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ScienceDirect
Phytochemistry 68 (2007) 1219–1226
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Xylogalacturonan exists in cell walls from various tissues of *Arabidopsis thaliana*

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Received 4 July 2006; received in revised form 20 January 2007
Available online 2 March 2007

Abstract

Evidence is presented for the presence of xylogalacturonan (XGA) in *Arabidopsis thaliana*. This evidence was obtained by extraction of pectin from the seeds, root, stem, young leaves and mature leaves of *A. thaliana*, followed by treatment of these pectin extracts with xylogalacturonan hydrolase (XGH). Upon enzymatic treatment, XGA oligosaccharides were primarily produced from pectin extracts obtained from the young and mature leaves and to a lesser extent from those originating from the stem of *A. thaliana*. The oligosaccharide $GalA_3Xyl$ was predominantly formed from these pectin extracts. No XGA oligosaccharides were detected in digests of pectin extracts from the seeds and roots.

A low number of XGA oligosaccharides was obtained from pectins of *A. thaliana*. This indicates a uniform distribution of xylose in XGA from *A. thaliana*. The predominant production of $GalA_3Xyl$, as well as the release of linear GalA oligosaccharides pointed to a lower degree of xylose substitution in XGA from *A. thaliana* than in XGA from apple and potato.

The estimated amount of XGA accounted for approximately 2.5%, 7% and 6% (w/w) of the total carbohydrate in the pectin fraction of the stem, young leaves and mature leaves, respectively.

Keywords: *Arabidopsis thaliana*; Pectin; Xylogalacturonan hydrolase; Xylogalacturonan

1. Introduction

Primary cell walls are the major textural components of plant-derived foods. The most important polysaccharides that account for 90–100% of the structural polymers of these cell walls are cellulose, hemicellulose and pectin (Albersheim et al., 1996).

Cellulose is comprised of β-(1 → 4)-linked D-glucan while hemicelluloses primarily consists of xyloglucan and arabinoxylan (Albersheim et al., 1996). Pectin is a heteropolysaccharide, which contains α-(1 → 4)-linked D-galacturonic acid chains (also known as the smooth regions of pectins) and the branched polysaccharides rhamnogalacturonan I, rhamnogalacturonan II, and xylogalacturonan (referred to as the "hairy" regions) (Benen et al., 2002; Schols and Voragen, 1996; Vincken et al., 2003).

Abbreviations: MALDI-TOF MS, matrix assisted laser desorption ionization time of flight mass spectrometry; HPAEC, high performance anion exchange chromatography; PAD, pulsed amperometric detection; XGH, xylogalacturonan hydrolase; XGA, xylogalacturonan; CWM, cell wall material; CWM-s, saponified cell wall material; RG-I, rhamnogalacturonan I; AIR, alcohol insoluble residue; UA, uronic acid.

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0031-9422/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2007.01.016

The cellulose–hemicellulose network is embedded in a matrix of pectic polysaccharides, which form a hydrated and crosslinked three-dimensional network (Gibeaut and Carpita, 1994; Knox, 2002).

Xylogalacturonan (XGA) is a chain of α-(1 → 4)-linked D-galacturonic acid, which is substituted with β-D-xylose at the O-3 position. It is suggested that this biopolymer is a side chain of RG-I (rhamnogalacturonan I) in the "hairy" regions of pectin (Vincken et al., 2003).

The presence of XGA in plants has been reported in storage tissues or reproductive organs such as in cell walls of peas, soybeans, watermelons, apples, pears, onions, potato's, pine pollen, and cotton seeds (Albersheim et al., 1996; Le Goff et al., 2001; Nakamura et al., 2002; Schols et al., 1995; Thibault and Ralet, 2001; Vincken et al., 2003; Voragen et al., 2001; Zandleven et al., 2006; Huisman et al., 1999). Its presence has also been reported in exudates from trees, such as gum tragacanth from the *Astralagus species* (Aspinall and Baillie, 1963).

Previous studies demonstrated that leaf primary cell walls of *A. thaliana* contain homogalacturonan, RG-I, RG-II, xylan, xyloglucan and cellulose (Zablackis et al., 1995). These polymeric structures were also suggested to be present in cell walls of the stem of this plant species (Gardner et al., 2002). Additionally, mannan polysaccharides have also been reported in the cell walls of leaves and stem of *A. thaliana* (Handford et al., 2003).

So far, the presence of other polysaccharides, such as XGA, has not been demonstrated in *A. thaliana* (Zablackis et al., 1995), although it has been suggested that this polymer exists in root caps of this plant species. This was based on immunocytochemical analysis using an LM8 antibody that was raised against pea XGA (Willats et al., 2004). Also Gardner et al. (2002) mentioned XGA as a probable xylose-containing polysaccharide in the stem of *A. thaliana*; however this was only based on the sugar composition of the alcohol insoluble residue (AIR) of the stems.

Recently, a gene (At5g33290) expected to encode a β-xylosyl transferase was identified in *A. thaliana*. Pectin isolated from a T-DNA insertion line having a T-DNA insertion in this particular gene was found to contain less xylose compared to the wild type (Sørensen et al., unpublished data). We therefore hypothesized that pectin from *A. thaliana* may contain regions of XGA. To investigate this, pectin was extracted from cell wall material, prepared from the seeds, roots, young leaves and mature leaves of *A. thaliana*. The pectin fractions were analysed for their sugar composition and subsequently treated with xylogalacturonan hydrolase (XGH) to determine the presence of XGA. The enzyme XGH is known to degrade the galacturonic acid backbone and has a requirement for xylosylation (Beldman et al., 2003). Based on the action of XGH towards a XGA derived from gum tragacanth (XGA-29; Zandleven et al., 2005) and XGA from the saponified modified "hairy" regions of apple and potato (Zandleven et al., 2006), this enzyme has a preference to cleave between two xylosylated galacturonic acid units as deduced from the high production of the di-saccharide GalAXyl. XGH can also act between two GalA residues of which only one is xylosylated as shown from the production of XGA oligosaccharides which have no xylose substitution at the non-reducing end or at the reducing end.

Based on the obtained XGA hydrolysis products from the pectin fractions of *A. thaliana*, some structural characteristics of XGA from this species are hypothesized. Also the amount of XGA liberated from these pectin fractions was estimated.

2. Results

2.1. Analysis of CWM

CWM was prepared from the seeds, roots, stem, young leaves and mature leaves of *A. thaliana*. In line with other reports (Zablackis et al., 1995), polysaccharides possibly lost during some of the preparation steps (i.e. starch and protein removal) were not considered as cell wall components.

These CWM was saponified to ensure full removal of methyl- and acetyl esters, which could interfere with subsequent enzymatic treatment. The sugar composition of the saponified CWM (CWM-s) samples is shown in Table 1. All the samples contained high amounts of glucose and significant amounts of xylose, which suggests the presence of cellulose, and possibly xylan and/or xyloglucan. The presence of these polysaccharides was indicated in cell walls of *A. thaliana* leaves before (Zablackis et al., 1995).

Table 1
Sugar composition (mol%) and total carbohydrate content (w/w %) of CWM-s from mature leaves, young leaves, stem, root and seeds of *A. thaliana*

	Rha (mol%)	Fuc (mol%)	Ara (mol%)	Xyl (mol%)	Man (mol%)	Gal (mol%)	Glc (mol%)	UA (mol%)	Total carbohydrate (w/w%)
Mature leaves	3	1	8	9	5	7	33	35	49
Young leaves	2	1	7	8	6	8	37	33	47
Stem	1	0	6	20	7	5	43	19	57
Root	2	1	18	14	4	7	35	20	52
Seeds	8	1	18	8	3	11	27	25	20

The total carbohydrate content was calculated from GC and AUA data.

Besides glucose and xylose, also rhamnose, arabinose, galactose and in particular high amounts of uronic acid were detected, which implies that these samples also contain pectin. The major sugars, glucose and uronic acid have also been demonstrated previously for CWM of *A. thaliana* stems (Gardner et al., 2002) and *A. thaliana* leaves (Zablackis et al., 1995) although different pectin extraction techniques were used. The total carbohydrate content of the CWM-s from the different plant organs (Table 1) was relatively low, compared to our experiences on the analysis of cell wall material from several fruits and vegetables (Voragen et al., 1983). The low carbohydrate content in these CWM-s is probably caused by the presence of non-polysaccharide materials, such as salts and proteins. Also the pectin fractions as well the pellet fractions, derived from these CWM-s, have a low total carbohydrate content (see Tables 2 and 3). This is probably due to similar reasons as well as the use of EDTA in the extraction buffer. Apparently, non-polysaccharide material (including EDTA) is not easily removed from the pectin and pellet fractions by dialysis. Despite the presence of non-polysaccharide materials in these fractions, XGH was able to act on its substrate (see Section 2.3).

2.2. Analysis of pectin extracts

To investigate the presence of xylogalacturonan (XGA) in the cell walls of seeds, roots, stem, young leaves and mature leaves of *A. thaliana* it was required to extract the pectin from CWM-s of these plant organs first. The solubilized material, as extracted from these CWM-s, which contain the pectic polysaccharides will be referred to as ''pectin fractions''. Fig. 1 shows an example of a pectin extraction scheme (mature leaves). The amounts of dry matter and total carbohydrate in the CWM-s and the pectin fraction are also indicated. The calculated yields of dry matter of the pectin fractions were 52%, 28%, 31%, 42% and 35% (w/w) of the CWM-s of the seeds, roots, stem, young leaves and mature leaves, respectively.

Based on the dry matter yields and the sugar compositions it was estimated that the yield of carbohydrate in the pectin fractions were 8%, 16%, 17%, 21% and 20% (w/w) for the seeds, roots, stem, young leaves, and mature leaves, respectively.

As expected for the method used (Voragen et al., 1983), the sugar composition of all the pectin fractions shows that uronic acid (most probably galacturonic acid; GalA) is the major sugar (Table 2). Compared to the sugar composition of the CWM-s samples, the amount of glucose is significantly reduced in the corresponding pectin fractions, which illustrates that pectin was selectively extracted.

Arabinose and galactose are the major neutral sugars in pectin fractions from the root, stem and leaves. This was also found in pectin extracts from several fruit and vegetables (Voragen et al., 1983). The arabinose and galactose contents suggest that RG-I with side chains of arabinan and/or arabinogalactan exists in these pectin fractions.

A relatively low content of galactose was observed in the pectin fraction from the seeds. On the other hand a high amount of rhamnose was found in this pectin fraction. This

Table 2
Sugar composition (mol%) and total carbohydrate content (w/w%) of pectin extracts of CWM-s from mature leaves, young leaves, stem, root and seeds of *A. thaliana*

Pectin extracts	Rha (mol%)	Fuc (mol%)	Ara (mol%)	Xyl (mol%)	Man (mol%)	Gal (mol%)	Glc (mol%)	UA (mol%)	Total carbohydrate (w/w%)
Mature leaves	3	1	9	7	5	9	5	64	28
Young leaves	4	0	9	6	5	9	5	64	23
Stem	3	0	16	13	2	10	4	52	31
Root	2	1	10	6	5	10	3	63	29
Seeds	15	0	17	5	3	4	7	70	14

The total carbohydrate content was calculated from GC and AUA data.

Table 3
Sugar composition (mol%) and total carbohydrate content (w/w%) of pellet fractions after pectin extraction of CWM-s from mature leaves, young leaves, stem, root and seeds of *A. thaliana*

Pellets	Rha (mol%)	Fuc (mol%)	Ara (mol%)	Xyl (mol%)	Man (mol%)	Gal (mol%)	Glc (mol%)	UA (mol%)	Total carbohydrate (w/w%)
Mature leaves	2	1	9	12	4	6	48	19	41
Young leaves	1	0	8	13	5	7	51	17	38
Stem	2	1	3	23	4	3	57	9	53
Root	2	2	18	14	3	7	44	10	41
Seeds	4	1	19	7	3	12	28	27	27

The total carbohydrate content was calculated from GC and AUA data.

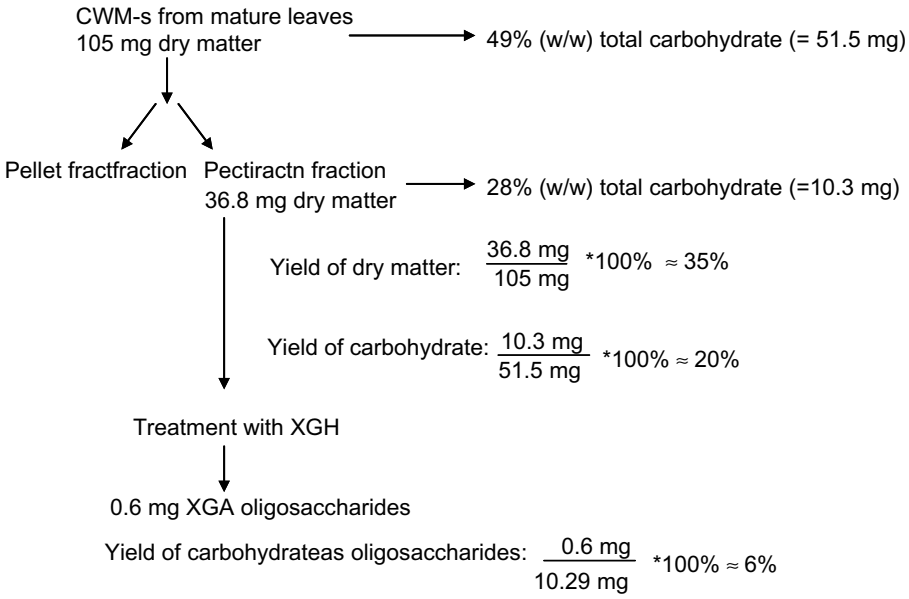

Fig. 1. Pectin extraction scheme of the mature leaves from *A. thaliana*, which includes the yield of total carbohydrate in the CWM-s and pectin fraction, as well as the yield of XGA oligosaccharides derived from the pectin fraction.

has also been reported before for the ammonium oxalate extracted mucilage from the seeds of *A. thaliana* (Usadel et al., 2004). This implies that RG-I is a major component in pectin from the seeds of *A. thaliana*.

The presence of both uronic acid and xylose in all pectin fractions (Table 2) indicate that these fractions may contain XGA. This is further investigated by the use of XGH as described in Section 2.3.

The major sugars in the pellet fractions of the different CWM-s extracts from roots, stem, young leaves and mature leaves were glucose and xylose (Table 3), which points to the presence of cellulose, and possibly xylan and/or xyloglucan. Besides glucose and xylose, these pellet fractions also contain uronic acid. Apart from glucose, the pellet fraction of the seeds is particularly rich in this monosaccharide. Based on the sugar composition of the pellet fractions it is possible that uronic acid occurs as glucuronic acid as well as galacturonic acid. Table 3 shows the presence of arabinose, xylose and uronic acid which indicate that these fractions contain hemicellulosic polysaccharides in the form of (glucurono-)arabinoxylan.

A similar conclusion was made by Zablackis et al. (1995). Besides this, also pectin related sugars were found in these pellet fractions, which point at the possible presence of galacturonic acid. Again, the total carbohydrate content of the pellet fractions was relatively low, which is probably due to reasons already mentioned above.

The pellet fraction from the seeds has a relatively higher content of rhamnose and galactose, compared to other pellet fractions, which indicates that this fraction contains a relatively high amount of rhamnogalacturonan. The relative galactose content of the pellet fraction from the seeds was 3 times higher than its corresponding pectin fraction, while the relative rhamnose content in this pellet fraction was approximately 4 times lower. Also an equal proportion of arabinose was found in the pellet, compared to its corresponding pectin fraction. These results indicate that, although a major part of the pectin was extracted, the pellet fraction of seeds still contains rhamnogalacturonan I with side chains of arabinans and/or arabinogalactans.

2.3. Evidence for XGA in A. thaliana

The presence of XGA in the pectin fractions was investigated using the enzyme XGH as analytical tool. The pectin fractions were treated with XGH for 16 h, prior to analysis by HPAEC and MALDI-TOF MS.

As demonstrated by HPAEC (Fig. 2), 10 different oligosaccharides were identified in the mature leaf digest. The main product was $GalA_3Xyl$, while significant amounts of GalA, GalAXyl, $GalA_2Xyl$, $GalA_4$, $GalA_5$ and $GalA_6$ and minor quantities of $GalA_2$, $GalA_2Xyl_2$ and $GalA_2Xyl'$ were observed. The accentuated oligosaccharide $GalA_2Xyl'$ is an isomer in which the xylose residue is probably linked to the O-2 of the reducing GalA (Zandleven et al., 2005).

Comparable results were acquired for young leaves and stem (results not shown), although a significantly lower level of the oligosaccharides $GalA_4$, $GalA_5$ and $GalA_6$ was released from pectin originating from the young leaves.

The formation of a series of oligosaccharides produced from the pectin extract of the mature leaves (Fig. 2) was confirmed by MALDI-TOF MS (Fig. 3), although some differences were observed. The monomer GalA and the di-saccharide GalAXyl could not be observed by MALDI-TOF MS because their corresponding mass peaks were disturbed by the matrix peaks (results not shown). The oligosaccharides $GalA_5$ and $GalA_6$ were also hardly detected by this method. It is our experience that an increased degree of polymerization of GalA oligosaccharides is accompanied with a lower signal intensity during

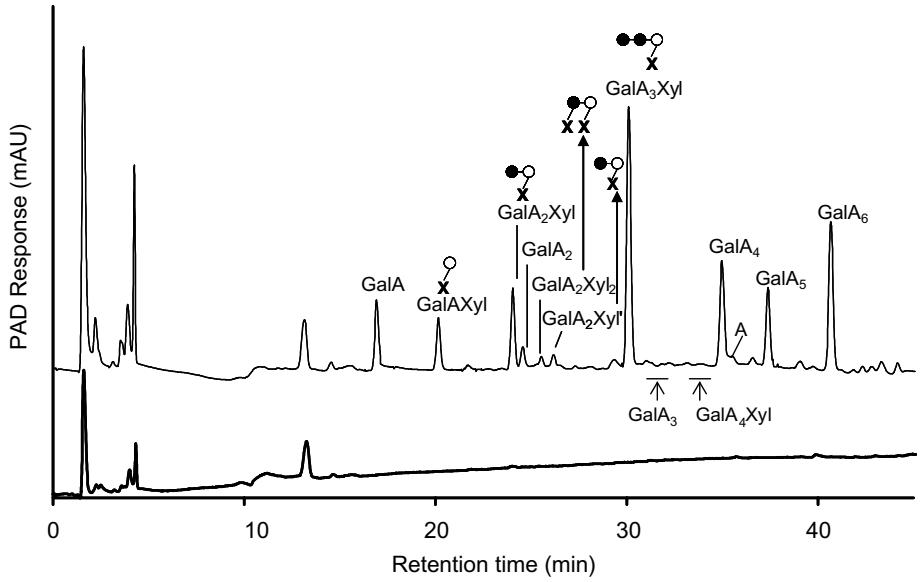

Fig. 2. HPAEC of pectin from mature leaves of *A. thaliana*, untreated (bold line) and treated for 20 h with XGH (thin line). The structures of the XGA oligosaccharides, as characterized previously (Zandleven et al., 2005), are also shown. The accentuated oligosaccharide $GalA_2Xyl'$ is an isomer in which the xylose residue is probably linked to the O-2 of the reducing GalA, instead of the O-3 in $GalA_2Xyl$. The expected elution times of the oligosaccharides $GalA_3$ and $GalA_4Xyl$ are indicated by arrows. The peak which may correspond to $GalA_4Xyl'$ is indicated by an "A". ●, GalA; ○, reducing GalA; X, xylose.

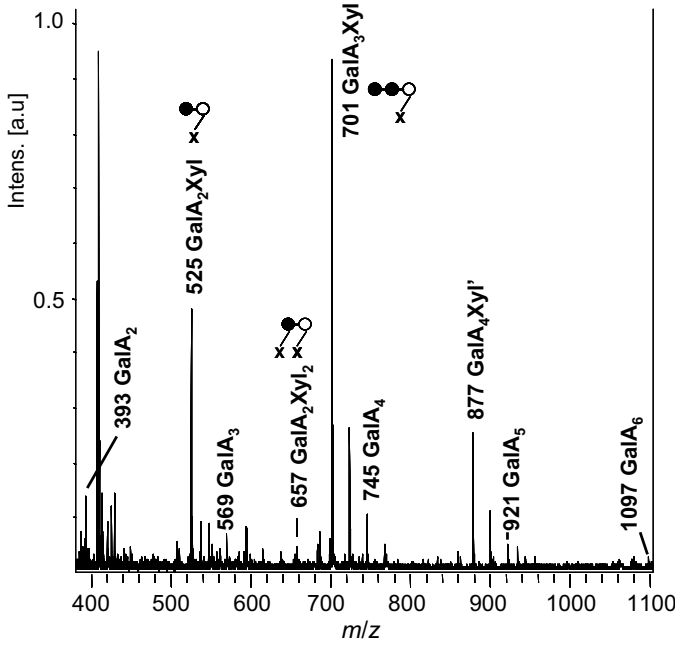

Fig. 3. Positive mode MALDI-TOF MS spectrum of pectin fragments from the mature leaves of *A. thaliana* obtained by treatment with XGH for 20 h. The *m/z* masses of the XGA oligosaccharides include H^+ and Na^+. ●, GalA; ○, reducing GalA; X, xylose.

MALDI-TOF MS analysis. This shows that HPAEC data are preferred for quantification of the produced XGA oligosaccharides from these *A. thaliana* pectins.

Two other oligosaccharides, $GalA_3$ and $GalA_4Xyl$, were also observed by MALDI-TOF MS analysis. Although no clear peak corresponding to $GalA_3$ was found by HPAEC analysis, an indication of the elution time of this oligosaccharide is illustrated in Fig. 2. This was based on our experiments with $GalA_3$ in a mixture of XGA oligosaccharides derived from gum tragacanth (XGA-29; Zandleven et al., 2005).

With respect to $GalA_4Xyl$, it is known that two isomers exist, namely $GalA_4Xyl$ and $GalA_4Xyl'$, which have a different structure and a different elution behavior upon HPAEC analysis (Zandleven et al., 2005). As indicated in Fig. 2, no peak could be observed which could correspond to $GalA_4Xyl$. However a shoulder peak, indicated as shoulder A (see Fig. 2), exists next to $GalA_4$, which corresponds to the elution time for $GalA_4Xyl'$. From this, it is likely that the mass peak at *m/z* 877 (Fig. 3) corresponds to $GalA_4Xyl'$.

Comparable MALDI-TOF MS results were obtained for pectin digests from the young leaves and the stem (results not shown). From this it can be concluded that XGA is present in the stem, young leaves and mature leaves of *A. thaliana*.

Compared to the number of different oligosaccharides from XGA-29 (Zandleven et al., 2005), a low number of different XGA oligosaccharides were produced from the *A. thaliana* pectins, which was similarly observed for digests of XGA from apple and potato pectins (Zandleven et al., 2006). This implies that XGA from *A. thaliana* has a relative conserved pattern of xylose side chain distribution compared to the random pattern of xylose side chain distribution in XGA-29.

The degree of xylose substitution in XGA from pectin from the stem and the leaves is lower than that of XGA from apple pectin and potato pectin. While XGH mainly released the disaccharide GalAXyl from these latter two

sources, a predominant production of $GalA_3Xyl$ was seen for the *A. thaliana* pectins. Also the relative amount of linear oligosaccharides in relation to branched GalA oligosaccharides was higher in the *A. thaliana* pectins than in pectins from apple and potato (Zandleven et al., 2006).

The enzyme XGH was also able to release linear GalA oligosaccharides from the *A. thaliana* pectin fractions. Based on the fact that XGH degrades neither polygalacturonic acid (Beldman et al., 2003) nor galacturonic acid oligosaccharides (Zandleven et al., 2005) it is concluded that these linear GalA oligosaccharides are also products from XGA. Linear GalA oligosaccharides can be released, by XGH, from unsubstituted regions of XGA by hydrolysis of the linkage in the galacturonan back-bone, next to a xylosylated GalA.

Although we already illustrated that subsites −1 and +1 of XGH can accommodate xylosylated GalA units (Zandleven et al., 2005), this study indicates that these subsites show a preference for these xylosylated residues. This can be seen from the formation of XGA oligosaccharides, which all have a xylose substitution at the reducing end. It is possible that subsites, other than −1 and +1, accept GalA residues that do not require or are not allowed to have xylosylation. This can be deduced from the significant formation of GalA oligosaccharides, especially from pectin of the mature leaves (Fig. 1). The production of the linear oligosaccharides $GalA_4$, $GalA_5$ and $GalA_6$ (which was especially high from the pectin of the mature leaves) implies that short regions of HG together with XGA exist in pectin from *A. thaliana*. The indication that XGA segments are interrupting the HG structural elements was also mentioned by Vincken et al. (2003).

By using HPAEC and MALDI-TOF MS analysis (results not shown), the presence of XGA could neither be demonstrated in pectin from the seeds nor from the roots of *A. thaliana*, although it has been indicated that XGA exists in root caps of *A. thaliana* (Willats et al., 2004). Also the pellet fractions from the different plant organs contained some residual pectin material, but no XGA oligosaccharides were detected by HPAEC and MALDI-TOF MS analysis (results not shown) of these XGH-treated fractions. It is possible that the concentration of XGA is too low in these pectin samples to be detected by our methods. If this is the case, it is possible that xylose in these samples originates from xylose containing polysaccharides other than XGA. Alternatively, it is also possible that XGH-resistant XGA exists in these pectin fractions. The occurrence of XGH-resistant XGA has also been observed before for pea XGA (Beldman et al., 2003).

The total amount of liberated XGA from the pectin fractions of the stem, young leaves and mature leaves was estimated. For this, the amount of each XGA oligosaccharide in the different pectin digests was quantified and the total amount determined by summation of these values. Linear GalA oligosaccharides, which were regarded as products from XGA, were also included in the calculations. An example of the yield of XGA oligosaccharides (% w/w) from the mature leaves is illustrated in Fig. 1.

Approximately 2.5%, 7% and 6% (w/w) of XGA related products could be liberated from the total carbohydrate present in the pectin fraction from the stem, young leaves and mature leaves, respectively. Only the degradable part of XGA is taken into account for the calculation of the yield of XGA oligosaccharides. It cannot be ruled out that XGH resistant parts of XGA are also present in these pectin samples, which imply an underestimation of these values.

The results from this study clearly demonstrate the presence of XGA in the stem, young leaves and mature leaves of *A. thaliana*, although a relative lower amount of this polymer was detected in the stem compared to the leaves.

To our knowledge this is the first time that the occurrence of XGA has been demonstrated in plant material other than storage or reproductive tissues and root caps. The outcome of this study may initiate investigations on the presence of XGA in other plant varieties, as well as the relationship between XGA structure and its function in different plant tissues.

3. Experimental

3.1. Plant material

Arabidopsis thaliana (L) Heyn. Ecotype Col-0 was used for all experiments.

Plants were grown in peat at an 8 h photoperiod at 100–120 μmol photons m^{-2} s^{-1}, 20 °C, 70% relative humidity and watered using tap water when necessary.

Plant material (green tissue) was harvested as young leaves (small rosettes 10–12 leaf stage), mature leaves (12 weeks), stems (including flowers and silliques) and seeds. Roots were obtained from plants grown hydroponically according to Husted et al. (2002).

3.2. Preparation of cell wall material (CWM)

Cell wall material was prepared as follows: alcohol insoluble residue (AIR) was prepared as described (Fry, 1988) with adaptations. Tissue of interest was ground in liquid nitrogen with a mortar and pestle and boiled in 96% ethanol for 30 min. The supernatant was removed after centrifugation at 10,000*g* for 5 min. The pellet was washed with 70% ethanol with subsequent centrifugation until it appeared free of chlorophyll. A final wash with 100% acetone was performed and the pellet was dried under vacuum.

Alcohol insoluble residue (AIR) was treated with enzymes for the removal of starch. For this, AIR was suspended in 10 volumes of a solution that had been preheated to 95 °C containing 10 mM potassium phosphate buffer (pH 6.5), 1 mM $CaCl_2$, and 0.05% NaN_3. Starch was allowed to gelatinize for 30 s before addition of thermostable α-amylase (Megazyme, Bray, Ireland) to a final

concentration of 1 U/ml. The suspension was incubated at 85 °C for 15 min. After the incubation the sample was cooled to 25 °C and amyloglucosidase and pullulanase (both from Megazyme) were added to a final concentration of 1 U/ml. The α-amylase, amyloglucosidase and pullulanase preparation were investigated by SDS–PAGE and appeared to give one band only, except for an extra faint band for the pullulanase preparation. Additionally, no pectinolytic side activities were detected in these enzyme preparations. The suspension was incubated for 16 h at 25 °C, with continuous shaking at 500 rpm. The suspension was centrifuged for 10 min at 6000*g*. The pellet was washed with 50 ml of a solution containing 10 mM potassium phosphate (pH 6.5), 1 mM $CaCl_2$, and 0.05% NaN_3, centrifuged again at 6000*g* for 10 min, and finally dried under vacuum. The de-starched cell wall material was extracted with phenol:acetic acid:water (2:1:1, v/v/v) for 3 h (1:10 (w/v) ratio between AIR and phenol:acetic acid:water 2:1:1) at room temperature followed by centrifugation at 6000*g* for 5 min. The pellet (CWM) was washed three times with water to remove phenol and extracted proteins and finally dried under vacuum.

3.3. Saponification of CWM

Pectin, as present in CWM from seeds, root, stem, young leaves and mature leaves was saponified in 0.1 M sodium hydroxide for 24 h at 4 °C and subsequently neutralized with 0.1 M acetic acid. After neutralization, samples were dialyzed overnight against MilliQ water and freeze-dried until further use. Saponified CWM is referred to as CWM-s.

3.4. Pectin extraction from CWM-s

Pectin was extracted from 200 mg, 100 mg, 130 mg, 20 mg, and 105 mg of CWM-s from seeds, root, stem, young leaves and mature leaves of *A. thaliana* according to the method of Voragen et al. (1983). For this extraction a cold solution of 5 mM EDTA in 50 mM NaOH (4 °C) was used.

The obtained pectin and pellet fractions from each type of CWM-s were dialyzed overnight against distilled water. Aliquots of these fractions were freeze-dried prior to analysis of their sugar compositions (see Section 3.5). The total concentration of soluble polysaccharides in the pectin fractions as well as the total concentration of the residue in the pellet fractions was calculated using the yield of dry material from the freeze dried aliquots of these fractions.

The remainder of the pectin fractions as well as the pellet fractions were dialyzed against 50 mM NaOAc (pH 3.5) prior to treatment by XGH, see Section 3.7.

3.5. Neutral sugar composition and uronic acid content

CWM-s, as well as the corresponding pectin and pellet fractions, were analyzed for their neutral sugar compositions using gas chromatography, after derivatization to alditol acetates (Englyst and Cummings, 1984). Inositol was used as internal standard. The samples were hydrolyzed in 72% (w/w) H_2SO_4 for 1 h at 30 °C, followed by a treatment in 1 M H_2SO_4 for 3 h at 100 °C. The uronic acid content of the samples was determined by an automated m-hydroxydiphenyl assay (Blumenkrantz and Asboe-Hansen, 1973; Thibault and Robin, 1975). Analysis of the neutral sugar composition and uronic acid content of the samples were performed in duplicate.

3.6. Substrates and enzymes

Xylogalacturonan (XGA-29) was prepared from gum tragacanth by treatment with alkali and trifluoro acetic acid (TFA) as described (Beldman et al., 2003). This XGA had a Xyl:GalA ratio of 0.29.

A set of XGA oligosaccharides with known structures and with different GalA/Xyl ratios were obtained as described (Zandleven et al., 2005).

The enzyme xylogalacturonan hydrolase (XGH) from *Aspergillus tubingensis* was cloned (Van der Vlugt-Bergmans et al., 2000) and expressed in the *A. niger* "PlugBug" (DSM Food Specialities, Delft, the Netherlands). This enzyme was purified as described (Beldman et al., 2003) and had a specific activity of 150 U/mg.

3.7. Enzyme incubations

One ml from the dialyzed pectin or pellet fraction (ranging in concentration between 1 and 6 mg/ml) from the different plant organs was incubated with XGH for 20 h at 30 °C. The final enzyme concentration was 0.35 μg/ml, which should be able to degrade all XGA possibly present in these samples during the incubation period. Subsequently, the enzyme was inactivated by heating the reaction mixtures for 10 min at 100 °C. The XGH treated samples were analyzed by HPAEC using a set of known XGA-oligosaccharides for identification (Zandleven et al., 2005). GalA, $GalA_2$, and $GalA_3$, (all 10 mM) were taken as standards to calculate the concentration of the corresponding oligosaccharides, as well as of the xylogalacturonan oligosaccharides. For the calculation of the XGA oligosaccharides it was assumed that xylose substitution has no significant effect on the response factor of galacturonan oligosaccharides (Sakamoto et al., 2002). The standard $GalA_3$ was also used to calculate concentrations of oligosaccharides with a degree of polymerization (DP) of 4 and higher, based on the experience that the differences in response factors for these larger oligosaccharides are relatively small.

3.8. MALDI-TOF mass spectrometry

XGH-treated pectin samples were desalted by treatment with H^+-Dowex AG 50W X8 (Biorad, Hercules, CA), using a final concentration of 350 mg H^+-Dowex per ml digest. The desalted digests were mixed with a matrix

solution (1 μl of sample in 1 μl of matrix) and applied on a MALDI sample plate. The matrix solution was prepared by dissolving 9 mg of 2,5-dihydroxybenzoic acid in a 1-ml mixture of acetonitrile:water (300 μl: 700 μl). MALDI-TOF MS analysis was performed using an Ultraflex workstation (Bruker Daltonics, Hamburg, Germany) equipped with a nitrogen laser of 337 nm. The mass spectrometer was selected for positive ions, which were accelerated by an electric field of 12 kV, after a delayed extraction time of 200 ns. The ions were detected in the reflector mode. External calibration of the mass spectrometer was performed using a mixture of maltodextrines (mass range 365–2309).

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