Construction of a Homologous Selectable Marker Gene for *Lentinula edodes* Transformation

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We cloned a gene for the iron sulfur protein (Ip) subunit from an edible mushroom, *Lentinula edodes*, and introduced a point mutation that confers carboxin resistance into it. The mutant gene successfully transformed *L. edodes* with high efficiency (9 transformants/2.5 μg vector DNA). Restriction enzyme-mediated integration (REMI) increased the transformation efficiency by about two-fold.

**Key words:** *Lentinula edodes*; *Pleurotus ostreatus*; homobasidiomycetes; iron-sulfur protein (Ip) subunit

Mushroom cultivation is an economically significant and rapidly expanding global industry. The homobasidiomycete *Lentinula edodes* is a widely cultivated mushroom throughout the world.1) *L. edodes* gills turn brown during post-harvest preservation, which is commercially undesirable since it causes an unpleasant appearance.2) Techniques for a DNA-mediated transformation system are valuable tools for investigation of the genes responsible for the phenomenon and for strain improvement. Though several systems have been developed for *L. edodes*, all of them use the *Escherichia coli* hygromycin B resistant gene (*hph*) with homologous promoters as selective markers.3,4) However, Japanese customer show a tendency to dislike foods containing transgenes, especially those from non-edible organisms and other selective markers that confer different drug resistances are required for the more complicated protocols of the transformation.

Previously, we successfully transformed another edible homobasidiomycetous mushroom, *Pleurotus ostreatus*, using a homologous drug resistant marker, Cbx that encodes a mutant Ip subunit of complex II and confers dominant resistance to a systemic fungicide, carboxin.5) However we did at least 5 independent experiments of *L. edodes* transformation using the *P. ostreatus* Cbx according to the procedure of *L. edodes* transformation (described below), but no transformants were obtained (data not shown). We considered that the cause of the failure was gene incompatibility between *P. ostreatus* and *L. edodes*. To overcome the obstacle, we cloned a Ip subunit gene from *L. edodes* and constructed a homologous Cbx.

The genomic library prepared by Hirano et al. (1999)6) was used to screen the Ip subunit gene from *L. edodes*. It was constructed using a Lambda EMBL3/BamHI Vector Kit (Stratagene) and the genomic DNA of strain 57. Genomic DNA fragments digested with Sau3AI were ligated into the BamHI site of the lambda EMBL3 cloning vector and packaging extracts. Degenerate oligonucleotide primers, IP-N (5′-GGI ATI TGC GGI TCI TGC GCI ATG AA-3′) and IP-C (5′-CAG CAI GCG CAI AGI ATG CAI TCG TA-3′) were designed based on the deduced amino-acid sequences of an Ip subunit from *Schizophyllum commune*.7) A DNA fragment of 412 bp was amplified using the primer molecules and the genomic DNA. The amplification conditions were as follows: denaturation at 93°C for 1 min; annealing at 50°C for 90 sec; extension at 72°C for 2 min. This cycle was repeated 30 times. The amplified DNA fragment was used as a probe for the cloning of the Ip subunit gene. For screening of the positive clones, 5×105 plaques from the genomic DNA library were transferred to Hybond N+ nylon membranes (Amer- sham Pharmacia Biotech). Labeling of the DNA probe, hybridization, and signal detection were done by means of the ECL Direct Nucleic Acid Labelling and Detection System (Amersham Pharmacia Biotech). Three positive clones were isolated from the genomic library. Sequencing analysis revealed that they contained the same sequence, coding a putative Ip subunit. One positive clone, gIP15, was used for further study. The sequence of the Ip subu-
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Fig. 1. Construction of pL-Cbx.
Black boxes represent signal regions and white boxes represent ORF regions. The small arrows indicate the positions and directions of primer binding. The misted boxes and the line represent the pUC19 region.

The Ip subunit mutation that confers carboxin resistance is caused by a single amino-acid substitution.\(^8,9\) A point mutation (CAC to CTC) that causes an amino-acid substitution, His243 to Leu, was introduced into the Ip subunit gene of \emph{L. edodes} (Fig. 1). As a first step, 2.4-kb DNA fragments containing the Ip subunit promoter and coding sequence were amplified using primer molecules, LeSD-U (5'-TAT TCT TGT CGG TGT CTC TC-3') and LeSDM-L (5'-GAA GAT AGT G AG GCA TCG GTA CAT GCT CAA CTC GTT TTG C-3'), where A identifies the base substitution), and the genomic DNA clone, gIP15 as a template. LeSD-U binds between \(-1389\) nt. and \(-1369\) nt., LeSDM-L binds between \(1026\) nt. and \(986\) nt. in the \emph{L. edodes} Ip subunit gene. A 4-kb DNA fragment containing the Ip subunit coding and terminator sequence was amplified using primer molecules, LeSDM-U (5'-TGT CTT GC TCA CTT CAG GAG CAT GTA CGG ATG TCT CAC TAT CTT GC-3', where T identifies the base substitution) and LeSD-L (5'-GCT TTT GCC GCA CTT GC-3'), and the genomic DNA clone, gIP15 as a template. LeSDM-U binds between \(968\) nt. and \(1026\) nt. in the \emph{L. edodes} Ip subunit gene. The exact binding position of LeSD-L cannot be identified because the terminator sequencing is incompletely. An overlap of 40 bp between the promoter-coding region fragment and the terminator-coding region fragment was generated. The amplification conditions were as follows: denaturation at \(94^\circ\text{C}\) for 30 sec.; annealing at \(55^\circ\text{C}\) for 30 sec.; extension at \(72^\circ\text{C}\) for 1 min. This cycle was repeated 25 times. DNA fragments were separated on a 0.8% agarose gel and purified. Subsequently, the two fragments were combined by PCR to form one 6.4-kb fragment using LeSD-U and LeSD-L primers and the two purified fragments as templates. The amplification conditions were as follows: denaturation at \(94^\circ\text{C}\) for 30 sec.; annealing at \(55^\circ\text{C}\) for 30 sec.; extension at \(72^\circ\text{C}\) for 2 min. This cycle was repeated 25 times. The DNA fragment was separated on a 0.8% agarose gel and purified. The fragment was digested with \emph{StuI} and \emph{SalI} to generate 3.1-kb fragment, which was then separated on a 0.8% agarose gel and purified. The 3.1-kb fragment was cloned into pUC19 digested with \emph{SmaI} and \emph{SalI}. The base substitution and the fragment combination were confirmed by nucleotide sequencing. The resulting plasmid was designated as pL-Cbx.

Preparation of \emph{L. edodes} protoplasts for the transformation was done by the method of Kawasumi \emph{et al.} (1987).\(^10\) \emph{L. edodes}, strain SR-1, previously designated as strain S-1\(^3\) was grown on MYPG agar medium for two weeks at \(25^\circ\text{C}\), after which mycelia were transferred to MYPG liquid medium and grown for a further 1–2 weeks at \(25^\circ\text{C}\) with shaking. The cultivated mycelia were collected, homogenized, and filtered through a 100 \(\mu\text{m}\) length. The filtrates were further grown in MYPG liquid medium at \(25^\circ\text{C}\) for 5–6 days without shaking. One g of the cultivated mycelia was collected, washed twice with SM buffer (50 mm suc-
cinate (pH 5.6), 0.6 M mannitol), and treated with 10 ml of enzyme solution (2.5% cellulase Onozuka RS (Yakult), 0.1% chitinase (Sigma) in SM buffer) at 28°C for 3–4 h. The protoplasts were filtered through 40-μm nylon mesh, washed with STC buffer (10 mM Tris-Cl (pH 7.5), 10 mM CaCl₂, 1.2 M sorbitol), and suspended in STC buffer. About 1 × 10⁸ protoplasts were obtained from 1 g of mycelia.

Transformations by the conventional PEG method and by the REMI method (PEG treatment-2) were done as described by Sato et al. (1998), based on the procedure of Yanai et al. (1996). Protoplasts of *L. edodes* (0.5–1.0 g) were transformed with 2.5 μg of a vector plasmid. After transformation and preculture in MS medium (2% malt extract, 0.6 M sucrose), the protoplasts were plated onto minimal medium containing 0.5 μg/ml carboxin and incubated at 25°C for five days. The plates were then overlaid with MYPG containing 2 μg/ml carboxin and 1.5% agar, and incubated at 25°C. Individual transformants that appeared on the plates were purified twice by subculturing on MYPG agar medium containing 2 μg/ml carboxin. The transformation efficiency was 9 transformants/2.5 μg DNA using the conventional PEG method (Table 1). This is more efficient than that in the vector pLG-hph was used to confer hygromycin B resistance (4)

<table>
<thead>
<tr>
<th>vector</th>
<th>restriction enzyme</th>
<th>transformants/2.5 μg DNA</th>
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<tbody>
<tr>
<td>pLG-hph</td>
<td>—</td>
<td>1.0</td>
</tr>
<tr>
<td>pL-Cbx</td>
<td>—</td>
<td>9.0</td>
</tr>
<tr>
<td>pLG-hph</td>
<td>Bgl II(5U)</td>
<td>18.0</td>
</tr>
<tr>
<td>pL-Cbx</td>
<td>Kpn I(25U)</td>
<td>16.5</td>
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When intact DNA extracted from the transformants was probed with the Ip subunit gene sequence, it was demonstrated that the Ip subunit gene sequence migrated with the high molecular weight DNA (Fig. 2). On analysis with the SalI and StuI double digested DNA, the transformants showed single additional bands located just above the 3.1-kb band of the endogenous Ip subunit gene sequence of the host strain. These results demonstrated that the introduced sequence was integrated the chromosomal DNA with one or more copy numbers in these transformants. In T3 and T4, single additional bands located just under 3.1 kb were also observed, indicating the gene rearrangement.

No transformants were obtained using *P. ostreatus* Cbx⁸, though the amino acid sequence of it showed homology (66%) to that of *L. edodes* Cbx⁸. The homology might be insufficient for gene substitution. Alternately, the promoter or the signal peptide might be incompatible. Of course, there is some possibility that *P. ostreatus* Cbx⁸ can transform *L. edodes* using a more efficient protocol exploited in the future. Anyway, the system of *L. edodes* Cbx⁸ is only the alternative to the hph system in *L. edodes* transformation at this stage. It will bring many benefits to the molecular analysis and strain improvement of *L. edodes*.

![Fig. 2. Southern Hybridization of Transformed L. edodes Genomic DNA.](image-url)

(A) Southern blot analysis of the genomic DNA of the pL-Cbx transformant, T1-T5, probed with ECL-labeled Cbx⁸ from *L. edodes*. Lane wt indicates genomic DNA of wild type strain, SR-1. Lane V indicates SalI-KpnI digested pL-Cbx. (B) Maps of Ip subunit gene and Cbx⁸ from *L. edodes*. Black boxes represent signal regions and white boxes represent ORF regions. The arrow indicates the point of mutation.
References