Mycoscience 37: 459–461, 1996

Short Communication

Isolation and characterization of *Pleurotus ostreatus* mutant strains resistant to a carboxin-derived fungicide, flutolanil

Yoichi Honda, Toshikazu Irie, Mina Atsuji, Takashi Watanabe and Masaaki Kuwahara

Wood Research Institute, Kyoto University, Gokasho, Uji 611, Japan

Accepted for publication 11 October 1996

To develop a dominant genetic marker in *Pleurotus ostreatus*, mutant strains resistant to a carboxin-derived fungicide, flutolanil, were isolated. These mutants included strains which showed resistance to 50-fold higher concentration of flutolanil than the wild-type strain, even after successive cultivations in the absence of the drug. Dominance of the phenotype was confirmed by back-crossing between the resistant and wild-type monokaryons. The flutolanil-resistance was also shown to be stably inherited by the basidiospore-derived progenies of the mutant strains.

Key Words——carboxin; fungicide; genetic marker; *Pleurotus ostreatus*; resistant mutant.

Basidiomycetous fungi have received considerable attention from various biological and industrial points of view. For instance, the abilities of white rot fungi to degrade plant cell wall lignin and a variety of environmental pollutants, including dioxins and PCBs, have been the focus of intense research interest in the last decade (Eaton, 1985; Spadaro et al., 1992; Valli et al., 1992a, b; Gold and Alic, 1993). To elucidate the mechanism of such specific properties of basidiomycetes at the molecular level, it is necessary to introduce gene engineering techniques, such as cloning, expression, disruption and replacement of a gene of interest, into the biological analysis of basidiomycetes. For development of a useful vector system convenient for DNA-mediated transformation in basidiomycetes, it is desired to isolate a dominant marker gene which requires no particular genetic background of the host strain. Flutolanil (α,α,α-trifluoro-3′-isopropoxy-*O*-toluanilide) is a systematic fungicide derived from carboxin and widely used in the fields to control smut diseases (Edgington et al., 1966; Ackrell et al., 1977). Carboxin and its derivatives are highly active against basidiomycetous fungi and inhibit respiration by preventing the oxidation of succinate by a tricarboxylic acid cycle enzyme, succinate dehydrogenase (Sdh: EC 1.3.99.1). In this report, we describe the isolation and genetic characterization of flutolanil-resistant mutants of a white rot fungus, *Pleurotus ostreatus* (Jacq.: Fr.) Kummer, which should meet the prerequisite conditions for developing a vector system based on flutolanil resistance in the basidiomycete.

Pleurotuo ostreatus dikaryotic strains #261 (ATCC 66376) and IS1 (our laboratory stock) were used as flutolanil-sensitive wild-type strains (Peng et al., 1992). Maintenance and growth-rate measurement of *P. ostreatus* strains were done using 3.9% potato-dextrose agar (PDA) medium, Nissui, at 25°C. The appropriate concentration of flutolanil to inhibit mycelial growth was measured by culturing several wild-type *P. ostreatus* strains on PDA medium containing 1–10 μg/ml of flutolanil. Mycelial growth of all strains tested was completely inhibited at 2.5 μg/ml of flutolanil. The drug resistance thereafter was assayed on PDA media containing 3 μg/ml of flutolanil unless otherwise stated.

To isolate flutolanil-resistant *P. ostreatus* mutant strains, basidiospores from the fruiting body of #261 and IS1 were subjected to ultraviolet (UV) irradiation on PDA plates containing 3 μg/ml flutolanil, then incubated at 25°C for 12 h in the dark, followed by additional incubation at 25°C for 3–4 wk. Colonies grown on the first screening plate were isolated for subculture and reincubated on fresh flutolanil-containing PDA medium. After the second screening on flutolanil-containing plates, a total of 29 isolates were proved to have acquired increased resistance to the drug. On microscopic observation, some of these resistant mutants were shown to have the clamp connections on their mycelium. It was plausible that these dikaryotic strains originated from matings between flutolanil-resistant mutant monokaryons and other monokaryons on the first screening plate.

Five dikaryotic mutant strains derived from strain #261, namely MA203, MA206, MA219, MA245 and MA246, showed higher drug resistance, up to 100 μg/ml of flutolanil, and were analyzed in further experiments. After five successive cultures on PDA plates in the absence of flutolanil, these mutant strains retained their resistance to the drug, indicating that the flutolanil-resistant phenotype is maintained stably during mitotic cell division. The mycelial growth rates of the dikaryotic mutant strains were measured on PDA plates in the presence or absence of flutolanil and compared with that

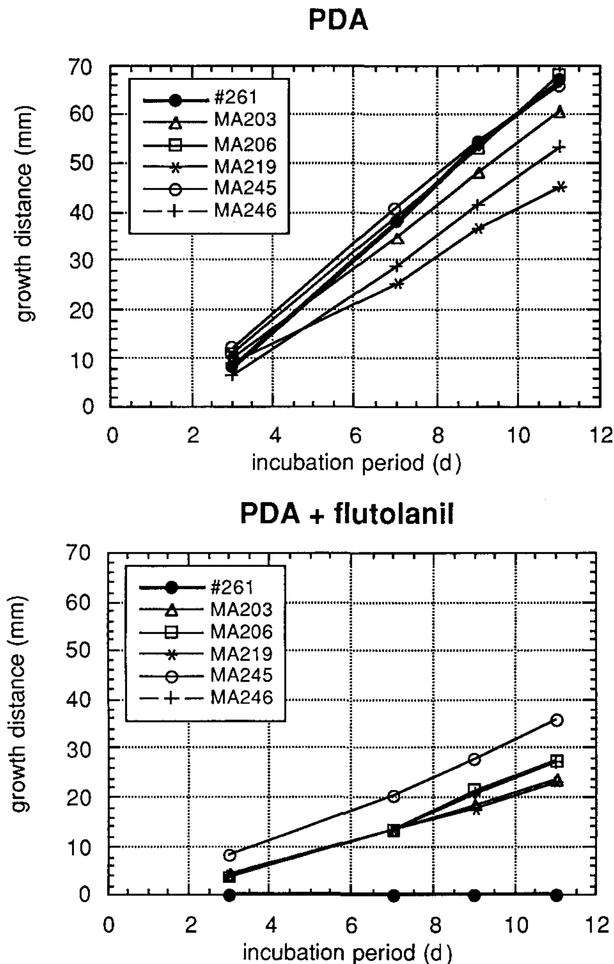

Fig. 1. Mycelial growth of mutant and wild-type strains in the absence and presence of flutolanil.
Symbols represent mycelial growth distances of each strain after the inoculation of a 5-mm plug of precultured mycelium on PDA plates containing no or 3 μg/ml of flutolanil. All values are the average of three independent determinations.

of wild-type strain #261 (Fig. 1). On PDA medium, mycelial growth rates of these mutants were similar to or slightly slower than that of strain #261. In contrast, in the presence of flutolanil, these mutant strains continued to grow, while strain #261 showed no mycelial growth. The growth rates of the mutant strains on the flutolanil-containing medium were up to 40–55% of their growth rates on the normal PDA medium.

The phenotypes of monokaryotic strains obtained from regenerated protoplasts of the dikaryotic mutant strain, MA203, were investigated. Preparation and regeneration of protoplasts were performed following the method of Eguchi et al. (1990) with minor modifications. Regenerated colonies were isolated and transferred to fresh PDA medium. Isolates with no clamp connections were selected and assayed as protoplast-derived monokaryons. A total of 20 monokaryons were obtained. Of these, 13 were shown to be resistant and 7 to be sensitive to flutolanil. The appearance of both of resistant and sensitive phenotypes in monokaryons from MA203 protoplasts strongly suggests that this dikaryotic strain originated from mating between flutolanil-resistant and -sensitive monokaryons and that the drug-resistant phenotype is dominant.

To confirm the dominance of the drug resistance in the mutant strains, the phenotype of dikaryotic strains obtained from matings between resistant and sensitive monokaryons was investigated. As expected, dikaryons between the flutolanil-resistant and -sensitive monokaryons from MA203 protoplasts were found to be resistant to the drug. Moreover, back-crossings of the resistant monokaryons from MA203 and MA206 protoplasts with wild-type monokaryons derived from germinated basidiospores of strain #261 also resulted in flutolanil-resistant dikaryons. These data indicate that the flutolanil-resistant phenotypes of these two mutants are dominant.

Drug sensitivity of the F_1 progenies obtained from the basidiospores of the mutant dikaryotic strains was tested to analyze meiotic stability of the resistant phenotype. Among 32 and 31 F_1 progenies from MA203 and MA206, 16 and 10 were proved to be able to grow on PDA plates containing 10 μg/ml of flutolanil, respectively. These data strongly suggest that the flutolanil-resistant phenotype is controlled by genomic element(s) and segregated stably during meiotic cell divisions in these mutant strains.

The experimental results presented here demonstrate that the flutolanil resistance should be a useful genetic marker which is dominant and maintained stably during mitotic and meiotic cell divisions. The dominant genetic marker could serve in positive screening strategies in which, for example, protoplast fusion might be used for the breeding of basidiomycetes. For development of a marker gene for use in the genetic transformation of *P. ostreatus*, the gene(s) conferring resistance to flutolanil needs to be isolated. In the maize smut pathogen, *Ustilago maydis* (DC) Corda, a mutant gene encoding a modified form of the Sdh Iron-sulphur (Ip) subunit protein, *oxr-1B*, was cloned and used as an efficient marker gene in the construction of a transformation vector plasmid (Keon et al., 1991). In this mutant gene, an amino-acid change (His253 to Leu) in the third cystein-rich cluster of the Ip subunit protein has been proved to confer resistance to carboxin (Broomfield and Hargreaves, 1992). Cloning and analysis of the gene encoding Sdh Ip subunit protein from both of the wild-type and flutolanil-resistant *P. ostreatus* strains are now in progress.

Acknowledgements——We are extremely grateful to Dr. M. Endo, Nihon Nohyaku Co. Ltd., for the gift of flutolanil. We also acknowledge Dr. T. Sato, Iwate Biotechnology Research Center, and Mr. M. Nakaya, Hokkaido Forest Products Research Institute, for their help and advice. This work was supported partially by a grant from Nogeikagaku Kenkyu Shoreikai and a Grant-in-Aid for Encouragement of Young Scientist from the Ministry of Education, Science and Culture, Japan (to Y.H).

Literature cited

Ackrell, B. A. C., Kearney, E. B., Coles, C. J., Singer, T. P., Beinert, H., Wan, Y.-P. and Folkers, K. 1977. Kinetics of the reoxidation of succinate dehydrogenase. Arch. Biochem. Biophys. **182**: 107–117.

Broomfield, P. L. E. and Hargreaves, J. A. 1992. A single amino-acid change in the iron-sulphur protein subunit of succinate dehydrogenase confers resistance to carboxin in *Ustilago maydis*. Curr. Genet. **22**: 117–121.

Eaton, D. C. 1985. Mineralization of polychlorinated biphenyls by *Phanerochaete chrysosporium*, a ligninolytic fungus. Enzyme Microb. Technol. **7**: 1194–196.

Edgington, L. V. Watson, G. S. and Miller, P. M. 1966. Fungicide selective for basidiomycetes. Science **153**: 307–308.

Eguchi, F., Tashiro, M., Suzuki, T. and Higaki, M. 1990. Preparation and regeneration of protoplasts of edible mushrooms. Mokuzai Gakkaishi **36**: 232–240

Gold, M. H. and Alic, M. 1993. Molecular biology of the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. Microbiol. Rev. **57**: 605–622.

Keon, J. P. R., White, G. A. and Hargreaves, J. A. 1991. Isolation, characterization and sequence of a gene conferring resistance to the systematic fungicide carboxin from the maize smut pathogen, *Ustilago maydis*. Curr. Genet. **19**: 475–481.

Peng, M., Singh, N. K. and Lemke, P. A. 1992. Recovery of recombinant plasmids from *Pleurotus ostreatus* transformants. Curr. Genet. **22**: 53–59.

Spadaro, J. T., Gold, M. H. and Renganathan, V. 1992. Degradation of azo dyes by the lignin-degrading fungus *Phanerochaete chrysosporium*. Appl. Environm. Microbiol. **58**: 2397–2401.

Valli, K., Brock, B. J., Joshi, D. K. and Gold, M. H. 1992a. Degradation of 2,4-dinitrotoluene by the lignin-degrading fungus *Phanerochaete chrysosporium*. Appl. Environm. Microbiol. **58**: 221–228.

Valli, K., Wariishi, H. and Gold, M. H. 1992b. Degradation of 2,7-dichlorodibenzeo-*p*-dioxin by the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. J. Bacteriol. **174**: 2131–2137.