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Expression and enzymatic properties of rice (*Oryza sativa* L.) monolignol β-glucosidases

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ABSTRACT

Monolignol glucosides and their β-glucosidases are found in monocots, but their biological roles are unclear. Phylogenetic analysis of rice (*Oryza sativa* L.) glycoside hydrolase family GH1 β-glucosidases indicated that Os4BGlu14, Os4BGlu16, and Os4BGlu18 are closely related to known monolignol β-glucosidases. An optimized Os4BGlu16 cDNA and cloned Os4BGlu18 cDNA were used to express fusion proteins with His_6 tags in *Pichia pastoris* and *Escherichia coli*, respectively. The secreted Os4BGlu16 fusion protein was purified from media by immobilized metal affinity chromatography (IMAC), while Os4BGlu18 was extracted from *E. coli* cells and purified by anion exchange chromatography, hydrophobic interaction chromatography and IMAC. Os4BGlu16 and Os4BGlu18 hydrolyzed the monolignol glucosides coniferin (k_{cat}/K_M, 21.6 mM^{-1} s^{-1} for Os4BGlu16 and for Os4BGlu18) and syringin (k_{cat}/K_M, 22.8 mM^{-1} s^{-1} for Os4BGlu16 and 24.0 mM^{-1} s^{-1} for Os4BGlu18) with much higher catalytic efficiencies than other substrates. In quantitative RT-PCR, highest Os4BGlu14 mRNA levels were detected in endosperm, embryo, lemma, panicle and pollen. Os4BGlu16 was detected highest in leaf from 4 to 10 weeks, endosperm and lemma, while Os4BGlu18 mRNA was most abundant in vegetative stage from 1 week to 4 weeks, pollen and lemma. These data suggest a role for Os4BGlu16 and Os4BGlu18 monolignol β-glucosidases in both vegetative and reproductive rice tissues.

1. Introduction

Plants have a variety of biochemical solutions to reinforce their cell walls and fulfill different adaptive strategies. Lignification is an important component of the cell wall strengthening process in land plants [1]. The lignin polymer is a major component of many plant cell walls, such as cell wall of tracheids, vessels and fibers, and has a significant impact on the pulp and paper industry, for which removal of lignin is a major process. The functions of lignin were found to include promoting the strength of woody stems and water proofing of water conductive elements within the xylem [2].

Lignins are complex, three-dimensional aromatic polymers derived from three hydroxycinnamyl alcohol monomers that differ in the number of methoxyl groups: *p*-coumaryl (M1H, with no methoxyl groups), coniferyl (M1G, with one methoxyl group), and sinapyl (M1S, with two methoxyl groups) alcohols [3]. These monolignols are incorporated in lignin as *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units, respectively. These monomers can also be joined into smaller, defense-related compounds, the lignans [4]. Lignin amounts and monolignol composition varies with the species of plant, tissue, developmental stage and environmental factors [5]. In general, dicotyledonous angiosperm (hardwood) lignins are mainly made up of G and S units with traces of H units, whereas gymnosperm (softwood) lignins are composed mostly of G units with low levels of H units. Lignins from monocots such as grasses contain similar levels of G and S units, and more H units than dicots [6].

It has been suggested that lignin production could be regulated by both monolignol synthesis and by the transport of monolignol precursors to the wall and their release from inactive forms [8]. The 4-*O*-β-D-glucosides of monolignols, namely *p*-coumaryl alcohol glucoside, coniferin and syringin have been suggested to serve as inactive transport or storage forms of monolignols. They are synthesized by uridine diphosphate-glucose (UDPGlc)-utilizing

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glucosyltransferases and subsequently hydrolyzed by monolignol-specific β-glucosidases [11]. A coniferin β-glucosidase was described in *Pinus banksiana* [12]. Subsequent characterization of a coniferin β-glucosidase (CBG) from *Pinus contorta* allowed cDNA cloning and the predicted amino acid sequence suggested that CBG is an extracellular glycoprotein belonging to glycoside hydrolase family 1 (GH1) [10,11]. CBG was also found to be localized in the lignification zone in the tree stem.

Although monolignol glucosides are found in all gymnosperms, not all angiosperms appear to have them. However, in poplar, coniferin β-glucosidase activity has been histochemically localized to lignifying cells [11] and radiolabeled monolignol glucosides are efficiently incorporated into lignin [13]. These data suggest that the monolignol glucosides may be hydrolyzed by β-glucosidases in lignifying tissues of angiosperms. In fact, monolignol-specific glycosyltransferase activity was detected in all angiosperm species tested, and a β-glucosidase that could hydrolyze monolignol glucosides was originally isolated from cell walls of chick pea cell suspension cultures [14]. While the chick pea monolignol β-glucosidase had higher hydrolysis activity against coniferin than syringin, a β-glucosidase purified from cell cultures of soybean (*Glycine max*) hypocotyls and roots had identical V_{max} values for the two substrates and a K_m for coniferin two times higher than that for syringin [15,16]. In a more recent study of monolignol glucosidases in *Arabidopsis*, the proteins encoded by the loci At1g61810 (BGLU45), At1g61820 (BGLU46), and At4g21760 (BGLU47) were found to cluster with *P. contorta* coniferin β-glucosidase in protein sequence-based phylogenetic analysis, leading to the hypothesis that the respective gene products may hydrolyze monolignol glucosides [17]. Among the natural substrates tested, BGLU45 exhibited specific activity toward the monolignol glucosides syringin, coniferin, and *p*-coumarol glucoside, while BGLU46 exhibited broader substrate specificity, cleaving salicin, *p*-coumarol glucoside, phenyl-β-D-glucoside, coniferin, syringin, and arbutin. RT-PCR showed that BGLU45 and BGLU46 expression was highest in *Arabidopsis* organs that are major sites of lignin deposition. Recently, Chapelle et al. [18] reported that the T-DNA insertions in the *Arabidopsis thaliana BGLU45* and *BGLU46* genes resulted in a significant increase in coniferin content in stem extracts, while syringin content was not changed. No change was observed in plants with a T-DNA insert in the *BGLU47* gene, suggesting it is not involved in the phenylpropanoid pathway or lignification in the stem [18]. The lack of large changes in the lignin content and composition in these *Arabidopsis* gene knockout lines suggested that the monolignol glucosides may be storage forms, but are not required transport forms of monolignols or direct precursors of lignin.

Little has been reported about monolignol β-glucosidases in monocots. Opassiri et al. [20] identified GH1 genes from the rice (*Oryza sativa* L.) genome, and their structures, predicted protein products and evidence of expression were evaluated. As shown in Fig. 1, phylogenetic analysis of predicted protein sequences of rice and *Arabidopsis* GH1 genes showed that Os4BGlu14, Os4BGlu16 and Os4BGlu18 are clustered with *Arabidopsis* BGLU45 and BGLU46 β-glucosidases. Since BGLU45 and BGLU46 have been shown to hydrolyze lignin precursors [17], Os4BGlu14, Os4BGlu16 and Os4BGlu18 were hypothesized to have similar activities. However, Os4BGlu14 may be inactive as a β-glucosidase, since it is one of the 2 of the 34 putative active rice β-glucosidase genes identified by Opassiri et al. [20] in which the conserved catalytic acid/base glutamate residue, is replaced by a nonionizable glutamine residue.

In the current work, the enzymatic properties and substrate specificities of recombinant Os4BGlu16 and Os4BGlu18 enzymes were determined to demonstrate that they are in fact monolignol β-glucosidases. Additionally, quantitative RT-PCR was used to investigate the relative expression of monolignol β-glucosidase gene in different rice tissues.

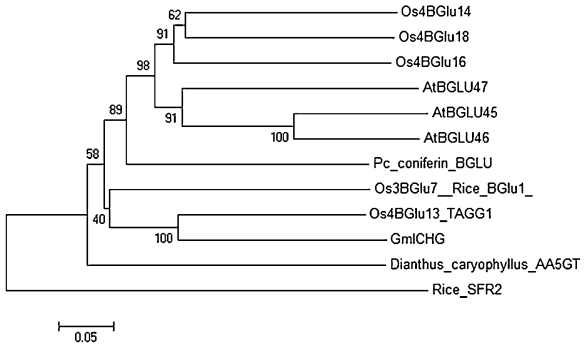

Fig. 1. Phylogenetic tree of predicted monolignol β-glucosidase protein sequences. The protein sequences include the putative monolignol β-glucosidases rice Os4BGlu14 (accession number: NP_001053302), Os4BGlu16 (Q7XSK2), Os4BGlu18 (Q7XSK0), *Arabidopsis* AtBGLU45 (NP_176374), AtBGLU46 (NP_850968), AtBGLU47 (NP_193907), pine coniferin β-glucosidase (Q9ZT64), and other GH1 enzymes, including Os3BGlu7 (BGlu1) (NP_001051013), Os4BGlu13 (OsTAGG1) (NP_001053071), *Glycine max* isoflavone conjugate hydrolyzing β-glucosidase (GmICHG) (NP_001237501), *Dianthus caryophyllus* acyl-glucose-dependent anthocyanin 5-O-glucosyltransferase (AA5GT) (E3W9M2) and Os11BGlu36 (OsSFR2) (NP_001068463), a putative galactolipid galactolipid galactosyltransferase. The tree was developed by neighbor-joining method with default parameters in MEGA 6.

2. Materials and methods

2.1. Materials

Initially, monolignol glucosides, *p*-coniferol glucoside, coniferin, and syringin were kindly provided by Norman Lewis and Laurence Davin, Washington State University, USA. Additional *p*-coniferol glucoside was synthesized by the method of Terashima et al. [9], while additional coniferin and syringin were purchased from IMAM International Group (Pharmaceutical) Co., Ltd. (Tianjian, China). Other glycoside substrates were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Cloning of Os4BGlu14, Os4BGlu16 and Os4BGlu18

Os4BGlu14 was cloned from the Genbank accession number AK067841 cDNA clone plasmid provided by the Rice Genome Resource full-length cDNA project [21]. A fragment of the cDNA that encoded the predicted mature rice Os4BGlu14 gene with Os4BGlu14Matstrf (5′-CACCATGGCCGTCGACCGCAGCCAG-3′) and Os4BGlu14Stop (5′-GAGGATCCTTAATAAGAGTTAAACTTATGAAGC-3′) primers with *Pfu* DNA polymerase was amplified. The PCR product encoding the mature Os4BGlu14 gene and pET32a(+) expression vector were digested with *Nco*I and *Bam*HI, then ligated and cloned in *E. coli* DH5α selected on 50 μg/ml ampicillin. Because the acid/base of Os4BGlu14 is replaced by glutamine residue 191, it was mutated from glutamine to glutamate in the pET32a/Os4BGlu14 vector with the QuikChange site-directed mutagenesis method and Os4BGlu14AB2Ef (5′-CGAATAAAATTCTGGACAACATTTAATGAGCCGAATTTGTCCATAAAGTTCAGTTAC-3′) and Os4BGlu14AB2Er (5′-GTAACTGAA CTTTATGGACAAATTCGGCTCATTAAA TGTTGTCCAGAATTTTATTCG-3′) primers.

Because of difficulty in cloning a functional cDNA for *E. coli* expression, a gene optimized for Os4BGlu16 (Genbank accession number KJ579205) expression in *Pichia pastoris* was synthesized and inserted into the pUC57 vector by GenScript Corporation (Piscataway, NJ, USA). The optimized Os4BGlu16 gene was cut with *Pst*I and *Xba*I and cloned into the same sites in the