Korea*
[2]*National Institute of Agricultural Biotechnology, R.D.A., Suwon 441-707, Korea*
[3]*National Institute of Agricultural Science and Technology, R.D.A., Suwon 441-707, Korea*

Received: October 30, 2006
Accepted: January 7, 2007

Abstract To assess the effects of a proton beam on oyster mushrooms (*Pleurotus ostreatus*), the genetic diversity and phylogenetic relationships among strains induced by a proton beam were investigated based on a clustering analysis. According to an AFLP DNA polymorphism analysis, the induced strains were divided into four groups that coincided with the dose. When applying proton-beam radiation, the dissimilarity among the induced strains increased when increasing the dose. When using more than 400 Gy, the genetic dissimilarity of the irradiated strains was 46–58%. Thus, evaluating the induced strains using the AFLP technique was effective in revealing the mutation effect of the proton beam.

Keywords: AFLPs, mutation, oyster mushroom (*Pleurotus ostreatus*), polymorphism, proton beam

It is well known that some mushrooms contain biologically active substances, exhibiting medicinal effects such as antitumor and antihypertensive activities [3, 7, 11]. The oyster mushroom, *Pleurotus ostreatus*, is commercially important in the world mushroom market, and especially appreciated in East Asia. In addition to its importance for food production, *P. ostreatus* also has effects on increasing macrophage and lymphocyte activities [8], reducing cholesterol levels [1], increasing antihepatoma and antisarcoma activities [16], and contains anticomplementary properties of polysaccharides [9].

Several recent studies have shown that gamma-ray radiation can change the genomic structure. For example, in diploid mutants of rice derived from gamma-ray irradiated tetraploids, the rDNA intergenic spacers (IGSs) were rearranged [4], and gamma-ray radiation induced positive or negative mutants of specific genes in yeast [13] and certain mushrooms [2, 17]. Similarly, proton-beam radiation can also change the genomic structure. For example, in *E. coli*, the optimum irradiating dose to obtain ion beam-induced mutations was approximately 1,000 Gy, where the cell cytotoxicity was dependent on the RecA protein. Among several selected mutant strains, the *ldh* mutant exhibited a genetically disrupted lactate production pathway and effectively accumulated PHB (polyhydroxybutyrate) (Cheong and Kim. 2005. Abstr. Annu. Meet. Kor. Soc. Biotechnol. Bioeng. Seoul, Korea).

Accordingly, this study was carried out to assess the effects of a proton beam on the radiation sensitivity of the mycelia of oyster mushrooms, and report on the genetic diversity and phylogenetic relationships among selected mutants based on a clustering analysis.

Chunchu-2, a commercial strain of *P. ostreatus* stocked at the National Institute of Agricultural Science and Technology, was used in the experiment. The mycelia of Chunchu-2 were treated with a proton beam (MC-50 Cyclotron, KIRAMS, Korea) at a dose of 10–2,000 Gy, following the same methodology as previously reported by Kwon and Kong [10]. Mutants treated with the proton beam were then selected based on their mycelial growth on Potato Dextrose Agar (PDA, Difco, pH 5.6) at room temperature. Ten strains for each dose from 10 to 750 Gy (R10 to R750) were isolated from the irradiated mycelia. There were no viable mycelia at ≥1,000 Gy.

To determine the fruiting phenotype, the selected colonies were cultivated in 570 g of a poplar sawdust substrate containing 20% rice bran and 65% deionized water that had been autoclaved for 90 min at 121°C. Each bottle was inoculated with 4 agar plugs and incubated at 25°C for 25 days in the dark. After the mycelia had covered the sawdust media, the conditions for fruiting were promoted by opening the bottles, removing the old spawn, and placing the bottles in a growth chamber at 16±2°C with about 93% relative humidity. The cultures were illuminated at 500 Lux for 14 h

*Corresponding author
Phone: 82-31-290-0377; Fax: 82-31-290-0209;
E-mail: wskong@rda.go.kr

Table 1. Sequences of adaptors and primers selected from 10-primer combination DNA fingerprinting by AFLP analysis.

Adaptors and primers	Nucleotide sequences (5'-3')	Application
MseI adapter	5'-GACGATGAGTCCTGAG TACTCAGGACTCAT-3'	Adapter ligation
PstI adapter	5'-CTCGTAGACTGCGTACATGCA CATCTGACGCATGT-3'	Adapter ligation
M00 (MseI+0)	5'-GATGAGTCCTGAGTAA-3'	Preamplification
M40 (MseI+3)	5'-GATGAGTCCTGAGTAA CAG-3'	Selective
M61 (MseI+3)	5'-GATGAGTCCTGAGTAA CTG-3'	Amplification
M70 (MseI+3)	5'-GATGAGTCCTGAGTAA GCT-3'	
M78 (MseI+3)	5'-GATGAGTCCTGAGTAA GTT-3'	
M80 (MseI+3)	5'-GATGAGTCCTGAGTAA TAC-3'	
M92 (MseI+3)	5'-GATGAGTCCTGAGTAA TTC-3'	
P00 (PstI+0)	5'-GAGCTGCGTACATGCAG-3'	Preamplification
P63 (PstI+3)-6FAM*	5'-GAGCTGCGTACATGCAGGAA-3'	Selective
P64 (PstI+3)-HEX	5'-GAGCTGCGTACATGCAG GAC-3'	Amplification
P65 (PstI+3)-NED	5'-GAGCTGCGTACATGCAG GAG-3'	
P75 (PstI+3)-6FAM	5'-GAGCTGCGTACATGCAG GTA-3'	
P76 (PstI+3)-HEX	5'-GAGCTGCGTACATGCAG GTC-3'	
P77 (PstI+3)-NED	5'-GAGCTGCGTACATGCAG GTG-3'	
Primer combinations for selective amplification	P63-M40, P63-M78, P63-M80, P63-M92, P64-M92, P75-M61, P75-M92, P76-M70, P76-M92, P77-M80 (Total 10 combinations)	

*6FAM, HEX, and NED: Fluorescent dye.

per day. Primordia appeared after a further 4–8 days of growth, and basidiocarps were harvested 5 days later. The fruiting phenotypes of the variants were examined at the most advanced stage of fruiting. Three strains were selected from the 400, 500, and 750 Gy proton-beam treatments, their DNA extracted using the method described by Kim *et al.* [6], and an AFLP analysis conducted with ten primer sets.

The Amplified Fragment Length Polymorphism (AFLP) analysis was performed according to Vos *et al.* [15], and GenoGrapher software (Benham, J. J. 2001 Ver.1.6.0 Montana State Univ., Boston, U.S.A.) displayed a sized and straightened gel image reconstructed from the lanes. The polymorphic AFLP bands were denoted as 1 or 0 for presence or absence, respectively, and transferred into a data matrix. A dendrogram was then constructed based on the coded data matrix and a Cluster analysis performed among the accessions within each treatment using the NTSYS computer program [14] for the grouping based on qualitative characters.

The AFLP patterns for the 9 treatments were analyzed using 10 primer combinations (Table 1). For 8 treatments, the genetic dissimilarities among the irradiated mycelia increased in the range of 6–58% compared with the control (Table 2), with a 46–58% genetic dissimilarity observed for the groups irradiated with 400 Gy or more and a 6–33% genetic dissimilarity for the groups irradiated with ≤200 Gy, suggesting that the genetic dissimilarity alteration was proportional to the dose of proton-beam radiation. The dendrogram based on the AFLP gel images revealed two major clusters (Fig. 1), where treatment with more than 200 Gy (R200) comprised the second cluster, with more severe changes in the genomic structures compared with the first cluster.

Previously, Lee and Chang [12] reported that the genetic similarity of mycelia and basidiospores was altered according to the dose of gamma-ray radiation, with 22–25% changes for basidiospores and 23–36% for mycelia. Kwon and Kong [10] also found that the optimum proton-beam dose

Table 2. Genetic dissimilarity coefficient matrix among strains of *Pleurotus ostreatus* irradiated by proton-beam radiation based on AFLP markers.

Treatments	Control	R10*	R20	R50	R100	R200	R400	R500	R750
Control	0.000								
R10	0.061	0.000							
R20	0.208	0.186	0.000						
R50	0.309	0.248	0.128	0.000					
R100	0.092	0.075	0.223	0.273	0.000				
R200	0.334	0.318	0.294	0.206	0.304	0.000			
R400	0.584	0.512	0.456	0.428	0.399	0.184	0.000		
R500	0.463	0.394	0.422	0.366	0.426	0.170	0.046	0.000	
R750	0.447	0.439	0.431	0.401	0.328	0.107	0.249	0.346	0.000

*R10–R750: Proton-beam dose from 10 to 750 Gy.

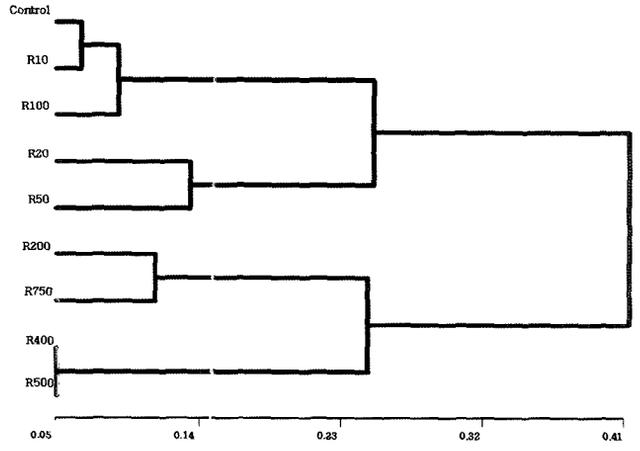

Fig. 1. Dendrogram showing cluster analysis of genetic dissimilarity estimates for *Pleurotus ostreatus* accessions from AFLPs.
R10–R750: Proton-beam dose from 10 to 750 Gy.

as a mutation source was between 500 Gy and 750 Gy, and 400 Gy and 1,000 Gy, for the basidiospores and mycelia of oyster mushrooms, respectively. The present results also showed wider dissimilarities between 400 Gy and 750 Gy. The mycelia did not survive at ≥1,000 Gy.

Ten strains from each treatment were cultivated in 850-ml bottles filled with poplar sawdust. Some of the strains grew very slowly, whereas others failed to form fruiting bodies, even with complete mycelial growth in the bottle. The developmental process of *P. ostreatus* is divided into two stages; the primordia and fruiting body stages. Primordia usually develop into adult fruiting bodies within a few days. Yet, some strains did not mature, referred to as maturationless mutants. The other morphological mutants varied in terms of the color and shape of the fruiting body (Fig. 2), where the color ranged from grey to brown and dark grey, and the shapes ranged from convex to funnel

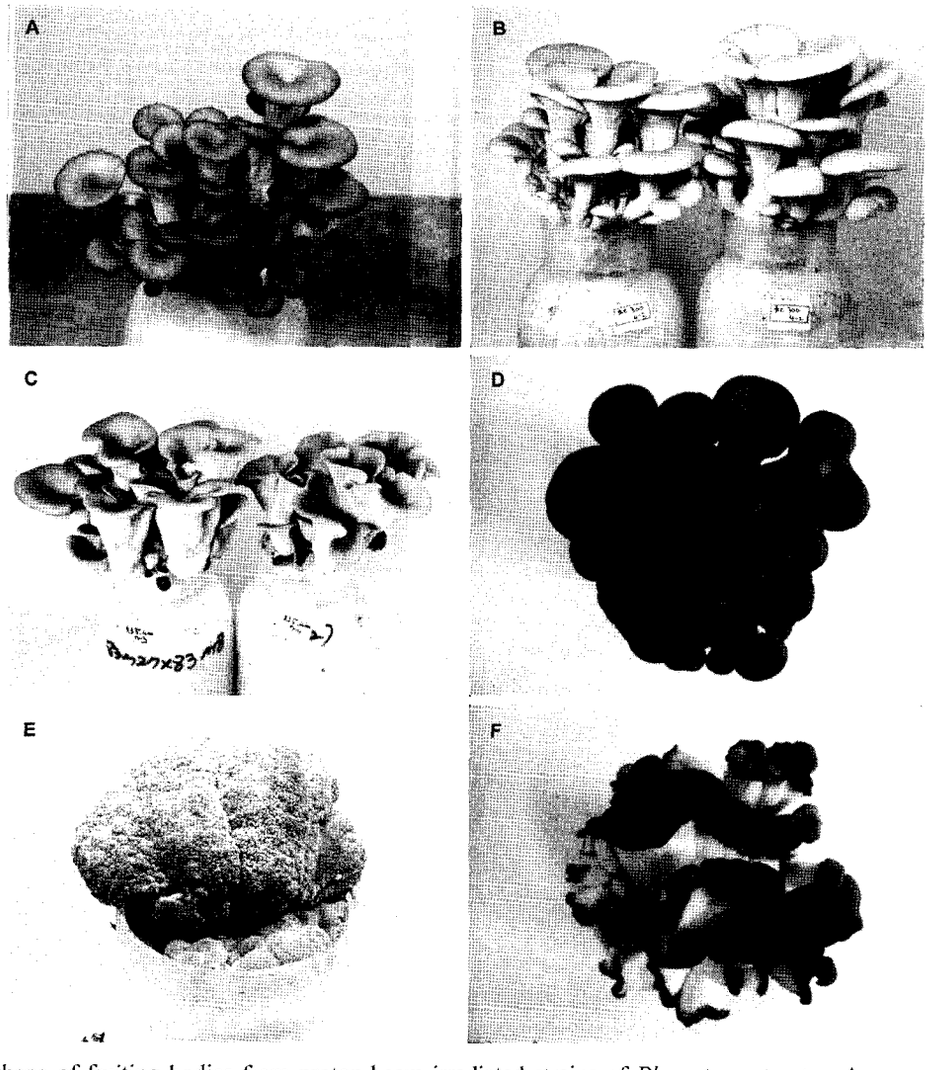

Fig. 2. Color and shape of fruiting bodies from proton-beam-irradiated strains of *Pleurotus ostreatus*: **A**, normal fruiting body; **B**, blueish-grey mutant; **C**, brown mutant; **D**, dark-grey mutant; **E**, maturationless mutant; and **F**, malformed mutant.

and some were malformed. However, since the morphological traits of color and shape are usually very flexible according to the cultivation environment, a genetic mutation can only be confirmed in future generations. Three strains with normal fruiting bodies after R400, R500, and R750 treatment were collected and analyzed by AFLP to determine any changes to their genomic structure (data not shown). Despite the formation of normal fruiting bodies, the band patterns showed a wide variability. However, these patterns could not explain the diversity of each accession, plus the discrepancy may have been due to the use of restricted morphological information. Nonetheless, the results still indicated some changes in the genomic structure that did not affect the morphology of the mushroom fruiting bodies and that may include some latent possibilities. Furthermore, mutants were generated from all the treatments, yet no tendency was noted relative to the beam strength, suggesting that the mutation patterns of the proton-beam-irradiated *P. ostreatus* were random and diverse, rather than progressive.

Joh *et al.* [5] developed mutants from 3,000 clones of *P. ostreatus* protoplasts using UV light and classified 6 groups: auxotrophic strains, abnormal vegetative strains, primordiumless strains, maturationless strains, specifically colored strains, and poorly spored strains. In the present experiment, the mutant spectrum was almost the same as that for the UV mutants, in spite of the small population, as only ten strains were tested from each treatment. With a large population, more diverse mutants would be expected. In conclusion, the mutant strains from the present study will be very useful for genetic breeding programs and studies on fungal development and genomics.

Acknowledgment

This work was supported by the Korean Ministry of Science and Technology through the Proton Accelerator User Program (No. M202AK010021-04A1101-02110) of the Proton Engineering R&D Project from the Nuclear R&D Program and 21st Century Frontier R&D Program.

References

1. Bobek, P., S. Galbavy, and L. Ozdin. 1998. Effect of oyster mushroom (*Pleurotus ostreatus*) on pathological changes in dimethylhydrazine-induced rat colon cancer. *Oncol. Rep.* **5:** 727–730.
2. Boominathan, K., S. B. Dass, T. A. Randall, and C. A. Reddy. 1990. Nitrogen-deregulated mutants of *Phanerochaete chrysosporium* - a lignin-degrading basidiomycete. *Arch. Microbiol.* **153:** 521–527.
3. Chihara, G., J. Hamuro, Y. Maeda, Y. Arai, and F. Fukuoka. 1970. Fractionation and purification of the polysaccharides with marked antitumor activity, especially lentinan, from *Lentinus edodes* (Berk.) Sing. (an edible mushroom). *Cancer Res.* **30:** 2776–2781.
4. Fukuoka, H., Y. Kageyama, K. Yamamoto, and G. Takeda. 1994. Rapid conversion of rDNA intergenic spacer of diploid mutants of rice derived from gamma-ray irradiated tetraploids. *Mol. Gen. Genet.* **243:** 166–172.
5. Joh, J. H., B. G. Kim, W. S. Kong, Y. B. Yoo, K. S. Chu, N. K. Kim, H. R. Park, B. G. Cho, and C. S. Lee. 2004. Isolation and characterization of dikaryotic mutants from *Pleurotus ostreatus* by UV irradiation. *Mycobiology* **32:** 88–94.
6. Kim, K. J., S. H. Eom, S. P. Lee, H. S. Jung, S. Kamoun, and Y. S. Lee. 2005. A genetic marker associated with the A1 mating type locus in *Phytophthora infestans*. *J. Microbiol. Biotechnol.* **15:** 502–509.
7. Koo, K. C., D. H. Lee, J. H. Kim, H. E. Yu, J. S. Park, and J. S. Lee. 2006. Production and characterization of antihypertensive angiotensin I-converting enzyme inhibitor from *Pholiota adipose*. *J. Microbiol. Biotechnol.* **16:** 757–763.
8. Kurashige, S., Y. Akuzawa, and F. Endo. 1997. Effects of *Lentinus edodes*, *Grifola frondosa*, and *Pleurotus ostreatus* administration on cancer outbreak, and activities of macrophages and lymphocytes in mice treated with a carcinogen, N-butyl-N-butanolnitrosoamine. *Immunopharmacol. Immunotoxicol.* **19:** 175–183.
9. Kweon, M. H., H. Jang, W. J. Lim, H. I. Chang, C. W. Kim, H. C. Yang, H. J. Hwang, and H. C. Sung. 1999. Anti-complementary properties of polysaccharides isolated from fruit bodies of mushroom *Pleurotus ostreatus*. *J. Microbiol. Biotechnol.* **9:** 450–456.
10. Kwon, H. J. and W. S. Kong. 2006. Proton beam sensitivity of basidiospore and mycelium in *Pleurotus ostreatus*. *Kor. J. Mycol.* **34:** 34–38.
11. Lee, J. S., H. S. Baik, and S. S. Park. 2006. Purification and characterization of two novel fibrinolytic proteases from mushroom, *Fomitella fraxinea*. *J. Microbiol. Biotechnol.* **16:** 264–271.
12. Lee, Y. and H. Chang. 1999. Radiation sensitivity of basidiospore and mycelium in *Pleurotus ostreatus*. *J. Kor. Nuclear Soc.* **31:** 287–293.
13. Nam, K. S. 2002. Low-dose gamma irradiation as means of isolating carotenoid-hyperproducing yeast mutant. *J. Microbiol. Biotechnol.* **12:** 1010–1012.
14. Rohlf, F. J. 1992. Numerical taxonomy and multivariate analysis system, NTSYS-PC program. Applied Bio-statistics Inc., 3 Heritage Lane, Setauket, New York 11733.
15. Vos, P., R. Hogers, M. Bleeker, M. Reijans, T. van de Lee, M. Hornes, A. Frijters, J. Pot, J. Peleman, M. Kuiper, *et al.* 1995. AFLP: A new technique for DNA fingerprinting. *Nucleic Acids Res.* **23:** 4407–4414.
16. Wang, H., J. Gao, and T. B. Ng. 2000. A new lectin with highly potent antihepatoma and antisarcoma activities from the oyster mushroom *Pleurotus ostreatus*. *Biochem. Biophys. Res. Commun.* **275:** 810–816.
17. Zolan, M. E., C. J. Tremel, and P. J. Pukkila. 1988. Production and characterization of radiation-sensitive meiotic mutants of *Coprinus cinereus*. *Genetics* **120:** 379–387.