Preliminary

Genetic Transformation of white Rot Fungus *Pleurotus ostreatus* to Carboxin Resistance using Electroporation*[1]

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Introduction

White rot basidiomycetes, such as *Phanerochaete chrysosporium* and *Pleurotus ostreatus*, are known to be able to degrade plant cell wall lignin extensively. Extracellular enzymes secreted by these fungi, including lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Lac), have been demonstrated to be major components of the lignin biodegradation systems[1,2]. The fungal lignin degrading systems are also the focus of considerable research attention because of their ability to degrade a variety of organic pollutants including PCBs and dioxins[3,4].

Although expression of genes encoding ligninolytic enzymes has been studied using various host organisms, including *Esherichia coli*, yeast, *Aspergillus* spp, and Bacuro virus system, recombinant enzymes obtained with these heterologous systems had unusual properties such as elevated molecular weights, requirements of re-folding and low specific activities[5–7]. A homologous gene expression system can provide an enzyme in both active and native form, which makes it possible to characterize the enzyme properties and to elucidate its physiological role in the biological functions. Recently, we have developed a DNA-mediated transformation system using a drug resistant marker gene to a systemic fungicide, carboxin. And a homologous gene expression of *P. ostreatus* MnP3 isozyme was achieved using the transformation system[8]. Besides its potential as an effective producer of MnP enzyme, the MnP-overproducing transformants have possibilities for advantages in mycelial treatment of lignin or aromatic pollutants.

While molecular breeding by genetic transformation itself is a powerful tool in investigation and development of specific functions of wood rot fungi, to achieve a gene targeting or functional cloning, new transformation protocol with even higher transformation efficiency should be developed. Here we reprot on DNA-mediated transformation of *P. ostreatus* using electroporation procedure.

Materials and Methods

P. ostreatus dikaryotic strain #261(ATCC 66376)was used as a host strain. Protoplasts were prepared by digesting liquid-cultured mycelium with a lysing enzyme solution containing 0.1% protoplasting enzyme (Mushroom Experimental Station, Horst, The Netherlands), 0.5% Zymolyase 20 T (SEIKAGAKU Corp., Tokyo), and 0.2% Chitinase (Sigma, St, Louis, MO, USA). Protoplasts were mixed with 1–10 μg of pTM1 DNA[9] in SMYM (1% sucrose, 1% malt extract, 0.4% yeast extract and 0.5 M mannitol) liquid medium. Electroporation experiments were conducted on SMYM-suspended protoplasts using Gene Pulser II (Bio-Rad Laboratories, Richmond, CA, USA). Following delivery of the electric pulse, the suspension was added to 5 ml of SMYM liquid medium and incubated at 28°C for 2–5 days prior to plating on SMYM agar plates containing 1 μg/ml of carboxin (AccuStandard Inc., New Haven, CT, USA). Drug resistant colonies were subcultured individually on PDA (potato-dextrose-agar, Nissui, Tokyo) plate containing 1 μg/ml of carboxin for the secondly screening. Detection of the introduced sequence in the drug resistant strains was carried out by specific amplification of the Cbx^R marker sequence with PCR. The primers used in the PCR were M13 (5′-GTAAAACGACGGCCAGT-3′) and SDH7 (5′-CACACA AATCATT GAACC-3′). Each PCR cycle

Table 1. Number of the drug-resistant colonies.

Exp.	Protoplasts ($\times 10^8$)	Cuvette gap (cm)	Voltage (V)	τ (msec)	No. of colonies
1	1.0	0.4	1,020	6.12	1
2	2.0	0.4	1,020	7.74	12
3	1.6	0.4	1,030	7.74	27
4	4.0	0.4	1,030	6.46	1
5	4.0	0.2	490	2.68	1
6	1.5	0.2	490	3.28	2
7	2.0	0.2	480	3.20	2
8	1.3	0.4	500	3.98	2
9	1.2	0.4	500	3.14	2
10	3.2	0.4	1,030	7.52	6
11	3.2	0.4	2,030	5.72	1
12	3.2	0.4	1,510	6.40	2

τ = capacitance × resistance.

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consisted of template denaturation at 98°C for 30 sec, primer annealing at 52°C for 1 minm and DNA extension at 72°C for 1 min. After 25 cycles, an additional extension step of 10 min at 72°C was included. All the PCR-amplified fragments were analysed on a 0.8% agarose gel.

Results and discussion

Electroporation experiments were conducted on several conditions summerized in Table 1, and in each experiments, germinating colonies were observed on the first screening plates containing 1 μg/ml of carboxin. Total 59 drug resistant colonies were obtained and, in the best condition, 27 drug resistant colonies were obtained at field strength of 2.58 kV/cm and time constant of 7.74 msec. These colonies were isolated and subcultured on PDA plates containg the same concentration of the drug. All the isolated colonied showed continuous growth in the presence of carboxin suggesting that they were not transient transformants with unstable drug resistance.

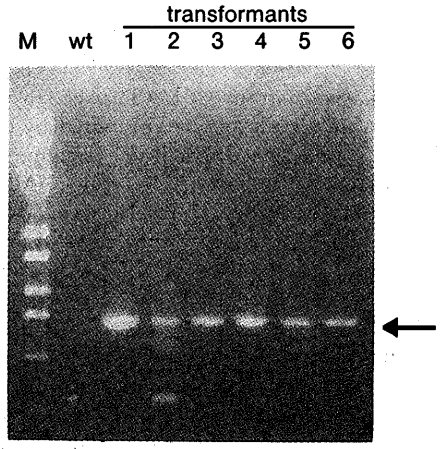

Fig. 1. Detection of *Cbx*^{R} sequence in the transformants by specific amplification with PCR. Lanes are: M, *Eco*T14I-digestd lambda phage DNA as a molecular standerd; wt, wild-type #261 strain as a negative control; lane 1–6, transformants. Bands with expected size (1.3 kb) were indicated by an arrow.

The presence of the introduced DNA sequence was checked by PCR using primers which specifically anneals to the *Cbx*^{R} construct[9] (Fig. 1). No fragments were amplified when genomic DNA extracted from wild type host strain was used as a template (lane wt). Whereas fragments of the expected size (1.3 kb) were amplified when DNA from the drug resistant strains were used as a temple (lane 1–6). These results indicated that the *Cbx*^{R} sequence was introduced and maintained in the drug resistant strains. Preliminary results of Southern blot analysis suggested that the *Cbx*^{R} DNA sequence was integrated ectopically into the chromosomal DNA with multiple copy numbers in these drug resistant strains (data not shown).

It was demonstrated that electroporation method is an alternative procedure for transformation of *P. ostreatus* to carboxin resistant, however, its efficiency is as high as that obtained by transformation of the PEG/$CaCl_2$ method.

References

1) M.H. Gold and M. Alic: *Microbiol.Rev.*, **57**, 605–622 (1993).
2) D. Cullen: *J. Biotechnol.*, **53**(2–3), 273–289 (1997).
3) J.A. Bumpus, M. Tien, D. Wright and S.D. Aust: *Science* **228**, 1434–1436 (1985).
4) K. Valli, H. Wariishi and M.H. Gold: *J. Bacteriol.*, **174**(7), 2131–2137 (1992).
5) R.E. Whitwam, I.G. Gazarian and M. Tien: *Biochem. Biophys. Res. Commun.*, **216**(3), 1013–1017 (1995).
6) P. Stewart, R.E. Whitwam, P.J. Kersten, D. Cullen and M. Tien: *Appl. Environ. Microbiol.*, **62**(3), 860–864 (1996).
7) F.J. Ruiz-duenas, M.J. Martinez and A.T. Martinez: *Appl. Environ. Microbiol.*, **65**(10), 4705–4707 (1999).
8) T. Irie, Y. Honda, T. Watanabe and M. Kuwahara: *Appl. Microbiol. Biotechnol.*, **55**(5), 566–570 (2001).
9) Y. Honda, T. Matsuyama, T. Irie, T. Watanabe and M. Kuwahara: *Curr. Genet.*, **37**(3), 209–212 (2000).