Improvement of Manganese Peroxidase Production by the Hyper Lignin-Degrading Fungus Phanerochaete sordida YK-624 by Recombinant Expression of the 5-Aminolevulinic Acid Synthase Gene

Original Paper

Improvement of manganese peroxidase production by the hyper lignin-degrading fungus *Phanerochaete sordida* YK-624 by recombinant expression of the 5-aminolevulinic acid synthase gene

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Running title: Manganese peroxidase production by *P. sordida* YK-624

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Abstract

The manganese peroxidase (MnP) gene (*mnp4*) promoter of *Phanerochaete sordida* YK-624 was used to drive expression of 5-aminolevulinic acid synthase (*als*), which is a key heme biosynthesis enzyme. The expression plasmid p*MnP4pro-als* was transformed into *P. sordida* YK-624 uracil auxotrophic mutant UV-64, and 14 recombinant *als* expressing-transformants were generated. Average cumulative MnP activities in the transformants were 1.18-fold higher than that of control transformants. In particular, transformants A-14 and A-61 showed significantly higher MnP activity (approximately 2.8 fold) than wild type. RT-PCR analysis indicated that the increased MnP activity was caused by elevated recombinant *als* expression. These results suggest that the production of MnP is improved by high expression of *als*.

Introduction

A small group of basidiomycetes, the white-rot fungi, has the ability to break down and mineralize lignin, an aromatic polymer that is the most resistant component of plant cell walls. To degrade lignin, white-rot fungi secrete a large number of heme-containing peroxidase isozymes [7], which are grouped into two families: the lignin peroxidase (LiP) and manganese peroxidase (MnP) families. LiPs are characterized by their high redox potential with hydrogen peroxide enabling oxidation of non-phenolic aromatic compounds [16, 17] and by a long-range electron transfer pathway, which enables the oxidation of polymers such as lignin [6, 9]. MnP is able to chelate and oxidize Mn(II) to Mn(III), thereby acting as a diffusing oxidizer [21]. Recently, a new versatile peroxidase, the third ligninolytic peroxidase described in fungi from the genera *Pleurotus* and *Bjerkandera*, has been characterized as a hybrid MnP-LiP peroxidase [2, 11, 13].

All living cells biosynthesize heme through several steps, wherein 5-aminolevulinic acid (ALA) is the first committed intermediate. Two alternative routes for the formation of ALA have been proposed: one in which the condensation of succinyl CoA and glycine is catalyzed by ALA synthase (ALAS, EC 2.3.1.37) in mitochondria, and the second, called the 5-carbon route, which occurs in the stroma of plastids [1]. In the white-rot fungus *Phanerochaete chrysosporium*, whose 30-million-bp genome was completely sequenced by a whole-shotgun approach [10], both the expression of the ALAS-encoding gene and the production of MnP is increased by vanillin [18], suggesting that ALAS participates in the biosynthesis of heme by white-rot fungi. In the white-rot fungus *Phanerochaete sordida* YK-624, which was isolated from rotten wood and has much higher ligninolytic activity and ligninolytic selectivity than *P. chrysosporium* or *Trametes versicolor* [3], the major extracellular ligninolytic enzymes are MnP [3, 4] and LiP [5, 8, 19], and the expression of the gene encoding ALAS corresponded with the expression and activity of MnP in our previous report [14]. These results indicate that the high expression of the ALAS gene is needed to produce large amounts of MnP by white-rot fungi. In the present study, we determined whether the production of MnP is improved by high expression of ALAS gene in *P. sordida* YK-624.

Materials and Methods

Strains

P. sordida YK-624 (ATCC 90872) and uracil auxotrophic strain UV-64 [22] were used in this study. All strains and transformants were maintained on potato dextrose agar (PDA) slants at 4 °C.

Construction of an ALAS gene expression vector, co-transformation of UV-64, and screening of regenerated clones

Genomic DNA was isolated from *P. sordida* YK-624 mycelium using ISOPLANT II (Nippon Gene). TAIL-PCR was performed using the degenerate primers TAIL1-6, as described previously [22], to obtain the 5'-flanking region of *mnp4* [14]. Nested primers MnP R1, R2, and R3 were used as gene-specific primers. Inverse PCR was performed to obtain the further upstream of the 5'-flanking region using the primer sets MnPproF1-MnPproR1 and MnPproF2-MnPproR2 and the restriction enzyme *Pst* I (New England Biolabs) for the construction of the inverse-PCR library, as previously described [22]. The full-length 5'-flanking region of *mnp4* (1165 bp) containing the *mnp4* promoter was amplified using the primer sets MnPproF3/MnPproR3.

The procedure for constructing the ALAS gene (*als*) [14] expression plasmid, p*MnP4pro-als*, is described in detail in the Supplementary Material. The procedure is schematically illustrated in Supplementary Figure 1, and the primers are listed in Supplementary Table 1.

UV-64 protoplasts were prepared by standard technique using cellullases and then transformed with p*PsURA5* and p*MnP4pro-als* using PEG method [22]. The co-transformed clones were selected by PCR, as described previously [19], with the following modifications: primers ALASF2 and ALASR2 were designed to amplify the *als* gene fused with the *mnp4* promoter. A total of 14 transformants containing the two plasmids were isolated.

Enzyme assay

The 14 obtained transformants were incubated on PDA plates at 30 °C for 3 days, and 10-mm-diameter disks were then punched out from the growing edge of mycelia with a sterile cork borer. Two mycelial disks for each transformant were placed into a 100-mL Erlenmeyer flask containing 10 mL nitrogen-limited Kirk medium [20], which was then statically incubated at 30 °C for 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20 days. As a control experiment, transformants containing only p*PsURA5* were similarly cultured.

MnP activity was measured by monitoring the oxidation of 2,6-dimethoxyphenol to coerulignone ($\varepsilon 470$ = 49.6 $mM^{-1}cm^{-1}$) [15]. The reaction mixture (1 ml) contained 2,6-dimethoxyphenol (1 mM), MnSO4 (1 mM), and H2O2 (0.2 mM) in 50 mM malonate, pH 4.5. One katal (kat) was defined as the amount of enzyme producing 1 mol of product per second.

Transcriptional analysis

Two mycelial disks punched from the growing edge of mycelium were added to a 100-ml Erlenmeyer flask containing 10 ml nitrogen-limited Kirk medium, which was then statically incubated at 30°C for 4 days. The mycelia were then collected and stored at -80 °C until needed for transcriptional analysis. One hundred milligrams of mycelium mat was ground to a fine powder using a mortar and pestle under liquid nitrogen, and then total RNA was isolated from the mycelium using an RNeasy Plant Mini kit (Invitrogen). RT-PCR was performed using 200 ng of total RNA with a PrimeScript RT-PCR Kit and the gene-specific primer sets ALASF3-ALASR3 (native *ala*), ALASF2- ALASR2 (recombinant *ala*), and ActinF-ActinR (actin gene, *act*). The PCR was performed for 30 cycles of template denaturation at 95°C for 30 s, primer annealing at 58°C for 1 min, and DNA extension at 72°C for 1 min using Ex Taq DNA Polymerase (TaKaRa Bio).

Nucleotide sequence accession numbers

The nucleotide sequences of the 5'-flanking region of *mnp4* derived from *P. sordida* YK-624

have been deposited in the DDBJ database (http://www.ddbj.nig.ac.jp/) under accession no. AB818895.

Results and Discussion

Construction of recombinant *als*-expressing transformants

To simultaneously express the MnP and *als* genes in *P. sordida* YK-624, we first cloned the gene promoter of MnP4, which is the main isozyme of MnP of *P. sordida* YK-624. We obtained 1165 bp 5'-flanking region of *mnp4* containing a TATAA element, three inverted CCAAT elements, six putative heat-shock elements, and four putative metal response elements [12]. To confirm whether the promoter region is responded by Mn(II), we constructed an expression plasmid which the enhanced green fluorescent protein (EGFP) was driven by the promoter, and the plasmid-introducing transformants were obtained. The transformant which was growing on the plate of Kirk medium containing Mn(II) showed high fluorescence originated by the production of EGFP although no fluorescence was observed in the trasnformant growing on the of Mn-defficient Kirk medium (data not shown). Next, a plasmid for the recombinant expression of *als* was constructed from p*PsGPD-EGFP* [22] by inserting the *als* gene between the *mnp4* promoter and *gpd* terminator. The resulting expression plasmid, p*MnP4pro-als*, was introduced into strain UV-64 using p*PsURA5* as a marker plasmid. The presence of the *mnp4* promoter-*als* gene fusion in each uracil prototrophic clone was confirmed by PCR using genomic DNA as the template (data not shown). We obtained 14 regenerated clones (strains A1-A14) that were co-transformed with p*MnP4pro-als* and p*PsURA5*.

MnP production by transformants expressing recombinant *als*

The production of MnP by strains A1-A14 was compared with that by control transformants, which contained only p*PsURA5*. The average cumulative MnP activity in the culture medium of 14 co-transformants was 77.2 nkat/flask, whereas that in the culture medium of control transformants was only 65.7 nkat/flask (Fig. 1). Although some transformants indicated lower MnP activities than control

transformants, it is thought that the MnP production and/or the expression of *als* are disturbed by the introduction of recombinant *als* since the target gene is randomly introduced into genomic DNA in the gene transformation system used in the present study. This result indicates that the introduction of recombinant *als* had a positive effect on the production of MnP by *P. sordida* YK-624. We have previously reported that the expression of *als* corresponds with the expression and activity of MnP [14]. Moreover, both the expression of the ALAS-encoding gene and production of MnP are increased by vanillin in *P. chrysosporium* [18]. These findings, together with our present results, suggest that ALAS, which participates in the biosynthesis of heme, also plays an important role in MnP production.

MnP productivity and transcriptional analysis among higher MnP-producing transformants

Among the co-transformed strains, A-14 and A-61 showed the highest cumulative MnP activity. Therefore, we analyzed the time course of MnP production by A-14 and A-61 (Fig. 2). The highest MnP activity was detected after 4 days incubation for both transformants, after which the MnP activities gradually decreased until 8 days incubation and remained relatively stable until 20 days incubation. After 4 days of culture, the MnP activities of both transformants were approximately 2.8 fold-higher than that of wild type.

We next analyzed the transcription of *als* and *mnp4* by the strains A-14 and A-61 by RT-PCR (Fig. 3). The analysis indicated that native *mnp4* and *als* were similarly transcribed in A-14, A-61, and wild type, whereas recombinant *als* was only detected in A-14 and A-61. These results suggest that ALAS activities in A-14 and A-61would be increased by the recombinant expression of *als*, and that the increase of MnP activity in the co-transformants was due to the increase of recombinant *als* expression. As the transcriptional levels of *mnp4* in strains A-14 A-61were similar to that in wild type, the expression of recombinant *als* likely increased the active form of MnP by supplying heme, as opposed to increasing the apoprotein of MnP.

To our knowledge, this is the first report to describe an improvement of MnP production by a white-rot fungus through recombinant expression of *als*. Our next target is the molecular breeding of a

MnP-overexpressing transformant of *P. sordida* YK-624 by the simultaneous expression of *als* and *mnp4*.

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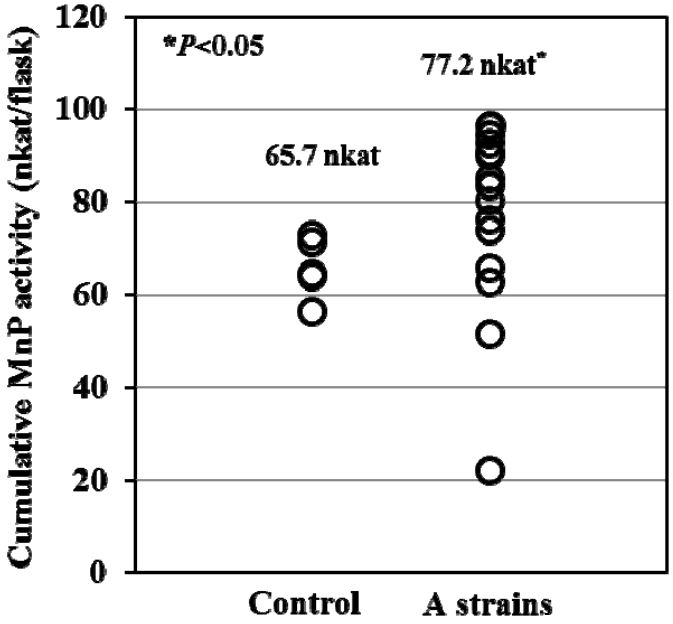

Fig. 1. Cumulative MnP activity by the *als*-expressing (A strains) and control strains.

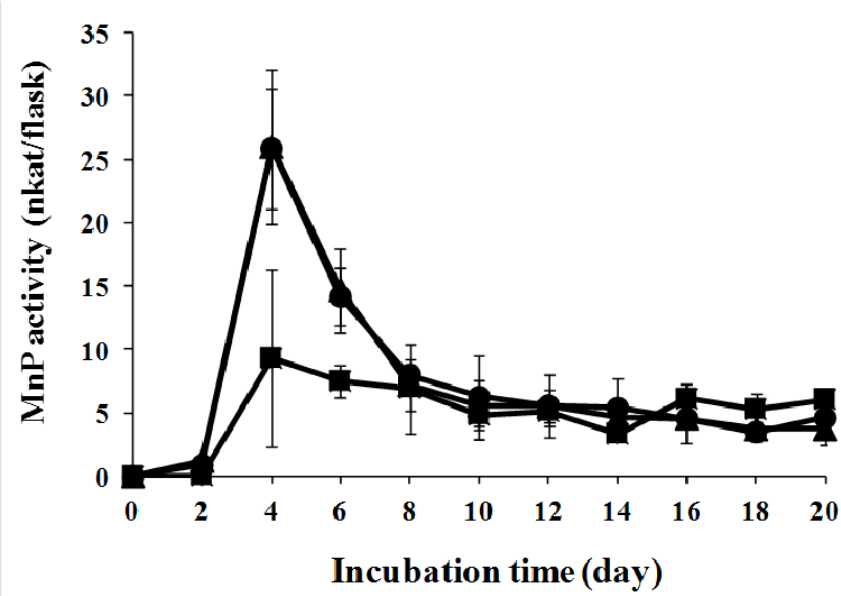

Fig. 2. Time course of MnP activity in strains A-11 (circles), A-61 (triangles), and wild type (squares).

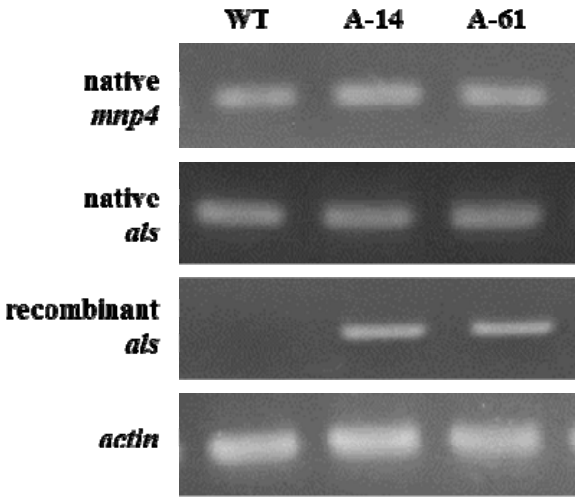

Fig. 3. RT-PCR analyses of *mnp4* and *als* in the wild-type, A-14, and A-61 strains. The expression of *actin* was used as an internal control.