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NOTE

Coniferin β-glucosidase is ionically bound to cell wall in differentiating xylem of poplar

Taku Tsuyama[1] · **Keiji Takabe**[1]

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Abstract Lignin is crucial for the growth and persistence of vascular plants in diverse environments. Although biosynthesis and polymerization of lignin precursors have been subjected to in-depth studies, only limited information exists on the ways in which these precursors are transported. Recent research has demonstrated that V-ATPase-dependent transport of coniferyl alcohol glucoside (coniferin) is a major metabolic function in differentiating xylem of both angiosperms and gymnosperms. Localization of β-glucosidase involved in the cleavage of coniferin to coniferyl alcohol and glucose is important to clarify the role of coniferin in lignification; nevertheless, only little has been reported in angiosperms. Here, we demonstrated the presence of coniferin β-glucosidase activity in a cell wall ionically bound protein fraction extracted from differentiating xylem of poplar. Coniferin β-glucosidase localization is very likely in cell wall similar to previous reports in conifers. We identified a putative poplar coniferin β-glucosidase through phylogenetic analysis and named this protein "PtrBGL6". Immunoprecipitation assays showed that the anti-PtrBGL6 antibody recognizes a coniferin β-glucosidase in a cell wall ionically bound protein fraction. Conserved coniferin β-glucosidases localized in cell wall in both angiosperm and gymnosperm implies their important roles in the formation of lignified cell wall.

Keywords Coniferin β-glucosidase · Poplar · Differentiating xylem · Cell wall

Introduction

Lignin is crucial for the growth and persistence of vascular plants in diverse environments. Lignification proceeds in three steps: biosynthesis of lignin precursors in the cell, transport of the precursors to the cell walls, and dehydrogenative polymerization of the precursors in cell walls [1]. Although biosynthesis and dehydrogenative polymerization of lignin precursors have been well studied [2–6], much less information has been published on the way in which these precursors are transported.

The transport mechanism of lignin precursors has been investigated previously by biochemical analyses. In rosette leaves of *Arabidopsis thaliana* (hereafter, Arabidopsis), ABC-like transporters mediate the translocation of monolignol and monolignol glucosides [7]. An ABC transporter of Arabidopsis involved in the transport of *p*-coumaryl alcohol has been characterized [8]. However, rosette leaves of Arabidopsis contain only small amounts of lignin and lignifying tissue, and H lignin derived from *p*-coumaryl alcohol is a minor lignin component. More recently, Tsuyama et al. [9] demonstrated that transport of coniferyl alcohol glucoside (coniferin) is a major transport mechanism in differentiating xylem undergoing vigorous lignification. The process is dependent on a proton gradient created by V-ATPase, and the mechanism occurs in both angiosperm and gymnosperm. Localization of V-ATPase is

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✉ Keiji Takabe
kjtakabe@kais.kyoto-u.ac.jp

[1] Laboratory of Tree Cell Biology, Division of Forest and Biomaterials Science, Graduate School of Agriculture, Kyoto University, Kitashirakawa oiwake-cho, Sakyo-ku, Kyoto 606-8502, Japan

observed not only to mature vacuoles, but also to endosomes and the trans-Golgi network [10, 11] that are trafficking to and from the plasma membrane. Coniferin might be transported to cell wall by small vesicles that have V-ATPase and coniferin transporter [9]. Knowledge of activity and localization of coniferin β-glucosidase (BGL) is important to investigate whether coniferin is transported to cell wall and involved in lignification of differentiating xylem.

Coniferin BGL has been examined in conifers [12–14], soybean (*Glycine max*) [15], poplar cell suspension cultures [16], silver birch (*Betula pendula*) [14], and Arabidopsis [17]. Arabidopsis has 47 BGLs; both AtBGL45/BGLU45 and AtBGL46/BGLU46 have been identified as coniferin BGLs [17]. Coniferin BGL from lodgepole pine (*Pinus contorta*) (PcBGL) was studied in detail and characterized by Dharmawardhana et al. [13, 18]. Samuels et al. [19] showed that this enzyme is localized in secondary walls of tracheids. However, scarce information exists on coniferin BGL in differentiating xylem of angiosperms. Only low levels of coniferin BGL activity have been reported for differentiating xylem in silver birch [14].

Here, we report the presence of coniferin BGL activity in cell wall ionically bound protein (WIB) fraction of poplar differentiating xylem. By phylogenetic analysis, we showed that PtrBGL6 is a putative coniferin BGL in poplar. Our immunoprecipitation assay demonstrated that anti-PtrBGL6 antibody recognizes coniferin BGL. Conserved coniferin β-glucosidases localized in cell wall in both angiosperm and gymnosperm implies their important roles in the formation of lignified cell wall.

Materials and methods

Chemicals

Chemicals were purchased from Nacalai Tesque (Kyoto, Japan). Coniferin and syringin were provided by Dr. N. Terashima (Nagoya University, Japan).

Plant materials

Differentiating xylem of an approximately 40-year-old hybrid poplar (*Populus sieboldii* × *P. grandidentata*) was obtained as described in Tsuyama et al. [9].

Enzyme extraction

Frozen xylem tissue (ca. 25 g) was extracted in 50 mL of ice-cold extraction buffer A (10 % (v/v) glycerol, 0.5 % (w/v) polyvinylpolypyrrolidone, 5 mM ethylenediaminetetraacetic acid (EDTA), and 50 mM MES–KOH adjusted to pH 6.0) for 15 min. Prior to use, a 3.3 mM dithiothreitol (DTT), and a 1 % (v/v) protease inhibitor cocktail (P9599; Sigma-Aldrich, St. Louis, MO, USA) were added to the buffer. The homogenate was filtered through Miracloth (Merck, Rahway, NJ, USA) and the filtrate was recognized as a soluble protein (Sol) fraction. Cell wall remnants were extracted in 50 mL of extraction buffer B (10 % (v/v) glycerol, 5 mM EDTA, 2 M NaCl, 3.3 mM DTT, 1 % (v/v) protease inhibitor cocktail, and 50 mM MES–KOH adjusted to pH 6.0) for 1 h. Wall remnants in buffer B were filtered through Miracloth and the extracted solution was recognized as the WIB fraction. Sol and WIB fractions were centrifuged at 122,000×*g* for 30 min. Supernatants were concentrated to approximately 1 mL volume with a centrifugal filter unit [10,000 NMWL (nominal molecular weight limit); Millipore, Billerica, MA, USA]. All of the above procedures were performed on ice or at 4 °C.

Glucosidase assays

The activity of BGL was determined by measuring the rate of nitrophenol liberation from *p*-nitrophenyl-β-D-glucoside (pNPG) in 100-μL assays containing 10 mM pNPG, 100 mM sodium acetate buffer (pH 5.5), and enzyme. After incubation at 30 °C for 30 min, reactions were terminated by adding 1 mL of 0.47 M Na_2CO_3; the *p*-nitrophenol released was determined spectrophotometrically at 400 nm in conjunction with a standard curve. To determine hydrolase activity toward non-chromogenic substrates, such as coniferin, the fractions were incubated for 30 min at 30 °C in 200-μL assays that contained 5 mM of substrate in 100 mM of sodium acetate buffer (pH 5.5). After terminating the reaction by boiling for 10 min, liberated glucose was determined by the glucose oxidase procedure (G3293; Sigma-Aldrich). Glucosidase activity of fractions incubated without substrates were used as background.

Phylogenetic analysis

A BLAST search demonstrated that *P. trichocarpa* has 48 putative BGLs or glycosyl hydrolase family 1 proteins; this protein group was named as "PtrBGL1-48" (Table 1). Further searches identified 13 PtrBGLs with high BLAST scores (>400) when the query sequence entered was AtBGL45. We constructed a phylogenetic tree showing the relationships among 13 PtrBGLs from *P. trichocarpa*, PcBGL from lodgepole pine, and 47 AtBGLs from Arabidopsis by aligning amino acid sequences with Clustal Omega software (http://www.clustal.org/). A maximum-likelihood phylogeny was constructed using PhyML 3.0 software (http://www.atgc-montpellier.fr/phyml/) and viewed with Mobyle software (http://mobyle.pasteur.fr/cgi-bin/portal.py).

Table 1 Putative BGLs from *Populus trichocarpa*

Name	NCBI accession	Name	NCBI accession
PtrBGL1	XP_002305597	PtrBGL25	XP_002330966
PtrBGL2	XP_002335958	PtrBGL26	XP_002333462
PtrBGL3	XP_002337816	PtrBGL27	XP_002299647
PtrBGL4	XP_002305595	PtrBGL28	XP_002299645
PtrBGL5	XP_002305594	PtrBGL29	XP_002299646
PtrBGL6	XP_002329151	PtrBGL30	XP_002302853
PtrBGL7	XP_002305596	PtrBGL31	XP_002332805
PtrBGL8	XP_002336575	PtrBGL32	XP_002332916
PtrBGL9	XP_002319794	PtrBGL33	XP_002305313
PtrBGL10	XP_002316169	PtrBGL34	XP_002330983
PtrBGL11	XP_002299251	PtrBGL35	XP_002330984
PtrBGL12	XP_002330881	PtrBGL36	XP_002305312
PtrBGL13	XP_002330882	PtrBGL37	XP_002331891
PtrBGL14	XP_002330884	PtrBGL38	XP_002330982
PtrBGL15	XP_002330883	PtrBGL39	XP_002338192
PtrBGL16	XP_002330885	PtrBGL40	XP_002330886
PtrBGL17	XP_002334229	PtrBGL41	XP_002338665
PtrBGL18	XP_002338830	PtrBGL42	XP_002316058
PtrBGL19	XP_002305150	PtrBGL43	EEF09517
PtrBGL20	XP_002298155	PtrBGL44	XP_002311330
PtrBGL21	XP_002298156	PtrBGL45	XP_002316096
PtrBGL22	XP_002328320	PtrBGL46	XP_002322085
PtrBGL23	XP_002328321	PtrBGL47	ABK95221
PtrBGL24	XP_002337115	PtrBGL48	XP_002322086

Expression analysis

Total RNA was extracted from approximately 100 mg of leaf or tangential cryo-section from differentiating xylem of poplar by CTAB method [20]. Total RNA was purified by RNeasy Plant Mini Kit (Quiagen, Hilden, Germany) and treated with DNase I (Promega, Madison, USA). First-strand cDNA was synthesized from 0.5 μg total RNA using Transcriptor High Fidelity cDNA Synthesis Kit (Roche Applied Science, Mannheim, Germany) according to the manufacture's instructions. Primers specific for the *PtrBGL6* gene or control 18S rRNA were then used to amplify each transcript using 20 PCR cycles (18S rRNA) or 31 cycles (*PtrBGL6*). The primers used were as follows: *PtrBGL6* (forward, 5′-GGTACCCTCCAGCTCACTGT-3′; reverse, 5′-TCCATTCCCTCTGGGACCAC-3′) and 18S rRNA (forward, 5′-AAACGGCTACCACATCCAAG-3′; reverse; 5′-CCTCCAATGGATCCTCGTTA-3′).

Raising antibodies

The peptide sequences NIKNNDNGDIADNH and GFSPPQQNDQVQA were selected from a database of amino acid sequences for PtrBGL6. The peptides were synthesized, conjugated to carrier protein keyhole limpet hemocyanin (KLH), and used as antigens to raise polyclonal antibodies in rabbits (Operon Biotechnology, Tokyo, Japan). The antibody raised was purified on a column together with the peptides NIKNNDNGDIADNH and GFSPPQQNDQVQA.

Immunoprecipitation

Seventy microliters of Sol and WIB fractions were separately incubated overnight at 4 °C with 20 μL of the antibody (1 μg protein/μL). Sol or WIB fractions incubated with distilled water served as controls. Ten microliters of Protein G Mag Sepharose (GE Healthcare, Pittsburgh, PA, USA) were added, followed by incubation for 1 h at room temperature. Beads were precipitated with a magnet, and the supernatant was used for the glucosidase assay.

Results and discussion

Low levels of coniferin BGL activity have been reported for differentiating xylem in silver birch, an angiosperm [14]. However, the protein fraction was extracted as mixture of Sol and WIB fractions in that study. In contrast, coniferin BGL activity was detected through a procedure in which Sol and WIB fractions were extracted separately (Fig. 1). The presence of coniferin BGL activity was demonstrated in differentiating xylem of poplar, particularly in the WIB fraction. Activity in the WIB fraction clearly increased and became saturated with increasing substrate concentration, whereas activity in the Sol fraction did not track any obvious trend in relation to the

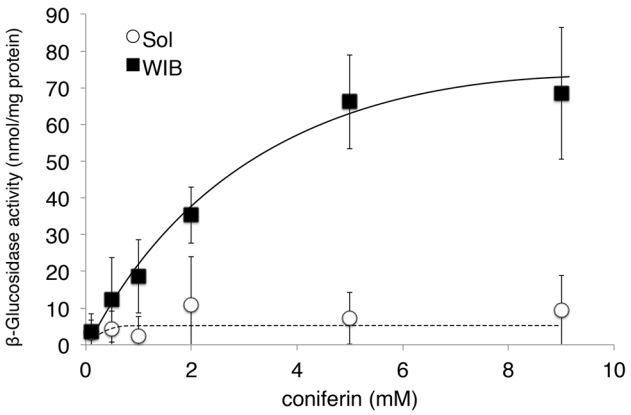

Fig. 1 Coniferin BGL activity in Sol and WIB fractions of differentiating xylem from poplar. Both fractions were incubated in each concentration of coniferin for 30 min. Fractions incubated without substrates served as background. *Sol* soluble protein fraction, *WIB* cell wall ionically bound protein fraction. Values are mean ± SD ($n = 4$ biological replicates)

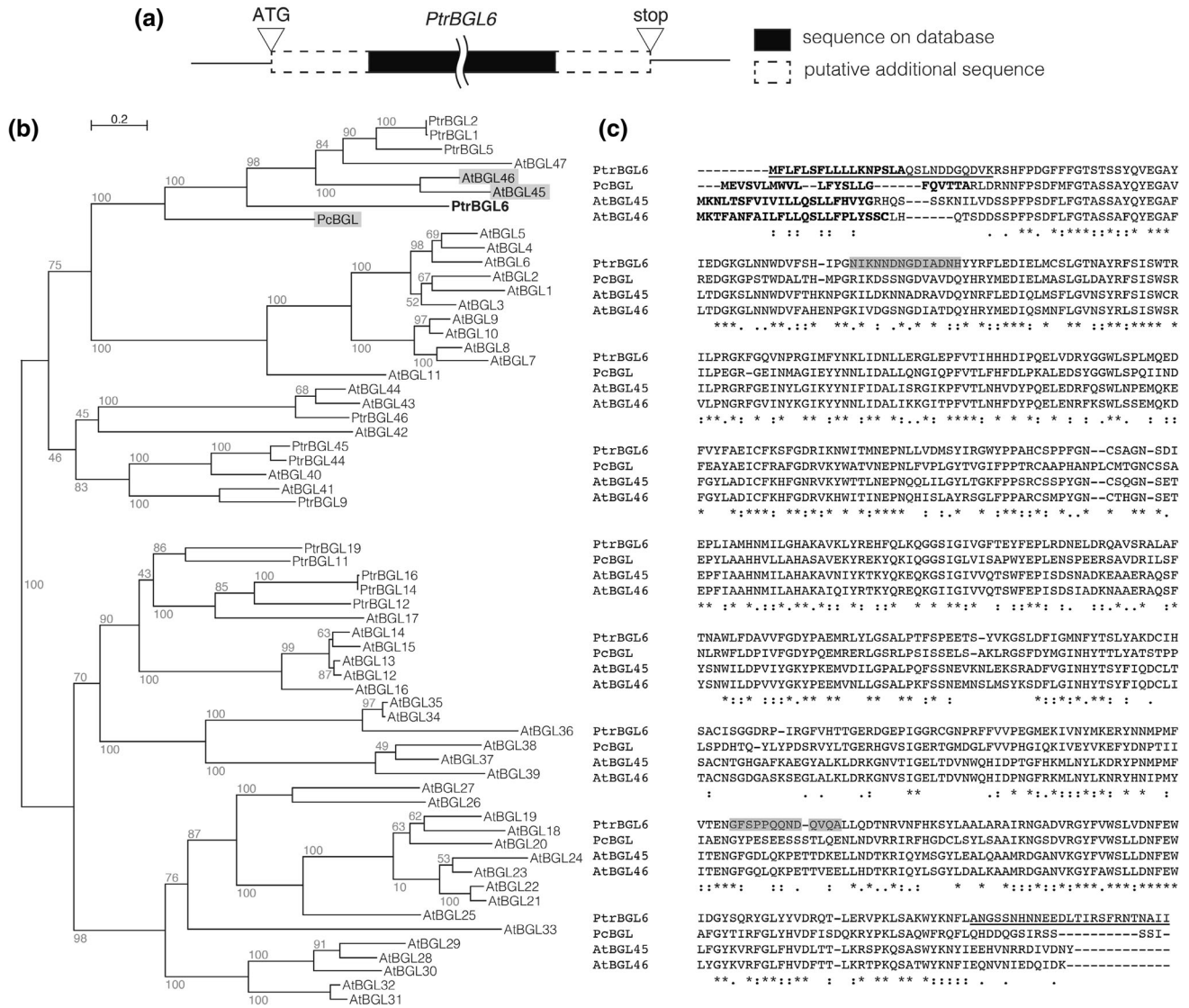

Fig. 2 Phylogenetic analysis of coniferin BGL in angiosperm and gymnosperm. **a** Diagram of *PtrBGL6* composed by sequence on database and putative additional sequences to begin with start codon (ATG) and end by stop codon. The putative additional sequences were expected by DNA sequence on database without considering additional intron. **b** Maximum-likelihood phylogeny of BGLs in *Arabidopsis thaliana* (At), *Pinus contorta* (Pc) and *Populus trichocarpa* (Ptr). AtBGL45, AtBGL46, PcBGL (hatched) have been identified as coniferin BGL [17, 18]. PtrBGL6 (*bold*) has similarity with identified coniferin BGLs. Bootstrapping was performed with 100 replicates. Bootstrap values are in *gray* type. **c** Alignment of amino acid sequences of PtrBGL6 and identified coniferin BGLs. All sequences were predicted to have signal peptide by SignalP (http://www.cbs.dtu.dk/services/SignalP/), which are shown in *bold*. Peptides of hatched sequence were used to raise anti-PtrBGL6 antibody. *Underlined* sequences indicate the putative additional sequences shown in **a**

concentration of coniferin. This result indicates that poplar has a coniferin BGL localized in cell wall.

To characterize coniferin BGL further, putative BGL proteins in poplar were searched using amino acid sequences from *Populus trichocarpa*. A BLAST search demonstrated that *P. trichocarpa* has 48 putative BGLs or glycosyl hydrolase family 1 proteins; this protein group was named as "PtrBGL1-48" (Table 1). Phylogenetic analysis showed that characterized coniferin BGLs clustered in a single clade that contained a putative BGL protein of poplar (PtrBGL6) (Fig. 2), which suggests that coniferin BGLs are conserved in both gymnosperm and angiosperm. PtrBGL1, PtrBGL2, and PtrBGL5 were also members of the clade, but clustered in a subclade containing AtBGL47/BGLU47, which seem to have no function in the hydrolysis of coniferin [21]. Thus, PtrBGL6 was focused for further analyses. PtrBGL6 was 50 % identical and 66 % similar to AtBGL45, and 50 % identical and 68 % similar to PcBGL. Expression of *PtrBGL6* in differentiating xylem was confirmed by semi-quantitative RT-

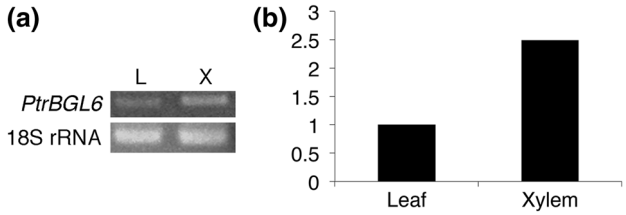

Fig. 3 *PtrBGL6* expression analysis in poplar. **a** Transcript abundance measured via semi-quantitative RT-PCR of *PtrBGL6*. 18S rRNA was used as control to ensure equal RNA loading. *L* leaf, *X* xylem. **b** Data are normalized with respect to 18S rRNA expression and standardized relative to leaf value which have been arbitrarily set to 1

PCR (Fig. 3), supporting that PtrBGL6 is involved in formation of xylem.

To pursue our line of enquiry on PtrBGL6, a candidate coniferin BGL from poplar, an antibody was raised using specific peptides of PtrBGL6 (Fig. 2b). The activity of BGL on pNPG was monitored as a general BGL; this activity was reduced in the supernatant after immunoprecipitation by the anti-PtrBGL6 antibody (Fig. 4a, b), indicating that the antibody recognized a BGL. Coniferin BGL activity in the WIB fraction also fell in the supernatant after immunoprecipitation (Fig. 4c), which suggests that the anti-PtrBGL6 antibody recognizes a coniferin BGL. Glucosidase activities on various substrates were measured in the supernatants before or after immunoprecipitation using WIB fraction (Fig. 5). Only BGL activities for coniferin or syringin were significantly inhibited after immunoprecipitation, and syringin was less favored as a substrate than coniferin. Thus, the antibody specifically recognized a coniferin BGL which may also hydrolyze syringin. Further research on cloning and characterizing, or silencing PtrBGL6 is required to confirm that PtrBGL6 is a homolog of coniferin BGL or the importance of PtrBGL6 in lignification. Still, higher BGL activity for coniferin than other substrates tested in this study (Fig. 5) suggests that hydrolysis of coniferin in cell wall has an important physiological function in differentiating xylem of poplar. Besides, very limited amount of coniferin in poplar xylem (ca. 0.2 nM) [22] and increased level of coniferin content in *bglu45* Arabidopsis mutant [21] imply fast consumption of coniferin by coniferin BGL in angiosperms.

Adding to the biochemical analyses, an in silico analysis predicted that a putative full sequence for PtrBGL6 as well as characterized coniferin BGLs would have a signal peptide (Fig. 2b), suggesting that coniferin BGL is secreted to the cell walls. Localization of coniferin BGL in poplar is consistent with coniferin BGL in lodgepole pine that localized in the secondary walls of tracheids [19] and in Arabidopsis that localized in the cell walls of xylem or interfascicular fibers [21]. Localization of coniferin BGLs

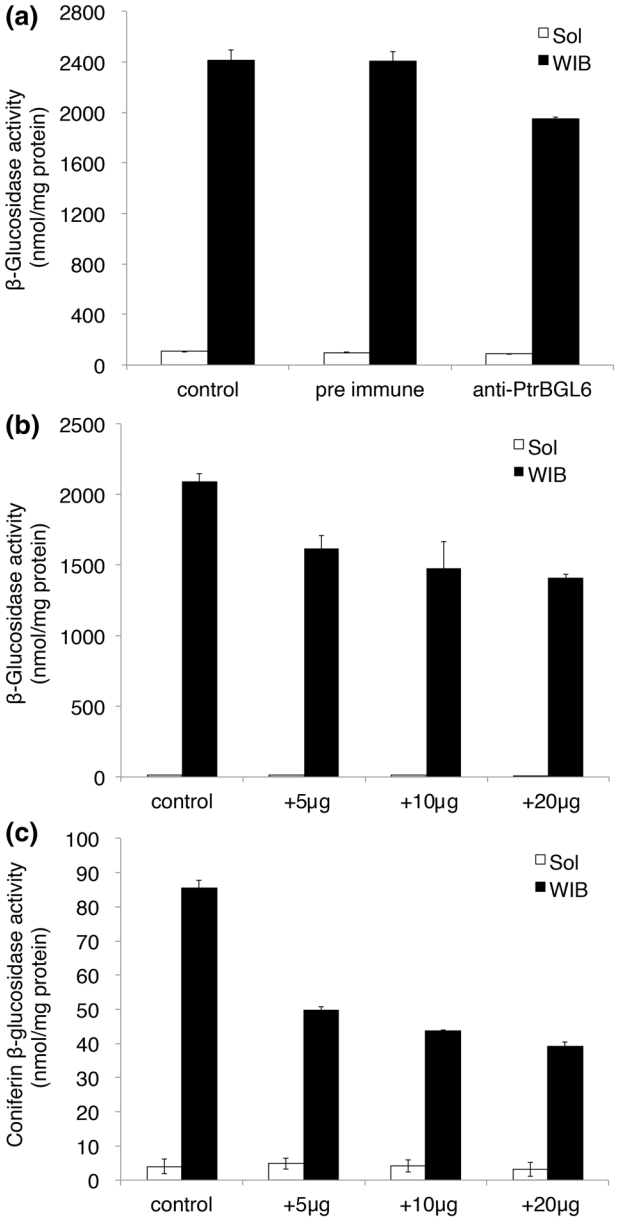

Fig. 4 Glucosidase activities in the supernatant after immunoprecipitation with the anti-PtrBGL6 antibody. Glucosidase substrates were 10 mM *p*-nitrophenyl-β-glucoside (**a**, **b**) and 5 mM coniferin (**c**). Glucosidase activity was measured after immunoprecipitation without serum as control. The amounts of antibody indicated were used for immunoprecipitation in **b** and **c**. Following immunoprecipitation, supernatant fractions were incubated with substrate for 30 min. Fractions incubated without substrates served as background. *Sol* soluble protein fraction, *WIB* cell wall ionically bound protein fraction. Values are mean ± SD ($n = 3$ technical replicates)

in lignifying cell walls seems to be a conserved trait in several plant species, implying that this localization of coniferin BGL is important for the formation of lignified secondary walls in both angiosperms and gymnosperms. Coniferin might be transported to lignifying cell walls [9] and hydrolyzed by coniferin BGL to supply coniferyl

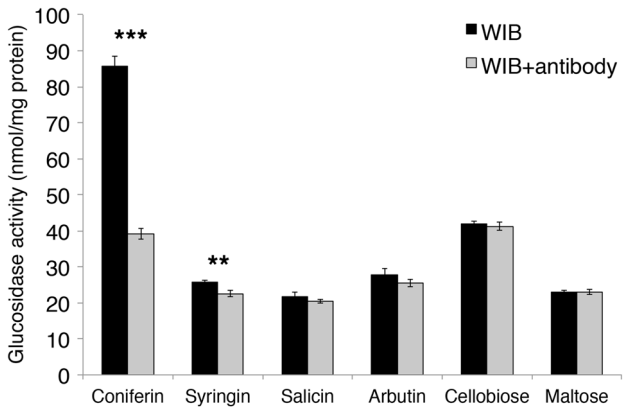

Fig. 5 Specificity of the anti-PtrBGL6 antibody. Glucosidase activities for six substrates in supernatant before and after immunoprecipitation with 20 μg of anti-PtrBGL6 antibody. Protein fractions were incubated with 5 mM of each substrate for 30 min. Fractions incubated without substrates served as background. *WIB* cell wall ionically bound protein fraction. Values are mean ± SD ($n = 3$ technical replicates). $^{**}P < 0.01$, $^{***}P < 0.001$, significant difference in activity with/without antibody (*t* test)

alcohol for lignification. This hypothesis is congruent with a putative mechanism proposed for gymnosperms [13, 19, 23, 24].

Several routes may exist for the transport of lignin precursors, which might contribute to the plasticity in lignin accumulation. In lodgepole pine, radiolabeled phenylalanine is incorporated into coniferyl alcohol and lignin; it is not incorporated into coniferin [25], although several studies indicate the incorporation of labeled phenylalanine into coniferin [26–28]. In Arabidopsis, single mutants of coniferin BGLs are not lignin-deficient, but double mutants have not been checked [21]. Thus, other mechanisms of lignin precursor transport may well exist in xylem. Further research will certainly be crucial for a better understanding of all lignin transport mechanisms that have evolved as plants have adapted to diverse terrestrial environments.

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