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Discovery of pinoresinol reductase genes in sphingomonads

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ABSTRACT

Bacterial genes for the degradation of major dilignols produced in lignifying xylem are expected to be useful tools for the structural modification of lignin in plants. For this purpose, we isolated *pinZ* involved in the conversion of pinoresinol from *Sphingobium* sp. strain SYK-6. *pinZ* showed 43–77% identity at amino acid level with bacterial NmrA-like proteins of unknown function, a subgroup of atypical short chain dehydrogenases/reductases, but revealed only 15–21% identity with plant pinoresinol/lariciresinol reductases. PinZ completely converted racemic pinoresinol to lariciresinol, showing a specific activity of 46 ± 3 U/mg in the presence of NADPH at 30 °C. In contrast, the activity for lariciresinol was negligible. This substrate preference is similar to a pinoresinol reductase, AtPrR1, of *Arabidopsis thaliana*; however, the specific activity of PinZ toward (±)-pinoresinol was significantly higher than that of AtPrR1. The role of *pinZ* and a *pinZ* ortholog of *Novosphingobium aromaticivorans* DSM 12444 were also characterized.

1. Introduction

Lignin is a major component of plant cell walls and produced from hydroxycinnamyl alcohols through radical coupling. It consists of phenylpropane units connected by various types of C—C and C—O—C bonds, and is important for mechanical support, water transport, and defense in vascular plants [1]. Despite the physiological importance of lignin in plants, the presence of lignin inhibits the saccharification process in biofuel production from cellulosic biomass [2]. Therefore, the development of genetically engineered plants with reduced lignin recalcitrance is greatly anticipated [3]. To date, a number of genetic modification of specific genes involved in the lignin biosynthesis have been reported to reduce lignin content, however, they often resulted in undesired phenotypes [4]. In order to reduce recalcitrance of lignin, the introduction and expression of microbial catabolic genes responsible for the degradation of lignin-derived aromatics in plant cells appeared to be an alternative strategy [5–7].

Sphingobium sp. strain SYK-6, one of the best characterized degraders of lignin-derived aromatics, is a unique bacterium capable of utilizing various types of lignin-derived biaryls, including β-aryl ether, biphenyl, phenylcoumaran, and diarylpropane, and monoaryls such as syringaldehyde, vanillin, and ferulate as the sole source of carbon and energy [8]. We have characterized the genes for the catabolism of β-aryl ether [9,10], biphenyl [11–13], and monoaryls [8]; however, the genes and enzymes involved in the degradation of pinoresinol in addition to phenylcoumaran and diarylpropane have not yet been characterized. Since β-aryl ether, pinoresinol, and phenylcoumaran (Fig. 1) are major dilignols produced in lignifying xylem [14,15], SYK-6 genes for the conversion of major dilignols are anticipated to be particularly useful for the structural modification of lignin in plants.

Although the degradation of syringaresinol by *Fusarium solani* M-13-1 was reported [16], to date, microbial enzymes and genes for the degradation of pinoresinol and syringaresinol have not been isolated. M-13-1 oxidized syringaresinol at the α-position of the side chain to generate a hemiketal, which was then transformed to a ketoalcohol. These products were converted to a γ-lactone derivative via alkyl-aryl cleavage. In addition to this report, it was documented that pinoresinol diglucoside (PDG) was converted to (−)-enterolactone by human intestinal microflora through (+)-pinoresinol, (+)-lariciresinol, and (−)-secoisolariciresinol [17]. This conversion is partially similar to plant lignan biosynthetic pathways [18]. From a human fecal suspension, *Enterococcus faecalis*

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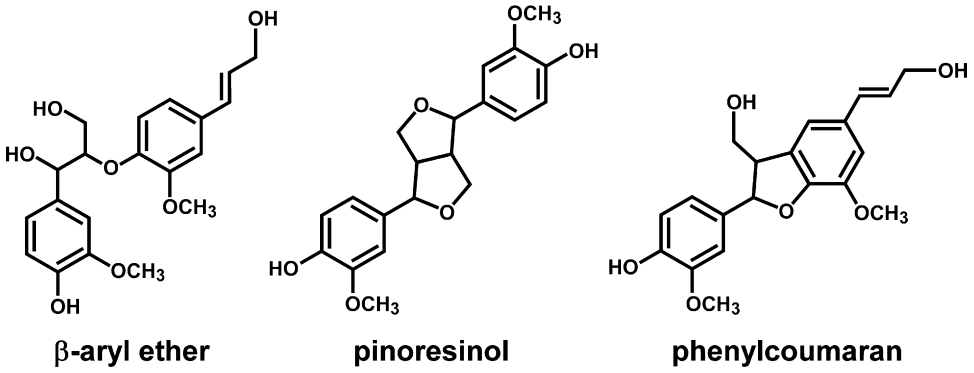

Fig. 1. Major dilignols produced in lignifying xylem.

PDG-1 was isolated as a bacterium converting (+)-pinoresinol to (+)-lariciresinol.

In this study, we found that *Sphingobium* sp. strain SYK-6 has the ability to convert (±)-pinoresinol, although this strain could not utilize (±)-pinoresinol as the sole source of carbon and energy. Here we isolated a novel pinoresinol reductase gene, *pinZ*, from SYK-6, and characterized enzyme properties of the gene product. In addition, a *pinZ* ortholog found in *Novosphingobium aromaticivorans* DSM 12444 was also characterized. This is the first report on the isolation and characterization of the bacterial pinoresinol reductase genes.

2. Materials and methods

2.1. Bacterial strains, plasmids, and culture conditions

Strains and plasmids used in this study are listed in Table S1. *Sphingobium* sp. strain SYK-6 was routinely grown in Luria–Bertani (LB) medium or Wx minimal salt medium [19] containing 10 mM vanillate or SEMP (10 mM sucrose, 10 mM glutamate, 0.13 mM methionine, and 10 mM proline) at 30 °C. Cells of *pinZ* mutant, SME042, *N. aromaticivorans* DSM 12444, and *Pseudomonas putida* PpY1100 were grown in LB medium at 30 °C. If necessary, 30 mg of chloramphenicol/liter or 50 mg of kanamycin/liter were added to the cultures. *Escherichia coli* strains were grown in LB medium at 37 °C. For cultures of cells carrying antibiotic resistance markers, the media for *E. coli* transformants were supplemented with 100 mg of ampicillin/liter or 25 mg of kanamycin/liter.

2.2. Preparation of substrates

(±)-Pinoresinol was prepared by oxidative coupling of coniferyl alcohol, and similarly, (±)-syringaresinol was prepared by oxidative coupling of sinapyl alcohol. (±)-Lariciresinol and (±)-secoisolariciresinol were synthesized through hydorogenation of (±)-pinoresinol by palladium on activated carbon. Preparation of substrates is described more fully in the supplementary method.

The authentic compounds were analyzed by high-performance liquid chromatography (HPLC; ACQUITY UPLC system; Waters) coupled with an ACQUITY TQ detector (Waters) using a TSKgel ODS-140HTP column (2.1 by 100 mm; Tosoh) as described previously [20]. The mobile phase of HPLC system was a mixture of water (75%) and acetonitrile (25%) containing formic acid (0.1%) at a flow rate of 0.3 ml/min. Pinoresinol, lariciresinol, and secoisolariciresinol were detected at 280 nm. Syringaresinol was detected at 271 nm. In the ESI-MS analysis, mass spectra were obtained by using the negative-ion mode with the settings described in a previous study [21].

2.3. Resting cell assays

Cells of SYK-6, SME042, and DSM 12444 grown in LB medium were washed with Wx medium and resuspended in LB medium to an absorbance at 600 nm (A_{600}) of 0.2. After 8 h of incubation, cells were collected by centrifugation, washed twice with 50 mM Tris–HCl buffer (pH 7.5), and resuspended to give an A_{600} of 5.0 in 1 ml of the same buffer. After the addition of (±)-pinoresinol (100 μM), resting cells were incubated at 30 °C with shaking. Portions of the cultures were collected at various sampling time points. The reactions were stopped by centrifugation, and the supernatants were filtrated and analyzed by LC–MS as described above.

2.4. Cloning of the pinZ gene

A partially SalI digested gene library of SYK-6 constructed with pVK100 was introduced into a host strain *P. putida* PpY1100 by triparental mating. Cells of transconjugants were grown in LB medium containing kanamycin until the A_{600} of the cultures reached 0.5, and then the cells were collected by centrifugation, washed with Wx medium, and resuspended in the same medium to an A_{600} of 5.0. After the addition of (±)-pinoresinol (1 mM), the cells were incubated at 30 °C with shaking. Portions of the cultures were collected at various sampling time points and centrifuged. The supernatants were filtrated and analyzed by HPLC. A cosmid, pVKPR150, was obtained from a transconjugant, which showed pinoresinol degradation activity. Each SalI fragment included in pVKPR150 was cloned into pBluescript II KS(+), and the pinoresinol degradation activity of cells of *E. coli* JM109 harboring each subclone grown in LB medium containing 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) as described below was examined. DNA manipulations and homology searches were performed as described in a previous study [21].

2.5. Expression of His-tag-fused pinZ and Saro_2808 in E. coli and purification

The coding region of *pinZ* was amplified by PCR using Ex*Taq* DNA polymerase (Takara Bio Inc.) together with pKSPR35 as a template and the pino-F and R primer pair (Table S2). The 1.0-kb PCR product was cloned into pT7Blue and sequenced. The 1.0-kb NdeI-BamHI fragment of the resulting plasmid was inserted into the corresponding sites of pET-16b to generate pETPZ. The coding region of *Saro_2808* was amplified by PCR using total DNA from *N. aromaticivorans* DSM 12444 as a template and the NmrA-F and R primer pair (Table S2). The 1.0-kb PCR product was cloned into pT7Blue and sequenced. The 1.0-kb NdeI-BamHI fragment carrying *Saro_2808* of pTPZNV was inserted into the corresponding sites of pET-16b to construct pETPZNV.

E. coli BL21(DE3) cells harboring pETPZ or pETPZNV were grown in 100 ml of LB medium at 30 °C. When A_{600} of the cultures reached 0.5, expressions of the genes were induced for 4 h by adding 1 mM IPTG. Cells were washed with NB buffer (50 mM Tris–HCl, 250 mM NaCl, 100 mM imidazole, and 10% glycerol; pH 7.0), resuspended in the same buffer, and broken by an ultrasonic disintegrator (UD-201; Tomy Seiko Co.) in a crushed-ice bath at an output level of 5 and a 50 duty cycle for 1 min with 0.5 s interval every second. After centrifugation (19,000 × *g*, 20 min, at 4 °C), the supernatants were applied to His Spin Trap columns (GE Healthcare) previously equilibrated with NB buffer. After centrifugation at 100 × *g* for 30 s, samples were washed twice with 500 μl of NB buffer. His-tag fused PinZ (ht-PinZ) and Saro_2808 (ht-Saro_2808) were eluted with 500 μl of NE buffer (50 mM Tris–HCl, 250 mM NaCl, 500 mM imidazole, and 10% glycerol; pH 7.0), and resultant fractions were subjected to desalting, buffer exchange (TG buffer [50 mM Tris–HCl, 10% glycerol; pH 7.0]), and centrifugal filtration with Amicon Ultra-0.5 30k filter units (Millipore). The protein concentration was determined by the Bradford method with bovine serum albumin as the standard [22].

2.6. Analytical methods

The purity of the enzyme preparations was examined by sodium dodecyl sulfate-12% polyacrylamide gel electrophoresis (SDS-PAGE). The molecular mass of the ht-PinZ was determined by gel filtration chromatography using a Superdex200 10/300GL column (GE Healthcare) with the BioAssist eZ system (Tosoh). Elution was performed with 50 mM Tris–HCl buffer containing 400 mM NaCl (pH 7.0) at a flow rate of 0.5 ml/min. Gel filtration calibration kits (HMW and LMW; GE Healthcare) were used as the molecular mass standard. In vitro cross-linking of ht-PinZ and ht-Saro_2808 were performed as descried in a previous study [23].

2.7. Enzyme assay

The pinoresinol and lariciresinol reductase activities of ht-PinZ and ht-Saro_2808 were determined by the oxidation of NAD(P)H. The enzyme reaction