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REGULAR PAPER

Monoclonal antibody-based analysis of cell wall remodeling during xylogenesis

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Abstract Xylogenesis, a process by which woody tissues are formed, entails qualitative and quantitative changes in the cell wall. However, the molecular events that underlie these changes are not completely understood. Previously, we have isolated two monoclonal antibodies, referred to as XD3 and XD27, by subtractive screening of a phage-display library of antibodies raised against a wall fraction of *Zinnia elegans* xylogenic culture cells. Here we report the biochemical and immunohistochemical characterization of those antibodies. The antibody XD3 recognized (1→4)-β-D-galactan in pectin fraction. During xylogenesis, the XD3 epitope was localized to the primary wall of tracheary-element precursor cells, which undergo substantial cell elongation, and was absent from mature tracheary elements. XD27 recognized an arabinogalactan protein that was bound strongly to a germin-like protein. The XD27 epitope was localized to pre-lignified secondary walls of tracheary elements. Thus these cell-wall-directed monoclonal antibodies revealed two molecular events during xylogenesis. The biological significance of these events is discussed in relation to current views of the plant cell wall.

Keywords Arabinogalactan protein · Cell wall · Germin-like protein · Pectic galactan · Monoclonal antibody · Xylogenesis

Introduction

Growth of many land plants involves xylogenesis, a process by which woody tissues develop to conduct water and provide mechanical support. During this process, the tracheary elements differentiate by reorganization of the cell wall. However, to date, the molecular processes underlying this reorganization are not fully clarified.

The *Zinnia elegans* cell culture system developed by Fukuda and Komamine (1980) has proven useful for the analysis of xylogenesis. In this system, mesophyll cells are mechanically isolated and suspended in liquid culture medium containing auxin and cytokinin (Fukuda and Komamine 1980). After a few days of culture, tracheary elements differentiate synchronously to constitute 30–40 % of the culture cells (Fukuda and Komamine 1980). Without auxin or cytokinin, this in vitro differentiation does not occur, thus serving as a non-induced control (Fukuda and Komamine 1980).

Monoclonal antibodies directed against cell wall molecules are valuable tools for the analysis of cell wall modification (Knox 2008). Stacey et al. (1995) analyzed the in vitro *Z. elegans* xylogenesis with previously isolated cell-wall-directed monoclonal antibodies, including the antibody JIM13, which was raised against secreted glycoproteins of a carrot embryogenic cell line (Knox et al. 1991). This analysis revealed that an arabinogalactan

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protein (AGP) reactive to JIM13 accumulates in cell walls and culture medium of induced cells, but is absent in those of non-induced cells (Stacey et al. 1995).

Recently the collection of cell-wall-directed monoclonal antibodies, similar to JIM13, has been expanded (Pattathil et al. 2010). Such cell-wall-directed monoclonal antibodies have been isolated by immunization of either highly purified molecules or, alternatively, crude materials, which generally require subsequent retrospective characterization of antigen molecules. To isolate more types of cell-wall-directed monoclonal antibodies for the analysis of xylogenesis, we have used a crude immunogen—a wall fraction of *Z. elegans* xylogenic culture cells, and constructed a phage-displayed antibody library to screen for clones that specifically adhere to induced cells (Shinohara et al. 2000; Shinohara and Fukuda 2002).

By using the xylogenesis-oriented method, we have previously isolated a monoclonal antibody, referred to as CN8, which recognizes a hemicellulosic antigen localized to differentiating tracheary elements and xylem parenchyma cells (Shinohara et al. 2000). Optimization of the screening procedure resulted in isolation of two additional monoclonal antibodies (Shinohara and Fukuda 2002; former #3 and #27, now named XD3 and XD27 for *X*ylem *D*ifferentiation; their accession numbers are LC034240 and LC034241). In this paper, we report detailed biochemical and immunohistochemical characterization of these two antibodies, and show that these are good tools for the analysis of xylem cell walls.

Materials and methods

Cell culture

Xylogenesis-induced culture of *Z. elegans* mesophyll cells was performed as described by Fukuda and Komamine (1980). To culture non-induced cells, no cytokinin was applied.

Fractions prepared from *Z. elegans* cells

To prepare a cell-wall fraction, *Z. elegans* cells, 72 h in culture, were harvested and disrupted by sonication in 500 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES)-Na buffer (pH 7.0) containing 1 % (w/v) Triton X-100 and 1 mM dithiothreitol whilst the buffer was kept on ice. The disrupted cells were repeatedly washed with 50 mM HEPES-Na buffer (pH 7.0) by centrifugation (500 g, 2 min, 4 °C), so that an orange-colored pulp-like material was obtained. To prepare hot-water and *trans*-1,2-cyclohexanediamine-N,N,N′,N′-tetraacetic acid (CDTA) extracts, the cell-wall fraction was heated in water at 95 °C for 15 min followed by centrifugation (2000 g, 5 min); the sediment was incubated with 50 mM CDTA in 50 mM sodium acetate buffer at 4 °C for 3 h followed by centrifugation; those two supernatants were dialyzed against water with 1,000 Da cutoff membrane and lyophilized. An AGP fraction was prepared from culture medium of *Z. elegans* cells with β-glucosyl Yariv reagent as described by Motose et al. (2004).

(1→4)-β-D-galacto-oligosaccharides

Potato galactan (Megazyme; 10 mg mL^{-1} in 100 mM ammonium acetate buffer, pH 4.5) was incubated with 0.1–1 unit mL^{-1} *Aspergillus niger* endo-(1→4)-β-D-galactanase (Megazyme) at 50 °C for 1 h, and subsequently lyophilized. Then, 20 mg of the lyophilized hydrolysate was dissolved in 10 mL water, and passed through Q FF (CO_3^{2-} form) and SP FF (H^+ form) Sepharose resins (GE healthcare) in HR 16/10 columns (GE healthcare). After lyophilization, the dried flow-through was dissolved at 50 mg mL^{-1} in 50 mM ammonium formate, and then separated with a Superdex Peptide HR 10/30 column (GE healthcare) equilibrated with 50 mM ammonium formate. For thin-layer chromatography (TLC), a silica gel-coated glass plate was used; development was repeated three times with solvent mixture of *n*-butanol, ethanol, and water (3:3:2, v/v/v); detection was made by spraying sulfuric acid and charring. Glycosyl linkage analysis was performed as described by York et al. (1986).

ELISA

Polysaccharide preparations were purchased from Megazyme and Sigma-Aldrich (details in Supplementary Table S1); 96-well ELISA plates (Universal Bind), from Corning. Extracts from a *Z. elegans* cell wall fraction and polysaccharide preparations from commercial sources were dissolved in deionized water. A 100-μL aliquot of the aqueous solution was applied to each well. Then, the filled well was subjected to UV cross-linking as described in the manufacturer's instructions (Corning). After removal of the solution, the well was washed with phosphate-buffered saline (PBS), and blocked with PBS containing 2 % (w/v) bovine serum albumin (BSA). This blocking solution was also used for antibody dilution. Primary antibody was 1 μg mL^{-1} of affinity-purified recombinant antibody (single chain fragment variable, scFv); secondary antibody, 1:8,000 diluted anti E-tag (residues 7–19 of human osteocalcin) antibody conjugated with horseradish peroxidase (GE healthcare); peroxidase substrate, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) solution (BioFx). Color development was done at room temperature for

20–40 min, and measured using a plate reader. Absorbance of a primary antibody omitted sample was used as a blank. Periodate oxidation on ELISA plates was performed as described by Woodward et al. (1985). For galactanase treatment, 10 μg mL^{-1} of the CDTA extract was incubated with 1 unit mL^{-1} *Aspergillus niger* endo-(1→4)-β-D-galactanase (Megazyme) in 50 mM ammonium acetate (pH 4.5) at 50 °C for 1 h. As a control, an aliquot of the enzyme solution was heated at 100 °C for 10 min and mixed with the CDTA extract. Indirect ELISA for measuring affinity was performed as described by Friguet et al. (1985). Fractions obtained by anion-exchange chromatography of the hot-water extract were applied to an ELISA plate without dilution.

Protein analysis

Ion exchange chromatography of the *Z. elegans* hot-water extract was performed with Q FF Sepharose in HR 16/10 column equilibrated with 20 mM bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane (Bis–Tris; pH 6.4); elution was made with linear gradient of 0–1 M NaCl in 20 mM Bis–Tris (pH 6.4). Deglycosylation with trifluoromethanesulfonic acid (TFMS) was performed as described by Sojar and Bahl (1987). SDS-PAGE, silver stain, immunoblot, and protein sequencing by Edman degradation were performed according to standard procedures.

In-silico analysis

Previously reported germin-like protein (GLP) sequences were retrieved from the literature (Carter and Thornburg 2000; Kishi-Kaboshi et al. 2014; Nakata et al. 2004), and are listed in Supplementary Table S2. Multiple sequences were aligned using Clustal Omega (Sievers et al. 2011) for motif comparison and MUSCLE (Edgar 2004) for phylogenetic analysis. The phylogenetic tree was constructed by the algorithm PhyML (Guindon et al. 2010) implemented in the graphical user interface SeaView (Gouy et al. 2010).

Immunolabeling

Unsectioned specimens of culture cells and paraffin-sections of *Z. elegans* plants were immunolabeled as described previously (Shinohara et al. 2000). Sections of culture cells were prepared and immunolabeled as described by Nakashima et al. (2004) with minor modifications; Vector Blue Substrate Kit (Vector Laboratories) was used for visualizing the signal, and Safranin O was used for counterstaining. Phloroglucinol staining was performed as described by Zhong et al. (2000).

Statistical analysis

Comparison of two sample means was performed by the two-tailed Student's *t* test, and results are shown in graphs in the conventional manner: not significant (n.s.), $P > 0.05$; significant, * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. When more than two samples were included in the analysis, a one-step multiple comparison procedure was performed by Tukey's test; different letters in graphs denote significant differences at $P < 0.05$.

Results

Extraction of antigens from the cell wall

Previously, XD3 and XD27 have been isolated by subtractive screening of a phage-display library of antibodies raised against a wall fraction of *Z. elegans* xylogenic culture cells (Shinohara and Fukuda 2002). To characterize the XD3 and XD27 antigens by their extractability from the cell wall, we incubated the wall fraction of *Z. elegans* culture cells with hot water and subsequently with CDTA to obtain two extracts. ELISA against those extracts showed that the XD3 signal was significantly higher ($P < 0.01$, Student's *t* test) in the CDTA extract than in the hot-water extract

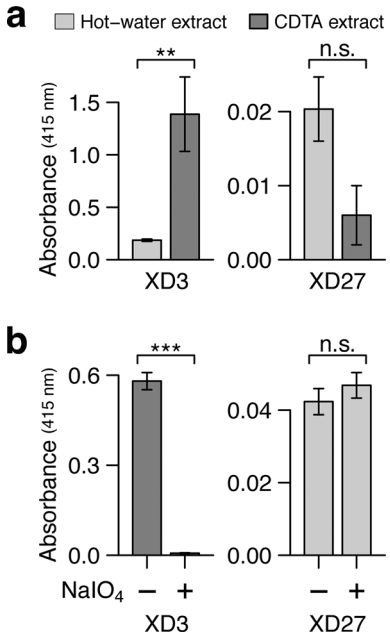

Fig. 1 Extraction of XD3 and XD27 antigens. **a** ELISA against hot-water and CDTA extracts (each 100 ng $well^{-1}$) from a *Z. elegans* wall fraction. **b** ELISA against CDTA extract (25 ng $well^{-1}$; *left*) and hot-water extract (500 ng $well^{-1}$; *right*) with or without periodate oxidation (30 mM $NaIO_4$). Mean ± SE of triplicate samples are shown. Sample means were compared by Student's test

(Fig. 1a). The XD27 signal was not significantly different between those two extracts, but the XD27 signal in the hot-water extract was significantly higher than the background level ($P < 0.01$, Student's t test). Periodate oxidation nullified the XD3 signal in the CDTA extract and did not alter the XD27 signal in the hot-water extract (Fig. 1b). Thus, XD3 recognized a polysaccharide that has vicinal diol groups, whilst XD27 recognized a structure resistant to periodate oxidation.

Recognition of (1→4)-β-D-galactan by XD3

We examined the reactivity of XD3 to different types of commercially available polysaccharides by a direct ELISA in which the polysaccharide concentration was set at 10 or 100 ng well^{-1}. XD3 did not recognize polygalacturonic acid from citrus fruit, rhamnogalacturonan from soy bean, arabinan from sugar beet, arabinogalactan from larch wood, or gum arabic from acacia sap (Fig. 2a). However, XD3 did recognize galactan from lupin seed and that from potato tuber (Fig. 2a). Because both of these preparations mainly consist of (1→4)-β-D-galactan (Hirst et al. 1947; Jarvis et al. 1981), we examined the susceptibility of the XD3 antigen to purified fungal *endo*-(1→4)-β-D-galactanase by enzymatic treatment of the CDTA extract.

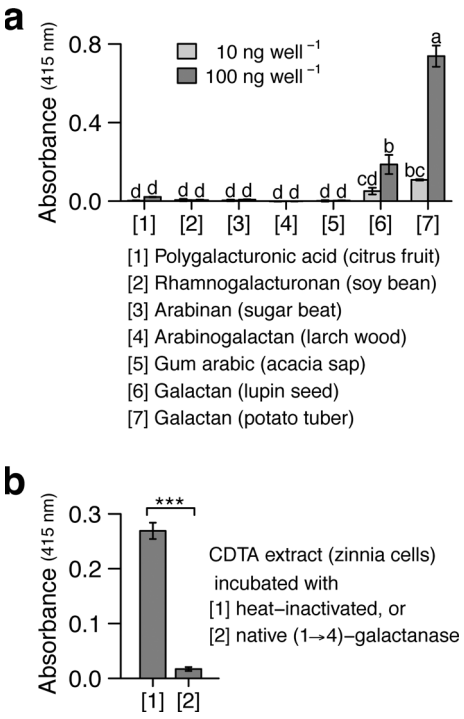

Fig. 2 Characterization of the XD3 antigen. **a** ELISA against types of polysaccharide preparation. **b** ELISA against CDTA extract (25 ng well^{-1}) incubated with heated (100 °C, 10 min) and non-heated (1→4)-β-D-galactanase. Mean ± SE of triplicate samples are shown. Sample means in **a** were compared by Tukey's test, and those in **b** were compared by Student's test

ELISA revealed that treatment with a non-heated aliquot of this enzyme almost nullified the XD3 signal, whilst treatment with a pre-heated aliquot did not (Fig. 2b). Thus XD3 recognized (1→4)-β-D-galactan in a pectic fraction.

XD3 recognized potato galactan at concentrations as low as 10 ng well^{-1} in a direct ELISA (Fig. 2a). To measure the affinity more precisely, we performed an indirect competitive inhibition ELISA. Fifty percent inhibition of XD3 binding was achieved at a potato galactan concentration of 1.7 (±0.6) ng mL^{-1}. Given the average molecular mass of 150 kDa for this polysaccharide preparation (Michalak et al. 2012), the dissociation constant was calculated to be 12 pM. This affinity is comparable to high-affinity scFv antibodies whose binding sites are optimized by multiple screening cycles of random mutagenesis and affinity selection (e.g., Chen et al. 1999; Schier et al. 1996), even though we did not subject XD3 to such an optimization. This finding motivated us to explore the mode of molecular recognition of XD3.

In general, the apparent binding affinity of the molecular recognition increases with multivalency—the number of an identical binding site in one molecule (Badjić et al. 2005), and polysaccharide recognition by antibody molecule can be categorized mainly into two modes: recognition of non-reducing ends of polysaccharides and recognition of non-terminal locations along linear polysaccharides (Kabat 1976). It is possible that XD3 recognizes a non-terminal location of (1→4)-β-D-galactan as its epitope, thereby strongly binding to the polysaccharide due to the high multivalency of the epitope. To explore this possibility, we prepared a series of fractions (named Gal2 to Gal8) that contained (1→4)-β-D-galacto-oligosaccharides with different degrees of polymerization (Fig. 3a, b).

The affinity of XD3 to those fractions as well as methyl-β-D-galactopyranoside was determined by an indirect competitive inhibition ELISA (Fig. 3c). The affinity to fractions Gal2 to Gal6 increased with degree of polymerization. However, the affinity to fractions Gal6 and Gal8 stayed unchanged. This result indicates that the XD3 epitope was composed of six contiguous (1→4)-β-D-galactosyl residues. The affinity to methyl-β-D-galactopyranoside was 12 % of the affinity to Gal6, whilst the affinity to Gal2 was 47 % of the affinity to Gal6. Thus XD3 did not recognize the non-reducing end of (1→4)-β-D-galacto-oligosaccharides. The affinity to Gal6 was in the micromolar range (18 ± 5 μM), whilst the affinity to potato galactan was in the picomolar range. This may be due to high multivalency of the XD3 epitope in the potato galactan molecule.

Recognition of an AGP by XD27

To characterize the XD27 antigen, we separated the hot-water extract by SDS-PAGE and performed an immunoblot

using XD27. The XD27 antigen migrated as a smear with a molecular mass ranging from 100 to 400 kDa (Fig. 4a, lane1). Next, we chromatographed the hot-water extract with a Q-Sepharose anion-exchange column, and analyzed the separated fractions by ELISA. The XD27 antigen was eluted as one major peak, and the corresponding fractions were pooled (Fig. 4b). Immunoblotting of this pooled sample revealed that a small protein of 23 kDa was co-purified together with the XD27 antigen (Fig. 4a, lane 2). When the pooled sample was boiled with 3 % SDS and subjected to ultrafiltration over a 50-kDa-cutoff membrane, the co-purified 23 kDa protein stayed in the retentate along

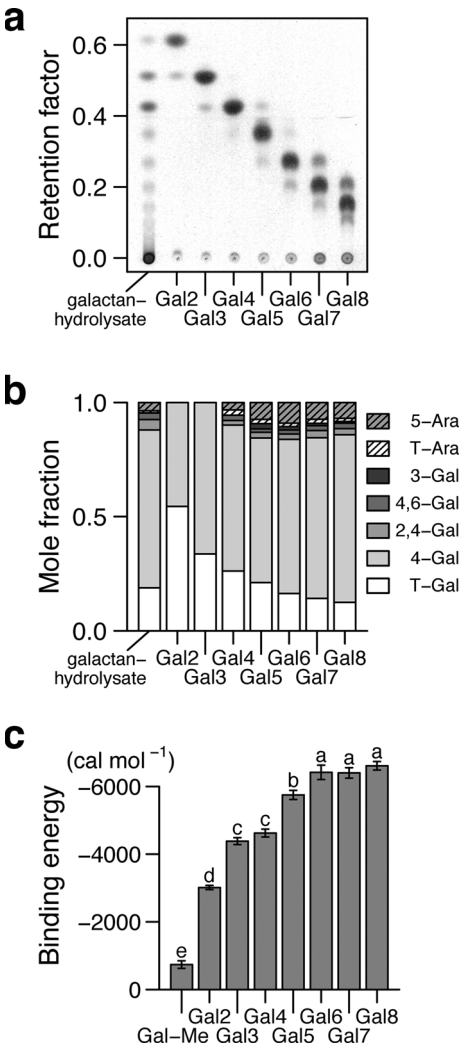

Fig. 3 Characterization of the XD3 epitope. **a** TLC and **b** glycosyl-linkage analysis of potato galactan hydrolysate and Gal2 to Gal8, fractions obtained by size-exclusion chromatography of the hydrolysate. **c** Binding energy of XD3 to methyl-β-D-galactopyranoside (Gal-Me) and Gal2 to Gal8 was determined by indirect competitive ELISA. In **a**, *Gal* galactopyranosyl residue, *Ara* arabinofuranosyl residue, *T* terminal; each number denotes the position of a carbon atom next to a substituted hydroxyl group. In **c**, mean ± SE of triplicate experiments are shown; sample means were compared by Tukey's test

with the XD27 antigen (Fig. 4a, lanes 4 and 5). Deglycosylation of the pooled sample by TFMS treatment erased the smear of the XD27 signal on the immunoblot and slightly lowered the molecular mass of the co-purified protein (Fig. 4a, lane 3).

To explore the structure of the XD27 antigen, we performed an ELISA against different types of commercially available polysaccharides at 100 μg well^{-1} (Fig. 4c). This concentration of galactan from potato tuber led to a near-saturated level of the XD3 signal. However, the XD27 signal was below detectable levels for galactan from potato tuber, rhamnogalacturonan from soy bean, arabinan from sugar beet, pectin from apple fruit, arabinoxylan from wheat flour, arabinogalactan from larch wood, and gum arabic from acacia sap.

AGPs are a large group of plant-specific proteoglycans that are highly decorated with diverse sugar chains. These proteins can be co-precipitated by using β-glucosyl Yariv reagent (Seifert and Roberts 2007). Considering the hot-water extractable nature and electrophoretic pattern of the XD27 antigen, we hypothesized that the antigen might be an AGP. To test this possibility, we prepared an AGP fraction from culture medium of *Z. elegans* cells by using β-glucosyl Yariv reagent. An ELISA against the AGP fraction revealed that XD27 recognized the fraction, whilst XD3 did not (Fig. 4d). Therefore, XD27 antigen was a type of AGP. The XD27 epitope was susceptible to TFMS-treatment (Fig. 4a) and could not be detected by an ELISA against gum arabic (Fig. 4b), which abundantly contains AGP (Randall et al. 1989). These findings indicate that XD27 recognized a carbohydrate motif occurring only in a subset of AGPs.

To examine the co-purified protein, we subjected the SDS-PAGE-separated bands of 23 kDa (Fig. 4a, lane 2) and 21 kDa (Fig. 4a, lane 3) to N-terminal sequencing, and obtained shorter and longer reads of an identical sequence. Those reads were GVQDFXVADL and GVQDFCVADLKGPDTPAGXI, where X is any amino acid residue. Using the latter as a query, we searched our in-house normalized cDNA library of *Z. elegans*, and found one full-length cDNA sequence that matched (Fig. 5a). Subsequently the predicted amino acid sequence was compared with those available in public databases—the nr database at NCBI (http://www.ncbi.nlm.nih.gov) and an Arabidopsis genome database (https://www.arabidopsis.org/). The amino acid sequence shared significant similarity to GLPs, indicating that the co-purified protein was a GLP. We named this protein ZeGLP1 (accession number: LC034242).

GLPs are encoded by a multigene family conserved in virtually all land plants (Dunwell et al. 2008). All members of this family include putative signal peptides (Bernier and Berna 2001), and many members catalyze the production of H_2O_2 (Davidson et al. 2009). The wheat protein germin, which is the first identified member of this family, and its

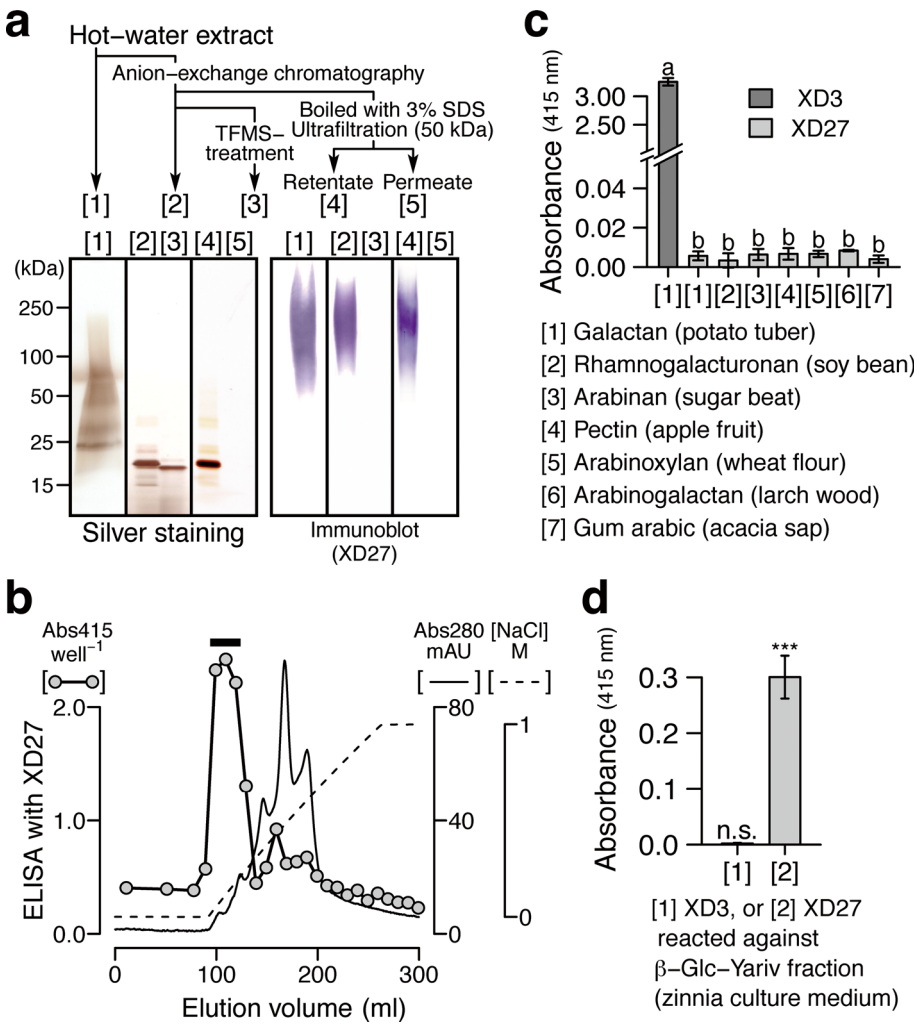

Fig. 4 Characterization of the XD27 antigen. **a** Hot-water extract separated by SDS-PAGE before silver stain (*left*) or immunoblot with XD27 (*right*); sample preparation procedure is represented at the *top*. **b** Anion-exchange chromatography of hot-water extract, whose chromatogram was monitored by ELISA with XD27 and by a UV-monitor with 2-mm optical path length (mAU: 10^{-3} arbitrary unit of absorbance at 280 nm), **c** ELISA against types of polysaccharide preparation at 100 μg well^{-1}. **d** ELISA against a fraction prepared from culture medium of *Z. elegans* cells with β-glucosyl Yariv reagent. In **b**, *horizontal bar* indicates pooled fractions. In **c** and **d**, mean ± SE of triplicate samples are shown. In **c**, sample means were compared by Tukey's test. In **d**, XD3- and XD27-treated samples were compared with primary antibody omitted control by Student's test

barley counterpart have oxalate oxidase (OXO) activity (Hurkman et al. 1994; Lane et al. 1993), converting oxalic acid into H_2O_2 and CO_2. Moreover, many members have superoxide dismutase (SOD) activity (Carter and Thornburg 2000; Godfrey et al. 2007; Gucciardo et al. 2007; Ohmiya 2002; Yamahara et al. 1999), which converts superoxide radicals into H_2O_2. We compared the amino acid sequence of ZeGLP1 with those of other known GLPs (listed in Supplementary Table S2). The most similar sequence in BLAST search was ABP19, a peach GLP that can bind to the synthetic auxin 2,4-D (Ohmiya et al. 1998) and has SOD activity (Ohmiya 2002). Phylogenetic analysis showed that ZeGLP1 and ABP19 resided in the same subfamily (Fig. 5b)—subfamily 3 in the report of Carter and Thornburg (2000).

In the crystal structure of germin, three His and one Glu residues interact with a manganese ion, presumably to form its active site (Woo et al. 2000). These tetrad residues are well conserved in catalytically active members of the GLP family (Bernier and Berna 2001). When the ZeGLP1 sequence was aligned with sequences of these catalytically active members, it turned out that ZeGLP1 contained all the tetrad residues (Fig. 5a).

Localization of XD3 and XD27 antigens in *Z. elegans* cell cultures

To examine the developmental changes of XD3 and XD27 antigens during in vitro xylogenesis, we harvested *Z. elegans* cells in induced and non-induced cultures over time. After fixation and immunolabeling, the percentage of signal-positive cells in total cell counts was determined by fluorescence microscopy. Both XD3- and XD27-positive cells increased more notably in the induced culture than in the non-induced culture (Fig. 6a), and in the induced culture the XD3-positive cells appeared earlier than the XD27-positive cells (Fig. 6a). A minor subpopulation of the XD3-positive cells showed patterned secondary wall thickening of the tracheary elements (Fig. 6a, b), whereas most of the XD27-positive cells showed that thickening (Fig. 6a, b). XD27-positive tracheary elements often had thin secondary walls and contained plastids (Fig. 6b).

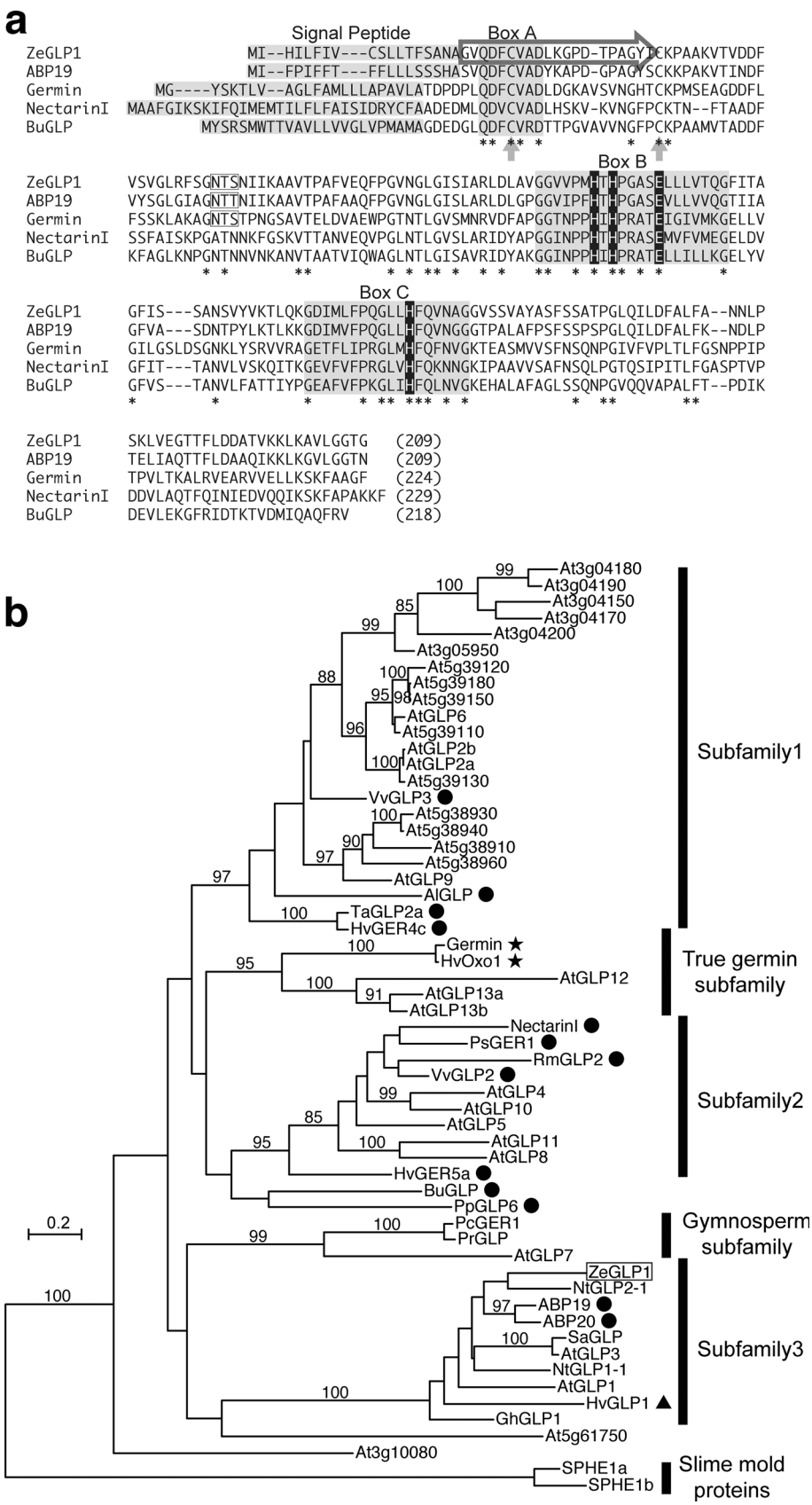

Fig. 5 Sequence comparison of ZeGLP1 and other known GLPs. **a** Multiple sequence alignment. *Grey line* predicted signal peptide, *right arrow* amino acid residues determined by N-terminal sequencing in this study; *grey box* germin boxes annotated according to the report of Bernier and Berna (2001); *upward arrow* two Cys residues that putatively form an intramolecular disulfide bridge; *boxed text* putative N-glycosylation site; *white letter* His and Glu residues that bind manganese in germin and are conserved in many germin-like proteins; *asterisk* conserved amino acid residues. **b** A maximum likelihood phylogenetic tree. Bootstrap values above 80 out of 100 resampling replicates are shown. Subfamilies were annotated according to the report of Carter and Thornburg (2000). ZeGLP1 is boxed for emphasis. *Closed circle* superoxide dismutase activity; *black star* oxalate oxidase activity; *closed triangle* ADP glucose pyrophosphatase/phosphodiesterase activity

To examine the subcellular localization of the XD3 and XD27 antigens, we prepared sections of *Z. elegans* cells in induced culture and incubated them with XD3 and XD27. The XD3 antigen was localized to the primary wall and intracellular compartments of a subset of the cells, and absent in tracheary elements (Fig. 6c). The XD27 antigen was exclusively localized to the secondary wall of tracheary elements (Fig. 6c).

Localization of XD3 and XD27 antigens in soil-grown *Z. elegans* plants

We examined the developmental changes of XD3 and XD27 antigens during xylogenesis using the shoot of soil-grown *Z. elegans* plants. In cross-sectioned vascular bundles, the XD3 antigen was localized to the primary wall of tracheary-element precursor cells (Fig. 7a, left panel, arrows), but was absent in fully differentiated tracheary elements (Fig. 7a, left panel, arrowheads). The XD3 antigen was also localized to xylem parenchyma cells (Fig. 7a, left panel, triangles). The XD27 antigen was localized to a subset of tracheary elements (Fig. 7a, right panel, arrowheads). Using serial sections stained with either XD27 or phloroglucinol, we confirmed that the XD27 antigen was localized to pre-lignified secondary walls of tracheary elements (Fig. 7b). Thus, the XD3 antigen was localized to tracheary-element precursor cells, and the XD27 antigen was localized to nascent tracheary elements.

To examine whether the XD3 antigen accumulates at one end of the cell in plant tissues as observed in culture cells (Fig. 6b, left panel), longitudinal sections of vascular bundle in the shoot were treated with XD3. Some of the tracheary-element precursor cells exhibited the XD3 antigen at the shootward end (Fig. 7c, left panel), whilst others exhibited XD3 antigen throughout the wall of the cell (Fig. 7c, right panel). Thus, some tracheary-element precursor cells exhibited XD3 antigen in a cell-polarized manner, irrespectively of whether they differentiated *in planta* or in vitro.

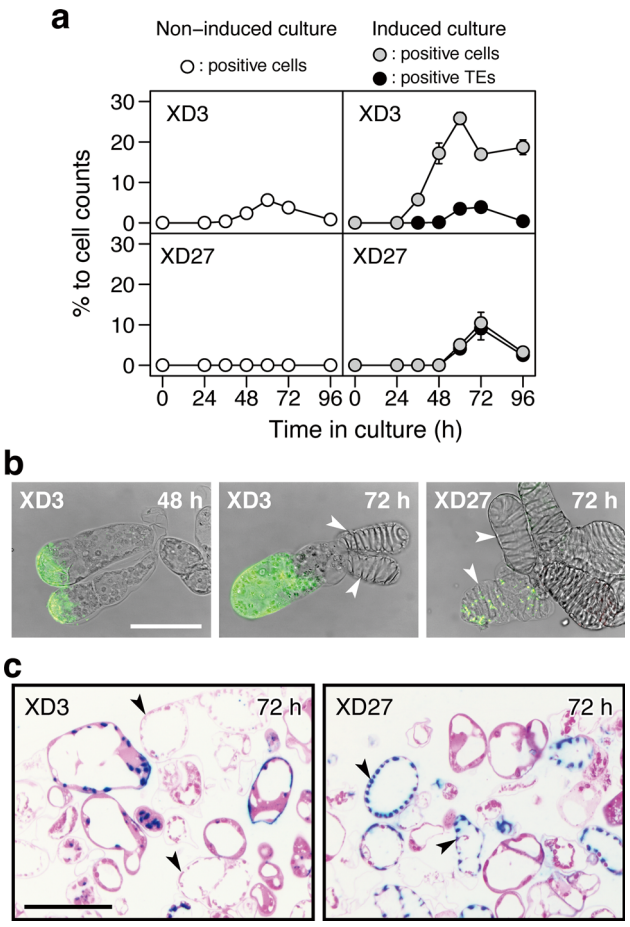

Fig. 6 Immunolocalization analysis of *Z. elegans* culture cells. **a** XD3-positive (*top*) and XD27-positive (*bottom*) cells in induced (*right*) and non-induced (*left*) cultures; *closed circles* represent tracheary elements that show the antibody signal; mean percentages ± SE of triplicate samples are shown; approximately 500 cells were observed for each data point. **b** Representative microscopic images of XD3-positive (*left* and *middle*) and XD27-positive (*right*) cells at 48 h (*left*) and 72 h (*middle* and *right*) in induced culture. **c** XD3-labeled (*left*) and XD27-labeled (*right*) sections of cells at 72 h in induced culture. In **b** and **c**, *arrowheads* indicate tracheary elements; *scale bar* 50 μm

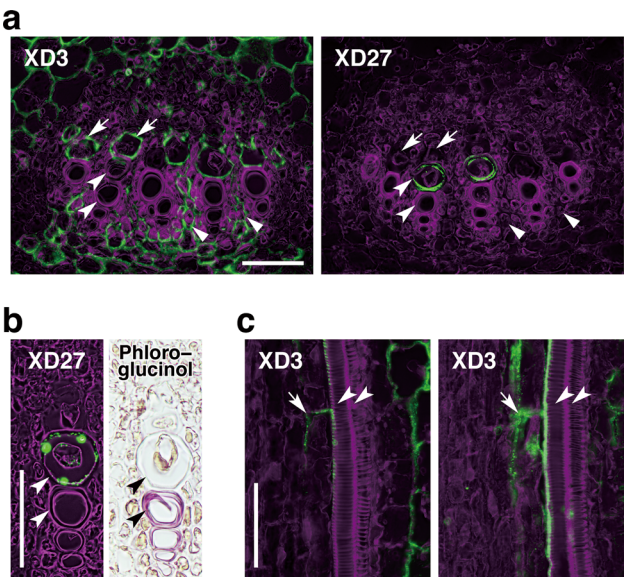

Fig. 7 Immunolocalization analysis of soil-grown *Z. elegans* plants. **a** XD3-labeled (*left*) and XD27-labeled (*right*) cross sections of the vascular bundle in the shoot of 14-day-old *Z. elegans* plants. **b** XD27-labeled (*left*) and phloroglucinol-stained (*right*) serial cross-sections of the vascular bundle. **c** XD3-labeled longitudinal sections of the vascular bundle; the *left panel* shows a tracheary-element precursor cell with the signal at the shootward end, and the *right panel* shows a tracheary-element precursor cell with a wider distribution of the signal. *Arrows* tracheary-element precursor cells, *arrowheads* tracheary elements, *triangles* xylem parenchyma cells. *Scale bar* 50 μm

Discussion

To understand the molecular changes that occur in the cell wall during xylogenesis, we characterized the two monoclonal antibodies XD3 and XD27. The results indicate that XD3 recognizes a pectic (1→4)-β-D-galactan that is localized to the primary wall of tracheary-element precursor cells. XD27 recognizes an AGP that is strongly associated with a GLP and was found to be localized to pre-lignified secondary walls of tracheary elements.

Affinity of XD3 to (1→4)-β-D-galactan

Probably the most widely used monoclonal antibody specific to (1→4)-β-D-galactan is LM5, isolated by Jones et al. (1997). Similar to LM5, XD3 is an anti-(1→4)-β-D-galactan monoclonal antibody. However, there are some differences between these two antibodies. With respect to antibody structure, XD3 is a monovalent antibody (scFv), whereas LM5 is a divalent antibody (IgG; Jones et al. 1997). Generally, binding of monovalent antibodies is much weaker than that of multivalent counterparts (Hornick and Karuch 1972). Nevertheless, the dissociation constant of XD3 to (1→4)-β-D-galactohexaose is 18 μM, whereas that of LM5 to (1→4)-β-D-galactotetraose is 87 μM (Jones et al. 1997). Hence, the monovalent binding of XD3 to its epitope is stronger than the divalent binding of LM5 to its epitope. With respect to affinity to the long (1→4)-β-D-galactan chain, the 50 % inhibition concentration of XD3 for potato galactan is 1.7 ng mL^{-1}, whereas that of LM5 for lupine galactan is 0.7 μg mL^{-1} (Jones et al. 1997). Such high affinity of XD3 can be explained by high multivalency of the epitope in the long (1→4)-β-D-galactan chain.

Immunogenicity of (1→4)-β-D-galactan

Our results shed light on the immunogenicity of (1→4)-β-D-galactan. It has been reported that immunization of a (1→4)-β-D-galactan-rich fraction from tomato fruit results in isolation of low-affinity monoclonal antibodies, whereas immunization of (1→4)-β-D-galactotetraose-conjugated BSA leads to isolation of high-affinity monoclonal antibodies, one of which is LM5 (Jones et al. 1997). These findings suggest that (1→4)-β-D-galactan is poorly immunogenic unless processed into a neoglycoprotein. However, XD3 and many other cognate clones have been isolated from a phage-displayed scFv library constructed from splenic mRNA of a mice immunized with a wall fraction of *Z. elegans* culture cells (Shinohara and Fukuda 2002), indicating that (1→4)-β-D-galactan is actually immunogenic as long as it resides in cell walls of *Z. elegans*. Although the generality of this finding for other polysaccharides and plant materials awaits further study, our results show that crude cell-wall materials could be a suitable immunogen for isolating high-affinity monoclonal antibodies that recognize cell wall polysaccharides.

Cellular localization of pectic (1→4)-β-D-galactan

Immunolocalization analysis with XD3 revealed that (1→4)-β-D-galactan accumulates on the primary wall of precursor cells of tracheary elements. Glycosyltransferases that elongate the (1→4)-β-D-galactan side chain of the pectic domain rhamnogalacturonan-I are expressed in various tissues, including xylem (Liwanag et al. 2012). This expression data combined with our results that the XD3 epitope was found in a pectic fraction, suggest that (1→4)-β-D-galactan is likely actively elongated as a side chain of rhamnogalacturonan-I during xylogenesis. It is known that (1→4)-β-D-galactan accumulates at the onset of cell elongation in carrot suspension culture cells and in the root of Arabidopsis (McCartney et al. 2003; Willats et al. 1999). Because the cell elongates considerably during differentiation from cambial initials to tracheary elements (Chaffey et al. 2002), the synthesis of (1→4)-β-D-galactan side chains may also take place at the onset of cell elongation in xylogenesis. The XD3 epitope was localized to the shootward end of some tracheary-element precursor cells. The elongation during differentiation from cambial initials to tracheary elements occurs in a cell-polarized manner (Larson 1994). That growing tip might be rich in rhamnogalacturonan-I with (1→4)-β-D-galactan side chain.

Possible function of (1→4)-β-D-galactan during xylogenesis

Pectic polysaccharides associate with each other by building Ca^{2+}-bridges to form a network coextensive with the cellulose-hemicellulose network (Carpita and Gibeaut 1993). Because (1→4)-β-D-galactan directly associates with cellulose (Zykwinska et al. 2005), (1→4)-β-D-galactan side chain can potentially tether cellulose microfibrils as an adhesive domain of the pectic network, thereby constituting a load-bearing structure in the cell wall (Zykwinska et al. 2007). This notion is consistent with a recent report that shows the existence of tethering polysaccharides other than xyloglucan (Park and Cosgrove 2012), which has long been believed to serve as the major load-bearing tether in the cell wall of eudicots. In mathematical modeling, an increase in the number of tethers can be captured as enhancement of the elastic, robust nature of the cell wall (Dyson et al. 2012; Passioura and Fry 1992). Given this prediction, the elongation of (1→4)-β-D-galactan side chains may impart mechanical strength to the cell wall by increasing the number of tethers. This notion is consistent with previous functional studies of (1→4)-β-D-galactan. In fact, transgenic potato tubers that express fungal (1→4)-β-D-

galactanase are more fragile (Ulvskov et al. 2005), and pea cotyledons become more durable to external mechanical force at the time of (1→4)-β-D-galactan accumulation (McCartney et al. 2000). In tracheary-element precursor cells, (1→4)-β-D-galactan may provide mechanical strength to the cell and enable it to enlarge rapidly without bursting before the secondary wall is formed and starts to thicken.

A cell wall fraction isolated from *Z. elegans* cells in xylogenic culture shows autolytic activity, abundantly releasing galactose (Ohdaira et al. 2002). In addition, during the maturation of tracheary elements, the primary wall undergoes dynamic reconstruction and becomes an enzymatically stable structure that contains glycine-rich proteins and rhamnogalacturonan-I (Ryser et al. 2004). The XD3 epitope was absent from mature tracheary elements, suggesting that the (1→4)-β-D-galactan side chains of rhamnogalacturonan-I are actively degraded in the course of tracheary-element differentiation. Seed mucilage effectively rehydrates when (1→4)-β-D-galactan side chains are removed (Dean et al. 2007; Macquet et al. 2007). Therefore, another function of (1→4)-β-D-galactan side chains during xylogenesis might be to make the cell wall more hydrophobic.

XD27 as an anti-AGP monoclonal antibody

Our results indicate that XD27 recognizes an AGP. The anti-AGP monoclonal antibody JIM13 is long known to mark secondary walls of tracheary elements as well as primary walls of other types of cell, such as endodermal cells and future sclerenchyma cells (Dolan and Roberts 1995; Schindler et al. 1995; Stacey et al. 1995). The XD27 epitope is not identical to JIM13 because the susceptibility to periodate oxidation differs between the two epitopes. Further analysis will clarify the structure of the XD27 epitope and the core protein of the XD27 antigen.

Interaction between an AGP and a GLP

The XD27 antigen was co-purified with ZeGLP1, a protein of 23 kDa. These two proteins could not be separated by 50 kDa-cutoff ultrafiltration after boiling in the presence of 3 % SDS. These findings are in line with known characteristics of the GLP multigene family. Many members of this family form 130 kDa homohexamers that are tolerant to many types of denaturing treatments, such as protease digestion and boiling with SDS (Dunwell et al. 2008), and are associated with extracellular polysaccharides (Bernier and Berna 2001).

Possible function of the AGP-GLP interaction in pre-lignified secondary walls

In-silico analysis revealed that ZeGLP1 is phylogenetically closely related to ABP19, a peach GLP that has SOD activity (Ohmiya 2002). In addition, the ZeGLP1 protein contains the conserved tetrad residues that putatively form the active site. Therefore, ZeGLP1 may have SOD activity. Germin, the first identified member of the GLP family, has OXO activity (Lane et al. 1993). In a germin-containing extract, OXO activity is elevated more than one order of magnitude by addition of a polysaccharide fraction (Lane 2000), which is thought to consist mainly of a polysaccharide co-purified with germin (Lane et al. 1992). On the basis of these findings, Lane (2000) proposed that OXO activity of germin may be regulated by binding to the co-purified polysaccharide. Given this hypothesis, our results raise the possibility that the association with the XD27 antigen may activate the enzymatic activity of ZeGLP1. SOD is considered to enhance peroxidase-mediated lignification by supplying H_2O_2 (Ogawa et al. 1997) and by preventing inactivation of peroxidase (Barr and Aust 1994). Because the XD27 antigen was localized to pre-lignified secondary walls of tracheary elements, interaction between the XD27 antigen and ZeGLP1 may lead to activation of SOD to enhance lignification.

Conclusions

Biochemical and immunolocalization analyses of the two monoclonal antibodies XD3 and XD27 have revealed dynamic changes in extracellular molecules during xylogenesis. XD3 recognizes pectic (1→4)-β-D-galactan in enlarging cells that were differentiating into tracheary element. This polysaccharide may confer the elastic and hydrophobic nature to the cell wall and enable those cells to enlarge rapidly (Fig. 8). XD27 recognizes an AGP that is strongly associated with a GLP and localized to pre-lignified secondary walls of tracheary elements. This AGP-GLP interaction may be involved in lignification by activating the GLP (Fig. 8). Our results show that XD3 and XD27 are

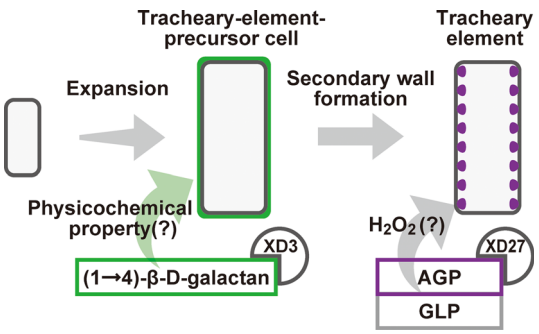

Fig. 8 Schematic representation of developmental regulation of XD3 and XD27 antigens during xylogenesis. The *green color* shows the XD3-antigen molecule and its localization; the *purple color* shows the XD27-antigen molecule and its localization. *AGP* arabinogalactan protein, *GLP* germin-like protein

useful tools, allowing further analysis of their antigen molecules and interactors thereof.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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