Mycoscience (2011) 52:431–435
DOI 10.1007/s10267-011-0122-7

NOTE

Molecular breeding of a novel *Coprinopsis cinerea* strain possessing a heterologous laccase gene, *lccK*, driven by a constitutive promoter

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Received: 21 February 2011 / Accepted: 6 May 2011 / Published online: 21 May 2011

Abstract Genomic DNA encoding the *Pleurotus ostreatus* LccK laccase was fused with the *Coprinopsis cinerea* β-tubulin promoter and terminator, and introduced into a *C. cinerea* strain. Linkage analysis, native PAGE separations, substrate specificity investigations and expression profiling indicated that *C. cinerea* transformants secrete *P. ostreatus* LccK, suggesting that the introns of the *lccK* gene are correctly spliced and the signal peptide for secretion is functional in *C. cinerea*. Transformants constitutively expressing laccase may be useful for the degradation of aromatic compounds.

Keywords Heterologous expression · *Pleurotus ostreatus* · Laccase secretion

Laccases (EC 1.10.3.2) are copper-containing polyphenol oxidases that are widely found in plants, fungi and bacteria. Although laccases are narrowly defined as oxidoreductases of *p*-diphenol, they exhibit low substrate specificity and can oxidize a wide range of aromatic compounds, with the concomitant reduction of molecular oxygen to water. When laccases oxidize phenolitic hydroxyl substrates, the radicals produced can polymerize substrates to produce pigments (Bavendamm 1928), allowing us to easily detect laccase activity (Tanabe et al. 1989).

The laccase secreted by *P. ostreatus* mycelia has been purified and characterized (Okamoto et al. 2000). Cloning and characterization of the *P. ostreatus lccK* gene suggested that this encodes the major extracellular laccase secreted by mycelia of *P. ostreatus* K16-2 (Okamoto et al. 2003). *Pleurotus ostreatus* laccase is constitutively expressed and secreted into culture media under normal cultural conditions (Okamoto et al. 2000). In contrast, laccase secretion by most wild-type strains of *C. cinerea* is not observed under normal cultural conditions, but appears to occur in response to environmental stresses. The different expression profiles of laccases in these basidiomycetes allowed us to detect heterologous expression of *P. ostreatus* laccase in *C. cinerea.* This has been suggested in a previous report, where *P. ostreatus* genomic DNA fragments digested with the restriction enzyme *Eco* RI were introduced into *C. cinerea* without cloning (Okamoto et al. 1995).

To express the *P. ostreatus* laccase, LccK, in *C. cinerea*, the *C. cinerea* β-tubulin promoter and terminator were fused to the genomic fragment encoding LccK. Genomic DNA was extracted from strain 39 of *P. ostreatus* (Mori Sangyo Co., Ltd., Gunma, Japan) for amplification of the laccase gene. Primers were designed based on the sequence of the *lccK* gene (Genbank accession no.: AB089612). Primer sequences used are listed in Table 1. The promoter of the *C. cinerea* β-tubulin gene (Genbank accession no.: AB000116) was amplified using primers 1 and 2, and the terminator was amplified using primers 5 and 6, from pPHT1; this carries a bacterial hygromycin B resistance gene fused to the promoter and terminator regions of the *C. cinerea* β-tubulin gene (Cummings et al. 1999). Amplifications were performed using *PfuUltra* high-fidelity DNA

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Table 1 Primer sequences used

Primer name	Sequence
#1-*Hin*dIII-t(P)-For	GCGCAAGCTTCATTTAAACGGCTTC *Hin*dIII
#2-POt(P)-Rev	TGCGCCTGGAAACATGCTGGGAACGCGAGG
#3-t(P)PO-For	CCTCGCGTTCCCAGCATGTTTCCAGGCGCA
#4-t(T)PO-Rev	GAACTAACGAATCATTTAGGACGGAACGAT
#5-POt(T)-For	ATCGTTCCGTCCTAAATGATTCGTTAGTTC
#6-t(T)*Hin*dIII-Rev	GCGCAAGCTTCAATATTCATCTCTC *Hin*dIII

The *Hin*dIII site is indicated by underlining

polymerase (Stratagene, La Jolla, CA) following the manufacturer's instructions. A genomic region coding for *P. ostreatus* laccase was amplified using primers 3 and 4, based on the *P. ostreatus* K16-2 *lccK* sequence (Okamoto et al. 2003). DNA fragments were separated on an 0.8% agarose gel, and the desired fragments were purified using a GENECLEAN II kit (BIO 101, Vista, CA). Purified fragments were used to provide template DNA for subsequent amplifications. The promoter and the laccase coding region were mixed and fused via amplification with primers 1 and 4, and then gel-purified. This fused fragment and the terminator were then mixed and fused via amplification with primers 1 and 6, and gel-purified. The final fusion product was digested with *Hin*dIII, gel-purified and ligated into the *Hin*dIII site of the pBACTZ vector that was constructed by insertion of the *C. cinerea trp1* gene into the pBeloBAC11 vector (Muraguchi et al. 2005). Ligation samples were used to electroporate 20 µl of electro-competent *Escherichia coli* DH10B cells; transformed cells were selected as described previously (Muraguchi et al. 2005), yielding pB-glccK.

Protoplasts were prepared from oidia of *C. cinerea trp*$^{-}$ strain 292, as previously described (Zolan et al. 1992), with the modification of 50 mg/ml of lysing enzyme (L1412, Sigma-Aldrich, St. Louis, MO) instead of Novozyme 234. Transformation of protoplasts was performed as previously described (Binninger et al. 1987). One hundred microliters of protoplasts (10^9/ml) was transformed by 10 µl of pB-glccK isolated with alkaline lysis.

Three trp^{+} transformants were obtained using pB-glccK and examined for expression of laccase using the Bavendamm test (Bavendamm 1928), involving the inoculation of mycelia onto MYG medium (Rao and Niederpruem 1969) containing 1 mM guaiacol. Two of the transformants exhibited the red phenotype signifying laccase activity, as in the original *P. ostreatus* strain (Fig. 1). To examine the dominance of laccase expression in *C. cinerea*, transformants were crossed with a wild-type monokaryon to obtain dikaryons. The resulting dikaryons also showed the red phenotype when grown on guaiacol medium (Fig. 1). Since phenol oxidase activity has been linked to fruit-body

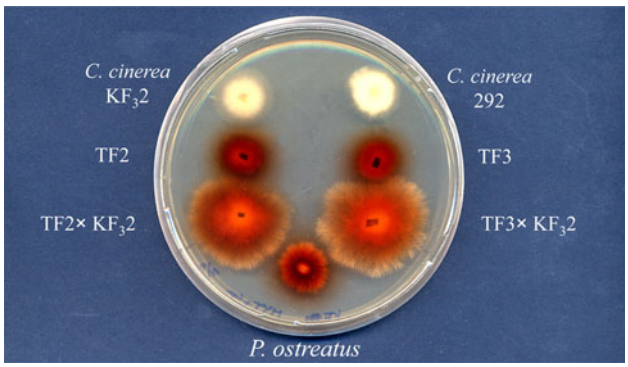

Fig. 1 Laccase activity of transformants in guaiacol medium. Mycelia of the indicated strains were grown on MYG medium containing guaiacol in order to monitor laccase activity. KF_32 is a wild-type strain. 292 was transformed with the LccK gene cassette inserted into the pBACTZ vector, yielding TF2 and TF3 transformants. *Red coloration* indicates laccase activity. The dikaryons formed by crossing transformants with KF_32 exhibit the dominant phenotype with laccase activity

formation in *C. cinerea* (Vnenchak and Schwalb 1989), we observed fruiting of the dikaryons. These were able to form normal fruit bodies, suggesting that the laccase activity of LccK does not affect physiological processes in *C. cinerea* reproduction.

To confirm that the laccase activity of transformants was due to expression of the *P. ostreatus lccK* gene integrated into the *C. cinerea* genome, we firstly examined whether the red phenotype in F_1 progeny cosegregates with insertion of the vector sequence into the genome. In F_1 progeny derived from a cross between transformant TF2 and a wild-type, the red phenotype was segregated (Fig. 2a). Ten white strains and ten red strains were selected from the F_1 progeny; their genomic DNAs were extracted and subjected to Southern analysis. Five micrograms of genomic DNA was digested with *Hin*dIII and fractionated by electrophoresis in 0.8% agarose gels. Fractionated fragments were transferred onto nylon membranes (Hybond-N^{+}, Amersham) by capillary blotting, using 0.4 N NaOH as the transfer solution, and subjected to Southern hybridization (Sambrook and Russell 2001). Probe labeling and hybridization were performed according to the Gene Images (GE

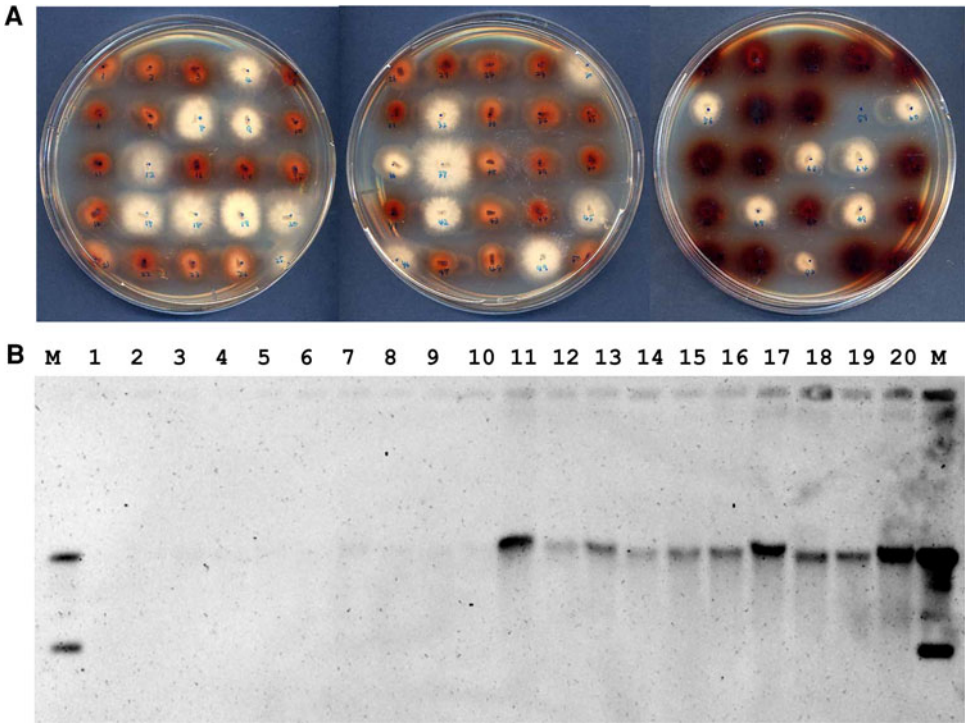

Fig. 2 Cosegregation in F_1 progeny. **a** Segregation of the red phenotype in F_1 progeny derived from crossing TF2 and KF_32. **b** Southern analysis of 10 white progeny and 10 red progeny using pBeloBAC11 as a probe. *Lanes 1–10* white progeny, *lanes 11–20* red progeny. *M* indicates a size marker, λ DNA digested with *Hin*dIII. The hybridized bands in the *marker lane* indicate 23 and 4.4 kb

Healthcare) instructions. The DNA of the pBeloBAC11 vector, which was part of pBg-lccK and did not contain *C. cinerea* DNA, was labeled and used as a probe to detect the introduced vector DNA (Muraguchi et al. 2005). All white strains showed no signal, and all red strains showed signals on the Southern blots (Fig. 2b), suggesting that the red phenotype is due to integration of pBeloBAC11 carrying the *P. ostreatus lccK* gene.

Secondly, we used native polyacrylamide gel electrophoresis (PAGE) (Davis 1965) to examine the properties of laccases secreted by mycelia into liquid media. Five agar cubes containing mycelia were inoculated into 20 ml of liquid MYG medium and incubated for 3 days at 28°C. Four milliliters of the liquid medium was dialyzed against 200 ml of Tris-HCl buffer (pH 6.8) at 4°C overnight. Dialyzed solutions were concentrated 40 times using a Mizubutori-kun kit (Atto, Tokyo, Japan). Samples were subjected to native PAGE (Fig. 3). A single band with laccase activity was detected in the *P. ostreatus* sample, as reported previously (Okamoto et al. 2000). The laccase secreted by TF2 had the same mobility as the *P. ostreatus* laccase, suggesting that TF2 secreted the *P. ostreatus* laccase, LccK.

We isolated tsh (temperature-sensitive defect in hyphal growth) mutants (Muraguchi et al. 2008) and found that when the temperature was increased from 28 to 42°C, the *tsh3-1* mutant (324) expressed the red phenotype in MYG medium containing guaiacol. We therefore used native PAGE to examine the laccases secreted by the *tsh3-1*

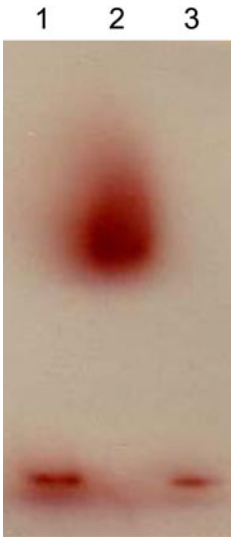

Fig. 3 Native PAGE analysis of secreted laccases. Laccases secreted into MYG liquid media were concentrated and subjected to native PAGE analysis. *Lane 1 P. ostreatus*, *lane 2 C. cinerea* 324 (*tsh3-1*), *lane 3 C. cinerea* TF2. Strain *C. cinerea* 324 (*tsh3-1*) is a temperature-sensitive mutant defective in hyphal growth at 42°C, which secretes intact laccase during incubation at 42°C. The laccase of 324 (*tsh3-1*) was collected after overnight culturing at 42°C

mutant using native PAGE. The *tsh3-1* mutant secreted a single laccase with a mobility different from either TF2 or the *P. ostreatus* strain (Fig. 3). Although we could not rule out the possibility that TF2 secreted another endogenous laccase with the same mobility as the *P. ostreatus* laccase, TF2 secreted a laccase different from that secreted by strain 324 under heat-shock stress, and it seems likely that it secreted the LccK laccase.

Thirdly, we examined the substrate specificity of the secreted laccases using 15 different substrates (Table 2). Mycelia of *P. ostreatus*, TF2, and 324 (*tsh3-1*) were

inoculated onto plates of each substrate, and the pigments produced by secreted laccases were observed. To encourage secretion of the intact *C. cinerea* laccase from the *tsh3-1* mutant, culture plates were transferred from 28 to 42°C. At 28°C, the substrate specificity of TF2 was the same as that of the *P. ostreatus* strain. After incubation at 42°C for 24 h, the pigments produced by TF2 at 28°C disappeared from some substrates (Table 2). This may have been due to degradation of the pigments by proteins secreted by TF2. The substrate specificity of the laccase at 28°C suggests that the laccase secreted by TF2 is the *P. ostreatus* laccase, LccK.

Finally, the physiological modes of secretion of laccases from TF2, 292 and *P. ostreatus* strains were observed in liquid media for 40 days. Five agar cubes with mycelia were inoculated into 20 ml of MYG liquid medium and cultured. One hundred microliters of liquid medium was sampled and added to 1 ml of reaction mixture containing 1 mM guaiacol and 50 mM of potassium phosphate buffer (pH 6.5). The mixture was incubated at 30°C for 30 min in the dark, and absorbance at 470 nm was measured. The laccase activity of TF2 peaked at 4 days after inoculation and then decreased. The laccase activity of *P. ostreatus* increased for 10 days and peaked at double the level of TF2 (Fig. 4). The laccase activity of *P. ostreatus* then declined and had disappeared by 30 days, while the activity of TF2 was maintained at low levels until 40 days. The expression property of TF2 suggested that the secreted laccase was constitutively expressed by the β-tubulin promoter. Transformants with constitutive expression of laccase may be useful for the degradation of aromatic compounds in the future.

Thus, four lines of evidence, linkage analysis, native PAGE separations, substrate specificity investigations and expression profiling strongly suggested that the laccase activities of transformants were due to the introduced genomic *lccK* gene. *Coprinopsis cinerea* laccases and their genes have been extensively characterized, and the genome project has revealed that this fungus contains a large family of laccases (Kilaru et al. 2006). We were not able to rule out the possibility that TF2 secreted an endogenous laccase with the same mobility as *P. ostreatus* laccase. This could be caused by the integration of the introduced DNA fragment containing the β-tubulin promoter into the 5′ region of the endogenous laccase. Further study using antibodies against LccK is required to confirm that the laccase secreted by TF2 is the *P. ostreatus* laccase, LccK.

If the laccase secreted by TF2 is indeed the *P. ostreatus* laccase, LccK, this suggests that the introns of the *P. ostreatus lccK* gene are correctly spliced and the signal peptide for secretion is functional in *C. cinerea*. Our results are consistent with previous research in which *P. ostreatus*

Table 2 Substrate specificity of laccases secreted by mycelia

Substrate	*P. ostreatus*		TF2		324	
	28°C	42°C	28°C	42°C	28°C	42°C
Guaiacol	+	+	+	−*	−	+
α-Naphtol	+	+	+	+	−	−
Catecol	+	+	+	+	−	−
Pyrogallol	+	+	+	+	−	+
Vanillin	−	−	−	−	−	+
Vanillic acid	+	+	+	−*	−	+
Gallic acid	+	+	+	+	−	+
3,4-Dihydroxy cinnamic acid	+	+	+	+	−	+
p-Hydroxy benzoic acid	−	−	−	−	−	+
Tannic acid	+	+	+	−*	−	+
p-Hydroxy benzaldehyde	−	−	−	−	−	+
4-Hydroxy-3-methoxycinnamic acid	+	+	+	+	−	+
p-Coumaric acid	−	−	−	−	−	+
Protocatechuic acid	+	+	+	−*	−	+
Syringic acid	+	+	+	+	−	+

Mycelia were inoculated onto plates containing different substrates (1 mM) and incubated for 3 days. After incubation at 28°C, plates were transferred to 42°C and incubated for 1 or 2 days

"+" indicates coloration based on laccase activity

"*" at 42°C may be due to degradation of pigments produced at 28°C

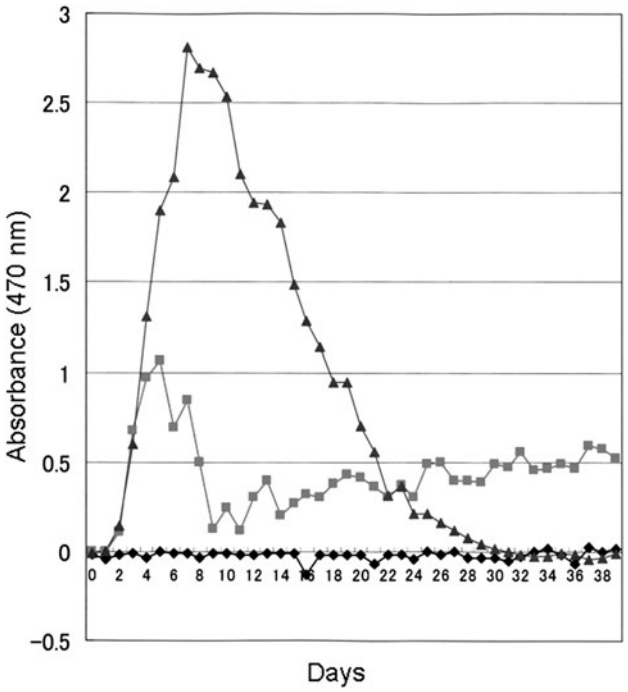

Fig. 4 Changes in laccase activity in liquid culture. Mycelia of *P. ostreatus* (*filled triangle*), *C. cinerea* TF2 (*filled square*) and 292 (*filled diamond*) were cultured in MYG liquid media at 28°C for 40 days. Laccase activities in the liquid media were measured daily

genomic fragments digested with *Eco* RI were introduced into *C. cinerea*, and *C. cinerea* strains that appeared to express the *P. ostreatus* lccK emerged (Okamoto et al. 1995). It has also been reported that the heterologous promoter and terminator function in *C. cinerea* to express native laccase genes with introns (Kilaru et al. 2006) and heterologous manganese (II) peroxidase (MnP) cDNA (Ogawa et al. 1998). It would appear that *C. cinerea* could be used as a host to express genes in heterologous genomic DNAs. Since some mushrooms are extremely difficult to grow in pure culture, functional analyses using easily cultivated hosts such as *C. cinerea* seem significant for both basic and practical purposes.

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