Preliminary

Development of a Transformation System in White-rot Fungus *Pleurotus ostreatus*[*1]

Takuro MATSUYAMA[*2], Yoichi HONDA[*2],
Takashi WATANABE[*2], Masaaki KUWAHARA[*2]

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Introduction

Pleurotus ostreatus is a white-rot basidiomycete fungus which is considered to be a good model for understanding the biochemical processes involved in lignin degradation. It is also a commercially important edible mushroom. In this research we aimed to establish a useful transformation system in *P. ostreatus* by developing a dominant selection marker gene. This technique can be used for molecular breeding and also permit us to combine molecular and biochemical analysis of the enzymes involved in lignin biodegradation.

It has been reported that *P. ostreatus* was transformed using a recombinant bacterial hygromycin resistant gene fused to *Aspergillus nidulans* expression signals[1,2]. However, this event does not show reproductivity in other laboratories including ours. In the present research, we aimed to develop a drug-resistant marker gene which is homologous to *P. ostreatus* itself. In the plant pathogenic fungus *Ustilago maydis*, a gene called Cbx^R which encodes a mutant iron-sulfur protein (Ip) subunit of succinate dehydrogenase (SDH) has been shown to confer dominant resistance to a systemic fungicide, carboxin[3]. It was demonstrated that the mutation causes substitution of a histidine residue (His257) in the cysteine-rich cluster III to leucine[4]. We have cloned and analyzed genomic and cDNA sequences of *sdi1* encoding Ip subunit of SDH from wild-type *P. ostreatus*[5,6]. It was shown that the corresponding histidine residue (His239) was conserved in *P. ostreatus sdi1* sequence. In this research, we demonstrated that a similarly modified *P. ostreatus sdi1* works as a dominant selection marker for transformation of this fungus to carboxin resistance.

Materials and Methods

A monokaryotic strain of *P. ostreatus*, #261-23 was isolated from single basidiospore of wild-type dikaryon #261 (ATCC66376) and used as a host strain for transformation unless otherwise stated. Transformants were grown on PDA medium containing 2 μg/ml carboxin,

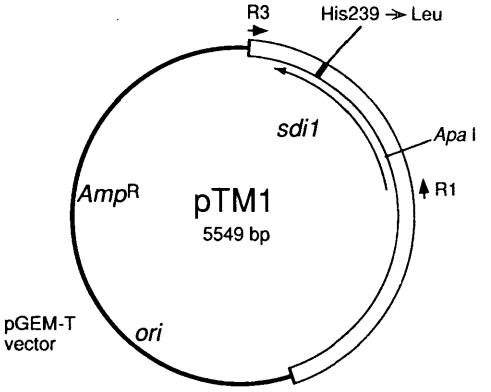

Fig. 1. Physical map of pTM1. Open box represents DNA sequence of *P. ostreatus sdi1* and direction of the transcription is indicated by an arrow. Position of the point mutation and the recognition site for *Apa*I were indicated. Small arrows represent location of the primer molecules used for amplification of *sdi1* sequence.

Wako chemicals.

A point mutation (His239 to Leu) which will cause the same amino acid substitution as in *U. maydis* Cbx^R was introduced into a allele of *P. ostreatus sdi1* gene, using a special kit for *in vitro* site-directed mutagenesis, Mutan-Super Express Km, *Takara*. The mutant *sdi1* fragment was cloned on pGEM-T vector, Promega, to produce pTM1 (Fig. 1) and used for transforming *P. ostreatus*. This sequence contains a recognition site of the restriction enzyme *Apa*I which does not exist in different allelic *sdi1* sequence of #261-23[6].

Transformation was carried out using PEG/$CaCl_2$ procedure. Protoplasts of *P. ostreatus* were mixed with 1 μg of pTM1 DNA in 25% PEG buffer containing 25 mM $CaCl_2$ under osmotic stabilizer. After incubation on ice for 20 min, all cells were poured onto regeneration medium plates containing 2 μg/ml of carboxin.

Detection of the introduced sequence in the transformants was carried out as follows. The *sdi1* sequence was amplified using total DNA extracted from the transformants as a template DNA. Amplification conditions were : 98°C for 30 sec, 52°C for 1 min and 68°C for 2 min. This cycle was repeated 25 times. The primers used were R1, 5′-CACACAAATCATTGAACC-3′ and R3, 5′-AGCATCGCAAGTGAAACCGA-3′. The

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[*2] Laboratory of Biomass Conversion.

amplified fragments were digested with *Apa*I and subjected to a 0.7% agarose gel electrophoresis.

Results and Discussion

After the incubation at 28°C for 4–5 days, colonies were observed on the regeneration medium containing carboxin. They were isolated and checked for the drug-resistance on secondly screening plates. *Apa*I-digestion of the PCR-amplified *sdi1* sequence demonstrated that the carboxin-resistant colonies contained pTM1 sequence in the mycelium as well as the endogenous *sdi1* allelic sequence of the host strain (Fig. 2). DNA sequence determination of the amplified fragments confirmed the presence of the introduced base substitution in the *Apa*I-sensitive *sdi1* fragment from the colonies (data not shown). These

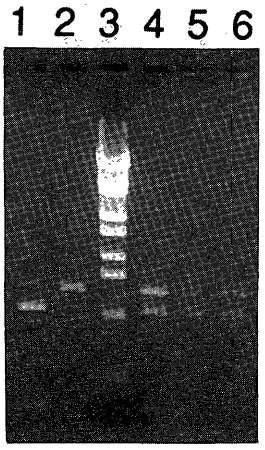

Fig. 2. Detection of the introduced sequence in the transformants. Samples are: lane 1, pTM1; lane 2, the host strain #261-23; lanes 4-6, the transformants. *Eco*T14I-digested lambda phage DNA was loaded in lane 3 as a size marker.

results indicated that *P. ostreatus* #261-23 was transformed to carboxin resistance by the introduction of the mutant *sdi1* gene. Efficiency of the transformation was 1–2 transformants per 1 μg of pTM1 DNA. Dikaryotic strain #261 was also transformed successfully with pTM1 using the same procedure (data not shown).

The drug-resistance of the transformants was maintained over several subcultures on non-selecting medium. And among single basidiospore isolates of the transformants crossed with a wild-type monokaryon #261-22, there are many carboxin resistant monokaryons. These data indicated that the acquired resistance is dominant and maintained stably during mitotic and meiotic cell divisions. Southern hybridization analysis showed that pTM1 sequence was integrated in high-molecular weight DNA with one or more copy numbers and that insertion of the sequence happened ectopicaly *via* non-homologous recombination mechanism in these transformants (data not shown).

This is the first report of a homologous drug-resistant marker gene available in transformation of a white-rot fungus and edible mushroom. It will be expected that many aspects of studies surrounding the fungus will be proceeded by utilizing this system.

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