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Heterologous Expression and Characterization of a Laccase from *Laccaria bicolor* in *Pichia pastoris* and *Arabidopsis thaliana*

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Laccases can oxidize a variety of phenolic and non-phenolic substrates including synthetic dyes. In this research, a laccase gene *Lcc9* from *Laccaria bicolor* was chemically synthesized and optimized to heterogeneous expression in *Pichia pastoris* and *Arabidopsis thaliana*. The properties of recombinant laccase expressed by *P. pastoris* were investigated. The laccase activity was optimal at 3.6 pH and 40°C. It exhibited K_m and V_{max} values of 0.565 mmol l^{-1} and 1.51 μmol l^{-1} min^{-1} for ABTS respectively. As compared with untransformed control plants, the laccase activity in crude extracts of transgenic lines exhibited a 5.4 to 12.4-fold increase. Both laccases expressed in transgenic *P. pastoris* or *A. thaliana* could decolorize crystal violet. These results indicated that *L. bicolor* laccase gene may be transgenically exploited in fungi or plants for dye decolorization.

Keywords: Laccase, *Laccaria bicolor*, heterologous expression, *Pichia pastoris*, *Arabidopsis thaliana*

Introduction

Laccases (E.C. 1.10.3.2) are oxygen oxidoreductases belonging to the blue multicopper oxidase family, which also includes ferroxidases, ascorbate oxidases and ceruloplasmins. Laccase was first found in latex obtained from the tree *Rhus vernicifera* by Yoshida. In 1894, the enzymes were isolated and purified from lacquer trees of Southeast Asia by Bertrand [1]. Laccase is widely distributed in plants, bacteria, fungi and insects. Among them, fungal laccase has been the most widely studied. In 1986, the presence of laccase was observed in fungi for the first time [2]. So far, a large number of fungi have been confirmed as producers of laccases, especially *Basidiomycetes* and *Ascomycetes* fungi. Four acidic laccase isoforms have been detected in Ganoderma lucidum BEOFB 431 [3], and recently, an extracellular laccase (LccH) from the newly-isolated Basidiomycetous fungus *Hexagonia hirta* MSF2 was also reported [4].

Laccases catalyze the oxidation of a wide variety of phenolic and non-phenolic compounds using molecular oxygen as the sole electron acceptor and generate water as the by-product. During wheat straw fermentation, laccases were also the dominant ligninolytic enzymes in some fungal species [5]. Tetracycline can be degraded by immobilized laccase and the mechanism was proposed [6]. Synthetic dyes were also decolorized by laccases from different organisms [7, 8]. The scope of laccase substrates can be widened to higher-redox potential compounds than laccase itself with the help of diffusible electron carriers like 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic) (ABTS) that constitute the laccase-mediator system (LMS) [9].

Thus, laccase and LMS have been found to have potential applications in bioremediation, biofuel cells, biosensors, textiles, pulp and paper, and food and organic synthesis. Interest in the bioremediation of laccases has increased recently because of their potential use in the detoxification of pollutants and in bioremediation of phenolic compounds [10]. Discovery and characterization of novel laccases are still important for industrial application.

Laccaria bicolor is a ubiquitous, ectomycorrhizal symbiotic fungus of hardwood and conifer roots found in different ecological niches. The genome sequence of *L. bicolor* has been reported and the 65-megabase genome contains ~20,000 predicted protein-encoding genes [11]. There have been 11 multi-copper oxidase genes of *L. bicolor* divided into two distinct subfamilies to laccases *sensu stricto* (*lcc1 to lcc9*) and ferroxidases (*lcc10 and lcc11*). Transcript profiling using whole-genome expression arrays and quantitative reverse transcriptase-polymerase chain reaction has revealed a specific function of these enzymes [12]. We have heterologous expression and characterization of one laccase (LbLCC3) from *L. bicolor.* But the function and properties of the other laccases from *L. bicolor* are still unclear. In this study, we synthesized a gene according to the amino acid sequence of LbLCC9, and transferred this gene into *P. pastoris* and *A. thaliana*. The characteristics of the recombinant laccase from *P. pastoris* were investigated and the dye decolorization ability of both laccases expressed in transgenic *P. pastoris* and *A. thaliana* were verified.

Materials and Methods

Organisms, Reagents and Plant Growth Conditions

The *Escherichia coli* strain DH5α (Invitrogen, USA) was applied to DNA and plasmid manipulations. *P. pastoris* strain GS115 (Invitrogen, USA) was used for heterologous expression of the laccase. pMD18-T (TaKaRa, China) and modified pPIC9K vectors (our lab) were used as cloning and expression vector respectively. All enzymes of DNA manipulation were purchased from TaKaRa (China). Bradford protein quantitative test kit was purchased from Shanghai Generay Biotech Co., Ltd. ABTS (2-2′-azino-bis-3-ethylbenzothiazoline-6-sulfonate) and crystal violet (hexamethyl-pararosaniline chloride) were obtained from Shanghai Sangon Co. Ltd. (China). Unless otherwise stated, all other chemicals were obtained from commercial sources and of analytical grade. The media used for the expression of recombinant protein were prepared following the Multi-Copy Pichia Expression Kit (Invitrogen, USA). *Agrobacterium tumefaciens* GV3101 and *A. thaliana* (ecotype Columbia L) plants were sourced from our laboratory. Seeds of *A. thaliana* were surface sterilized with bleaching powder (5%, w/v) for 20 min, washed with sterile water three times, placed in Petri dishes that contained Murashige and Skoog medium with 0.8% agar, and then placed in the dark at 4°C for 3 days. All of the plants were cultivated in a growth room at 22°C under standard long-day conditions (16:8 h day-night cycle, ~120 μM photons $m^{-2}s^{-1}$ light intensity).

Synthesis, Cloning and Vector Construction

According to the amino acids sequence of LbLCC9 (GenBank: ACN49096.1), *LbLCC9I* was designed and synthesized according to preferential codon usage in yeast. The synthetic gene was cloned into the pMD18-T vector and sequenced. The amino acid sequences used for analysis were retrieved from the database of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) and protein domain was conducted using NCBI conserved domain database (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml). The DNA fragment of *LbLCC9I* gene was subcloned into a modified pPIC9K vector and pCAMBIA1301 vector.

Transformation, Screening and Expression of LbLCC9I in *P. pastoris*

The expression vector pPIC9K/*LbLCC9I* was transformed into *E. coli* DH5α and extracted according to the standard method. Ten microliters of plasmid was digested into two fragments with restriction enzyme *Bgl* II, and then transformed into *P. pastoris* GS115 competent cells by electroporation (Bio-Rad Genepulser, USA). After being pulsed, *P. pastoris* cells were spread on selective RDB plates and incubated at 28°C until colonies appeared, and these were picked and streaked on BMMY plates containing ABTS. If the *LbLCC9I* gene is recombined into yeast genome DNA and expressed successfully, the medium around the yeast colony should turn blue. Therefore, a blue colony was selected and further confirmed by PCR.

A single colony was picked and inoculated in 50 ml of BMGY medium at 28°C in a shaking incubator until the culture reached to OD_{600} = 2-6. The cells were harvested by centrifuging and resuspended in 100 ml of BMMY medium supplemented and returned to an incubator to grow for 3 days. Methanol was added to a final concentration of 1% every 24 h.

Purification and Characterization of Recombinant Laccase in *P. pastoris*

The crude enzyme in the supernatant of methanol-induced culture was precipitated by 40% and 70% ammonium sulfate. PBS (pH 6.0) was used to dissolve and dialysis the precipitation. Then the desalted solution was purified by nickel-charged iminodiacetic acid column according to the operation manual. The molecular mass of the laccase was estimated by SDS-PAGE with a 12% polyacrylamide gel. The concentration of purified protein was quantified by Bradford Protein Assay with bovine serum albumin as a standard. The activity measurement was performed with 0.5 mM ABTS in 0.2 M citrate-phosphate buffer at 37°C in a total of 200 μl. The assay mixture was incubated at 37°C for 10 min

before the enzyme was added. The reaction was terminated by addition of NaN_3 after 120 min. The absorbance of reactant was measured at 420 nm (ε = 12,000 $M^{-1}cm^{-1}$). One unit (U) of laccase activity was defined as the amount of enzyme catalyzing the formation of 1.0 μmol of product per minute under the assay conditions. The optimal pH and temperature, stability at different pH and temperature were determined. K_m and V_{max} were assayed using ABTS from 0.025 to 1.5 mM. Kinetic parameters were determined using Lineweaver–Burk plots. There were three replicates of each treatment.

Transformation of the LbLCC9I Gene into *A. thaliana*

The vector pCAMBIA1301/*LbLCC9I* was introduced into the *A. tumefaciens* GV3101 by electroporation and subsequently used to transform *A. thaliana* by a previously described floral dip method to generate transgenic plants [13]. Transgenic plants were obtained from plants grown on a medium containing 40 mg hygromycin l^{-1}. Genomic DNA extracted from *A. thaliana* seedlings was used for PCR to confirm the transfection of a foreign gene. The actin2 gene of *A. thaliana* (*ACT2*, GenBank Accessin No. U41998) was used as a reference. PCR reactions were carried out in 30 cycles lasting 30 sec at 94°C, 30 sec at 55°C, 30 sec at 72°C, with a final extension at 72°C for 10 min with the following primers: *ACT2* (*ACT2F*: 5′-AGTAAGGTCACGTCCAGCAAGG-3′; *ACT2R*: 5′-GCACCCTGTTCTTCTTACCGAG-3′) and *LbLCC9I* (*LbLCC9I*F: 5′-ACCATTGTAGGTTGGACACAG-3′; *LbLCC9I*R: 5′-ATTGGT GGTCCACATCCACTG-3′). The PCR products were separated on 1% (w/v) agarose gels.

Expression of the LbLCC9I Gene in Transgenic *A. thaliana*

The 3-week-old transgenic and wild-type (WT) seedlings were used to extract total RNA according to the manufacturer's instructions of the Multisource Total RNA Miniprep Kit (Axygen, USA). A reverse transcription system was conducted with 5 μg of total RNA as the template (Promega, USA), and the products were qualitatively amplified by RT-PCR. PCR and electrophoretic procedures are the same as described above.

Similar 3-week-old seedlings were used to extract crude protein. Transgenic and WT plants were ground in liquid nitrogen in a mortar with pestle and homogenized with a 0.2 M citrate-phosphate buffer (pH 3.6). Plant tissue was suspended in this buffer according to 100 mg/ml. The homogenate was centrifuged at 12,000 ×*g* (4°C). The supernatant was used to detect the laccase activity (added ABTS to 0.2 mM). Native-PAGE was performed according to the reported method [14].

Heterologous Expression of Laccases in *P. pastoris* and *A. thaliana* for Decolorization of Crystal Violet

The decolorization of crystal violet was assessed using freshly prepared enzyme from *P. pastoris* and the extract crude protein from *Arabidopsis*. The reaction mixture for the decolorization assay contained 0.1 mM of crystal violet, McIlvaine buffer and 50 μl of the above enzyme in a total of 200 μl. ABTS, as the mediator, was added if necessary. Reaction was initiated by adding enzyme to the assay mixtures and then subsequently incubated in dark for 24 h. The amount of dye was determined by measuring the absorbance at 590 nm and calculated from standard curves of absorbance versus dye concentration.

Results and Discussion

Synthesis of LbLCC9I and Genetic Transformation of *P. pastoris*

Amino acid sequence analysis showed 56.1 % homology between LbLCC9 and a laccase (PDB accession: 1HFU) from *Coprinopsis cinerea*. LbLCC9I had the fungal laccase signature sequences L1-L4 [15]. Three cupredoxin-like domains corresponding to the laccase from *Trametes versicolor* were predicted. In the phylogenetic analysis of basidiomycete laccases *sensu stricto*, Lcc9 and four other laccases from *L. bicolor* were clustered with members of the large *C. cinerea* laccase family [12]. To improve the expression in yeast, the sequence of *LbLCC9* was modified, synthesized and renamed *LbLCC9I*. The nucleotide sequences of *LbLCC9I* and *LbLCC9* were 78.23% identical. The DNA fragment was inserted into pPIC9K to construct expression vector after PCR amplification. Linearized expression vector was transformed into *P. pastoris* by electroporation. The transformants confirmed by PCR were chosen for further investigation.

Characterization of LbLCC9I Expressed by *P. pastoris*

After three consecutive days of fermentation, the enzyme LbLCC9I was purified by affinity chromatography with Ni-column. The purified laccase appeared as a single protein band on SDS-PAGE gel and the molecular mass of the purified laccase was consistent with its theoretical molecular weight (Fig. 1). This is similar to another laccase in *L. bicolor* as we reported earlier, the molecular mass of that laccase is 56 kDa [16]. The normal laccase enzymes have molecular weight ranging from 50 to 130 kDa [17]. Difference between the molecular weight predicted from the peptide sequence and the experimentally obtained molecular weight is caused by glycosylation, which typically accounts for about 10−20% of the total MW [18]. The *C. cinereus* laccase CcLCC5I purified from yeast can be glycosylated, as proved by the treatment of deglycosylation enzyme Endo H [19].

Gallic acid, tannic acid, syringaldazine, guaiacol and ABTS have been used as laccase indicators for the detection

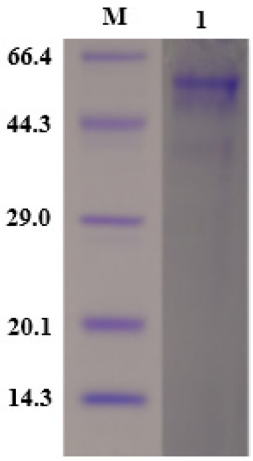

Fig. 1. SDS-PAGE of recombinant LbLCC9I expressed via *P. pichia*.
The gel was stained with Coomassie brilliant blue. Lanes M, molecular weight marker, Lane 1, purified LbLCC9I.

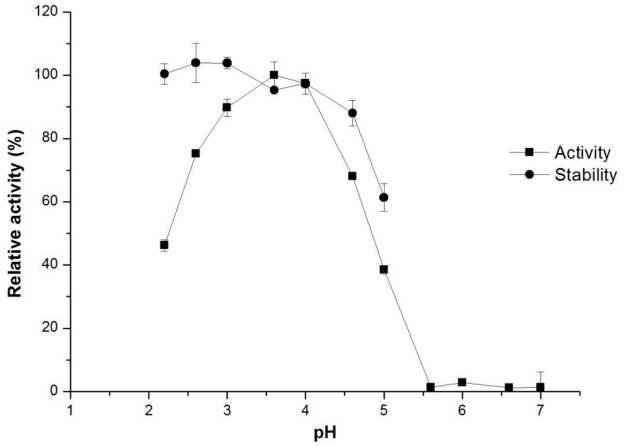

Fig. 2. Effects of pH on laccase activity and stability.
The optimum pH was determined spectrophotometrically with ABTS in 0.2 M citrate-phosphate buffer (pH 2.2–7.0) at 37°C. The stability of LbLCC9I was done by enzyme incubation at 4°C in various pH buffers for 24 h. Enzyme activity at starting time was assumed as 100% stability. Enzyme assays were performed in triplicates.

of laccase producers. They can be oxidized by laccases to produce a color. ABTS is one of the most common and electron substrates used for the measurement of laccase activity and the definition of the international unit of laccase activity is based on ABTS oxidation [20]. With ABTS as a substrate, the *Km* and *Vmax* values of the heterologous laccase were 0.565 mM and 1.51 μM min^{-1} respectively at pH 3.6 and 40°C. The *Km* values for a large number of laccases have been described. This parameter has great variance depending on the enzyme source and the substrates [18]. The *Km* of LbLCC9I is slightly higher than that of LbLCC3I from the same organism [16].

Using ABTS as a substrate, the optimum pH value of catalytic reaction by LbLCC9I was 3.6, which was similar to the laccases from *Trametes modesta* [21], *Panus rudis* [22], and *Albatrella dispansus* [23]. Moreover, the activity of LbLCC9I was only 46.2% and 38.5% when the catalytic reaction was carried out at pH 2.2 and pH 5.0 respectively. It became completely inactive at pH 5.6–7.0 (Fig. 2). The stability of the enzyme also decreased with the increase of pH values. The decrease of laccase activity in neutral or alkaline pH may be because of the inhibition by hydroxide anion. After incubation at 4°C for 24 h, the residual activity was 61.3% at pH 5.0. The enzyme was stable at acidic pH (Fig. 2). This was consistent with the optimum pH and probably indicated the laccase function under acidic physiological conditions.

The optimum reaction temperature and thermal stability of LbLCC9I were determined with ABTS as the substrate at pH 3.6. The laccase had maximum activity at 40°C (Fig. 3).

The enzyme retained most activity at 20–40°C after 60 min (Fig. 4). It was not stable at higher temperatures than 60°C during prolonged incubation. The residual activities were reduced to 7.4% and 4.3% after incubation at 60 and 70°C for 60 min, respectively. The optimum temperature for laccases is generally between 30°C and 50°C [24]. But the optimum temperature of a novel laccase from the thermophilic ascomycete fungus *Scytalidium thermophilum* was 80°C using DMP as a substrate [14].

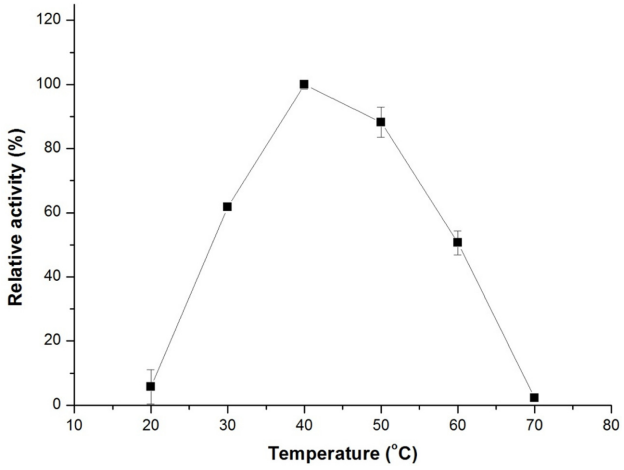

Fig. 3. Effects of temperature on laccase activity.
The laccase activity was determined spectrophotometrically with ABTS in 0.2 M citrate-phosphate buffer (pH 3.6) at different temperatures (20–70°C) for 2 h. Enzyme assays were performed in triplicates.

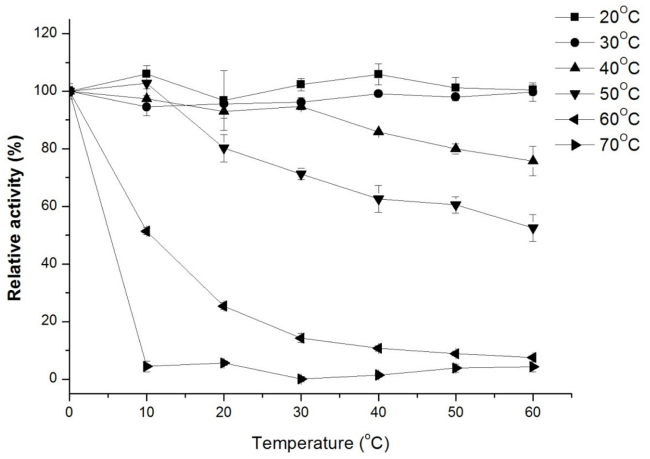

Fig. 4. Effects of temperature on laccase stability.
The enzyme stability was checked by enzyme incubation at various temperatures (20–70°C) with different durations (10 min increments). Enzyme activity at start of incubation time was assumed as 100% stability. Enzyme assays were performed in triplicates.

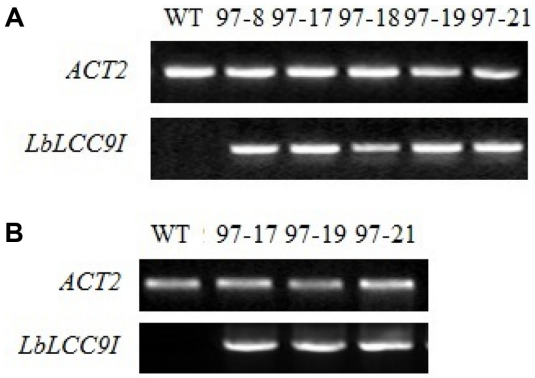

Fig. 5. The recombination and transcription of *LbLCC9I* in transgenic lines (97-17, 97-19 and 97-21) were confirmed by PCR (**A**) and RT-PCR (**B**).
The *A. thaliana* actin2 gene was used as the control to show the normalization of the amount of template in PCR.

Transformation and Expression of the LbLCC9I Gene in Arabidopsis

The LbLCC9I gene was transformed into *A. thaliana* by an *A. tumefaciens* (GV3101)-mediated transformation and verified through PCR (Fig. 5A). Genomic DNA extracted from *A. thaliana* seedlings was used for PCR to confirm the insertion of a foreign gene. After segregation analyses, three T_2 lines were selected for further analysis. Wild-type plants were also grown to be used as controls. Expressions of the LbLCC9I gene were determined from T_2 plants by using RT-PCR. The specific DNA fragment (300 bp) of LbLCC9I was amplified from three transgenic lines. However, no signals were detected in wild-type plants (Fig. 5B). Reverse transcriptase PCR demonstrated that the LbLCC9I gene was successfully transcribed in the transgenic plants. As compared with untransformed control plants, the laccase activity in crude extracts of transgenic plants 97-17, 97-19 and 97-21 exhibited a 5.4 to 12.4-fold increase (Fig. 6). This indicates the transgenic *Arabidopsis* could express active laccase from *L. bicolor*.

Laccases were encoded by multigene families in plants and *A. thaliana* contained 17 laccases with different physiological functions including lignin synthesis [25]. A laccase gene *lac3* from poplar was essential for normal cell wall structure and integrity in xylem fibers [26]. Reduced expression of another laccase of *Populus deltoids* resulted in transgenic plants with changes in syringyl/guaiacy ratios as well as altered sugar release phenotypes [27]. Two laccase genes of carrot had different responses to abiotic and metal ion stresses [28]. But in this study, all the transgenic lines were phenotypically indistinguishable from the wild-type line when they were grown on MS agar plates. This indicates that the insertion of the LbLCC9I gene in these plants produced no visible morphological changes.

CV Decolorization by Recombinant Laccases from *P. pastoris* and *A. thaliana*

Triphenylmethane dyes were employed in several industrial dyeing processes, but they are toxic, mutagenic and carcinogenic in mammalian cells. Study about the biodegradation of these dyes helps to eliminate the threat to humans. So the decolorization ability of laccases expressed in *P. pastoris* and transgenic *Arabidopsis* on crystal violet were investigated. ABTS is necessary for the decolorization of crystal violet by the two laccases. A similar result could be found in a heterologous laccase from *Colletotrichum lagenarium* [29]. In the presence of ABTS, the decolorization rates of crystal violet by laccases in *P. pastoris* or *A. thaliana* reached 90.7% and 83.6%, respectively (Fig. 7). Expression of *LbLCC9I* gene in *P. pastoris* was helpful for production of this laccase to meet the requirements of industrial applications. Phytoremediation was a low-cost method for removal of various organic pollutants. The application of laccase in pollution remediation has attracted many researchers. Transgenic *A. thaliana* overexpressing a laccase gene from cotton exhibited enhanced resistance to 2,4,6-trichlorophenol [23]. The laccase of *Coriolus versicolor* was introduced into tobacco plants and one transgenic plant was able to remove bisphenol A and pentachlorophenol [30]. Crystal violet was

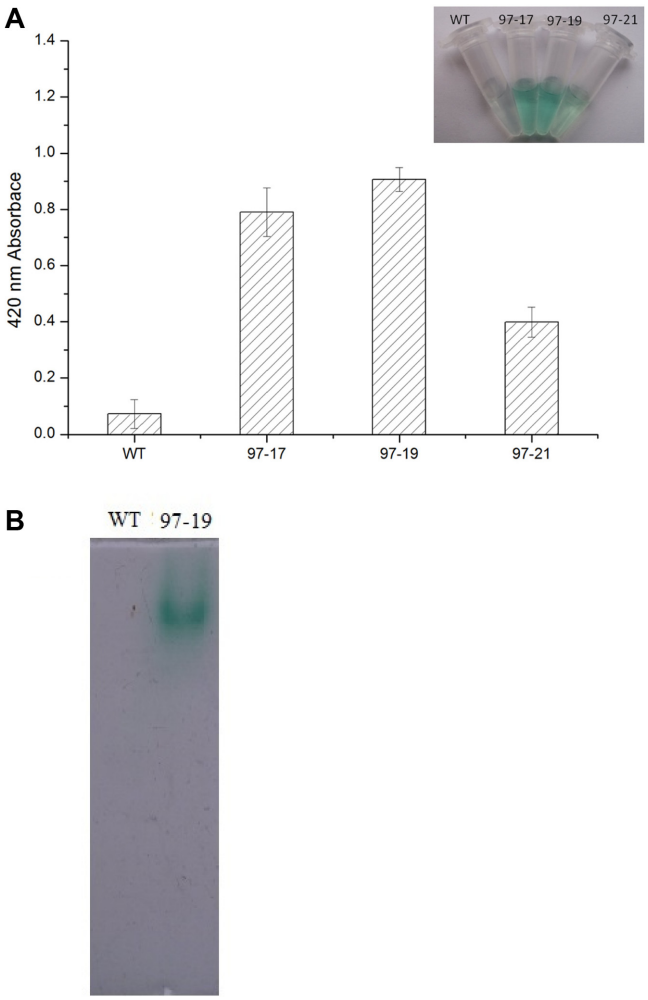

Fig. 6. The laccase activity in crude protein extracts of transgenic and wild-type (WT) plants.

(**A**) The transgenic and WT plants (fresh weight 100 mg) were ground in liquid nitrogen in a mortar with pestle and homogenized with 1 ml 0.2 M citrate-phosphate buffer (pH 3.6). The homogenate was centrifuged and the supernatant was used as crude extracts. Adding ABTS to 0.2 mM, the reactions were done at 37°C for 1 h. (**B**) Native-PAGE of crude protein extracts of transgenic line (97-19) and wild-type plants (WT).

converted to non-toxic leucocrystal violet by transgenic *Arabidopsis* expressing a *Citrobacter* sp. triphenylmethane reductase [31]. In this study, the transgenic *Arabidopsis* could express a fungal laccase from *L. bicolor* and this laccase could effectively decolorize crystal violet. These results indicated that fungal laccase genes may be transgenically exploited into plants for bioremediation. Our report provided an alternative approach to dye decolorization in the environment.

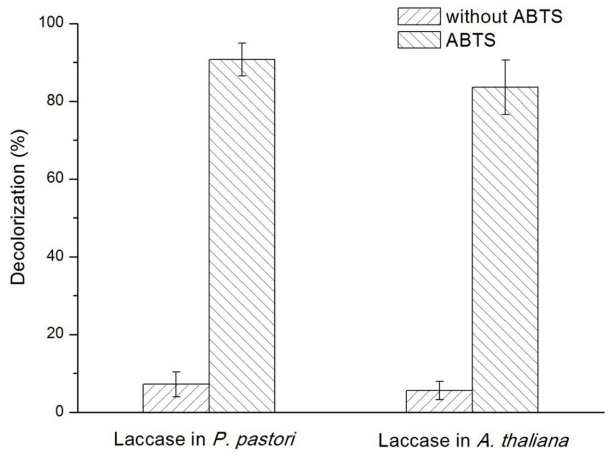

Fig. 7. Decolorization of crystal violet by laccases expressed in *P. pastoris* and transgenic *Arabidopsis*.

Decolorization was detected with or without 0.1 mmol l^{-1} ABTS as a mediator by different laccases.

Acknowledgements

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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