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María Moneo-Sánchez[1]; Andrea Vaquero-Rodríguez[1]; Josefina Hernández-Nistal[2]; Lucía Albornos[1]; Paul Knox[3]; Berta Dopico[1]; Emilia Labrador[1]; Ignacio Martín[1*].

Pectic Galactan Affects Cell Wall Architecture During Secondary Cell Wall Deposition.

[1]Departamento de Botánica y Fisiología Vegetal, Centro Hispano Luso de Investigaciones Agrarias (CIALE), Universidad de Salamanca. Salamanca 37007. Spain.

[2]Departamento de Biología Funcional. Universidad de Santiago de Compostela. Lugo 27002. Spain.

[3]Centre for Plant Sciences, Faculty of Biological Sciences, University of Leeds, LS2 9JT (United Kingdom).

[*]Corresponding Author: Ignacio Martín. Email: a56562@usal.es; Fax: +34 923 294515.

Main conclusion

β-(1,4)-galactan determines the interactions between different matrix polysaccharides and cellulose during the cessation of cell elongation

Abstract

Despite recent advances regarding the role of pectic β-(1,4)-galactan neutral side chains in primary cell wall remodelling during growth and cell elongation, little is known about the specific function of this polymer in other developmental processes. We have used transgenic Arabidopsis plants overproducing chickpea βI-Gal β-galactosidase under the 35S CaMV promoter (35S::βI-Gal) with reduced galactan levels in the basal non-elongating floral stem internodes to gain insight into the role of β-(1,4)-galactan in cell wall architecture during the cessation of elongation and the beginning of secondary growth. The loss of galactan mediated by βI-Gal in 35S::βI-Gal plants is accompanied by a reduction in the levels of KOH-extracted xyloglucan and an increase in the levels of xyloglucan released by a cellulose specific endoglucanase. These variations in cellulose-xyloglucan interactions cause an altered xylan and mannan deposition in the cell wall that in turn results in a deficient lignin deposition. Considering these results, we can state that β-(1,4)-galactan plays a key structural role in the correct organization of the different domains of the cell wall during the cessation of growth and the early events of secondary cell wall development. These findings reinforce the notion that there is a mutual dependence between the different polysaccharides and lignin polymers to form an organized and functional cell wall.

Keywords: β-(1,4)-galactan, β-galactosidase, Arabidopsis, Cell Wall, Hemicellulose

Introduction

Plant cell walls are highly organized and dynamic structures primarily composed of a mixture of polysaccharides, proteins and phenolic compounds. The main polysaccharides of the cell wall, namely cellulose, hemicelluloses and pectins, interact with each other and with other cell wall components through covalent and non-covalent bonds. These complex interactions, along with the relative proportions of the different polymers, define the physicochemical properties of the wall and the distinct features of individual cells throughout development (Carpita and Gibeaut 1993; Park and Cosgrove 2015).

The specific nature of these interactions and the precise cell wall polymers involved have not been completely elucidated in all developmental processes. However, several models have been proposed to explain the structural organization of the cell wall, mainly focusing on primary cell wall structure and its remodelling during cell elongation. Although classical cell wall models assume that the cellulose-xyloglucan network is the main load-bearing structure, more recent studies suggest pectins play a major role in the maintenance of cell wall architecture (Carpita and Gibeaut 1993; Cosgrove 2005; Peaucelle et al. 2012). Several interactions between pectic polysaccharides and cellulose, hemicelluloses and cell wall proteins have been proposed (Peaucelle et al. 2012; Broxterman and Schols 2018; Cornuault et al. 2018).

More specifically, among pectic polysaccharides, rhamnogalacturonan-I (RG-I) neutral galactan and arabinan side chains seem to play crucial roles in cell wall architecture. In addition, they have been proposed to be determining factors in interactions with other components, such as cellulose and hemicelluloses, during various processes (Popper and Fry 2008; Zykwinska et al. 2008; Wang et al. 2019). Most of these studies are aimed at determining the role of these pectic side chains in primary cell wall architecture and cell elongation. In a recent study Moneo-Sánchez et al. (2019), by means of altering β-(1,4)-galactan levels in *Arabidopsis thaliana* elongating organs, provided evidence that this polymer is directly involved in etiolated hypocotyl and apical floral stem internode elongation. Moreover, it was shown that the amount of this pectic side chain may be controlling the degree of interaction between cellulose and XG.

The prior results suggest that pectic neutral side chains, and more specifically β-(1,4)-galactans, have a role in primary cell wall remodelling during growth and cell elongation. However, little is known about the specific function of these polymers in the cell wall and during the cessation of elongation and the onset of secondary growth and extensive secondary wall deposition. Depending on the organ, and even on the specific cell type, the cessation of elongation occurs in a series of phases, starting from the first moments of the transition from primary to secondary growth to the synthesis and deposition of cell wall components. This therefore implies the marked reorganization of the cell wall, including a shift in the pectin/cellulose balance, increased deposition of xylan and mannan polysaccharides, and in some cases, lignification of the cell wall (Hao et al. 2014; Hao and Mohnen 2014; Hernández-Gómez et al. 2015).

Given the aforementioned involvement of β-(1,4)-galactan in XG interactions with cellulose (Moneo-Sánchez et al. 2019), and taking into account previous reports about the possible

interactions of pectic polysaccharides with other cell wall components, the main objective of this work was to study the function of pectic galactan in cell wall remodelling during the transition between primary and secondary cell wall formation. For this purpose, we have used previously generated transgenic Arabidopsis plants overproducing chickpea βI-Gal β-galactosidase, under the control of the 35S CaMV promoter (35S::βI-Gal), with reduced galactan levels in apical stem internodes (Moneo-Sánchez et al. 2019). The characterization of the basal stem internodes precisely at the moment of cell elongation cessation, during the first stages of secondary growth and secondary cell wall deposition, has shed light on the interactions between the cell wall polymers at this transition stage.

Materials and Methods

Plant material and growth conditions

Arabidopsis thaliana Columbia-0 (Col-0) ecotype and transgenic Arabidopsis plants overproducing chickpea βI-Gal β-galactosidase (coded by *CarBGal1*) under the 35S CaMV promoter (35S::βI-Gal plants) were used. The 35S::βI-Gal plants were generated in previous work. Detailed information on vector construction, plant transformation and line selection is available in Moneo-Sánchez et al. (2019). Seeds from WT and 35S::βI-Gal plants were surface sterilized and grown in solid MS medium (Murashige and Skoog 1962) as described in Izquierdo et al. (2018) in a growth chamber (Aralab, Portugal) at 22ºC with 16-h photoperiod (80-100 µmol m^{-2} s^{-1} irradiance). Ten-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 until basal floral stem internodes were collected from 32-d-old plants, when elongation of the basal internode has already ceased. Pieces of 1 cm were cut using a razorblade from the most basal zone of the floral stem, and were immediately frozen in liquid nitrogen for cell wall analyses or immersed in a fixative solution for carrying out the staining/immunolocalization studies.

For agroinfiltration experiments, *Nicotiana benthamiana* seeds were sown in potting soil and allowed to grow for 6 weeks in a growth chamber (Aralab, Portugal) at 25°C and 16-hour (h) photoperiod.

Preparation of cell wall extracts

Basal internodes from WT and 35S::βI-Gal plants were used for cell wall extracts preparation, according to the method described by Cornuault et al. (2014). Freeze-dried material was ground for 2 min in a Retsch mixer mill MM400 (Sarstedt, Germany) at 30 oscillations/s and washed with 70% and 90% ethanol/H_2O (v/v), chloroform methanol (1:1) and acetone to obtain the alcohol insoluble residue (AIR) material. Cell wall components were extracted sequentially from 1 mg of AIR with 500 µl H_2O during 20 min in a mixer at 30 oscillations per second. After centrifugation at 14000 g for 15 min, the supernatant was collected and the remaining material was further extracted with 500 µl of 50 mM CDTA, pH 7.5 and 4 M KOH containing 1% w/v $NaBH_4$ with the same conditions. The pH of KOH extract was neutralized with 80% v/v acetic acid. All

extracts were stored at 20ºC until use. The remaining residue was considered the cellulosic fraction.

Analysis of the cellulosic fraction

The cellulosic fraction was washed sequentially two times with 70% ethanol and H_2O for 20 min and centrifuged at 14000 g for 12 min after each wash. The cellulosic residue was incubated with 18 U of a cellulose-specific endoglucanase from *Aspergillus niger* (Megazyme, Ireland) 0.1 M Na-acetate buffer pH 4.5 (containing 0.02% sodium azide) at 37ºC for 48 h. For all samples, a control with no enzyme was performed under the same conditions. The sugars released to the incubation media were collected (14000 g for 20 min), neutralised with 1 M Na_2CO_3 and analysed by ELISA as described below.

Anion-exchange epitope detection chromatography (AE-EDC)

For anion exchange epitope detection chromatography (AE-EDC) of the polysaccharides present in H_2O, CDTA and KOH extracts, the method described in Cornuault et al. (2014) was followed. For anion-exchange chromatography, 50 µl aliquots of the H_2O, CDTA or KOH extracts were diluted in 2.5 ml H_2O and eluted through a 1 ml Hi-Trap ANX FF column (GE Healthcare, UK) using a BioLogic LP system (Bio-Rad, USA). Samples were eluted with 20 mM sodium acetate buffer, pH 4.5, from 0-2 min, followed by a linear gradient from 0% to 50% 0.6 M NaCl in 50 mM sodium acetate buffer, pH 4.5 (25 min), followed by 50% to 100% 0.6 M NaCl (31 min) and 0.6 M NaCl to 48 min. All steps were conducted at 1 ml/min flow-rate. The fractions were neutralized with 50 µl of 1 M Na_2CO_3. One hundred µl aliquots were incubated in NUNC Maxisorp microtiter plate wells (Thermo Fisher Scientific, USA) overnight at 4°C and used in ELISA analysis. ELISA assays were conducted as described in Moneo-Sánchez et al. (2019) using a 1:25 dilution of primary antibodies and 1:1000 dilution of secondary antibody (anti-rat or anti-mouse horseradish peroxidase-conjugated IgG; Sigma, USA) in 5% w/v milk powder/PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4). Plates were developed using 100 µl of substrate per well (0.1 M sodium acetate buffer, pH 6, 1% tetramethyl benzidine, 0.006% H_2O_2). The reaction was stopped with 50 µl of 2.5 M H_2SO_4 and the absorbance read at 450 nm.

Antibodies used

Monoclonal antibodies against pectic polysaccharides used in this study include LM5, which recognizes a minimum of three sugar residues of β-(1,4)-galactan (Jones et al. 1996; Andersen et al. 2016) and JIM7, which recognizes methyl-esterified epitopes of homogalacturonan (HG) but does not bind to un-esterified homogalacturonan (Verhertbruggen et al. 2009). Regarding xyloglucan (XG), three antibodies were used: anti-fucosylated XG CCRC-M1, that recognizes terminal fucosyl residues linked α-(1,2) to a galactosyl residue (Puhlmann et al. 1994); LM25, that binds the XG backbone motif XXXG and galactosylated XXLG/XLLG epitopes (Pedersen et al. 2012); and CCRC-M100, which recognizes the unsubstituted motif XXXG in XG backbone (Pattathil et al 2010; Zabotina et al. 2012). The nomenclature proposed by Fry et al. (1993) for XG substitutions is used in this work. For xylan analyses we used two antibodies specific to the

xylan backbone (CCRC-M139 and LM11; McCartney et al. 2005; Ruprecht et al. 2017) and the glucuronoxylan specific antibody LM28 (Cornuault et al. 2015). Finally, we used the anti-mannan LM21 antibody, that binds to β-(1,4)-manno-oligosaccharides from mannan, glucomannan and galactomannan polysaccharides (Marcus et al. 2010). All these antibodies are available on CarboSource Services (http://www.carbosource.net) and PlantProbes (www.plantprobes.net) websites.

Immunolocalization of βI-Gal protein and cell wall polysaccharides

Sample preparation and incubation with antibodies for immunofluorescence labelling of cell wall polysaccharides were performed as described in Moneo-Sánchez et al. (2018). All monoclonal antibodies were used at 1:5 dilution, and the corresponding secondary antibodies, both anti-rat and anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC) (Sigma, USA), were applied at 1:300. Prior to immunolocalization of hemicelluloses, sections were treated with a pectate lyase from *Cellvibrio japonicus* (Megazyme, Ireland), according to Marcus et al. (2008). When necessary, the sections were stained with Calcofluor White (0.2 µg/mL) (Fluorescent Brightner 28, Sigma, USA) and mounted in Citifluor AF1 (Agar Scientific, UK). Images were taken using a Leica DM 4000 LED microscope equipped with a Leica DFC550 camera and HCX PL Fluotar 20x/0.5 objective (Leica Microsystems, Germany).

Generation of anti-βI-Gal antibodies and protein immunolocalization studies were conducted as described in Martín et al (2011).

Cellulose determination

To determine the cellulose content, 1 mg of AIR was treated with 2 N TFA at 120°C for 2 h. The insoluble residue was hydrolysed with H_2SO_4 for 90 min at RT, and subsequently diluted with H_2O to a H_2SO_4 concentration of 1 N and hydrolysed at 100°C for 2 h. After neutralizing with $Ba(OH)_2$, the amount of cellulose was determined by the phenol-sulphuric method (Dubois et al. 1956) using glucose as a standard.

Lignin analyses

For acetyl bromide soluble lignin (ABSL) determination, the method described in Yan et al. (2012) was followed, using 1 mg of AIR in 25% acetylbromide (v/v in glacial acetic acid). Samples were incubated for 30 min 70ºC and then treated with 2 M NaOH, glacial acetic acid and 7.5 M hydroxylamine hydrochloride. After centrifuging at 4000 g for 10 min, the supernatant was diluted 50-fold with glacial acetic acid and the absorbance determined at 280 nm.

The 2D HSQC (Heteronuclear Single-Quantum Correlation) NMR (nuclear magnetic resonance) experiments were conducted by the General Service of RMN of the University of Salamanca (Spain) on a Bruker Avance Neo 400 MHz equipped with a Prodigy Cryoprobe. The ABSL was extracted twice with ethyl acetate, neutralized with sodium sulphate, vacuum-dried and dissolved in DMSO/d6. The spectral widths were 5000 and 20000 Hz for the ^{1}H and ^{13}C dimensions, respectively. The number of collected complex points was 1024 for the ^{1}H dimension with a recycle delay of 1.5 seconds. The number of transients was 64, and 256 time increments were

always recorded in the ^{13}C dimension. The 1JCH used was 145 Hz. Prior to Fourier transformation, the data matrices were zero filled to 1024 points in the ^{13}C dimension. Data processing was performed using standard Bruker Topspin-NMR software. Chemical shifts are given in ppm and peaks were assigned by comparison of published spectra and available databases (Ralph et al. 2009; Yuan et al. 2011; Chong et al., 2014). Lignin staining by the safranin/astra blue method was conducted according to Srebotnik and Messner (1994) with 1% (w/v) aqueous safranin-O and 1% (w/v) aqueous astra-blue.

Saccharification assays

Saccharification assays on basal stem internodes were performed according to Van Acker et al. (2014) with 10 mg of total fresh weigh biomass. Plant material was either pre-treated with 1 N HCl at 80°C for 2 h or 62.5 mM NaOH at 90 °C for 3 h. After extensive washing with 70% ethanol overnight at 55°C and two additional washes with 70% ethanol at RT for 5 min, plant material was air dried and incubated with the enzyme mix consisting of desalted cellulase from *Trichoderma reseei* ATCC 26921 (Sigma, USA) and β-glucosidase from *Aspergillus niger* (Sigma, USA) in a 5:3 ratio (desalted β-glucosidase was 50-fold diluted prior to mixing with cellulase). Samples were dissolved in acetic acid buffer (pH 4.8) and the enzyme mix was added with an activity of 0.04 filter paper units. Glucose released was calculated at 1, 2, 3, 4, 5, 6, 12, 24, 48, and 60 h with the D-Glucose Assay Kit from Megazyme (Ireland).

Agroinfiltration of *Nicotiana benthamiana* leaves and activity assays

For heterologous expression in *N. benthamiana* leaves, *CarBGal-1* ORF was PCR amplified adding the attB1 and attB2 sequences at 5′ - and 3′ -ends and cloned into the pDONR201 vector. The entry clones were used in LR reaction with the pEAQ-HT-DEST1 vector (Sainsbury et al. 2009; provided by Plant Bioscience Ltd, Norwich, UK). All constructs were verified by sequencing. The primers used are listed on Supplementary Table S1. The expression constructs and the GFP-containing pEAQ-GFP-HT vector used as control (also provided by Plant Bioscience Ltd, Norwich, UK) were electroporated into *Agrobacterium tumefaciens* strain AGL1 and *N. benthamiana* leaves were agroinfiltrated as indicated in Izquierdo et al. (2018).

Cell wall proteins were isolated from *N. benthamiana* leaves 6 d after the inoculation according to Izquierdo et al. (2018) and quantified with the Protein Assay from Bio-Rad (USA). The hydrolytic activity of the purified protein was tested using p-nitrophenyl (pNP) derivatives as substrates as described in Dopico et al. (1989) pNP-α-L-arabinofuranoside, pNP-α-L-fucopyranoside, pNP-α-D-galactopyranoside, pNP-β-D-galactopyranoside, pNP-α-D-glucopyranoside, pNP-β-D-glucopyranoside, pNP-β-D-mannopyranoside, pNP-β-D-xylopyranoside, from Sigma (USA) were used. Activity was also assayed against lupin β-(1,4)-galactan pre-treated with α-L-arabinofuranosidase and a mix of xyloglucan oligosaccharides containing β-(1,2)-galactose (Megazyme, Ireland); β-(1,4)-galactobiose, larch wood arabinogalactan and lactose (Sigma, USA); β-(1,3)-galactan, β-(1,3)(1,6)-galactan, β-(1,3)-galactobiose, β-(1,3)-galactotriose, β-(1,6)-galactobiose and β-(1,6)-galactotriose (kindly supplied by Dr. T. Kotake, Saitama University, Japan). The reaction mixtures were prepared

according to Izquierdo et al. (2018). The reaction products were separated by thin layer chromatography on silica gel plates (Merck, Germany) and the galactose released was quantified in a CS-9000 Dual-wavelength Flying-spot scanner densitometer (Shimadzu, Japan), using commercial galactose (Sigma, USA) as standard. βI-Gal activity was estimated by subtracting the activity of leaves transformed with the GFP construct.

To analyse β-galactosidase activity *in planta*, the chromogenic substrate Magenta-Gal (Sigma, USA) was used following the protocol described by Pichon et al. (1994), except the step involving paraformaldehyde fixation was omitted. Once the reaction was conducted, the basal internodes were fixed and included in paraffin according to the method described by Martín et al. (2011).

Results

Phenotypic characterization of 35S::βI-Gal basal internodes

As an approach to elucidate the function of β-(1,4)-galactan during the end of cell elongation and the onset of secondary growth, we used previously generated Arabidopsis 35S::βI-Gal plants overexpressing *C. arietinum* βI-Gal β-galactosidase. Cell wall characterization was conducted using the basal floral stem internodes harvested from 32-day-old wild-type (32-d-old WT) and transgenic plants, the timepoint at which elongation has already ceased (Fig. 1a). 35S::βI-Gal plants showed no significant differences from WT plants regarding the length of the basal internodes (Fig. 1a). After transverse sectioning of the basal internode, a slight decrease (approximately 20%) in the diameter of stems of transgenic plants was detected (Fig. 1b) with no other visible phenotypic alterations in plant structure (Fig. 1c).

Analysis of cell wall polysaccharides in H_2O and CDTA extracts

The accumulation of βI-Gal protein in 35S::βI-Gal basal stem internodes was determined by immunolocalization studies with anti anti-βI-Gal antibodies (Supplementary Fig. S1). After confirmation of the increase in β-galactosidase activity in 35S::βI-Gal internodes using the chromogenic substrate Magenta-Gal (Supplementary Fig. S2), we checked for any possible variations in β-(1,4)-galactan levels, and whether the variations detected could cause alterations in the rest of the polysaccharides.

After sequential extraction of cell wall polysaccharides with H_2O and CDTA, the levels of the different polysaccharides present in these extracts were evaluated, both in WT and in transgenic plants by anion-exchange epitope detection chromatography (AE-EDC) using the epitope-specific monoclonal antibodies indicated in the Materials and Methods section. Elution gradient and conductivity curves are shown in Supplementary Fig. S3.

The pectic β-(1,4)-galactan epitope, recognized by LM5, is reduced in 35S::βI-Gal with respect to WT, with a greater reduction in the H_2O than in the CDTA extract (Fig. 2a). The other pectic epitopes analysed exhibited no significant changes, except for an increase in the methyl-

esterified homogalacturonan (HG) epitope recognized by the JIM7 antibody in the CDTA extract from 35S::βI-Gal internodes (Fig. 2b).

Among all the hemicelluloses analysed in these fractions, only the xyloglucan (XG) epitope recognized by LM25 antibody was detected, both in the H_2O and CDTA extracts (Fig. 2c). Despite the neutral character of this polysaccharide, the main elution peak was detected in the later elution zone, coinciding with the galactan peak. This suggested that all the XG present in these fractions is attached to the pectic macromolecule, including RG-I and HG. The epitope recognized by LM25, in the H_2O and CDTA extracts, decreased in 35S::βI-Gal plants with respect to the WT.

Analysis of KOH-extracted hemicelluloses

Once the reduction of β-(1,4)-galactan in the basal internodes of the 35S::βI-Gal plants was established, and taking into account the reduction of the XG epitope recognized by LM25, we analysed the hemicellulose levels present in WT and 35S::βI-Gal cell walls. To this end, the hemicellulose-enriched cell wall fraction extracted with KOH was analysed by AE-EDC with specific antibodies against different XG structural motifs: CCRC-M1 (fucosylated XG), LM25 (XXXG and XXLG/XLLG), and CCRC-M100 (unsubstituted XG motif XXXG). Likewise, antibodies against xylan backbone (CCRC-M139, LM11), glucuronoxylan (LM28), and mannan (LM21) were used.

As seen in Fig. 3, all XG epitopes were detected in the first eluted fractions. Transgenic 35S::βI-Gal plants show a marked reduction in XG epitopes recognized by CCRC-M1 and LM25. It should be noted that although this reduction was also observed with the CCRC-M100 antibody, the signal was quite low in both transgenic and WT plants.

As in the case of XG epitopes, the rest of the hemicelluloses analysed showed a marked decrease in 35S::βI-Gal extracts when compared to the WT, even in the case of the xylan epitope recognized by CCRC-M139, despite its low levels (Fig. 4a). This decrease is especially pronounced for LM11, with almost undetectable levels in the internodes of the transgenic plants (Fig. 4a). The mannan epitope recognized by LM21 also showed lower levels in 35S::βI-Gal extracts (Fig. 4b). All these hemicelluloses were mainly detected in the early eluting fractions (Fig. 4), except for glucuronoxylan (LM28) which elutes mainly in the intermediate zone of the gradient (coinciding with the increase in conductivity) reflecting its slightly acid character. However, a peak coinciding with the XG elution zone and a later elution peak in the pectin elution zone were also detected.

Substrate specificity of *C. arietinum* βI-Gal

Due to the evident changes in galactose substituted xyloglucan epitopes, we decided to transiently produce *C. arietinum* βI-Gal in *N. benthamiana* leaves, and establish its substrate specificity to discard any possible action of this enzyme on XG.

As expected, when tested against synthetic pNP substrates (Table 1), the produced βI-Gal was mainly active against pNP-β-D-galactopyranoside substrate, although trace levels of activity were detected against pNP-α-D-galactopyranoside and pNP-α-D-glucopyranoside.

Activity analyses against galactose-containing oligo- and polysaccharides (Table 2) pointed to a high specificity of βI-Gal against β-(1,4) linkages, with the highest activity against β-(1,4)-galactan and β-(1,4)-galactobiose. No activity was detected against XG oligosaccharides (containing β-(1,2)-linked galactose), or against arabinogalactan, gum arabic, β-(1,3)-galactan and β-(1,3)(1,6)-arabinogalactan purified from AGP; although trace levels of activity were detected against β-(1,3) galactobiose and β-(1,6) oligosaccharides.

Analysis of hemicelluloses released after endoglucanase treatment

To determine if the reduction of hemicelluloses in H_2O, CDTA and KOH cell wall extracts from 35S::βI-Gal plants could be related to variations in hemicellulose interactions with cellulose microfibrils, the cellulose fraction obtained after the sequential extraction with H_2O, CDTA and KOH was treated with a cellulose-specific endoglucanase. In addition, the hemicelluloses released after this treatment were analysed by ELISA using the antibodies mentioned above.

In the case of XG epitopes (Fig. 5a), a slight reduction in the epitope recognized by CCRC-M1 (fucosylated XG) was observed, although the most notable differences were detected with the LM25 antibody which showed a marked increase in 35S::βI-Gal plants with respect to the WT. When heteroxylan and mannan were analysed (Fig. 5b), the most remarkable change~~s~~ were detected with the LM28 antibody (glucuronoxylan), with a notable reduction in 35S::βI-Gal basal internodes.

Immunolocalization of cell wall polysaccharides

Immunolabelling of cross sections of the basal internodes (Fig. 6) with the LM5 antibody (β-(1,4)-galactan) showed that in 35S::βI-Gal plants this epitope was less abundant throughout the whole section. However, this reduction is more noted in the interfascicular region, with almost no detection, and in the pith parenchyma.

Regarding the hemicelluloses analysed, both the epitopes recognized by CCRC-M100 (unsubstituted XG motif XXXG) and CCRC-M139 (xylan backbone) were not detected, most probably due to the low levels shown in the AE-EDC studies.

XG epitopes recognized by CCRC-M1 (Fig. 6) were also reduced in 35S::βI-Gal internodes, especially in the pith parenchyma and the interfascicular region, while the epidermis, cortex and vascular tissue showed a more subtle reduction. This decreased signal in the transgenic line was not observed in the case of LM25, showing similar levels in WT and in 35S::βI-Gal sections.

Xylan and glucuronoxylan-containing epitopes (recognized by LM11 and LM28, respectively) were exclusively located in xylem cells, both in WT and 35S::βI-Gal internodes, and showed decreased levels in the transgenic line (Fig. 6). Mannan (recognized by LM21) was mainly detected in the xylem, with slight fluorescence also being observed in cortical parenchyma cells; although the reduced signal in 35S::βI-Gal was not as evident as for the xylan epitopes.

Lignin content in 35S::βI-Gal plants

To determine whether other characteristic events occurring during the cessation of elongation and the onset of secondary cell wall deposition were affected in 35S::βI-Gal internodes, and considering the altered hemicellulose content and the implication of these polymers in the correct deposition of lignin, we decided to analyse the lignin content in WT and transgenic internodes by the acetyl bromide soluble lignin (ABSL) method (Fig. 7).

Despite the low levels of solubilized lignin, quantification of the ABSL content indicated that the lignin content was significantly decreased in 35S::βI-Gal internodes (Fig. 7a), being almost 50% lower than in the WT. In a double staining with safranin (which stains lignified tissues in red) and astra blue (which gives blue coloration of cellulose only in the absence of lignin), the red coloration in xylem elements was only slightly more intense in WT than in 35S::βI-Gal section. Conversely, the blue staining was more evident in the interfascicular region of the transgenic internodes when compared to the WT (Fig. 7b).

The analysis of ABSL by 2D NMR spectroscopy supports the reduction of lignin in guaiacyl (G), syringyl (S) and *p*-hydroxyphenyl (H) subunits in transgenic plants as compared to the WT, as seen in the HSQC spectrum of aromatic region (Supplementary Fig. S4). More interestingly, the HSQC spectrum of the polysaccharide anomeric region (Fig. 7c) showed the same pattern in WT and 35S::βI-Gal, although a notable reduction in the cross signals corresponding to phenyl glycoside linkages (δC/δH 99.54/5.08-100.44/4.99) and 2-O-acetyl and 3-O-acetyl β-D-xylose (101.60/4.84-102.11/8.80) was observed in the transgenic line. This indicates a loss of interaction between the lignin and the polysaccharidic component of the cell wall.

Saccharification assays

The reduction in ABSL and the lack of variation in cellulose content in 35S::βI-Gal plants with respect to the WT (Supplementary Fig. S5) could result in an increase in cellulose accessibility that could in turn improve the saccharification efficiency. To check this possibility, we quantified the glucose released after hydrolysis of the basal internodes with a cellulase/β-glucosidase enzyme mix, both without any previous treatment and with acid (HCl) and alkali (NaOH) pre-treatments (Fig. 8). When no pre-treatment was applied, the glucose yield was similar in WT and transgenic internodes (Fig. 8). Conversely, both acid and alkali pre-treatments induced an increase of released glucose during the first 5 hours of the reaction (with the effect being more pronounced in the case of HCl treated samples). However, the final glucose yield was similar in WT and 35S::βI-Gal samples, which also showed similar levels to those reached in the untreated samples (Fig. 8).

Discussion

β-(1,4)-galactan is one of the main side chains of RG-I, which has been implicated as having a role in cellulose-xyloglucan interactions in the cell wall during elongation (Zykwinska et al. 2008; Moneo-Sánchez et al. 2019). However, the specific function of these side chains in other developmental processes has not been completely established.

The main objective of this work was to determine the function of β-(1,4)-galactan in the structural organization of the cell wall during the cessation of elongation and the onset and early steps of secondary growth. To do so, alterations of the cell wall polysaccharides in the basal floral stem internodes of Arabidopsis plants overproducing the β-galactosidase βI-Gal from *C. arietinum* (35S::βI-Gal) were studied. It has been shown that these plants have reduced levels of β-(1,4)-galactan with respect to the WT, when elongation has ceased (Fig. 1) and prior to an evident secondary cell wall thickening in the interfascicular region.

The transgenic 35S::βI-Gal plants, which display an increase in β-galactosidase activity (Supplementary Fig. S2), have reduced levels of β-(1,4)-galactan in the cell wall, both in the H_2O and the CDTA extracted fractions (Fig. 2a) of these basal internodes. This reduction in galactan is most evident in the pith parenchyma and especially in the interfascicular region (Fig. 6), the area of differentiation of interfascicular fibres, a tissue characterized by a thickened secondary wall. In similar tissues, such as flax, cotton and poplar fibres or even in chickpea phloem fibres in which βI-Gal is localized (Martín et al. 2011), a highly active galactan metabolism is necessary for the complete differentiation of these cell types (Gorshkova and Morvan 2006; Martín et al. 2008; Singh et al. 2009; Gorshkova et al. 2015).

The reduction of β-(1,4)-galactan levels in the cell wall is only compensated by an increase in methyl-esterified HG, as evidenced by the results of the AE-EDC studies (Fig. 2b). Similar results were observed in potato tubers (Martín et al. 2005), where this compensating mechanism is supposed to be responsible for the lack of severe growth phenotypes. Thus, the fact that only a reduction of 20% in stem diameter is observed in 35S::βI-Gal internodes (Fig. 1), as previously observed in Arabidopsis stems with reduced RG-I neutral side-chains (Obro et al. 2009), may be a consequence of the increase in HG.

Besides this connection between the levels of β-(1,4)-galactan and HG, our results also implicate β-(1,4)-galactan neutral side chains in pectin-XG interactions. In H_2O and CDTA extracts from cell walls of WT basal stem internodes, only a XG subpopulation (recognized by LM25) (Fig. 2c) is detected. This coincides with the elution peak of the pectic macromolecule, indicating an association between pectins and XG *in muro* as previously described by Cornuault et al. (2014, 2018). In addition, this XG population markedly decreases in the cell wall extracts of 35S::βI-Gal basal internodes, coinciding with the reduction of the galactan subpopulation that co-elutes with the pectic macromolecule. This result supports previously published data that

proposes there is a direct interaction between the neutral side-chains of the pectins and XG (Cumming et al. 2005; Popper and Fry 2008).

β-(1,4)-galactan controls not only XG-pectin interactions, but also influences the levels of all epitopes of alkali soluble XG, which are also notably reduced in 35S::βI-Gal internodes when compared to the WT (Fig. 3). The fact that all these XG populations in KOH extracts are detected as a neutral peak, which indicates they are not interacting with pectins, suggests that the observed decrease in β-(1,4)-galactan induces changes in XG partitioning in the cell wall. Taking this into account, it seems likely that XG-cellulose interactions are affected. To clarify this point, the cellulosic residue obtained after sequential extraction with H_2O, CDTA and KOH was treated with a cellulose specific endoglucanase, which releases strongly interacting hemicelluloses or those trapped between cellulose microfibrills (Pauly et al. 1999; Park and Cosgrove 2015). The XG released shows a higher proportion of the epitopes recognized by LM25 (XXXG and galactosylated XXLG/XLLG) in the cell wall of basal internodes of the 35S::βI-Gal plants when compared to the WT (Fig. 5). This finding suggests a shift in the distribution of this XG epitope, with diminished levels in the KOH extracts of the cell wall. This could explain the lack of changes in the immunolocalocalization experiments involving LM25 (Fig. 6).

Thus, the β-(1,4)-galactan side chains determine the proportion of the XG that binds to the surface of the microfibrills (extracted with KOH) and the amount of XG trapped between them (released by endoglucanase). These results reinforce the notion, based on *in vitro* studies, that pectins and XG are in direct competition to bind cellulose (Zykwinska et al. 2008), and confirm similar results obtained using Arabidopsis elongating apical stem internodes and etiolated hypocotyls (Moneo-Sánchez et al. 2019). According to our results, this competition occurring in elongating tissues is also maintained during the cessation of elongation and is determined by pectic β-(1,4)-galactan and affects at least the XG epitope recognized by LM25. Moreover, this shift in the distribution of XG could also be responsible for the lack of a stronger phenotype in 35S::βI-Gal internodes and in turn could be reflecting the importance of XG partitioning in maintaining the structural integrity of the cell wall.

One of the most remarkable events occurring during the cessation of elongation and the beginning of secondary cell wall formation is a marked deposition of heteroxylans and mannans (Hernández-Gómez et al. 2015; Peralta et al. 2017). However, our AE-EDC studies on the hemicellulose rich KOH fraction (Fig. 4) show a reduction of the xylan backbone (CCRC-M139, LM11), glucuronoxylan (LM28) and mannan (LM21, which recognizes mannan, glucomannan and galactomannan) in the 35S::βI-Gal plants when compared to the WT, indicating that transgenic internodes with reduced levels of β-(1,4)-galactan are impaired in one of the key features of the end of elongation.

Several authors have proposed, based mainly on *in vitro* studies, that heteroxylan chains may bind XG during their deposition in the cell wall (Brett et al. 1997; Rizk et al. 2000; Kerr and Fry 2004). Considering the diminished XG levels detected in KOH extracts from 35S::βI-Gal cell walls (Fig. 3), we can propose that the reduction of the levels of heteroxylan and mannan epitopes

in 35S::βI-Gal plants (Fig. 4) is attributable to the alteration of the levels of XG. According to this, the correct organization of XG would be conditioning the adequate deposition of the rest of the hemicelluloses, which is supported by the fact that the main elution peaks of all hemicelluloses analysed co-elute in the same zone of the gradient in AE-EDC studies (Figs. 3 and 4). Moreover, the relationship between the levels of XG and the rest of the hemicelluloses would also occur at the level of the polysaccharides with stronger interaction with cellulose, released by the endoglucanase treatment (Fig. 5). In this case, the increase of XG could be limiting the access of other hemicelluloses and is responsible for reduced levels of glucuronoxylan and to a lesser extent of mannan in this fraction of the cell wall. In this way, the altered heteroxylan and mannan levels in 35S::βI-Gal plants would not be a direct consequence of the β-(1,4)-galactan reduction observed in the transgenic internodes, but an indirect effect of the abnormal XG distribution induced by the loss of these pectic β-(1,4)-galactan side chains, although some authors have described a physical interaction between heteroxylan and pectins (Tan et al. 2013; Ralet et al. 2016),

Considering these data, it seems clear that some of the key events affecting cell wall structure during the cessation of elongation are altered in 35S::βI-Gal internodes. Therefore, we decided to investigate whether these plants present some alteration in the lignin levels, considering the strong implication of the adequate organization of the hemicelluloses in the subsequent deposition of lignin in the secondary walls (Mortimer et al. 2010) and the reduced levels of heteroxylan in 35S::βI-Gal plants, especially in lignified xylem elements (Fig. 6). As expected, the acetyl bromide soluble lignin (ABSL) content is markedly reduced in the cell wall of 35S::βI-Gal internodes (Fig. 7a and Supplementary Fig. S4). Furthermore the 2D NMR data (Fig. 7c) show decreased signals in the region of phenyl glycoside linkages and acetylated xylose units, confirming that the decreased levels of heteroxylan induced by the lack of pectic galactan has impaired lignin-hemicellulose interactions and lignin deposition. Also, the increased staining in the interfascicular region with astra blue in 35S::βI-Gal internodes (Fig. 7b) indicates that lignin deposition in this region may occur early in development, before an evident thickening of the interfascicular fibres. This would also suggest that proper cell wall organization and adequate hemicellulose levels are required for this process, as previously proposed (Hao et al. 2014; Hao and Mohnen 2014).

Since the lignin content is one of the key factors controlling biomass recalcitrance and may be determining the saccharification properties of the lignocellulosic component (Li et al. 2016), we decided to check whether 35S::βI-Gal internodes are affected in the saccharification efficiency. Our results indicate that the reduced lignin content does not reduce the need for alkali or basic pre-treatment of the biomass (Fig. 8), which are supposed to break lignin and ester bonds or glycosidic bonds in hemicellulose and amorphous cellulose, respectively (Van Acker et al. 2014). However, the saccharification efficiency is increased during the first hours after pre-treatment, especially with acid pre-treatment (Fig. 8). This could reflect the higher accessibility of the hydrolytic enzymes to cellulose, thus opening a new research channel for future actions on pectins to modify cell wall structure with the aim to improve the effectiveness of saccharification.

In conclusion, and taking into account all of our results, we can state that β-(1,4)-galactan plays a key structural role in the correct organization of the different domains of the cell wall during the cessation of elongation. This in turn determines the interactions, not only between pectins and XG, but also between the rest of hemicelluloses and cellulose. The lack of galactan during early events of secondary cell wall development alters the interactions between the different matrix polysaccharides and reinforces the notion that there is a mutual dependence between the different polysaccharides and lignin polymers to form an organized and functional cell wall.

Author contribution statement

IM, BD, and EL conceived and designed the research. MM-S, AV-R and PK conducted the immunohistochemistry and staining analyses. BD, LA and JH-N conducted the *N. benthamiana* transformation and activity experiments. IM and JH-N performed the lignin analyses. BD and PK helped with the discussion of the results. IM and EL wrote the manuscript.

Compliance with ethical standards

Conflict of interest

No conflict of interest has been declared.

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Table 1 Substrate specificity of βI-Gal toward pNP substrates (nkat/mg prot.). nd: not detected.

Substrate	Activity
pNP-β-D-galactopyranoside	2.78±0.52
pNP-α-D-galactopyranoside	0.41±0.24
pNP-β-D-glucopyranoside	n.d.
pNP-α-D-glucopyranoside	0.17±0.09
pNP-α-L-arabinopyranoside	n.d.
pNP-β-D-fucopyranoside	n.d.
pNP-β-D-mannopyranoside	n.d.
pNP-β-D-xylopyranoside	n.d.

Table 2 Substrate specificity of βI-Gal toward galactose poly- and oligosacarides (nkat/mg prot.). nd: not detected.

Substrate	Activity
β-(1,4)-galactan	0.199±0.001
β-(1,3)-galactan	n.d
β-(1,3)(1,6)-galactan	n.d.
Gum arabic	n.d.
Arabinogalactan	n.d.
XG oligosaccharides	n.d.
β-(1,4)-galactobiose	0.047±0,005
Lactose	n.d.
β-(1,3)-galactobiose	0.023±0.001
β-(1,3)-galactotriose	n.d.
β-(1,6)-galactotriose	0.021±0.002
β-(1,6)-galactotriose	0.027±0.017

Figure legends

Fig. 1 Morphological characterization of WT and 35S::βI-Gal basal floral stem internodes. a: length of the basal internodes of 23-35 d-old-plants. b: diameter of basal stem internodes 0.5 cm above the rosette leaves. c: calcofluor-stained cross sections of basal stem internodes. Scale bars = 200 µm. The stars indicate the levels of significance (Student's t test): *p < 0.05.

Fig. 2 Anion exchange epitope detection chromatography (AE-EDC) profiles of H_2O and CDTA soluble polysaccharides from WT and 35S::βI-Gal basal stem internodes. Profiles are shown for LM5 anti-β-(1,4)-galactan (a), JIM7 anti-methyl-esterified HG (b) and LM25 anti-XG (c) antibodies. Values are the means of three biological replicates (±SD). Main galactan elution peaks are marked using dashed lines.

Fig. 3 Anion exchange epitope detection chromatography (AE-EDC) profiles of reactivity of anti-XG antibodies to KOH cell wall extracts from WT and 35S::βI-Gal basal stem internodes. Profiles are shown for CCRC-M1 (fucosylated XG), LM25 (XXXG, XXLG and XLLG) and CCRC-M100 (XG with no xylose-linked substitutions). Values are the means of three biological replicates (±SD).

Fig. 4 Anion exchange epitope detection chromatography (AE-EDC) profiles of KOH soluble hemicelluloses from WT and 35S::βI-Gal basal stem internodes. a: profiles of epitope recognition by xylan (CCRC-M139 and LM11) and glucuronoxylan (LM28) antibodies. b: profile of epitope recognition by LM21 (mannan). Values are the means of three biological replicates (±SD).

Fig. 5 ELISA analysis of hemicelluloses released after treatment of the cellulosic fraction from WT and 35S::βI-Gal basal stem internodes with a cellulose-specific endoglucanase. a: signal for XG antibodies CCRC-M1 (fucosylated XG), LM25 (XXXG, XXLG and XLLG) and CCRC-M100 (XG with no xylose-linked substitutions). b: signal for xylan (CCRC-M139 and LM11), glucuronoxylan (LM28) and mannan (LM21) antibodies. The stars indicate the levels of significance (Student's t test): *p < 0.05; **p < 0.01.

Fig. 6 Immunolocalization of β-D-(1,4)-galactan (LM5), XG (CCRC-M1 and LM25), xylan (LM11), glucuronoxylan (LM28) and mannan (LM21) in basal floral stem internodes of WT and 35S::βI-Gal plants. c, cortex; ep, epidermis; ir, interfascicular region; p, pith; vt, vascular tissue; x, xylem

Fig. 7 Analysis of lignin content in WT and 35S::βI-Gal basal stem internodes. a: acetyl bromide soluble lignin (ABSL) content expressed as % of cell wall AIR (alcohol insoluble residue). b: Cross sections stained with safranin/astra blue. Scale bars = 200µm. c: 2D HSQC spectrum of anomeric regions of ABSL. The stars indicate the levels of significance (Student's t test): **p < 0.01.

Fig. 8 Glucose release in saccharification assays from WT and 35S::βI-Gal basal stem internodes after no pre-treatment, acid (HCl) pre-treatment and alkali (NaOH) pre-treatment. Values are means of three biological replicates (±SD). The stars indicate the levels of significance (Student's t test): *p < 0.05; **p < 0.01.