



RESEARCH PAPER

Heterologous expression and characterization of an *Arabidopsis* β -L-arabinopyranosidase and α -D-galactosidases acting on β -L-arabinopyranosyl residues

Chiemi Imaizumi¹, Harumi Tomatsu¹, Kiminari Kitazawa¹, Yoshihisa Yoshimi¹, Seiji Shibano¹, Kaoru Kikuchi¹, Masatoshi Yamaguchi¹, Satoshi Kaneko², Yoichi Tsumuraya¹ and Toshihisa Kotake^{1,*}

¹ Graduate School of Science and Engineering, Saitama University, 255 Shimo-okubo, Sakura-ku, Saitama 338–8570, Japan

² Faculty of Agriculture, University of the Ryukyus, 1 Senbaru, Nishihara, Okinawa 903-0213, Japan

* Correspondence: kotake@mail.saitama-u.ac.jp

Received 9 March 2017; Editorial decision 10 July 2017; Accepted 11 July, 2017

Editor: Björn Usadel, RWTH Aachen University

Abstract

The major plant sugar L-arabinose (L-Ara) has two different ring forms, L-arabinofuranose (L-Araf) and L-arabinopyranose (L-Arap). Although L-Ara mainly appears in the form of α -L-Araf residues in cell wall components, such as pectic α -1,3:1,5-arabinan, arabinoxylan, and arabinogalactan-proteins (AGPs), lesser amounts of it can also be found as β -L-Arap residues of AGPs. Even though AGPs are known to be rapidly metabolized, the enzymes acting on the β -L-Arap residues remain to be identified. In the present study, four enzymes, which we call β -L-ARAPASE (APSE) and α -GALACTOSIDASE 1 (AGAL1), AGAL2, and AGAL3, are identified as those enzymes that are likely to be responsible for the hydrolysis of the β -L-Arap residues in *Arabidopsis thaliana*. An *Arabidopsis apse-1* mutant showed significant reduction in β -L-arabinopyranosidase activity, and an *apse-1 agal3-1* double-mutant exhibited even less activity. The *apse-1* and the double-mutants both had more β -L-Arap residues in the cell walls than wild-type plants. Recombinant APSE expressed in the yeast *Pichia pastoris* specifically hydrolyzed β -L-Arap residues and released L-Ara from gum arabic and larch arabinogalactan. The recombinant AGAL3 also showed weak β -L-arabinopyranosidase activity beside its strong α -galactosidase activity. It appears that the β -L-Arap residues of AGPs are hydrolysed mainly by APSE and partially by AGALs in *Arabidopsis*.

Key words: α -galactosidase, β -L-arabinopyranosidase, arabinogalactan-protein, cell wall, glycoside hydrolase 27 family, recombinant enzyme.

Introduction

The physiologically important plant sugar L-arabinose (L-Ara) exists in both pyranose and furanose ring forms, which are respectively called L-arabinopyranose (L-Arap; sugars other than L-Ara are in pyranose form unless stated otherwise) and L-arabinofuranose (L-Araf). L-Ara mainly exists as α -L-arabinofuranosyl (α -L-Araf) residues of cell wall

Abbreviations: AG, arabinogalactan; AGAL1, α -galactosidase 1 from *Arabidopsis*; AGAL2, α -galactosidase 2 from *Arabidopsis*; AGAL3, α -galactosidase 3 from *Arabidopsis*; AGAL3-OX, AGAL3-overexpressing; AGP, arabinogalactan-protein; L-Ara, L-arabinose; L-Araf, L-arabinofuranose; α -L-Araf, α -L-arabinofuranosyl; β -L-Araf, β -L-arabinofuranosyl; APSE, β -L-ARAPASE from *Arabidopsis*; APSE-OX, APSE-overexpressing; L-Arap, L-arabinopyranose; α -L-Arap, α -L-arabinopyranosyl; β -L-Arap, β -L-arabinopyranosyl; GlcA, glucuronic acid; GUS, β -glucuronidase; HPAEC-PAD, high-performance anion-exchange chromatography with pulsed amperometric detection; 4-Me-GlcA, 4-O-methyl-GlcA; OsAPSE, β -L-arabinopyranosidase from rice; PNP- β -L-Arap, *p*-nitrophenyl- β -L-arabinopyranoside; PNP- α -Gal, *p*-nitrophenyl- α -galactoside; RT, reverse-transcriptase; rAGAL2, recombinant AGAL2; rAGAL3, recombinant AGAL3; rAPSE, recombinant APSE; SaArap27A, β -L-arabinopyranosidase from *Streptomyces avermitilis*.

© The Author 2017. Published by Oxford University Press on behalf of the Society for Experimental Biology.

This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/4.0/>), which permits unrestricted reuse, distribution, and reproduction in any medium, provided the original work is properly cited.

polysaccharides and proteoglycans such as pectic α -1,3:1,5-arabinan (pectic arabinan), arabinoxylan, and arabinogalactan-proteins (AGPs). However, it can also be found in lesser amounts in the form of β -L-arabinopyranosyl (β -L-Arap) residues in the AGPs of plants (Tryfona *et al.*, 2010; Kotake *et al.*, 2016), and as α -L-arabinopyranosyl (α -L-Arap) residues in pectin rhamnogalacturonan II, xylan in the primary cell walls of monocots and some dicots, and β -L-arabinofuranosyl (β -L-Araf) residues in glycan chains of extensin (Lamport *et al.*, 1973; McNeill *et al.*, 1984; Kieliszewski *et al.*, 1995; O'Neill *et al.*, 2001; Mohnen, 2008; Mortimer *et al.*, 2015; Peña *et al.*, 2016).

AGPs are a family of extracellular proteoglycans implicated in growth, cell differentiation, cell-to-cell signaling, cell adhesion, stress responses, pollen tube growth, and fertilization (Fincher *et al.*, 1983; Cheung *et al.*, 1995; Wu *et al.*, 1995; Majewska-Sawka and Nothnagel, 2000; van Hengel *et al.*, 2002; Shi *et al.*, 2003; van Hengel and Roberts, 2003; Motose *et al.*, 2004; Seifert and Roberts, 2007; Mizukami *et al.*, 2016). AGPs consist of hydroxyproline-rich core proteins and large arabinogalactan (AG) chains. The basic structure of the AG moiety, the so-called type II AG, comprises a β -1,3-galactan main chain and β -1,6-galactan side chains. The side chains are substituted with auxiliary sugars such as L-Araf, glucuronic acid (GlcA – the D-prefix is omitted for sugars belonging to the D-series), and 4-O-methyl-GlcA (4-Me-GlcA) (Tsumuraya *et al.*, 1987, 1988; Tan *et al.*, 2010). In several plants, including wheat (*Triticum aestivum*), larch (*Larix dahurica*), and Scots pine (*Pinus sylvestris*), β -L-Arap residues can be found as non-reducing terminal residues joined to α -L-Araf residues through 1,3-linkages (Odonmazig *et al.*, 1994; Ponder and Richards, 1997; Willför *et al.*, 2002; Tryfona *et al.*, 2010), although they are less abundant than the β -Gal, α -L-Araf, GlcA, and 4-Me- β -GlcA residues found in the AGPs of many other plants (Tan *et al.*, 2010; Tryfona *et al.*, 2012; Shimoda *et al.*, 2014). Mung bean hypocotyls exhibit β -1,3-arabinopyranosyltransferase activity, probably catalysing the synthesis of the β -L-Arap residues (Ishii *et al.*, 2005).

The structures of the AG moieties differ depending on tissue, organ, and plant (Tsumuraya *et al.*, 1987, 1988). It is highly probable that the specific structures are determined by glycoside hydrolases (GHs) trimming and degrading the AG moieties, and do not solely depend on the glycosyltransferases that are involved in the synthesis. Indeed, extremely rapid turnover of the AG moieties has been reported in proso millet cells (Gibeaut and Carpita, 1991). As the carbohydrate moieties of AGPs consist of many types of sugar residues, such as β -Gal, α -L-Araf, and 4-Me- β -GlcA, various GHs participate in the hydrolysis (Knoch *et al.*, 2014). Land plants have β -galactosidases that prefer β -1,3- and β -1,6-Gal residues in type II AG to β -1,4-Gal residues in pectic β -1,4-galactan (Kotake *et al.*, 2005; Eda *et al.*, 2014, 2016). On the basis of amino acid sequence and structural similarity, these β -galactosidases are categorized as members of the GH35 family (Henrissat, 1991; Henrissat and Bairoch, 1993).

Because the hydrolysis of β -1,3:1,6-galactan by β -galactosidases never occurs in an endo-manner in plants, it is highly probable that non-reducing terminal residues,

such as α -L-Araf, β -L-Arap, and β -GlcA, must be hydrolysed first. In other words, the hydrolysis of these auxiliary sugar residues partially regulates the degradation and metabolism of the AG moieties. Several GHs hydrolysing the auxiliary sugar residues have been identified. It is known that α -L-arabinofuranosidases from the GH3 family act on non-reducing terminal α -L-Araf residues of AG moieties (Hata *et al.*, 1992; Kotake *et al.*, 2006). The non-reducing terminal β -GlcA and 4-Me- β -GlcA residues are presumably hydrolysed by endogenous β -glucuronidase(s), designated AtGUS1, 2, and/or 3, belonging to the GH79 family, as the overexpression of *AtGUS2* reduces these residues in AGPs in *Arabidopsis thaliana* (Eudes *et al.*, 2008). This interpretation of the function of these AtGUSs is also supported by significant sequence similarity to fungal β -glucuronidases belonging to the GH79 family that are active on β -GlcA and 4-Me- β -GlcA residues of AGP (Konishi *et al.*, 2008; Michikawa *et al.*, 2012). As well as α -L-Araf and β -GlcA residues, β -L-Arap residues are also probably removed prior to the hydrolysis of β -1,3:1,6-galactan in the metabolism of AG moieties. Although an enzyme with activity toward *p*-nitrophenyl- β -L-arabinopyranoside (PNP- β -L-Arap) has previously been detected and purified from pigeon pea (*Cajanus indicus*) (Dey, 1973, 1983), its properties, including its action on natural substrates, remain to be determined.

AG moieties also undergo active hydrolysis by bacteria and fungi in nature. Several β -L-arabinopyranosidases hydrolysing β -L-Arap residues of AGP have been found and characterized in *Streptomyces avermitilis*, *Fusarium oxysporum*, *Geobacillus stearothermophilus*, and *Chitinophaga pinensis* (Ichinose *et al.*, 2009; Sakamoto *et al.*, 2010; Salama *et al.*, 2012; McKee and Brumer, 2015). These microbial β -L-arabinopyranosidases belong to the GH27 family and have amino acid sequences similar to those of α -galactosidases in this family, because the β -L-Arap residue and the α -Gal residue are structurally similar (Ichinose *et al.*, 2009; Kotake *et al.*, 2016) (Fig. 1).

For the present study, four members of the GH27 family, possibly responsible for the hydrolysis of the β -L-Arap residues in *Arabidopsis*, were identified. On the basis of the phenotypes of *Arabidopsis* mutants with defects in these enzymes and the properties of recombinant enzymes expressed in the yeast *Pichia pastoris*, one of them, designated β -L-ARAPASE (APSE), appeared to be a β -L-arabinopyranosidase specifically acting on β -L-Arap residues of AGP. In addition, one α -galactosidase, designated α -GALACTOSIDASE 3 (AGAL3), was found to have weak β -L-arabinopyranosidase activity and may participate in the hydrolysis of the β -L-Arap residues in *Arabidopsis*.

Materials and methods

Plant materials

Arabidopsis thaliana ecotype Columbia (Col) was used in this study. The T-DNA insertion lines, SALK_016141 (*apse-1*), SALK_083934 (*agal-1*), SALK_134497 (*agal2-1*), and SALK_012366C (*agal3-1*), were provided by the *Arabidopsis* Biological Resource Center (The Ohio State University, USA; Supplementary Fig. S1 at JXB online). The *apse-1 agal3-1* double-mutant was generated by a cross between

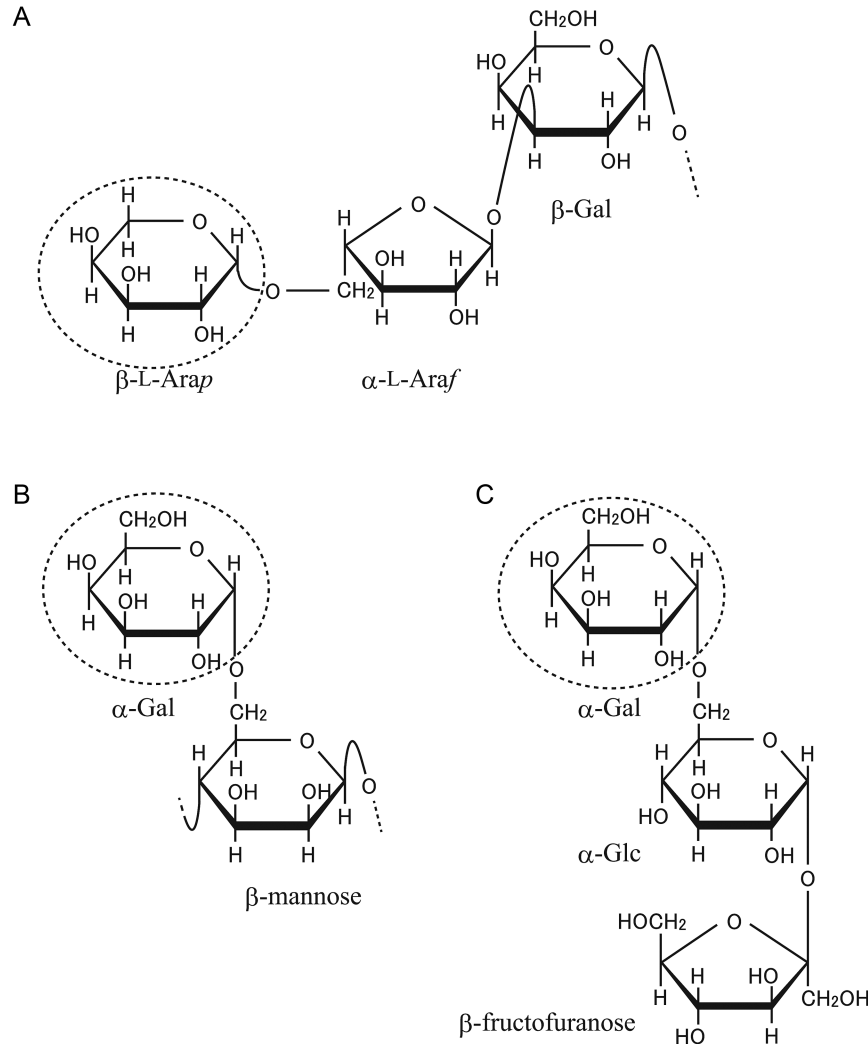


Fig. 1. Partial structures of β-L-Arap- and α-Gal-containing molecules. (A) Type II AG, (B) galactomannan, and (C) raffinose. The β-L-Arap and α-Gal residues are ringed by dashed lines. Sugars are drawn using a Haworth projection.

homozygous *apse-1* and homozygous *agal3-1*. The genotypes of the mutants were determined using specific primers (Supplementary Table S1). The Arabidopsis seedlings were germinated and grown on Murashige–Skooog (MS) medium in 0.8% (w/v) agar under continuous light at 23 °C for 2 weeks, then on rock wool fibers (Nittobo, Tokyo, Japan) under the same conditions (Murashige and Skoog, 1962).

For overexpression of *APSE* and *AGAL3*, the cDNAs were amplified using the sets of primers shown in Supplementary Table S1 and cloned between the *Bam*HI site and *Sac*I site of pBI121 (Clontech, Palo Alto, CA, USA). The plasmids were introduced into Arabidopsis (ecotype Columbia) by an Agrobacterium- (*Rhizobium radiobacter*, EHA105 strain) mediated method (Clough and Bent, 1998). For the analysis of enzyme activity, T2 populations of the *APSE*-overexpressing (*APSE*-OX) line 2 (#2) and *AGAL3*-overexpressing (*AGAL*-OX) #7 were used. The transgenic plants including homozygous or heterozygous transgenes were selected based on the resistance to kanamycin.

Oligo- and polysaccharides

The following were purchased from Sigma (St. Louis, MO, USA): PNP-α-L-arabinofuranoside (PNP-α-L-Araf), PNP-β-L-Arap, PNP-α-L-fucoside (PNP-α-L-Fuc), PNP-α-galactoside (PNP-α-Gal), PNP-β-galactoside (PNP-β-Gal), PNP-α-glucoside (PNP-α-Glc), PNP-β-glucoside (PNP-β-Glc), PNP-β-glucuronide (PNP-β-GlcA),

guar gum (galactomannan from *Cyamopsis tetragonoloba*), gum arabic from acacia (*Acacia senegal*), locust bean gum (galactomannan from *Ceratonia siliqua*), laminarin (β-1,3:1,6-glucan) from *Laminaria digitate*, and β-1,4-xylan from birchwood. β-1,4-Galactan from lupin (*Lupinus albus*) was from Megazyme (Wicklow, Ireland). Larch wood (*Larix laricina*) AG was from Tokyo Kasei (Tokyo, Japan).

Analytical methods

The concentration of protein was determined by the method of Bradford (1976) with bovine serum albumin as the standard. Reducing sugars were estimated by the method of Dygert *et al.* (1965). Total sugars were determined by the phenol-sulfuric acid method (Dubois *et al.*, 1956). Sugar composition analysis was carried out by high-performance anion-exchange chromatography (HPAEC) with pulsed amperometric detection (PAD) using a Dionex DX-500 liquid chromatograph fitted with a CarboPac PA-1 column and a pulsed amperometric detector, as described previously (Ishikawa *et al.*, 2000).

Phylogenetic analysis

The amino acid sequences of GH27 enzymes were collected at NCBI BLAST (blast.ncbi.nlm.nih.gov/Blast.cgi). The alignments of

amino acid sequences were performed using the MUSCLE program in the MEGA software package (version 6; Tamura *et al.*, 2013). Phylogenetic trees were generated by the neighbor-joining method with 1000 bootstrap replications. The accession numbers and abbreviations used are listed in Supplementary Table S2.

Preparation of recombinant enzymes

Total RNA was extracted from 2-week-old *Arabidopsis* seedlings with an Isogen kit (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. Single-strand cDNA was synthesized from 1 μ g of total RNA from the seedlings using a reverse-transcriptase, ReverTra Ace- α - (Toyobo, Osaka, Japan) and oligo(dT)₁₂₋₁₈ primer (Invitrogen, Carlsbad, CA, USA). The cDNA fragments corresponding to Arg33-Ala647 of APSE (At3g26380.1), Phe26-Ala396 of α -GALACTOSIDASE 2 (AGAL2, At5g08370.1), and Gly31-Val437 of AGAL3 (At3g56310.1) were amplified using sets of specific primers listed in Supplementary Table S1. The cDNA fragments were subcloned between the *Eco*RI and *Spe*I sites that are preceded by yeast α -factor of pPICZ α C (Invitrogen). The methylotrophic yeast *P. pastoris* strain KM71 was transformed with the linearized plasmid construct with a multicopy *Pichia* expression kit (Invitrogen). The transformants resistant to zeocin were screened according to the manufacturer's instructions.

The transformants were cultured in medium containing 1% (w/v) yeast extract, 2% (w/v) peptone, and 1% (w/v) glycerol at 28 °C with shaking at 100 rpm for 2 d. The cells were harvested by centrifugation, washed with ice-cold distilled water, then suspended in 50 ml of the medium containing 1% (w/v) yeast extract, 2% (w/v) peptone, and 1% (v/v) methanol. The yeast cells were cultured for another 5 d at 28 °C, during which time 0.5 ml of methanol was added each day, to induce the recombinant enzyme.

The culture medium of the *Pichia* cells was centrifuged, and the supernatant was collected. The sample was desalted with a PD-10 column (GE Healthcare, Tokyo, Japan) for recombinant APSE (rAPSE) or dialysed against 20 mM sodium acetate buffer (pH 5.0) for rAGAL2 and rAGAL3, and passed through a TOYOPEARL HW65 (Tosoh, Tokyo, Japan) column to remove cell debris. rAPSE was purified by cation-exchange chromatography using a CM-Sepharose Fast Flow (GE Healthcare) column. rAGAL2 was purified by cation-exchange chromatography using CM-Sepharose Fast Flow and then by gel-permeation chromatography using a Sephacryl S-200 (GE Healthcare) column. rAGAL3 was purified by anion-exchange chromatography using a DEAE-Sepharose Fast Flow (GE Healthcare) column and then by gel-permeation chromatography using Sephacryl S-200. The purity of the recombinant enzymes was determined on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970).

Enzyme assays

The β -L-arabinopyranosidase activity of recombinant proteins was determined using a reaction mixture (100 μ l) consisting of the enzyme, 1 mM PNP- β -L-Arap, and 50 mM sodium acetate buffer (pH 4.0 for rAPSE, pH 5.5 for rAGALs). After incubation at 37 °C for the appropriate reaction time, the reaction was terminated by addition of 200 mM Na₂CO₃ (900 μ l) and monitored at 420 nm. One unit of enzyme activity liberates 1 μ mole of *p*-nitrophenol per minute.

For the determination of the substrate specificity toward poly- and oligosaccharides, enzyme activity was measured using reaction mixtures (100 μ l) consisting of the enzyme, 0.25% (w/v) poly- or oligosaccharide, and 50 mM sodium acetate buffer (pH 4.0 for rAPSE, pH 5.5 for rAGALs). After incubation at 37 °C, the liberated sugars were detected and quantified by HPAEC-PAD.

Measurement of enzyme activity in plants

Arabidopsis plants grown for 35 d on rock wool fibers were homogenized with mortar and pestle in 20 mM sodium acetate buffer (pH

5.0). After centrifugation at 10 000 g for 5 min, the supernatant was collected (the soluble fraction). The precipitate (the cell wall fraction) was washed once with the same buffer and then suspended in the buffer with addition of 1 M NaCl to release cell wall-bound protein. After centrifugation at 10 000 g for 5 min, the supernatant was collected (the cell wall-bound fraction). The β -L-arabinopyranosidase activity was measured using a reaction mixture (100 μ l) consisting of the soluble or cell wall-bound fraction, 1 mM PNP- β -L-Arap, and 50 mM sodium acetate buffer (pH 5.0) at 37 °C.

Quantification of L-Arap residues in cell walls

The cell wall fraction prepared as described above was used. The cell wall was treated with microbial β -L-arabinopyranosidase (SaArap27A; obtained in our previous study; Ichinose *et al.*, 2009) at 37 °C for 24 h to release L-Arap residues remaining in the cell walls. The released L-Ara was measured by HPAEC-PAD.

Results

GH27 enzyme genes in *Arabidopsis*

Although plant β -L-arabinopyranosidase remains to be identified, several microbial β -L-arabinopyranosidases belonging to the GH27 family have been reported (Ichinose *et al.*, 2009; Sakamoto *et al.*, 2010; Salama *et al.*, 2012; McKee and Brumer, 2015). These β -L-arabinopyranosidases share amino acid sequences similar to those of α -galactosidases in the GH27 family, as the structure of β -L-Arap is similar to that of α -Gal (Fig. 1). The genome of *Arabidopsis* includes four genes for GH27 family enzymes. Among them, two proteins (At5g08380.1 and At5g08370.1) have been characterized as α -GALACTOSIDASE 1 (AGAL1) and AGAL2 with activity toward α -Gal residues of raffinose oligosaccharides (Tapernoux-Lüthi *et al.*, 2004). In the present study, together with AGAL1 and AGAL2, two other proteins (At3g26380.1 and At3g56310.1), APSE and AGAL3, respectively, were identified as candidate enzymes responsible for the hydrolysis of β -L-Arap residues in *Arabidopsis*. In contrast to α -galactosidases belonging to GH family 36, such as AtSIP2 in *Arabidopsis* (Peters *et al.*, 2010), AGALs and APSE have a signal peptide at the N-terminus, suggesting that these enzymes are secreted.

In our previous studies, an amino acid residue in GH27 family enzymes was shown to be involved in the recognition of β -L-Arap and α -Gal residues (Fujimoto *et al.*, 2003; Ichinose *et al.*, 2009). A rice α -galactosidase, Os α Gal II, with Asp in the position prefers α -Gal residues as its substrate. This Asp residue is highly conserved in many GH27 α -galactosidases, whereas it is replaced with bulkier amino acids such as Glu and Ile in microbial β -L-arabinopyranosidases (Fig. 2) (Ichinose *et al.*, 2009; Salama *et al.*, 2012; McKee and Brumer, 2015). Among the four GH27 enzymes in *Arabidopsis*, only APSE has a bulky residue, Tyr, at the corresponding position. Therefore, APSE was presumed to be a β -L-arabinopyranosidase, whereas AGAL3, with the Asp residue in this position, was viewed, like AGAL1 and AGAL2, as an α -galactosidase. In the phylogenetic tree, APSE was located near a microbial β -L-arabinopyranosidase (GsAbp from *Geobacillus stearothermophilus*) together with close

APSE	GYQYVVVDYLWYR	88
OsAPSE	GYEFAVIDYLWYR	88
CpArap27	GWQYIIVDIRWYV	77
GsAbp	GWEYVVVDIQWYE	71
FoAp1	GKFFQIDCGWAS	73
FoAp2	GYSIVTVDCGWPS	70
SaArap27A	GYTYINIDEGWWQ	103
↓		
AGAL1	GYNYVNI DDCWAE	101
AGAL2	GYKYINIDDCWGE	87
AGAL3	GYIHVNI DDCWSN	120
OsαGal I	GYQYVNI DDCWAE	111
AfAg1B	GYEYINIDDCWSV	80
CcGal27A	GYVYLNLD DNNWMA	83
CjAga27A	GYEYVNL D DNNWMA	89
SeAgaB2	G QYVVVD DDCWFD	94

Fig. 2. Amino acid residues important for substrate specificity. Partial amino acid sequences of GH27 enzymes were aligned by the pairwise method using the ClustalW program. The amino acid residue corresponding to Glu99 in SaArap27A involved in the recognition of the α-Gal or β-L-Arap residue is indicated by an arrow. As in microbial β-L-arabinopyranosidases, there is a bulky amino acid residue, Tyr, at this point in APSE and OsAPSE, whereas AGALs tend to have Asp, which is conserved for many α-galactosidases in the GH27 family. Residues conserved in all proteins are highlighted in black.

homologues in plants (Fig. 3, Supplementary Table S2). On the other hand, AGAL1, AGAL2, and AGAL3 were grouped into the plant α-galactosidase subfamily with known plant α-galactosidases including OsαGal II (Fujimoto *et al.*, 2003; Li *et al.*, 2007).

Decreased β-L-arabinopyranosidase activity in the *apse-1* mutant

To examine the participation of APSE and AGALs in the hydrolysis of β-L-Arap residues *in vivo*, β-L-arabinopyranosidase activity in the seedlings of *apse-1*, *agal1-1*, *agal2-1*, and *agal3-1* mutants was measured. It is probable that the AGALs also have β-L-arabinopyranosidase activity as well as α-galactosidase activity, because the structures of β-L-Arap and α-Gal are similar (Fig. 1). In the present study, β-L-arabinopyranosidase activity in the soluble fraction obtained as the supernatant of the homogenate and the cell wall-bound fraction extracted from the residual precipitate with 1 M NaCl were measured using PNP-β-L-Arap as the substrate. As shown in Fig. 4, the *apse-1* mutant showed a significant reduction in the activity mainly in the cell wall-bound fraction compared with wild-type (WT) plants. The total activity in *apse-1* was approximately 50% of the WT. The *agal3-1* mutants showed approximately 30% reduction in total

activity. In addition, the *apse-1 agal3-1* double-mutant exhibited further reduction in activity compared with the *apse-1* mutant. These results indicate that β-L-arabinopyranosidase activity in the seedlings can be mainly attributed to APSE and AGAL3. The reduction of the activity in the *agal2-1* mutant was not obvious compared with *agal3-1*. According to expression data obtained from Genevestigator (Hruz *et al.*, 2008), *AGAL3* has a higher expression level than *AGAL2*. Hence, the *agal3-1* mutation probably affected the total β-L-arabinopyranosidase activity more than the *agal2-1* mutation (Supplementary Fig. S4).

Effect of overexpression of APSE and AGAL3 on β-L-arabinopyranosidase activity

The β-L-arabinopyranosidase activity of APSE and AGAL3 was confirmed by overexpression of *APSE* and *AGAL3* genes. As shown in Fig. 4 and Supplementary Fig. S2, the *APSE*-OX plants exhibited higher β-L-arabinopyranosidase activity than WT plants in terms of the total activity. Because the activity was quite high in the cell wall-bound fraction compared with that in the soluble fraction, we suggest that APSE probably binds to cell wall components. The overexpression of *AGAL3* also significantly raised β-L-arabinopyranosidase activity, although higher activity was mainly observed in the soluble fraction, suggesting that *AGAL3* is secreted but not tightly bound to cell wall components. These results strongly support the hypothesis that APSE and *AGAL3* function as enzymes with β-L-arabinopyranosidase activity in Arabidopsis.

Increased β-L-Arap residues in cell walls in the *apse-1* mutant

If β-L-Arap residues are mainly hydrolysed by APSE, more β-L-Arap residues should remain in the *apse-1* mutant. The amount of β-L-Arap residues in the AGP-rich fraction prepared from cell walls was therefore measured. In this experiment, remaining β-L-Arap residues were released using SaArap27A, which effectively acts on β-L-Arap residues, and were quantified by HPEAC-PAD (Ichinose *et al.*, 2009). The amount of remaining β-L-Arap residues was significantly higher in *apse-1* and the double-mutants compared with the WT (Fig. 5), indicating that the β-L-Arap residues are indeed mainly hydrolysed by APSE.

Properties of rAPSE, rAGAL2, and rAGAL3

In order to understand the properties of APSE and AGALs, rAPSE, rAGAL2, and rAGAL3 were prepared. Based on the SignalP 3.0 program for the prediction of signal sequences, APSE, AGAL2, and AGAL3 have a signal sequence (Bendtsen *et al.*, 2004). Hence, each cDNA – but excluding the sequence corresponding to the signal peptides – was isolated by RT-PCR, subcloned to an expression vector, and introduced into the methylotrophic yeast *P. pastoris*. rAPSE, rAGAL2, and rAGAL3 were purified by conventional chromatography (Supplementary Tables S3–S5). As the yield of rAPSE was quite low compared to rAGAL2 and rAGAL3,

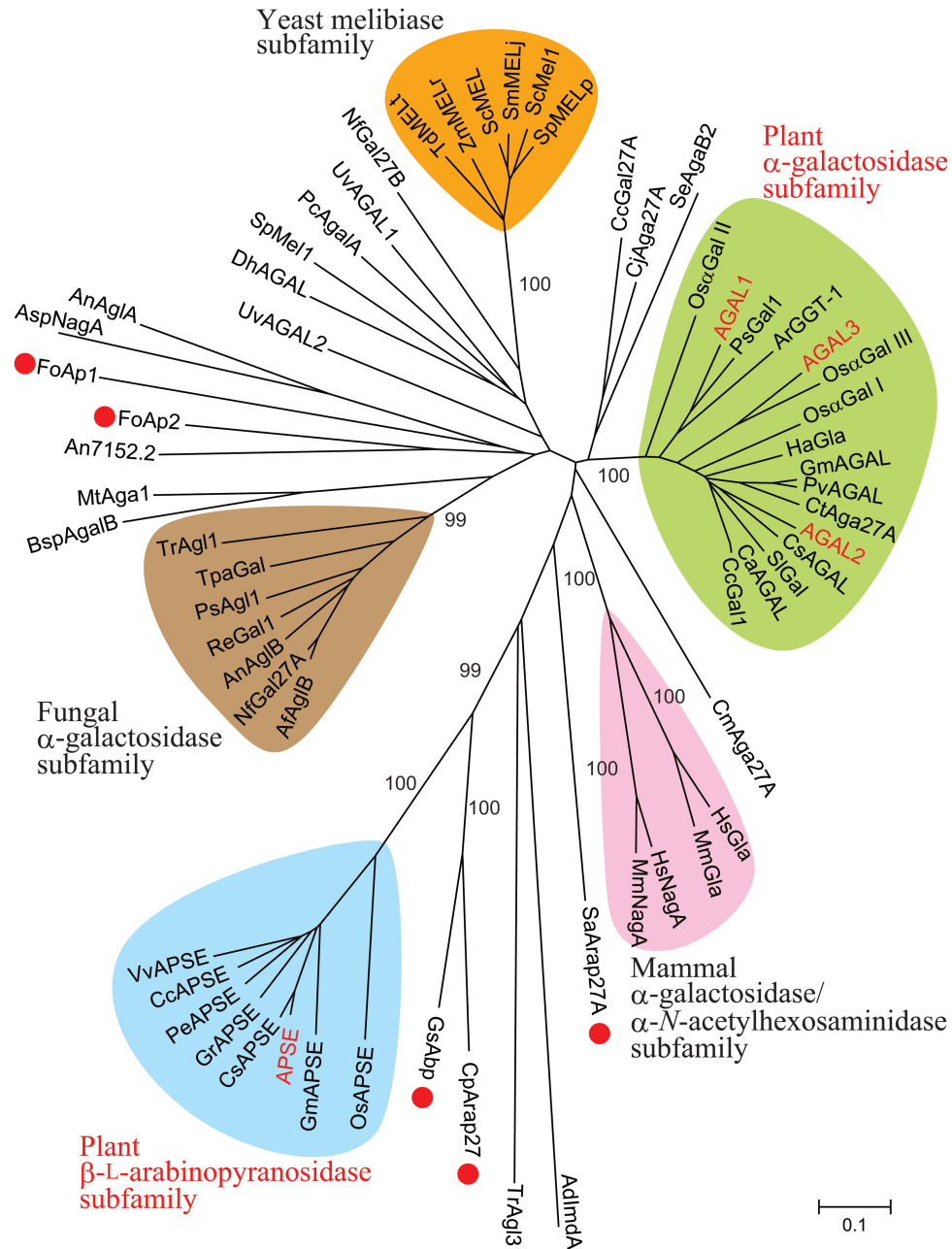


Fig. 3. The phylogenetic relationships of β -L-arabinopyranosidases and α -galactosidases belonging to the GH72 family. β -L-Arabinopyranosidases are marked with red circles. The analysis was performed using MEGA software (version 6.0, Tamura *et al.*, 2013). The scale bar indicates substitutions per site. Numbers at nodes represent bootstrap values. The accession numbers for amino acid sequences are listed in Supplementary Table S1.

we suspect that rAPSE may be unstable in *P. pastoris* cells. The purity of the recombinant enzymes was confirmed on SDS-PAGE (Fig. 6). The apparent sizes observed for rAPSE (72 kDa), rAGAL2 (46 kDa), and rAGAL3 (57 and 53 kDa) on SDS-PAGE were slightly larger than those calculated based on the deduced amino acid sequences (APSE, 68 863 Da; AGAL2, 41 282 Da; AGAL3, 45 096 Da), which may be explained by *N*-glycosylation(s). Indeed, NetNGlyc 1.0 (www.cbs.dtu.dk/services/NetNGlyc/, Joshi and Gupta, 2015), predicts that APSE, AGAL2, and AGAL3 have two possible *N*-glycosylation sites each. The two protein bands of rAGAL3 presumably derive from variations in the *N*-glycosylation in *P. pastoris* cells.

The basic properties of rAPSE were examined using PNP- β -L-Arap as the substrate, and those of rAGAL2 and rAGAL3 using PNP- α -Gal. rAPSE showed maximum activity at pH 4, whereas rAGAL2 and rAGAL3 were most active at pH 5.5 (Supplementary Fig. S3).

Substrate specificity of the recombinant enzymes

The substrate specificity of rAPSE, rAGAL2, and rAGAL3 toward sugar residues was examined using various PNP-substrates. rAPSE specifically acted on PNP- β -L-Arap (Table 1). Importantly, the strong α -galactosidase activity of rAGAL2 and rAGAL3 was accompanied by weak

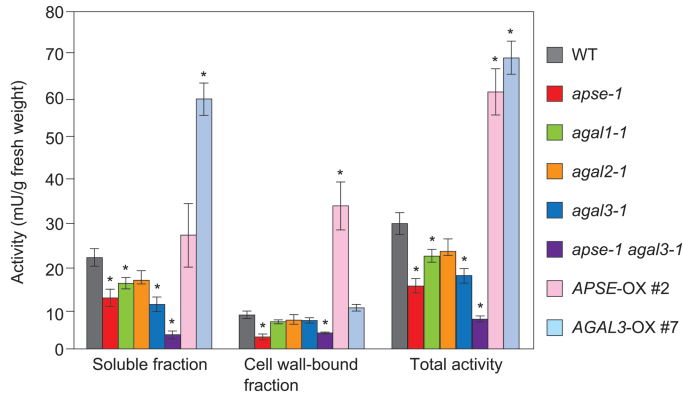


Fig. 4. β-L-Arabinopyranosidase activity in the mutants and in APSE- and AGAL3-OX plants. The soluble and cell wall-bound fractions were prepared from Arabidopsis seedlings grown for 2 weeks on MS-agar medium. Data are mean values with ±SE (*n*=3 biological replicates). Asterisks indicate significant differences from the wild-type (WT) plants (Student's *t*-test, *P*<0.05). The activities of APSE-OX #2 and AGAL3-OX #7 are shown here; results for the other APSE- and AGAL3-OX lines are shown in Supplementary Fig. S2.

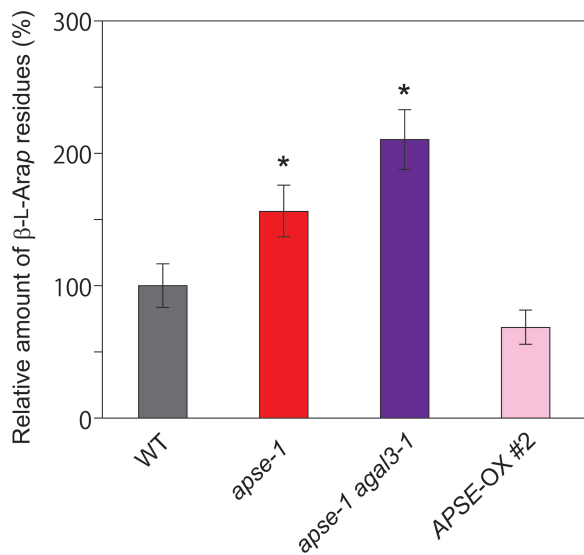


Fig. 5. Amount of β-L-Arap residues remaining in the cell walls. β-L-Arap released from the cell wall fraction by SaArap27A, which is known to act effectively on residues in AGPs, was measured by HPAEC-PAD. Data are mean values ±SE (*n*=3 biological replicates). Asterisks indicate a significant difference from the wild-type (WT) plants (Student's *t*-test, *P*<0.05).

β-L-arabinopyranosidase activity. The results strongly suggest that not only APSE but also AGAL2 and AGAL3 participate in the hydrolysis of β-L-Arap residues in Arabidopsis.

Action on natural substrates

Larch wood AG and gum arabic have large (3.9%) and small amounts (1.9%) of β-L-Arap residues, respectively (Akiyama *et al.*, 1984; Ponder and Richards, 1997; Willför *et al.*, 2002). However, the β-L-Arap residue is absent or very rare in AGPs from radish roots and leaves (Tsumuraya *et al.*, 1987, 1988). The activity of rAPSE and rAGALs toward these AG and AGPs was also examined (Table 2). rAPSE

substantially acted on larch wood AG, releasing free L-Ara. rAGAL2 and rAGAL3 also released L-Ara, but the amount was low compared with rAPSE. As rAGAL2 has weak α-L-arabinofuranosidase activity as well as α-galactosidase and β-L-arabinopyranosidase activities (Table 1), it is possible that rAGAL2 acted on α-L-Araf residues, rather than β-L-Arap residues. Because released L-Araf may spontaneously convert to more stable L-Arap, we were not able to determine which sugar was hydrolysed. rAGAL3 substantially released Gal from guar gum and locust bean gum, in which Gal residues occupy approximately 30% and 20% of the weight, respectively, suggesting that the main substrate for AGAL3 is galactomannan or galactoglucomannan (Daas *et al.*, 2000).

Growth of hypocotyls of mutants

The growth of hypocotyls of *apse* and *agal* mutants were examined. The *apse-1* and the double-mutants showed significantly shorter hypocotyls than the WT plants (Fig. 7). Because the hypocotyls of mutants did not reach the length of those of WT plants even after extended incubation (data not shown), we conclude that the shorter hypocotyl is caused by reduced growth, rather than a delay of germination. The mutants did not show any other phenotype significantly different from the WT plants.

Discussion

Plant GH27 enzymes acting on β-L-Arap residues

AG moieties undergo rapid hydrolysis *in vivo* (Gibeaut and Carpita, 1991) and to date, β-galactosidase, α-L-arabinofuranosidase, and β-glucuronidase have been identified as enzymes involved (Kotake *et al.*, 2005, 2006; Eudes *et al.*, 2008; Eda *et al.*, 2014). The present study has identified APSE as a plant β-L-arabinopyranosidase participating in the hydrolysis of AG moieties of AGPs. The wide distribution of genes with high similarity to APSE in land plants suggests that β-L-arabinopyranosidases are conserved and physiologically important. It is worth mentioning that pigeon pea (*Cajanus cajan*) has a gene encoding a GH27 protein (accession number XP_020221274.1) highly related to APSE in its genome (Dey, 1973, 1983). On the basis of reduced β-L-arabinopyranosidase activity in *agal* mutants and weak but significant β-L-arabinopyranosidase activity (as well as strong α-galactosidase activity) of rAGAL2 and rAGAL3, AGALs can also be assumed to hydrolyze β-L-Arap residues in AGPs. Like other non-reducing terminal residues including α-L-Araf and β-GlcA residues, β-L-Arap residues probably affect the hydrolysis of the β-1,3:1,6-galactan, because plant β-galactosidases act on the galactan in an exo-manner (Kotake *et al.*, 2005; Eda *et al.*, 2014). Therefore, it seems likely that APSE and AGALs affect the metabolism of AGP both directly and indirectly. It is conceivable that APSE and AGALs act on different β-L-Arap residues in AGPs: we observed that the recombinant enzymes have different limits of hydrolysis for both gum arabic and larch AG (Table 2).

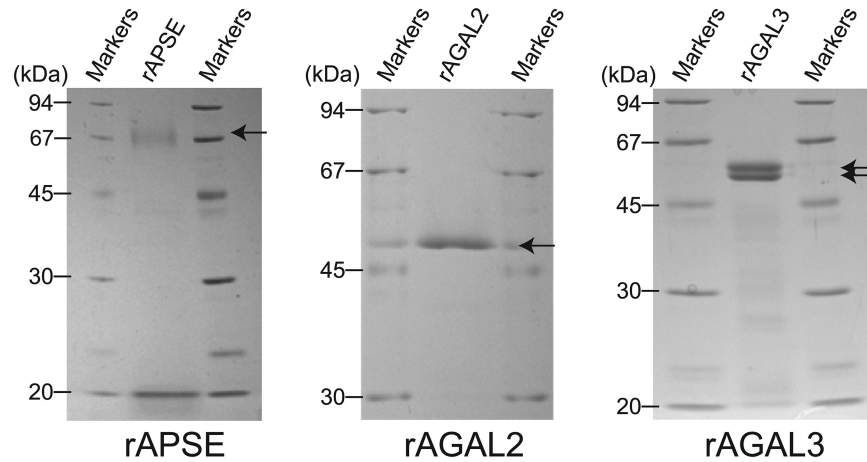


Fig. 6. SDS-PAGE of recombinant enzymes. AGAL2, rAGAL3, and rAPSE were prepared by heterologous expression in *P. pastoris* and purified by conventional chromatography. Proteins in the gel were stained with Coomassie Brilliant Blue R-250. The arrows indicate the purified recombinant enzymes.

Table 1. Substrate specificity of recombinant enzymes toward PNP-sugars

PNP-Sugar ^a	rAPSE ^b	rAGAL2 ^c	rAGAL3 ^c
PNP- α -L-Araf	<0.1	0.2	<0.1
PNP- β -L-Arap	100.0	10.6	7.0
PNP- α -L-Fuc	<0.1	<0.1	<0.1
PNP- α -Gal	<0.1	100.0	100.0
PNP- β -Gal	<0.1	<0.1	<0.1
PNP- α -Glc	<0.1	<0.1	<0.1
PNP- β -Glc	<0.1	<0.1	<0.1
PNP- β -GlcA	<0.1	<0.1	<0.1

^aThe enzymes were incubated with 1 mM PNP-sugars.

^bActivity is expressed as % of that toward PNP- β -L-Arap.

^cActivity is expressed as % of that toward PNP- α -Gal.

Substrate specificity of GH27 β -L-arabinopyranosidase and α -galactosidase

In plant GH27 α -galactosidases, a conserved Asp residue in the catalytic pocket provides the space for the C-6 hydroxylmethyl group of α -Gal. This Asp residue is replaced with bulkier residues such as Glu and Ile at the corresponding site in GH27 β -L-arabinopyranosidases (Fujimoto *et al.*, 2003; Li *et al.*, 2007; Ichinose *et al.*, 2009; Salama *et al.*, 2012; McKee and Brumer, 2015). The bulkier residues can be presumed to change the interaction of the enzyme with α -Gal residues, which affects the substrate specificity toward β -L-Arap and α -Gal residues. The results in the present study substantially support the importance of the residue for the recognition of β -L-Arap and α -Gal residues. In fact, while rAGAL2 and rAGAL3 with the conserved Asp residue preferred α -Gal to β -L-Arap residues, rAPSE with Tyr at the corresponding position specifically acted on β -L-Arap residues. However, in many GH27 β -L-arabinopyranosidases and α -galactosidases – including SaArap27A, FoAp1, and FoAp2 from *Fusarium oxysporum* and AGALs – the substrate specificity is not completely determined by this residue (Ichinose *et al.*, 2009;

Sakamoto *et al.*, 2010). These facts suggest that there are other amino acid residues affecting the substrate specificity toward β -L-Arap and α -Gal residues.

Substrates for APSE and AGALs

It has been reported that several AGPs and type II AGs have β -L-Arap residues in their carbohydrate moieties (Odonmazig *et al.*, 1994; Ponder and Richards, 1997; Willför *et al.*, 2002; Tryfona *et al.*, 2010). However, there may be β -L-Arap-containing molecules serving as substrates for APSE and AGALs other than these AGPs and type II AGs. In fact, β -L-Arap residues are sometimes seen in other cell wall components. For instance, β -L-Arap exists as a non-reducing terminal residue in pectic α -1,3:1,5-arabinan in the common marsh mallow (*Althaea officinalis*) and pigeon pea (*C. cajan*) (Capek *et al.*, 1983; Swamy and Salimath, 1991). β -L-Arap also constitutes the non-reducing terminus of type I AG of soybean (*Glycine max*) (Huisman *et al.*, 2001). Therefore, we cannot exclude the possibility that APSE and AGALs also contribute to the hydrolysis of β -L-Arap residues of pectic α -1,3:1,5-arabinan and type I AG.

The rAGALs showed α -galactosidase activity toward raffinose and stachyose (Supplementary Table S6), but these oligosaccharides are not expected to be their main substrates *in vivo*, because, unlike GH36 enzymes such as AtSIP2 acting on α -Gal residues of raffinose and stachyose, AGALs have a signal peptide that causes them to localize to cell walls, suggesting that AGALs act on cell wall components with α -Gal and β -L-Arap residues. To explore the *in vivo* substrate for AGALs, α -galactosidase activity of rAGALs was examined on seed mucilage and cell wall fractions prepared from different tissues (Supplementary Table S7). As shown in Supplementary Fig. S5, the rAGALs released Gal from seed mucilage, indicating that AGALs act on a polysaccharide included in the mucilage. Importantly, it has been reported that Arabidopsis synthesizes galactoglucomanan as a component of seed mucilage, although this polysaccharide is either not present or is rare in other

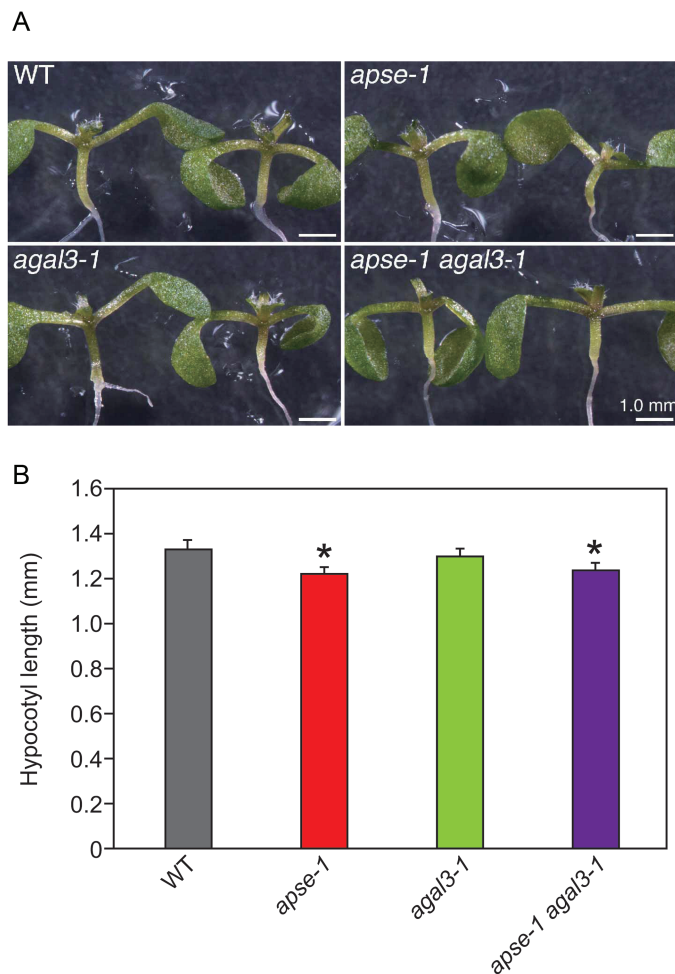


Fig. 7. Growth of hypocotyls. (A) Representative pictures of seedlings of the wild-type (WT), *apse-1*, *agal3-1*, and the double-mutants. Scale bars indicate 1.0 mm. To show the hypocotyls clearly, some main leaves were excised. (B) Hypocotyl lengths of seedlings grown on MS-agar medium for 7 d under continuous light. Data are mean values ±SE (n=10 biological replicates). Asterisks indicate significant differences from WT plants (Student's *t*-test, *P*<0.05).

Table 2. Actions of recombinant enzymes on natural substrates

Substrate ^a	Limit of hydrolysis (%) ^a		
	rAPSE	rAGAL2	rAGAL3
AGP from radish roots ^b	<0.1	<0.1	0.1
AGP from acacia (gum arabic) ^b	6.0	0.1	0.7
AG from larch wood ^b	10.6	0.5	2.9
Galactomannan from locust bean ^c	<0.1	<0.1	<0.1
Galactomannan from guar ^c	<0.1	<0.1	<0.1
β-1,4-Xylan	<0.1	<0.1	<0.1
Pectic galactan ^c	0.2	0.8	0.2
β-1,3:1,4-Glucan	<0.1	<0.1	<0.1

^aThe reaction was carried out using 1 mUnit of recombinant enzyme and 2.5 mg ml⁻¹ polysaccharide substrate at 37 °C for 24 h. Released L-Ara was detected and quantified by HPAEC-PAD.

^bProportion (%) of released L-Ara to total L-Ara residues included in the substrate.

^cProportion (%) of released Gal to total Gal residues included in the substrate.

tissues (Yu *et al.*, 2014; Voiniciuc *et al.*, 2015). Based on the data for gene expression patterns (Winter *et al.*, 2007), *AGAL2* and *AGAL3* are highly expressed in imbibed seeds (Supplementary Fig. S6). These facts suggest that one of the *in vivo* substrates for AGALs is galactoglucomannan included in the mucilage.

Contribution of APSE to the growth of hypocotyls

The *apse-1* mutation significantly inhibited elongation growth of hypocotyls (Fig. 7), an observation reminiscent of a report that the loss of the *AtGUS* gene, which encodes a β-glucuronidase acting on GlcA residues of AGPs, also reduced elongation (Eudes *et al.*, 2008). These observations suggest that the hydrolysis of non-reducing terminal sugar residues of AGPs affects hypocotyl growth by changing AGP metabolism. Rapid hydrolysis of AG moieties might be required for the formation and modification of cell walls, as monomer L-Ara and Gal released from AGPs are recycled to respective UDP-sugars that serve as donor substrates in the synthesis of cell wall polysaccharides (Bar-Peled and O'Neill, 2011). It has also been proposed that AGPs render cell wall components soluble in the Golgi apparatus and secretion vesicles until they arrive at their proper sites at the cell surface (Gibeau and Carpita, 1991). AGPs may have to be degraded immediately after they have performed their function. The physiological importance of the rapid metabolism of AGPs in plants should be further clarified in future studies.

Supplementary Data

Supplementary data are available at *JXB* online.

Table S1. Sequences of primers used in the present study.

Table S2. Accession numbers for amino acid sequences.

Table S3. Purification of rAPSE expressed in *P. pastoris*.

Table S4. Purification of rAGAL2 expressed in *P. pastoris*.

Table S5. Purification of rAGAL3 expressed in *P. pastoris*.

Table S6. Actions of rAGALs on oligosaccharides.

Table S7. Actions of rAGALs on α-Gal residues in cell walls.

Fig. S1. Schematic diagram of T-DNA insertion sites in *apse*, *agal1*, *agal2*, and *agal3* mutants.

Fig. S2. β-L-Arabinopyranosidase activity in *APSE*- and *AGAL3*-OX plant lines.

Fig. S3. The effect of pH on the activity of rAPSE, rAGAL2, and rAGAL3.

Fig. S4. The expression levels of *AGAL1*, *AGAL2*, *AGAL3*, and *APSE*.

Fig. S5. Action of rAGAL2 and rAGAL3 on mucilage.

Fig. S6. The expression patterns of *AGAL2* and *AGAL3*.

Acknowledgements

The authors thank Prof. Paul Dupree for a critical reading of the manuscript. This research was supported in part by a Grant-in-aid for Scientific Research to TK (no. 16K07391) from the Japan Society for the Promotion of Science and to YT and TK (no. 24114006) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

References

- Akiyama Y, Eda S, Kato K.** 1984. Gum arabic is a kind of arabinogalactan-protein. *Agricultural and Biological Chemistry* **48**, 235–237.
- Bar-Peled M, O'Neill MA.** 2011. Plant nucleotide sugar formation, interconversion, and salvage by sugar recycling. *Annual Review of Plant Biology* **62**, 127–155.
- Bendtsen JD, Nielsen H, von Heijne G, Brunak S.** 2004. Improved prediction of signal peptides: SignalP 3.0. *Journal of Molecular Biology* **340**, 783–795.
- Bradford MM.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248–254.
- Capek P, Toman R, Kardosova A, Rosik J.** 1983. Polysaccharides from the roots of the Marsh Mallow (*Althaea officinalis* L): structure of an arabinan. *Carbohydrate Research* **117**, 133–140.
- Cheung AY, Wang H, Wu HM.** 1995. A floral transmitting tissue-specific glycoprotein attracts pollen tubes and stimulates their growth. *Cell* **82**, 383–393.
- Clough SJ, Bent AF.** 1998. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *The Plant Journal* **16**, 735–743.
- Daas PJ, Schols HA, de Jongh HH.** 2000. On the galactosyl distribution of commercial galactomannans. *Carbohydrate Research* **329**, 609–619.
- Dey PM.** 1973. Beta-L-arabinosidase from *Cajanus indicus*: a new enzyme. *Biochimica et Biophysica Acta* **302**, 393–398.
- Dey PM.** 1983. Further characterization of β -L-arabinosidase from *Cajanus indicus*. *Biochimica et Biophysica Acta* **746**, 8–13.
- Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F.** 1956. Colorimetric method for determination of sugars and related substances. *Analytical Chemistry* **28**, 350–356.
- Dygert S, Li LH, Florida D, Thoma JA.** 1965. Determination of reducing sugar with improved precision. *Analytical Biochemistry* **13**, 367–374.
- Eda M, Ishimaru M, Tada T, Sakamoto T, Kotake T, Tsumuraya Y, Mort AJ, Gross KC.** 2014. Enzymatic activity and substrate specificity of the recombinant tomato β -galactosidase 1. *Journal of Plant Physiology* **171**, 1454–1460.
- Eda M, Matsumoto T, Ishimaru M, Tada T.** 2016. Structural and functional analysis of tomato β -galactosidase 4: insight into the substrate specificity of the fruit softening-related enzyme. *The Plant Journal* **86**, 300–307.
- Eudes A, Mouille G, Thévenin J, Goyallon A, Minic Z, Jouanin L.** 2008. Purification, cloning and functional characterization of an endogenous beta-glucuronidase in *Arabidopsis thaliana*. *Plant & Cell Physiology* **49**, 1331–1341.
- Fincher GB, Stone BA, Clarke AE.** 1983. Arabinogalactan-proteins: structure, biosynthesis, and function. *Annual Review of Plant Physiology* **34**, 47–70.
- Fujimoto Z, Kaneko S, Momma M, Kobayashi H, Mizuno H.** 2003. Crystal structure of rice alpha-galactosidase complexed with D-galactose. *The Journal of Biological Chemistry* **278**, 20313–20318.
- Gibeaut DM, Carpita NC.** 1991. Tracing cell wall biogenesis in intact cells and plants: selective turnover and alteration of soluble and cell wall polysaccharides in grasses. *Plant Physiology* **97**, 551–561.
- Hata K, Tanaka M, Tsumuraya Y, Hashimoto Y.** 1992. alpha-L-Arabinofuranosidase from radish (*Raphanus sativus* L.) seeds. *Plant Physiology* **100**, 388–396.
- Henrissat B.** 1991. A classification of glycosyl hydrolases based on amino acid sequence similarities. *The Biochemical Journal* **280**, 309–316.
- Henrissat B, Bairoch A.** 1993. New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. *The Biochemical Journal* **293**, 781–788.
- Hruz T, Laule O, Szabo G, Wessendorp F, Bleuler S, Oertle L, Widmayer P, Gruissem W, Zimmermann P.** 2008. Genevestigator v3: a reference expression database for the meta-analysis of transcriptomes. *Advances in Bioinformatics* **2008**, 420747.
- Huisman MM, Brül LP, Thomas-Oates JE, Haverkamp J, Schols HA, Voragen AG.** 2001. The occurrence of internal (1→5)-linked arabinofuranose and arabinopyranose residues in arabinogalactan side chains from soybean pectic substances. *Carbohydrate Research* **330**, 103–114.
- Ichinose H, Fujimoto Z, Honda M, Harazono K, Nishimoto Y, Uzura A, Kaneko S.** 2009. A beta-L-arabinopyranosidase from *Streptomyces avermitilis* is a novel member of glycoside hydrolase family 27. *The Journal of Biological Chemistry* **284**, 25097–25106.
- Ishii T, Konishi T, Ito Y, Ono H, Ohnishi-Kameyama M, Maeda I.** 2005. A beta-(1→3)-arabinopyranosyltransferase that transfers a single arabinopyranose onto arabino-oligosaccharides in mung bean (*Vigna radiata*) hypocotyls. *Phytochemistry* **66**, 2418–2425.
- Ishikawa M, Kuroyama H, Takeuchi Y, Tsumuraya Y.** 2000. Characterization of pectin methyltransferase from soybean hypocotyls. *Planta* **210**, 782–791.
- Joshi HJ, Gupta R.** 2015. Eukaryotic glycosylation: online methods for site prediction on protein sequences. *Methods in Molecular Biology* **1273**, 127–137.
- Kieliszewski MJ, O'Neill M, Leykam J, Orlando R.** 1995. Tandem mass spectrometry and structural elucidation of glycopeptides from a hydroxyproline-rich plant cell wall glycoprotein indicate that contiguous hydroxyproline residues are the major sites of hydroxyproline O-arabinosylation. *The Journal of Biological Chemistry* **270**, 2541–2549.
- Knoch E, Dilokpimol A, Geshi N.** 2014. Arabinogalactan proteins: focus on carbohydrate active enzymes. *Frontiers in Plant Science* **5**, 198.
- Konishi T, Kotake T, Soraya D, Matsuoka K, Koyama T, Kaneko S, Igarashi K, Samejima M, Tsumuraya Y.** 2008. Properties of family 79 beta-glucuronidases that hydrolyze beta-glucuronosyl and 4-O-methyl-beta-glucuronosyl residues of arabinogalactan-protein. *Carbohydrate Research* **343**, 1191–1201.
- Kotake T, Dina S, Konishi T, Kaneko S, Igarashi K, Samejima M, Watanabe Y, Kimura K, Tsumuraya Y.** 2005. Molecular cloning of a β -galactosidase from radish that specifically hydrolyzes β -(1→3)- and β -(1→6)-galactosyl residues of arabinogalactan protein. *Plant Physiology* **138**, 1563–1576.
- Kotake T, Tsuchiya K, Aohara T, Konishi T, Kaneko S, Igarashi K, Samejima M, Tsumuraya Y.** 2006. An alpha-L-arabinofuranosidase/beta-D-xylosidase from immature seeds of radish (*Raphanus sativus* L.). *Journal of Experimental Botany* **57**, 2353–2362.
- Kotake T, Yamanashi Y, Imaizumi C, Tsumuraya Y.** 2016. Metabolism of L-arabinose in plants. *Journal of Plant Research* **129**, 781–792.
- Laemmli UK.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Lampert DT, Katona L, Roerig S.** 1973. Galactosylserine in extensin. *The Biochemical Journal* **133**, 125–132.
- Li S, Kim WD, Kaneko S, Prema PA, Nakajima M, Kobayashi H.** 2007. Expression of rice (*Oryza sativa* L. var. Nipponbare) alpha-galactosidase genes in *Escherichia coli* and characterization. *Bioscience, Biotechnology, and Biochemistry* **71**, 520–526.
- Majewska-Sawka A, Nothnagel EA.** 2000. The multiple roles of arabinogalactan proteins in plant development. *Plant Physiology* **122**, 3–10.
- McKee LS, Brumer H.** 2015. Growth of *Chitinophaga pinensis* on plant cell wall glycans and characterisation of a glycoside hydrolase family 27 β -L-arabinopyranosidase implicated in arabinogalactan utilisation. *PLoS ONE* **10**, e0139932.
- McNeil M, Darvill AG, Fry SC, Albersheim P.** 1984. Structure and function of the primary cell walls of plants. *Annual Review of Biochemistry* **53**, 625–663.
- Michikawa M, Ichinose H, Momma M, et al.** 2012. Structural and biochemical characterization of glycoside hydrolase family 79 β -glucuronidase from *Acidobacterium capsulatum*. *The Journal of Biological Chemistry* **287**, 14069–14077.
- Mizukami AG, Inatsugi R, Jiao J, et al.** 2016. The AMOR arabinogalactan sugar chain induces pollen-tube competency to respond to ovular guidance. *Current Biology* **26**, 1091–1097.
- Mohnen D.** 2008. Pectin structure and biosynthesis. *Current Opinion in Plant Biology* **11**, 266–277.
- Mortimer JC, Faria-Blanc N, Yu X, Tryfona T, Sorieul M, Ng YZ, Zhang Z, Stott K, Anders N, Dupree P.** 2015. An unusual xylan in

- Arabidopsis primary cell walls is synthesised by GUX3, IRX9L, IRX10L and IRX14. *The Plant Journal* **83**, 413–426.
- Motose H, Sugiyama M, Fukuda H.** 2004. A proteoglycan mediates inductive interaction during plant vascular development. *Nature* **429**, 873–878.
- Murashige T, Skoog F.** 1962. A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiologia Plantarum* **15**, 473–497.
- Odonmazig P, Ebringerová A, Machová E, Alföldi J.** 1994. Structural and molecular properties of the arabinogalactan isolated from Mongolian larchwood (*Larix dahurica* L.). *Carbohydrate Research* **252**, 317–324.
- O'Neill MA, Eberhard S, Albersheim P, Darvill AG.** 2001. Requirement of borate cross-linking of cell wall rhamnogalacturonan II for *Arabidopsis* growth. *Science* **294**, 846–849.
- Peña MJ, Kulkarni AR, Backe J, Boyd M, O'Neill MA, York WS.** 2016. Structural diversity of xylans in the cell walls of monocots. *Planta* **244**, 589–606.
- Peters S, Egert A, Stieger B, Keller F.** 2010. Functional identification of Arabidopsis AT5G57520 (At3g57520) as an alkaline α -galactosidase with a substrate specificity for raffinose and an apparent sink-specific expression pattern. *Plant & Cell Physiology* **51**, 1815–1819.
- Ponder GR, Richards GN.** 1997. Arabinogalactan from Western larch, Part III: alkaline degradation revisited, with novel conclusions on molecular structure. *Carbohydrate Polymers* **34**, 251–261.
- Sakamoto T, Tsujitani Y, Fukamachi K, Taniguchi Y, Ihara H.** 2010. Identification of two GH27 bifunctional proteins with β -L-arabinopyranosidase/ α -D-galactopyranosidase activities from *Fusarium oxysporum*. *Applied Microbiology and Biotechnology* **86**, 1115–1124.
- Salama R, Alalouf O, Tabachnikov O, Zolotnitsky G, Shoham G, Shoham Y.** 2012. The *abp* gene in *Geobacillus stearothermophilus* T-6 encodes a GH27 β -L-arabinopyranosidase. *FEBS Letters* **586**, 2436–2442.
- Seifert GJ, Roberts K.** 2007. The biology of arabinogalactan proteins. *Annual Review of Plant Biology* **58**, 137–161.
- Shi H, Kim Y, Guo Y, Stevenson B, Zhu JK.** 2003. The Arabidopsis SOS5 locus encodes a putative cell surface adhesion protein and is required for normal cell expansion. *The Plant Cell* **15**, 19–32.
- Shimoda R, Okabe K, Kotake T, Matsuoka K, Koyama T, Tryfona T, Liang HC, Dupree P, Tsumuraya Y.** 2014. Enzymatic fragmentation of carbohydrate moieties of radish arabinogalactan-protein and elucidation of the structures. *Bioscience, Biotechnology, and Biochemistry* **78**, 818–831.
- Swamy NR, Salimath PV.** 1991. Arabinans from *Cajanus cajan* cotyledon. *Phytochemistry* **30**, 263–265.
- Tamura K, Stecher G, Peterson D, Filipowski A, Kumar S.** 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology and Evolution* **30**, 2725–2729.
- Tan L, Varnai P, Lampion DT, Yuan C, Xu J, Qiu F, Kieliszewski MJ.** 2010. Plant O-hydroxyproline arabinogalactans are composed of repeating trigalactosyl subunits with short bifurcated side chains. *The Journal of Biological Chemistry* **285**, 24575–24583.
- Tapernoux-Lüthi EM, Böhm A, Keller F.** 2004. Cloning, functional expression, and characterization of the raffinose oligosaccharide chain elongation enzyme, galactan:galactan galactosyltransferase, from common bugle leaves. *Plant Physiology* **134**, 1377–1387.
- Tryfona T, Liang HC, Kotake T, et al.** 2010. Carbohydrate structural analysis of wheat flour arabinogalactan protein. *Carbohydrate Research* **345**, 2648–2656.
- Tryfona T, Liang HC, Kotake T, Tsumuraya Y, Stephens E, Dupree P.** 2012. Structural characterization of Arabidopsis leaf arabinogalactan polysaccharides. *Plant Physiology* **160**, 653–666.
- Tsumuraya Y, Hashimoto Y, Yamamoto S.** 1987. An L-arabino-D-galactan and an L-arabino-D-galactan-containing proteoglycan from radish (*Raphanus sativus*) seeds. *Carbohydrate Research* **161**, 113–126.
- Tsumuraya Y, Ogura K, Hashimoto Y, Mukoyama H, Yamamoto S.** 1988. Arabinogalactan-proteins from primary and mature roots of radish (*Raphanus sativus* L.). *Plant Physiology* **86**, 155–160.
- van Hengel AJ, Roberts K.** 2003. AtAGP30, an arabinogalactan-protein in the cell walls of the primary root, plays a role in root regeneration and seed germination. *The Plant Journal* **36**, 256–270.
- van Hengel AJ, Van Kammen A, De Vries SC.** 2002. A relationship between seed development, arabinogalactan-proteins (AGPs) and the AGP mediated promotion of somatic embryogenesis. *Physiologia Plantarum* **114**, 637–644.
- Voiniciuc C, Schmidt MH, Berger A, Yang B, Ebert B, Scheller HV, North HM, Usadel B, Günl M.** 2015. MUCILAGE-RELATED10 produces galactoglucomannan that maintains pectin and cellulose architecture in Arabidopsis seed mucilage. *Plant Physiology* **169**, 403–420.
- Willför S, Sjöholm R, Laine C, Holmbom B.** 2002. Structural features of water-soluble arabinogalactans from Norway spruce and Scots pine heartwood. *Wood Science and Technology* **36**, 101–110.
- Winter D, Vinegar B, Nahal H, Ammar R, Wilson GV, Provart NJ.** 2007. An “Electronic Fluorescent Pictograph” browser for exploring and analyzing large-scale biological data sets. *PLoS ONE* **2**, e718.
- Wu HM, Wang H, Cheung AY.** 1995. A pollen tube growth stimulatory glycoprotein is deglycosylated by pollen tubes and displays a glycosylation gradient in the flower. *Cell* **82**, 395–403.
- Yu L, Shi D, Li J, Kong Y, Yu Y, Chai G, Hu R, Wang J, Hahn MG, Zhou G.** 2014. CELLULOSE SYNTHASE-LIKE A2, a glucomannan synthase, is involved in maintaining adherent mucilage structure in Arabidopsis seed. *Plant Physiology* **164**, 1842–1856.