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Knockout mutants of *Arabidopsis thaliana* β-galactosidase. Modifications in the cell wall saccharides and enzymatic activities

M. MONEO-SÁNCHEZ[1], L. IZQUIERDO[1], I. MARTÍN[1], J. HERNÁNDEZ-NISTAL[2], L. ALBORNOS[1], B. DOPICO[1], and E. LABRADOR[1]*

Dpto de Botánica y Fisiología Vegetal, Instituto Hispano Luso de Investigaciones Agrarias, Universidad de Salamanca, Campus Miguel de Unamuno E-37007, Salamanca, Spain[1]
Dpto de Biología Funcional, Universidad de Santiago de Compostela, Campus de Lugo E-27002, Spain[2]

Abstract

This work studied the six β-galactosidases (BGALs) of the subfamily a1 of *Arabidopsis*, that have been proposed to play important roles in the cell wall remodelling during plant development, although their precise functions are still unknown. Knockout mutants *bgal1*, *bgal2, bgal3*, *bgal4*, *bgal5*, and *bgal12* of *Arabidopsis* and their wild type (WT) plants were analysed to determine their morphology and composition of their cell walls. The gas chromatography and the Fourier transform infrared spectroscopy revealed differences between the mutants and their WT such as in the proportions of glucose, galactose, or xylose in *bgal2* and *bgal4* and in cell walls polysaccharides in *bgal1*, *bgal3*, and *bgal5*. However, these slight changes did not result in morphological variations during plant development. None of the mutant seedlings displayed a clear reduction in β(1,4)-galactan content, analysed by immunolocalization. The absence of significant phenotypic changes in the β-galactosidase subfamily a1 mutants could indicate possible β-galactosidases functional redundancy. Future studies will focus on the construction of multiple mutants that help to establish the precise function of each member of the β-galactosidase subfamily a1.

Additional keywords: FTIR, β(1,4)-galactan, galactose, glucose, glycosyl hydrolase family 35, immunolocalization, xylose.

Introduction

Plant β-galactosidases (EC 3.2.1.23) are included in the glycosyl hydrolase (GH) family 35 and are thought to be involved in cell wall biogenesis and modification (Jamet *et al.* 2006, Vervelen and Vissenberg 2007). The β-galactosidases are encoded by multigene families in several plant species (Smith and Gross 2000, Esteban *et al.* 2005, Ahn *et al.* 2007).

Each β-galactosidase isoform from a given plant species could present hydrolytic activity against different substrates and could be involved in different developmental processes. Thus, *Solanum sculentum* β-galactosidase TBG4 hydrolysed pectins and hemicelluloses from tomato cell walls and commercial galactan (Smith and Gross 2000). A β-galactosidase from radish (RsbGal) specifically hydrolysed β(1,6) and β(1,3)-linkages in arabinogalactan protein (Kotake *et al.* 2005). A β-galactosidase I purified from ripe carambole was active against β(1,4)-linked spruce galactan and β(1,6)/β(1,3)-linked Arabic gum galactan and alkali-soluble hemicelluloses of carambole cell wall (Balasubramaniam *et al.* 2005). The five isoforms of β-galactosidase from mungbean differ in their enzymatic characteristics and substrate specificity (Li *et al.* 2001). Papaya β-galactosidase isoforms differentially hydrolysed cell wall during fruit ripening (Lazan *et al.* 2004). Among the four members of the *Cicer arietinum* β-galactosidases (Esteban *et al.* 2005, Hernández-Nistal *et al.* 2014), βI-Gal is involved in the polymer

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Abbreviations: BGAL - β-galactosidase; FTIR - Fourier transform infrared spectroscopy; RGI - rhamnogalacturonan I.
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* Corresponding author; e-mail: labrador@usal.es

modification leading to the hardening of the wall (Martin *et al.* 2011), βIII-Gal protein with hydrolytic action on β(1,4)-galactan neutral rhamnogalacturonan I (RGI) side chains (Vincken *et al.* 2003, Esteban *et al.* 2003) contributes to reinforce the cell walls in the phloem differentiation in roots, epicotyls, and stems (Martin *et al.* 2013), βIV-Gal is present specifically in the vascular tissue and the sclerenquimatic cell layer surrounding the vascular cylinder in organs whose cell elongation rate is low or has ceased (Martin *et al.* 2008), and βV-Gal modifies the cell wall during the final stages of cell proliferation and the establishment of an expanding wall (Martin *et al.* 2009).

In *Arabidopsis thaliana*, a family of 17 genes encoding β-galactosidases, further subdivided into two groups and seven subfamilies, was identified (Ahn *et al.* 2007). Most of the subfamilies contain one to three members, except for the largest subfamily a1, which consists of six genes, *AtBGAL1* to *5* and *AtBGAL12* (Gantulga *et al.* 2009). It has been predicted that all the encoded β-galactosidases from subfamily a1 have signal peptides and basic isoelectric points (7.2 - 8.6) consistent with a cellular destination to the cell wall (Ahn *et al.* 2007).

Studies of the activity of the promoters of the *AtBGAL* genes from the subfamily a1 reported that temporal and spatial expression of those β-galactosidases genes are differentially regulated (Albornos *et al.* 2012) indicating that these β-galactosidases could be involved in the cell wall metabolism during many processes such as growth (AtBGAL1, 2, 3 and 4), differentiation (AtBGAL12), or development of trichomes (AtBGAL5)**.** Our study of the *AtBGAL* transcript accumulation along plant development indicated that all *AtBGAL* transcripts appeared in initial stages of development, both in dark- and light-grown seedlings, the *AtBGAL1*, *AtBGAL2*, and *AtBGAL3* transcripts being the predominant in light-grown ones, mainly in the aerial part (Moneo-Sánchez *et al.* 2016). However, the function of each isoenzyme in the different developmental processes and at the different growth stages of the plant is not completely established.

The significance of β-galactosidases to modify the pectic polysaccharides of the cell walls that could play important roles in cell wall remodelling during plant development led us to study the subfamily a1 of *Arabidopsis* β-galactosidases. It has been reported that they are able to act on the cell wall pectic polysaccharides (Gantulga *et al.* 2009), although their precise functions have not yet been determined. Previously, we studied the expression of the a1 subfamily genes by analysing the activity of their promoters and their transcript accumulation. Therefore, the aim of this work was to study the function of these cell wall β-galactosidases by using knockout mutants of *Arabidopsis* ecotype Col-0 in the loci *At3g1375* (AtBGAL1), *At3g52840* (AtBGAL2), *Atg36360* (AtBGL3), *At5g56870* (AtBGAL4), *At1g45130* (AtBGAL5), and *At4g26140* (AtBGAL12). The analysis of the mutant phenotypes compared with their corresponding wild types and the putative changes in cell wall composition and enzymatic activities can help to deep inside the function of these β-galactosidase isoenzymes in plant development.

Materials and methods

Plants and growth conditions: Seeds from *Arabidopsis thaliana* L. *bgal* mutants were obtained through the Nottingham *Arabidopsis* Stock Centre. The *bgal1* (SALK_032664), *bgal2* (SALK_022121), *bgal3* (SALK_019604), *bgal4* (SALK_022796), and *bgal12* (SALK_087787) mutants were generated by the Salk Institute Genomic Analysis Laboratory (http:// signal.salk.edu) (Alonso *et al.* 2003). The *bgal5* (SAIL_1245_H07) was selected from the *Syngenta Arabidopsis Insertion Library* (Sessions *et al.* 2002). Homozygous lines for the T-DNA insertion and a WT line for each mutant (indicated as WT(*bgal*)) were selected by using gene-specific primers in combination with the T-DNA left border-specific primer (listed in Table 1 Suppl.).

Seeds from WT and *bgal* mutants were surface sterilized as described by Albornos *et al.* (2012) and cold treated at 4 ºC for 3 d before sowing. Seeds were grown in Petri dishes on one-half-strength Murashige and Skoog (1962) agar medium with 1 % (m/v) sucrose. These plates were maintained in a growth chamber (*Aralab*, Sintra, Portugal) at 22 ºC with either a 16-h photoperiod (provided by cool-white fluorescent tubes, an irradiance of approximately 80 - 100 μmol m^{-2} s^{-1}), or in darkness to obtain etiolated seedlings. To obtain adult plants, 10-d-old green seedlings were transferred to plastic pots containing a 3:1 mixture of potting soil and *Vermiculite* and grown under the same conditions.

Reverse-transcription semi quantitative polymerase chain reaction (RT-sqPCR): For RT-sqPCR analyses, RNA was extracted from 10-d-old green plants with the *Nucleospin*® RNA plant kit (*Macherey-Nagel*, Düren, Germany) according to the manufacturer's instructions. First strand complementary DNA (cDNA) was synthesized from 1 μg of RNA with a mixture of hexanucleotides (final concentration 20 μM) and oligo dT (final concentration 2.5 μM) using *PrimerScript*® RT reagen kit (*Takara*, Kusatsu, Japan). Once obtained, cDNA was diluted 5-fold for the subsequent PCR analysis. PCR was performed with the *GoTaq*® DNA polymerase (*Promega*, Fitchburg, WI, USA) using 2 mm^3 of the 5-fold diluted cDNA. The reaction consisted of the following steps: 1 × 95 ºC for 5 min, 30 × 95 ºC for 1 min, 60 ºC for 1 min, 72 ºC for 30 s, and 1 × 72 ºC for 10 min. PCR-amplified fragments (all raging from 150 to

300 bp in lenght) were separated in agarose gels containing ethidium bromide, and images were acquired with a *ChemLite 400 FA* (*Avegene Life Sciences*, Taipei, Taiwan). Band quantification was performed by *Total Lab Quant 1D* gel analysis software *v. 10* (*Avegene Life Sciences*, Taipei, Taiwan). Quantification was done relative to Actin2 (*ACT2,* AT3G18780), used as internal control, which was assigned a value of 100 %. The specific primers used are listed in Table 2 Suppl.

Cell wall and protein extraction: Two different methods were used to obtain the cell walls. In the first method, cell walls were isolated from 10 d-old-mutants and WT plants. Freeze-dried plant material (3 g) was grinded (30 Hz for 1 min) using a *retschmill MM400* (*Sarstedt*, Nümbrecht, Germany). After addition of 15.0 cm^3 of 50 mM NaCl at 4 ºC to the ground material and vortex thoroughly, the suspension was centrifuged (3 000 *g*, 4 ºC, 15 min). The pellet was washed with acetone at -20 ºC and cold 50 mM NaCl and centrifuged after each wash. For protein extraction, the cell wall material obtained was suspended in 1 M NaCl + 10 mM Na-citrate phosphate buffer, pH 5.5, at 4 ºC. After 48 h, the suspension was centrifuged (3 000 *g*, 15 min), and the supernatant was dialysed for 48 h using 20 mM Na-acetate buffer, pH 5.0. The protein extract was concentrated by ultrafiltration in *Vivaspin 4* tubes (*Sartorius Stedim*, Göttingen, Germany) to a final volume of 0.2 - 0.3 cm^3. Both the protein dialysation and the subsequent concentration were carried out at 4 ºC. After protein extraction, the cell wall material was washed with distilled water, acetone, and diethyl ether and dried at room temperature to a constant mass.

In the second method, cell walls were obtained from the alcohol insoluble residue (AIR) of 3-d-old mutants and WT seedlings grown under a 16-h photoperiod or in the darkness. The material was frozen in liquid nitrogen and ground using a *retschmill MM400* as indicated above. Methanol was added to each sample and boiled for 10 min to obtain the AIR. The suspension was centrifuged at 8 000 *g* for 20 min and the supernatant discarded. The obtained residue was washed 3 times with methanol-chloroform in a 1:2 proportion and twice with diethyl ether and centrifuged after each wash at 8 000 *g* for 20 min. The walls thus obtained were dried at room temperature to a constant mass.

Cell wall monosaccharides, proteins, and enzymatic activity: The neutral monosaccharide composition of the cell walls was analyzed by gas phase chromatography after hydrolysis with 2 M trifluoroacetic acid at 121 °C for 90 min. The hydrolyzed sugars were converted into alditol acetates according to Albersheim *et al*. (1967). A *Konik 3000-HRGC* device equipped with a column of 3 % *ECNSS-M* on *Gas Chrom P* (*Konik Instruments*, Miami, FL, USA) was employed. Inositol was used as internal standard.

The total amount of proteins was assayed by the *Protein assay* (*Bio-Rad*, Hercules, USA) based on the method of Bradford (1976). The β-D-galactosidase activity was assayed using the p-nitrophenylgalactoside as substrate, basically as described in Dopico *et al.* (1989). The assay was performed with 1 µg of cell wall protein extract. Incubation took place at 34 ºC for 90 min. Free *p*-nitrophenol concentration was determined by measuring the absorbance at 400 nm using a *Jenway 7315* spectrophotometer (Stone, UK).

Fourier transform infrared spectroscopy (FTIR) and multivariate analysis: For the FTIR spectroscopic measurement, 3 mg of cell walls (obtained as described in the 2nd method) were mixed with KBr (100 mg) in a *Specadie* kit (*Smiths Industries*, London, UK) to prepare the tablets. The spectra were recorded on a *Bomen MB 160* FTIR spectrometer (*Hartmann and Braun*, Frankfurt, Germany) at a resolution of 4 cm^{-1} in the spectral range of 2 000 to 900 cm^{-1} to identify changes in cell wall polysaccharides. All spectra were normalized using *Perkin-Elmer* (Waltham, MA, US) *IR Data* software, baseline corrected and area-normalized using the application *Win-DAS* (*John Wiley and sons*, Hoboken, NJ, USA). Then, the spectra were exported to *Microsoft Excel 2010* for digital subtraction of mutant and corresponding WT. Cluster analysis of mutants and WT seedlings grown under a 16-h photoperiod or in the darkness were performed according to the UPGMA (unweighted pair group method with arithmetic mean) method. Principal component analysis (PCA) were performed using the software *SPSS Statistics v. 20* (*IBM*) to elucidate differences among spectra of each type of plants.

Immunofluorescence labelling of β(1,4)-galactan in *Arabidopsis* cell wall: Immunofluorescence labelling of β(1,4)-galactan was conducted with monoclonal antibody LM5, specific for four consecutive β(1,4)-galactan residues (Jones *et al.* 1997) on floral stem internodes from 24 to 30-d-old *Arabidopsis* plants and in rosette leaves from 17-d-old plants. Sample preparation and incubation with antibodies were performed as described by Martín *et al.* (2013). LM5 and secondary antibody (goat anti rat-IgG conjugated with FITC) were applied at 1.25 and 1.50 dilutions, respectively. Images were taken using a *DM4000* microscope equipped with a *DFC550* camera (*Leica*, Wetzlar, Germany).

Statistical analysis: The data are presented as means ± standard errors (SEs) of at least three biological replicates. The *SPSS Statistics v. 20* (*IBM*) was used for statistical analysis. Student's *t*-test was applied to compare means between mutants and wild type plants. A significant level was set at $\alpha = 0.05$.

Results

Knockout mutants *bgal1*, *bgal2, bgal3*, *bgal4*, *bgal5*, and *bgal12* of *Arabidopsis* ecotype Col-0 and their corresponding wild type [WT(*bgal*)] plants were analysed to determine differences in their morphology and in the structure and composition of their cell walls by analyzing the content of monosaccharides and the distribution of polysaccharides by FTIR spectroscopy. Once selected the homozygous mutants by PCR and verified the absence or strong reduction of transcripts of the mutated gene by RT-sqPCR in the knockout mutants, we performed the subsequent analyses.

Phenotypic studies revealed no significant morphological differences between the subfamily a1 *bgal* knockout mutants and their corresponding WT along plant development both in vegetative and reproductive stages (Fig. 1 Suppl. shows photographs of 17-d-old rosettes from mutants and their corresponding wild type as an example). Also the proportion of the cell wall fresh mass in the whole fresh mass and the amounts of cell wall proteins were similar in *bgal* mutants and WT(*bgal*) plants (Table 3 Suppl.). However, cell walls from *bgal1, bgal2, bgal4,* and *bgal5* mutants showed higher β-galactosidase specific activity when compared to corresponding WT, although the difference was statistically significant only for *bgal4* (Fig. 1). This higher specific activity was also reflected in total β-galactosidase activity

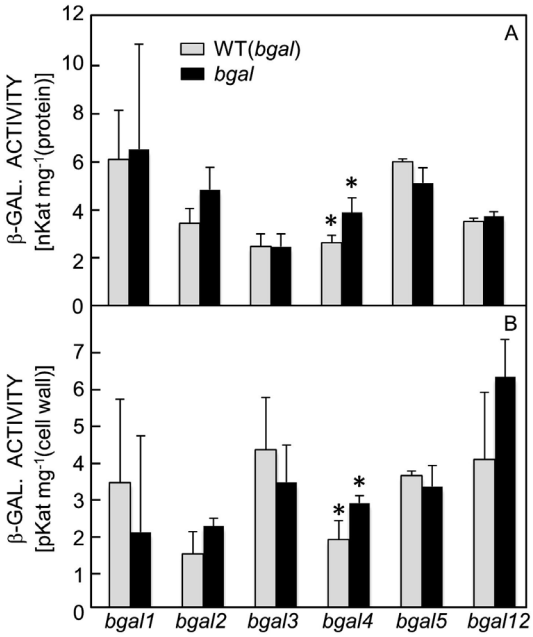

Fig. 1. The β-galactosidase activity [expressed per mg of protein (*A*) and mg of cell wall (B)] in 10-d-old *bgal* and WT(*bgal*) seedlings. Means ± SEs, n = 3. The statistically significant differences ($\alpha = 0.05$) are indicated by *asterisks*.

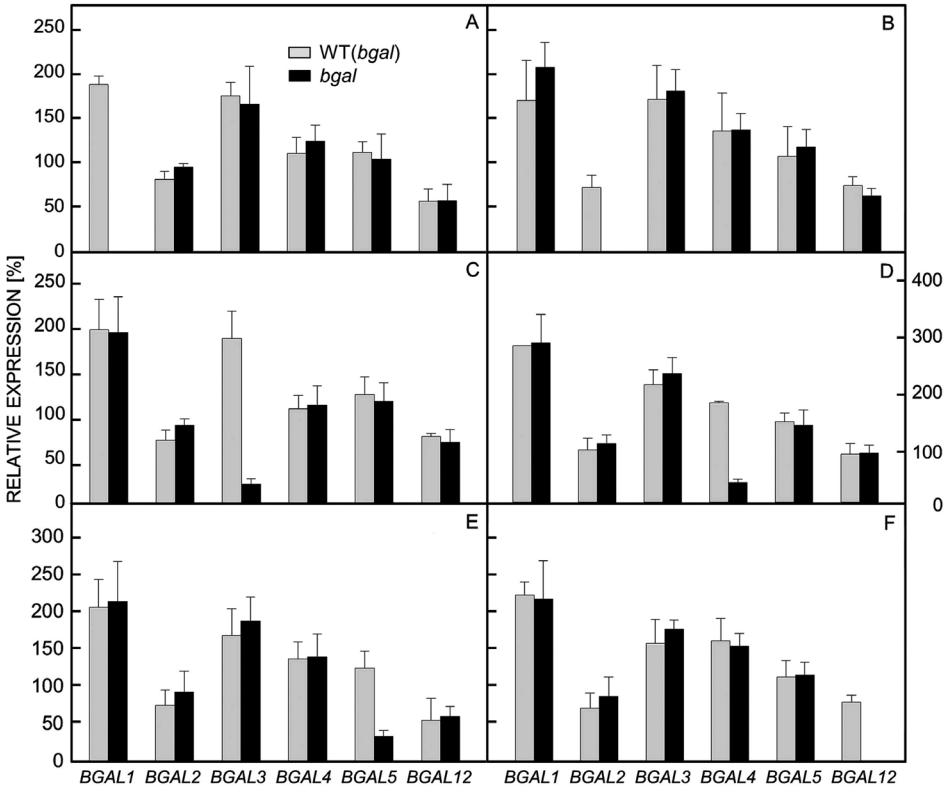

Fig. 2. *AtBGAL* transcript accumulation estimated by RT-sqPCR in 10-d-old *bgal1* (*A*), *bgal2* (*B*), *bgal3* (*C*), *bgl4* (*D*), *bgal5* (*E*), and *bgal12* (*F*) mutants and in their corresponding WT(*bgal*) plants. The relative expression is the ratio between the expression of each *BGAL* gene and the *Actin2* gene used as internal control. Means ± SEs, n = 3.

per unit of cell wall. No differences could be observed on the other mutants (Fig. 1). The differences observed among the WT(*bgal*) are due to the fact that the analyses were carried out using a segregated WT for each mutant to avoid the interference of endogenous variability of the different *Arabidopsis* seed stocks.

Since the β-galactosidase activity detectable in *bgal* lines could be due to one or more of the other members of the family of β-galactosidases (Ahn *et al.* 2007), we analysed whether the absence of a specific *Arabidopsis* β-galactosidase was compensated by increased transcript accumulation of genes encoding other a1 subfamily enzymes. However, no changes in amount of transcripts were found in the six *bgal* seedlings with respect to their WT, except for the expected lack of the corresponding *BGAL* transcript in each mutant (Figs. 2, 3).

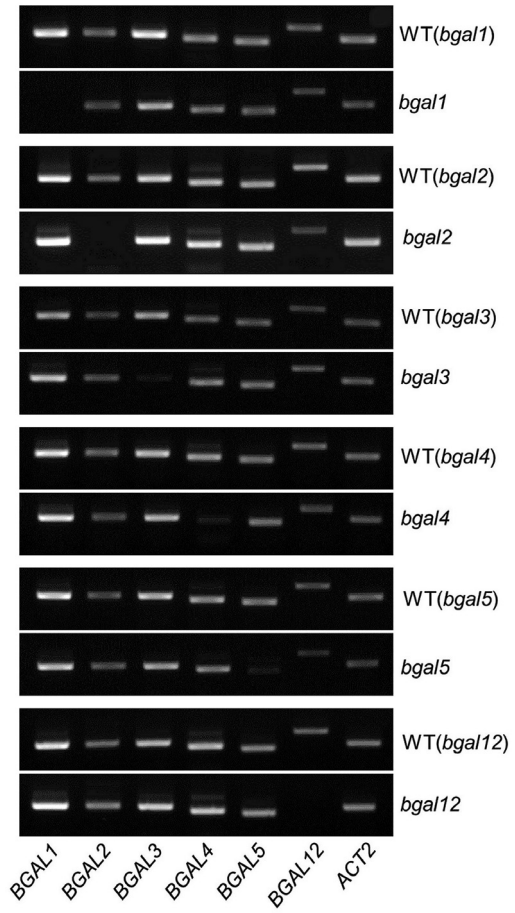

Fig. 3. Agarose gel electrophoresis of PCR products with the specific primer pair of each *AtBGAL* transcript (*BGAL*) and the internal control *ACT2*.

To check whether the lack of one *AtBGAL* from the subfamily a1 induces changes in the structure and/or composition of the cell wall, we determined its monosaccharide composition in cell walls purified from 10-d-old mutant green seedlings and corresponding WT. The galactose and glucose were the main cell wall sugars. Statistically significant differences between WT and mutants have been determined for xylose in mutants *bgal2* and *bgal4*, for the galactose and glucose in *bgal2*, and only for glucose in *bgal3* (Fig. 4).

The FTIR cell wall spectra obtained from the alcohol insoluble residue (AIR) of 3-d-old mutants and WT plants growing under a 16-h photoperiod or in the darkness showed no evident variability for the region between 2000 and 900 cm^{-1} (Fig. 2 Suppl.). The profiles were very similar although it is worthy to note the lower absorbance of the cell walls of both *bgal2* and WT in green plants as compared to etiolated ones. The spectra were compared by multivariate analysis. In all cases the dendrograms obtained (Fig. 3 Suppl.) indicated that it was possible to establish different groups that separate the analyzed plants depending on growing conditions and whether they are mutant or WT plants, which could indicate differences in their cell walls. Principal components 1 and 2 (PC1 and PC2) could be used to separate the four groups of spectra (Fig. 4 Suppl.). The PC1, which explains more than 95 % of total covariance of dates (Table 4 Suppl.) in *bgal2*, *bgal4*, and *bgal12,* did not allow separate the groups of these mutants from the WT, which confirms that PC1 is not suitable for this purpose. Regarding *bgal1*, *bgal3*, and *bgal5*, when a PCA analysis was performed on the corresponding spectra, green *bgal* plants could be split from green WT plants along the PC2 axis (with a cumulative variance of less than 15 % in all cases; Table 4 Suppl.), showing negative values for *bgal1* and *bgal3* and positive for *bgal5*.

Fig. 5 Suppl. analyses the loadings plots corresponding to PC2 according to the absorption assigned to different cell wall polysaccharides in the literature (Kačuráková and Mathlouthi 1996, Coimbra *et al.* 1999, Kačuráková and Wilson 2001, Černá *et al.* 2003). Our results point to a higher proportion of RGI (989 cm^{-1}), glucan linkages (1026 cm^{-1}), and cellulose (1040 cm^{-1}) in the cell walls of WT(*bgal1*) green seedlings when compared to *bgal1* green seedling, and a higher proportion of pectic polymers (1101 and 1113 cm^{-1}) in *bgal1* (Fig. 5 Supp *A*). The *bgal3* seedlings show an increase of peaks at 1011, 1032 and 1132 cm^{-1} associated to uronic acids, HG, and cellulose (Fig. 5 Suppl.). Finally, in *bgal5* a peak at 1014 cm^{-1} associated to glycosidic linkages in pectic polysaccharides was observed (Fig. 5 Suppl*.)*.

In addition to mutant morphological characterizations and mutant cell wall analysis, we decided to conduct histological analysis to check for possible anatomical changes and perform immune-localization studies to establish changes in specific polysaccharides, namely those containing four consecutive β(1,4)-galactan residues, specifically recognized by LM5 antibodies (Jones *et al.* 1997). We used internodes with different growth activity, the first (basal) internode and the third (apical) internode from 28 to 32-d-old plants and the second pair of rosette leaves of 17-d-old plants. The labelling with LM5 in the stem changed with the development, decreasing from young toward old tissues, being the strongest labelling in the

apical internode (Fig. 5*A*), especially in epidermal cells and vascular tissue. No significant differences could be observed between WT and mutant plants [as an example, the immunolocalization of LM5 in *bgal3* and WT(*bgal3*) is represented in Fig. 5]. In leaves, the high content of β(1,4)-galactan was found in epidermis and vascular tissues, and no differences were detected in mutant plants regarding to their corresponding WT, as can be seen for *bgal3* and WT(*bgal3*), as an example (Fig. 5*C*).

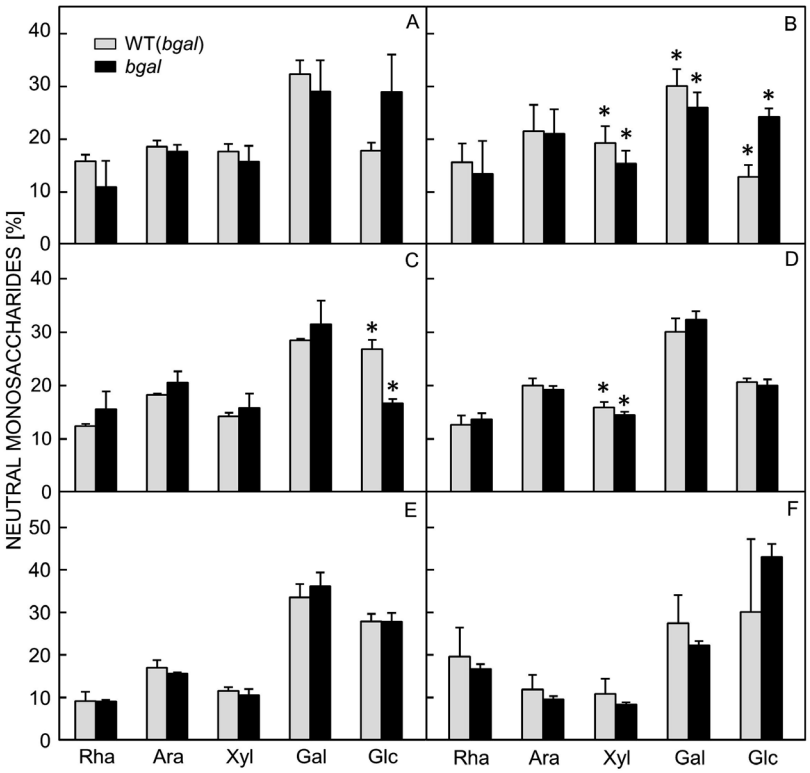

Fig. 4. Percentage of rhamnose (Rha), arabinose (Ara), xylose (Xyl), galactose (Gal), and glucose (Glc) in cell wall monosaccharides in 10-d-old *bgal1* (*A*), *bgal2* (*B*), *bgal3* (*C*), *bgl4* (*D*), *bgal5* (*E*), *bgal12* (*F*) mutants and their corresponding WT(*bgal*) seedlings. Means ± SEs, $n = 3$. The statistically significant differences ($\alpha = 0.05$) are indicated by *asterisks*.

Discussion

In *Arabidopsis thaliana,* the β-galactosidases are encoded by a multigene family. It is possible that each isoform presents different function in the plant development. This work focuses on the six members of the subfamily a1 (namely AtBGAL1, 2, 3, 4, 5, and 12). The aim was to study the phenotypic changes caused by the lack of each β-galactosidase by using the respective knockout mutant. Once the homozygous lines both for mutants and WT plants were obtained and the absence of transcripts of the mutated genes was checked, a complete characterization of their morphology and cell wall composition was performed. The mutant lines were always compared to their WT type obtained after segregation of a heterozygous mutant. Further, green seedlings grown under a 16-h photoperiod with an irradiance of 80 - 100 µmol m^{-2} s^{-1} were compared with etiolated seedlings grown in the darkness.

Three- and 10-d-old seedlings were used for cell wall studies, according to *AtBGAL* transcript accumulation in initial stages of development (Moneo-Sánchez *et al.* 2016). Some significant differences were observed between the mutants and their corresponding WT such as a higher β-galactosidasic activity in cell wall protein extracts of 10-d-old seedlings of *bgal4* mutant compared to its WT (Fig. 1) or slight variations in the proportions of glucose, galactose, or xylose in *bgal2* and *bgal4* mutants (Fig. 4). However, these slight changes did not result in morphological changes along the plant development. By methods, such as FTIR spectroscopy, it was not possible to establish differences in cell walls between *bgal2*, *bgal4*, and *bgal12* and their corresponding WT (Fig. 4 Suppl), and only small variations were founds between *bgal1*, *bgal3*, and *bgal 5* and their WT (Fig. 5 Suppl.): a higher proportion in pectic polymers (Kačuráková *et al.* 2000, Černá *et al.* 2003, Morris *et al*. 2003, Alonso-Simón *et al.* 2011, Dokken and Davis 2011) in the cell wall of *bgal1* and *bgal5* seedlings and an increase of uronic acids in homogalacturonan (HG) and cellulose

(Coimbra *et al*. 1999, Wilson *et al.* 2000, Kačuráková and Wilson 2001) in the *bgal3* seedlings. None of the mutant seedlings displayed a clear reduction in galactan content or in other possible substrates of β-galactosidases, but we have to keep in mind that complete seedlings were used in this study, so the absence of more marked changes could be due to overlapping absorbance derived from the complex mixture of polysaccharides present in the starting material (Kačuráková and Wilson 2001, Szymanska-Chargot and Zdunek 2013).

Taking in mind that the absence of the different

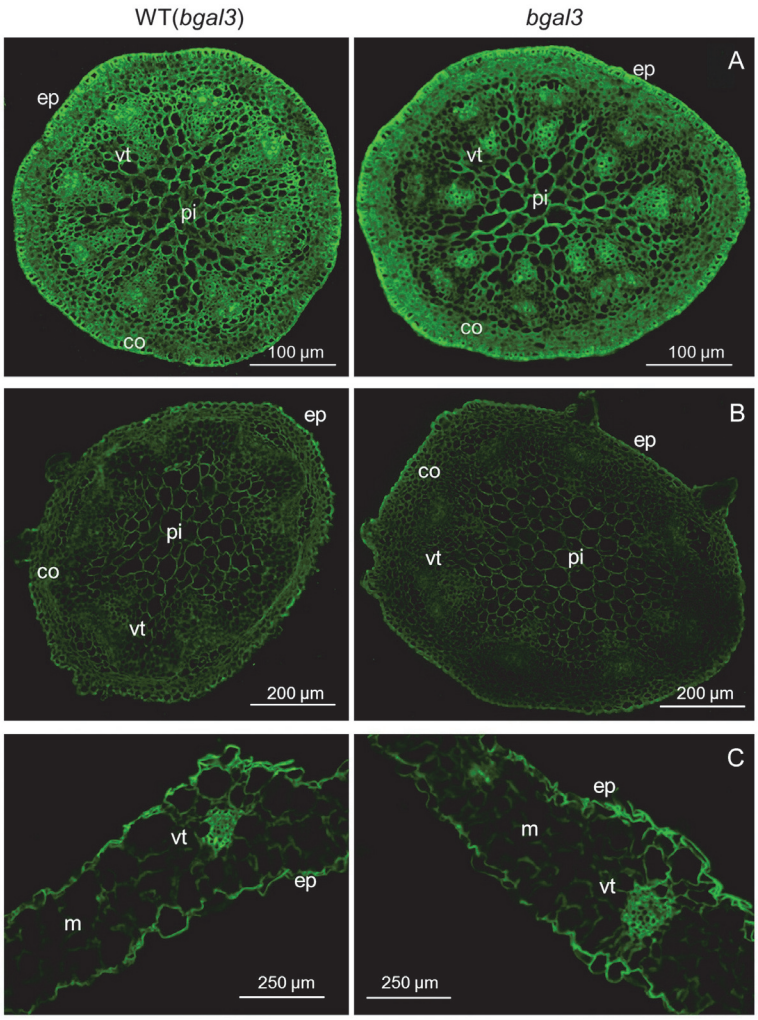

Fig. 5. Immunolocalization of β-(1-4)-galactan in the 3rd (*A*) and 1st internodes (*B*), and in rosette leaves (*C*) of *bgal3* and *WT*(*bgal3*) plants with LM5 antibody (co - cortex, ep - epidermis, m - mesophyll, pi - pith, vt - vascular tissue).

β-galactosidases of the a1 subfamily in the mutants could be related to changes in the distribution of the β(1,4)-galactan side chains of RGI, one of the natural substrates reported for these proteins, we carried out immunolocalization of the β(1,4)-galactan chains by using LM5 antibodies (Fig. 5). Specific labelling with LM5 was detected when cross sections were analysed, with a decreasing intensity from young towards old tissues in floral stems and a high labelling in epidermis and vascular tissues in leaves. However, no significant differences could be observed in the distribution of LM5 labelling between WT and mutant plants.

The absence of significant phenotypic changes in the β-galactosidase subfamily a1 mutants lead us to think that some other β-galactosidases, probably from the same subfamily a1, could compensate for the loss of function of these proteins, suggesting functional redundancy between different members within this multigene family.

The phenomenon of functional redundancy is not uncommon in the plant kingdom and has been described many times in different multigene families encoding proteins related to the cell wall metabolism, as in the cellulose synthases (CESA) or xyloglucan endotransglucosylase/hydrolase (XTH) families (Osato *et al.* 2006, Desprez *et al.* 2007, Persson *et al.* 2007, Hara *et al.* 2014). In this work, we could not find any phenomenon of redundancy among the BGAL included in the *Arabidopsis* subfamily a1 (Fig. 2), which could explain

the lack of phenotypic changes between mutants and WT plants. However, we cannot discard the possibility of redundancy among other BGALs different from the a1 subfamily.

The absence of phenotypic changes in the mutants analysed in this work could be explained if we consider the profiles of transcript accumulation of each member of the a1 subfamily (Moneo-Sánchez *et al.* 2016) whose expressions overlaps at certain developmental stages and organs. Thus, these facts could involve a functional compensation among the six members of the subfamily a1, when one of them is not present or is no longer functional, which happens in the mutants. This phenomenon of overlapping was also established in the activity of the promoters of subfamily a1 β-galactosidase genes in several developmental processes (Albornos *et al.* 2012).

As a result of these findings, the need arises to generate multiple mutants for different locus of genes *AtBGAL* from this subfamily, aiming that the possible phenotypic differences in the multiple mutants will help to establish the precise function of the β-galactosidase subfamily a1 in the physiology of plants.

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