Isolation and transformation of uracil auxotrophs of the edible basidiomycete *Pleurotus ostreatus*

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Abstract

Uracil auxotrophs of *Pleurotus ostreatus* were isolated using the selectable marker, resistance to 5'-fluoro-orotic acid (5'-FOA). Two of the nine uracil auxotrophs obtained were transformed to prototrophy using plasmid pTRura 3-2 that contains the orotidine monophosphate decarboxylase (*ura3*) gene from *Trichoderma reesei*. Southern blot analyses of the transformants showed that the transforming DNA had integrated into the genome of the protoplasts. Using $2 \times 10^7$ protoplasts, this system gave a transformation efficiency of about 30 transformants per µg of DNA. Normal fruiting bodies were induced in the transformants by crossing them with wild-type monokaryons, and the basidiospores collected from these fruiting bodies showed a biased segregation rate to prototrophy. These results indicate the integrated DNA was stably inherited. © 1999 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

*Pleurotus ostreatus* (Fr.) Kummer, the oyster mushroom, is one of the most widely cultivated edible mushrooms. Various strains of this wood-rotting fungus are found all over the world, this mushroom is especially appreciated in Europe and Asia for its edibility [1]. The production of oyster mushrooms has risen rapidly in the last decade to the point where *P. ostreatus* production now ranks second to production of the common mushroom, *Agaricus bisporus*. However, despite the popularity of *P. ostreatus*, there have been few studies of the molecular genetics of the species, partly due to the lack of an efficient transformation system. Recently considerable efforts have been spent on developing transformation systems for *P. ostreatus* using drug resistance as the dominant selectable marker [2,3].

In a previous study, *P. ostreatus* was transformed to hygromycin B resistance, but the introduced DNA was extrachromosomally replicated and unstably inherited [2,4]. Recently, *P. ostreatus* has also been transformed to bialaphos resistance [3], and in this...
study, the transformed DNA was successfully integrated into the chromosome. Meanwhile, a transformation system based on selection of an auxotrophic marker has not been developed yet. The development of these transformation systems, however, can be problematic as isolation of auxotrophic mutants can be time-consuming and tedious, and the gene for complementation of the auxotrophic mutant needs to be cloned. Positive selection of uracil auxotrophs using the pyrimidine analogue, 5'-fluoro-orotic acid (5'-FOA), has been reported in *Saccharomyces cerevisiae* and filamentous fungi [5,6]. We have also developed a rapid and easy method for isolating *Pleurotus sajor-caju* uracil auxotrophs. The corresponding genes *ura3* and orotate phosphoribosyl transferase (*ura5*) genes were cloned in *Trichoderma reesei* [7] and *Aspergillus niger* [8], respectively.

In this study, we report the development of an easy and efficient transformation system for *P. ostreatus* that selects uracil auxotrophs by 5'-FOA resistance and complementation with a vector containing the heterologous *ura3* from *T. reesei* [7].

2. Materials and methods

2.1. Strains, plasmids and culture conditions

*P. ostreatus* strain ASI2029 was obtained as a stock culture from the Korean National Institute of Agricultural Science and Technology, and the plasmids, pTRura 3-2 and pTRura 5-3, were obtained from Dr. Christian Barreau [7]. These plasmids contain the *ura3* and orotate phosphoribosyl transferase (*ura5*) genes from *T. reesei*. Vegetative cultures of monokaryotic and dikaryotic mycelia were made on MCM (0.2% yeast extract, 0.2% bacto-peptone, 2% glucose, 0.05% MgSO₄·7H₂O, 0.05% KH₂PO₄, and 0.1% K₂HPO₄) at 25°C. Fruiting bodies were induced in a 1 l bottle culture containing 570 g poplar tree sawdust, 120 g rice bran and 65% water. The cultures were incubated at 25°C under dark conditions for 25-40 days and then transferred to conditions that induced fruiting (12-15°C, over 85% humidity, and light). The fruiting bodies were harvested just before sporulation and the spores were collected for meiotic segregation analysis.

2.2. Isolation of 5'-FOA resistant mutants from *P. ostreatus*

*P. ostreatus* uracil auxotrophs were obtained using a method described for *P. sajor-caju* [9]. After ultraviolet (UV) irradiation for 120 s which gave about 5% survival rates, 10⁸ basidiospores of strain ASI2029 were plated on minimal medium supplemented with uracil (50 g ml⁻¹) and 5'-FOA (1.5 mg ml⁻¹). Colonies that grew on this medium were transferred to minimal medium to select for the uracil auxotrophs.

2.3. Transformation procedures

The mycelia were harvested from the liquid cultures by filtration. After being washed in 0.6 M sucrose, the mycelia were resuspended in 10 ml lysis buffer (2 mg ml⁻¹ cellulase ‘ONOZUKA’ RS (Yakult Pharmaceutical), 0.5 mg ml⁻¹ zymolyase (Kirin Brewery), 0.4 mg ml⁻¹ chitinase (Calbiochem, Boehringer), and 3 μl ml⁻¹ gluconidase (Sigma)), and gently shaken at 25°C for 3 h. The protoplasts were filtered through a glass filter (G3), centrifuged at 2000 × g, and resuspended in 10 ml of STC (0.6 M sucrose, 10 mM Tris-HCl, pH 7.5, and 10 mM CaCl₂). The protoplasts were then centrifuged again at 2000 × g and resuspended in STC to a concentration of 5 × 10⁷ protoplasts ml⁻¹.

For transformation, 5 μg of plasmid DNA and 50 μl PTC (60% PEG 3350, 10 mM Tris-HCl, pH 7.5, and 10 mM CaCl₂) was added to 200 μl of the protoplast suspension. The mixture was kept on ice for 20 min. A further 600 μl of PTC was then added to the mixture, followed by incubation for 20 min at room temperature. The protoplasts were directly plated on minimal media to allow them to regenerate.

2.4. Southern blot analysis

Genomic DNA was prepared from lyophilized mycelia according to the method described by Raeder [10]. Southern blot analysis was performed with 5 μg DNA per sample. Labelling of the *EcoR*I-digested pTRura 3-2 vector, and the detection of signals, was performed using the DIG system according to
the manufacturer’s instructions (Boehringer Mannheim).

3. Results and discussion

The screening of pyrimidine auxotrophs with 5'-FOA has been successfully applied to the isolation of ura3 and ura5 mutants from several fungi, including S. cerevisiae [5] and T. reesei [6]. In a previous study, we showed that the 5'-FOA method is also efficient for isolating uracil auxotrophs of P. sajor-caju. As positive selection could also facilitate the isolation of auxotrophs from more important Pleurotus species, we applied this method to the isolation of P. ostreatus.

In order to obtain uracil auxotrophs from the P. ostreatus strain ASI2029, 10^8 basidiospores were mutagenized and plated on 5'-FOA media. Among the 5'-FOA resistant colonies that emerged, 125 were selected for further characterization. Nine P. ostreatus uracil auxotrophs were isolated from these colonies.

Since no *ura* genes have been cloned from *P. ostreatus*, *ura* genes from *T. reesei* were used for transformation to see whether these vectors could complement the uracil mutants of *P. ostreatus*. Transformation of five of the nine *P. ostreatus* uracil auxotrophs was attempted using the PEG/CaCl2 method with both the pTRura 3-2 and pTRura 5-3 vector. From these five auxotrophs, two strains were transformed to prototrophs using the pTRura 3-2 vector, while no prototrophs were recovered using the pTRura 5-3 vector. One of the two strains transformed with pTRura 3-2 was used as the host for further experiments. This strain was designated ‘ASI2029-8’ and gave a transformation efficiency of about 30 colonies per μg plasmid vector from 2 × 10^7 protoplasts.

Two previous reports have described the use of the *ura* marker for transformation of basidiomycetes: the transformation of uracil auxotrophs of *Phanerochaete chrysosporium* [11] and the homologous transformation of an *Agrocybe aegerita* *ura* auxotrophic mutant [12]. In the latter case, the integrated vector sequences were excised and maintained within the host as extrachromosomal DNA.

In the present study, an extrachromosomal form of the introduced DNA was not detected when undigested total DNA was hybridized with DIG-labelled pTRura 3-2 (data not shown). pTRura 3-2 is a vector which gives very high transformation effi-
ciency in *T. reesei* (>10^4 transformants per μg DNA). When we used this vector to transform *P. ostreatus*, however, a much lower transformation efficiency was observed. In fact, the transformation rates obtained with this vector were similar to the rates observed for *P. chrysosporium ura2* transformed with the heterologous *ura* gene from *Podospora anserina* (1–25 transformants per μg DNA).

Studies have shown that the transformation efficiency of *P. chrysosporium ura2* can be greatly increased when the *ura3* gene from *Schizophyllum commune* is used for complementation (100–600 transformants per μg DNA). In contrast, low transformation efficiencies are obtained when *A. aegerita* (1–26 transformants per μg DNA) is transformed using a homologous *ura* transformation system. These results suggest that the efficiency of *ura* transformation cannot be predicted by whether the transforming DNA is heterologous or homologous.

The total DNA extracted from *P. ostreatus* transformants obtained in this study was digested with EcoRI and hybridized with DIG-labelled pTRura 3-2 (Fig. 1). The different Southern hybridization profiles of the three transformants indicated that the DNA was inserted into different sites in the genome. Many hybridizing bands of high intensity were detected in transformant T1, indicating a great number of insertions or tandem repeat insertions within the T1 genome. Transformants T2 and T3 showed only two major hybridizing bands, indicating one or two insertions. ASI2029-8 strain didn’t show the hybridizing bands, suggesting that this gene has a low level of nucleotide homology between *P. ostreatus* and *T. reesei*.

For fruiting body induction in the three *P. ostreatus* transformants, we crossed them with the compatible wild-type monokaryon strain, ASI2018-247. The resulting fruiting bodies showed normal morphologies (data not shown). To determine if the transformed DNA was stably inherited, basidiospores were obtained from the basidiocarps of the transformants, and the segregation ratio of uracil auxotrophic basidiospores to prototrophic basidiospores formants, and the segregation ratio of uracil auxo-

References


