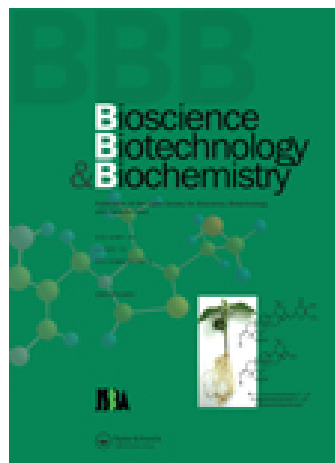




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Improvement of ligninolytic properties by recombinant expression of glyoxal oxidase gene in hyper lignin-degrading fungus *Phanerochaete sordida* YK-624

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Glyoxal oxidase (GLOX) is a source of the extracellular H₂O₂ required for the oxidation reactions catalyzed by the ligninolytic peroxidases. In the present study, the GLOX-encoding gene (*glx*) of *Phanerochaete chrysosporium* was cloned, and *bee2* promoter of *P. sordida* YK-624 was used to drive the expression of *glx*. The expression plasmid was transformed into a *P. sordida* YK-624 uracil auxotrophic mutant (strain UV-64), and 16 clones were obtained as GLOX-introducing transformants. These transformants showed higher GLOX activities than wild-type *P. sordida* YK-624 and control transformants harboring marker plasmid. RT-PCR analysis indicated that the increased GLOX activity was associated with elevated recombinant *glx* expression. Moreover, these transformants showed higher ligninolytic activity than control transformants. These results suggest that the ligninolytic properties of white-rot fungi can be improved by recombinant expression of *glx*.

Keywords: *Phanerochaete sordida* YK-624; lignin degradation; glyoxal oxidase; molecular breeding

Lignin is one of the most abundant and recalcitrant natural polymers, consisting of various nonphenolic phenylpropanoid units complexed into a three-dimensional network. Therefore, lignin degradation is a key step for carbon recycling in forest ecosystems, and the process is a central issue in lignocellulose-based industries (e.g. in paper pulp manufacture and production of bioethanol and other renewable chemicals)¹. In nature, white-rot fungi (members of the *basidiomycete* group) are able to degrade recalcitrant lignin efficiently². The biodegradation process is initiated via a one-electron oxidation of lignin units, a step that is mediated by extracellular heme peroxidases (including lignin peroxidase (LiP), manganese peroxidase (MnP), and versatile peroxidase), enzymes that are of keen interest as industrial biocatalysts^{3,4}. Activation

of molecular oxygen to hydrogen peroxide by extracellular oxidases represents a common step in the fungal decay strategy, as shown by biochemical^{2,5} and genomic^{6,7} evidence. White-rot decay is based on peroxide activation of high-redox-potential fungal heme peroxidases that depolymerize lignin, leaving a cellulose-rich residue⁸. The mechanism of enzymatic attack on lignin by these fungi has been extensively investigated because of its biotechnological interest^{1,9}.

The white-rot fungus *Phanerochaete chrysosporium* secretes three known classes of extracellular enzymes under ligninolytic (secondary metabolic) conditions in defined glucose medium: glyoxal oxidases (GLOXs), LiPs, and MnPs³. GLOX represented a family of radical copper oxidases produced by filamentous fungi, mainly organisms belonging to the group of white-rot fungi; the GLOX from *P. chrysosporium* is the most extensively studied of this enzyme family. GLOX acts as a source of the extracellular hydrogen peroxide that is required for the oxidations catalyzed by the LiPs and MnPs involved in lignin degradation^{10,11}. The enzyme catalyzes the oxidation of aldehydes to the corresponding carboxylic acids by coupling the reaction to the reduction of dioxygen to H₂O₂. Although GLOX activity was detected under ligninolytic conditions^{10,11}, there is no evidence for GLOX involved in lignin degradation.

The white-rot fungus *Phanerochaete sordida* YK-624, which was isolated from rotted wood, exhibits greater ligninolytic activity and selectivity than either *P. chrysosporium* or *Trametes versicolor*¹². In our previous study, we demonstrated that *P. sordida* YK-624 produces MnP^{12,13} and LiP^{14,15} as ligninolytic enzymes. Moreover, we previously identified a protein (BUNA2) that accumulated to high levels in cultures of *P. sordida* YK-624 under wood-rotting conditions. The promoter region of the BUNA2-encoding gene, designated *bee2*, was successfully cloned and demonstrated to be a useful regulator for the high expression of genes under ligninolytic conditions¹⁶. More recently, we have reported that the production of MnP was

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increased by the recombinant expression of a 5-amino-levulinic acid synthase-encoding gene¹⁷.

In the present study, we report the transformation of *P. sordida* YK-624 with a recombinant *glx* gene under regulation of the *bee2* promoter, yielding elevated transcript accumulation and GLOX activity under wood-rotting conditions. Moreover, we demonstrate that these transformants exhibit improved ligninolytic properties.

Materials and methods

Strains. *P. sordida* strain YK-624 (ATCC 90872), uracil auxotrophic *P. sordida* strain UV-64¹⁸, and *P. chrysosporium* ME-446 (ATCC 34541) were used in this study. All strains and transformants were maintained on potato dextrose agar (PDA) slants at 4 °C.

Construction of a GLOX-encoding gene expression vector, co-transformation of UV-64, and screening of regenerated clones. Genomic DNA was isolated from *P. chrysosporium* ME-446 mycelium using ISOPLANT II (Nippon Gene, Tokyo). Gene sequences for the GLOX-encoding gene were obtained from the DOE Joint Genome Institute and used to design PCR primers. The full-length *glx* (1907 bp) gene was amplified using the primer pair PcGLOX F1-PcGLOX R1. The procedure for constructing the *glx* expression plasmid, pBUNA2pro-GLOX, is described in Fig. 1. The plasmid pBUNA2pro, which was generated in our previous study¹⁶, was used for the construction of the *glx*

expression plasmid. Primers GLOX F1 and Pc GLOX R1 were designed to amplify the *glx* gene and to introduce a *Bgl* II site just before the first methionine codon and to add a *Bgl* II site just after the stop codon, respectively (step 1). The amplified DNA fragment was digested with *Bgl* II (New England Biolabs) and cloned into *Bgl* II-digested pBUNA2pro (step 2), yielding plasmid pBUNA2pro-GLOX (step 3). pBUNA2pro-GLOX was sequenced to verify the absence of PCR errors.

UV-64 protoplasts were prepared by a standard technique using cellulases and then co-transformed with pPsURA5 and pBUNA2pro-GLOX using the PEG method¹⁸. The co-transformed clones (G strains) were screened by PCR, as described previously¹⁹, with the following modifications: primers PcGLOX F2 and PsGPD R1 were designed to amplify the *glx* gene fused with the *bee2* promoter.

Enzyme assay. *P. sordida* YK-624, G strains, and U strains (UV-64 protoplasts were singly transformed with pPsURA5, yielding transformants designated as U strains) 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 the mycelia using a sterile cork borer. For determining the activities in the extracellular culture fluids, two mycelial disks from each source plate were placed into a 100-mL Erlenmeyer flask containing 10 mL nitrogen-limited Kirk medium²⁰, which then was statically incubated at 30 °C for 2, 4, 6,

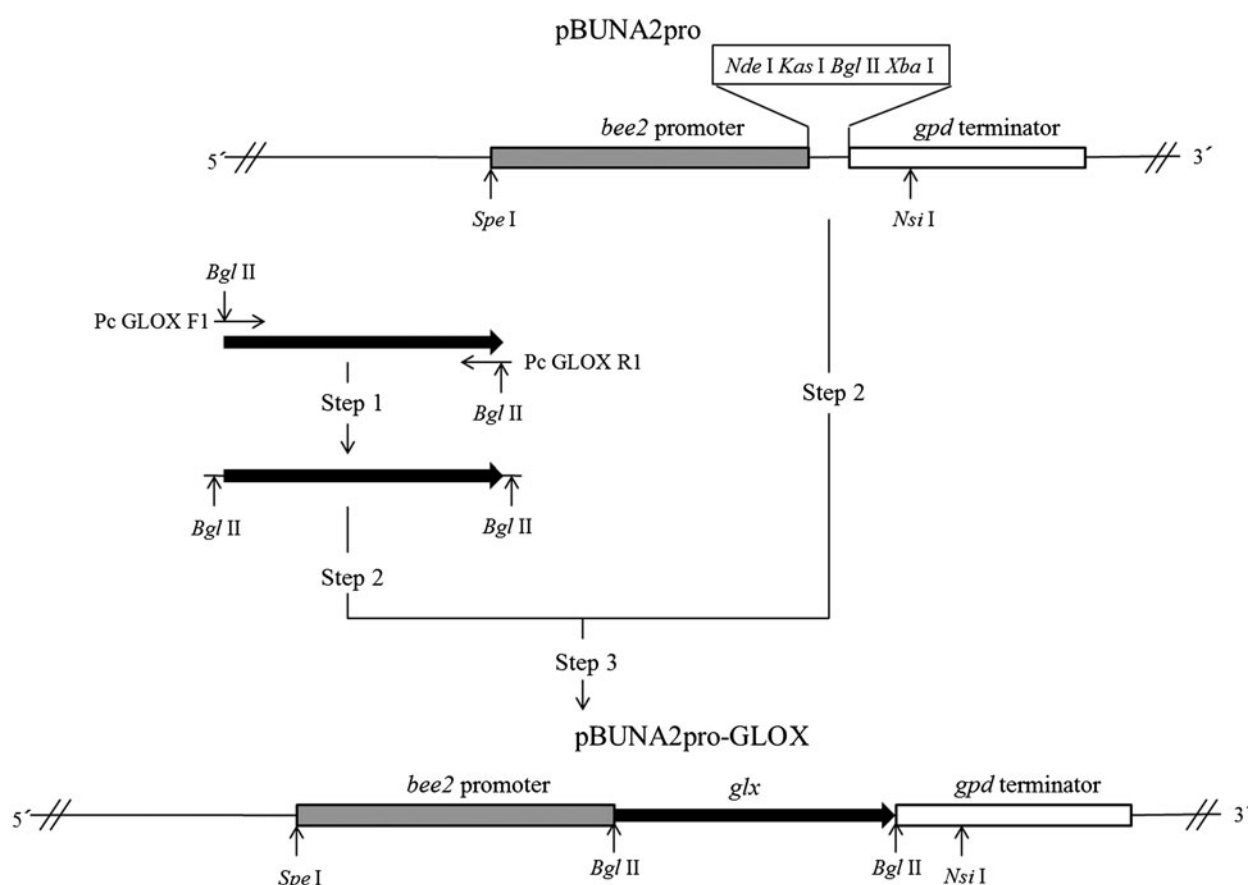


Fig. 1. Plasmid map of the *glx* expression plasmid pBUNA2pro-GLOX.

and 8 days. Meanwhile, for detecting the activities in fungal-treated beech wood meals, these strains were cultured in 50-mL Erlenmeyer flasks containing 0.5 g of extractive-free beech wood meal (80–100 mesh) and 1.25 mL of distilled water, and incubated at 30 °C for 5, 10, 15, and 20 days. Following the culture period, the method described by Hirai *et al.*¹²⁾ was modified for enzyme extraction. Briefly, fungal-treated wood meal was homogenized with 25 mL of 50 mM malonate (pH 4.5) or phosphate (pH 6.0) buffer containing 0.05% Tween 20 (Wako) using a Polytron PT1200E (Kinematica, Canada) homogenizer for a total of 5 min (20 s blending with 10-min intervals) at 4 °C.

MnP activity was measured by monitoring the oxidation of 2,6-dimethoxyphenol to coeruleinone (extinction coefficient at 470 nm ($\epsilon_{470} = 49.6 \text{ mM}^{-1} \text{ cm}^{-1}$).²¹⁾ The reaction mixture (1 mL) contained 2,6-dimethoxyphenol (1 mM), MnSO_4 (1 mM), and H_2O_2 (0.2 mM) in 50 mM malonate buffer (pH 4.5). GLOX activity was measured by monitoring the oxidation of guaiacol to the tetramer ($\epsilon_{470} = 26.6 \text{ mM}^{-1} \text{ cm}^{-1}$). The reaction mixture (1 mL) contained guaiacol (1 mM), 0.08 nkat of horseradish peroxidase, and 2-methylglyoxal (1 mM) in 50 mM phosphate buffer (pH 6.0). A standard curve was constructed for horseradish peroxidase (*Y*-axis: production of the tetramer; *X*-axis: H_2O_2 concentration) and used to calculate the production of H_2O_2 in the reaction system. One katal (kat) was defined as the amount of enzyme producing 1 mol of product per second.

Determination of ligninolytic properties. G strains, U strains, and *P. sordida* YK-624 were cultured in 50-mL Erlenmeyer flasks containing 0.5 g of extractive-free beech wood meal (80–100 mesh) and 1.25 mL of distilled water, which then were incubated at 30 °C for 28 days. After incubation, weight loss, Klason lignin content, and acid-soluble lignin content of the fungal-treated wood meal were determined, as previously described¹²⁾. The selection factor (SF), which is an indicator of ligninolytic selectivity, was calculated as follows: $\text{SF} = \text{lignin loss} / \text{holocellulose loss}$. Holocellulose loss was calculated as follows: total weight loss – lignin loss.

Transcriptional analysis of strain G-22. Strain G-22 was inoculated in liquid medium as described above and then statically incubated at 30 °C for 2, 4, 6, and 8 days. The mycelia were then collected and stored at –80 °C pending transcriptional analysis. One hundred milligrams of mycelial mat was ground to a fine powder using a mortar and pestle under liquid nitrogen, and then total RNA was isolated from the powder using an RNeasy Plant Mini kit (Invitrogen). RT-PCR was performed using 200 ng of total RNA with a PrimeScript RT-PCR Kit (TaKaRa Bio, Otsu) and the gene-specific primer sets PcGLOX F3-PsGPD R1 (to amplify recombinant *glx*) and ActinF-ActinR (to amplify the actin-encoding gene, *act*). PCR was performed for 28 cycles of template denaturation at 95 °C for 30 s, primer annealing at 56 °C for 1 min, and DNA extension at 72 °C for 1 min using Ex Taq DNA Polymerase (TaKaRa Bio).

Nucleotide sequence accession number. The nucleotide sequences of *glx* derived from *P. chrysosporium* have been deposited in the DDBJ database (<http://www.ddbj.nig.ac.jp/>) under accession no. AB910536.

Results and discussion

Isolation of recombinant *glx*-expressing transformants

P. chrysosporium was cultured in Kirk liquid medium for 5 days, and then genomic DNA was extracted from the mycelia. Based on the genome database of *P. chrysosporium*, two primers were designed to amplify the genomic DNA encoding GLOX. Genomic DNA of *glx* obtained by genome PCR was inserted between the *bee2* promoter and *gpd* terminator by restriction enzyme digestion and ligation (Fig. 1). This expression plasmid, pBUNA2pro-GLOX, was introduced into strain UV-64 together with pPsURA5 as a marker plasmid. The presence of the *bee2* promoter-*glx* fusion gene in each uracil prototrophic clone was confirmed by PCR using genomic DNA as the template (data not shown). We obtained 16 of these “G” strains that were co-transformed with pBUNA2pro-GLOX and pPsURA5.

GLOX production and ligninolytic properties of recombinant *glx*-expressing transformants

In our previous reports, *P. sordida* YK-624 produces only LiP and MnP as ligninolytic enzymes,^{12–15)} and LiP activity has been hardly detected in the culture containing Mn ion.¹²⁾ Therefore, we focused MnP among ligninolytic enzymes, and the production of GLOX and MnP by G strains was compared with those by singly transformed (pPsURA5 alone) “U” strains. The mean GLOX activity in the culture medium inoculated with G strains for 5 days was 0.66 nkat/flask, whereas that in the culture medium of U strains was 0.19 nkat/flask (Fig. 2(a)). The mean MnP activity in the culture medium inoculated with G strains was 8.51 nkat/flask, whereas that in the culture media of U strains was 7.78 nkat/flask (Fig. 2(b)). These results indicate that G strains showed higher GLOX activities than U strains, although MnP activities of G strains did not apparently differ from those of U strains.

The effect of recombinant *glx* expression on the ligninolytic properties of G strains was investigated. G strains showed 1.08-fold higher ligninolytic activity (Fig. 3(a)) and 1.18-fold higher SF values than U strains (Fig. 3(b)). In particular, strain G-22 showed 1.22-fold higher ligninolytic activity and 1.25-fold higher SF values than U strains (data not shown). These results indicated that the ligninolytic properties were improved by the recombinant expression of *glx* using the *bee2* promoter, with strain G-22 exhibiting the highest lignin degradation activity among the 16 screened co-transformants. In our previous study, the promoter region of *bee2* was demonstrated to be a useful regulator for the high expression of genes under ligninolytic conditions.¹⁶⁾ The results of the present study are consistent with that previous work. The proposed role of GLOX in lignin biodegradation is to generate H_2O_2 for LiP- and MnP-mediated reactions.²²⁾ Moreover, the identification of *glx* transcripts in decayed

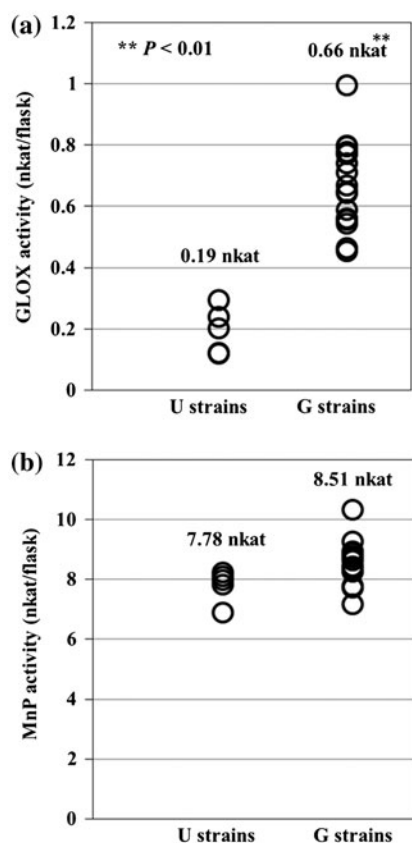


Fig. 2. GLOX (a) and MnP (b) activities in the extracellular culture fluids inoculated with U strains and G strains for 5 days.

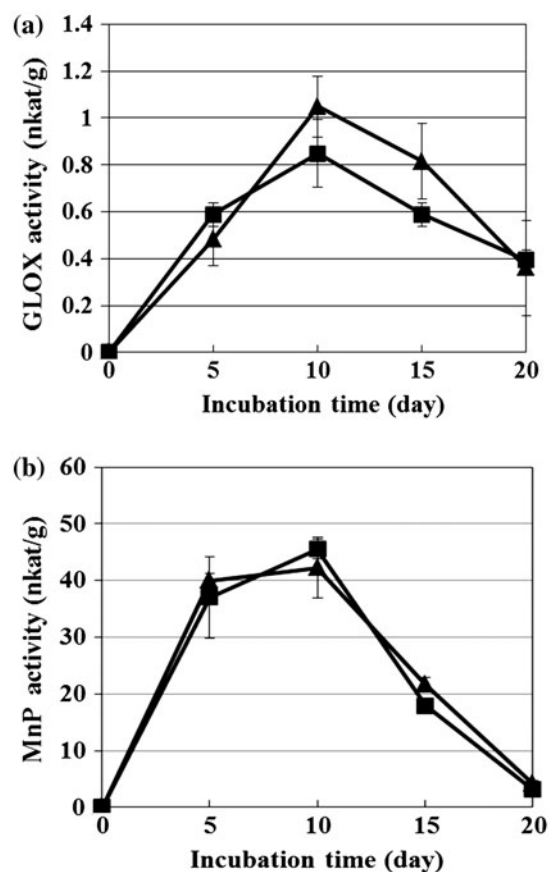


Fig. 4. GLOX (a) and MnP (b) activities detected in fungal-treated beech wood meals. G-22 (triangles) and WT (squares).

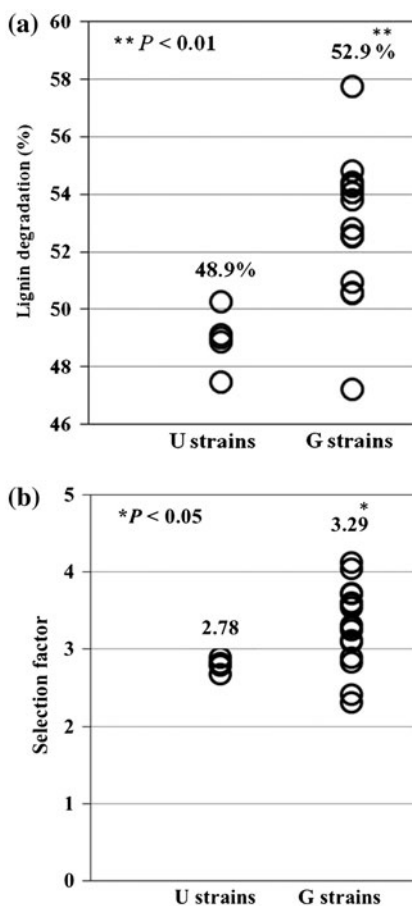


Fig. 3. Ligninolytic properties of U strains and G strains. Lignin degradation (a) and SF (b) by each strain for 28 days.

wood is consistent with a close physiological connection between extracellular peroxidases and GLOX.²³⁾ These reports, together with our present results, suggest that GLOX plays an important role in lignin degradation by white-rot fungi.

To confirm whether the improvement of the ligninolytic properties resulted from an increase in GLOX production, GLOX and MnP activities produced in beech wood meals inoculated with G-22 and wild type (WT) were determined. The MnP activity of G-22 was similar to that of WT, and no drastic fluctuations were observed (Fig. 4(b)). In contrast, higher GLOX activities were detected at 10 and 15 days in G-22, although similar GLOX activities for each strain were detected at 5 days (Fig. 4(a)). These results indicate that the improvement of the ligninolytic properties correlates with the higher production of GLOX.

GLOX production and transcriptional analysis of G-22

Transcriptional analysis was performed by RT-PCR to confirm whether the increment of GLOX activity reflected increased accumulation of *glx* transcript. Specifically, we analyzed a time course of GLOX activity in G-22 and WT. GLOX activity in G-22 increased gradually after 4 days, remaining relatively stable through 8 days (Fig. 5(a)). GLOX activity in G-22 was higher than that in WT during the incubation. The highest GLOX activity in G-22 was observed at 8 days, when activity was 7.2-fold higher than that in WT.

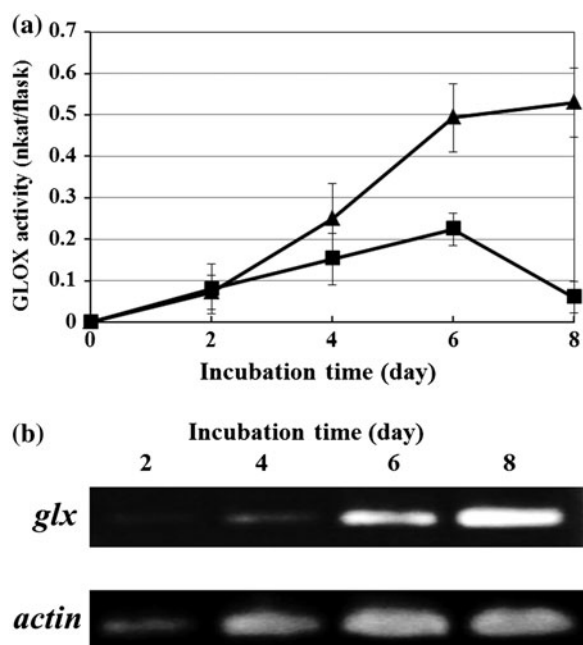


Fig. 5. Production of GLOX and expression of *glx* by strain G-22. Notes: (a) Time course of GLOX activity in strain G-22 (triangles) and WT (squares). (b) RT-PCR analysis of *glx* in strain G-22. The expression of actin was used as an internal control.

We next analyzed the transcription of recombinant *glx* in G-22 by RT-PCR (Fig. 5(b)). The analysis indicated that high levels of recombinant *glx* transcript were detected after 4–8 days. These results suggest that the increase of GLOX activity in G-22 was caused by *bee2*-driven expression of the recombinant *glx*.

To conclude, we found that the recombinant expression of *glx* under the control of the *bee2* promoter is effective for the improvement of the ligninolytic properties in white-rot fungi. We also provide the evidence that physiologic productivity of GLOX in white-rot fungi is not enough under ligninolytic condition. In our next study, we propose to investigate the molecular breeding of superior lignin-degrading fungi by the simultaneous expression of *glx* and a MnP-encoding gene (*mnp4*). To our knowledge, this is the first report to demonstrate that GLOX is involved in lignin degradation.

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