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Changes in homogalacturonans and enzymes degrading them during cotton cotyledon expansion

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Abstract

Changes in homogalacturonans (HGs) and enzymes degrading them have been investigated during cotton (*Gossypium hirsutum* L.) cotyledon expansion. Using an *in vivo* assay for pectin-degrading enzymes that involves fluorescent labeled oligomers of GalA as substrate and capillary electrophoresis for product analysis, we found that endo- and exo-polygalacturonases are present in the cotyledon extracellular spaces, and there are dramatic changes in the levels of both activities as the cotyledons change their rate of expansion. Capacity for endo-polygalacturonase activity was highest during the initial stages of cotyledon expansion. However, for exo-polygalacturonase activity it was highest in the later stages of expansion. Cell walls were prepared from 3-, 5-, and 7-day-old cotton cotyledons and treated with liquid HF at −23 °C. This treatment cleaves the glycosidic linkages of most neutral sugars in the walls without degrading HGs. HGs with a relatively high degree of esterification can then be solubilized with water, and those with low esterification can be solubilized with concentrated imidazole buffer. The majority of HGs were obtained in the water extracts. The degrees of esterification were 57%, 47%, and 47% in water extracts and 34%, 25%, and 27% in imidazole extracts, in 3-, 5-, and 7-day-old cotton cotyledons, respectively. Using a PA100 ion-exchange column, the members of a GalA homologous series up to approximately 70 residues can be separated. The results from HG molecular length distribution analysis indicated that the HG at 3 days was on average shorter than that in the older cotyledons, perhaps reflecting the higher level of endo-polygalacturonase activity at this stage of more rapid growth.

Keywords: Cell walls; Endo-polygalacturonase; Exo-polygalacturonase; *Gossypium*; Malvaceae; Growth; Pectin

1. Introduction

Investigating the biochemistry of plant cell wall polymers and wall enzymes is crucial to an understanding of tissue expansion. Plant cells and organs may expand tremendously in volume before reaching maturity. The cell wall can accommodate this enormous expansion without losing mechanical integrity and generally without getting thinner (Cosgrove, 1997). Thus, expansion of plant cells plus formation of intercellular spaces must involve the breakage and reformation of linkages within cell walls and middle lamellae (Albersheim et al., 1996; Taiz, 1984; Willats et al., 2001b), at least in part by enzymes that degrade and reform wall polymers (Fry, 1995). In primary cell walls of growing tissues, a xyloglucan–cellulose network is embedded in a pectin matrix, and both are major polysaccharide components in these walls. Expansion of the xyloglucan–cellulose network involves interaction with xyloglucan endo-transglycosylases and expansins (Campbell and Braam, 1999; Cosgrove, 1999; Darley et al., 2001), while loosening and remodeling of the pectin matrix requires action of pectic enzymes.

Homogalacturonan (HG) is one of the main components of pectic polysaccharides of the intercellular matrix in plant primary cell walls. HG is a homopolymer of 1–4

Abbreviations: ANTS, 8-aminonaphthalene-1,3,6-trisulphonate; DE, degree of esterification; EPG, endo-polygalacturonase; Exo-PG, exo-polygalacturonase; HF, hydrogen fluoride; HG, homogalacturonan; PG, polygalacturonase.

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linked α-D-galacturonic acid residues, which are frequently esterified through their carboxyl group to methanol. Kim and Carpita (1992) and McCann et al. (1994) have inferred the presence of additional, unidentified alcohols esterified to the carboxyl groups in some HGs. The O-2 and O-3 positions of HG are sometimes esterified with acetate (Perrone et al., 2002). HG is acted on by hydrolases (both exo- and endo-polygalacturonases), lyases (preferring either pectin or pectate as substrate), and esterases (e.g., pectin methylesterases and pectin acetylesterases). The extent to which different pectic fractions are esterified can vary greatly (McCann et al., 1990). Evidence suggests that newly synthesized pectin is deposited in a highly esterified form (Mohnen, 1999; Zhang and Staehelin, 1992). Variation in degree and pattern of methyl-esterification as influenced by pectin methylesterases with distinct modes of action is thought to influence the location and extent of cell wall remodeling during tissue expansion (Willats et al., 2001a). In expanding vegetative organs, stretches of un-esterified (or de-esterified) galacturonic acid residues crosslinked via calcium bridges are believed to contribute to wall strength and regulation of growth (Knox et al., 1990; Yamaoka and Chiba, 1983). De-esterified stretches of the pectin matrix are also sites where polygalacturonases and pectate lyases can act. A pectate lyase gene was found to be expressed in particular locations (vascular bundles and some shoot primordia) in *Zinnia* plants rather than throughout the growing regions (Domingo et al., 1998). The interplay of HG structure and polygalacturonase activity during growth is largely unknown. When endo-polygalacturonase (EPG) levels are high, pectin molecular size may be expected to be smaller, as that is what has been observed during fruit ripening (Huber, 1983, 1992; Huber and O'Donoghue, 1993; Koch and Nevins, 1989). The average size of pectin molecules was smaller, presumably as a result of EPG action, in the early stage compared to the later stages of expansion growth of primary bean leaves (Arribas et al., 1991).

Another major component in the pectin network is rhamnogalacturonan, which has a repeating disaccharide backbone of rhamnose and galacturonic acid with frequent sidechains attached to the rhamnose residues. Recently Naran et al. (2007) reported that there is a low level of rhamnogalacturonan lyase activity in expanding cotton cotyledons, which decreases as the rate of expansion decreases.

The majority of previous work from this laboratory on detailed analysis of cell wall polysaccharide structures has used cultured cells, because of the ready availability of large amounts of relatively uniform tissue, and has concentrated on cotton (*Gossypium hirsutum* L.) suspension cells (e.g., Komalavilas and Mort, 1989). For this project, cotton cotyledons were chosen because they undergo leaf-like expansion growth, but are easy to infiltrate, having few veins, and a single cell type (mesophyll) predominates. The goal was to relate PG activities and structural characteristics of HGs to the relative growth rate of the cotyledons. A sensitive assay for quantitating hydrolase enzyme activity in complex media (Zhang et al., 1996), such as living cotyledons, was developed with this project in mind. Here we show the cell wall matrix contains exo- and endo-polygalacturonase activities, the relative quantities of which change reciprocally during cotyledon expansion. Capacity for EPG activity is high during early, rapid growth, and is correlated with HG that, compared to the more mature cotyledons, is of a higher degree of methyl-esterification and is of a smaller average length.

2. Results and discussion

2.1. Cotton cotyledon growth rate

Each cotton plant has a smaller, more delicate cotyledon and a larger, more rugged one. The areas of the smaller and of the larger cotyledons were measured for a set of plants using an area meter and plotted separately. The time course of cotyledon expansion and the relative growth rates of those cotyledons are shown in Fig. 1. The relative growth rate ($cm^2\ cm^{-2}\ h^{-1}$) was calculated with respect to the leaf area at the start of each 24-h period for the larger sized cotyledons and expressed as a percentage; i.e., the average percent increase in area per hour over a 24-h period. This method of presenting the data highlights the much more rapid expansion rate of the younger cotyledons (Van Volkenburg et al., 1985). The relative growth rate of the

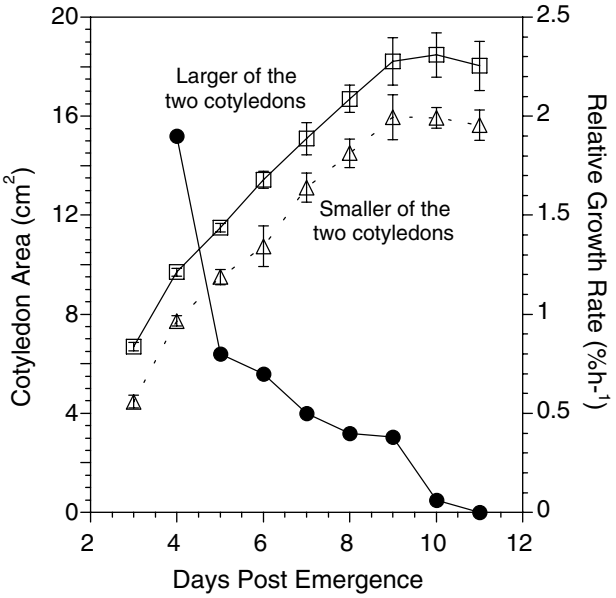

Fig. 1. The areas of both the larger (□) and smaller (Δ) cotyledons on several cotton seedlings were measured with an area meter and plotted against the number of days after emergence from the soil. Each value is the mean (±s.d.) of five cotyledons. Before three days the cotyledons were often still trapped within the seed coats, so their area could not easily be measured. Relative growth rate (●) was calculated as 100 times the average increase in area per hour over a 24-h period divided by the area at the beginning of the time period based on the group of larger cotyledons. The experiment was performed three times with similar results.

cotyledons dramatically decreased until day 5 and then remained low until the cotyledons reached mature size on day 9.

2.2. Enzyme activities in vivo

PG activities were determined by the measurement of *in vivo* degradation of a fluorescent-tagged hexamer of pectic acid (Zhang et al., 1996). Injection of substrate soaked about 2 cm^2 of a cotyledon's intercellular space. The total surface area of the larger-sized cotyledons ranged from approximately 7 to 18 cm^2 from 3 to 9 days after seedling emergence (Fig. 1). Thus, only approximately 30% of the tissue in the cotyledons from 3-day-old seedlings down to 11% in 9-day-old seedlings was exposed to the substrate. Estimating the amount of enzyme activity in a living cotyledon is not a highly controlled experiment. We do not know exactly what volume of intercellular space was infiltrated with substrate, or how the number of cells that were exposed to substrate changed as the cotyledons expanded. We do not know exactly the pH of the extracellular space, nor whether the enzyme activities are fixed in the walls or mobile. A potential problem is that there could be more efficient rinsing out of the cotyledons of small oligomers compared to larger ones. Despite all of the unknowns, we obtained consistent results using the procedure given in Section 4. Incubation times with the substrate were adjusted empirically (5 min–3 h between the injection time and immersion of the cotyledon in cold infiltration buffer) to allow the reaction to proceed only until about 75% of the fluorescent-labeled substrate had been converted to products. Rather than assuming complete recovery of reactants and products, we calculated the percent of the recovered reactants plus products that was represented by products, and expressed activity as percent conversion per unit of time. This percent conversion could be converted to units of enzyme activity by comparing with a standard curve.

EPG and exo-polygalacturonase (exo-PG) activities were clearly distinguished by the products formed from the labeled hexamer. As noted previously (Zhang et al., 1996), cotton exo-PG activity only produced labeled pentamer by removal of a single GalA residue from the non-reducing end. (In terms of enzyme specificities, the labeled hexamer behaves as a pentamer.) As was found for exo-PG from carrots and peaches and rose cell cultures (Garcia-Romera and Fry, 1995; Pressey and Avants, 1975), the enzyme displayed little activity on oligomers of less than five residues. In contrast, the EPG activity produced labeled dimer and trimer from the substrate hexamer.

Fig. 2 shows the relative amounts of exo-PG and EPG activity throughout the cotton cotyledon expansion. The results indicated that there are dramatic changes in the levels of exo- and endo-PG activities as the cotyledons change their rate of expansion. The capacity for EPG activity was highest during the initial stages of the expansion and showed a very good correlation with relative growth rate (Fig. 1 compared to Fig. 2). This may be because the pectin must be degraded to allow expansion, and/or perhaps the middle lamella must be weakened to allow cell separation for the production of the air spaces needed for gas diffusion. The capacity for exo-PG activity increased steadily in the cotyledons between 3 and 8 days after emergence (Fig. 2), and dropped abruptly on day 9 as the cotyledons reached their final size (Fig. 1). The exo-PG activity may be needed for salvage from the pieces of polygalacturonic acid released during the expansion phase and/or, as suggested by Garcia-Romero and Fry (1995), it may serve to control the concentrations of bio-active oligogalacturonides.

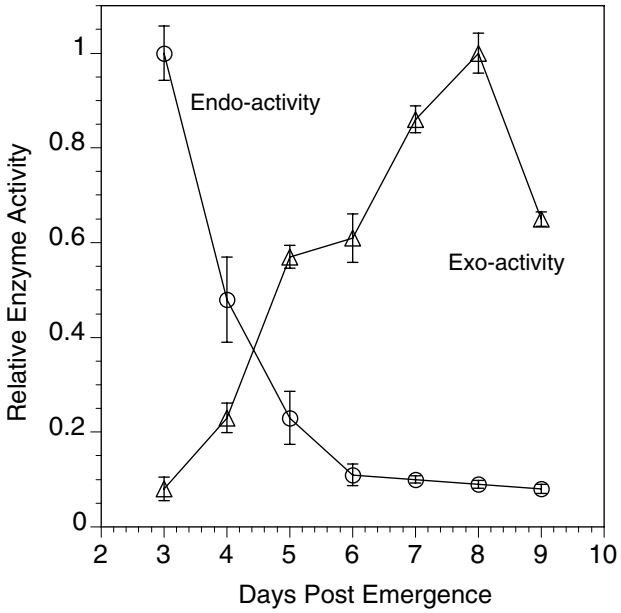

Fig. 2. The mean and standard deviation of the EPG (O) and exo-PG (Δ) enzyme activities detected in the intercellular spaces of cotton cotyledons from three to nine days after emergence from the soil. Only the larger of the two cotyledons on each plant was used for these measurements. Values are normalized to the maximum for each enzyme (0.0035 U cm^{-2} for EPG; undetermined for exo-PG). In all cases, measurements were made over a time span in which approximately 75% of the substrate was converted to products.

Hadfield and Bennett (1998) have suggested a role for polygalacturonases in growing tissues, particularly in xylogenesis. They cited the gradient from higher to lower levels of exo-PG from the rapidly growing tips of coleoptiles downward in oat seedlings (Pressey and Avants, 1977) and an association of transcripts for PG with developing vascular tissue. Sitrit et al. (1999) suggested an involvement of PG with vascular tissue differentiation in the radicle tip of germinating tomato seeds. Dubald et al. (1993) found low levels of exo-PG in young maize leaves, but they were higher than in expanded leaves. PGs represent a very large gene family (Hadfield and Bennett, 1998; Kim et al., 2006), and their roles in plant development are likely widespread and numerous even within expanding vegetative tissues of different sorts. Cotton cotyledons have few veins, so the

exo-PG, particularly the increased exo-PG activity that we found in cotton cotyledons as their growth rate slowed, was unlikely to be associated with xylogenesis. Since the enzymic capacity dropped concomitant with the cessation in expansion, the exo-PG in the cotyledons during expansion is probably involved with rapid turnover of the oligomers generated by the high EPG activity earlier in the expansion phase. Despite the potential milli-units per cm^2 of EPG reported here, Miranda (1993) detected GalA oligomers of lengths less than 15 residues in the intercellular spaces of mature cotton cotyledons at only 1–100 picomole per cm^2 levels.

We estimate that about 700 μg of HG cm^{-2} h^{-1} could be digested by EPG in 3-day-old cotyledons, but only about 60 μg of HG cm^{-2} h^{-1} could be digested in 9-day-old cotton cotyledons. Taking into account the cell wall dry weight (~6 mg cm^{-2} in 3-day-old cotyledons) and that GalA accounts for at least 20% of the cell wall dry weight (see the following section), we estimate an average of 1 mg of HG cm^{-2} in expanding cotton cotyledons. There appears to be excess enzyme over substrate just as in ripening tomato fruit (Tucker and Seymour, 2002). It will be of interest to find out what factors in addition to partial methylesterification, such as enzyme inhibitors or enzyme immobilization, prevent catastrophic digestion.

2.3. Carbohydrate composition of cotyledon walls

Yield of dried cell walls from cotton cotyledons was about 3.0 g 100 g^{-1} fresh weight. Iodine staining indicated that the wall preparations were essentially free of adherent starch (data not shown). The sugar compositions of cell walls from deveined 3-, 5-, and 7-day-old cotton cotyledons are presented in Tables 1–3, respectively. They did not vary in a statistically significant way. As the cotyledons expanded, there may have been a small decrease in arabinose and xylose along with an increase in GalA per unit weight of wall.

For the sugar analysis, we used methanolysis and trimethylsilylation prior to separation and quantitation by gas–liquid chromatography. By this procedure, the glucose from cellulose is not detected, and some of the non-esterified polygalacturonic acid is not converted to monomers and, therefore, not quantitatively recovered. Nevertheless, measured sugars accounted for 36% of the dry weight of the sample, and GalA represented over 50% of the sugar recovered. For cotton suspension culture walls we obtained similar compositions except that there was about half as much rhamnose and two-thirds as much arabinose per gram dry weight in the suspension culture walls (Komalavilas, 1988; Qi, 1992). This probably reflects a higher proportion of HG and lower proportion of rhamnogalacturonan with arabinan sidechains in the suspension cultures.

Table 1
Weight of sugars (mg) detected by gas–liquid chromatography in extracts from 500 mg of cell walls prepared from 3-d-old cotton cotyledons ($n = 5$)

	Ara	Rha	Xyl	GalA	Gal	Glc	Total
Intact walls	27 ± 1	10 ± 3	20 ± 3	95 ± 8	21 ± 1	9 ± 2	182
Extracts after −23 °C HF							
Ether extract	20 ± 3	4 ± 1	10 ± 2	2 ± 1	4 ± 1	2 ± 1	42
Water extract	4 ± 1	5 ± 1	4 ± 1	55 ± 1	15 ± 1	5 ± 1	88
Water extract[a]	5 ± 1	6 ± 1	5 ± 1	72 ± 1	19 ± 1	4 ± 1	111
Imidazole extract	0 ± 0	0 ± 0	1 ± 0	23 ± 4	0 ± 0	0 ± 0	24
Residue	1 ± 0	0 ± 0	4 ± 0	4 ± 2	1 ± 0	1 ± 1	11
Recovery[b] (%)	93	90	95	88	95	89	91
Recovery[c](%)	96	100	100	106	114	78	103

[a] Values obtained when the water extract was treated with EPG prior to sugar analysis.
[b] Sum from all extracts plus residue compared to intact walls when the water extract was not treated with EPG.
[c] Sum from all extracts plus residue compared to intact walls when the water extract was treated with EPG prior to sugar analysis.

Table 2
Weight of sugars (mg) detected by gas–liquid chromatography in extracts from 500 mg of cell walls prepared from 5-d-old cotton cotyledons ($n = 5$)

	Ara	Rha	Xyl	GalA	Gal	Glc	Total
Intact walls	25 ± 6	12 ± 2	16 ± 2	97 ± 6	20 ± 2	10 ± 3	180
Extracts after −23 °C HF							
Ether extract	19 ± 2	6 ± 1	10 ± 1	3 ± 0	5 ± 0	1 ± 1	44
Water extract	4 ± 1	5 ± 1	4 ± 0	43 ± 3	8 ± 0	1 ± 1	65
Water extract[a]	2 ± 1	3 ± 1	5 ± 1	82 ± 5	11 ± 0	3 ± 0	106
Imidazole extract	0 ± 0	0 ± 0	1 ± 0	17 ± 1	0 ± 0	1 ± 0	19
Residue	0 ± 0	0 ± 0	2 ± 0	4 ± 1	1 ± 0	7 ± 1	14
Recovery[b] (%)	92	92	106	69	70	100	79
Recovery[c] (%)	84	75	113	109	85	120	102

[a] Values obtained when the water extract was treated with EPG prior to sugar analysis.
[b] Sum from all extracts plus residue compared to intact walls when the water extract was not treated with EPG.
[c] Sum from all extracts plus residue compared to intact walls when the water extract was treated with EPG prior to sugar analysis.

Table 3
Weight of sugars (mg) detected by gas–liquid chromatography in extracts from 500 mg of cell walls prepared from 7-d-old cotton cotyledons ($n = 5$)

	Ara	Rha	Xyl	GalA	Gal	Glc	Total
Intact walls	24 ± 4	14 ± 1	15 ± 1	102 ± 6	21 ± 1	13 ± 3	189
Extracts after −23 °C HF							
Ether extract	21 ± 2	5 ± 1	9 ± 2	4 ± 1	6 ± 1	6 ± 1	51
Water extract	1 ± 1	2 ± 1	3 ± 0	46 ± 3	8 ± 1	5 ± 1	65
Water extract[a]	2 ± 0	4 ± 1	2 ± 1	76 ± 3	10 ± 1	4 ± 0	98
Imidazole extract	0 ± 0	0 ± 0	0 ± 0	12 ± 3	0 ± 0	0 ± 0	12
Residue	1 ± 1	0 ± 0	2 ± 0	4 ± 1	1 ± 0	8 ± 0	16
Recovery[b] (%)	96	50	93	65	71	146	76
Recovery[c] (%)	100	64	87	94	81	138	94

[a] Values obtained when the water extract was treated with EPG prior to sugar analysis.
[b] Sum from all extracts plus residue compared to intact walls when the water extract was not treated with EPG.
[c] Sum from all extracts plus residue compared to intact walls when the water extract was treated with EPG prior to sugar analysis.

To focus directly on the HGs (since they are the target for EPG) rather than on the whole cell wall, we solubilized the HGs for compositional and size analysis. We have found previously (Mort et al., 1991) that a treatment with hydrogen fluoride (HF) at −23 °C breaks the rhamnosyl linkages in pectins as well as most other neutral sugar linkages without cleaving GalA linkages, thus making it possible to completely solubilize highly methyl esterified HG in water and little-esterified HG in 500 mM imidazole buffer.

The HF procedure results in a fractionation of the cell walls: monosaccharides released by the HF are soluble in the HF/ether mixture left at the end of the treatment. This fraction is designated "Ether Extract" in the tables. The water extract of the ether-insoluble fraction contains mainly methyl esterified HG and some neutral sugar oligosaccharides. Subsequent imidazole extraction of the residue contains HG with a low degree of esterification. The final residue contains mostly cellulose. Tables 1–3 show the sugar compositions and amounts of the various extracts from three different ages of cotyledons. A greater yield of GalA was obtained from the water-soluble fraction if it was treated with EPG before methanolysis. There were not any striking changes in sugar composition as the cotyledons expanded.

2.4. Molecular size of HGs

The lengths of the HG molecules, both in the water extract and in the imidazole extract, from HF-treated cotyledon walls were estimated by anion-exchange chromatography (Figs. 3 and 4). The HGs from cotyledons of different ages and from commercial pectic acid (totally deesterified HG), as a standard, were labeled with 2-aminopyridine and separated using a PA100 anion-exchange column with fluorescence detection. Prior to labeling, the cotton HGs were saponified so that every GalA residue in the chain would contribute to the interaction with the anion exchange column. The chromatogram resulting from commercial pectic acid showed separation of the pectic oligomers up to about 70 GalA residues. The identification of peaks from short oligomers was carried out by co-chromatography with commercial standards (i.e., dimer and trimer), and the identity of those from longer ones was inferred by assuming that the commercial pectic acid contained only a homologous series of GalA oligomers. The peaks on HG chromatograms were identified by comparison with those from the commercial pectic acid. Comparing the HGs from the water extract (Fig. 3) and the imidazole extract (Fig. 4) among 3-, 5-, and 7-day-old cotyledons, shows that HGs from younger cotyledons appear to be somewhat smaller (more degraded?) in both extracts. This is particularly evident when one takes into account

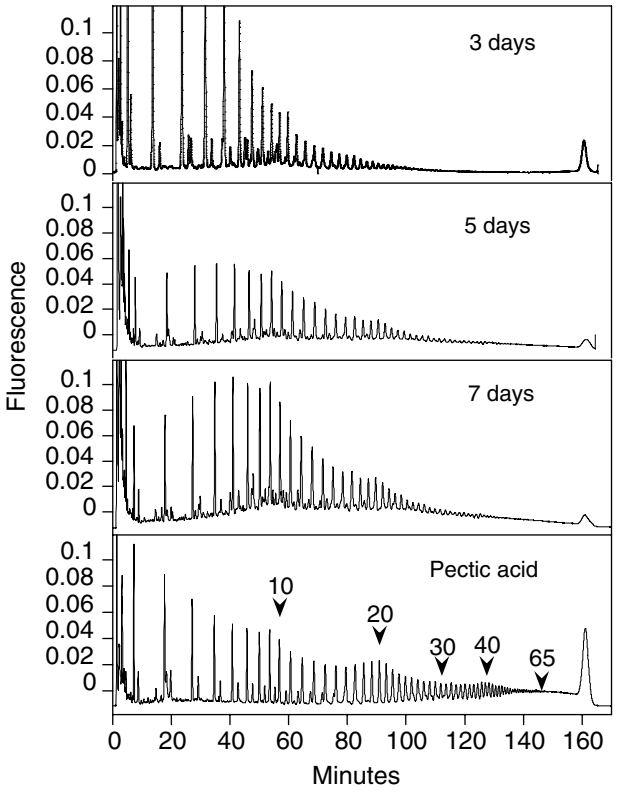

Fig. 3. PA100 anion-exchange chromatography molecular size profiles of homogalacturonans in the water extract from cell walls of 3-day-old, 5-day-old, and 7-day-old cotton cotyledons. The bottom panel shows the results obtained from pectic acid (from citrus pectin) for comparison. The numbers indicate the number of GalA residues per molecule for material which elutes at the corresponding time.

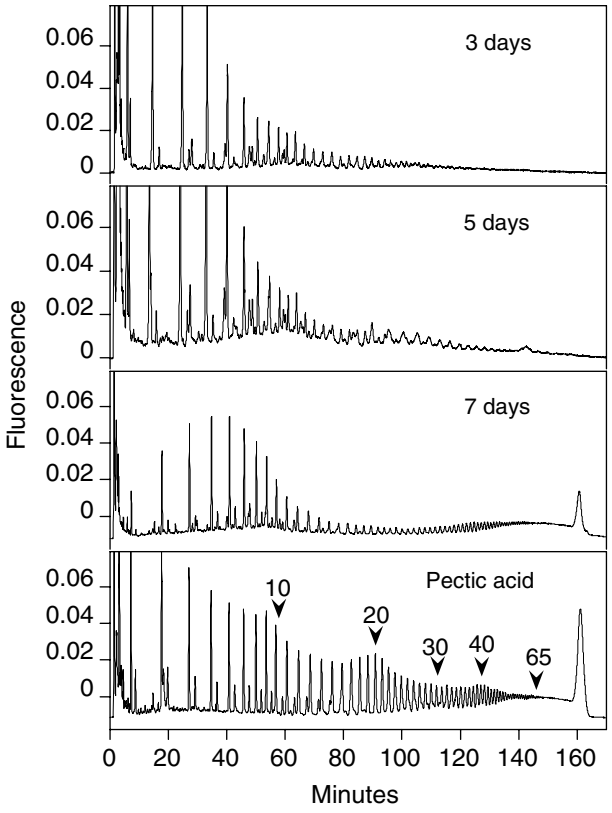

Fig. 4. PA100 anion-exchange chromatography molecular size profiles of homogalacturonans in the imidazole extract from cell walls of 3-day-old, 5-day-old, and 7-day-old cotton cotyledons. The bottom panel shows the results obtained from pectic acid (from citrus pectin) for comparison. The numbers indicate the number of GalA residues per molecule for material which elutes at the corresponding time.

that the area of each peak is proportional to the number of moles of the oligomer since only the reducing end is labeled. Thus, it takes 50 μg of a 50-mer to give the same response as 1 μg of monomer. Since the HF treatment used to solubilize HGs was the same in all cases, the changes in HG size indicate less degradation by EPG (Fig. 2) as the cotyledons expand, or that the HG is synthesized as shorter chains in rapidly expanding cotyledons.

2.5. Esterification of HGs

Although the O-2 and O-3 positions of HG are sometimes esterified with acetate, ^{1}H NMR spectroscopy of the water and imidazole extracts indicated a negligible amount of acetylation of cotton cotyledon HGs.

The degrees of esterification (DE) of the carboxyl groups of HGs in the water extracts were 57%, 47%, and 47%, and in the imidazole extracts they were 34%, 25%, and 27% from the 3-, 5-, and 7-day-old cotton cotyledons, respectively. There was no significant change to the DE between 5- and 7-day-old cotyledons, not only in imidazole-soluble HGs but also in water-soluble HGs. The DE of HGs of 3-day-old cotyledons was dramatically higher than for older cotyledons. A high DE prevents pectic polymers from associating with divalent calcium ions and may cause lower cohesion of middle lamellar pectins. The trend in decreasing DE with decreasing growth rate parallels results of Goldberg et al. (1986) in mung bean hypocotyls.

3. Conclusion

A sensitive, *in vivo* assay has been used in cotton cotyledons to quantitate the capacity for endo- and exo-polygalacturonase activity, and the results have been correlated to homogalacturonan structural properties in 3-, 5-, and 7-day-old cotyledons. High levels of EPG capacity were found in young, rapidly expanding cotyledons, and their HG contained a higher degree of esterification and was of a shorter average length than in the older cotyledons. We suggest from these results that the HG structure reflects the high EPG activity, with degradation of de-esterified stretches of the polymer resulting in shorter polymers and leaving them more esterified. As the cotyledons expand, either new HG is deposited in a less highly methyl-esterified form than earlier, or the HG in the more mature cotyledons has lost some of its esterification without being as degraded, which may reflect the much lower capacity for EPG activity in the older cotyledons. In contrast, exo-PG activity greatly increased as the cotyledons aged, providing capacity for re-cycling of the oligogalacturonides produced during cotyledon expansion.

4. Experimental

4.1. Plant materials

The upland cotton (*Gossypium hirsutum* L.) line used was Ac44E, a single plant selection made by M. Essenberg of our department (unpublished) from bacterial-blight susceptible cultivar Ac44. Plants were grown in flats or clay pots of Jiffy Mix-Plus in a Conviron E15 growth chamber as described by Pierce et al. (1993). Cotyledons, which in cotton are leaf like, were used for the experiments. The areas of detached cotyledons were measured by a leaf area meter (LAMBDA Instruments Corporation, USA). The relative growth rate was calculated as 100 times the average increase in area per hour over a 24-h period divided by the area at the beginning of the time period.

4.2. PG activity in vivo

PG activities *in vivo* were measured as described by Zhang et al. (1996) with slight modifications. The injection of 8-aminonaphthalene-1,3,6-trisulphonate (ANTS)-labeled substrate dissolved in distilled water into different aged cotyledons was carried out in the growth chamber in the middle of the photoperiod as follows. About 100 μg (5μg μl^{-1}) of ANTS-labeled hexamer of GalA, purified and labeled as described by Zhang et al. (1996), was injected into the intercellular space by using a 10-μl

gas-tight syringe (Hamilton Co., Reno, NV, USA) fitted with a needle made of a 15-cm section of 0.17-mm o.d. fused silica capillary (Alltech Associates, Inc., Deerfield, IL, USA). Two injections of 10 μl were made per cotyledon to avoid leakage of the solution out of the cotyledon. At a certain time, cotyledons were excised from the plants and placed in an Erlenmeyer flask (125 ml) containing about 30 ml of ice-cold extracting solvent (25 mM sodium acetate buffer, pH 5.2). The following steps were carried out at 4 °C. Vacuum was applied for 2 min from a water aspirator. Release of vacuum caused infiltration of the cotyledon's intercellular space by the extracting solvent. Infiltration of the cotyledon was completed by 10-s applications and release of the vacuum two to three times. An infiltrated cotyledon was removed, transferred to paper towels and blotted with tissue. The cotyledon was rolled with a taper and put into a 2-ml Reacti-Vial (Supelco, Inc., Bellefonte, PA, USA) reaching only half way to the bottom to avoid contact of the cotyledon with the intercellular wash fluid during centrifugation. The cotyledon was centrifuged at 1500*g* for 15 min. About 0.3 ml of intercellular wash fluid per cotyledon was collected and lyophilized. Before the enzyme activity assessment, the freeze-dried intercellular wash fluid of the cotyledon was dissolved in 3 μl of deionized water and centrifuged at 1500*g* for 10 min. Two microliters of supernatant was taken for CZE.

Enzyme activities were assessed by using capillary zone electrophoresis, as described by Zhang et al. (1996). A 60-cm fused silica capillary (Polymicro Technologies, Phoenix, AZ) of 50-μm i.d. (365-μm o.d.) was used as the separation column. The length was 34 cm from injection end to the detection window. New capillary was treated with running buffer (0.1 M phosphate buffer, pH 2.5) overnight. The capillary was rinsed with running buffer after each run and allowed to equilibrate for 2 min before each injection. Samples were introduced hydrodynamically, and electrophoresis was conducted at 17 kV with the negative electrode on the injection side. The capillary was stored in deionized water for long-term storage. The custom-made instrumental setup for capillary electrophoresis was comprised of a Spellman Model CZE 1000R high voltage power supply (Plainview, NY, USA) with positive and negative polarity and a Model FL-750 HPLC Plus Spectrofluorescence Detector (McPherson Instrument, Acton, MA, USA) equipped with a cell for on-column capillary detection and a 200 W Xenon–Mercury lamp with the excitation wavelength set to 364 nm; a cut-off filter permitted detection of emission beyond 440 nm. Data were collected using custom-built data loggers (Merz and Mort, 1992) and viewed and quantitated using the programs Analog Connection Chrom (Strawberry Tree Computer, Inc.) and KaleidaGraph (Abelbeck Software).

ANTS-labeled oligomers of GalA resulting from the incubation (*in vivo* digestion of the hexamer) were well separated by CZE and were identified by comparing their electrophoretic mobilities to those of ANTS-labeled authentic $GalA_1$, $GalA_2$, and $GalA_3$, and/or to the mobilities of an ANTS-labeled partial hydrolysate of pectic acid, which contained oligomers of from 1 to 23 residues of GalA. *In vivo* enzyme activity was calculated as peak area ratio per unit time of substrate incubation in the cotyledon at 30 °C. The peak area ratio was calculated by dividing the sum of the areas of the peaks representing the products of a particular enzyme activity by the sum of all peak areas (reactant plus products). In other words, the peak area ratio for exo-PG was the peak area for the pentamer divided by the sum of the peak areas of the pentamer plus the substrate hexamer, and for EPG it was the sum of peak areas for the dimer and trimer divided by that sum plus the peak area of the substrate hexamer. Units of enzyme activity could be estimated from the peak area ratios by reference to a standard curve produced in the assay with known units of commercial EPG.

4.3. Enzyme activity standard curve

Aliquots of 10 μg of ANTS-labeled hexamer of GalA in 2 μl of 25 mM sodium acetate buffer, pH 4.0, were incubated with 2 μl of a dilution series of EPG enzyme from *Aspergillus niger* (Megazyme International Ireland Ltd., County Wicklow, Ireland) at 40 °C for 5 min. The enzyme products were analyzed by CZE as described in the previous section. The standard curve was plotted as the peak area ratio per 5 min vs. EPG units. A linear relationship was obtained between 0% and 75% conversion/5 min vs. 0–0.012 units of activity ($R = 0.997$). One unit of activity was defined as the amount of enzyme required to release one μmol of reducing-end equivalents from the substrate per min at pH 4 and 40 °C.

4.4. Cell wall preparation

Cotyledons (~100 g fresh weight per batch) were excised, rinsed, and the main veins were removed. The cotyledons were immediately immersed in liquid nitrogen and crushed into a fine powder using a pestle and mortar. The frozen powders were homogenized with 100–200 ml of PAW buffer (phenol:acetic acid:water = 2:1:1, v/v/v) (Ring and Selvendran, 1978) in a polytron (Brinkmann Instruments, Inc., Westbury, NY, USA) at high speed for 9 min with three or four interruptions to avoid overheating. The cells were viewed under the microscope to check for complete rupture. The mixture was diluted with PAW buffer to a final volume of 500 ml and placed at 4 °C overnight. The mixture was then centrifuged at 4000*g* for 30 min, and the supernatant was discarded. The residue was washed three times with deionized water and placed in 10 vol of dimethyl sulfoxide in the cold room overnight to remove the starch (Ring and Selvendran, 1978). The mixture was filtered on nylon cloth (pore size of 35 μm) (Small Parts Inc., Miami Lakes, FL, USA). The residue was washed twice with dimethyl sulfoxide and three times with 5 vol of choloroform:methanol (1:1, v/v); the organic solvent was removed by a gentle suction to a coarse sin-

tered-glass funnel and the cell walls were washed with 5 vol of acetone at least three times, air-dried, and stored at room temperature until use.

4.5. Hydrogen fluoride solvolysis and HG isolation

Almost all of the HG of a plant cell wall can be extracted after the preparation is treated with HF at −23 °C (Mort et al., 1991), a treatment which cleaves the rhamnosyl linkages in the pectin while leaving those of GalA and methyl esters on them intact (Komalavilas and Mort, 1989). The cell walls were treated with anhydrous liquid HF as described by Mort (1983) with the modifications described by Qi et al. (1993). In the experiment, 500 mg of dry cell walls were placed in a Teflon reaction vessel. The vessel was pre-incubated in 95% ethanol at −23 °C for at least 20 min. The cooled cell walls were treated with 20 ml of HF at −23 °C for 30 min. To stop the reaction, the reaction vessel was immersed in liquid nitrogen until the HF solidified, and cold ether (cooled by adding liquid nitrogen to the ether) was added to the reaction mixture. The quenched reaction mixture was allowed to come to room temperature and filtered on a Teflon filter (pore size of 1–2 μm). The filtrate from the reaction mixture was designated "Ether Extract," and the HF-ether was evaporated from it under vacuum through a liquid nitrogen trap. The residue from the reaction mixture was placed in deionized water at 4 °C overnight. The mixture was filtered with a Teflon filter, and the residue was washed with water and filtered at least two times. The aqueous filtrates were combined and freeze-dried (Water Extract). The residue was placed in 0.5 M imidazole buffer, pH 7.0, at 4 °C overnight. The suspension was filtered and washed with the same buffer two times. The filtrates were combined and freeze-dried (Imidazole Extract). The residue was washed with water and freeze-dried (Residue).

4.6. Sugar composition analysis

Carbohydrate compositions were determined by gas chromatographic analysis of the trimethylsilyl methyl glycosides. Methanolysis and derivatization were carried out by a modification of the protocol of Chaplin (1982). Briefly, about 50 μg of dry samples were weighed on a CAHN 29 electrobalance (Instrument Group Walnut Creek Division, Walnut Creek, CA, USA) and placed in screw-cap glass vials fitted with Teflon-lined lids containing 100 nmol of inositol as an internal standard. Two hundred μl of 1.5 N methanolic HCl and 100 μl of methylacetate were added and incubated in a 80 °C heating block overnight. After adding five to seven drops of *t*-butanol, the samples were evaporated to dryness under a stream of nitrogen gas. Fifty microliters of freshly prepared trimethylsilylating reagent, one part of Tri-Sil (Pierce Chemical Company, Rockford, IL, USA) to three parts of dry pyridine (Pierce Chemical Company), was applied and allowed to react at room temperature for at least 15 min. The derivatized samples were evaporated under nitrogen just to dryness, and redissolved in 50–100 μl isooctane. A 1-μl aliquot was injected into a DB-1 fused silica capillary column (0.25-μm film thickness; J&W Scientific, Inc., Rancho Cordova, CA) installed in a Varian 3300 gas–liquid chromatograph. The sample was injected at 105 °C; the temperature was immediately raised to 160 °C, held for 4 min, and then raised at 2 °C min^{-1}–220 °C. The column was purged at 240 °C before being returned to initial conditions. Peak integration was performed using a Varian 4290 integrator.

4.7. Degree of esterification (DE) of HGs

The DEs of HGs were determined by the method described by Maness et al. (1990). Esterified GalA residues were specifically reduced to galactose and the percentages of GalA converted to Gal were used to calculate the degrees of esterification. Briefly, approx. 100 μg of HG was suspended in 40 μl of 1.0 M imidazole-HCl buffer, pH 7.0, and cooled on ice. About 4 mg of sodium borohydride was added and the sample vials were loosely capped and placed on ice for at least 1 h. Excess borohydride was decomposed by slow addition of 20 μl of glacial acetic acid. An equal volume (60 μl) of distilled water was added, and the reduced HG was obtained by precipitation with 4 vol of 95% ethanol. The sample was resuspended in water, precipitated with ethanol two times for removing the salts, and dried in a vacuum centrifuge. The glycosyl compositions of both the reduced HG and the native HG were determined by gas chromatographic analysis. DE was calculated by:

$$\mathrm{DE} = \frac{\text{galactose(R)} - \text{galactose(N)}}{\text{galactose(R)} - \text{galactose(N)} + \text{galacturonic acid(R)}} \times 100$$

where galactose(R), galactose(N), and galacturonic acid(R) represent galactose in the reduced sample, galactose in the native sample, and galacturonic acid in the reduced sample, respectively.

4.8. Size analysis of HGs by ion-exchange chromatography

Totally de-esterified HG oligomers yield a regular series of peaks in ion-exchange chromatography, which shows a predictable pattern of elution time versus residue number (Yu and Mort, 1996). HGs were dissolved in 0.1 N NaOH. The pH was monitored with pH paper and maintained for 15 min at 12 by adding 0.1 N NaOH. The pH was adjusted to between 6 and 7 with 0.1 N acetic acid. The de-esterified HGs were dialyzed overnight against water in 1000 MW-cutoff tubing (Spectrum Medical Industries, Inc., Los Angeles, CA, USA) and freeze-dried. Only GalA oligomers of one or two residues in length are lost from the dialysis tubing under these conditions (Mort et al., 1991). De-esterified HGs were labeled with 2-aminopyridine at their reducing end by a condensation reaction, and the excess labeling reagent was removed as described by Maness et al. (1991).

The 2-aminopyridine-labeled HGs were chromatographed on a CarboPac PA100 HPLC anion-exchange column (4×250 mm) using a Dionex Bio-LC Carbohydrate System (Sunneyvale, CA) at an eluent flow rate of 1.0 ml min^{-1}. Separation was carried out using a gradient consisting of solvent 1 (water) and solvent 2 (1.0 M phosphate buffer, pH 7.0, containing 0.25 M NaCl) (Yu and Mort, 1996). Sample components were injected into the system equilibrated with 50 mM phosphate buffer and eluted after a 3-min lag period using a linear gradient of phosphate buffer from 50 mM to 270 mM over 47 min, to 350 mM over 40 min, to 430 mM over 65 min, then to 500 mM over 5 min, with a final 5-min hold at 500 mM to wash the column. The system was allowed to equilibrate at initial conditions for at least 10 min prior to another injection. The 2-aminopyridine-labeled HGs were detected by a Shimadzu RF 535 fluorescence detector (Kyoto, Japan), with the exciting wavelength set at 290 nm and emission wavelength at 350 nm. Data were collected and quantitated as described for CZE.

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