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Original Paper

Characterization of α-L-arabinofuranosidase related to the secondary cell walls formation in *Arabidopsis thaliana*

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Abstract A gene encoding a family 51 α-L-arabinofuranosidase (At3g10740) is specifically expressed at the stage of xylem vessel formation in *Arabidopsis thaliana*. To investigate the role of the enzyme in the xylem vessel formation, the recombinant protein was expressed in *Pichia pastoris* and the properties were characterized. The enzyme showed optimal activity at pH 4.5 and 50°C and was stable over the pH range of 4.0–7.0 under 30°C. The enzyme released L-arabinose from *p*-nitrophenyl-α-L-arabinofuranoside, synthetic arabinofuranobiosides, arabinoxylo-oligosaccharides and arabinose-containing polysaccharides. The enzyme hydrolyzed *p*-nitrophenyl-α-L-arabinofuranoside but did not hydrolyze any other *p*-nitrophenyl-glycosides, and the specific activity for *p*-nitrophenyl-α-L-arabinofuranoside was 1.2 units mg^{-1}. Among the synthetic regioisomers of arabinofuranobiosides, the enzyme hydrolyzed all linkages that can occur between two α-L-arabinofuranosyl residues in the following order: α-1,5-linkage > α-1,2-linkage > α-1,3-linkage. The enzyme hydrolyzed arabinan, gum arabic, corn hull arabinoxylan, and wheat arabinoxylan. The enzyme showed higher activity for oligosaccharides than for polysaccharides. Furthermore, the enzyme preferentially hydrolyzed arabinoxylo-oligosaccharides such as *O*-α-L-arabinofuranosyl-1,3-*O*-β-D-xylopyranosyl-1,4-D-xylopyranoside and *O*-β-D-xylopyranosyl-1,4-[*O*-α-L-arabinofuranosyl-1,3]-*O*-β-D-xylopyranosyl-1,4-*O*-β-D-xylopyranoside, which were the hydrolysis products of xylan generated by a family 10 xylanase, in comparison to its activity for arabinofuranobiosides. These data suggest that the enzyme is involved in the modification of the structure of xylan together with family 10 xylanases during the xylem vessel formation.

Key words: *Arabidopsis thaliana*, glycoside hydrolase family 51, secondary wall formation, xylan-degradation, α-L-arabinofuranosidase.

Plant cell walls undergo dynamic changes during plant growth and development. Carbohydrate active enzymes are involved in the formation and modification of the carbohydrate matrix of plant cell walls. Among them, glycoside hydrolases (GHs) have been classified into 115 families according to the similarity of their amino acid sequences, which implies both structural and mechanistic relationships (Cantarel et al. 2009). GHs are responsible for the hydrolysis and/or transglycosylation of glycosidic bonds of carbohydrates.

α-L-Arabinofuranosidases (EC 3.2.1.55) hydrolyze non-reducing α-L-arabinofuranosyl residues in hemicellulose, releasing L-arabinose as the only hydrolysis product. L-Arabinose residues are widely distributed in plant cell walls, where they are present in polymers such as arabinoxylans, arabinans, arabinogalactans and arabinogalactan-proteins (Carpita and Gibeaut 1993). α-L-Arabinofuranosidases are classified into five GH families, GH3, GH43, GH51, GH54, and GH62 (see the CAZy server at http://www.cazy.org/). In previous work, we purified some α-L-arabinofuranosidases and elucidated their substrate specificities towards structurally defined substrates (Ichinose et al. 2008; Kaneko et al. 1993; Kaneko et al. 1994; Kaneko and Kusakabe 1995; Kaneko et al. 1998; Matsuo et al. 2000; Yang et al. 2006). GH54 enzymes remove arabinofuranosyl moieties from arabinose-containing polysaccharides, whereas GH51, by

Abbreviations: AtAraf51A, α-L-arabinofuranosidase from *Arabidopsis thaliana*; GH, glycoside hydrolase; HPAEC-PAD, high-performance anion-exchange chromatography with a pulsed amperometric detection system; PNP, *p*-nitrophenol; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; YFP, yellow fluorescent protein.

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This article can be found at http://www.jspcmb.jp/

contrast, exhibit very little or no activity towards polymers. The properties of α-L-arabinofuranosidases tell us the precise role of each enzyme which might be playing in the modification of plant cell walls.

Carbohydrate active enzyme genes have been extensively studied at the genomic and transcriptomic levels in *Arabidopsis thaliana*. *A. thaliana* has approximately 900 genes encoding carbohydrate active enzymes (Geisler-Lee et al. 2006; Henrissat et al. 2001). Which enzymes are responsible for control of cell differentiation in plants remain to be solved. In the case of secondary wall formation, there is an in vitro xylem vessel element inducible system from *A. thaliana* suspension cells established to gain an expression profile of xylem-cell differentiation-related genes in *A. thaliana* (Kubo et al. 2005). Microarray analysis with the *A. thaliana* full-genome GeneChip array ATH1 (Affymetrix) using the system indicated that 1705 genes showed more than an eightfold change in expression over the time course (Kubo et al. 2005). Over 150 genes showed up-regulated expression just when the xylem vessel elements were actively forming, and included a gene encoding a putative glycoside hydrolase family 51 a-L-arabinofuranosidase (At3g10740) (Figure 1; Kubo et al. 2005).

The gene product of At3g10740 showed high similarity with AXAH-II, barley arabinoxylan arabinofuranohydrolase (Lee et al. 2001). The expression of the gene is intense in zones of cell proliferation, developing and regressing floral tissues, floral abscission zones, and the vascular system (Fulton and Cobbett 2003). The gene product seemed to act on polysaccharides at the plant cell wall, and play an important role for cell wall modification (Minic et al. 2004). Analysis of sugar composition of transgenic *A. thaliana* plants and immunolocalization patterns proposed that arabinans are potential in vivo substrates of the enzyme (Montes et al. 2008).

To date, little is known regarding the function of plant α-L-arabinofuranosidase. In the present work, we cloned the gene At3g10740 from *A. thaliana* and expressed the gene in *Pichia pastoris* to obtain the recombinant protein. The substrate specificity of recombinant protein was investigated to predict the enzyme function in the secondary wall formation. The results suggest that the enzyme is involved in the modification of the structure of xylan together with family 10 xylanases during the secondary cell wall formation.

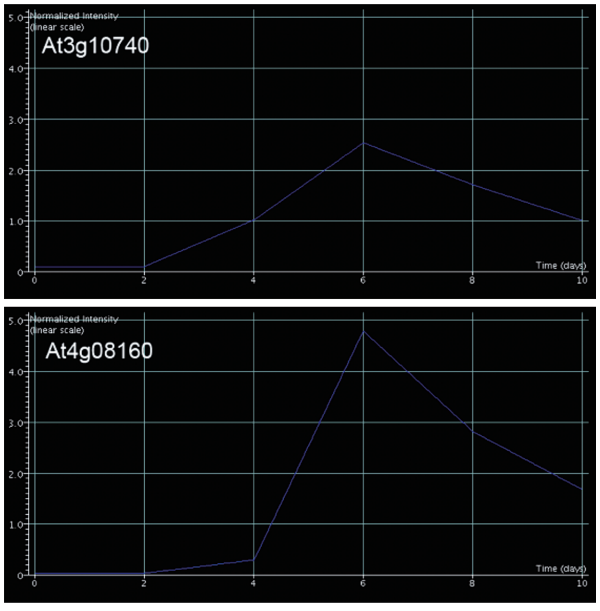

Figure 1. Gene expression profiles during in vitro xylem vessel element formation. The original data from Kubo et al. (2005).

Materials and methods

Substrates

p-Nitrophenyl-glycosides including *p*-nitrophenyl-α-L-arabinofuranoside (PNP-α-L-Ara*f*) and *p*-nitrophenyl β-D-xylopyranoside (PNP-β-D-Xyl*p*), larch wood arabinogalactan, oat spelts xylan, birch wood xylan were purchased from Sigma (St. Louis, MO, USA). Debranched arabinan and wheat arabinoxylan were obtained from Megazyme (Wicklow, Ireland). Gum arabic was obtained from Nacalai Tesque (Kyoto, Japan). Nihon Syokuhin Kakoh (Fuji, Japan) supplied corn hull arabinoxylan. Sugar-beet arabinan and arabinoxylo-oligosaccharides, and methyl 2-*O*-, methyl 3-*O*- and methyl 5-*O*-α-L-arabinofuranosyl-α-L-arabinofuranosides (arabinofuranobiosides) were prepared as described previously (Kawabata et al. 1995; Kusakabe et al. 1995; Yoshida et al. 1990).

Plant materials and plasmid construction

The *Arabidopsis thaliana* Columbia ecotype (Col-0) was used for this study. Transgenic plants carrying *AtAraf51Ap:YFP* [At3g10740 promoter with yellow fluorescent protein (YFP)] and *At4g08160p:YFP* (At4g08160 promoter with YFP) were generated. For the expression pattern of the gene, a 2,003 bp region upstream from the start codon of AtAraf51A gene and a 1,858 bp region upstream from the start codon of At4g08160 gene were amplified by PCR (AtAraf51A-F, 5′-cac cgt ctc tgt ctt cct tta gcg g-3′; AtAraf51A-R, 5′-aga ctc cat atc cat aat cac c-3′) (At4g08160-F, 5′-cac cat tgg ttg ctg atg gaa gtg-3′; At4g08160-R, 5′-ctt ctc cat agc aag ttc caa-3′) from *A. thaliana* genomic DNA, subcloned into pENTR/D-TOPO vector and transferred into pBGYN (Kubo et al. 2005). The binary vector constructs were introduced into *A. thaliana* plants by the *Agrobacterium tumefaciens* (GV3101 :: pMP90)-mediated floral dip method. Arabidopsis seeds were sterilized by 70% (v/v) ethanol with tween20 and were rinsed by 90% (v/v) ethanol, then after dried seeds placed on germination medium containing half-strength Murashige and Skoog basal medium (Sigma), 0.5% (w/v) sucrose and 0.3% (w/v) phytagel (Sigma). They were treated at 4ºC for 2 days, and then incubated in growth chamber under continuous illumination at 22ºC.

Histology

Assays of YFP signal were performed with T1 and T2 generation of transgenic plants. For observation of the *promoter:reporter* (YFP) lines, 7 days old seedling were used. The seedlings were mounted with 0.1% (w/v) sucrose on a slide glass. Fluorescent and DIC images were observed under a microscope (BX51; Olympus) and photographed with a digital DP70 camera (Olympus).

Expression of recombinant AtAraf51A

The cDNA encoding a mature region of putative a-L-arabinofuranosidase (At3g10740) was amplified from *A. thaliana* cDNA pools of the Col-0 ecotype by PCR with the following primers: forward, 5′-GAA TTC CAA GAA GAT CCA AAA CCA-3′; reverse, 5′-GCG GCC GCT CAC ACA GTG GTA GTT TTC TGA TG-3’. PCR was performed for 25 cycles of 98°C for 10 s, 55°C for 10 s, and 72°C for 60 s, using Phusion DNA polymerase (Finnzymes, Espoo, Finland). The amplified DNA was digested with *Eco*RI and *Not*I (underlined) and then ligated into the corresponding site of the *Pichia* expression vector pPICZαA (Invitrogen, Carlsbad, CA, USA). The plasmid was linearized with *Bpu*1102I (Takara Bio, Otsu, Japan) prior to transformation of *Pichia pastoris* strain KM71H (Mut^s) cells. Electroporation and selection of transformants were carried out according to the instruction manual of the EasySelect^{TM} *Pichia* expression kit (version G; Invitrogen). The selected clone was cultured in YPG medium consisting of 1% (w/v) yeast extract, 2% (w/v) peptone, and 1% (v/v) glycerol at 30°C for 1 day. The cells were harvested by centrifugation at 2,000×*g* for 5 min, then suspended in YPM medium containing 1% (w/v) yeast extract, 2% (w/v) peptone, and 1% (v/v) methanol as an inducer, and cultivated at 20°C for 5 days with constant shaking. Methanol was supplemented at a 1% concentration every 24 h during the period of induction.

Purification of recombinant AtAraf51A

The recombinant enzyme was purified from the culture supernatant of the *P. pastoris* transformant carrying the AtAraf51A gene. The crude enzyme preparation was dialyzed against 10 mM acetate buffer, pH 4.5, and applied to a SP-Sepharose Fast Flow (GE Healthcare, Buckinghamshire, UK) column (5×50 mm) at a flow rate of 1 ml/min. The column was washed with the same buffer to remove unbound materials, then the bound proteins were eluted with a linear gradient of sodium chloride (0–1 M). The eluate was fractionated into 1-ml portions. The purity was AtAraf51A was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the relevant fractions were pooled and dialyzed against deionized water. The final preparation thus obtained was used as the purified enzyme.

Enzyme assay and measurement of protein

The enzyme assay mixture contained 25 μl 2 mM PNP-α-L-Ara*f*, 20 μl McIlvaine buffer, pH 4.5 (0.2 M Na_2HPO_4/0.1 M citric acid) and 5 μl of the enzyme solution. The reactions were carried out at 50°C for 10 min, and were terminated by the addition of 50 μl of 0.2 M Na_2CO_3. The amount of PNP released was determined at 400 nm with an extinction coefficient of 19,200 $M^{-1}\,cm^{-1}$. One unit of enzyme activity is defined as the amount of enzyme that released 1 μmol of PNP per min from PNP-α-L-Ara*f* under these conditions. The protein concentration was determined using the BCA Protein Assay Kit (PIERCE, Rockford, IL) with bovine serum albumin as the standard. The effects of pH and temperature on enzyme activity were investigated as described previously (Ichinose et al. 2005; Ichinose et al. 2008).

Substrate specificity

The substrate specificity of AtAraf51A was determined using various PNP-glycosides. Each assay mixture contained 25 μl of 2 mM PNP-glycoside solution, 20 μl of McIlvaine buffer, pH 4.5, and 5 μl of enzyme solution. The reactions were performed at 50°C for 10 min and terminated by adding 50 μl of 0.2 M Na_2CO_3.

The substrate specificity of the enzyme for polysaccharides and oligosaccharides was determined at 30°C in McIlvaine buffer, pH 4.5, with 0.1 % (w/v) substrate and 2 mM enzyme. After incubation for the appropriate reaction time, the released L-arabinose was quantified by high-performance anion-exchange chromatography with a pulsed amperometric detection system (HPAEC-PAD) using L-fucose as an internal standard as reported previously (Ichinose et al. 2005). The samples were analyzed using a CarboPac^{TM} PA-1 column (4×250 mm, Dionex Corp., Sunnyvale, CA) and elution with 0.1 M NaOH (0–5 min), followed by a linear gradient (5–35 min) of sodium acetate (0–0.2 M) at a flow rate of 1 ml min^{-1} (Ichinose et al. 2008).

Results and discussion

Gene expression of AtAraf51A

As reported by Kubo et al., an expression profile of xylem cell-differentiation-related genes in *A. thaliana* was examined using in vitro xylem vessel element inducible system from *A. thaliana* suspension cells

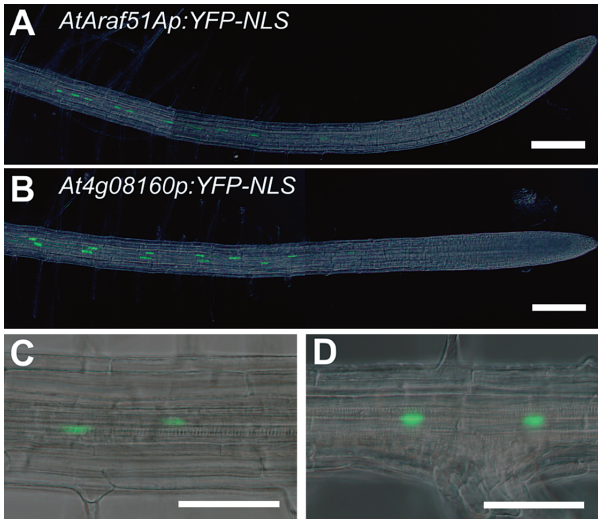

Figure 2. Expression of *AtAraf51Ap:YFP-NLS* and *At4g08160:YFP-NLS* in *A. thaliana* root. Image of differential interference contrast (DIC) and YFP Fluoresence was merged. Bar indicates 100 μm. Expression of *AtAraf51Ap:YFP-NLS* (A, C, D) and *At4g08160:YFP-NLS* (B). (C), protoxylem formaion; (D), metaxylem formation.

(Kubo et al. 2005). The expression level of the At3g10740 gene was up-regulated during the xylem vessel element formation (Figure 1). When the gene expression was investigated by the promoter::*YFP* reporter gene expression, the signal intensity was observed in the xylem vessels in roots at the secondary cell wall formation (Figure 2). These results reveal that AtAraf51A is correlated with xylem vessel differentiation. There is one possibility that the enzyme is involved in the modification of the structure of xylem vessels.

Expression and characterization of recombinant AtAraf51A

To predict the target of the enzyme, we cloned the gene and performed the characterization of the recombinant protein. The cDNA sequence of At3g10740 (2,037 bp) encodes a putative α-L-arabinofuranosidase (AtAraf51A, 678-amino-acid protein). In order of decreasing similarity, the sequence resembled the following plant sequences: a hypothetical protein from *A. thaliana* (79% identity and 86% similarity; AGI code At5g26120), a hypothetical protein from *Populus trichocarpa* (73% identity and 85% similarity; GenBank accession number EEE91463), a hypothetical protein from *P. trichocarpa* (71% identity and 84% similarity; EEF04345), a hypothetical protein from *Oryza sativa* (67% identity and 80% similarity; AK065240), and a hypothetical protein from *O. sativa* (67% identity and 80% similarity, AK072576) (Figure 3). AtAraf51A showed 66% identity and 80% similarity with an arabinoxylan arabinofuranohydrolase from *Hordeum vulgare*, AXAH-II, and also showed 65% identity and 78% similarity with AXAH-I (Lee et al. 2001).

The sequence at the N terminus (amino acids 1 to 33) was predicted to be a secretion signal sequence (SignalP program, http://www.cds.dtu.dk/services/SignalP/). The mature region of AtAraf51A was successfully expressed in *P. pastoris*, and purified recombinant enzyme was obtained by cation-exchange chromatography. The purified AtAraf51A appeared as smear bands on SDS-PAGE (Figure 4, lane 1). AtAraf51A has four *N*-glycosylation sites, so that it seems to be expressed as a glycoprotein. After endoglycosidase H treatment, AtAraf51A appeared as smaller bands of approximately expected size (71 kDa) on SDS-PAGE (Figure 4, lane 2).

AtAraf51A showed activity for PNP-α-L-arabinofuranoside, and the specific activity was 1.2 U mg^{-1}. The enzyme achieved maximal activity at pH 4.5 and 50°C, and was stable over the pH range of 4.0–7.0 under 30°C.

Substrate specificity of AtAraf51A

The activity of AtAraf51A was tested for various PNP-glycosides. The enzyme showed activity only toward PNP-α-L-arabinofuranoside and did not show any activities toward other PNP-glycosides (Table 1). Subsequently, activity of the enzyme towards arabinose-

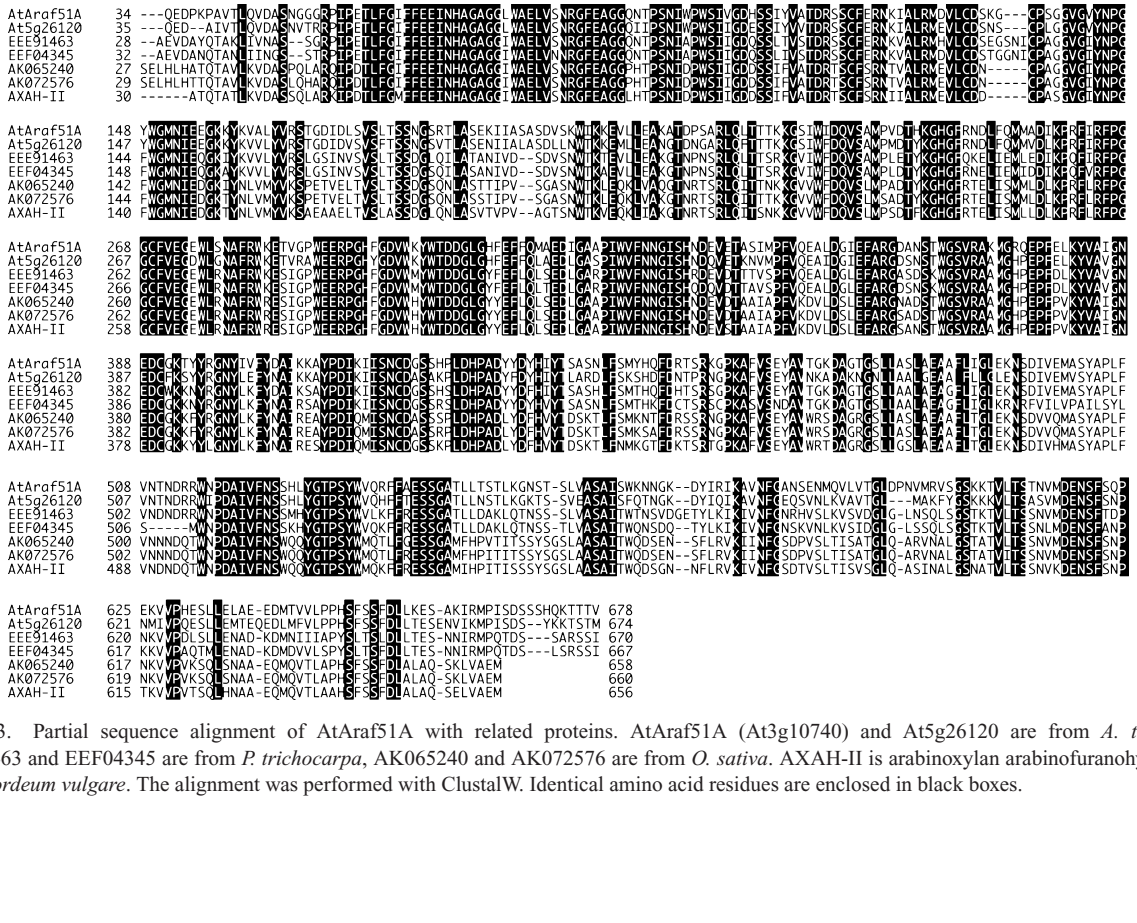

Figure 3. Partial sequence alignment of AtAraf51A with related proteins. AtAraf51A (At3g10740) and At5g26120 are from *A. thaliana.* EEE91463 and EEF04345 are from *P. trichocarpa*, AK065240 and AK072576 are from *O. sativa*. AXAH-II is arabinoxylan arabinofuranohydrolase from *Hordeum vulgare*. The alignment was performed with ClustalW. Identical amino acid residues are enclosed in black boxes.

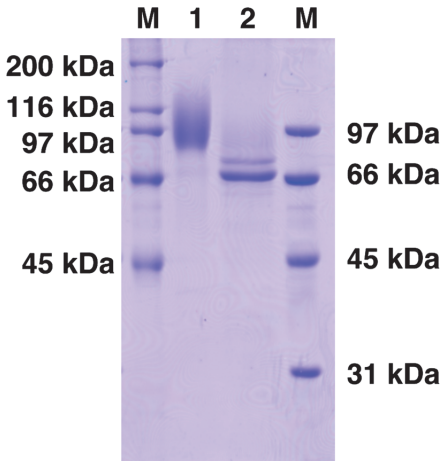

Figure 4. SDS-PAGE analysis of recombinant AtAraf51A. Lane M, molecular mass markers (1 μg each); lane 1, recombinant AtAraf51A; lane 2, endoglycosidase H-treated recombinant AtAraf51A. Each sample was separated on 10% polyachrylamide gel.

Table 1. Substrate specificity of AtAraf51A toward PNP-glycosides

Substrate	Activity (μmol of PNP per min)
PNP-α-L-arabinofuranoside	18.6
PNP-α-L-arabinopyranoside	0
PNP-β-L-arabinopyranoside	0
PNP-α-D-xylopyranoside	0
PNP-β-D-xylopyranoside	0
PNP-α-D-galactopyranoside	0
PNP-β-D-galactopyranoside	0
PNP-α-D-mannopyranoside	0
PNP-β-D-mannopyranoside	0
PNP-α-L-fucopyranoside	0
PNP-β-D-fucopyranoside	0
PNP-α-D-glucopyranoside	0
PNP-β-D-glucopyranoside	0
PNP-α-L-rhamnopyranoside	0
PNP-β-D-glucuronide	0
PNP-β-D-galacturonide	0

The enzyme was incubated in a mixture containing 1 mM PNP-glycoside and McIlvaine buffer, pH 4.5, at 30°C.

Table 2. Substrate specificity of AtAraf51A toward arabino-oligosaccharides and arabinose-containing polysaccharides

Substrate	Activity (μmol of arabinose per min)
Arabinoxylo-oligosaccharides	
$A1X_2$	70
$A1X_3$	44
Methyl arabinofuranobiosides	
α1,5-linked biose	7.8
α1,2-linked biose	5.7
α1,3-linked biose	1.9
Polysaccharides	
Arabinan	3.9
Gum arabic	0.7
Corn hull arabinoxylan	0.6
Wheat arabinoxylan	0.5

The enzyme was incubated in a mixture containing 0.1% (w/v) substrate and McIlvaine buffer, pH 4.5, at 30°C.

containing oligosaccharides and polysaccharides was tested (Table 2). The enzyme was incubated with methyl 2-*O*-, methyl 3-*O*- or methyl 5-*O*-α-L-arabinofuranosyl-α-L-arabinofuranosides. Among these synthetic regioisomers of arabinofuranobiosides, the enzyme hydrolyzed all linkages that can occur between two α-L-arabinofuranosyl residues in the following order: α-1,5-linkage>α-1,2-linkage>α-1,3-linkage. The activity of AtAraf51A toward arabinoxylo-oligosaccharides such as A_1X_2 (*O*-α-L-arabinofuranosyl-1,3-*O*-β-D-xylopyranosyl-1,4-D-xylopyranose) and A_1X_3 (*O*-β-D-xylopyranosyl-1,4-[*O*-α-L-arabinofuranosyl-1,3]-*O*-β-D-xylopyranosyl-1,4-*O*-β-D-xylopyranose) was also investigated. The enzyme showed higher activity for these substrates than for arabinofuranobiosides. The enzyme released 70 and 44 μmol of arabinose/min from A_1X_2 and A_1X_3, respectively. These data suggested that the enzyme preferentially hydrolyzed arabinoxyo-oligosaccharides, in comparison to its activity with arabinofuranobiosides. In case of the enzyme activity for polysaccharides, the hydrolysis rate of the polysaccharides was much lower than that of oligosaccharides. AtAraf51A hydrolyzed arabinan, gum arabic, corn hull arabinoxylan, and wheat arabinoxylan, but not debranched arabinan and larch arabinogalactan. From the results, it is indicated that AtAraf51A prefers oligosaccharides than polysaccharides and the arabinoxylooligosaccharides are the best substrate for the enzyme.

AtAraf51A was reported as a bifunctional enzyme which possessed α-L-arabinofuranosidase/β-D-xylosidase activity in the previous report (Minic et al. 2004). However, our results clearly indicated that the recombinant AtAraf51A hydrolyzed PNP-α-L-arabinofuranoside but did not hydrolyze any other PNP-glycosides including PNP-β-D-xyloside. Additionally, only L-arabinose was detected as the hydrolysis products of arabinoxylo-oligosaccharides by AtAraf51A (Figure 5), so that we concluded the enzyme was α-L-arabinofuranosidase without β-xylosidase activity.

Function of AtAraf51A

So far GH51 arabinofuranosidases reported as the enzymes prefer oligosaccharides to arabinose-containing polysaccharides (Matsuo et al. 2000; Taylor et al. 2006). Like these enzymes, AtAraf51A showed higher activity for oligosaccharides than polysaccharides such as arabinan, gum arabic and arabinoxylan (Table 2). GH51 enzymes have been shown to remove both α-1,2- and α-1,3- arabinofuranosyl moieties from xylan, so that the enzymes hydrolyze arabinoxylo-oligosaccharides such as A_1X_2 and A_1X_3 derived from xylanase breakdown of xylan. The family 51 enzyme from *Clostridium thermocellum* hydrolyzes A_1X_2, although the activity for A_1X_2 was almost same for α-1,5-linked arabinobiose (Taylor et al. 2006). AtAraf51A showed the highest

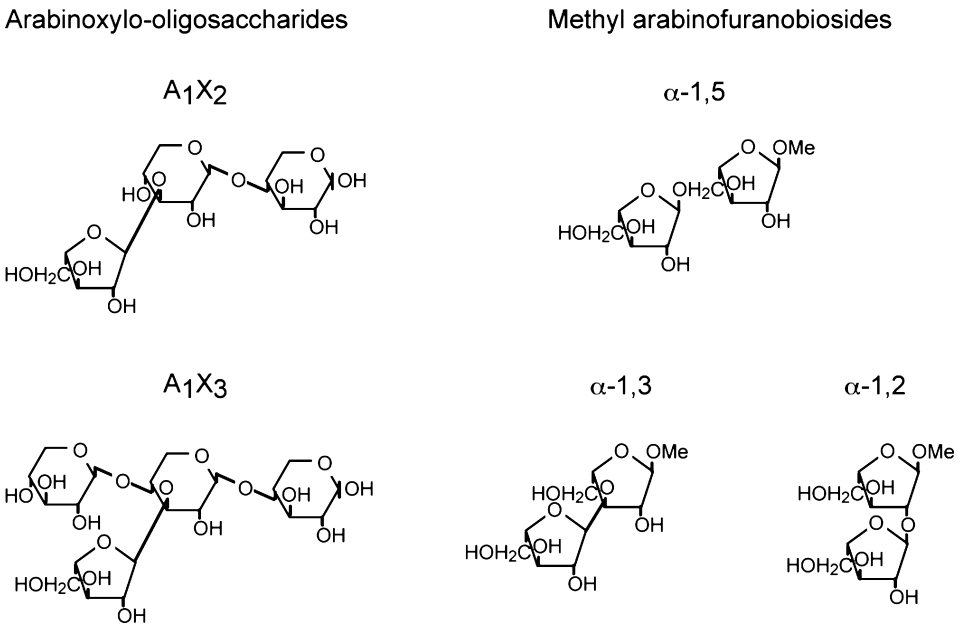

Figure 5. Structure of substrates.

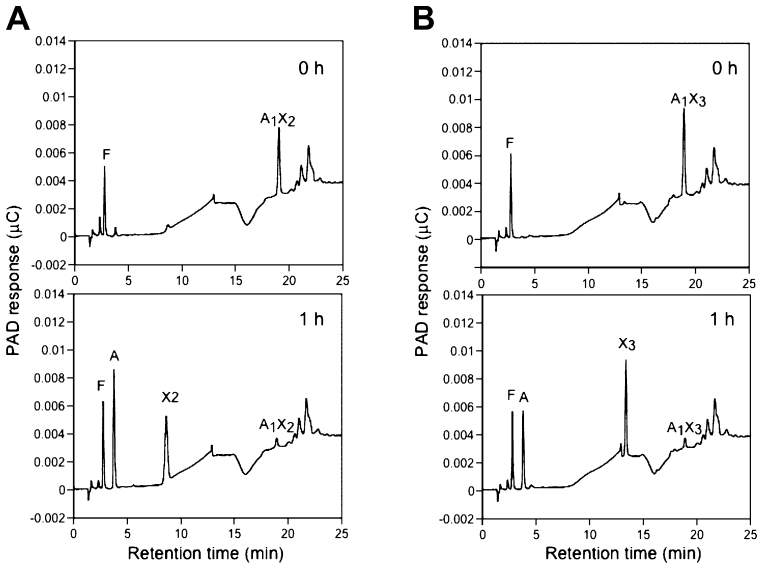

Figure 6. Hydrolysis products of arabinoxylo-oligosaccharides by AtAraf51A. A_1X_2(A) and A_1X_3(B) were used as substrates. The enzyme (0.015 unit) was incubated in a mixture containing 0.1% (w/v) substrate and McIlvaine buffer, pH 4.5, at 30°C for the appropriate time; the samples were subjected to HPAEC-PAD analysis. F, fucose; A, arabinose; X2, xylobiose; X3, xylotriose.

activity for arabinoxylo-oligosaccharides, in comparison to arabinofuranobiose and arabinose-containing polysaccharides. These results suggest that AtAraf51A prefers arabinoxylan as the substrate, and plays important roles in xylan modification together with xylanases.

Endo-β-1,4-xylanases are mostly belonging to GH families 10 and 11. These two families of xylanases differ in their patterns of cleavage of various heteroxylans (Biely et al. 1997). The arabinoxylo-oligosaccharides used as substrate in this study were prepared by treating a xylan with a GH10 xylanase. GH10 enzymes produce mainly A_1X_2 in which arabinose residues exist in non-reducing end of the substrate, but GH11 enzymes produce only branched arabinoxylooligosaccharides. AtAraf51A showed the highest activity toward A_1X_2 (Table 2). The data strongly supported the idea that AtAraf51A works together with GH10 xylanases. According to the CAZy database, no sequence from plants is classified into GH11, and twelve GH10 sequences are found in *A. thaliana* genome. This is consistent with our experimental evidence.

Xylan is the major component of hemicellulose (Timell 1967). Two types of monoclonal antibodies are

available for detection of xylans (McCartney et al. 2005). LM10 binds only to no- or low-substituted xylans such as birch wood xylan and oat spelts xylan. In contrast, LM11 binds wheat arabinoxylan in addition to the low-substitution xylans. Immunocytochemical analysis on *Silene* stem sections using the monoclonal antibodies specific for xylan indicated that xylans were restricted to secondary cell wall. This was the same in *A. thaliana.* These data imply that xylanases are likely to act in secondary cell walls. One gene of GH10 xylanase (At1g58370) was isolated from *A. thaliana*, and its expression and localization was investigated (Suzuki et al. 2002). The deduced protein named AtXyn1 consists of GH10 catalytic module and three carbohydrate-binding modules. The observation of the fluorescence of transgenic *A. thaliana* expressing enhanced green fluorescent protein-fused AtXyn1 suggested that AtXyn1 is localized in the cell wall. Additionally, the results of β-glucuronidase reporter assay said AtXyn1 gene is expressed in vascular bundles that are tissues rich in secondary cell walls. It suggests that xylanase is likely to be involved in secondary cell wall metabolism as well as AtXyn1.

Microarray analysis demonstrated in Kubo et al. revealed that the expression profiles of two GH10 genes were the same as that of AtAraf51A (Kubo et al. 2005). When these GH10 gene expressions were investigated by the promoter :: *YFP* reporter gene expression, the same signal intensity as AtAraf51A was observed. The expression pattern of At4g08160 was shown in Figure 1. The expression was specifically in the xylem vessel formation in root as same as in case of AtAraf51A. The results backed up the association of co-expression pattern between AtAraf51A and GH10 in *A. thaliana*. The immunolocalization studies performed by Montes et al. revealed that the distributions of anti-arabinan LM6 signal of the T-DNA insertional mutant and overexpressing mutant of AtAraf51A plants were almost the same as that of wild type. However, the intensity of the signal was increased in the T-DNA insertional mutant plants, suggesting that the absence of AtAraf51A causes the accumulation of LM6 epitopes. They proposed that pectic arabinans are potential substrates of AtAraf51A in vivo. However, they also reported that the immunolocalization studies with anti-xylan antibodies such as LM10 and LM11 showed that the intensities of both signals were enhanced in overexpressing plants. According to their findings, arabinofuranosidase-treatment did not alter the signal intensity of LM10 and LM11. Moreover, overexpressing lines of AtAra51A exhibited an increase in xylose content in roots and stems. However, our results suggest that major candidate as the substrate of AtAraf51A is arabinoxylan, and AtAra51A has a role to modify the structure of arabinoxylan together with a family 10 xylanase during the xylem vessel formation. Further study such as functional characterization of At4g08160 would be necessary to understand the detail of the function of these enzymes in the xylem vessel formation.

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