Regular Paper

Heteromannan and Heteroxylan Cell Wall Polysaccharides Display Different Dynamics During the Elongation and Secondary Cell Wall Deposition Phases of Cotton Fiber Cell Development

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(Received March 17, 2015; Accepted June 27, 2015)

The roles of non-cellulosic polysaccharides in cotton fiber development are poorly understood. Combining glycan microarrays and in situ analyses with monoclonal antibodies, polysaccharide linkage analyses and transcript profiling, the occurrence of heteromannan and heteroxylan polysaccharides and related genes in developing and mature cotton (*Gossypium* spp.) fibers has been determined. Comparative analyses on cotton fibers at selected days post-anthesis indicate different temporal and spatial regulation of heteromannan and heteroxylan during fiber development. The LM21 heteromannan epitope was more abundant during the fiber elongation phase and localized mainly in the primary cell wall. In contrast, the AX1 heteroxylan epitope occurred at the transition phase and during secondary cell wall deposition, and localized in both the primary and the secondary cell walls of the cotton fiber. These developmental dynamics were supported by transcript profiling of biosynthetic genes. Whereas our data suggest a role for heteromannan in fiber elongation, heteroxylan is likely to be involved in the regulation of cellulose deposition of secondary cell walls. In addition, the relative abundance of these epitopes during fiber development varied between cotton lines with contrasting fiber characteristics from four species (*G. hirsutum*, *G. barbadense*, *G. arboreum* and *G. herbaceum*), suggesting that these non-cellulosic polysaccharides may be involved in determining final fiber quality and suitability for industrial processing.

Keywords: Cotton • Fiber cell development • *Gossypium* spp • Heteromannan • Heteroxylan • Plant cell walls.

Abbreviations: CBM, carbohydrate-binding module; CDTA, 1,2-diaminocyclohexanetetraacetic acid; CesA, cellulose synthase; Csl, cellulose synthase-like; dpa, days post-anthesis; ELISA,enzyme-linked immunosorbent assay; GC, gas chromatography; GC/MS, gas chromatography/mass spectrometry; GO, gene ontology; HG, homogalacturonan; PBS, phosphate-buffered saline.

Introduction

Cotton is one of the most important textile fibers, with around 25 Mt produced each year (International Cotton Advisory Committee, ICAC). The genus *Gossypium* includes approximately 50 species, and four of them (*G. barbadense*, *G. hirsutum*, *G. arboreum* and *G. herbaceum*) have been domesticated for cotton fiber production. Two of these cultivated species (*G. hirsutum* and *G. barbadense*) are allotetraploids (AADD) resulting from the natural hybridization of two parental diploid lineages (the A and D genome groups) that probably occurred during the Cretaceous period (Wendel and Cronn 2002). Efforts are being made towards the full sequencing of the cotton genome and, recently, the D genome from *G. raimondii* and the A genome from *G. arboreum* have been completed (Wang et al. 2012, Li et al. 2014a). Cotton fibers are not only important for textile and derivative industries but they also represent an excellent single-cell model to study cell wall synthesis and cell expansion as they have an extraordinary capacity for both cell elongation (independent of cell division) and secondary cell wall synthesis.

A mature cotton fiber is composed of approximately 95% cellulose; however, cellulose only accounts for approximately 35–50% of the primary cell wall of expanding fibers (Tokumoto et al. 2002). Other main polysaccharides are pectins, other non-cellulosic glycans, and glycoproteins. These cell wall polymers and glycoproteins undergo active synthesis, modification and turnover during fiber development. Fiber development is a finely regulated process that completes in 50–60 d and is often divided into five sequential and overlapping events: initiation, elongation, transition, secondary cell wall deposition and dessication. The timing of these events and final fiber qualities vary between germplasms, and are also greatly influenced by temperature cycles during fiber growth (Roberts et al. 1992, Thaker et al. 1989, Liakatas et al. 1998). Fiber development has been described in detail for the commercial *G. hirsutum* lines. Fiber initiation takes place from 0 to 5 days post-anthesis

(dpa) when selected epidermal cells emerge from the seed surface forming the fiber initials. Fiber diameter (usually between 11 and 22 μm) is determined during this stage (Paiziev and Krakhmalev 2004, Wilkins and Arpat 2005). During the elongation phase, fibers grow in a spiral-like manner, gathered in bundles by the cotton fiber middle lamella (CFML), so that the space inside the very packed environment of the locule is optimized (Paiziev and Krakhmalev 2004, Singh et al. 2009). By 17–20 dpa, the transition phase occurs and it is defined as the end of cell elongation and the beginning of secondary cell wall deposition. This phase is characterized by an abrupt increase in the rate of cellulose synthesis (Meinert and Delmer 1977) and the formation of the winding layer (similar to the S1 layer in xylem vessels) which is the first of the secondary cell wall layers to be deposited. The cellulose microfibrils in the winding layer are orientated at an angle of 20–30° to the elongation axis and in opposite helical gyre to the inner secondary cell wall layer where cellulose microfibrils are placed at a 70° angle to the elongation axis (Flint 1950). The winding layer confers a significant degree of the final fiber strength (Stiff and Haigler 2012). More layers of cellulose are formed during the secondary cell wall deposition stage, each one with a steeper helical gyre compared with the previous outer layer. A significant feature of cotton fibers is the change of direction of cellulose microfibrils called reversals (Flint 1950, Yatsu and Jacks 1981, Seagull 1986), which happen several times along the fiber. Around 55 days after flowering, the boll opens, and fibers desiccate and become suitable for harvest. Cotton fibers twist during desiccation due to their reversals, making possible the spinning of cotton fibers into yarn. At the final stage, the secondary cell wall can be around 4 μm thick (Kim and Triplett 2001) and the remaining outer primary wall that surrounds the secondary wall is a thin layer of approximately 200 nm (Singh et al. 2009).

Extensive cell wall remodeling takes place during fiber development. Changes in terms of monosaccharide composition, degree of polymerization of cellulose and changes in the molecular weight of pectic fractions and xyloglucan have been reported (Meinert and Delmer 1977, Timpa and Triplett 1993, Tokumoto et al. 2002). However, little is known about other cell wall matrix glycans such as heteromannan and heteroxylan. The monosaccharide mannose was detected in cotton fibers long ago (Meinert and Delmer 1977), and incorporation of radioactivity from GDP-mannose was shown to be correlated with that from GDP-glucose in particle fractions at the primary cell wall stage (Buchala and Meier 1985). However, the analysis of these radioactive products was not explicitly related to heteromannan polysaccharide, and no direct evidence of its presence in the cotton fiber cell wall has been reported. Glycan microarray analysis has indicated small quantities of the LM10 and LM11 xylan epitopes in an alkali extract of developing fiber cell walls (Singh et al. 2009, Avci et al. 2013), and cotton linters xylan has been analyzed by nuclear magnetic resonance (Kim and Ralph 2014). Using a range of approaches, we describe here the developmental changes in occurrence of heteromannan and heteroxylan polysaccharides, and associated transcripts, in cotton fibers from four *Gossypium* species

with distinctive physical properties in relation to textile processing.

Results

Cotton fiber cell wall heteromannan and heteroxylan epitopes show species-dependent developmental profiles

In order to study the occurrence of the heteromannan and arabinoxylan epitopes in developing and mature fibers, cotton fiber cell walls were sequentially extracted and screened by glycan microarrays providing semi-quantitative data (Moller et al. 2007). 1,2-Diaminocyclohexanetetraacetic acid (CDTA) and NaOH extractions of *G. barbadense* (PimaS7), *G. hirsutum* (FM966), *G. herbaceum* (Krasnyj) and *G. arboreum* (JFW15) fibers at 11 different developmental stages resulted in the detection of the LM21 heteromannan and AX1 heteroxylan epitopes in the alkali cell wall extracts (**Fig. 1**). The JIM7 high-ester homogalacturonan (HG) probe was included as control for the CDTA cell wall extraction efficiency.

The heteromannan LM21 epitope was present in all four cotton species and peaked at early developmental stages (8–10 dpa) except for the *G. barbadense* line PimaS7 which showed a slightly delayed occurrence, peaking between 10 and 13 dpa. The persistence of the LM21 epitope throughout fiber development has clear line-dependent patterns. In JFW15 (*G. arboreum*), the LM21 signal dropped between 10 and 15 dpa whereas in FM966 (*G. hirsutum*) and PimaS7 (*G. barbadense*) similar low levels were only reached at 30 dpa. The *G. herbaceum* line shows a trend between *G. arboreum* and the other two species. Analysis of FM966 (*G. hirsutum*) and JFW15 (*G. arboreum*) fibers using enzyme-linked immunosorbent assay (ELISA) corroborated the dynamics found in the glycan microarray and also demonstrated that LM21 binding was sensitive to mannanase action (**Supplementary Figure S1**).

The arabinoxylan AX1 epitope showed a very different developmental pattern in comparison with the LM21 epitope. The AX1 signal increased during the primary cell wall stage, reaching a maximum at 17 dpa for all cotton species and decreasing later during development, with the levels in mature fiber being below the detection limit. JFW15 (*G. arboreum*) had the lowest relative amounts of the AX1 epitope. These experiments show the presence of the LM21 heteromannan and AX1 heteroxylan epitopes in all four domesticated cotton species and suggest species-dependent profiles of the LM21 epitope and a time-dependent profile of the AX1 epitope.

Heteromannan- and heteroxylan-specific monosaccharide linkages are detected in cotton fibers

To corroborate the presence and relative abundance of heteromannan and heteroxylan polysaccharides in the cotton fiber, the same fiber samples were analyzed for monosaccharide composition and polysaccharide linkages. The data for FM966

	CDTA				NaOH			
DPA	PimaS7 (G. barbadense)	FM966 (G. hirsutum)	Krasnyj (G. herbaceum)	JFW15 (G. arboreum)	PimaS7 (G. barbadense)	FM966 (G. hirsutum)	Krasnyj (G. herbaceum)	JFW15 (G. arboreum)
AX1								
8	0	0	0	0	0	14	26	23
10	0	0	0	0	29	31	29	26
13	0	17	0	0	34	51	49	34
15	0	20	26	20	43	71	60	46
17	17	0	0	0	71	100	80	57
20	0	0	0	0	71	77	71	43
23	0	0	0	0	60	63	60	26
25	0	0	0	0	54	46	51	26
30	0	0	0	0	37	37	34	26
40	0	0	0	0	26	29	0	0
Mat	0	0	0	0	0	0	0	0
LM21								
8	0	0	0	0	53	83	80	80
10	0	0	0	0	95	82	82	87
13	0	0	0	0	100	80	68	55
15	0	0	0	0	88	55	50	20
17	0	0	0	0	75	50	35	0
20	0	0	0	0	53	62	37	12
23	0	0	0	0	72	37	18	0
25	0	0	0	0	53	45	20	0
30	0	0	0	0	8	10	12	8
40	0	0	0	0	20	0	15	0
Mat	0	0	0	0	40	0	0	8
JIM7								
8	51	79	100	51	0	0	0	0
10	56	77	67	65	0	0	0	0
13	69	81	55	48	0	0	0	0
15	64	41	48	17	0	0	0	0
17	71	49	51	0	0	0	0	0
20	32	43	43	8	0	0	0	0
23	27	23	21	0	0	0	0	0
25	21	21	28	0	0	0	0	0
30	0	0	8	0	0	0	0	0
40	0	0	9	0	0	0	0	0
Mat	0	0	0	0	0	0	0	0

Fig. 1 Glycan microarray of CDTA and NaOH extractions of cotton fibers at 11 different developmental stages from four different cotton species. Monoclonal antibodies used were AX1 for heteroxylan, LM21 for heteromannan and JIM7 for esterified homogalacturonan as a control for the CDTA extraction. DPA, days post-anthesis; Mat, mature. Values are means of nine technical replicates scaled to one maximum value of 100 for each antibody.
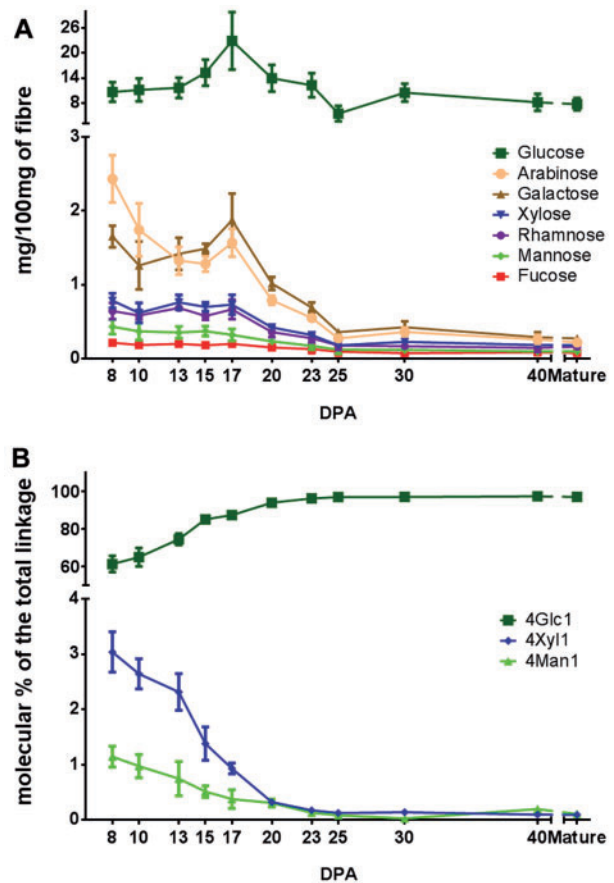
Fig. 2 Monosaccharide composition and linkage analysis of cotton fiber polysaccharides during development of FM966 (G. hirsutum). (A) Monosaccharide composition of extracted polysaccharides from FM966 fibers during development. (B) Quantification of selected sugar linkages indicating xylan, mannan and cellulose polysaccharides from a total sugar linkage analysis of FM966 cotton fibers during development. Error bars: SD ($n = 6$).

(G. hirsutum) are shown in **Fig. 2**, but the same monosaccharides and linkages were found in all cotton species (**Supplementary Figs. S2**, **S3**). The analysis of the alditol acetates by gas chromatography (GC) showed glucose as the major monosaccharide (**Fig. 2A**), doubling its amount from 8 dpa (9.2 mg 100 mg^{-1} of fiber) to 17 dpa (18.8 mg 100 mg^{-1} of fiber). It also confirmed the presence of mannose (up to 0.43 mg 100 mg^{-1} of fiber) and xylose (up to 0.78 mg 100 mg^{-1} of fiber) in FM966 (G. hirsutum) fibers. The amount of mannose

and xylose per mg of extracted fiber did not vary significantly during the early stages of fiber development up to 17 dpa. This is in contrast to arabinose that shows a decrease prior to 17 dpa. From 17 to 25 dpa, both mannose and xylose levels decreased and remained stable at very low levels subsequently. A similar pattern was observed for the other non-glucose monosaccharides. Additional evidence of the presence of mannan and xylan glycans in the cell wall was obtained by linkage analysis (**Fig. 2B**). As expected, the most abundantly detected linkage was the 1,4-glucosyl linkage that increased from 60 mol% at 8 dpa to 97 mol% at 25 dpa. Both mannan backbone- and xylan backbone-specific sugar linkages (1,4-linked mannose and 1,4-linked xylose, respectively) were detected at low levels in fiber cell walls. At 8 dpa, the mannan backbone linkage accounted for 1.1 mol% of the total extracted linkages, and the xylan backbone linkage for 3 mol%. The relative amount of these two linkages decreased as fiber development continued, and detection was negligible after 20 dpa. These data confirm the presence of both heteromannan and heteroxylan in cotton fibers.

Heteromannan- and heteroxylan-related genes are expressed in developing cotton fibers

The presence of mannan and xylan in cotton fiber cell walls suggests that mannan- and xylan-related genes are expressed in the developing fiber. Therefore, transcriptomic data from 10, 15 and 20 dpa cotton fibers were analyzed for the presence of mannan- and xylan-related transcripts. We identified five mannan synthesis-associated genes (Verhertbruggen et al. 2011, Yin et al. 2011) from the cellulose synthase-like (Csl) family (CslA2, CslA9, CslD2, CslD3 and CslD5) and three gene ontology (GO) annotated genes that showed expression in all the studied cotton lines (**Fig. 3A**). CslA2, CslD2 and CslD3 homologs appear to be expressed at all time points, whereas the CslA9 and CslD5 homologs are only expressed at 10 dpa. Amongst the genes expressed at all three time points, the two CslA2 homologs and one CslD3 homolog have a stronger expression at early days post-anthesis (particularly at 10 dpa). *Gossypium barbadense* and *G. hirsutum* showed a higher expression of the CslA2 homolog at both 10 and 15 dpa, while CslA2 is primarily expressed at 10 dpa in *G. arboreum*. The opposite is observed for the CslD3 homolog, which is expressed at both 10 and 15 dpa in *G. arboreum* but only at 10 dpa in the two other species. Xylan transcripts showed a completely different pattern of expression compared with mannan-related transcripts, with a strong signal only at 15 and 20 dpa (**Fig. 3B**). A block of expression is clearly visible in all species over 15 and 20 dpa, including genes homologous to IRX9, IRX10, GUX1, GUX2 and FRA8, and GO annotated genes. However, certain genes had a higher expression in *G. arboreum* compared with the two other species, and are more strongly expressed at 20 dpa in *G. barbadense*. Expression profiles of the cellulose synthase (CesA) genes were used as a positive control for the fiber transcriptome data (**Fig. 3C**). For the secondary cell wall-related CesA genes, including the CesA4, 7 and 8 homologs, a block of expression similar to that of xylan-related genes was observed at 15 and 20 dpa, with a stronger expression at 20 dpa in the *G. barbadense* lines.

The higher expression levels of several of the mannan-related genes at earlier time points and the later expression of the xylan-related genes provide complementary evidence supporting the differential regulation and different functions of heteromannans and heteroxylans during the development of cotton fibers.

JFW15 (*G. arboreum*) fibers mature earlier and have different mechanical properties compared with FM966 (*G. hirsutum*) fibers

For a more extensive analysis of the cotton lines studied here, we compared the mechanical properties of the mature fibers (**Table 1**). Among all the studied lines, JFW15 had the largest differences in mechanical fiber properties and suitability for textile processing when compared with the commercial FiberMax®-FM966. JFW15 produces a non-spinnable fiber that has poor fiber length (16.4 vs. 28.8 mm) and strength (20 vs. 35 g tex^{-1}) with low uniformity (72% vs. 85%) and very high micronaire (7.7 vs. 4.2). This micronaire value is

obtained by measuring the resistance to an airflow and depends on the fiber fineness and degree of maturation (Montalvo 2005, Montalvo et al. 2006). Breeders and cotton manufacturers select fibers with a micronaire between 3.8 and 4.5, as fibers that do not fall in this range cause problems for spinning and dyeing. To corroborate this higher degree of maturation of the JFW15 line, we measured the cell wall thickness in Calcofluor White-stained cross-sections of 10, 17, 25 dpa and mature fibers of PimaS7, FM966, Krasnyj and JFW15 (**Fig. 4**). Striking differences were found in the thickness of the JFW15 (*G. arboreum*) cell walls compared with those of FM966 (*G. hirsutum*). At 17 dpa, cell walls of JFW15 fibers are thicker (1.1 μm) than in FM966 (0.3 μm) and the secondary cell wall of JFW15 at 25 dpa (5 μm) is thicker than that of mature FM966 fibers. Cell walls of mature JFW15 fibers appeared four times thicker (8 μm) than in FM966 (2 μm). These data indicate that JFW15 produces an extra-thickened secondary cell wall the synthesis of which starts at an earlier day post-anthesis than in the other lines, as JFW15 fiber cell walls at 17 dpa are significantly thicker compared with FM966. The extra-thick cell walls of JFW15 are associated with this line having shorter, weaker fibers.

In situ analyses revealed the masking of the heteromannan and heteroxylan epitopes by pectic HG and their differential immunolocalization in cell walls throughout fiber development

The immunolocalization of heteromannan (LM21 and BS400-4) and heteroxylan (AX1, LM11, CBM2b2-1, CBM22 and UX1) epitopes in the cotton fibers was compared using cross-sections of resin-embedded material at several developmental stages. Masking of cell wall epitopes by non-related polysaccharides has been previously reported (Marcus et al. 2008, Marcus et al. 2010, Xue et al. 2013). On resin sections of mature cotton fibers, pre-treatment with sodium carbonate and pectate lyase uncovered heteromannan epitopes in the primary cell walls (**Fig. 5A**). Both the LM21 and BS400-4 heteromannan epitopes were found in the primary cell wall of mature fibers from all cotton lines and were highly masked by pectic HG (**Fig. 5A, B**). The BS400-4 probe showed higher binding to cotton fibers than the LM21probe (**Fig. 5A**), and PimaS7 (*G. barbadense*) had the highest fluorescence intensity at equivalent time exposures when compared with FM966 (*G. hirsutum*), Krasnyj (*G. herbaceum*) and JFW15 (*G. arboreum*) (**Fig. 5B**). This correlates well with the microarray data in which the LM21 epitope in mature fibers is detected at 40% of maximum signal in PimaS7 while it was below the detection limit in other lines. In situ labeling of developing PimaS7 fiber cross-sections (**Supplementary Fig. S4**) showed that the BS400-4 epitope is weakly detected in the secondary cell wall of 25 dpa fibers only, and pectate lyase treatment did not unmask the epitope in developing fibers.

The AX1 heteroxylan epitope was detected in a punctate pattern around the primary cell wall of 17 dpa fibers of FM966 and JFW15 (**Fig. 6**). The already thickened secondary cell wall of JFW15 displayed AX1 labeling around the inner cell wall
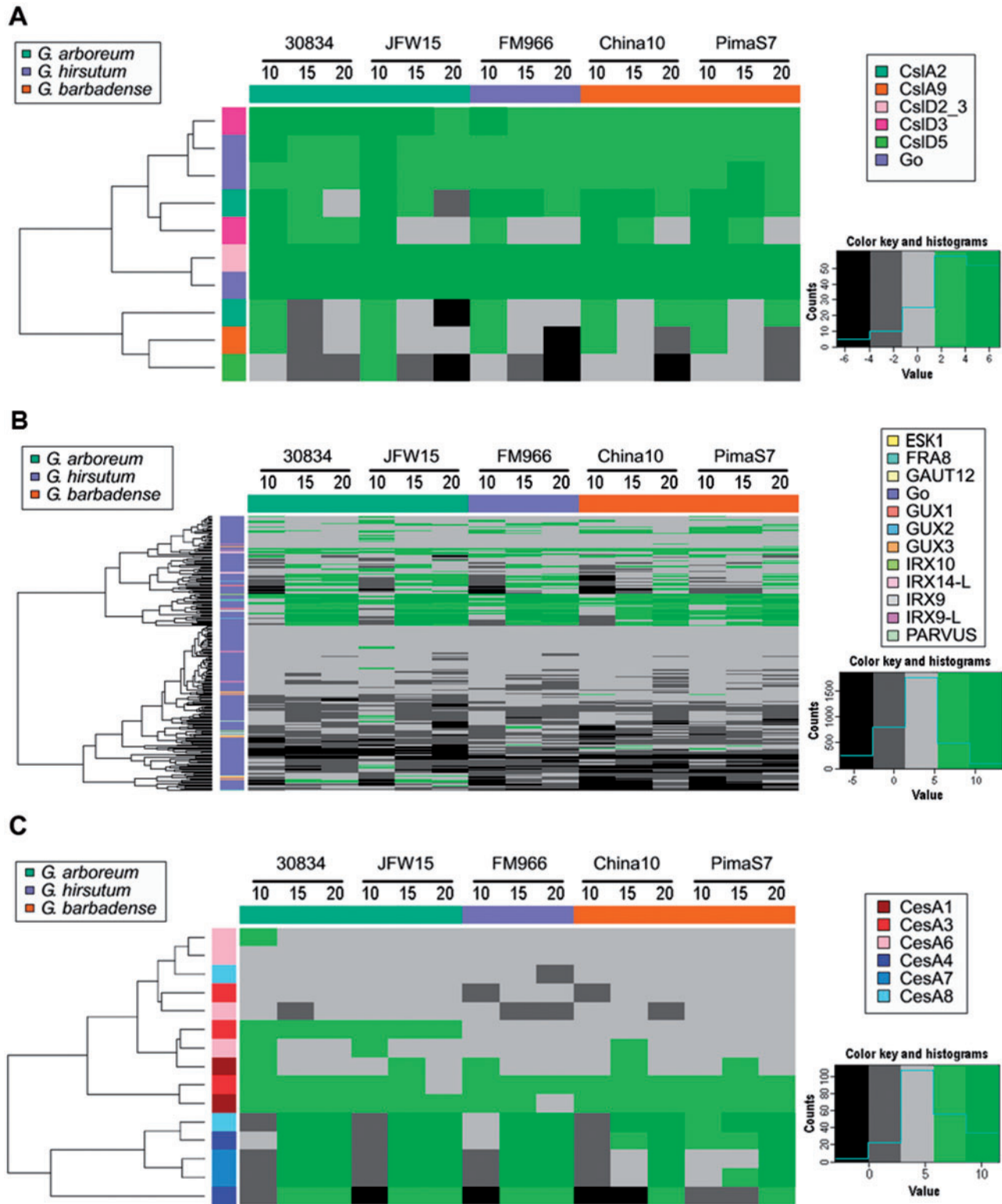
Fig. 3 Hierarchical clustering of transcripts associated with genes for mannan, xylan and cellulose synthases. RNA-seq data were filtered and clustered based on the expression level of transcripts identified as products of these genes based on GO annotations and publications. Right legend indicates identified homolog genes for the hits. The histogram on the right indicates the number of hits depending of their expression values (log2 transformed and split into five bins). (A) Clustering of mannan synthase gene expression. (B) Clustering of xylan synthase gene expression. (C) Clustering of CesA gene expression. Putative mannan or xylan synthase genes identified by gene ontology are named as GO for each cluster. CslD2_3 means that the gene was identified in our sample from two transcripts (splicing variants), one being a ortholog of CslD2 transcripts and one being a ortholog of CslD3.

Table 1 Comparison of the mature fiber characteristics and the mechanical properties of six lines obtained from four species of cotton

Name	Species	Length (mm)	Uniformity (% length)	Strength (g tex^{-1})	Elongation (%)	Micronaire
FM966	*G. hirsutum*	28.8	85	35.0	5.6	4.2
JFW15	*G. arboreum*	16.4	72	20.0	8.7	7.7
30834	*G. arboreum*	22.3	80	28.4	6.4	5.3
China10	*G. barbadense*	33.0	85	40.3	6.4	3.8
PimaS7	*G. barbadense*	32.3	86	44.4	5.5	4.1
Krasnyj	*G. herbaceum*	21.9	78	24.0	6.5	3.9

All values are the means of six measurements.
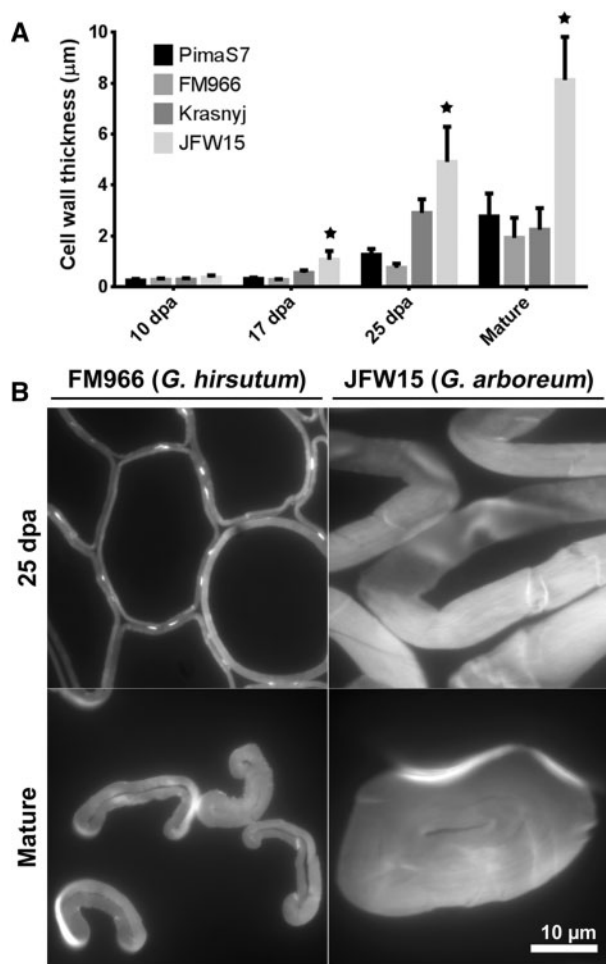
Fig. 4 Difference in cotton fiber cell wall thickness. (A) Measurements of cell wall thickness of 10, 17, 25 dpa and mature fibers from all lines (PimaS7, FM966, Krasnyj and JFW15). Error bars: SD ($n \geq 25$). Asterisk: Student *t*-test *P*-value <0.001 compared with FM966. (B) Representative Calcofluor White-stained cross-sections of 25 dpa and mature fibers from FM966 and JFW15.
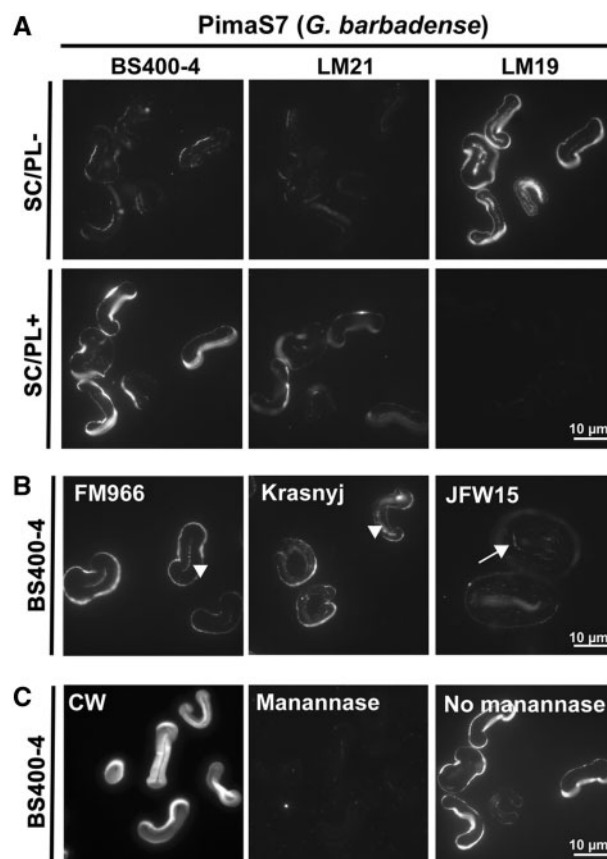

Fig. 5 Immunolocalization of heteromannans in cross-sections of mature cotton fibers. (A) Unmasking of the BS400-4 and LM21 heteromannan epitopes in equivalent sections of PimaS7 (*G. barbadense*) mature fibers after removal of pectic HG with pectate lyase. LM19 antibody is shown as a control of the pectate lyase enzymatic action. SC, sodium carbonate; PL–, no pectate lyase treatment (only buffer); PL+, pectate lyase treated. (B) The heteromannan BS400-4 epitope was found in all cotton species. Heteromannnans localized mainly at the primary cell wall and weakly in restricted regions of the secondary cell wall (arrowheads) and in the lumen (arrows) in FM966 (*G. hirsutum*), Krasnyj (*G. herbaceum*) and JFW15 (*G. arboreum*). All sections were SC/PL+ treated. (C) Mannanase treatment of PimaS7 mature fibers efficiently removed the heteromannan epitope BS400-4. All sections were SC/PL+ treated. All images in this figure were taken at the same time exposure (except for the Calcofluor White image in C). Exposure time in all BS400-4 and LM21 images = 1 s. The scale is the same for all the images.
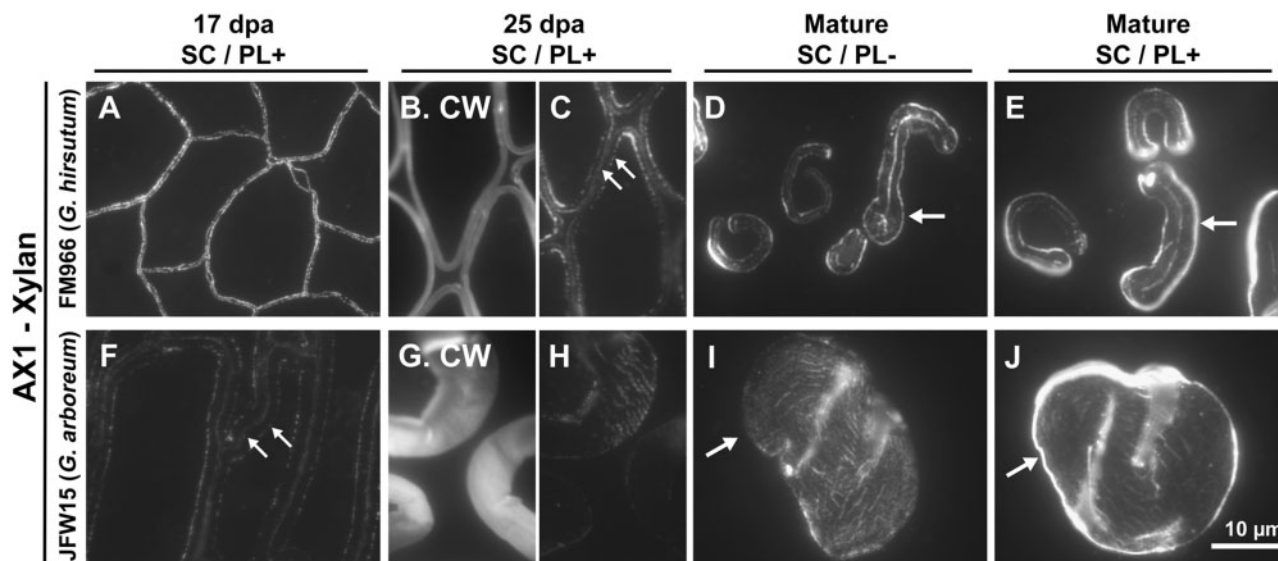
region next to the plasma membrane (**Fig. 6A, F**). This double localization of the AX1 epitope was also visible in FM966 at 25 dpa (**Fig. 6C**). In mature fibers, removal of pectin by pectate lyase uncovered the AX1 epitope in the primary cell wall (arrows in **Fig. 6D, E, I**, J). Additionally, in mature JFW15 fibers, the AX1 epitope was localized in concentric rings in

Fig. 6 Immunolocalization of the AX1 heteroxylan epitope in cross-sections of developing and mature cotton fibers of FM966 (*G. hirsutum*) and JFW15 (*G. arboreum*). A double immunolocalization of the AX1 epitope at the inner and outer part of the cell wall is indicated by arrows in (C) and (F). Unmasking of the AX1 epitope in the primary cell wall of mature fibers is shown by arrows in (D), (E), (I) and (J). Striations of AX1 labeling can be seen in secondary cell walls in (H), (I) and (J). SC, sodium carbonate; PL–, no pectate lyase treatment (only buffer); PL+, pectate lyase treated; CW, Calcofluor White-stained sections. The scale is the same for all the images.

the secondary cell wall (**Fig. 6I, J**). Similar detection of heteroxylan in the secondary cell wall of mature fibers was observed when using xylan-directed carbohydrate-binding modules (CBMs) CBM2b-1-2 and CBM22 (**Fig. 7**). The LM11 xylan antibody bound in a similar way, but the signal was weaker and the UX1 glucuronoxylan antibody bound mainly to the primary cell wall after pectin removal. The binding pattern in concentric rings was specific to the heteroxylan epitopes as verified by the LM19 HG antibody whose location was restricted to the primary cell wall of cotton fibers.

Discussion

Heteromannan and heteroxylan polysaccharide abundance and localization change throughout cotton fiber development.

Our results, using a combination of glycan microarray, in situ localization, monosaccharide composition and linkage analyses, demonstrate that heteromannan and heteroxylan polysaccharides occur in developing and mature cotton fibers of tetraploids *G. hirsutum* and *G. barbadense* as well as the diploids *G. herbaceum* and *G. arboreum*. Even though these polysaccharides are present at very low levels in mature cotton fibers relative to cellulose, they have specific time- and species-dependent patterns suggesting that they may influence specific cellular processes during fiber development, and in consequence impact the final fiber properties and their suitability for industrial purposes.

Heteromannan was present in all lines at early developmental stages (between 8 and 15 dpa) in the NaOH extract, as shown by glycan microarrays, but only detected in 25 dpa and mature fibers by in situ labeling. This suggests that

heteromannan is below the level of detection by immunocytochemistry at early stages or, perhaps more probably, that its context in relation to other cell wall molecules at early stages may block its detection in the intact cotton fiber cell walls and that it cannot be detected by the probes used here unless solubilized.

Heteroxylan was abundant in the cotton fiber at later developmental stages, and the AX1 epitope showed a clear time-dependent developmental profile. In situ labeling with AX1 showed the strongest and clearest signal at 17 dpa, detecting an AX1 epitope-rich layer in the innermost part of the forming secondary cell wall as well as the primary cell wall. This is in agreement with the glycan microarray data where the AX1 epitope showed a peak at 17 dpa for all lines, suggesting a later synthesis of this epitope compared with the heteromannan epitope and pointing to a role for this epitope during the transition phase. In contrast, the LM11 antibody only bound weakly to mature fibers after pectin removal, suggesting the recognition of different xylan epitopes by these probes. AX1 antibody was produced after immunization with arabinoxylan oligo-conjugates (Guillon et al. 2004), whereas LM11 was produced after immunization with a xylopentaose–bovine serum albumin (BSA) conjugate (McCartney et al. 2005). Although both AX1 and LM11 antibodies are expected to recognize arabinosyl-substituted xylan to some extent, LM11 in some instances does preferentially bind to unsubstituted xylan (Lovegrove et al. 2013). This differential binding of the two probes suggests that most of the cotton fiber cell wall heteroxylan is highly substituted and may differ structurally from the heteroxylan of dicot vascular tissue secondary cell walls. Interestingly, AX1 bound to the secondary cell wall of mature fibers, identifying concentric layers around the fiber elongation axis. Concentric layers of cellulose have been previously observed in *G. hirsutum* cross-sections after swelling with
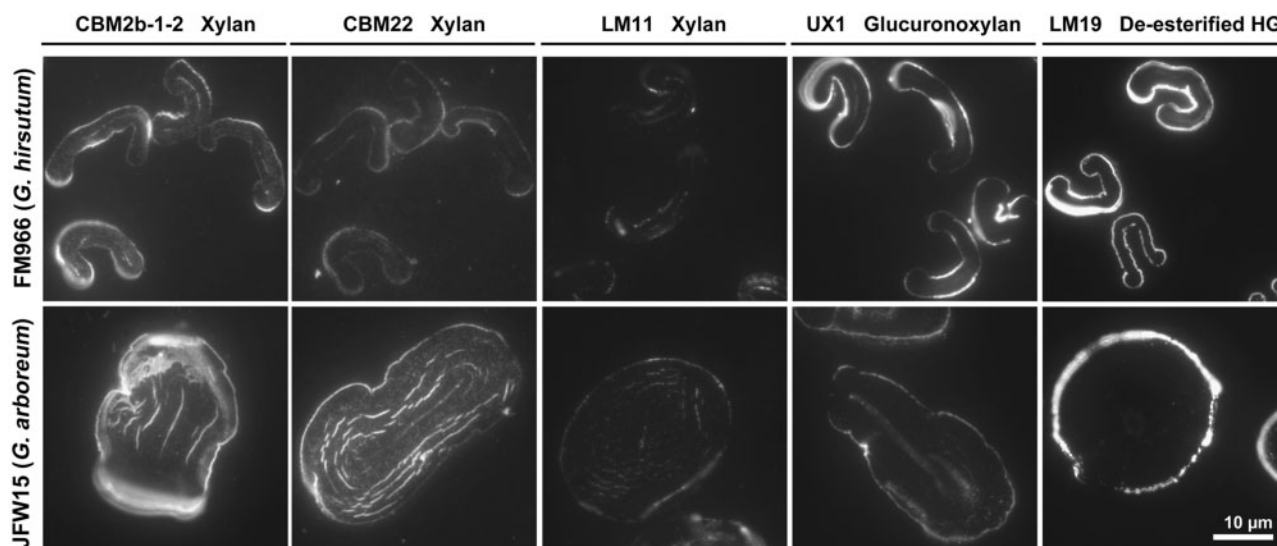
Fig. 7 Immunodetection of an extended set of xylan epitopes/ligands in cross-sections of mature fibers of FM966 (*G. hirsutum*) and JFW15 (*G. arboreum*) using CBM2b-1-2, CBM22, LM11 and UX1 probes. LM19 (de-esterified homogalacturonan) labeling is shown as a comparative epitope specific to the primary cell wall. In all cases for the xylan probes, sections were pre-treated with sodium carbonate and pectate lyase. The scale is the same for all the images.

NaOH (Balls 1919, Haigler et al. 1991, Roberts et al. 1992). These layers have been described as daily growth rings occurring due to a diurnal cycling of cellulose deposition that depends on day and night temperatures and can only be seen at the microscopic scale after fiber swelling. It is unclear whether the observed concentric layers in the JFW15 line are overpronounced growth rings of the same nature as those discussed above. It is possible that the cotton cell wall heteroxylan has a specific role in controlling aspects of this cyclic temperature-controlled cellulose microfibril deposition. In this regard, it has recently been observed that overexpression of *PtxtXyn10A*, a cell wall xylan endotransglyco-sylase gene, impacts cellulose microfibril angle in wood fibers (Derba-Maceluch et al. 2015), although the underlying cellular mechanism is unclear. A role for heteroxylan in cellulose micro-fibril deposition would also be consistent with the AX1-rich inner layer of secondary cell wall next to the plasma membrane.

In addition to the AX1 arabinoxylan epitope, the UX1 glucuronoxylan epitope was also present in mature fibers. Recently, it has been reported that two glucuronoxylan glycosyltransferases, GhGT43A1 and GhGT43C1, are preferentially expressed in 15 dpa and 20 dpa cotton fibers (Li et al. 2014b). Although glucuronoxylans are known to be a major component of secondary cell walls and only found in minor quantities in the primary cell walls of dicotyledonous plants (Zablackis et al. 1995), in our study UX1 bound mainly to the primary cell walls of both FM966 (*G. hirsutum*) and JFW15 (*G. arboreum*) cotton fibers upon removal of pectic HG, again suggesting a non-structural role for heteroxylan in cotton fibers unrelated to its role in vascular tissue secondary cell walls.

Fiber developmental pace impacts fiber quality in the JFW15 line (*G. arboreum*).

JFW15 is not suitable for textile processing due to its high micronaire and poor fiber length and strength. Our results demonstrate clear differences in the pace of cell development followed by JFW15 and that the relationship between days post-anthesis and the timing of developmental stages in JFW15 differs significantly from that of the other lines analyzed. When compared with a commercial line such as FM966, JFW15 shows an earlier start to secondary cell wall deposition (before 17 dpa) and a thicker secondary cell wall at equivalent days post-anthesis. These results suggest that the shorter and extra-matured fibers of this line are associated with a shorter elongation phase and an extensive secondary cell wall phase with a higher cellulose deposition rate. Moreover, the extra-thickened secondary cell walls could account for the earlier disappearance of epitopes such as the LM21 epitope during fiber development in the JFW15 line compared with other lines when analyzed by glycan microarrays, monosaccharide and linkage analyses. The large increase in cellulose content when the secondary cell wall is deposited would increase the dry weight of material and thus dilute the other polysaccharides per weight unit. Differences in cell wall thickness became noticeable at 17 dpa; nevertheless, the AX1 epitope peaked at 17 dpa even in the extra- matured JFW15 line. An extended analysis of other cell wall epitopes and a statistical correlation of epitope dynamics and fiber characteristics would be of interest in order to identify possible targets for fiber quality improvement.

JFW15 fiber has an extra-thick secondary cell wall and yet is very weak compared with fiber from other lines. The reason for this apparent contradiction may lie in the cell wall reversals. Reversals have been proposed as localized areas of cell expansion after the secondary cell wall is deposited, and their frequency along the fiber depends on the germplasm and developmental stage (Wakeham and Spicer 1951). Reversals have been traditionally considered as weak points prone to breakage (Raes et al. 1968, Seagull 1986); however, fiber strength can vary depending on the type of reversal and their frequency (Gould and Seagull 2002). In fact, it is known that *G. arboreum*

lines with thicker secondary cell walls have fewer reversals than any *G. hirsutum* line, and improved *G. arboreum* lines with thicker cell walls but higher reversal frequency have been achieved (Chandra and Sreenivasan 2011).

Functional significance of heteromannan and heteroxylan occurrence in cotton fiber cell walls

Heteromannans are highly conserved in plant evolution and are commonly found in the primary cell walls of charophytes and early land plants (bryophytes and lycophytes) but are less abundant in the later evolved gymnosperms and angiosperms (Nothnagel and Nothnagel 2007). In the latter, other hemicelluloses seem to have replaced heteromannans in their structural roles (Scheller and Ulvskov 2010). Nonetheless, heteromannan polysaccharides can be found in both primary and secondary cell walls of dicotyledonous plants. In Arabidopsis, mannans are abundant in the secondary cell walls of xylem cells and thickened epidermal walls (Handford et al. 2003), where they are proposed to be linkers of the hemicellulose–cellulose network (Schröder et al. 2009). Despite the extraordinary capacity of secondary cell wall formation of the cotton fiber, mannan polysaccharides were only detected in the primary cell walls of mature fibers, and linkage analysis showed that they occur at very low levels relative to cellulose. This suggests that a structural role for mannan in secondary cell walls of the cotton fiber cells is unlikely to be significant, and another function of mannan in cell signaling and the regulation of cell elongation or secondary cell wall deposition (Liepman et al. 2007, Zhao et al. 2013) is more likely.

Dhugga et al. (2004) reported mannan synthase activity in the Golgi apparatus by cellulose synthase-like (CSL) proteins from the CSLA family and, more recently, it has been shown that the CSLD family is also involved in mannan synthesis and acts in a multimeric complex (Verhertbruggen et al. 2011, Yin et al. 2011). Transcriptome profiling revealed the expression of putative mannan synthase and mannan glycosyltransferases genes with homology to CslA2 and 9 and CslD2, 3 and 5. Data presented here show that the expression of mannan synthase genes in cotton fibers occurs from at least 10 to 20 dpa, with an emphasis on the early stages. The global trend is the same across the species, with the exception of *G. arboreum*. This could be due to the fact that the *G. arboreum* species genome is diploid (genome A) while *G. hirsutum* and *G. barbadense* are tetraploid (combination of genome A and D) and they are likely to have developed differences in gene regulation during their respective evolution.

The suitability of our transcriptome profiling data to discriminate between genes expressed in early stages of development and genes expressed during the secondary cell wall deposition phase was verified using cellulose synthase genes. Indeed, homologs of CesA genes can be separated into two groups: CesA1, 3 and 6 that are proposed to be involved in primary cell wall synthesis; and CesA4, 7 and 8 that are proposed to be involved in secondary cell wall synthesis. This second group is strongly expressed at 15 and 20 dpa, which is consistent with the secondary cell wall thickening stage, and it

appears to be delayed for *G. barbadense* species in the same way as reported by Li et al. (2013). This secondary cell wall CesA expression profile is very similar to the expression profile observed for xylan synthesis genes. This is in accordance with a role for xylan synthesis genes in cellulose deposition as observed in Arabidopsis where several xylan synthesis mutants (e.g. *irx9* and *irx14*) have defects in cellulose deposition (Turner and Somerville 1997, Hao and Mohnen 2014). The function of heteroxylan is commonly related to the strengthening of cell walls and a load-bearing role as revealed by xylan mutants in the above-cited works. Cotton fibers have little need to cope with gravity or external forces during their development, but the strength of the fiber cell wall is crucial for its suitability for textile processing. The synchronicity between cellulose and heteroxylan in the cotton fiber cell wall points to a potential connection between these polysaccharides that modulates the construction of secondary cell walls in cotton fibers. As reported here and discussed above, such a connection is also evidenced by the fluorescence imaging of striations of heteroxylan epitopes reflecting cellulose deposition in the secondary cell walls.

In summary, the identification and study of cell wall heteromannan and heteroxylan in four species of cotton with differing fiber developmental rates and characteristics has confirmed the widespread presence of these non-cellulosic polysaccharides during fiber development and in mature fibers. These analyses in several species has also provided insights into the possible roles of heteromannan and heteroxylan in fiber development and provided a basis for the future elucidation of their molecular functions.

Materials and Methods

Plant materials

Six domesticated inbred cotton lines (FM966-FiberMax®, JFW15, 30834, Krasnyj, China10 and PimaS7) belonging to four different *Gossypium* species (*G. hirsutum*, *G. arboreum*, *G. herbaceum* and *G. barbadense*) were selected for this study. Seeds were sown in soil compost and grown at constant conditions in a greenhouse set at 26–28°C with a 16 h photoperiod. The sowing was done in two batches in February, and samples were collected during the months of April, May, June and July. Cotton flowers were tagged on the day of anthesis so that bolls could be harvested at the desired day post-anthesis. Immediately after collection, small regions of fiber tissues were carefully dissected from cotton bolls, causing minimal tissue disruption, and submerged in 4% (v/v) paraformaldehyde in PEM buffer (0.1 M PIPES pH 6.95, 2 mM EGTA, 1 mM MgSO$_4$) (Lee and Knox 2014) for microscopy analysis. For the study of cell wall components by glycan microarrays, ELISA, GC and GC/mass spectrometry (GC/MS) analysis, cotton bolls were frozen in liquid nitrogen immediately after collection and fibers from at least three different plants were detached from the seeds, pooled and boiled in 70% (v/v) ethanol for 30 min, air-dried and powdered using a nitrogen-cooled crusher (SPEX Sample Prep Freezer/mill 6870). For the RNA sequencing (RNA-seq) data, five bolls from three developmental stages, 10, 15 and 20 dpa, for each cotton line (except Krasnyj) were collected and immediately frozen in liquid nitrogen and stored at −70°C until extraction.

Determination of fiber characteristics

Fiber characteristics of length (mm), fiber uniformity (% length), strength (g tex^{-1}), elongation (%) and micronaire were determined with the High Volume

Instrument (HVI) (Suh and Sasser 1996) by CIRAD (France) from 5 g of mature cotton of each line.

Monoclonal antibodies and carbohydrate-binding modules

Monoclonal antibodies and CBMs used in this study were as follows: INRA-AX1 arabinoxylan (Guillon et al. 2004), LM11 unsubstituted xylan/arabinoxylan (McCartney et al. 2005), INRA-UX1 glucuronoxylan (Koutaniemi et al. 2012), LM21 heteromannan (Marcus et al. 2010), BS400-4 heteromannan (Biosupplies) (Pettolino et al. 2001), JIM7 esterified homogalacturonan (Knox et al. 1990, Willats et al. 2000, Clausen et al. 2003), LM19 de-esterified homogalacturonan (Verhertbruggen et al. 2009), CBM22 and CBM2b1-2 xylan-binding modules (McCartney et al. 2006).

Glycan microarrays

Microarray analyses were carried out essentially as described by Moller et al. (2007) but with minor modifications. Cotton fiber cell wall polymers were extracted sequentially in two solvents: 50 mM CDTA and 4 M NaOH with 1% (v/v) NaBH$_4$. For each of three replicates of each fiber at each day post-anthesis, 10 mg of fiber was extracted in 300 µl of solvents. The supernatant from each extraction was printed in four replicates and four dilutions [1 : 2, 1 : 6, 1 : 18 and 1 : 54 (v/v) dilutions], giving a total of 48 spots representing each developmental stage for each solvent. Three independent prints were used for each antibody.

Preparation of alditol acetates, partial methylation and GC acquisition parameters

Monosaccharide derivatization and partial methylation were carried out as described in Runavot et al. (2014). Cellulose, being a very resistant polysaccharide, is only partly analyzed by the methods used in this study; this particularly affects the results for older days post-anthesis. The results always refer to the extractable/accessible/hydrolyzable polysaccharides that we considered as representative of the cotton fibers at these different developmental stages. These analyses detected neutral sugars only.

Sugars were injected using the on-column mode of injection on a Trace GC ultra with an ISQ single quadrupole GC-MS (Thermo Scientific) and a flame ionization detector (FID) associated with a medium polarity, bonded phase DB-225 capillary column 30 m × 0.25 mm × 0.25 µm (Agilent) using hydrogen as the carrier gas with a flow rate of 4 ml min^{-1}. The MS transfer line was set at 240°C with 70 eV electron impact ionization mode, and the data from 41 to 450 *m/z* were acquired. The alditol acetates were separated by setting the oven to an initial temperature of 170°C, held for 0.5 min, and then ramped at 120°C min^{-1} to 210°C, ramped at 5°C min^{-1} to 220°C, ramped at 120°C min^{-1} to 230°C, ramped at 2°C min^{-1} to 240°C and then held for 0.5 min. The partially methylated alditol acetates were separated by setting the oven to an initial temperature of 150°C, held for 1 min, and then ramped at 120°C min^{-1} to 180°C, ramped at 5°C min^{-1} to 220°C, ramped at 120°C min^{-1} to 240°C and then held for 5.5 min.

Immunochemistry and in situ fluorescence imaging

Fixed developing fibers were dehydrated, resin-embedded and sectioned as described previously (Lee and Knox 2014). Mature fibers were neither fixed nor dehydrated but dewaxed before resin embedding: fibers were compressed to minimize air capture and placed inside glass jars. Solvents were used in a 75 : 1 ratio (v/w) and samples were vacuum infiltrated. Cotton fibers were treated with absolute ethanol for 90 min twice, followed by acetone for 60 min and ether for 60 min. Samples were left to air-dry overnight. All steps were carried out under a fume hood at room temperature.

Resin sections were used for light microscopy detection of cell wall epitopes. Phosphate-buffered saline (PBS) with 5% (w/v) milk protein was added for 30 min at room temperature to prevent non-specific binding. Primary antibodies were used at a 1 : 5 dilution, except the BS400-4 antibody that was used at 10 µg ml^{-1}, in 5% milk/PBS for 1.5 h. Goat anti-rat or anti-mouse IgG

Alexa Fluor488 (Life Technologies) were used as secondary antibody in a 1 : 100 dilution in 5% milk/PBS, and samples were incubated for 1 h. Calcofluor White (Sigma-Aldrich) was used at 0.02 mg ml^{-1} in PBS for 5 min for visualization of cell walls. Anti-fade reagent Citifluor glycerol/PBS (Agar Scientific) was added before covering with a coverslip. In some cases, sections were treated chemically and enzymatically prior to labeling. Pre-treatments included: 0.1 M sodium carbonate for 2 h at room temperature for removal of pectic methyl esters and recombinant pectate lyase from *Cellvibrio japonicus* (Megazyme) at 10 µg ml^{-1} in a 2 mM CaCl$_2$, 50 mM 3-(cyclohexylamino)-1-aminopropane sulfonic acid (CAPS) buffer, pH 10.0, for 2 h at room temperature for removal of pectic homogalacturonan.

Immunofluorescence imaging was performed using an Olympus BX61 microscope (http://www.olympus-global.com/) equipped with epifluorescence irradiation. Micrographs were obtained with a Hamamatsu ORCA285 camera (Hamamastu, http://www.hamamatsu.com) and PerkinElmer Volocity software. All related and comparative micrographs were captured using equivalent settings, and relevant micrographs were processed in equivalent ways for the generation of data sets.

Secondary cell wall measurements

Secondary cell walls were measured in micrographs of resin sections in ImageJ using high magnification Calcofluor White-stained cross-sections of 10, 17, 25 dpa and mature fibers from PimaS7, FM966, Kranyj and JFW15 lines.

RNA profiling

For each line and time point (10, 15 and 20 dpa), fibers were removed on dry ice from five bolls with RNase-free treated tweezers and stored at −70°C before extraction. RNA extraction was performed using a Spectrum™ plant total RNA kit (Sigma). Samples were checked for quality and amount using a NanoDrop ND-1000 spectrophotometer and sent to BGI (China) for RNA sequencing (RNA-seq quantification protocol, 100 nucleotide paired-end reads, Illumina Hiseq2000). The quality of the raw reads was checked with the FastQC tool (http://www. bioinformatics.babraham.ac.uk/projects/fastqc/) to set up parameters for the cleaning step with the FastX toolkit (mainly the first 15 nucleotides of the raw reads were removed to avoid nucleotides bias content and the last 5 nucleotides due to quality drop). The cleaned reads were used with RSEM (default parameters; http://deweylab.biostat.wisc.edu/rsem/) (Li and Dewey 2011) to assess the gene expression levels based on *Gossypium raimondii* (D5) genome JGI assembly v2.0 (annot v2.1; http://www.cottongen.org/species/Gossypium_raimondii/jgi_ genome_221). The TPM scores from RSEM were multiplied by a factor of 10^6 to obtain the expression values. The genes with no or low expression were filtered out, and only the genes with an expression value >5 were kept. The heatmap figures were generated by the 'heatmap.2' function from gplots_2.12.1 R package (R version 3.0.1) with default parameters for the gene expression profile clustering, and the expression values were log2-transformed for the display. For each gene, the GO annotation was added from the *Arabidopsis thaliana* gene ortholog to the GO annotation provided by JGI. The gene expression profile clustering of CesA gene homologs was performed as a positive control. For study of the both heteromannan and heteroxylan transcripts, gene association was conducted with GO search and individually identified genes in diverse publications on xylans or mannans. The GO annotated genes should only be considered as putative xylan or mannan genes as their roles in biosynthesis were not proven by specific experiments. All the IDs, GO association and expression level of the identified transcripts used in this study are available along with BLASTP scores and e-values in the **Supplementary data**. The data were derived from a search of the *G. raimondii* proteins corresponding to the transcripts of interest (deduced from genome assembly v2 and available on the CottonGen site (ftp://ftp.bioinfo. wsu.edu/species/Gossypium_raimondii/JGI_221_G.raimondii_Dgenome/genes/ G.raimondii_JGI_221_v2.1.proteins.fasta.gz) in the *A. thaliana* protein database (TAIR V10), with the aim of assessing the similarity between orthologs (annotation by JGI).

Supplementary data

Supplementary data are available at PCP online.

Disclosures

The authors have no conflicts of interest to declare.

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