Curr Genet (2000) 37: 209–212 © Springer-Verlag 2000

ORIGINAL PAPER

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Carboxin resistance transformation of the homobasidiomycete fungus *Pleurotus ostreatus*

Received: 24 August / 1 December 1999

Abstract A novel selection marker gene for transformation of the white-rot basidiomycete *Pleurotus ostreatus* was developed by introducing a point mutation in a gene which encodes the iron-sulfur protein (Ip) subunit of succinate dehydrogenase. The mutant gene, Cbx^R, encodes a modified Ip subunit with an amino-acid substitution (His239 to Leu) and confers resistance to the systemic fungicide, carboxin. The DNA sequence was integrated ectopically in the chromosome of the transformants. This is the first report of a homologous marker gene which is available for the molecular breeding of an edible mushroom.

Key words Basidiomycete · Transformation · White-rot fungi · Carboxin · Succinate:ubiquinone oxidoreductase

Introduction

Pleurotus ostreatus is a commercially important edible mushroom known as the oyster mushroom. It is, moreover, a white-rot basidiomycete fungus which is considered to be a good model for understanding the biochemical processes involved in lignin biodegradation (Kofujita et al. 1991). Techniques for DNA-mediated transformation are valuable tools for strain improvement and make it possible to combine molecular and biochemical analysis of the enzymes involved in lignin degradation.

A dominant drug resistance marker is very effective for developing a transformation system in many organisms. It permits one to avoid isolating auxotrophic strains and cloning the appropriate metabolic genes that will complement their defects. To-date, transformation events by plasmids harboring a recombinant hygromycin B resistance marker and a sequence from P1 bacteriophage have been reported in *P. ostreatus* (Peng et al. 1992, 1993a, b; Herzog et al. 1995). The marker gene was made up of the structural gene of *Echerichia coli* hygromycin B phosphotranferase (*hph*) fused to *Aspergillus nidulans gpd* promoter and *trpC* terminator sequences. Upon transformation, the plasmids were reported to have been maintained as autonomously replicating extrachromosomal plasmids. However, these transformation events were inefficient or very difficult to reproduce in other laboratories. Generally, it is considered that chimeric genes containing heterologous components are not expressed efficiently in homobasidiomycetes and the poor expression of the marker gene is a possible reason for the difficulty in transformation. In this context, it has been necessary to develop a reproducible transformation system for *P. ostreatus*. A homologous drug resistance marker gene, once developed, would be a promising candidate to this end, because it is expected to be maintained and expressed more stably in the mycelium.

In the heterobasidiomycete *Ustilago maydis,* mutant strains resistant to a systemic fungicide, carboxin, were isolated and studied. It was demonstrated that a mutant gene encoding the iron-sulfur protein (Ip) subunit of succinate dehydrogenase conferred dominant drug resistance (Keon et al. 1991; Broomfield and Hargreaves 1992). The mutation caused a single amino-acid substitution (His257 to Leu) in the third cysteine-rich cluster of the Ip subuit (Broomfield and Hargreaves 1992), and the mutant gene was used to develop vector plasmids for transformation of this species (Kinal et al. 1993). We have isolated the homolog gene, *sdi1*, from wild-type *P. ostreatus* (Irie et al. 1998a) and shown that the corresponding histidine residue (His239) is conserved in the *P. ostreatus* polypeptide. In the present report, we describe the successful utilization of an artificially mutagenized *P. ostreatus sdi1* gene as a valuable selection

Communicated by K. Esser

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marker for transformation of this fungus in order to impart carboxin resistance.

Materials and methods

Microbial strains and culture conditions

P. ostreatus (Jacq.: Fr.) Kummer monokaryotic strain, #261-23, was isolated from basidiospores of a dikaryotic strain #261 (ATCC 66376) and used as a host strain for the transformation experiments, unless otherwise stated. Another monokaryotic progeny, #261-22, was used to clone an allelic *sdi1* sequence to construct the transforming vector plasmid (see below). Maintenance of *P. ostreatus* strains was done using 3.9% potato-dextrose agar (PDA; Nissui, Tokyo, Japan) medium at 28 °C. Drug resistance was normally assayed on PDA media containing 2 μg/ml of carboxin from AccuStandard Inc., New Haven, Connecticut, unless otherwise stated. *E. coli* K-12 strain JM109 (Yanisch-Perron et al. 1985) was used as a bacterial host for general gene-engineering techniques.

Construction of the vector plasmid pTM1

A point mutation (CAC to CTC) which causes an amino-acid substitution (His239 to Leu) was introduced into an allelic *sdi1* sequence from *P. ostreatus* #261-22 (Irie et al. 1998b; GenBank accesion number AB009845). This sequence is slightly different from another allele of the *sdi1* sequence from #261-23 (Genbank accession number Ab007361) and they can be distinguished by the presence and absence of a recognition site for a restriction endonuclease, *Apa*I, respectively (Irie et al. 1998b). The properties of the allelic sequences, such as the coding sequence, expression signals and introns, have been described previously (Irie et al. 1998a, b).

As the first step, a 2.5-kp DNA fragment containing the allelic *sdi1* sequence was amplified using primer molecules, R3 (5′-AGCATCGCAAGTGAAACCGA-3′) and R4 (5′-TCCGATGACACTGCCAACGAC-3′), and genomic DNA was extracted from strain #261-22 as a template. Amplification conditions were: 98 °C for 20 s and 68 °C for 3 min. This cycle was repeated 30 times. Then the amplified fragment was cloned on the pGEM-T vector from Promega, Madison, Wis. The cloned sequence contains a 1.3-kb promoter region followed by a 1057-bp coding sequence and a 189-bp terminator region for the *sdi1* allele.

The point mutation was introduced using a kit for in vitro site-directed mutagenesis, Mutan-Super Express Km, from Takara Shuzo, Kyoto, Japan. As a pre-requisite, a 1.3-kb *Sma*I-*Sph*I fragment containing the *sdi1* coding sequence was cloned on a special vector plasmid pKF18 k supplied with the kit. Then the PCR reaction was done using a selection primer from the kit and an oligo-nucleotide primer TCCGCTGCCTCACAATCTTC, in which the underlined T identifies the base substitution. The reaction condition, cloning and selection of the mutagenised sequence followed the supplier's manual. The resulting *sdi1* coding sequence containing the point mutation was cloned back to the pGEM-T-based plasmid to produce pTM1 (Fig. 1). The base substitution was confirmed by nucleotide sequencing using an ABI 377 DNA sequencer. This plasmid was used for transforming *P. ostreatus*.

Transformation of *P. ostreatus*

Transformation was carried out basically according to a protocol developed for *Coprinus cinereus* (Binninger et al. 1987). Protoplasts were prepared by digesting liquid-cultured *P. ostreatus* mycelium with a lysing enzyme solution. The enzyme solution contains 2% Novozyme234 from Calbiochem-Novabiochem Corp., La Jolla, Calif., 0.5% Zymolyase 20 T from SEIKAGAKU Corp., Tokyo, Japan, 0.2% Chitinase from Sigma, St. Louis, Mo., 0.5 M mannitol and 0.2 M maleate buffer (pH 5.5). Then 2×10^7 protoplasts were mixed with 1–5 μg of pTM1 DNA in 25% PEG#4000 buffer containing 25 mM $CaCl_2$ and 0.5 M mannitol as an osmotic stabilizer. After incubation on ice for 20 min, all cells were poured onto SMY (1% sucrose, 1% malt extract and 0.4% yeast extract) regeneration-medium plates containing 0.6 M mannitol and 2 μg/ml of carboxin.

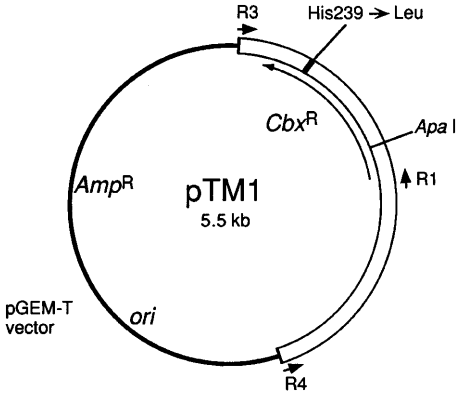

Fig. 1 Physical map of the plasmid pTM1. The open box represents a 2.5-kb DNA sequence from *P. ostreatus* which contains the mutant *sdi1* gene (Cbx^R). The location of the coding sequence and the direction of transcription are indicated by the *inside arrow*. The line represents the pGEM-T vector plasmid which contains the ampicilin-resistant marker (Amp^R) and origin (*ori*) for vegetative DNA replication in *E. coli*. Position of the amino acid substitution (His239 to Leu) and a recognition site for *Apa*I in the Cbx^R sequence are also indicated. Small arrows represent the location of the primer molecules, R1, R3 and R4, used for amplification and detection of the *sdi1* sequence

Detection of the introduced sequence in the transformants

Detection of the introduced sequence in the #261-23 transformants was carried out as follows. The *sdi1* sequence was amplified using total DNA extracted from the transformants as template DNA. The amplification conditions were: 98 °C for 30 s, 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 (described above). The amplified fragments were digested with *Apa*I and subjected to 0.7% agarose-gel electrophoresis. The introduced pTM1 sequence contains an *Apa*I site within the amplified region, whereas no *Apa*I sites exist in the endogenous allelic *sdi1* sequence of the host #261-23 strain.

Southern hybridization analysis of the transformants

Intact and *Eco*RI-digested total DNA isolated from the transformants was analyzed by Southern hybridization using the PCR amplified *sdi1* sequence as a probe. The labelling, hybridization and signal detection were done using a DIG DNA Labelling and Detection Kit from Boehringer Mannheim GmbH, Mannheim, Germany. Hybridization was for 12 h at 65 °C. Then membranes were rinsed three times in the washing buffer for 20 min at 65 °C.

Results and discussion

P. ostreatus protoplasts were prepared from liquid cultured mycelium of #261-23 and treated with pTM1 DNA in the presence of PEG/$CaCl_2$ (see Materials and methods). After the incubation at 28 °C for 4–5 days, colonies were observed on the regeneration medium

containing carboxin. No carboxin resistant colonies were observed in the control experiment in which protoplasts were treated with TE buffer instead of the pTM1 solution. Similar results were obtained when a dikaryotic strain #261 was used as host. The transformation efficiencies of the monokaryotic and dikaryotic strains were 1–2 and about five transformants per 1 μg of pTM1 DNA, respectively. Through analysis of the drug resistance, using a subcultured mycelium and basidiospores of the transformants, it was demonstrated that the acquired resistance was maintained stably during the mitotic and the meiotic cell divisions (data not shown).

The presence of the DNA sequence introduced via transformation was checked by *Apa*I-digestion of the PCR-amplified *sdi1* sequence from the #262–23 transformants. It was demonstrated that the carboxin-resistant colonies contained the *Apa*I-sensitive *sdi1* sequence as well as the *Apa*I-resistant endogenous *sdi1* sequence of the #261-23 host strain (Fig. 2). These results indicated that the fungus was made carboxin-resistant by the introduction of the mutant *sdi1* gene and that the introduced sequence exists in the transformants in addition to the endogenous *sdi1* sequence.

Southern-hybridization analysis of the transformants was carried out to analyze the mode of integration of the transforming DNA (Fig. 3). When intact DNA extracted from the transformants was probed with the *sdi1* sequence, it was demonstrated that the *sdi1* sequence migrated with the high-molecular-weight DNA. On analysis with the *Eco*RI-digested DNA, transformant T1 showed a single additional band located just below the 1.4-kb band of the endogenous *sdi1* sequence of the host strain. For the other transformants, several bands of various size were observed in addition to the host band. These results demonstrated that the introduced sequence was integrated ectopically into the chromosomal DNA with one or more copy numbers in these transformants.

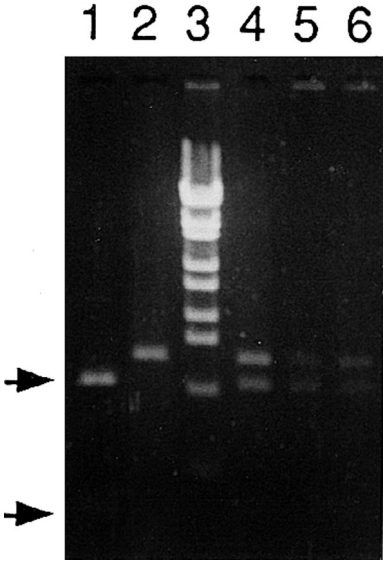

Fig. 2 Detection of the introduced sequence in the transformants with *Apa*I digestion. Agarose-gel electrophoresis of the *Apa*I-digested 1.3-kb *sdi1* fragments amplified from the transformants' DNA. The fragment amplified from the pTM1 sequence (*lane 1*) is *Apa*I-sensitive and has been divided into 0.2- and 1.1-kb fragments (indicated by *arrows*). Whereas the fragment amplified from the host #261-23 does not contain the *Apa*I site and showed resistance to *Apa*I digestion (*lane 2*). For the transformants (*lane 4* T1; *lane 5* T2; *lane 6* T3), both sensitive and resistant patterns were observed. *Eco*T14I-digested lambda DNA was loaded as a size marker (*lane 3*)

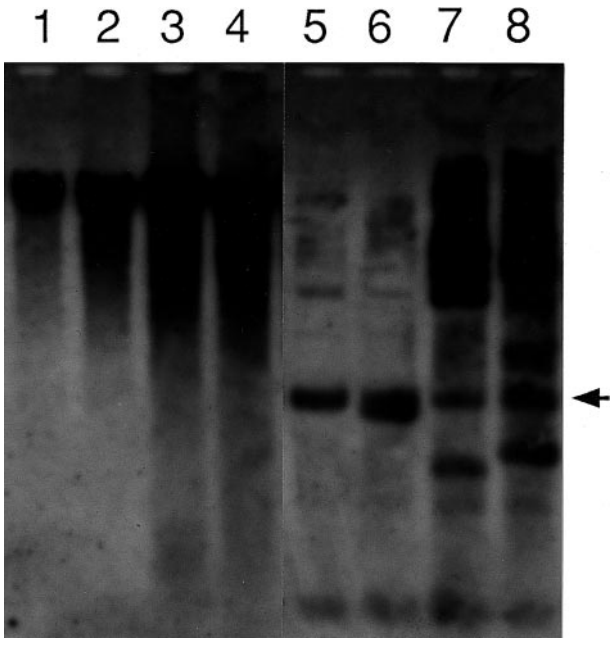

Fig. 3 Southern-hybridization analysis of the transformants. Intact (*lanes 1–4*) and *Eco*RI-digested (*lanes 5–8*) DNA extracted from the transformants was probed with the DIG-labeled *sdi1* sequence. Samples are: *lanes 1 and 5* the host strain #261-23 as a control; *lanes 2 and 6* T1; *lanes 3 and 7* T2; *lanes 4 and 8* T3. The position of the 1.4-kb *Eco*RI-fragment, originating from the endogeneous *sdi1* sequence, is indicated by an *arrow*

It was shown that the same amino-acid substitution in *P. ostreatus sdi1* imparts resistance to carboxin as in the *U. maydis* homolog. The drug resistance was dominant and used successfully as a selection marker in DNA-mediated transformation of the fungus. This is the first report of a homologous drug-resistant marker gene available for transformation of an edible and white-rot fungus. A self-cloning system makes it possible to over-express a gene encoding a protein of interest in a native and active form. Using the transformation system, we are now trying to over-express a manganese peroxidase homologously in *P. ostreatus*. The *P. ostreatus* Cbx^R gene also has the potential to be used as a selection marker for the transformation of other basidiomycetes, including edible mushrooms. At the very least, the strategy presented in this report would be effective for developing a transformation system in other organisms which are sensitive to carboxin.

Acknowledgments We are deeply grateful to Prof. S. Yanagi, Akita Prefectural University, and Dr. T. Sato, Iwate Biotechnology

Research Center, for valuable suggestions and advice. This work was supported partially by a Grant-in-Aid for Encouragement of Young Scientists from the Ministry of Education, Science and Culture, Japan (to Y.H.).

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