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Arabidopsis thaliana β-Glucosidases BGLU45 and BGLU46 hydrolyse monolignol glucosides

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Dedicated to Professor Rodney Croteau on the occasion of his 60th birthday.

Abstract

In higher plants, β-glucosidases belonging to glycoside hydrolase (GH) Family 1 have been implicated in several fundamental processes including lignification. Phylogenetic analysis of *Arabidopsis thaliana* GH Family 1 has revealed that At1g61810 (BGLU45), At1g61820 (BGLU46), and At4g21760 (BGLU47) cluster with *Pinus contorta* coniferin β-glucosidase, leading to the hypothesis that their respective gene products may be involved in lignification by hydrolysing monolignol glucosides. To test this hypothesis, we cloned cDNAs encoding BGLU45 and BGLU46 and expressed them in *Pichia pastoris*. The recombinant enzymes were purified to apparent homogeneity by ammonium sulfate fractionation and hydrophobic interaction chromatography. Among natural substrates tested, BGLU45 exhibited narrow specificity toward the monolignol glucosides syringin (K_m, 5.1 mM), coniferin (K_m, 7 mM), and *p*-coumaryl glucoside, with relative hydrolytic rates of 100%, 87%, and 7%, respectively. BGLU46 exhibited broader substrate specificity, cleaving salicin (100%), *p*-coumaryl glucoside (71%; K_m, 2.2 mM), phenyl-β-D-glucoside (62%), coniferin (8%), syringin (6%), and arbutin (6%). Both enzymes also hydrolysed *p*- and *o*-nitrophenyl-β-D-glucosides. Using RT-PCR, we showed that *BGLU45* and *BGLU46* are expressed strongly in organs that are major sites of lignin deposition. In inflorescence stems, both genes display increasing levels of expression from apex to base, matching the known increase in lignification. *BGLU45*, but not *BGLU46*, is expressed in siliques, whereas only *BGLU46* is expressed in roots. Taken together with recently described monolignol glucosyltransferases [Lim et al., J. Biol. Chem. (2001) 276, 4344–4349], our enzymological and molecular data support the possibility of a monolignol glucoside/β-glucosidase system in *Arabidopsis* lignification.

Keywords: *Arabidopsis thaliana*; Brassicaceae; Heterologous expression; Monolignol glucosides; β-Glucosidases; *Pichia pastoris*; Lignification

1. Introduction

Accounting for almost one-third of the organic carbon in the biosphere, lignin performs several functions in higher plants, including strengthening and water-proofing cell walls, providing mechanical support for the plant body, and contributing to defense against microbial attack (Boerjan et al., 2003). In angiosperms, this complex heteropolymer is derived principally from the monolignols *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol by dehydrogenative polymerization. Although the intracellular biosynthesis of these lignin monomers is largely understood (Anterola and Lewis, 2002; Humphreys and Chapple, 2002; Boerjan et al., 2003; Nair et al., 2004), their transport across the plasma membrane to sites of lignin

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deposition within the apoplast remains to be elucidated. It is generally believed that monolignols traverse the plasma membrane via Golgi-mediated secretion or ABC transporters (Samuels et al., 2002; Ehlting et al., 2005). Alternatively, these monolignols might be glucosylated by specific intracellular glucosyltransferases to their corresponding 4-O-β-D-glucosides (i.e. *p*-coumaryl alcohol glucoside (CAG), coniferin, and syringin, respectively) (Whetten et al., 1998). Serving as transport forms to the cell exterior, these glucosides would be subsequently hydrolysed at lignification sites in the apoplast by specific monolignol glucoside β-glucosidases. A transport mechanism involving monolignol glucosylation is attractive, because, by increasing the solubility and decreasing the reactivity of monolignols, it would facilitate their export while preventing their polymerization in undesirable locations.

Especially in the case of gymnosperms, considerable correlative evidence has accumulated for the participation of such a monolignol glucoside/β-glucosidase system in lignification. Monolignol glucosides have been isolated from many gymnosperms and angiosperms, predominantly from cambial tissues (for comprehensive list of references, see Steeves et al., 2001). Pulse-labeling studies revealed that coniferin is rapidly synthesized from radiolabeled L-phenylalanine in spruce (*Picea abies*) seedlings and turns over with a half-life of approximately 60 h (Marcinowski and Grisebach, 1977). Coniferin is a lignin precursor in several species (e.g. Brown, 1966; Kratzl et al., 1957; Terashima et al., 1997; Fukushima, 2001), and both coniferin and syringin stimulate lignification in different tissue cultures (Sarkanen and Ludwig, 1971). With respect to the enzymology of glucosylation–deglycosylation, UDP-Glc-dependent glucosyltransferases (EC 2.4.1.111) exhibiting pronounced specificities toward monolignols have been purified and characterized from Paul's scarlet rose cell cultures (Ibrahim and Grisebach, 1976), lignifying stem segments of *Forsythia ovata* (Ibrahim, 1977), spruce cambial sap (Schmid and Grisebach, 1982), and *Pinus banksiana* cambium (Savidge and Förster, 1998). Furthermore, in a survey conducted by Ibrahim (1977), coniferyl alcohol glucosyltransferase activity was detected in crude homogenates of all gymnosperms tested; among angiosperms, woody species exhibited higher enzyme activities than herbaceous species. As for the hydrolysis of monolignol glucosides, β-glucosidases (EC 3.2.1.126) showing specificity toward these substrates have been characterized from both gymnosperm and angiosperm sources, namely spruce (*P. abies*) seedlings (Marcinowski and Grisebach, 1978), chick pea (*Cicer arietinum*) cell suspension cultures and seedlings (Hösel et al., 1978; Surholt and Hösel, 1981), soybean (*Glycine max*) cell cultures, hypocotyls, and roots (Hösel and Todenhagen, 1980), and the differentiating xylem of *P. banksiana* (Leinhos et al., 1994) and lodgepole pine (*Pinus contorta*) (Dharmawardhana et al., 1995). In some cases (Marcinowski et al., 1979; Burmeister and Hösel, 1981; Samuels et al., 2002), their apoplastic location was demonstrated by immunofluorescent techniques. At the molecular level, Dharmawardhana et al. (1999) reported the cloning and heterologous expression of a cDNA encoding *P. contorta* xylem coniferin β-glucosidase. Belonging to glycoside hydrolase (GH) Family 1 (Coutinho and Henrissat, 1999), this hydrolase was active toward coniferin, syringin, and the chromogenic substrates *o*-nitrophenyl-β-D-glucoside (*o*NPGlc) and *p*-nitrophenyl-β-D-glucoside (*p*NPGlc).

With its genome completely sequenced (Arabidopsis Genome Initiative, 2000), *Arabidopsis thaliana* represents an excellent species with which to gain further information about the potential involvement of a monolignol glucoside/β-glucosidase system in angiosperm lignification. While *Arabidopsis* is not usually noted for monolignol glucoside accumulation, coniferin and syringin accumulated in light-grown primary and secondary roots (Hemm et al., 2004). Furthermore, using a functional genomic approach, Lim et al. (2001) showed that the genes *UGT72E2* and *UGT72E3* encode two UDP-Glc-dependent glucosyltransferases that glucosylate sinapyl alcohol and coniferyl alcohol in vitro. More recently, we reported that *Arabidopsis* GH Family 1 contains 48 members (Xu et al., 2004). Of these, 47 share a common evolutionary origin and were assigned new gene names (*BGLU1–BGLU47*), whereas the remaining family member (*sfr2*) belongs to a distinct lineage. Interestingly, our phylogenetic analysis indicated that BGLU45 (At1g61810), BGLU46 (At1g61820), and BGLU47 (At4g21760) cluster with the *P. contorta* β-glucosidase, suggesting that these gene products may play a role in lignification. To test this hypothesis, we expressed *BGLU45* and *BGLU46* in the *Pichia pastoris* expression system in order to analyze the substrate specificities of the corresponding recombinant hydrolases. Additionally, we investigated the organ-level expression of these genes by relative RT-PCR. Both the expression patterns of *BGLU45* and *BGLU46* and the substrate specificities of their encoded hydrolases are largely consistent with a role in *Arabidopsis* lignification.

2. Results and discussion

2.1. Heterologous expression of BGLU45 and BGLU46

Phylogenetic analysis of sequenced glycoside hydrolase 1 family members showed that the *Arabidopsis* proteins BGLU45, BGLU46, and BGLU47 cluster with the *P. contorta* β-glucosidase that exhibits pronounced specificity toward coniferin and syringin (Dharmawardhana et al., 1995, 1999; Xu et al., 2004). Therefore, we hypothesized that these enzymes might play a role in *Arabidopsis* lignin biosynthesis by hydrolysing monolignol β-glucosides. To test this hypothesis, our primary strategy was to express two of these putative hydrolases (BGLU45 and BGLU46) in an appropriate expression system, purify them to homogeneity, and assay their activities toward a spectrum of different β-glucosidic substrates including monolignol β-glucosides. Because our NetNGlyc predictions suggested that these enzymes might be glycosylated in planta and

therefore unlikely to be expressed in active and soluble form in *Escherichia coli* (Xu et al., 2004), we chose the *P. pastoris* expression system for this task. The latter system has found wide usage, because this yeast utilizes most of the post-translational modification pathways typically associated with higher eukaryotes and is easy to use (Cregg et al., 1993). Furthermore, it has been successfully employed to express several β-glucosidases, including the *Prunus serotina* cyanogenic β-glucosidases amygdalin hydrolase and prunasin hydrolase (Zhou et al., 2002), *Brassica napus* myrosinase (Hartel and Brandt, 2002), and the *Arabidopsis* β-mannosidase/β-glucosidase BGLU44 (Xu et al., 2004).

cDNAs encoding the mature proteins (i.e. excluding any signal peptide, as predicted by SignalP V2.0) were cloned into the expression vector pPICZαB. This vector encodes the *Saccharomyces cerevisiae* α-factor signal sequence allowing secretion of the desired recombinant protein into the culture medium. It also encodes the zeocin resistance selection marker, permitting selection of multicopy transformants. After integrating the constructs into the *P. pastoris* genome using electroporation as transformation method, we tested the expression levels of approximately 20 individual *P. pastoris* transformants for each gene by assaying the β-glucosidase activities of supernatants of 3–4-day-old, methanol-induced cultures toward the chromogenic substrates *p*NPGlc or *o*NPGlc. Culture supernatants from control transformants (containing the vector without insert) were assayed in parallel, because endogenous glycosidases are co-secreted into the culture medium. One of these contaminating *P. pastoris* hydrolases, an exo-1,3-β-glucanase (GH Family 5) that cleaves *p*NPGlc, has been purified to homogeneity and characterized (Xu et al., 2006). Those *BGLU45* and *BGLU46* transformants exhibiting highest β-glucosidase activities were chosen for culture scale-up and enzyme purification.

2.2. Purification and characterization of recombinant BGLU45 and BGLU46

Recombinant BGLU45 and BGLU46 were purified from their respective culture supernatants by $(NH_4)_2SO_4$ fractionation and hydrophobic interaction chromatography (HIC). Purification progress was monitored by SDS–PAGE with Coomassie Blue staining and by assaying protein content and β-glucosidase activity. These two steps achieved 8.8-fold and 13.7-fold purification for BGLU45 (Table 1) and BGLU46 (Table 2), respectively, and confirmed the usefulness of HIC for β-glucosidase purification (e.g. Cicek et al., 2000; Czjzek et al., 2001). However, both purified hydrolases appeared on SDS–PAGE gels as smears rather than sharp bands (Fig. 1). Peptide fingerprint analysis verified that different regions of each smear represented the same polypeptide but with different degrees of glycosylation (data not shown). Such differential glycosylation has been reported for several other heterologous proteins expressed in *P. pastoris* (Tschopp et al., 1987; Scorer et al., 1993; Montesino et al., 1998).

2.3. Analysis of substrate specificities of recombinant BGLU45 and BGLU46

The substrate specificities of the purified recombinant BGLU45 and BGLU46 were investigated with the ultimate goal of identifying the endogenous substrates of these

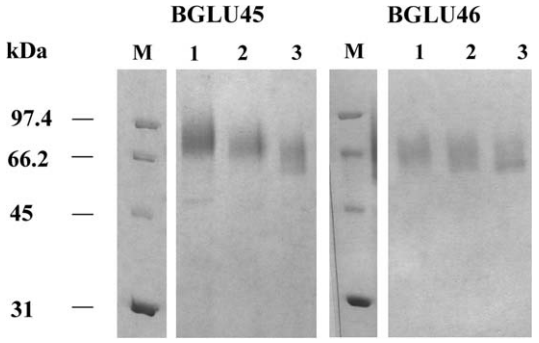

Fig. 1. SDS–PAGE analysis of purified recombinant BGLU45 and BGLU46. For each enzyme, lane M indicates the position of protein markers, while lanes 1–3 show three successive fractions (3–5 μg protein/lane) from the Toyopearl Butyl-650M linear 1–0 M $(NH_4)_2SO_4$ gradient. Resolved polypeptides were stained with Coomassie Blue.

Table 1
Purification of recombinant BGLU45 from *Pichia pastoris* culture supernatant

Purification step	Protein (mg)	Activity[a] (units)	Specific activity (units/mg)	Purification (fold)	Recovery (%)
Culture supernatant	29	119.5	4.1	1	100
50–70% $(NH_4)_2SO_4$	2.83	43.2	15.3	3.7	36.2
Toyopearl Butyl-650M	0.156	5.6	35.9	8.8	4.7

[a] One unit (nkat) of enzyme activity is defined as 1 nmol of liberated ONP/s.

Table 2
Purification of recombinant BGLU46 from *Pichia pastoris* culture supernatant

Purification step	Protein (mg)	Activity[a] (units)	Specific activity (units/mg)	Purification (fold)	Protein yield (%)
Culture supernatant	25.5	1116	43.8	1	100
40–60% $(NH_4)_2SO_4$	7.22	909	126	2.9	81.5
Toyopearl Butyl-650M	0.87	521	599	13.7	46.7

[a] One unit (nkat) of enzyme activity is defined as 1 nmol of liberated PNP/s.

enzymes and thereby their biological roles in vivo. First, the glycone and linkage specificities of BGLU45 and BGLU46 were examined by assaying their activities toward several β- and α-linked *p*-nitrophenyl (*p*NP)-sugars (Table 3). As expected, both enzymes showed nearly absolute preference for the glycone glucose. The sole exception was the hydrolysis of *p*NPGal by BGLU46 at 21% of the rate of *p*NPGlc degradation. *p*NPGal hydrolysis by plant β-glucosidases has been frequently noted (e.g. Hösel et al., 1978; Opassiri et al., 2004; Xu et al., 2004). Both recombinant enzymes exhibited negligible activity toward *p*NP-α-D-glucopyranoside and *p*NP 1-thio-β-D-glucopyranoside, reflecting a requirement for a β-linked O-glycosyl moiety in their substrates. Table 3 also shows that, among chromogenic substrates tested, *o*NPGlc was most rapidly hydrolysed by BGLU45 and BGLU46 with relative rates of 619% and 449%, respectively, of the rate of *p*NPGlc degradation. *p*NPGlc was also a substrate for other monolignol glucoside β-glucosidases, and, where tested, *o*NPGlc was hydrolysed 2–7-fold faster than *p*NPGlc (Marcinowski and Grisebach, 1978; Surholt and Hösel, 1981; Leinhos et al., 1994; Dharmawardhana et al., 1995).

To probe the aglycone specificities of the recombinant enzymes, their activities were tested toward a collection of β-glucosides largely of plant origin (Table 4). BGLU45 exhibited absolute specificity for the three monolignol glucosides, with syringin being most rapidly hydrolysed (72 nkat/mg protein = 100%) followed by coniferin (87%) and CAG (7%). No activity was detectable toward any other natural substrate tested. High activity toward syringin and coniferin with relatively poor utilization of CAG is a hallmark of known monolignol glucoside β-glucosidases (Marcinowski and Grisebach, 1978; Hösel et al., 1978; Surholt and Hösel, 1981; Hösel and Todenhagen, 1980; Dharmawardhana et al., 1995). Furthermore, this substrate preference reflects the composition of dicotyledonous angiosperm lignins, which consist principally of S and G units (derived from sinapyl and coniferyl alcohols, respectively) with traces of H units (derived from *p*-coumaryl alcohol) (Boerjan et al., 2003). Literature K_m values for monolignol glucosides range widely between 0.18 mM and 5 mM, so the observed BGLU45 K_m values for coniferin (7 mM) and syringin (5.1 mM) lie at the high end of that range. The close similarity in enzymatic properties between BGLU45 and other known monolignol glucoside β-glucosidases, together with the remarkable absolute specificity of BGLU45 toward monolignol glucosides among natural substrates tested, strongly supports our hypothesis that this enzyme plays a role in *Arabidopsis* lignification.

When tested against the same spectrum of potential β-glucosidic substrates, BGLU46 exhibited broader specificity than BGLU45, showing significant activity toward the phenolic glucosides salicin (328 nkat/mg protein = 100%), CAG (71%), phenyl-β-D-glucoside (62%), coniferin (8%), syringin (6%), and arbutin (6%) (Table 4). However, the enzyme failed to hydrolyse the glucosinolate sinigrin, methyl-β-D-glucoside, and the disaccharides cellobiose, β-gentiobiose, maltose, and sucrose. The K_m for CAG was 2.2 mM. Given the breadth of BGLU46 substrate specificity, it is currently difficult to identify unequivocally the biological role(s) played by this enzyme. However, several possibilities should be considered. First, BGLU46 may play a role in lignification. By its preference for CAG over syringin and coniferin, BGLU46 is unique among hydrolases reported to cleave monolignol glucosides, and it is tempting to speculate that CAG hydrolysis is the principal role for this

Table 3
Glycone and linkage specificities of recombinant *Arabidopsis* BGLU45 and BGLU46

Substrate	BGLU45 relative activity (%)	BGLU46 relative activity (%)
p-Nitrophenyl β-D-glucopyranoside	100	100
p-Nitrophenyl 1-thio-β-D-glucopyranoside	0	0
p-Nitrophenyl α-D-glucopyranoside	0	0.1
p-Nitrophenyl *N*-acetyl-β-D-glucosaminide	0	0
p-Nitrophenyl β-D-galactopyranoside	0.4	21
p-Nitrophenyl β-D-mannopyranoside	0	0
p-Nitrophenyl β-D-xylopyranoside	0	2
p-Nitrophenyl β-L-fucopyranoside	0	0
p-Nitrophenyl β-L-arabinopyranoside	0	0
o-Nitrophenyl β-D-glucopyranoside	619	449

The purified hydrolases were incubated at their optimum pH (pH 5.5) with potential substrates provided at 10 mM final concentration. Enzyme activity was determined by measuring the rate of PNP (or ONP) production spectrophotometrically at 400 nm with subsequent use of standard curves. Reaction rates are expressed here as a percentage of that observed with PNPG (BGLU45, 100% = 5 nkat/mg protein; BGLU46, 100% = 289 nkat/mg protein).

Table 4
Substrate specificity of recombinant BGLU45 and BGLU46 toward selected natural and non-chromogenic substrates

Substrate	BGLU45 relative activity (%)	BGLU46 relative activity (%)
Syringin	100	6
Coniferin	87	8
p-Coumaryl alcohol glucoside	6.9	71
Salicin	0	100
(*R*)-Amygdalin	0	5
Sinigrin	0	0
4-Methylumbelliferyl β-D-glucoside	0	9
Phenyl β-D-glucoside	0	62
Arbutin (hydroquinone β-D-glucoside)	0	6
Methyl β-D-glucoside	0	0
D(+)Cellobiose	0	0
β-Gentiobiose	0	0
D(+)Maltose	0	0
Sucrose	0	0

The purified hydrolases were incubated at their optimum pH (pH 5.5) with potential substrates provided at 10 mM final concentration. The rate of glucose production was assayed by the Megazyme glucose assay procedure in conjunction with a standard curve. For BGLU45, reaction rates are expressed as a percentage of the rate of syringin hydrolysis (100% = 72 nkat/mg protein). For BGLU46, reaction rates are expressed as a percentage of the rate of salicin hydrolysis (100% = 328 nkat/mg protein).

enzyme in vivo. If true, BGLU46 would be a key player in the biosynthesis of H units, which are present at low levels in *Arabidopsis* lignin (Dharmawardhana et al., 1992; Franke et al., 2002; Goujon et al., 2003a; Rogers et al., 2005a) and are usually laid down preferentially in early stages of secondary wall formation (Boerjan et al., 2003). Thus, this enzyme could functionally complement BGLU45, which prefers syringin and coniferin over CAG. However, one should keep in mind that, although BGLU46's low relative activities toward syringin and coniferin may lead one to discount them as significant substrates in vivo, the hydrolytic rates toward these substrates (20–26 nkat/mg protein) are of the same order of magnitude on a per mg protein basis as those seen with BGLU45 for the same monolignol glucosides. Secondly, it is possible that BGLU46 is involved in arbutin and/or salicin metabolism. The fact that these glucosides have not yet been demonstrated in *Arabidopsis* should not be regarded as conclusive proof of their absence. Indeed, two lines of evidence suggest that arbutin or related compounds may exist in *Arabidopsis*: (i) homologs of the plant glucosyltransferase arbutin synthase exist in this species (At1g01420 and At4g01070; Hefner et al., 2002), and (ii) both arbutin and salicin are transported by the *Arabidopsis* sucrose transporter AtSUC2 (expressed in *Xenopus* oocytes) with $K_{0.5}$ values similar to that of sucrose (Chandran et al., 2003). Finally, BGLU46 may play a role in the biosynthesis of the polyphenolic domain of suberin. In *Arabidopsis*, this polymer is found in the endodermis and periderm of roots (Franke et al., 2005), an organ in which *BGLU46* is highly expressed (Schmid et al., 2005; also, see below).

In addition to the three main monolignols, lignin may contain small amounts of other monomers, such as coniferyl aldehyde and sinapyl aldehyde (Boerjan et al., 2003; Kim et al., 2003). Interestingly, these hydroxycinnamyl aldehydes, which also serve as precursors of ferulic acid and sinapic acid (Nair et al., 2004), are glycosylated in vitro to their 4-O-glucosides by *Arabidopsis* glucosyltransferases UGT72E1 and UGT72E2 but not by UGT72E3 (Lim et al., 2005). Whether these glucosides serve as transport forms to the apoplast is unknown. In this context, it would have been informative to test these compounds as substrates for recombinant BGLU45 and BGLU46, but unfortunately they were not available for our studies.

To determine whether variation in protein glycosylation might alter the specific activity and/or the substrate specificity of these hydrolases, purified BGLU45 and BGLU46 with different glycosylation degrees (i.e. different HIC fractions) were tested against several substrates. No differences in the specific activity or substrate specificity were observed between fractions (data not shown), from which we conclude that the glycosylation patterns conferred by *P. pastoris* cells do not affect enzyme activity.

2.4. Organ-level expression of BGLU45 and BGLU46 genes

The localization and timing of lignin deposition in *A. thaliana* have been well documented (e.g. Chapple et al., 1992; Turner and Somerville, 1997; Zhong et al., 1997; Rogers and Campbell, 2004; Ehlting et al., 2005; Rogers et al., 2005b). In mature plants, inflorescence stems constitute the most lignified organ, with the extent of lignification increasing basipetally as the xylem vessels and interfascicular fibers become progressively more differentiated. Typical of herbaceous dicotyledonous angiosperms, *Arabidopsis* synthesizes guaiacyl-rich, syringyl-guaiacyl lignin, but the relative proportions of these units vary according to developmental stage and tissue type (Chapple et al., 1992; Meyer et al., 1998). For example, the syringyl lignin content of the rachis increases from <6 mol% in apical regions to >26 mol% near its base. Furthermore, histochemical staining of mature stems indicated that xylem elements of the vascular bundles contain only guaiacyl lignin, whereas the adjacent interfascicular fibers contain large amounts of syringyl units. Lignin is also found to lesser extent in the xylem of *Arabidopsis* roots, hypocotyls, and leaves (Dharmawardhana et al., 1992). This polymer plays a key role in fruit dehiscence and seed dispersal in the lignified valve and margin layers of the siliques (Spence et al., 1996; Liljegren et al., 2000; Liljegren et al., 2004). Finally, under certain growth conditions, a vascular cambium may form in the hypocotyl resulting in extensive amounts of lignified secondary xylem fibers and vessel elements (Chaffey et al., 2002).

Several recent studies have demonstrated that the expression of *Arabidopsis* lignin biosynthetic genes is strongly correlated with sites of lignin deposition (Goujon et al., 2003b; Raes et al., 2003; Ehlting et al., 2005). Unfortunately, *BGLU45* and *BGLU46* were not included in those investigations. If these two genes indeed participate in lignification, one might predict that their expression patterns would similarly reflect those of lignin deposition. To test this prediction, we used RT-PCR methodology with gene-specific primers to measure the relative steady-state mRNA levels of *BGLU45* and *BGLU46* in various *Arabidopsis* organs. Each inflorescence stem was divided into three regions reflecting different degrees of lignification. As Fig. 2 shows, *BGLU45* and *BGLU46* were expressed in stems with increasing levels of expression from apex to

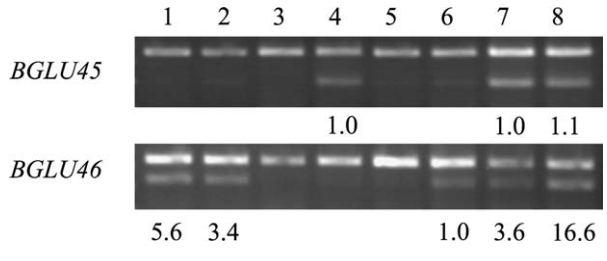

Fig. 2. Relative RT-PCR analysis of *BGLU45* and *BGLU46* expression patterns. Lane 1, light-grown roots; lane 2, dark-grown roots; lane 3, leaves; lane 4, siliques; lane 5, flowers; lane 6, stems (top); lane 7, stems (middle); lane 8, stems (bottom). In each lane, constitutive βATPase gene expression (upper band, 350 bp), target gene expression (lower band: *BGLU45*, 200 bp; *BGLU46*, 236 bp). The numbers under individual lanes indicate the relative intensities of the target bands (see Section 4).

base. For *BGLU45*, the relative expression levels in the top, middle, and basal sections were non-detectable, 1.0, and 1.1, respectively; for *BGLU46*, they were 1.3, 3.6, and 16.6, respectively. Correlating with the increase of lignification toward the base of the rachis (Meyer et al., 1998), the *BGLU45* and *BGLU46* expression patterns support our hypothesis that these genes participate in lignification. In roots and siliques, *BGLU45* and *BGLU46* exhibit organ-specific expression. Whereas *BGLU45* expression was not detectable in roots, the *BGLU46* gene expressed strongly in both light-exposed and dark-grown root cultures. In contrast, *BGLU45*, but not *BGLU46*, was expressed in the siliques, where it may be involved in lignification of the aforementioned lignified valve and margin layers of this organ (Spence et al., 1996). Surprisingly, we detected no expression of either gene in rosette leaves or flowers, despite the presence of xylem elements in these organs. This unexpected finding raises the questions whether *BGLU45* and *BGLU46* expression lies below the level of detection in our analyses or whether lignification in these organs involves other members of *Arabidopsis* GH Family 1. It should be noted that our expression data are supported by the recently reported gene expression map of *Arabidopsis* development, which showed that *BGLU45* was mainly expressed in stems and seeds, whereas *BGLU46* was mostly expressed in the roots and stems (Schmid et al., 2005).

3. Conclusions

Several lines of evidence support the involvement of a monolignol glucoside/β-glucosidase system in *Arabidopsis* lignification, including: (i) the demonstration of UDP-Glc-dependent glucosyltransferases (UGT72E2 and UGT72E3) that glucosylate the monolignols coniferyl alcohol and sinapyl alcohol (Lim et al., 2001), (ii) the hydrolysis of monolignol glucosides by BGLU45 and BGLU46; among natural substrates tested, monolignol glucosides are the sole substrates for BGLU45, and, although BGLU46 showed broader substrate specificity, it readily cleaved CAG and, to a lesser extent, coniferin and syringin, (iii) the predicted targeting of these hydrolases to the apoplast (Xu et al., 2004), and (iv) the demonstration of *BGLU45* and *BGLU46* expression in *Arabidopsis* organs that are major sites of lignin deposition. Future studies might utilize in situ hybridization to investigate whether BGLU45 and BGLU46 are expressed solely in lignifying cells within individual organs or whether, like the *P. abies* coniferin β-glucosidase (Marcinowski et al., 1979), they exhibit more generalized expression patterns. Immunocytochemical approaches could be used to confirm the predicted apoplastic location of these hydrolases. Finally, our current data does not allow us to conclude whether monolignol glucosides are obligatory lignin precursors in *Arabidopsis*. This issue might be addressed by investigating to what extent lignification is affected in single and double *BGLU45* and *BGLU46* T-DNA insertion lines.

4. Experimental

4.1. Plant materials and growth conditions

A. thaliana ecotype Columbia seeds were treated for 3 days with 10 mM KNO_3 at 4 °C in the dark before being sown in sterilized Jiffy-mix Plus (Jiffy Products of America, Batavia, IL):perlite (3:1 (v/v)). Seedlings were grown at 20 °C under continuous cool-white fluorescent light (100 μmol m^{-2} s^{-1}). At appropriate times, rosette leaves, flowers, stems, and siliques were harvested into liquid nitrogen and stored at −80 °C. To obtain root cultures, seeds were sterilized using a combination of washes with 70% (v/v) ethanol, 15% (v/v) sodium hypochlorite, and sterile water before receiving cold-dark treatment for 72 h in liquid culture medium (MS + 1% (w/v) sucrose). Subsequently, the seedlings were grown with continuous agitation (100 rpm) for 20 days at room temperature under continuous light or in darkness.

4.2. Biochemicals and chromatography materials

Toyopearl Butyl-650M was obtained from Tosoh Bioscience (Montgomeryville, PA), and glycosidic substrates were purchased from Sigma (St. Louis, MO).

4.3. Isolation and sequencing of cDNAs encoding BGLU45 and BGLU46

Total RNA was isolated from rosette shoots using the Trizol Reagent (Gibco-BRL, Cleveland) following manufacturer's instructions. Subsequently, poly(A^+) RNA was prepared by the Oligotex mRNA Mini Kit (Qiagen Inc., Valencia, CA) and used in double-stranded cDNA synthesis with the SMART cDNA Library Construction Kit (BD Biosciences Clontech, Palo Alto, CA). These cDNA populations served as templates in PCR amplification of regions encoding the mature β-glucosidases; such regions were based on GenBank-predicted sequences and amended for likely signal sequences. PCR primers were designed to include appropriate restriction sites to facilitate subcloning and, where required, additional bases to maintain the desired open reading frame (Table 5). Amplification was carried out using the KOD HiFi DNA Polymerase (Novagen, Madison, WI) according to the supplier's recommendations. For sequence verification, PCR products were cloned into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA) for transformation into *E. coli* TOP10 competent cells (Invitrogen). Prior to cloning, blunt-ended PCR products resulting from KOD HiFi DNA Polymerase amplification reactions were incubated for 30 min at 70 °C with 5 units of *Taq* DNA polymerase (Promega) and 0.2 mM dATP to add overhang adenine nucleotides. Plasmids were isolated using the Wizard Plus Minipreps DNA Purification System (Promega), and their inserts were sequenced in both directions at the Center for Comparative Genomics (University of Iowa) using a 373S Fluorescent Automated

Table 5
Sequences of primers used for cloning and RT-PCR

Gene name	Purpose	Sequence (5′ to 3′)
BGLU45	Cloning	F: CCCGGGTCGTCATCAAAGTTCTTCGAAG R: CCGCGGTTAATAATTATCTACGATATCTCTTCG
	RT-PCR	F: TGGTCGTCATCAAAGTTCTTC R: GCTCTATCTGCATTGTTCTTG
BGLU46	Cloning	F: CTGCAGGAACTTCAGATGATTCATCTCCATTTC R: CCGCGGTTATTTATCTATTTGATCTTCTATATTC
	RT-PCR	F: TTGCCAACTTTGCAATTCTG R: ATGTCTCCATTGCTTCCATC
βATPase	RT-PCR	F: GATCATGACATCTCTCGAGG R: TGGTAAGGAGCAAGGAGATC

F, forward primer; R, reverse primer. Underlined nucleotides indicate restriction sites.

Sequencer (Perkin–Elmer Applied Biosystems, Foster City, CA).

4.4. Construction of expression vectors and transformation of *P. pastoris*

After sequence confirmation, inserts encoding the mature β-glucosidases were excised from their respective TOPO vectors by appropriate restriction enzymes, gel purified using the GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences), and cloned into the *P. pastoris* expression vector pPICZαB (Invitrogen). Subsequent transformation of competent *E. coli* TOP10 cells yielded zeocin-resistant transformants, which were selected on low salt Luria–Bertani plates containing 25 μg/ml zeocin and verified by PCR screening. Desired plasmids were isolated using the Wizard Plus Minipreps DNA Purification System (Promega) and linearized by *Pme*I digestion, thereby allowing integration of the vector DNA into the *Pichia* chromosome by homologous recombination. Approximately 10 μg of linearized plasmid DNA were used to transform *P. pastoris* X-33 host cells (Invitrogen) by electroporation following supplier's recommendations using a Bio-Rad Gene Pulser (conditions: 1.5 kV, 25 μF, 200 Ω). Transformants were then cultured at 30 °C for 2–4 days on yeast extract peptone dextrose plates containing 100, 500, or 1000 μg/ml zeocin.

To identify transformants exhibiting highest β-glucosidase activity, 10–20 individual *Pichia* colonies for each gene (preferring the transformants obtained from the highest zeocin concentration plates) were cultured for 24 h in a rollodrum incubator (50 rpm) at 30 °C in buffered glycerol-complex medium (BMGY). Cells were collected by centrifugation and resuspended in buffered methanol-complex medium (BMMY) containing 0.5% MeOH (v/v) to induce β-glucosidase expression. Cultures were sampled at 24, 48, 72, and 96 h of expression. After removing cells by centrifugation, the supernatants were analyzed by SDS–PAGE and tested for β-glucosidase activity using *o*NPGlc and coniferin (BGLU45) or *p*NPGlc (BGLU46) as substrates. In addition to undertaking the usual boiled-enzyme and no-substrate controls, culture supernatants of cells transformed with empty vector (pPICZαB) were also assayed to assess endogenous β-glucosidase levels.

4.5. Expression and purification of recombinant BGLU45 in *P. pastoris*

A single colony was selected to inoculate 50 ml of BMGY medium containing 100 μg/ml zeocin. This starter culture was grown in a shaking incubator (275 rpm) at 30 °C, until the OD_{600} reached 16. Subsequently, 5 ml of this culture was used to inoculate 400 ml of BMGY that was incubated in a shaking incubator (225 rpm) at 30 °C, until the OD_{600} reached 15–20 (12–16 h). Cells were collected by centrifugation, washed with sterile H_2O, and resuspended in BMMY (400 ml) supplemented with 1% (w/v) of casamino acids containing 0.75% (v/v) methanol. The culture was incubated at 30 °C for 72 h in 2.8-liter Erlenmeyer flasks with addition of methanol to the same final concentration every 24 h. After removal of cells by centrifugation, recombinant BGLU45 was purified at 4 °C from 405 ml of the 72-h culture supernatant using β-glucosidase activity toward *o*NPGlc or coniferin to monitor purification progress. Ammonium sulfate fractionation was conducted according to standard methods to obtain the following saturation fractions: 0–50%, 50–70%, and 70–100%. Pellets were redissolved in HIC buffer (20 mM His–HCl buffer, pH 5.5). The 50–70% fraction, which exhibited the highest β-glucosidase activity was diluted twice with 2 M $(NH_4)_2SO_4$ and applied to a Toyopearl Butyl-650 M column (8 × 1.5 cm) pre-equilibrated with HIC buffer containing 1 M $(NH_4)_2SO_4$. After extensive washing with pre-equilibration buffer, bound proteins were eluted with a linear 1–0 M $(NH_4)_2SO_4$ gradient (150 ml, total volume) in HIC buffer.

4.6. Expression and purification of recombinant BGLU46 in P. pastoris

A single colony was selected to inoculate BMGY medium (3 ml) containing 100 μg/ml zeocin and incubated for 24 h in a rollodrum incubator (50 rpm) at 30 °C. An aliquot (1 ml) was taken to inoculate 100 ml of BMGY, which was held in a shaking incubator (275 rpm) at 30 °C until the OD_{600} reached 15–20. After collection of cells by centrifugation, they were washed with sterile water, resuspended in BMMY (100 ml) containing 0.75% (v/v) MeOH, and incubated at 30 °C for 48 h in 1-liter Erlenmeyer flasks with addition of methanol to the same final concentration every 24 h. After removal of cells by centrifugation, recombinant BGLU46 was purified at 4 °C from 170 ml of the 48-h culture supernatant, monitoring purification progress by assaying β-glucosidase activity toward *o*NPGlc. Ammonium sulfate fractionation was conducted according to standard methods to obtain the 40–60% fraction, which was subjected to HIC as described for BGLU45 purification.

4.7. Protein methodology

Protein samples were fractionated on 12% (w/v) SDS–polyacrylamide gels according to Laemmli (1970) and visualized by staining with Coomassie Brilliant Blue R-250 (Sigma). Protein determination was performed using a protein assay kit (Bio-Rad Laboratories) with bovine serum albumin serving as standard.

4.8. Verification of identity of recombinant BGLU45 and BGLU46

To verify their authenticity, purified recombinant BGLU45 and BGLU46 proteins were digested in excised gel bands with trypsin (Promega), and the resulting proteolytic fragments were analyzed by MALDI-TOF mass spectrometry using a Bruker Daltonics Biflex III mass spectrometer at the Molecular Analysis Facility of the University of Iowa. The search engine Mascot (Matrix Science Ltd.) was used to match the peptide mass fingerprint data with potential candidates in the protein databases.

4.9. BGLU45 and BGLU46 enzyme assays

During enzyme purification, β-glucosidase activity was determined by measuring the rate of nitrophenol liberation from *o*NPGlc (BGLU45) or *p*NPGlc (BGLU46) in 250-μl assays containing 10 mM glucoside, 100 mM sodium acetate buffer, pH 5.5 (pH optimum for both hydrolases), and enzyme. After incubation at 30 °C for 30 or 60 min, reactions were terminated by adding 1 ml of 0.47 M Na_2CO_3, and the released *o*- or *p*-nitrophenol was determined spectrophotometrically at 400 nm in conjunction with a standard curve. The sugar and linkage specificities of purified BGLU45 and BGLU46 were investigated using a range of α- and β-linked *p*NP-sugars (10 mM final concentration). To determine hydrolase activity toward non-chromogenic substrates, these enzymes were incubated for 30 or 60 min at 30 °C in 100-μl assays that contained 10 mM substrate and 100 mM sodium acetate buffer (pH 5.5). After terminating the reaction by boiling for 2 min, liberated glucose was determined by the glucose oxidase procedure (Megazyme International Ireland Ltd.) in conjunction with a standard curve. All assays were performed in duplicate or triplicate under conditions that ensured linearity with respect to both time and enzyme concentration.

4.10. RT-PCR analysis of BGLU45 and BGLU46 expression

Total RNA was extracted from various *Arabidopsis* organs using the Trizol method (Invitrogen, Carlsbad, CA). Subsequently, 2 μg of RNA was reverse transcribed using N_9 random primers and M-MuLV reverse transcriptase (New England Biolabs, MA). The PCRs (25 μl, total volume) contained 0.5 μM gene-specific primers, 0.2 mM each of dNTPs, 1.5 units of *Taq* DNA polymerase (New England Biolabs, MA), and buffer supplied with the enzyme. Gene-specific primers were designed using the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Products were separated on 2% (w/v) agarose gels and visualized under UV light after ethidium bromide staining. The control gene used was β*ATPase* (*At5g08680*). The intensities of the amplified product bands were determined using the program ImageJ 1.32 (NIH). The value of the target band in each line was divided by the value of the control band, and the lowest value among these ratios was set to unity.

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