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Short Communication

Determination of laccase gene expression during degradation of 2,4,6-trinitrotoluene and its catabolic intermediates in *Trametes versicolor*

Sujin Cheong, Sumin Yeo, Hong-Gyu Song, Hyoung T. Choi*

Department of Microbiology, Division of Life Sciences, Kangwon National University, Chunchon 200-701, Republic of Korea

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Summary

We have cloned a laccase gene fragment isolated from a *Trametes versicolor* strain in Korea. It showed high similarity in nucleotide sequences when compared with other fungal laccases. TNT (2,4,6-trinitrotoluene), a widely used explosive, was transformed rapidly by *T. versicolor*. When TNT and its catabolic intermediates were added to the fungal culture, they were transformed during the first few hours and the expression level of the laccase gene was increased during the early stage of cultivation.

Introduction

The biological degradation of an explosive such as 2,4,6-trinitrotoluene (TNT) is an interesting research area because TNT is not only widely used but also a cause of environmental contamination. Since TNT shows high toxicity and mutagenicity (Won et al., 1976), its degradation is very important to environmental safety. There are many reports about TNT degradation by bacteria (Breitung et al., 1996; French et al., 1998) and by fungi (Stahl and Aust, 1995; Van Aken et al., 1997). The lignin degrading enzyme system of white-rot fungi is known to be deeply involved in the degradation (Van Aken et al., 1999).

Lignin degrading enzyme system consists of laccase, lignin peroxidase and manganese peroxidase. Glucose oxidase is also necessary for supplying H_2O_2 for the peroxidase reaction. Laccases have a variety of functions including dye decolorization (Rodriguez et al., 1999), dechlorination of chlorophenolic compounds (Roy-Arcand and Archibald, 1991), and fungal pathogenicity (Salas et al., 1996). Many fungal laccases have been cloned, and their expression regulations have been analyzed in lignolytic basidiomycetes, *Ceriporiopsis*

*Corresponding author.
E-mail address: htchoi@kangwon.ac.kr (H.T. Choi).

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subvermispora (Karahanian et al., 1998) and *Lentinula edodes* (Ohga and Royse, 2001). *Trametes versicolor* is one of the most famous white-rot fungi, which shows high degrading activities for lignin as well as many different recalcitrant materials. Since there are many reports about its laccase genes (Ong et al., 1997; Cassland and Jönsson, 1999), we isolated a *T. versicolor* strain from Korea (Kim et al., 2002) to investigate its functions on the degradation of recalcitrant materials. We have reported the genetic transformation (Kim et al., 2002), and the degradation of phenanthrene by this fungus and purified laccase enzyme (Han et al., 2004). It is necessary to determine whether TNT and its degrading intermediates (= catabolites) such as 2,4-dinitrotoluene (DNT) and nitrobenzene (Samson et al., 1998) can be degraded by this Korean isolate. In this study, we cloned a fragment of a laccase gene in *T. versicolor*, and determined its expression under degradation conditions by TNT and its catabolites.

Materials and methods

Fungal strain and culture conditions

T. versicolor monokaryon (9522-1) was grown at 30 °C in YMG medium to get the mycelial lawn (yeast extract 4 g, malt extract 10 g, glucose 4 g, H_2O 1 l, agar 16 g). The fungus was grown in the same liquid medium (50 ml in 250 ml Erlenmeyer flask) in a shaking incubator at 30 °C by inoculating 20 pieces of fungal mycelia cut with a no. 1 cork borer (diameter 4 mm). Whole fungal cells were ground in a Waring blender and the homogenate was transferred (10%, v/v) to fresh liquid medium (50 ml). The fungus was grown in the liquid medium for 5 days and then TNT, DNT and nitrobenzene (Supelco, USA) were added to the fungal cultures to determine laccase expression under nitroaromatic degrading conditions. The stock solutions for TNT, DNT and nitrobenzene were prepared in methanol and added to the culture flasks at the concentration of 100 mg/l. Laccase activity was determined using *o*-tolidine as the substrate as previously reported (Ko et al., 2001).

Determinations of TNT removal from the culture supernatant

To analyze residual TNT at the indicated times, each fungal culture including fungal cells and the culture supernatant was mixed with an equal volume of methylene chloride in a 50 ml centrifuge tube. The mixture was separated by centrifugation at 24,900*g* for 15 min after vigorous mixing for 30 s. The methylene chloride phase containing the residual TNT was analyzed by Waters HPLC (Milford, Mass, USA) with a reverse-phase column (5 μm, 4.6 × 250 mm). Elution was performed with a linear gradient of 20% (v/v) acetonitrile in water, and was increased to 90% (v/v) after 60 min. Both solvents contained 1% (v/v) acetic acid. Flow rate was carried out 1 ml/min and column temperature was 38 °C. The retention time of TNT was determined by monitoring the effluent at 235 nm and confirmation of TNT was accomplished by comparison of the retention time of analytical-grade TNT (Supelco).

Cloning of a laccase gene

Fungal laccases generally have four conserved copper-binding regions, and the conserved DNA sequences have been used as the primers for the amplification of laccase genes from several fungi (D'Souza et al., 1996; Kim et al., 2001). Two degenerated primers, primer 1 as a forward primer 5′-CAYTGGCAYGGNTTYTTYCA-3′ and primer 2 as a reverse primer 5′-TGNCCGTGMARRTGSAANGG-3′, which represent the copper-binding domains I and III, respectively, were used. Genomic DNA of *T. versicolor* was isolated as the template for PCR by following the CTAB method (Möller et al., 1992). The PCR conditions for the amplification of laccase fragments were the same as Kim et al. (2001). The amplified DNA band was cloned into the pGEM-T vector for the sequence analysis.

Determination of the laccase gene expression by RT-PCR

T. versicolor monokaryon cells grown in YMG liquid medium (50 ml; with or without TNT and its catabolites) were harvested at the time indicated by filtration through Whatman filter paper #1, and the fungal cells were immediately frozen in liquid N_2 after washing with d-H_2O treated with 0.1% diethylpyrocarbonate. Cells were ground into powder in a mortar and pestle with liquid N_2, total RNAs were isolated using RNeasy Plant Mini kit (Qiagene). The first strand of cDNA was synthesized from 1 μg of total RNA using PowerScript Reverse Transcriptase (Promega) by following the manufacturer's instructions. PCR was performed using Taq polymerase with the two primers that were used for the amplification of the laccase gene fragment. The expected length of the amplified DNA fragments by

RT-PCR was 1.05 kb, and they were analyzed in 1% agarose gel.

Results and discussion

When *T. versicolor* was transferred to the medium containing TNT or its catabolites, the fungus could not grow because of their toxicity (Song, unpublished). Therefore, the fungal culture was incubated for 5 days without the aromatic compounds. After the indicated incubation, TNT and its catabolites were added to the culture flasks at concentrations of 100 mg/l. When TNT was added to the 5-day-old culture of *T. versicolor*, TNT was rapidly transformed via the degradation pathway under either static or shaking culture conditions (Fig. 1). There have been several reports that lignin degrading enzymes are synthesized more at the static culture condition than the shaking culture condition; however, our fungal strain showed the same removal rate under both conditions. Therefore, we used the shaking culture method hereafter to get more homogenous fungal cells. Samson et al. (1998) reported that 96% of TNT was transformed after 4 days of incubation in *Ceratocystis coerulescens*, and *Lentinus lepideus* showed similar results. In case of a white-rot fungus, *Irpex lacteus*, TNT was also removed more than 95% during the first 10 h, and many metabolic intermediates appeared sequentially (Kim and Song, 2003). Therefore, *T. versicolor* isolated in Korea showed very good activity in the early transformation of the nitroaromatics.

A laccase gene fragment (1.3 kb) was amplified by PCR with primers 1 and 2 which were complementary to the conserved copper-binding regions I and III (Fig. 2). The nucleotide sequence was determined and was reported to the EMBL nucleotide sequence database (accession number: AJ746240). When the deduced amino acid sequence was compared with those of other fungal laccases such as POX2 of *Pleurotus ostreatus* (Z34848), LCC1 of *T. versicolor* (X84683) and LCC4 of *T. villosa* (L78077), it showed 65.7%, 97.4% and 97.1% homologies, respectively.

The expression levels of the laccase gene in the cultures with TNT and its catabolites were higher

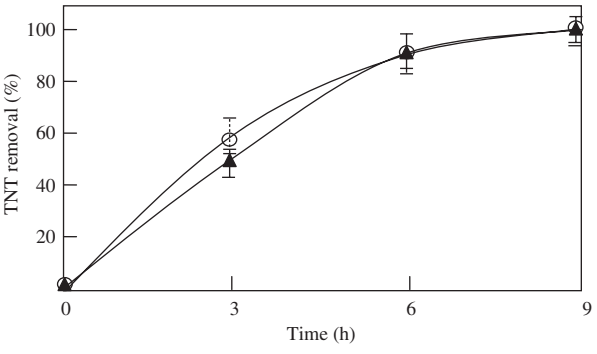

Figure 1. Removal of TNT in two different culture methods of *Trametes versicolor*. Experiments of static culture (▲) and shaking culture (○) with 50 ml media were conducted in triplicates.

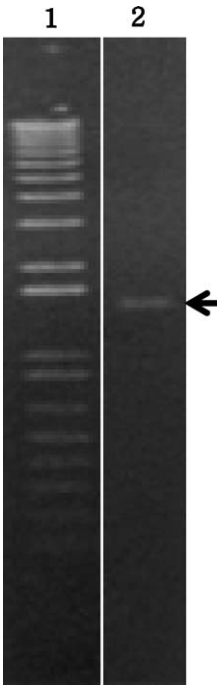

Figure 2. Agarose gel electrophoresis of the PCR-amplified fragment with primers 1 and 2, which represent the copper-binding domains I and III, respectively. Lane 1, 1 kb ladder; lane 2, laccase gene fragment (1.3 kb, arrow) amplified with primers 1 and 2.

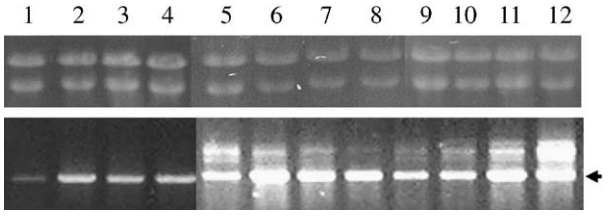

Figure 3. Induction of laccase gene expression by TNT, nitrobenzene and DNT. Upper panel, total RNA from cultures at the indicated times. Lower panel, agarose gel electrophoresis of RT-PCR products (1 kb, arrow) with laccase primers using RNAs isolated from cultures. Lane 1, control culture (6 h); lane 2, culture with TNT (6 h); lane 3, culture with nitrobenzene (6 h); lane 4, culture with DNT (6 h); lanes 5–8, same as lanes 1–4 but for 12-h-old cultures; lanes 9–12, same as lanes 1–4 but for 24-h-old cultures.

than that of the control culture (Fig. 3). The increase of the gene expression was most prominent at the early phase of addition (6 h), even though there was some increase at later culture periods. Kim and Song (2003) have reported that the nitro group of TNT was reduced to an amino group in the initial degradation pathway in *I. lacteus*. Furthermore, laccase induction has been observed after the addition of many materials such as cellulose, potato extracts and sawdust in *Lentinula edodes* (Zhao and Kwan, 1999). Therefore, the laccase gene in this fungus is considered to be implicated in the degradation of TNT and its catabolites because many fungal laccases are frequently expressed more under diverse recalcitrant material degrading conditions.

The pattern of increase of gene expression was parallel with the transformation phase of TNT, which removed more than 80% at 6 h after the addition of TNT (Fig. 1). When TNT and its catabolites were added to the 5-day-old culture of *T. versicolor*, laccase activities were induced up to 4-fold higher than the control culture in the presence of three nitroaromatics following 6 h of addition (results not shown). There was a high increase of laccase gene expression at the initial phase (6 h after addition) of TNT transformation, which implicated laccase in the transformation of nitroaromatics. *Trametes* sp. I-62 showed increased gene expression and enzyme activity of three laccase isozymes until 42 h in the presence of *p*-coumaric acid and guaiacol, when the chemicals were added at the beginning of cultivation (Terrón et al., 2004). *T. versicolor* and *P. ostreatus* showed increased laccase activities under mixed culture conditions with soil microorganisms (Baldrian, 2004). We are currently analyzing the degradation of TNT and its catabolites by our fungal strain grown in a sterilized soil, and this result will be used as a guideline for the degradation of nitroaromatics in natural soil in future experiments.

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