Available online at www.sciencedirect.com
ScienceDirect
Phytochemistry 69 (2008) 1661–1670
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

The *Arabidopsis* At1g45130 and At3g52840 genes encode β-galactosidases with activity toward cell wall polysaccharides

Dashzeveg Gantulga [a], Yusuf Turan [a,1], David R. Bevan [b], Asim Esen [a,*]

[a] *Department of Biological Sciences, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061, United States*
[b] *Department of Biochemistry, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061, United States*

Received 1 May 2007; received in revised form 15 January 2008
Available online 21 March 2008

Abstract

The *Arabidopsis* genes At1g45130 and At3g52840 encode the β-galactosidase isozymes Gal-5 and Gal-2 that belong to Glycosyl Hydrolase Family 35 (GH 35). The two enzymes share 60% sequence identity with each other and 38–81% with other plant β-galactosidases that are reported to be involved in cell wall modification. We studied organ-specific expression of the two isozymes. According to our western blot analysis using peptide-specific antibodies, Gal-5 and Gal-2 are most highly expressed in stem and rosette leaves. We show by dot-immunoblotting that Gal-5 and Gal-2 are associated with the cell wall in *Arabidopsis*. We also report expression of the recombinant enzymes in *P. pastoris* and describe their substrate specificities. Both enzymes hydrolyze the synthetic substrate *para*-nitrophenyl-β-D-galactopyranoside and display optimal enzyme activity between pH 4.0 and 4.5, similar to the pH optimum reported for other well-characterized plant β-galactosidases. Both Gal-5 and Gal-2 show a broad specificity for the aglycone moiety and a strict specificity for the glycone moiety in that they prefer galactose and its 6-deoxy analogue, fucose. Both enzymes cleave β-(1,4) and β-(1,3) linkages in galacto-oligosaccharides and hydrolyze the pectic fraction of *Arabidopsis* cell wall. These findings suggest that Gal-5 and Gal-2 could be involved in the modification of cell wall polysaccharides.
© 2008 Elsevier Ltd. All rights reserved.

Keywords: *Arabidopsis*; Cruciferae; Cell wall; Glycosyl hydrolase family 35; β-Galactosidase; β-Galactosides; Galactan

1. Introduction

Carbohydrates play many important biological and physiological roles in living organisms, and they occur in different forms such as glycoproteins, glycolipids, glycosides, and oligo- and polysaccharides. Selective hydrolysis of glycosidic bonds specifies the breakdown of these glycoconjugates and their function in subsequent biochemical processes. Living organisms have evolved a great diversity of glycosyl hydrolases (GH) with different substrate specificity and kinetic parameters, operating under different optimal conditions. (Davies and Henrissat, 1995). Glycosyl hydrolases are found in all animals, plants, and microorganisms, and they are classified into 111 families on the basis of amino acid sequence similarity (CAZY database, http://www.cazy.org; Coutinho and Henrissat, 1999). The GH family 35 includes plant β-galactosidases (EC 3.2.1.23) that are thought to be involved in cell wall biogenesis and modification (Jamet et al., 2006; Vervelen and Vissenberg, 2007). In plants, β-galactosidase isozymes are encoded by a multigene family. The presence of β-galactosidases as a moderately large enzyme family is indicative of the structural complexity and heterogeneity of cell wall polysaccharides. Depending on developmental stages and cell differentiation, the different isozymes may act on either the same or different cell wall polysaccharides (Smith and Gross, 2000; Perez

Abbreviations: *Arabidopsis thaliana*, *Arabidopsis*; Gal-5, (AtBGal5)–β-galactosidase-5; Gal-2, (AtBGal2)–β-galactosidase-2; *p*NP, *para*-nitrophenyl; GH, glycosyl hydrolase.

* Corresponding author. Tel.: +1 540 231 5894; fax: +1 540 231 9307.
E-mail address: aevatan@vt.edu (A. Esen).

[1] Yusuf Turan's contribution to this research is ~ 5%. Present address: Balıkesir University, Faculty of Arts and Science, Department of Biology, 10100, Balıkesir, Turkey.

0031-9422/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2008.01.023

Almeida, 2004; Kotake et al., 2005; Ishimaru et al., 2005; Jamet et al., 2006; Chantarangsee et al., 2007).

A growing body of evidence suggests that GH Family 35 β-galactosidases participate in loosening of the cell wall during growth and degrade cell wall components during ripening of fruits and senescence. Recently, many β-galactosidases have been identified and purified from different plant sources. For example, Smith and Gross (2000) showed that a family of at least seven β-galactosidases is expressed during tomato fruit development. Furthermore, these authors expressed the tomato β-galactosidase isozyme 4 (TBG4) in yeast and studied its natural substrate specificity. TBG4 hydrolyzed chelator-soluble pectin, alkali-soluble pectin, hemicellulose from tomato cell walls, and commercially prepared galactan. Li et al. (2001) purified five isoforms of β-galactosidases from mung bean and showed that they differ with respect to enzymatic characteristics and substrate specificities. Kotake et al. (2005) isolated a β-galactosidase from radish (RsbGal), which specifically hydrolyzed β-(1,6) and β-(1,3) linkages in arabinogalactan protein. β-Galactosidase I purified from ripe carambola (*Averrhoa carambola*) was active in hydrolyzing β-(1,4)-linked spruce and β-(1,6)/β-(1,3)-linked gum arabic galactan, and alkali-soluble hemicelluloses of carambola cell wall (Balasubramaniam et al., 2005). Furthermore, papaya β-galactosidase isoforms differentially hydrolyzed cell wall during fruit ripening (Lazan et al., 2004).

Despite the status of *Arabidopsis thaliana* as a model plant with a completely sequenced genome, not much is known about its β-galactosidases. There are 17 putative GH family 35 β-galactosidases (17 BGALs) in *Arabidopsis* (Ahn et al., 2007). According to a recent microarray study, the β-galactosidase genes are among 765 genes specific to cell wall dynamics (Imoto et al., 2005). Perez Almeida (2004) studied expression of β-galactosidases by promoter-GUS fusion and found that temporal and spatial expression of β-galactosidase genes are differentially regulated. She also reported that the galactosyl content of the cell wall increases and total β-galactosidase activity decreases in *Gal-1* knockout mutant. Iglesias et al. (2006) reported β-galactosidase activity on xyloglucan oligosaccharides in the apoplastic fluid. To corroborate further the role of β-galactosidases in cell wall modification, it is essential to determine their substrate specificity, by isolating and characterizing the proteins. To this end, Ahn et al. (2007) expressed Gal-4 (At5g56870), one of the seventeen β-galactosidases, in *Escherichia coli* and insect cells. It was shown that in addition to synthetic substrates, Gal-4 hydrolyzes β-(1,3) and β-(1,4) linked galacto-oligosaccharides (Ahn et al., 2007).

It is likely that the 17 BGALs differ in temporal and spatial expression and in their ability to hydrolyze various cell wall polysaccharides. For annotating them and understanding their function better, it is desirable to determine the expression profile and substrate specificity of all 17 BGALs. Currently, we are focused on the largest subfamily (Gal-1 through Gal-5 and Gal-12), which consists of six genes, amongst the 17 BGALs. Of these, we studied Gal-5 and Gal-2 for this paper. First, we conducted database analyses using microarray data available for these genes. Second, we studied spatial expression and subcellular localization by immunoblotting using peptide-specific antibodies. Third, we purified the recombinant proteins expressed in *Pichia pastoris* and investigated their substrate specificity using synthetic galactosides and natural polysaccharides derived from *Arabidopsis* cell wall.

2. Results and discussion

2.1. In silico analysis of Gal-5 and Gal-2

BLAST searches of the NCBI database using Gal-5 and Gal-2 sequences as queries show that the sequences of 20 other plant β-galactosidases share 38–81% identity with all *Arabidopsis* β-galactosidases except Gal-17. Gal-5 and Gal-2 proteins share 60% sequence identity with each other. The sequence differences among β-galactosidases suggest differences in their enzymatic properties (e.g., substrate specificity) and biological functions.

In silico characterization of predicted protein sequences of Gal-5 and Gal-2 using SignalP and TargetP shows that these proteins contain a signal peptide that targets them to the endoplasmic reticulum from which they can be secreted to the cell wall via vesicular pathways (Bendtsen et al., 2004; Emanuelsson et al., 2000). Phylogenetic analysis of Gal-5 and Gal-2 proteins shows that they cluster with other plant β-galactosidases that are known to be cell wall modifying enzymes (Fig. 1). In tomato fruit, the galactosyl content of cell wall and fruit firmness depend on two β-galactosidases, TBG6 and TBG4 (Smith et al., 2002), which are closely related to Gal-5 and Gal-2, respectively (Fig. 1).

2.2. Microarray data for expression of At1g45130 (Gal-5) and At3g52840 (Gal-2) transcripts

To study the expression profiles of the *Gal-5* and *Gal-2* genes, we used microarray data from the integrated database Genevestigator (Zimmermann et al., 2004). We analyzed the relative abundance of At1g45130 (*Gal-5*) and At3g52840 (*Gal-2*) transcripts for different tissues, developmental stages, and various stress conditions. Table 1 shows mean gene expression data across many microarray experiments carried out in different laboratories. Relatively small standard errors for the mean expression values indicate the consistency and reliability of the data. The mean expression of *Gal-5* is high in stem, root and silique, while the mean expression of *Gal-2* is high in the petiole of rosette leaves and stem. The transcript levels do not change notably throughout developmental stages from seedling to adult plants. *Gal-5* and *Gal-2* transcripts were absent in the pollen (data not shown). It is possible that other members of BGAL family are expressed in pollen to rescue Gal-5 and Gal-2. Indeed, Hruba et al. (2005) showed that *Gal-7*,

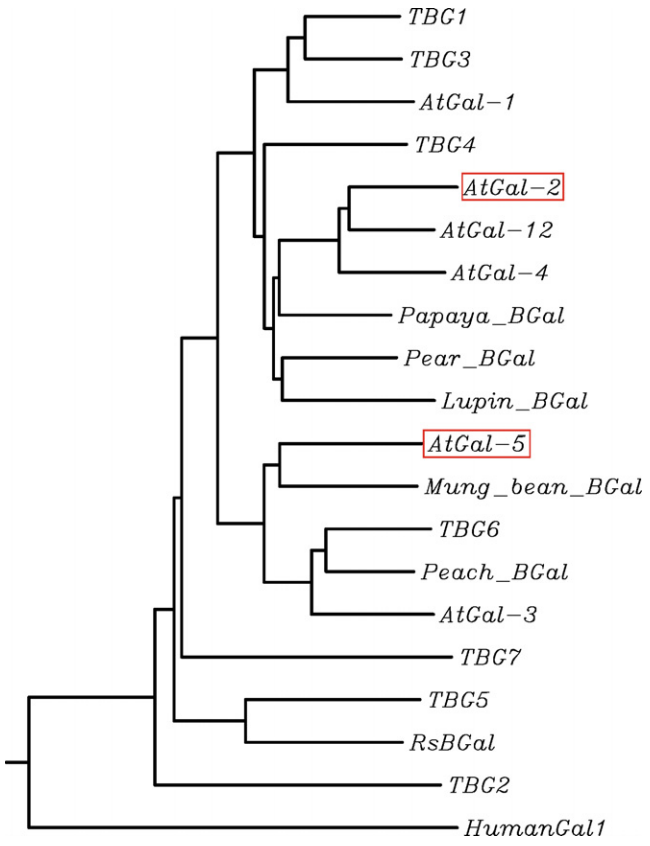

Fig. 1. Phylogenetic tree of selected plant β-galactosidases. The tree was constructed from the alignment of the amino acid sequences using Clustal W software in http://align.genome.jp/. AtGal-1, *A. thaliana* β-galactosidase-1/AtBGAL1 (NP187988); AtGal-2, *A. thaliana* β-galactosidase-2/AtBGAL2 (NP190852); AtGal-3, *A. thaliana* β-galactosidase-3/AtBGAL3 (NP849506); AtGal-4, *A. thaliana* β-galactosidase-4/AtBGAL4 (CAB64740); AtGal-5, *A. thaliana* β-galactosidase-5/AtBGAL5 (NP175127); AtGal-12, *A. thaliana* β-galactosidase-12/AtBGAL12 (NP849553); Pear BGal, *P. pyrifolia* (BAB21492); Papaya BGal, *C. papaya*(AAC77377); TBG1, *L. esculentum* (AAF21626); TBG2, *L. esculentum* (AAF70821); TBG3, *L. esculentum* (AAF70822); TBG4, *L. esculentum* (AAC25984); TBG5, *L. esculentum* (AAF70824); TBG6, *L. esculentum* (AAF70825); TBG7, *L. esculentum* (AAF70823); Mung bean BGal, *V. radiata* (AAF67341); Peach BGal, *P. persica* (AAW47739); Lupin BGal, *L. angustifolius* (CAA09467); RsbGal, *R. sativus* β-galactosidase 1 (BAD20774); Human BGal, *H. sapiens* (P16278).

Gal-11 and *Gal-13* are specifically expressed in the pollen. It is worth mentioning that microarray results are in good agreement with recent RT-PCR results for selected organs (Ahn et al., 2007).

Comprehensive microarray data are available for *Arabidopsis* genes expressed in shoots and roots under various stress conditions (http://www.weigelworld.org/resources/microarray/AtGenExpress). According to the microarray data, expression levels of *Gal-5* and *Gal-2* transcripts are not significantly different under most of the stress conditions, except UV–B light and osmotic (mannitol) stresses. In comparison to the control, maximal change (~7-fold increase) of *Gal-2* expression is in roots (harvested after 3 h) treated with UV–B light. For *Gal-5*, maximal change (~3-fold decrease) is in root (harvested after 24 h) treated by osmotic stress. A RT-PCR study of β-galactosidase gene expression in plant organs under various stress conditions and hormone treatments may further corroborate the role of BGALs in cell wall modification, by demonstrating coordinated expression of β-galactosidase genes and the genes whose products are known to be cell wall-specific (e.g. expansin, polygalacturonase).

Table 1
Expression profile of Gal-5 and Gal-2 using microarray data

Organ/tissue	Number of chips	At1g45130 Gal-5	At3g52840 Gal-2	At4g05320 UBQ10
Root	200	11.67 ± 0.04	8.55 ± 0.06	14.7 ± 0.02
Petiole	12	10.62 ± 0.11	12.82 ± 0.2	14.8 ± 0.07
Rosette	604	8.93 ± 0.05	11.14 ± 0.04	14.7 ± 0.01
Stem	7	12 ± 0.04	11.26 ± 0.22	14.6 ± 0.24
Cauline leaf	3	7.3 ± 0.33	10.69 ± 0.11	14.8 ± 0.04
Flower	58	9.94 ± 0.25	9.12 ± 0.11	14.5 ± 0.07
Silique	11	11.83 ± 0.33	10.12 ± 0.23	14.8 ± 0.04
Seedling	386	10.57 ± 0.05	10.9 ± 0.03	14.8 ± 0.01

Second column shows the number of microarray chips available for a given tissue. The organ/tissue specific expression levels for Gal-5 (At1g45130) and Gal-2 (At3g52840) as $\log_2(n)$ are shown along with the standard errors in columns three and four. Ubiquitin10 (*UBQ10*/Atg05320) used as controls for comparison are shown in column five. The Gene Atlas Tool of Genevestigator (https://www.genevestigator.ethz.ch/at/) was used for analysis.

2.3. Expression of Gal-5 and Gal-2 in Arabidopsis: western blotting studies using peptide-specific antibodies

Before the injection of immunogens, rabbit preimmune sera were tested by ELISA for immunoreactivity with total protein extracts of *Arabidopsis* and synthetic peptides (data not shown). Two rabbits with the lowest preimmune serum reactivity were chosen for the injection of Gal-5 and Gal-2 peptide conjugates, respectively. The peptide-specific antisera were tested for their specificity by dot blotting. Anti-Gal-2 peptide antibody recognized Gal-2 synthetic peptide and the recombinant Gal-2 protein (*E. coli* and *P. pastoris* expressed), and anti-Gal-5 peptide antibody recognized Gal-5 peptide and recombinant Gal-5. Preimmune sera did not show any detectable immunoreactivity with Gal-5 and Gal-2 on the blots.

The expression pattern of the two β-galactosidases in different *Arabidopsis* tissues was studied by western blotting using antibodies raised against Gal-5 and Gal-2 specific peptides. Immunoreactive bands of ~75 kD were detected in 4–5-week old plants (Fig. 2A), indicating Gal-5 and Gal-2 proteins do not undergo proteolytic processing that has been reported to produce 35–50-kD fragments for β-galactosidases in apple (Ross et al., 1994), lupin (Buckeridge and Reid, 1994) and radish (Kotake et al., 2005). Such fragments were not detected on immunoblots even after using high protein loads (Fig. 2B). Under our experimental conditions, levels of Gal-2 protein were similar in all organs (root, leaf, stem, flower, and silique), whereas Gal-5 levels were different. Gal-5 levels were higher in stem and leaves, but lower in roots and silique. We compared our results

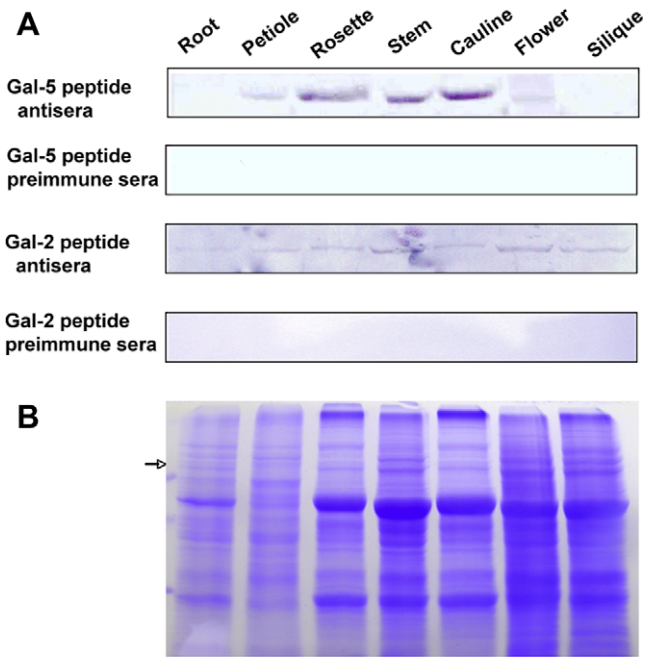

Fig. 2. Organ-specific expression of Gal-5 and Gal-2. (A) Western blot and (B) Coomassie Blue stained SDS–PAGE. Total proteins (30 μg) from *Arabidopsis* (4 weeks old) root, petiole of rosette leaves, rosette leaves, stems, cauline leaves, flowers, and siliques were separated by 10% SDS–PAGE and transferred to nitrocellulose membranes. Identical membranes were incubated with rabbit preimmune sera and immune antisera against Gal-5 and Gal-2 peptides. The arrow marks the position of immunoreactive bands.

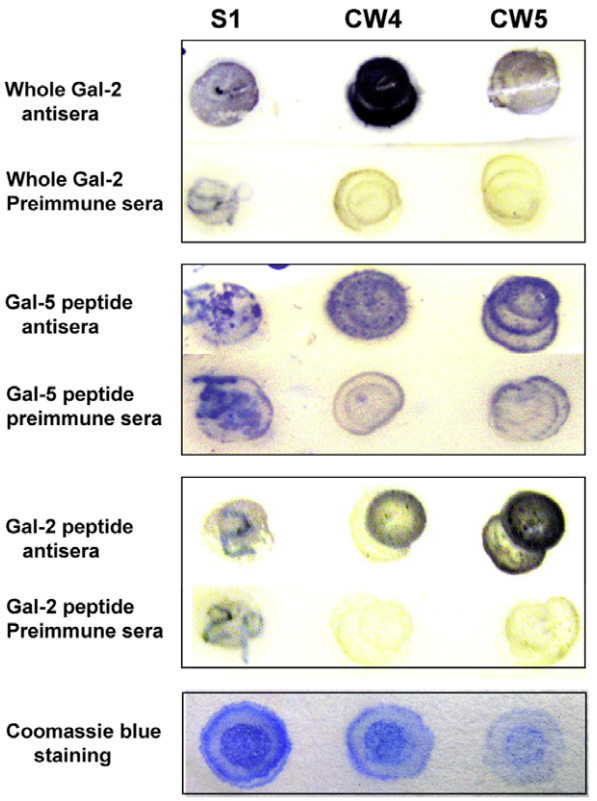

Fig. 3. Localization of Gal-5 and Gal-2 in cell wall fractions by dot blotting. Cell walls were isolated from *Arabidopsis* rosette leaves. Three fractions (S1, soluble; CW4, $CaCl_2$-soluble; CW5, LiCl-soluble) with β-galactosidase activity were spotted on nitrocellulose strips at the same place multiple times. Identical membranes with protein spots were incubated with anti-whole Gal-2, anti-Gal-5 peptide-, and anti-Gal-2 peptide-specific antisera. Total protein spots were stained with Coomassie Blue R-250. These dot-immunoblotting data show the presence of Gal-5 and Gal-2 in the cell wall.

with the previous studies on expression of β-galactosidase genes by Perez Almeida (2004) and Ahn et al. (2007). These authors found Gal-2 transcripts with moderate levels of expression in all organs, which is in agreement with our result for Gal-2. In the case of Gal-5, they found higher level of expression in roots than we did. We propose that this difference in Gal-5 expression is due to the difference in transcriptional and translational stages of regulation, though we do not exclude other factors, such as plant age, growth conditions, and extraction methods used in the experiments. It is worth mentioning that there is agreement between our data and those of Perez Almeida (2004) in that Gal-5 is not detectable in mature roots, although Perez Almeida (2004) found Gal-5 expression in root elongation and root hair zones of juvenile plants.

2.4. Cell wall localization of Gal-5 and Gal-2 proteins: dot blotting

We isolated cell wall from rosette leaves of *Arabidopsis* to confirm the presence of Gal-5 and Gal-2 proteins in the cell wall. Five different fractions (S1, soluble 1; S2, soluble 2; S3, soluble 3; CW4, extractable with $CaCl_2$; and CW5, extractable with LiCl) were obtained. These fractions were assayed for β-galactosidase activity using *p*NPGal as a substrate. S2 and S3 fractions did not have detectable activity. Specific activities of S1, CW4, and CW5 were 0.06, 0.12, and 20.1 nmole *p*NP/min/mg, respectively, indicating that fraction CW5 (LiCl-soluble) had the highest specific β-galactosidase activity. Fraction CW5 contained the lowest amount of protein (Fig. 3, bottom row) among the three fractions. The immunoblotting data showed that antiserum to intact Gal-2 protein had high immunoreactivity (Fig. 3, top blot) with fraction CW4 ($CaCl_2$-soluble) and weak immunoreactivity with fractions S1 and CW5. However, this antiserum is not specific for Gal-2; it recognizes also other cell wall-bound β-galactosidases. In contrast, the reactivity of the Gal-2 peptide-specific antiserum was strongest with fraction CW5 (Fig. 3, third blot), indicating that Gal-2 is enriched in CW5 and it requires LiCl for complete release from the cell wall. In the case of Gal-5, the preimmune serum from the rabbit immunized with Gal-5 peptide had considerable background activity with cell wall components (Fig. 3, second blot). Although the immune serum from the same rabbit reacted more strongly with fractions CW4 and CW5 than the preimmune serum, the difference between the specific and the background reactions was not as striking as for Gal-2. Taken together, our enzyme activity and dot-immunoblotting data indicate both Gal-5 and Gal-2 are present in, and tightly associated with, the cell wall in

Arabidopsis. ELISA data (not shown) also confirmed that Gal-5 and Gal-2 proteins were present in cell wall fractions, supporting our hypothesis that Gal-5 and Gal-2 proteins are bound to the cell wall.

2.5. Expression of Gal-5 and Gal-2 in *P. pastoris* and purification

The recombinant proteins were expressed under the control of the AOX (alcohol oxidase) promoter in *P. pastoris*. Gal-5, 700 amino acids long, (79-kD protein, calculated) and Gal-2, 719 amino acids long, (81-kD protein, calculated), were expressed and secreted into the culture medium. Optimization of induction and time-course studies of expression were done to obtain the best expression level for recombinant Gal-5 and Gal-2. Results from the induction time course (data not shown) showed that β-galactosidase activity was secreted into the culture medium and was detectable after 24 h of induction on 1% methanol, and it peaked after 72–96 h. While β-galactosidase activity of Gal-5 and Gal-2 transformants increased during the course of induction, no detectable activity was observed in the control *P. pastoris* transformed with an empty vector.

Recombinant Gal-5 and Gal-2 were purified from the culture medium by ion exchange chromatography. Cation exchange chromatography using Sulphoxyethyl (SE) cellulose was efficient in purification of these enzymes because *P. pastoris* culture medium contains low levels of secreted endogenous protein, and both enzymes bind SE due to their high positive net charge around pH 6. It allowed 2.7-fold purification of Gal-5 and 2-fold purification of Gal-2 in a single step. Purified Gal-5 and Gal-2 appeared on SDS–PAGE gel as single bands with an estimated monomeric molecular weight of ~75 kD (Fig. 4A and B),

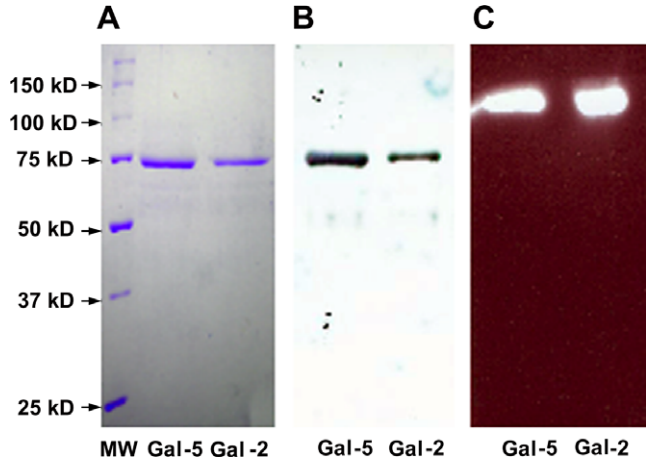

Fig. 4. SDS–PAGE (A), western blot (B), and zymogram (C) of purified Gal-5 and Gal-2. (A) 10% SDS gel was stained with Coomassie Blue R-250. (B) Purified proteins separated on 10% SDS–PAGE were transferred to a nitrocellulose membrane, which was incubated with rabbit anti-whole Gal-2 antiserum and (C) Proteins were subjected to 8% acidic native gel and stained with 4-methylumbelliferyl galactoside. Lane MW, molecular weight standard; Lane 1, Gal-5; Lane 2, Gal-2.

indicating their purification to near homogeneity. This result was supported by acidic native polyacrylamide gel electrophoresis that showed a single activity band on the zymogram for each enzyme (Fig. 4C). It should be noted that the monomeric molecular weight of Gal-5 and Gal-2 obtained by SDS–PAGE is slightly lower than the estimation based on amino acid sequence (79-kD for Gal-5 and 81-kD for Gal-2). Peptide mass fingerprinting (MALDI-TOF) analysis showed that both ends of the Gal-5 polypeptide sequence were present in the peptide mixture, which ruled out proteolytic modification of Gal-5 during purification. Thus, the lower experimentally estimated monomeric molecular weight of Gal-5 and Gal-2 is likely due to their high content of hydrophobic amino acids (Gal-5 40%, Gal-2 42% compared to BSA (bovine serum albumin) 34%). Hydrophobic amino acids bind SDS at a higher ratio compared to hydrophilic ones, thereby causing slightly faster mobility of hydrophobic proteins on SDS–PAGE (Bayreuther et al., 1980).

The fact that Gal-5 and Gal-2 bind to a cation exchanger at pH ~ 6 and migrate through acidic native gels indicates that these proteins have high p*I* values, consistent with the predicted p*I*s of 8.1 and 8.6 for Gal-5 and Gal-2, respectively. It is interesting to note that basic p*I* values were also observed for kiwifruit, tomato, and carambola β-galactosidases (Ross et al., 1994; Carey et al., 1995; Balasubramaniam et al., 2005). These results support the hypothesis that the cell wall-associated proteins interact ionically with the acidic cell wall matrix. Pectic polygalacturonic acid provides negative charges and contributes to the interaction with high p*I* proteins (Jamet et al., 2006).

2.6. Properties of recombinant Gal-5 and Gal-2

Recombinant Gal-5 and Gal-2 displayed optimal activity on *p*NP-β-D-galactopyranoside between pH 4.0 and 4.5. The pH-activity profile had a narrow range in that less than 50% of maximal activity was observed at pH values below 3.5 and above 6.0. These data are consistent with the pH optima of most of the well-characterized plant β-galactosidases, which range from 3.0 to 5.0... (Edwards et al., 1988; Carey et al., 1995; Kotake et al., 2005; de Alcantara et al., 2006). It is generally believed that the pH of the cell wall is acidic, around 5.5. During growth and expansion of the cell, the pH of the cell wall falls below 5.5 (Grebe, 2005), which is optimal for the activity of most cell wall modifying enzymes. When β-galactosidase activity was measured as a function of temperature, both Gal-5 and Gal-2 showed temperature optima at 40 °C and were stable up to 55 °C (data not shown). At temperatures above 55 °C, both enzymes were inactivated irreversibly.

2.7. Aglycone, glycone and natural substrate specificity

To determine the specificity of the enzymes for the aglycone moiety, β-galactosides with a variety of aglycones such as *ortho*-nitrophenyl (*o*NP), *para*-nitrophenyl (*p*NP),

4-methylumbelliferyl (4MU), 5-bromo-4-chloro-3-indolyl (X) and 6-bromo-2-naphthyl (6BN) were tested. All of them were hydrolyzed by both Gal-5 and Gal-2, albeit with different efficiencies (Table 2). These results indicate that they have a broad specificity with respect to the aglycone moiety. To investigate the specificity for the glycone moiety, *p*NP-β-D-galactopyranoside (*p*NPGal), *p*NP-β-D-mannopyranoside, *p*NP-β-D-fucopyranoside, *p*NP-β-D-xylofuranoside, *p*NP-β-D-arabinopyranoside, and *p*NP-α-L-arabinopyranoside (*p*NPAra) were tested. The results are summarized in Table 3, which show that only *p*NP-β-D-galactopyranoside and its 6-deoxy analogue *p*NP-β-D-fucopyranoside were hydrolyzed. Gal-5 and Gal-2 failed to hydrolyze *p*NPAra, showing that the glycone specificity of these two enzymes is strict. Thus, Gal-5 and Gal-2 are highly specific for β-galactopyranoside and discriminate sugars based on the configuration of the hydroxyl group at C4 and C3 positions.

Kinetic parameters of Gal-5 and Gal-2 were determined with *p*NP-β-D-galactopyranoside. K_m values for *p*NP-β-D-galactopyranoside for the two enzymes were similar (0.28 ± 0.06 mM for Gal-5 and 0.40 ± 0.02 mM for Gal-2), but k_{cat}values were different ($1.55\ s^{-1}$ for Gal-5 and $6.03\ s^{-1}$ for Gal-2). Their catalytic efficiencies (k_{cat}/K_m) differed to some extent ($5.54\ s^{-1}\ mM^{-1}$ for Gal-5 and $15.21\ s^{-1}\ mM^{-1}$ for Gal-2). K_m values for *o*NPGal and *p*NPFuc were 0.83 ± 0.015 and 3.75 ± 0.55 mM for Gal-5, respectively, and $0.72 \pm .016$ and 6.4 ± 2.8 mM for Gal-2, respectively.

Table 2
Aglycone specificities of Gal-5 and Gal-2

Aglycone	Relative activity[a] (%)	
	Gal-5	Gal-2
para-Nitrophenyl-(*p*NPGal)	100	100
ortho-Nitrophenyl-(*o*NPGal)	61	72
4-Methylumbelliferyl-(4MUGal)	18	20
5-Bromo-4-chloro-3-indolyl-(X-Gal)	43	56
6-Bromonaphthyl-(6BNGal)	22	30

[a] Activities of Gal-5 and Gal-2 were assayed in reaction mixtures containing 2.5 mM substrate in NaOAc buffer pH 4.6. Aglycone specificity is expressed as a percentage of activity against *p*NPGal (100% ~ 0.03 units (nkat)). For insoluble aglycones, amounts of galactose produced as a result of hydrolysis were measured by the galactose dehydrogenase assay.

Table 3
Sugar specificities of Gal-5 and Gal-2

Glycone	Relative activity[a] (%)	
	Gal-5	Gal-2
*p*NP-β-D-galactopyranoside	100	100
*p*NP-β-D-fucopyranoside	25	21
*p*NP-β-D-glucopyranoside	<1	<1
*p*NP-β-D-mannopyranoside	0	0
*p*NP-β-D-xylofuranoside	0	0
*p*NP-β-D-arabinopyranoside	0	0
*p*NP-α-L-arabinopyranoside	0	0

[a] Activities of Gal-5 and Gal-2 were assayed in reaction mixtures containing 2.5 mM substrate in NaOAc buffer pH 4.6. Sugar specificity is expressed as a percentage of activity against *p*NPGal (100% ~ 0.03 unit (nkat)).

Inhibitory effects of several sugars and sugar derivatives were tested using *p*NPGal as a substrate. γ-Galactonolactone and D-galactose were the most effective inhibitors for Gal-5 and Gal-2 activity. Their K_i values were 44 μM and 7.4 mM, respectively, for Gal-5 and 98 μM and 4.5 mM for Gal-2. Also D-fucose, methyl-α-D-galactoside and raffinose were weaker inhibitors for both enzymes while *p*NPGlc, *p*NPAra, lactose, IPTG, galacturonic acid, L-arabinose, and D-mannose did not show any inhibitory effects. Ag^{+}, Hg^{2+} and SDS strongly inhibited activity of both enzymes when *p*NPGal was used as a substrate.

β-Galactosidases from either different plants or within the same plant are known to differ considerably in their linkage specificity (Kotake et al., 2005; Ishimaru et al., 2005; Buckeridge et al., 2005). Using β-(1,4), β-(1,3) and β-(1,6) linked galacto-oligosaccharides, we investigated the linkage specificity of Gal-5 and Gal-2. As can be seen from Fig. 5, both Gal-5 and Gal-2 hydrolyze β-(1,4) (lanes 3–4 and 5–6) and β-(1,3) (lanes 8–9 and 10–11) linkages, whereas the β-(1,6) linkages in galacto-oligosaccharides were less susceptible to hydrolysis (lanes 13–16).

Ahn et al. (2007) showed that a member of the family, Gal-4, preferentially cleaves β-(1,4) and β-(1,3) linkages. Thus, the three *Arabidopsis* paralogs, Gal-5, Gal-2, and Gal-4, might act on the same natural substrates with β-(1,4) and β-(1,3) linkages. To probe the natural substrate specificity of Gal-5 and Gal-2, more complex oligo-/polysaccharides were tested and the results are shown in Table 4. L-Arafase (α-L-arabinofuranosidase) pretreated (to remove arabinose) lupin galactan, a polymer of β-(1,4)

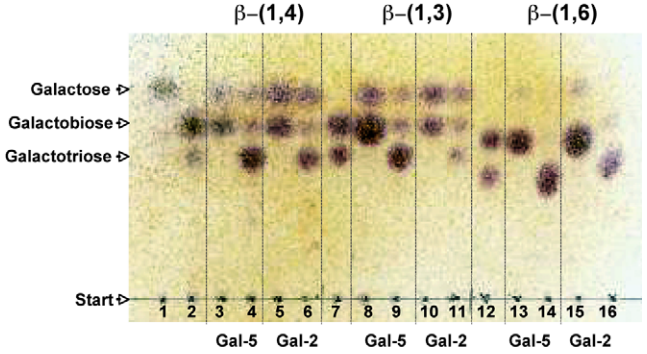

Fig. 5. Linkage specificity of Gal-5 and Gal-2. After hydrolysis of galactobioses and galactotrioses by Gal-5 and Gal-2, products were separated by TLC and developed with naphthoresorcinol (see methods). Lane 1, monogalactose, Lane2-β-(1,4)-linked galactobiose and galactotriose; Lane 3 and 4, hydrolysis product of β-(1,4)-linked galactobiose and galactotriose by Gal-5; Lane 5 and 6, hydrolysis product of β-(1,4)-linked galactobiose and galactotriose by Gal-2; Lane 7, β-(1,3)-linked galactobiose and galactotriose; Lane 8 and 9, hydrolysis product of β-(1,3)-linked galactobiose and galactotriose by Gal-5; Lane 10 and 11, hydrolysis product of β-(1,3)-linked galactobiose and galactotriose by Gal-2; Lane 12,β-(1,6)- linked galactobiose and galactotriose; Lane 13 and 14, hydrolysis product of β-(1,6)-linked galactobiose and galactotriose by Gal-5; Lane 15 and 16, hydrolysis product of β-(1,6)-linked galactobiose and galactotriose by Gal-2.

Table 4
Natural substrate specificities of Gal-5 and Gal-2

Substrate	Linkage of terminal residue	Activity[a]		
		Gal-5	Gal-2	Crude
Arabidopsis cell wall, hot water soluble pectin	Unknown	0.7	0.7	0.3
Arabidopsis cell wall, oxalate-soluble pectin	Unknown	0.6	1.3	0.6
Arabidopsis cell wall, alkali-soluble hemicellulose I	Unknown	tr	0.7	0.6
Arabidopsis cell wall, alkali-soluble hemicellulose II	Unknown	tr	tr	tr
Lupin galactan	Galβ-(1,4)Gal	1.7	2.5	N/A
Apple pectin	unknown	0.8	4.5	1.5
Gum Arabic	Galβ-(1,3), (1,6) Gal	0	0	N/A
Gum Guar	Manβ-(1,4)Man, Galα-(1,6)Gal	0	0	N/A

[a] – μg of Gal released from 2 mg of polysaccharide by 0.03 nkat (units) of enzyme at 25 °C in 24 h, tr – trace (poor hydrolysis), N/A – not tested.

linked galactose, was hydrolyzed to some extent, whereas gum arabic and gum guar were not hydrolyzed. Commercially prepared apple pectin (Sigma, P8471) was the best substrate among the polysaccharides tested. In the case of larchwood arabinogalactan and gum arabic galactan, we were unable to measure hydrolysis, since the negative controls had high background due to the presence of components that interfere with the galactose dehydrogenase assay (#2570-050, Interscientific, Hollywood FL). The fact that Gal-5 and Gal-2 hydrolyzed lupin galactan suggests that these enzymes have exo-galactanase activity. Exo-galactanase activity has been reported for lupin β-galactosidase (Buckeridge and Reid, 1994 and Buckeridge et al., 2005), a tomato β-galactosidase (TBG4) (Carey et al., 1995; Ishimaru et al., 2005), and apple β-galactosidase (Ross et al., 1994).

The strict specificity of Gal-5 and Gal-2 for galactose and their ability to hydrolyze β-(1,4) and β-(1,3) linkages in galacto-oligosaccharides and β-(1,4) linkages in lupin galactan suggest that cell wall polysaccharides rich in galactan are more likely to be natural substrates for these enzymes. Rhamnogalacturonan-I (RG-I) and rhamnogalacturonan-II (RG-II), the pectic polysaccharides of *Arabidopsis* cell wall, contain side chains rich in terminal galactose residues with β-(1,4) and β-(1,3) linkages. (Zablackis et al., 1995). Besides pectins, xyloglucans from hemicellulose are also known to have terminal β-(1,2) linked galactose residues that are susceptible to cleavage by β-galactosidases. Interestingly, xyloglucan oligosaccharides were resistant to the action of Gal-5 and Gal-2. Both enzymes released galactose only from the hot water-soluble and ammonium oxalate-soluble pectic fractions of *Arabidopsis* cell wall, though at a slow rate (Table 4). Gal-5 and Gal-2 (~0.03 nkat) released ~1 μg of galactose from 2 mg of *Arabidopsis* cell wall. The crude extract from rosette leaves released even less galactose under the same conditions. This observation is likely due to the complex structure of cell wall polysaccharides (e.g. RG-I) that contain side chains with linear and branched α-L-arabinose and β-D-galactose residues that sometimes can be substituted by α-L-fucose, β-D-glucuronic acid, and 4-*O*-methyl-β-D-glucuronic acid residues. Such substitutions make galactose residues inaccessible to the action of β-galactosidases and hence limit hydrolysis (Zablackis et al., 1995; Kotake et al., 2005; Iglesias et al., 2006). It should be noted that the limited action of Gal-5 and Gal-2 on the side chains of the pectic backbone can increase porosity of the matrix that creates microenvironments in cell walls in vivo, which in turn may control accessibility of other cell wall-degrading enzymes to their substrates (Smith et al., 2002; Vervelen and Vissenberg, 2007).

2.8. Conclusions

Most of the enzymes and structural proteins that are directly involved in construction and functioning of *Arabidopsis* cell wall are encoded by multigene families (Farrokhi et al., 2006). These families consist of members sharing structural similarity, but differing in their temporal and spatial expression profiles and physiological functions. GH family 35 enzymes, consisting of 17 putative β-galactosidases in *Arabidopsis*, are believed to be involved in cell wall dynamics (Imoto et al., 2005; Ahn et al., 2007; Vervelen and Vissenberg, 2007). We studied two members of the *Arabidopsis* β-galactosidase family, Gal-5 and Gal-2. Western blot analysis using peptide-specific antibodies revealed organ-specific expression of the two genes encoding these enzymes. We showed that Gal-5 and Gal-2 are present in and tightly associated with the cell wall in *Arabidopsis* by using peptide-specific antisera and dot blotting. Recombinant Gal-5 and Gal-2 expressed in *P. pastoris* hydrolyzed various synthetic galactosidases, galacto-oligosaccharides and cell wall-derived polysaccharides. Both enzymes preferentially cleaved galactosides containing β-(1,4) and β-(1,3) linkages. The properties of the enzymes and their natural substrate specificities suggest that they may have the potential to be involved in modification of pectic polysaccharides of cell wall matrices. Further studies are needed to understand their biological roles as pectin modifying enzymes.

3. Experimental

3.1. Materials

cDNAs for Gal-5 (pda05881 or pda06378) and Gal-2 (pda01770) in pBluescript vector were obtained from RIKEN, Institute of Physical and Chemical Research, Japan. Enzymes for the cloning were from Stratagene (La Jolla, CA) and NEB (Ipswich, MA). Easy select *Pichia* expression kit was from Invitrogen (Carlsbad, CA). Synthetic substrates and other chemicals were from Sigma (St. Louis, Mo). The imject maleimide activated BSA

conjugation kit was from Pierce (Rockford, IL). Total Galactose Neonatal Screening Test Kit was from Interscientific (Hollywood, FL). Galactose dehydrogenase was from Roche (Indianapolis, IN). Galacto-oligosaccharides were a gift from Dr. Yoichi Tsumuraya and Dr. Toshihisa Kotake of Saitama University, Japan. Lupin galactan was a gift from Dr. David Smith of USDA, Beltsville, MD.

3.2. Methods

3.2.1. Database analysis

Signal sequence predictions and subcellular targeting of predicted proteins were done using SignalP (Bendtsen et al., 2004) and TargetP (Emanuelsson et al., 2000). Amino acid sequences of β-galactosidases were aligned using software at http://align.genome.jp. A phylogenetic tree was constructed from the alignment using PAUP 4.0.

3.2.2. Microarray expression analysis

Expression profile analysis was done using*Arabidopsis* gene expression datasets from the Genevestigator website (http://www.genevestigator.ethz.ch). Using Gene Atlas Tool, the organ/tissue-specific expression levels for At1g45130 (*Gal-5*) and At3g52840 (*Gal-2*) were estimated by Genevestigator software along with Atg05320 (Ubiquitin10, *UBQ10*) as the control for comparison. A given gene was scored as "expressed" if data from the Digital Northern Tool gave signal values higher than 200 with $p < 0.06$ (Zimmermann et al., 2004). The p value for *Gal-5* and *Gal-2* was $p = 0.00164$. Response Viewer Tool was used to verify up- and down-regulated genes under different abiotic and biotic stresses. Reliability and reproducibility of analyses were evaluated by the number of chips and replicates in individual experiments.

3.2.3. Plant materials

Arabidopsis seeds (Col-O) were obtained from the Arabidopsis Biological Resource Center (ABCR), Ohio State University Seed Stock Center (Columbus, OH). For germination, seeds were surface-sterilized with 3% hypochlorite for 10 min followed by washes with dH_2O (three times) and finally suspended in 0.1% agarose. Sterilized seeds were kept at 4 °C for 3–4 days and seedlings were germinated on half strength Murashige–Skoog salt-agar plates for 10–14 days with 16 h day and 8 h night cycles. Seedlings were transferred to soil and grown at 16/8 h day/night cycle. Plants were harvested when 4–5 weeks old, and immediately frozen in liquid nitrogen and kept at −80 °C until use. For western blot analysis, *Arabidopsis* tissue was ground with sand (0.3 g/1 g tissue). Total proteins were solubilized in 6 M urea (1 g tissue: 2 ml solvent). Cell wall polysaccharide isolation was done as described in Li et al. (2001).

3.2.4. Expression of Gal-2 in E. coli and preparation of rabbit antisera

The Gal-2 mature protein coding sequence was cloned into pET21a vector and expressed in *E. coli* BL21 codon plus cells. Cells were suspended in lysis buffer (50 mM Tris–HCl pH 8.0, 100 mM NaCl, 0.02% SDS, 1 mM PMSF) and broken up using a French press. After extensive washing of soluble fractions with lysis buffer, insoluble proteins (the inclusion body fraction) were solubilized in 6 M urea and separated on a 10% SDS–PAGE preparative gel. The gel was stained (30 min) with Coomassie brilliant blue R-250 and the band corresponding to the Gal-2 polypeptide was excised. The excised band was destained in MeOH:H_2O (1:1, v/v) with several changes of solution and rehydrated in a minimum amount of 1X PBS at 4 °C overnight. After rehydration, the band was ground in a pre-chilled mortar. Ground powder was suspended in 1X PBS containing 0.2% SDS and 0.5% 2-mercaptoethanol (v/v) and heated at 75 °C for 15 min. After cooling, the suspension was mixed with 1 volume of Freund's Complete Adjuvant (Sigma) and used for immunization. Rabbit anti-Gal-2 sera were raised by repeated injection of antigen mixed with Freund's Incomplete Adjuvant at two-week intervals. Synthetic peptides (Gal-2: CSGKIRAPTILMKMIPTS and Gal-5: CSGVAFLTNYHMNAPAKVV) were conjugated to the BSA using an imject maleimide activated BSA kit (Pierce, Rockford, IL) according to the vendor's protocol. Peptide-specific antisera were raised by injecting BSA-conjugated synthetic peptides with Freund's adjuvants. A small volume of trial bleeding was taken at two-week intervals to monitor the change in antisera titer during the course of immunization. All antisera were diluted twice with glycerol and stored at −20 °C until usage.

3.3. Expression of recombinant Gal-5 and Gal-2 in P. pastoris and purification

The mature protein coding sequences of Gal-5 (S24 through N724) and Gal-2 (V28 through K727) cDNAs were amplified by the primer pair 5′-CACCGTGGTCACTTATGATCACAAAGC-3′ and 5′-CCAATGAAAGAGGGTAACAAAGGGC-3′ for Gal-2 and 5′-AGGTGAATTCCAGTGTAGTAGTGTAACCTACG-3′ and 5′-TTTGCGGCCGCAAGTTAG TTTACTGATCTCTTCACAAC-3′ for Gal-5 from cDNA inserts of plasmids obtained from RIKEN, using the high-fidelity *Pfu* Turbo DNA polymerase. The inserts were cloned into pPICZα *Pichia* expression vector to express Gal-5 and Gal-2 as the yeast α factor secretion signal fusion protein to facilitate secretion of recombinant proteins into culture medium. After confirming the accuracy of the sequence and the correct reading frame, linearized plasmids were transformed into *P. pastoris* by electroporation. Recombinant enzyme production was under the control of the alcohol oxidase (AOX) promoter induced by methanol. Production of recombinant proteins was monitored by assaying β-galactosidase activity toward *p*NPGal in culture supernatant samples taken every 24 h. After 72 h of induction, cells were pelleted and culture supernatant was used for further purification of the recombinant enzymes.

Gal-5 and Gal-2 were purified from culture supernatants by ion exchange chromatography using Sulphoxyethyl (SE) cellulose. In a typical experiment, 100 ml of culture supernatant was filtered, diluted five times with degassed dH_2O to reduce ionic strength of the medium and loaded onto the column (1.5 cm × 3 cm) pre-equilibrated with buffer A (20 mM potassium phosphate, pH 6.0). After washing with 10 column volumes of buffer A, bound proteins were eluted in one step with 150 mM NaCl in buffer A (flow rate 1 ml/min). All fractions were checked for β-galactosidase activity using *p*NPGal as substrate. Fractions with highest β-galactosidase activity were used for further experiments. Protein concentration was determined by the Bradford method (Bradford, 1976) (Bio-Rad Protein Assay Reagent kit) using BSA as a standard.

3.3.1. Cell wall isolation and extraction of cell wall-bound proteins

We isolated cell walls from rosette leaves of *Arabidopsis* using the procedure described by Feiz et al. (2006). Rosette leaves were ground in a blender whose cup was dipped at intervals into liquid nitrogen to maintain low temperature during grinding. The cell wall fraction was washed extensively with 3 L of wash buffer (5 mM NaOAc buffer, pH 4.6) on a metal net (75 μm pore size). After washing, the cell wall fraction was lyophilized. The lyophilized cell wall material was ground to a fine powder by grinding in a blender, and was then used to extract wall-bound proteins. Five different (S1, soluble 1; S2, soluble 2; S3, soluble 3; CW4, extractable with $CaCl_2$; and CW5, extractable with LiCl) fractions were obtained. These fractions were assayed for β-galactosidase activity. Of these, three fractions with β-galactosidase activity were further analyzed for immunoreactivity. They were spotted multiple times on nitrocellulose strips to increase antigen (Gal-5 and Gal-2) concentration and incubated with pre-immune (control) and immune sera from rabbits immunized with whole Gal-2 polypeptide and unique peptides derived from Gal-5 and Gal-2 sequences.

3.3.2. SDS–PAGE, native PAGE and western blotting

SDS–PAGE was performed as described by Laemmli (1970). Native PAGE was performed in acidic gels using the protocol on the website (http://wolfson.huji.ac.il/purification/Protocols/PAGE_Acidic.html). After electrophoresis, the gel was rinsed in a wash buffer (100 mM acetate buffer pH 4.6) for 2 × 15 min. The zymogram was developed by incubating the gel in 0.5 mM 4-MUGal in wash buffer at 37 °C for 20 min and photographed under UV light. For immunoblotting, the gel was soaked in a blotting buffer (10 mM CAPS, pH 11 with 10% (v/v) MeOH) for 2 × 15 min. Proteins were transferred onto a nitrocellulose (0.45 μm, Protran) membrane using a Bio-Rad Mini trans blot cell at 50 V, at 4 °C overnight following the vendor's protocol. For immunodetection, 2000-times dilution of anti-Gal-2 antiserum or 1000-times dilution of peptide-specific antiserum was used as primary antibody and 2000-times dilution of goat anti-rabbit antibody conjugated with peroxidase (A0545, Sigma, Saint Louis, MO) as secondary antibody. Immunoreactive bands were visualized by the deposition of 4-chloronaphthol after oxidation by HRP (horse radish peroxidase) using the substrate solution (21 ml of PBS pH 7.4, mixed with 5.5 ml of 3.3 mg/ml 4-chloronaphthol in 100% MeOH and 10 μl of 30% H_2O_2).

3.3.3. β-galactosidase activity assay

One hundred micro liters of 5 mM *p*NPGal in 100 mM NaOAc buffer pH 4.6, 80 μl H_2O and 20 μl of the diluted enzyme solution were mixed and incubated at 37 °C for up to 30 min. The reaction was stopped by adding 100 μl of 1 M Na_2CO_3. The absorbance was measured at 405 nm to quantify the amount of *p*NP released after hydrolysis. This standard protocol was used for all activity assays with *p*NPGal, if not otherwise stated. Boiled enzyme or buffer solution was used as a control. One unit (nkat) of enzyme activity is defined as an amount of enzyme that is able to produce 1 nmole of *p*NP per second at 37 °C.

For the determination of natural substrate specificity, galacto-oligosaccharides (20 mM), xyloglucan oligosaccharides (1 μg/μl) and polysaccharides (1% (w/v)) were prepared in H_2O. Final concentration of substrates was 4 mM for oligosaccharides and 0.5% for polysaccharides in 100 mM acetate buffer pH 4.6. Reaction mixture was incubated with 0.03 units/nkats enzymes at room temperature for 24 h. Reaction mixtures containing no enzyme and no substrate were used as controls. Reactions for polysaccharides were stopped by adding 1 ml of 100% EtOH to 0.4 ml of reaction mix to precipitate proteins and polysaccharides. After centrifugation, the supernatant was transferred into a new microfuge tube and vacuum dried. Dried mixtures were dissolved in 100 μl of dH_2O, and total galactose produced as a result of hydrolysis was quantified using galactose dehydrogenase assay kit (Interscientific, Hollywood, FL). Products of hydrolysis of oligosaccharides were analyzed by thin layer chromatography (TLC) on silica gel 60 F_{254} (EM Science, Germany) using 3:2:1 (v/v/v) n-BuOH:AcOH:H_2O as the solvent and detected by heating TLC plates after spraying with 0.2% (w/v) naphthoresorcinol in 1:19 H_2SO_4:EtOH (v/v). (Ahn et al., 2004).

Acknowledgements

We are grateful to Drs. Yoichi Tsumuraya and Dr. Toshihisa Kotake of Saitama University, Japan for providing galacto-oligosaccharides, and Dr. David Smith of USDA, Beltsville, MD for providing lupin galactan. The authors also wish to thank Dr. Farooqahmed Kittur of Virginia Tech for much help and critical reading of the manuscript. This research is funded by the *Arabidopsis* 2010 Project of the National Science Foundation (MCB-0115937) A. Esen et al., and a research grant award from the Virginia Academy of Science to D. Gantulga.

References

Ahn, Y.O., Mizutani, M., Hiromichi, S., Kanzo, S., 2004. Furcatin hydrolase from *Viburnum furcatum* blume is a novel disaccharide-specific acuminosidase in Glycosyl Hydrolase Family 1. J. Biol. Chem. 279, 23405–23414.

Ahn, Y.O., Zheng, M., Winkel, B., Bevan, D.R., Esen, A., Shin-Han, S., Benson, J., Peng, H., Miller, J.T., Cheng, C., Poulton, J.E., Shih, M., 2007. Functional genomic analysis of *Arabidopsis thaliana* Glycoside Hydrolase Family 35. Phytochemistry 68, 1510–1520.

Balasubramaniam, S., Lee, H., Lazan, H., Othman, R., Ali, Z.M., 2005. Purification and properties of a β-galactosidase from carambola fruit with significant activity toward cell wall polysaccharides. Phytochemistry 66, 153–163.

Bayreuther, K., Bieseler, D.J., Ehring, R., Griesser, H.W., Mieschendahl, M., Muller-Hill, B., Triesch, I., 1980. Investigation of structure and function of lactose permease of *Escherichia coli*. Biochem. Soc. Trans. 8, 675–676.

Bendtsen, J., Nielsen, H., von Heijne, G., Brunak, S., 2004. Improved prediction of signal peptides: SignalP 3.0. J. Mol. Biol. 340, 783–795.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Buckeridge, M.S., Reid, J.S., 1994. Purification and properties of a novel beta-galactosidase or *exo*-(1->4)-beta-D galactanase from the cotyledons of germinated *Lupinus angustifolius* L. seeds. Planta 192 (4), 502–511.

Buckeridge, M.S., Huntcheon, I.S., Grant Reid, J.S., 2005. The role of *exo*-(1-4)-β-galactanase in the mobilization of polysaccharides from the cotyledon cell wall of *Lupinus angustifolus* following germination. Ann. Botany 96, 435–444.

Carey, A.T., Holt, K., Picard, S., Wilde, R., Tucker, G.A., Bird, C.R., Schuch, W., Seymour, G.B., 1995. Tomato *exo*-(1-4)-D-galactanase. Isolation, changes during ripening in normal and mutant tomato fruit, and characterization of related cDNA clone. Plant Physiol. 108, 1099–1107.

Chantarangsee, M., Fujimura, T., Fry, S.C., Ketudat-Cairns, J.R., 2007. Molecular characterization of β-galactosidases from germinating rice (*Oryza sativa*). Plant Sci. 173, 118–134.

Coutinho, P.M., Henrissat, B., 1999. Carbohydrate-active enzymes: an integrated database approach. In: Gilbert, H.J., Davies, G., Henrissat, B., Svensson, B. (Eds.), Recent Advances in Carbohydrate Bioengineering. The Royal Society of Chemistry, Cambridge, pp. 3–12.

Davies, G., Henrissat, B., 1995. Structures and mechanism of glycosyl hydrolases. Structure 3, 853–859.

de Alcantara, P.H.N., Martim, L., Silva, C.O., Dietrich, S.M.C., Buckeridge, M.S., 2006. Purification of a β-galactosidase from cotyledons of *Hymenaea courbaril* L. (Leguminosae). Enzyme properties and biological function. Plant Physiol. Biochem. 44, 619–627.

Edwards, M., Bowman, Y., Dea, I., Reid, J., 1988. A β-D-galactosidase from Nasturtium (*Tropaeolum majus* L.) cotyledons. J. Biol. Chem. 9, 4333–4337.

Emanuelsson, O., Nielsen, H., Brunak, S., von Heijne, G., 2000. Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. J. Mol. Biol. 300, 1005–1016.

Farrokhi, N., Burton, R.A., Brownfield, L., Hrmova, M., Wilson, S.M., Bacic, A., Fincher, G.B., 2006. Plant cell wall biogenesis: genetic, biochemical and functional genomics approaches to the identification of key genes. Plant Biotechnol. 4, 145–167.

Feiz, L., Irshad, M., Pont-Lezica, R.F., Canut, H., Jamet, E., 2006. Evaluation of cell wall preparations for proteomics: a new procedure for purifying cell walls from *Arabidopsis* hypocotyls. Plant Methods 2, 10.

Grebe, M., 2005. Growth by Auxin: when a weed needs acid. Science 310, 60–61.

Hruba, P., Honys, D., Twell, D., Capkova, V., Tupy, J., 2005. Expression of β-galactosidase and β-xylosidase genes during microspore and pollen development. Planta 220, 931–940.

Iglesias, N., Abelenda, J.A., Rodino, M., Samredro, J., Revilla, G., Zarra, I., 2006. Apoplastic glycosidase active against xyloglucan oligosaccharides of *Arabidopsis thaliana*. Plant Cell Physiol. 47 (1), 55–63.

Imoto, K., Yokoyama, R., Nishitani, K., 2005. Comprehensive approach to genes involved in cell wall modifications in *Arabidopsis thaliana*. Plant Mol. Biol. 58 (2), 177–192.

Ishimaru, M., Smith, D.L., Gross, K.C., 2005. Yeast expressed tomato β-galactosidase 1,4, and 5 have activity against synthetic and plant-derived cell wall substrates. HortScience 40, 1092.

Jamet, E., Canut, H., Boudart, G., Pont-Lezica, R.F., 2006. Cell wall proteins: a new insight through proteomics. Trends Plant Sci. 11, 33–39.

Kotake, T., Dina, S., Konichi, T., Kaneko, S., Igarashi, K., Samejima, M., Watanbe, 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 Physiol. 138, 1563–1576.

Laemmli, U.K., 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 277, 680–685.

Lazan, H., Ng, S.Y., Goh, L.Y., Ali, Z.M., 2004. Papaya β-galactosidase/galactanase isoforms in differential cell wall hydrolysis and fruit softening during ripening. Plant Physiol. Biochem. 42, 847–853.

Li, S.C., Han, J.W., Chen, K.C., Chen, C.S., 2001. Purification and characterization of isoforms of β-galactosidases in mung bean seedling. Phytochemistry 57, 349–359.

Perez Almeida, I.B., 2004. *Arabidopsis* cell wall beta-galactosidase gene family: expression, catalytic activities and biological function in galactose dynamics. Dissertation. Purdue University ETD.

Ross, G.S., Wegrzyn, T., MacRae, E.A., Redgwell, R.J., 1994. Apple β-galactosidase activity against cell wall polysaccharides and characterization of related cDNA clone. Plant Physiol. 106, 521–528.

Smith, D.L., Gross, K.C., 2000. A family of at least seven β-galactosidase genes is expressed during tomato fruit development. Plant Physiol. 123, 1173–1183.

Smith, D.L., Abbott, J.A., Gross, K.C., 2002. Down-regulation of tomato β-galactosidase 4 results in decreased fruit softening. Plant Physiol. 129, 1755–1762.

Vervelen, J.P., Vissenberg, K., 2007. The expanding cell. Plant Cell Monographs, vol. 5. Springer.

Zablackis, E., Huang, J., Muller, B., Darvill, A.G., Albersheim, P., 1995. Characterization of the cell wall polysaccharides of *Arabidopsis thaliana* leaves. Plant Physiol. 107, 1129–1138.

Zimmermann, P., Hirsch-Hoffmann, M., Hennig, L., Gruissem, W., 2004. GENEVESTIGATOR. *Arabidopsis* microarray database and analysis tool. Plant Physiol. 136, 2621–2632.