Protoplasma (2011) 248:579–590
DOI 10.1007/s00709-010-0212-y

ORIGINAL ARTICLE

Polysaccharide and glycoprotein distribution in the epidermis of cotton ovules during early fiber initiation and growth

Andrew J. Bowling · Kevin Christopher Vaughn · Rickie B. Turley

Received: 17 September 2008 / Accepted: 14 September 2010 / Published online: 28 September 2010
© Springer-Verlag (outside the USA) 2010

Abstract The cotton fiber is a model system to study cell wall biosynthesis because the fiber cell elongates (~3 cm in ~20 days) without mitosis. In this study, developing cotton ovules, examined from 1 day before anthesis (DBA) to 2 days post-anthesis (DPA), that would be difficult to investigate via classical carbohydrate biochemistry were probed using a battery of antibodies that recognize a large number of different wall components. In addition, ovules from these same stages were investigated in three fiberless lines. Most antibodies reacted with at least some component of the ovule, and several of the antibodies reacted specifically with the epidermal layer of cells that may give clues as to the nature of the development of the fibers and the neighboring, nonfiber atrichoblasts. Arabinogalactan proteins (AGPs) labeled the epidermal layers more strongly than other ovular tissue, even at 1 DBA. One of the AGP antibodies, CCRC-M7, which recognizes a 1➔6 galactan epitope of AGPs, is lost from the fiber cells by 2 DPA, although labeling in the atrichoblasts remained strong. In contrast, LM5 that recognizes a 1➔4 galactan RGI side chain is unreactive with sections until the fibers are produced and only the fibers are reactive. Dramatic changes also occur in the homogalacturonans (HGs). JIM5, which recognizes highly de-esterified HGs, only weakly labels epidermal cells of 1 DBA and 0 DPA ovules, but labeling increases in fibers cells, where a pectinaceous sheath is produced around the fiber cell and stronger reaction in the internal and external walls of the atrichoblast. In contrast, JIM7-reactive, highly esterifed HGs are present at high levels in the epidermal cells throughout development. Fiberless lines displayed similar patterns of labeling to the fibered lines, except that all of the cells had the labeling pattern of atrichoblasts. That is, CCRC-M7 labeled all cells of the fiberless lines, and LM5 labeled no cells at 2 DPA. These data indicate that a number of polysaccharides are unique in quantity or presence in the epidermal cell layers, and some of these might be critical participants in the early stages of initiation and elongation of cotton fibers.

Keywords Cotton · Fiber initiation · Pectins · Arabinogalactan proteins · Esterification

Abbreviations

AGP	Arabinogalactan protein
BSA	Bovine serum albumin
DBA	Days before anthesis
DPA	Days post-anthesis

Handling Editor: Hanns H. Kassemeyer

A. J. Bowling · K. C. Vaughn
Crop Production Systems Research Unit,
US Department of Agriculture,
The Agricultural Research Service (USDA-ARS),
Stoneville, MS 38776, USA

R. B. Turley (✉)
Crop Genetics Research Unit,
USDA-ARS,
PO Box 345, Stoneville, MS 38776, USA
e-mail: Rick.Turley@ars.usda.gov

Present Address:
A. J. Bowling
Dow Agrosciences,
9330 Zionsville Road,
Indianapolis, IN 46268, USA

HG Homogalacturonan
IGS Immunogold–silver
PBS Phosphate-buffered saline
RGI Rhamnogalacturonan I

Introduction

Cotton fibers undergo one of the most dramatic changes of any cell type, arising from a tiny growth on the epidermal cell at 0 day post-anthesis (DPA) to become as long as 3 cm at 20–30 DPA (Lee et al. 2007). Although cotton fibers may (rarely) divide (Van't Hof and Saha 1997; Vaughn and Turley 2001), in most instances, the cellular growth results solely from elongation and differentiation of the fiber cell. Because of these unique properties, cotton is often used as a model system to investigate cell elongation and secondary wall biosynthesis.

Basra and Malik (1984) divide cotton fiber development into four stages, namely, (1) initiation, (2) elongation, (3) secondary wall biosynthesis/thickening, and (4) maturation. Because of the small size of the fibers at initiation and early stages of elongation, most of what we know chemically of the polysaccharide composition of fiber cells is from the later stages of the elongation and secondary wall stages of development (e.g., Tokumoto et al. 2002). A notable exception is the study of Meinert and Delmer (1977), which extends the analysis to 4 DPA fibers. These data support the idea that these early fibers are more like typical dicot walls until the beginning of secondary wall biosynthesis. However, most of our knowledge of the polysaccharide composition of cotton fiber at the earliest stages comes from microscopic studies (Weis et al. 1999; Vaughn and Turley 1999) as so many of the polysaccharides result from sets of genes or *in muro* modifications of wall components. Thus, even as valuable as protein (Turley and Ferguson 1996) and DNA studies are in identifying genes that might be involved in fiber initiation/early development (reviewed in Lee et al. 2007; Taliercio and Boykin 2007), they do not allow a thorough understanding of polysaccharide content and certainly not their distribution.

In a previous study, we determined that 1–2 DPA cotton fibers contained a pectinaceous sheath, with the cellulose/xyloglucan layer occupying the cell wall areas closest to the plasma membrane (Vaughn and Turley 1999). That study utilized antibodies to these polysaccharides and the detection of this pectin sheath would have been impossible without these immunolocalization techniques. A similar analysis of callose distribution was made by Salnikov et al. (2003), showing a pattern of distribution of callose that matched sucrose synthase, an enzyme involved in its synthesis. Since these studies, the number of antibodies and the variety of polysaccharides that they recognize has mushroomed to include virtually all of the major and many of the minor components of the cell wall (e.g., Bowling and Vaughn 2008a, b; Bowling et al. 2008), with the exception of cellulose, which is not antigenic. These antibodies have allowed us to probe tissues that would have been impossible to dissect out for biochemical analysis. In this study, the developing cotton fiber was revisited, using this increased arsenal of antibodies to determine the distribution of other polysaccharide and glycoprotein epitopes. In addition, we compared the results in wild-type cotton ovules with those from identical stages in three fiberless lines.

Materials and methods

Plant material

Six inbred lines, namely, SL1-7-1 (PI 528807), MD17 (PI 616493), pilose (PI 528521), DP5690 (PVP no. 9100116), XZ142w, and XZ wild type, were evaluated for changes in cell wall composition. DP5690 was obtained from Delta and Pine Land (Scott, MS). SL1-7-1 and pilose were obtained from the National Collection of Cotton Germplasm (Percival 1987). MD17 fiberless was developed in Stoneville, MS (Turley 2002) and XZ142w and XZ wild type were a generous gift from Dr. Tianzhen Zhang (Cotton Research Institute, Nanjing, China). All plants were grown in the greenhouse at Stoneville, MS, during the fall of 2007 to the spring of 2008. Plants were watered daily, fertilized every 2 weeks and supplemental lighting was used for 18 h light/6 h dark intervals.

Tissue preparation

Ovules from 1 day before anthesis (DBA), 0 DPA, 1 DPA and 2 DPA cotton capsules were removed and fixed in 5 ml of 3% glutaraldehyde in 50 mM Pipes (pH 7.4) for 2 h at room temperature. Samples were rinsed three times in 50 mM Pipes (pH 7.4) for 15 min. An additional two rinses were performed with deionized water. Samples were dehydrated at room temperature in 25%, 50%, and 75% (2 h each) ethanol and with absolute ethanol overnight at 4°C. A second change of absolute ethanol was made and the samples were left overnight at 4°C. LR white resin (Polysciences, Warrington, PA) was added in ethanol at 25%, 50%, 75%, and 100% (24 h) at 4°C. A subsequent addition of LR white resin was used and the samples were left overnight at −20°C. Specimens were placed onto a shaking platform for 24 h at 24°C, then placed into pucks as described by Bowling and Vaughn (2008a). Samples were polymerized at 55°C for 2 h.

IGS labeling

Sections (~0.35 μm) were cut with a Delaware Histoknife and mounted on chrome-alum subbed slides. A wax pen

was used to circle the sections (4–8/slide) to form a well for the subsequent incubation steps. The slides were transferred to an incubation chamber (Accurate Scientific) and solutions were applied as follows: 1% bovine serum albumin (BSA) fraction IV in 20 mM sodium phosphate buffer (pH 7.2) with 0.05 M sodium chloride [phosphate-buffered saline (PBS)=PBS–BSA], 30 min; primary antibody (obtained from either the Complex Carbohydrate Research Center, Athens GA or Plant Probes, Leeds, UK) diluted from neat to 1:8 in PBS–BSA, 3 h; three exchanges of PBS–BSA, 10 min; goat anti-mouse or anti-rat IgG coupled to 15 nm gold (EY Labs, San Mateo, CA) diluted 1:20 in PBS–BSA, 1 h. (When the 2F4 antibody was used, a 20 mM Tris buffer, pH 8.3, supplemented with 0.5 mM calcium and 150 mM NaCl, was substituted for the PBS–BSA in all the solutions.) After these stages, the slides were washed with a stream of double distilled water to remove excess chlorides. The sections were then incubated from 15 to 20 min in the IntenSE silver intensification kit (GE Biosciences) solutions (equal mixture of parts A and B) in the incubation chamber. The reaction was terminated by repeated washing of the slide in distilled water, the slides dried with compressed air and then mounted with Permount. Slides were dried on a slide warmer for 2 days before images were obtained on a Zeiss Axioskop with a digital acquisition capability. An Olympus Q-Color 3 digital camera (Olympus, Tokyo, Japan) was used to record images and the images manipulated with the Program Image J (National Institutes of Health, Bethesda, MS), and plates were constructed using the GNU Image Manipulation Program (GIMP, version 2.2; http://www.gimp.org/).

Controls included the absence of the primary antibody, and in the case of JIM5 and JIM7, a heat-killed primary antibody (boiling water bath for 30 s) was applied to the sections where it was substituted for the primary antibody.

Some sections were chemically de-esterified before the blocking step by treating the dried sections with 1 M sodium carbonate for 1 h prior to the blocking step. After the de-esterification, the slides were washed with distilled water, dried with compressed air, and then incubated in the blocking step. Subsequent steps were performed as for the nontreated sections.

Sections from the same block faces used for immunocytochemistry were stained with 1% toluidine blue in 1% sodium borate on a slide warmer and mounted and photographed as for the IGS-labeled slides.

Transmission electron microscopy

Sections from the same block faces used for IGS labeling were cut with a Delaware Diamond knife at ~100 nm and mounted on uncoated 300-mesh gold grids. Sections were floated on 4–10 μl drops of the following solutions: 1% PBS–BSA, 30 min; primary antibody diluted 1:8 in PBS–BSA, 3 h; 4× PBS–BSA, 2.5 min each; and 1:20 dilution of goat anti-mouse or anti-rat IgG coupled to 15 nm colloidal gold in PBS–BSA, 1 h. The sections were washed extensively with double distilled water and post-stained with 2% uranyl acetate (2 min) and Reynold's lead citrate (30 s), washed again with distilled water, and observed with a Zeiss EM10CR electron microscope. Negatives were obtained with Kodak electron-imaging film and then scanned to obtain digital images using an Epson 700 perfection scanner. The images were collected as inverted images and contrast adjusted manually. Plates were formed using the GIMP as above.

Results

Development of the fiber cells in DP5690

Development of fiber cells in cotton ovules of cotton cultivar DP5690 follows a well-known sequence of events described previously in other cultivars (Basra and Malik 1984; Ryser 1999; Fig. 1). At 1 DBA, the epidermal cells exhibit no protrusions, and all epidermal cells appear similar in cytoplasmic density (Fig. 1a). By 0 DPA, some cells at the chalazal end of the ovule begin to exhibit slight swelling, developing a slightly convex exterior compared to the neighboring (future) atrichoblasts (Fig. 1b). About ¼ of the cells in the epidermis display this swelling and eventually become fibers (Stewart 1975). At 1 DPA, the fibers are very distinct from the neighboring atrichoblasts, with a prominent nucleus and expanding central vacuole (Fig. 1c). Growth of the fiber cells is rapid, and by 2 DPA, the surface of the ovule is covered with elongating fiber cells, up to 100 μm in length (Fig. 1d). Thus, in just 3 days' time, the cotton ovule epidermis has changed from a completely smooth structure to one studded with fibers.

Immunocytochemistry of the 1 DBA–2 DPA cotton ovules

Cotton ovule sections from 1 DBA to 2 DPA were probed with a group of antibodies that recognizes a diverse array of polysaccharide and glycoprotein epitopes. In this study, we utilized immunogold–silver (IGS)-labeling protocols to label sections of cotton ovules. This allowed us to see tissue differences clearly and resolved the problem of the relatively high endogenous fluorescence of cotton tissue, due to the presence of high levels of terpenoids and phenolics in cotton cells. In addition, once the sections were processed, they were essentially immortal, allowing us to reexamine unusual localization patterns and to compare labeling from different days.

Antibodies to homogalacturonans (HGs: JIM5, JIM 7, 2F4, and CCRC-M38), rhamnogalacturonan I (RGI) and its

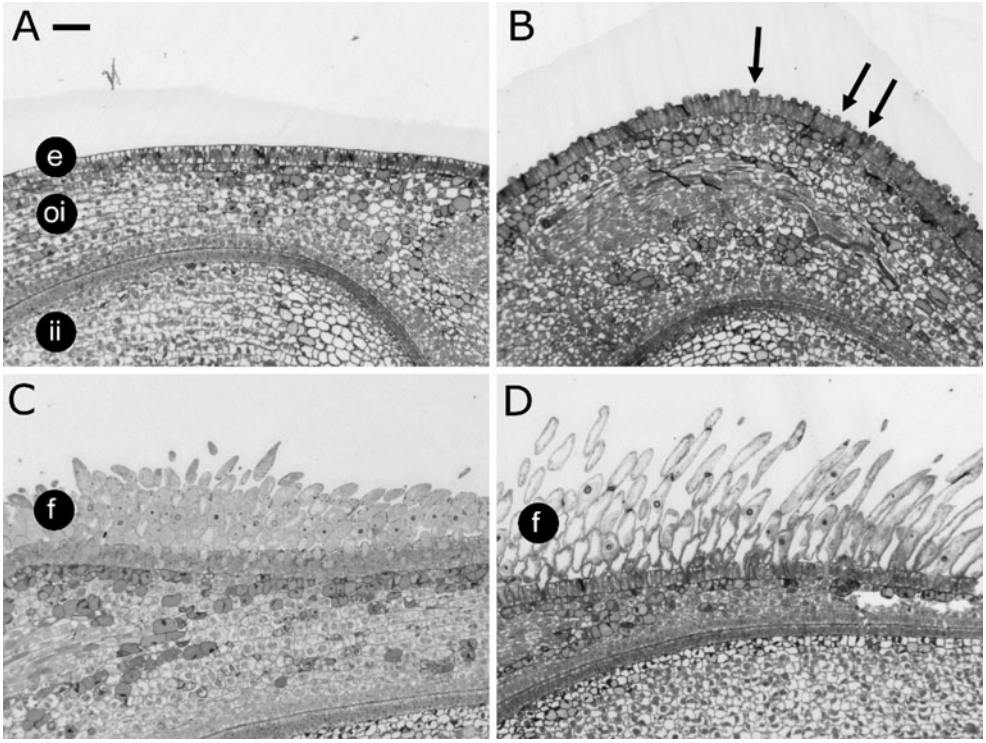

Fig. 1 Light micrographs of cotton ovules stained with toluidine blue at 1 DBA (**a**), 0 DPA (**b**), 1 DPA (**c**), and 2 DPA (**d**). Although all cells of the epidermis (*e*) seem identical at 1 DBA, there are small fiber initials at 0 DPA (*arrow*). These fiber cells expand greatly in 1 and DPA cotton ovules. Bar=50 μm. *oi* Outer integument, *ii* inner integument

side chains (LM5, LM6, CCRC-M2, CCRC-M10, and CCRC-M22), arabinogalactan proteins (AGPs: JIM13, JIM14, LM2, CCRC-M7), and xyloglucans (LM15 and CCRC-M1) were used to probe sections. All of these antibodies labeled at least some cell types in the ovule sections. Some sections were also probed with the LM1 antibody that recognizes extensin, and the LM10 and LM 11 antibodies that recognize xylans. No reaction was noted on these young ovule sections, although other cotton tissue, such as leaves and stems, reacted strongly with each of these antibodies (Cochran et al. submitted for publication). These data indicate that both xylans and extensin (or at least the epitopes recognized by these antibodies) are absent from 1 DBA to 2 DPA cotton ovules. In contrast, many of the epitopes recognized by the other antibodies were present in all sections and in virtually all cell types (Table 1). In some cases, the embryo and endosperm reacted differently than the immature seed coat and fiber tissues, generally reacting with less intensity than the cells in the fiber and developing seed coat.

Two control reactions were run for this set of experiments. One was a heat killed antibody solution run at the same concentration as the primary antibody and the other was a reaction with no primary antibody. Neither reaction gave any significant silver grain deposition even after prolonged development in the silver intensification reactions. In addition, the lack of reaction with the LM10 and LM11 antibodies on the ovule sections and their strong reaction on other cotton sections (Cochran et al. submitted) indicated the specificity of the labeling process. Thus, the antibodies were not adhering nonspecifically to the cotton fibers.

AGP antibodies on DP5690

One general trend that was noted is the much stronger reaction of antibodies to AGPs with the epidermal cell layer relative to the other cell layers, even at 1 DBA. These include the JIM13, JIM14, LM2, and CCRC-M7 antibodies. Reaction was not always confined to the wall/plasma membrane, as cytoplasmic vesicles and occasionally tonoplasts were also labeled (Figs. 2 and 3). CCRC-M7, which labels a 1➔ 6 galactan epitope that occurs on both RGI and AGPs (Stafstrom and Staehelin 1988), appears similar to the JIM13 antibody in its labeling pattern, indicating that AGPs are the epitopes principally labeled by this antibody in cotton ovules. Interestingly, CCRC-M7 labeled both atrichoblasts and fiber cells through 1 DPA, but by 2 DPA, the reaction in the fiber cells was much weaker to be virtually absent at the plasma membrane/wall interface. This was confirmed by EM localizations on serial sections of those used in the IGS-labeling protocols. Moreover, the transmission electron microscopy (TEM) studies revealed that the labeling of this antibody was at the cell-wall/plasma membrane interface, especially where cortical microtubules were observed in atrichoblasts (Fig. 4). Of the gold particles not found at the plasmalemma/wall interface, 73% (228) of the gold particles

Table 1 Antibodies employed in this study and their reactions with cotton fibers and ovule tissue

Antibody[a]	Reaction[a]
AGPs	
JIM13	Strong on epidermis, weaker on subepidermis
JIM14	Like weaker reaction of JIM13
LM2	Weak reaction but stronger in epidermis, lost in fiber
CCRC-M7	Strong on epidermis, lost from fiber cells at 1–2 DPA
Pectins	
JIM5	All cells but epidermis 1 DBA, pectin sheath at 0–2 DPA
JIM7	Strong reaction in epidermis throughout stages
2F4	Weak reaction unless sections treated with sodium carbonate
LM5	No reaction on 1 DBA–0 DPA, first noted on fibers at 1 DPA
LM6	Strong reaction throughout tissue at all stages
CCRC-M22	Strong reaction throughout tissue
CCRC-M38	Strong reaction except in fiber cell cross-walls, pectin sheath
Xyloglucans	
LM15	Uniform reaction throughout tissue
CCRC-M1	Uniform reaction throughout tissue
Xylans	
LM10	No reaction
LM11	No reaction
Extensin	
LM1	No or very weak reaction

[a] The CCRC series of monoclonal antibodies and their specificities are available from Carbosource at http://www.ccrc.uga.edu/mao/wallmab; the JIM, LM, and 2F4 antibodies and the reference to their specificities are available at http://www.plantprobes.net

were found within 0.2 μm of a recognized cortical microtubule. LM2 also showed lower reaction in the fiber cells, although the results were not as striking as with the CCRC-M7 antibody.

RGI and HG antibodies on DP5690

The patterns of labeling of both the RGI and HG epitopes (Fig. 5) are a bit more complex than what was noted for the

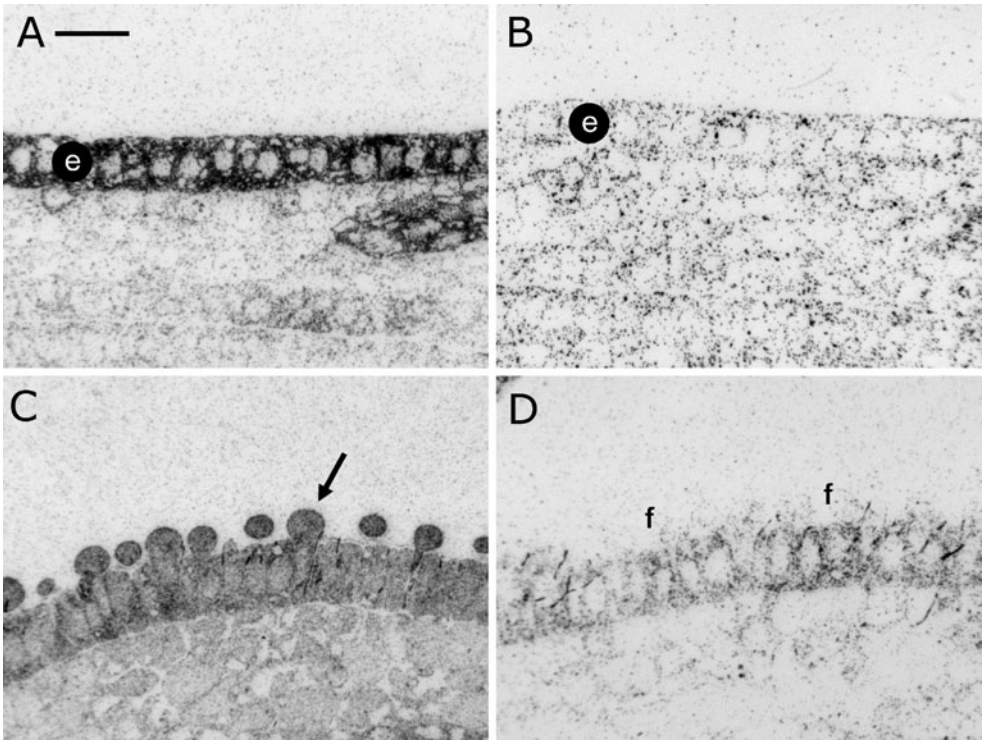

Fig. 2 Cotton ovule sections labeled with antibodies to AGPs. **a** JIM13 labeling at 1 DBA. **b** LM2 labeling at 1 DBA. **c** JIM 13 labeling at 0 DPA. The young fiber cells are strongly labeled; one is **d** LM2 labeling at 0 DPA. Note the strong labeling of the epidermis (*e*) and fibers with the JIM13 antibody but the lack of labeling of the fibers (*f*) with the LM2 antibody. Bar=25 μm

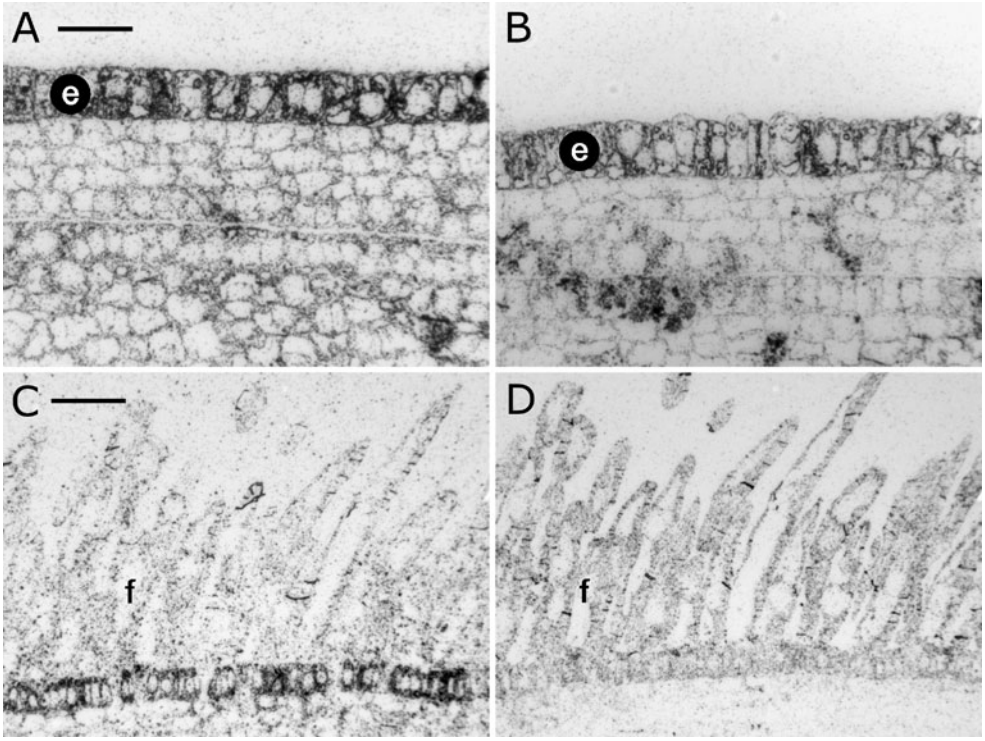

Fig. 3 Reaction of cotton ovules to AGP antibodies CCRC-M7 (**a**–**c**) and JIM 13 (**d**). Although cotton ovule epidermal cells are labeled strongly at 1 DBA (**a**) and 0 DPA (**b**) with CCRC-M7, at 2 DPA, the density of label is much weaker in the fiber (*f*) cells. In contrast, JIM 13 labeling is relatively the same in both fiber and epidermis. Bar=25 μm in **a** and **b**, 50 μm in **c** and **d**

AGP epitopes. Several of the antibodies to HG and RGI label the epidermis more strongly than the subepidermal cells throughout development [e.g. JIM7 (Fig. 5a) and CCRC-M22]. The other antibodies to these polysaccharides displayed distinctly different labeling patterns, however. Antibodies to the side chains of RGI are a prime example.

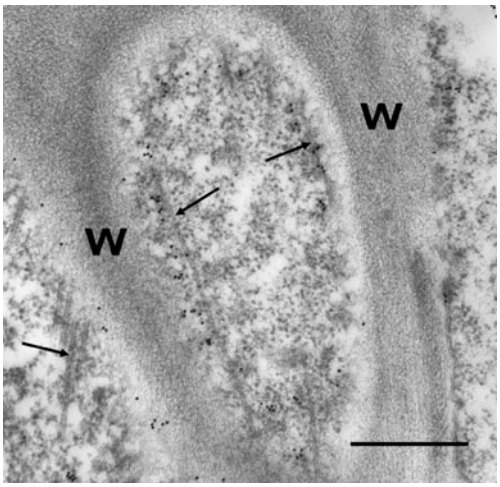

Fig. 4 TEM micrograph of atrichoblast cells of cotton ovules labeled with the CCRC-M7 antibody. Although the wall proper (*W*) is not labeled, areas of the cell at the plasmamembrane/wall interface and near the cortical microtubules (some marked with *arrow*) are labeled with this antibody. Bar=0.5 μm

In early stages of development, all of the tissues and developing fibers were labeled strongly with the LM6 antibody that recognizes the 1➔6 arabinan side chain of RGI (Fig. 5b and c). LM6 also labels 1–2 DPA fibers, but at this point, the LM5 antibody, which recognizes 1➔4 galactan, only appears in the fiber but not in other tissues (Fig. 5c). Even the fiber bases remain unlabeled. Thus, the fiber cells are already differentiated from the atrichoblasts at 1 DPA by the kind of RGI side chains that are present in their walls.

A similar dramatic change is noted for the HGs (Fig. 6). As mentioned above, JIM7, which recognizes highly esterified HGs, strongly labels the epidermal cells from 0 DPA to 2 DPA, indicating that a large amount of highly esterified HG is present in this cell layer. In contrast, JIM5, which recognizes weakly esterified HG, labels nothing in the epidermis in 1 DBA ovules and only weakly labels the 0 DPA fibers, which appears to be the start of the pectinaceous sheath (Fig. 6a and b). Labeling increases dramatically at 1 DPA, with the appearance of a pectinaceous sheath just at the base of the fibers (Fig. 6c). This label increases by 2 DPA, extending around the fiber. Label with JIM5 remains low in the walls of the atrichoblast and bases of the fibers. Pretreatment of the sections with sodium carbonate, which chemically de-esterifies HG, prior to labeling with JIM5 radically increases the labeling, giving a pattern similar to that observed with JIM7 on untreated sections (Fig. 6d). Thus, the labeling differences between

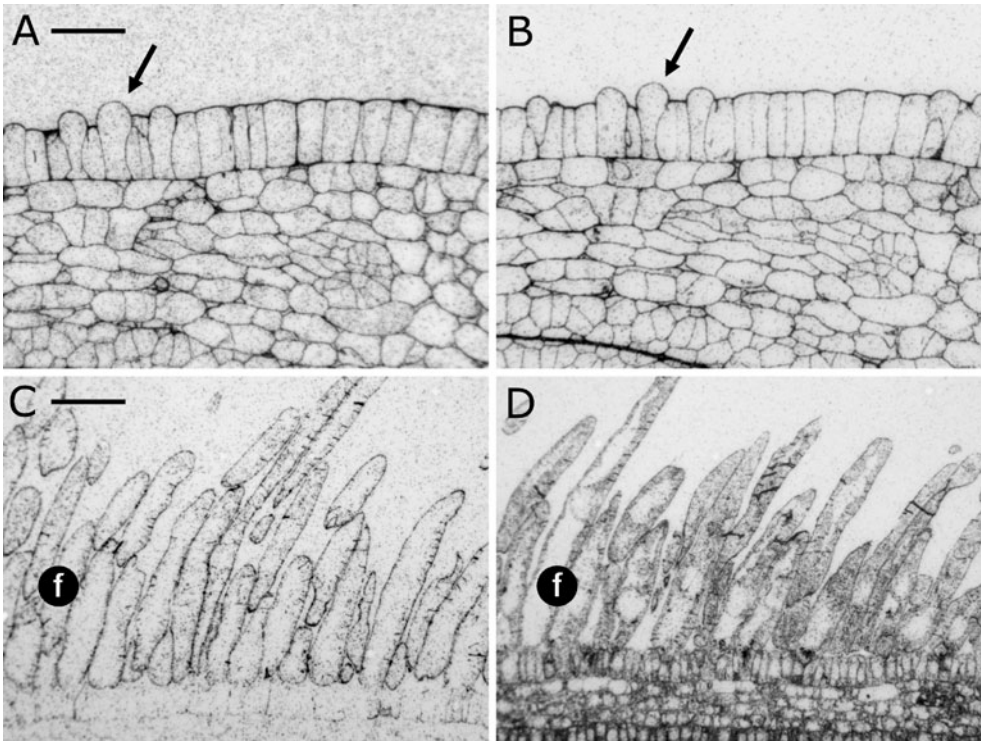

Fig. 5 0 DPA (**a**, **b**) and 2 DPA (**c**, **d**) cotton ovules reacted with JIM7 (**a**), LM6 (**b**, **d**) and LM5 (**c**). Although the JIM7 and LM6 label all of the cells, LM5 reactivity is not noted until the fibers (*f*) elongate at 2 DPA. Bar=25 μm in **a** and **b**, 50 μm in **c** and **d**. *Arrow* note fiber initials in **a** and **b**

JIM5 and JIM7 reflect the differences in HG esterification states between the earliest ovule stages monitored and the growing fiber. We obtained similar results with 2F4 as with JIM5, although the extent of labeling was always less except when the sections were pretreated with sodium carbonate (not shown). In these cases, they resembled JIM-labeling patterns with the exception of the lower amounts of reaction in the side walls. This antibody also strongly recognized cell corners. CCRC-M38, which recognizes a number of HG epitopes including fully de-esterfied and esterified epitopes (W. Willats, personal communication), labeled the side walls less than the epidermal side of the atrichoblasts and fibers but seemed to label other epidermal walls not labeled by JIM5 at 1 DBA–0 DPA (Fig. 6e). At 0 DPA–1 DPA, however, the bottom of the fiber, but not the top, portions were labeled, much as with JIM5 (Fig. 6e and f).

Comparisons with other strains

Besides the DP5690 strain that serves as our "wild-type" control, we also monitored two other strains, pilose and XZ wild type, which produce fibers similar to DP5690 but had other trichome/fiber traits. The pilose line had hirsute leaves, stems, and bolls, whereas the XZ wild type was near isogenic to XZ142w (a fiberless line). Both of these strains had an identical labeling pattern to that observed in DP5690 (Fig. 7). Thus, the pattern of labeling with the polysaccharide antibodies appears to be common to strains that are producing fibers, regardless of other traits that might be influenced by these genetic differences nor are the changes we observed unique to DP5690.

Fiberless mutants

We examined several strains of fiberless lines of cotton. Each of these lines [XZ142w (Zhang and Pan 1991), MD17 (Turley and Kloth 2002), and SL1-7-1 (Turley and Kloth 2008)], shares common loci; however, each is also genetically unique. Presently, there are five loci that are involved in the expression of trichome initiation on ovules (Turley and Kloth 2008). Surprisingly, much of the labeling patterns observed with DP5690 were also observed in the fiberless lines (Fig. 8), including the strong labeling of the epidermal cells with AGP antibodies and JIM7 and some increase in JIM5 labeling from 0 to 2 DPA. However, two of the reactions that distinguished fibers from atrichoblasts, the positive reaction of LM5 and the negative reaction with CCRC-M7 in 2 DPA fibers, is not observed in the fiberless lines. No LM5 reaction was noted in the 2 DPA ovules from fiberless lines and all cells of the epidermis reacted strongly with CCRC-M7 at 2 DPA (Fig. 8c and g). Similarly, 1 DBA ovules displayed the same staining patterns for JIM 5 (Fig. 8a), JIM7 (Fig. 8f), and other HG antibodies as described for the DP5690 strain (Fig. 8e and h). Thus, the epidermal cells of the fiberless lines have the same labeling pattern as atrichoblasts in the fibered lines.

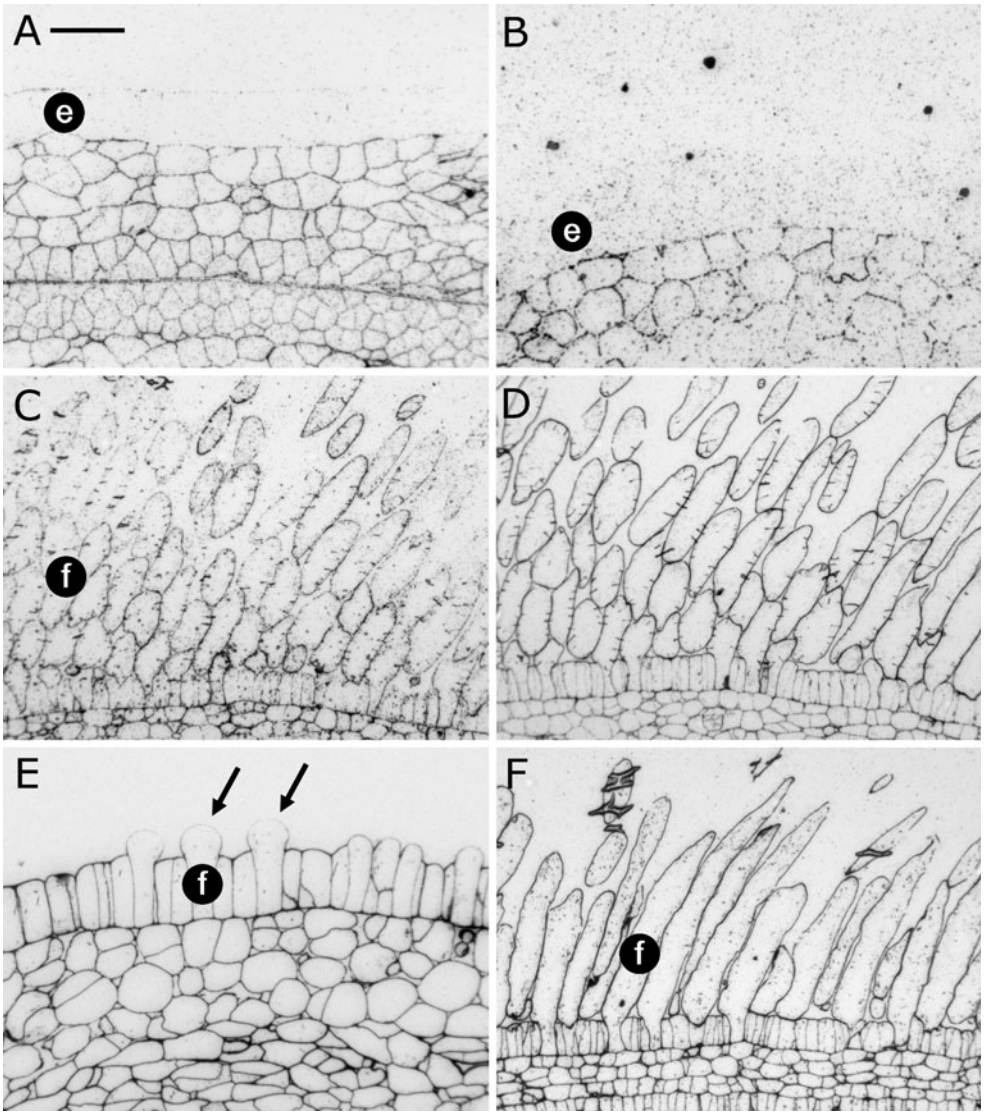

Fig. 6 Reaction of antibodies to HG in ovule sections of cotton. JIM 5 (**a**–**d**) and CCRC-M38 (**e**, **f**). **A** JIM5, which recognizes highly de-esterified HG, strongly labels the subepidermal cells, but only sparse label is found in the epidermal (*e*) cells (**a**). A bit more reaction is observed at 0 DPA, but the reaction in the epidermis (*e*) is still weak (**b**). However, at 2 DPA, the cotton fibers and atrichoblasts are strongly labeled both without (**c**) and with (**d**) sodium carbonate treatment. Only the cross-walls of epidermal cells reveal lower levels of reactivity. **e** Sections of cotton at 0 DPA reacted with CCRC-M38. Note ends of fibers are unreactive or weakly so (*arrow*), and lower reaction in cross-walls of epidermal cells are less reacted than the side walls. **f** 2 DPA fibers labeled with CCRC-M38. Strong reaction is noted along the pectin sheath of the cotton fiber. Weaker reaction is observed in cross-walls. Bar=25 μm in **a**, **b**, and **e** and 50 μm in **c**, **d**, and **f**

Discussion

Cotton fibers contain a variety of polysaccharide and glycoprotein epitopes

Cotton fiber is often described as "pure cellulose," as the secondary wall that commences ~16–20 DPA is predominantly composed of highly crystalline cellulose (Ryser 1999; Lee et al. 2007). However, the primary wall of the cotton contains an interesting assemblage of other polysaccharides and glycoproteins, including xyloglucans, HGs, RGIs, and AGPs. We observed no labeling with xylan and extensin antibodies, although we have found some low-level labeling in the past with a polyclonal extensin serum, indicating that at least some extensins may be present in the fiber, although not those recognized by LM1 (Vaughn and Turley 1999). Moreover, there are dramatic changes in this composition as the fiber cells begin to differentiate from the adjacent atrichoblasts.

Changes in pectin components during fiber development

Previously, we showed that by using an antibody that recognizes primarily a highly de-esterified HG, the primary wall of the cotton fiber developed a sheath of pectin, and the xyloglucan–cellulose components were present more in the areas of the wall adjacent to the plasma membrane (Vaughn and Turley 1999). Both of these antibodies were polyclonal antibodies and, despite their usefulness, are not as specific as the monoclonal antibodies used in these studies. In the current study, however, we could confirm the presence of the pectin sheath with the JIM5 and CCRC-M38 antibodies that recognize a range of HG epitopes. Both antibodies labeled an area at the bases of elongating fibers at 1 DPA that extended around the fiber by 2 DPA. Because essentially no reaction was observed in the cotton epidermis with JIM5 prior to 1 DPA and the change in JIM5 labeling parallels the initial events of fiber initiation, we would expect that a pectin methylesterase must be a key

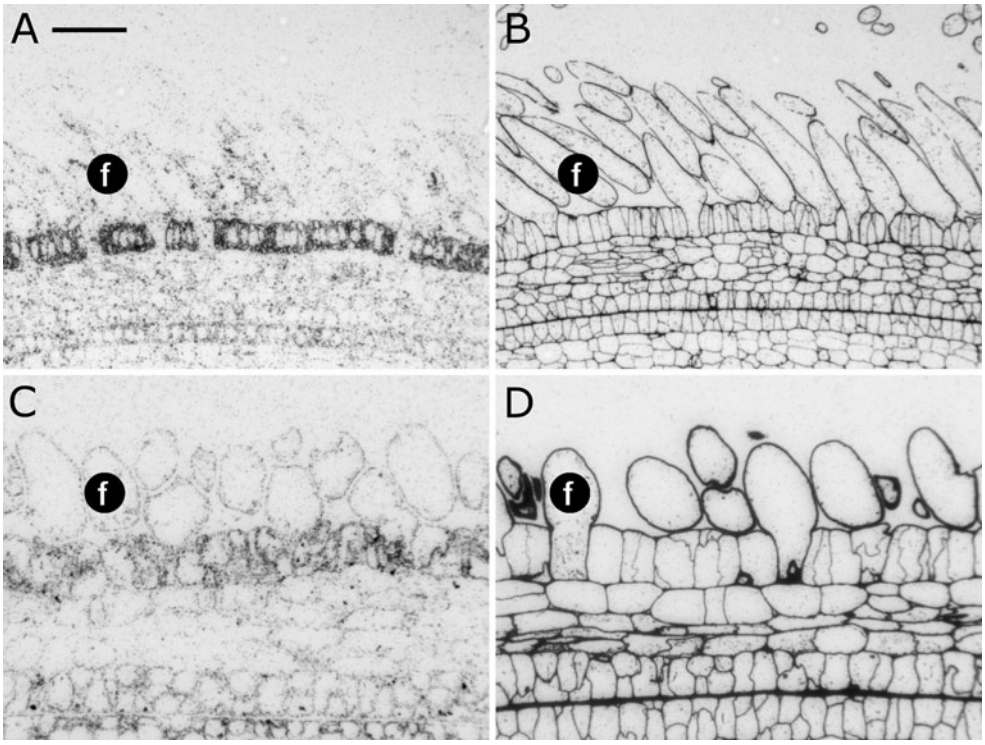

Fig. 7 Reaction of two other lines with diverse genotypes to DP5690 to the CCRC-M7 (**a**, **c**) and CCRC-M38 (**b**, **d**) to the fuzz (**a**, **b**) and pilose (**c**, **d**) lines. Bar=50 μm in **a** and **b**, 25 μm in **c** and **d** *f* Fiber

enzyme in the early stages of the differentiation of both the atrichoblasts and the fiber cells.

A different sort of change occurs in the RGI side chains. Initially, only LM6 labels the ovule sections, indicating that all of the RGIs have 1➔5 arabinan side chains. At 2 DPA, however, the fiber cells (but not the areas of the fiber cell not protruding beyond the epidermis) are strongly labeled with antibodies to the 1➔4 galactan RGI side chain. Many other tissues have been examined and they have revealed that LM6 seems to preferentially label younger tissue, while LM5 labels more mature tissue (e.g., Vaughn 2006). Thus, it is especially interesting that the highly elongating cotton fiber is enriched in this particular RGI side chain.

Moreover, antibodies to highly esterified HGs (JIM7) and RGI (CCRC-M22 and CCRC-M2) strongly label the xyloglucan/cellulose area of these primary fibers, which is similar to other primary walls in the cotton ovule and in many other tissues, indicating that minus the pectin sheath, the primary wall of cotton is very similar to other primary walls in composition and organization.

AGP changes and its possible significance in fiber development

An especially intriguing developmental change occurs with the reactivity of the sections to the CCRC-M7 antibody, which recognizes a 1➔6 galactan epitope that can be present as a side chain of both RGI and AGP in some plants (Steffan et al. 1998; Freshour et al. 1996). In cotton, the labeling pattern is more similar to what is observed for other AGPs, and at the TEM level, the label is clearly along the plasma membrane, especially where sites of cortical microtubules are located (Fig. 4). In contrast, RGIs, such as those labeled by CCRC-M10 and CCRC-M22, are found in the wall proper, not at the plasma membrane. Thus, it is fairly certain that CCRC-M7 principally labels AGPs in cotton ovules.

Developmentally, this epitope is quite interesting in that, while present in all of the epidermal cells at 1 DBA–1 DPA, this epitope is not present (or is present at very low quantities) in the 2 DPA fiber. This change was also noted in two other cotton lines with different epidermal qualities, so it is likely that these changes occur across many cotton genotypes. Obviously, the change in labeling might be related to a number of events that occur during the transition of the fiber cell from a mere epidermal cell to a highly elongated fiber cell, although other AGP antibodies, such as JIM13 and JIM14, do not show this change. LM2 shows no reaction with the fiber cells, but its reaction throughout the tissue is much less than that observed for CCRC-M7.

One of the more intriguing aspects of the CCRC-M7 antibody is the association of it with high levels of cortical microtubules in atrichoblasts (Fig. 4). Recent investigations on *Arabidopsis* may shed some light onto this observation. Nguema-Ona et al. (2007) have observed that the treatment of *Arabidopsis* cells with the Yariv reagent, which binds to AGPs, or the AGP antibodies JIM13 or JIM14, which bind AGP epitopes, causes changes not only in AGPs but also in the organization of cortical microtubules. In the *reb1-1*

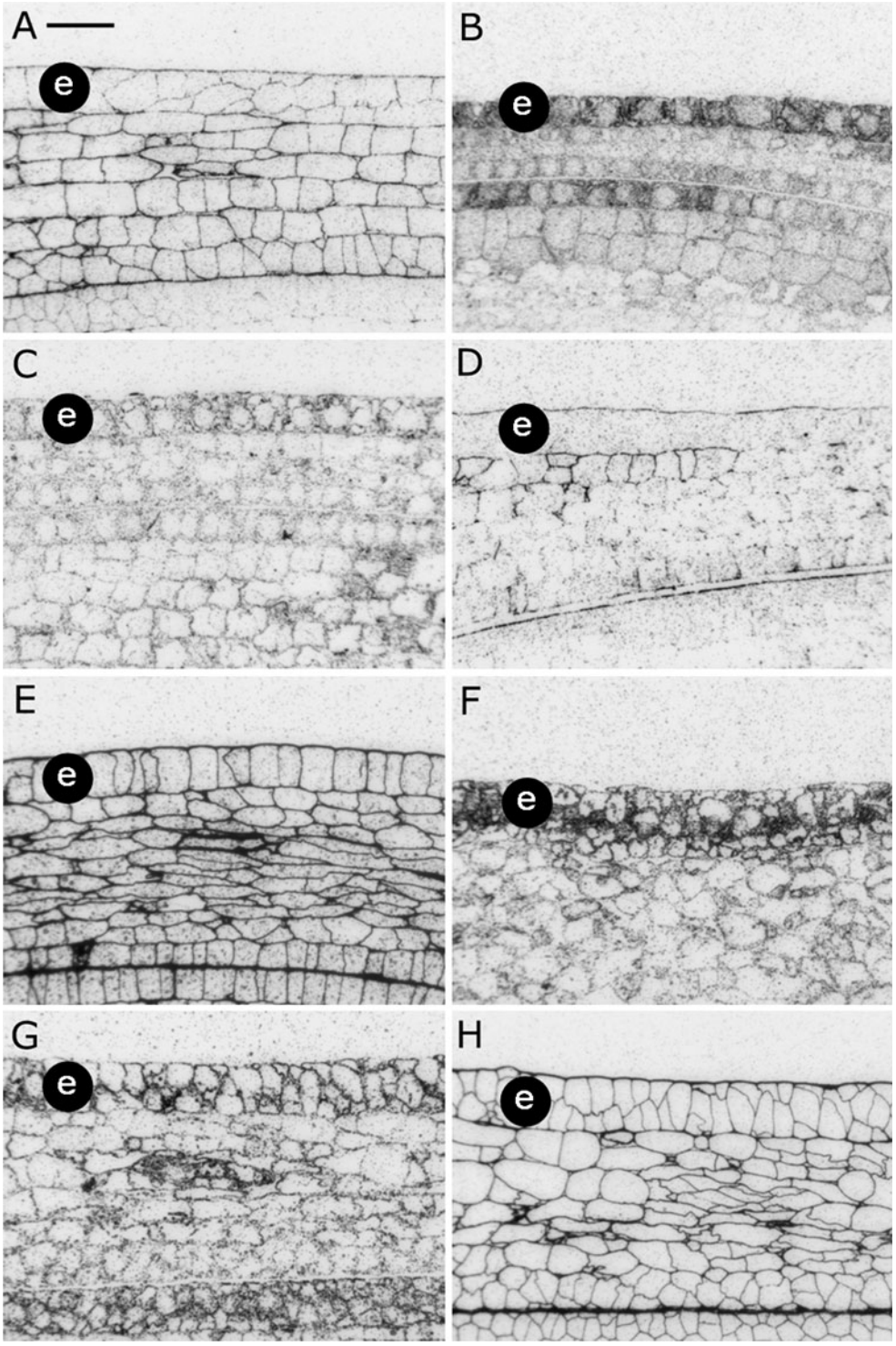

Fig. 8 Reaction of antibodies to tissue in fiberless lines. XZ142w (**a**–**c**) and SL1-7-1 (**d**–**h**) reacted with JIM5 (**a**, **d**), JIM13 (**b**), CCRC-M7 (**c**, **g**), CCRC-M38 (**e**, **h**), and JIM7 (**f**). Samples are from 2 DPA (**a**–**c**; **f**–**h**) or 1 DBA (**d**, **e**). Reactions in these sections are similar to atrichoblasts in DP5690. Bar=25 μm. *e* Epidermis

mutant of *Arabidopsis*, which has an abnormal AGP population, the trichome cells display aberrant morphology and have extremely aberrant cortical microtubule morphology (Andeme-Onzighi et al. 2002). These authors have argued that AGPs might serve as the source of communication between the wall and the microtubules by influencing the orientation of cortical microtubules. Interestingly, the changes in the AGP in cotton fibers also occur at a time when changes in microtubule organization are required. Cotton fibers grow by diffuse growth and have a pattern of microtubules that form a transverse array along the fiber which is much different than the more densely packed net of microtubules found in the neighboring atrichoblasts. Thus, it is possible that the change in CCRC-M7 labeling is reflective of this gross change in microtubule organization that accompanies the elongation of the fiber.

Fiberless lines

In the above, we have described changes that occur in the labeling pattern of both atrichoblasts and fibers at 2 DPA with the CCRC-MQ7 and LM5 antibodies. These changes clearly separate the fiber cell response from that of atrichoblasts. However, when the 2 DPA fiberless lines are observed, neither of these changes was found to occur. Rather, they were found to label the same as the atrichoblasts of DP5690 and other fibered lines (Fig. 7). Considerable data has accumulated on the potential role of sucrose synthase in causing the fiberless condition in the SL 1-7-1 line (Ruan and Chourey 1998; Ruan 2005, 2007), but in this study, we note that two other events associated with changing wall characteristics are also not found in the fiberless lines. Neither of these changes would directly or even indirectly involve sucrose synthase, however. Therefore, we think of the changes that occur in the fiberless lines as due to some regulatory protein(s) that might control the encoding of a number of genes, with those encoding the AGPs, enzymes in galactan biosynthesis and sucrose synthase, among others. This is also more in accordance with the genetic data on these lines.

In conclusion, we have found that the early stages of fiber initiation are associated with a number of changes in wall polysaccharides in the HG, RGI, and AGP components. These changes may be critical factors in the differentiation of the fiber and the atrichoblasts that feed the growing fiber cells.

Acknowledgments The authors would like to thank Grant Cochran for excellent technical assistance. AJB was supported in part by a headquarters-funded research associate position to KCV. Production of the monoclonal antibodies of the CCRC series was provided by NSF grants DBI-0421683 and RCN-0090281 to the Complex Carbohydrate Center, University of Georgia. Mention of a trademark, proprietary product, or vendor does not constitute an endorsement by the US Department of Agriculture.

Conflicts of Interest None

References

Andeme-Onzighi C, Sivaguru M, Judy-March J, Baskin TI, Driouich A (2002) The reb1-1 mutation of *Arabidopsis* alters the morphology of trichoblasts, the expression of arabinogalactan-proteins and the organization of cortical microtubules. Planta 215:949–958

Basra AS, Malik CP (1984) Development of the cotton fiber. Int Rev Cytol 87:65–113

Bowling AJ, Vaughn KC (2008a) A simple technique to minimize heat damage to specimens during thermal polymerization of LR White plastic in gelatin capsules. J Microsc 231:186–189

Bowling AJ, Vaughn KC (2008b) Immunocytochemical characterization of tension wood: gelatinous fibers contain more than just cellulose. Am J Bot 95:655–663

Bowling AJ, Maxwell HB, Vaughn KC (2008) Unusual trichome structure and composition in mericarps of catchweeed bedstraw (*Galium aparine*). Protoplasma 232:153–163

Freshour G, Clay RP, Fuller MS, Albersheim P, Darvill AG, Hahn MG (1996) Developmental and tissue-specific structural alterations of the cell wall polysaccharides of *Arabidopsis thaliana* roots. Plant Physiol 110:1413–1429

Lee JJ, Woodward AW, Chen ZJ (2007) Gene expression changes and early events in cotton fibre development. Ann Bot 100:1391–1401

Meinert MC, Delmer DP (1977) Changes in biochemical composition of the cell wall of the cotton fiber during development. Plant Physiol 59:1088–1097

Nguema-Ona E, Andeme-Onzighi C, Aboughe-Angone S, Bardor M, Ishii T, Lerouge P, Driouich A (2006) The *reb1-1* mutation of *Atabidopsis*. Effect on the structure and localization of galactose-containing cell wall polysaccharides. Plant Physiol 140:1406–1417

Percival AE (1987) The national collection of *Gossypium* germplasm. Southern Coop Ser Bull 321

Ruan YL (2005) Recent advances in understanding cotton fibre and seed development. Seed Sci Res 15:269–280

Ruan YL (2007) Rapid cell expansion and cellulose synthase regulated by plasmodesmata and sugar: insights from the single-celled cotton fibre. Funct Plant Biol 34:1–10

Ruan YL, Chourey PS (1998) A fiberless seed mutation in cotton is associated with lack of fiber cell initiation in ovule epidermis and alterations in sucrose synthase expression and carbon partitioning in developing seeds. Plant Physiol 118:399–406

Ryser U (1999) Cotton fiber initiation and histodifferentiation. In: Basra SA (ed) Cotton fibers. Hawthorne, Binghamton, pp 1–46

Stafstrom JP, Staehelin LA (1988) Antibody localization of extensin in cell walls of carrot storage roots. Planta 174:321-332

Salnikov VV, Grimson MJ, Seagull RW, Haigler CH (2003) Localization of sucrose synthase and callose in freeze-substituted secondary-wall-stage cotton fibers. Protoplasma 221:175–184

Steffan W, Kovac P, Albersheim P, Darvill AG, Albersheim P (1998) Characterization of a monoclonal antibody that recognizes an arabinosylated (1➔6) β-D-galactan epitope in plant complex carbohydrates. Carbohydr Res 275:295–307

Stewart JM (1975) Fiber initiation on the cotton ovule (*Gossypium hirsutum*). Am J Bot 62:261–288

Taliercio EW, Boykin D (2007) Analysis of gene expression in cotton fiber initials. BMC Plant Biol 7:22

Tokumoto H, Wakabayashi K, Kamisaka S, Hoson T (2002) Changes in the sugar composition and molecular mass distribution of matrix polysaccharides during cotton fiber development. Plant Cell Physiol 43:411–418

Turley RB (2002) Registration of MD171 fiberless upland cotton as a genetic stock. Crop Sci 42:994–995

Turley RB, Ferguson DL (1996) Changes in ovule proteins during early fiber development in a normal and a fiberless line of cotton (*Gossypium hirsutum* L.). J Plant Physiol 149:695–702

Turley RB, Kloth RH (2002) Identification of a third fuzzless seed locus in upland cotton (*Gossypium hirsutum* L.). J Hered 93:359–364

Turley RB, Kloth RH (2008) The inheritance model for the fiberless trait in upland cotton (*Gossypium hirsutum* L.) line SL 1-7-1: variation on a theme. Euphytica 164:123–132

Van't Hof J, Saha S (1997) Cotton fibers can undergo cell division. Am J Bot 84:1231–1235

Vaughn KC (2006) Conversion of the searching hyphae of dodder into xylic and phloic hyphae: A cytochemical and immunocytochemical investigation. Intern J Plant Sci 167:1099-1114

Vaughn KC, Turley RB (1999) The primary walls of cotton fibers contain an ensheathing pectin layer. Protoplasma 209:226–237

Vaughn KC, Turley RB (2001) Ultrastructural effects of cellulose biosynthesis inhibitor herbicides on developing cotton fibers. Protoplasma 216:80–93

Weis KG, Jacobsen KR, Jernstedt JA (1999) Cytochemistry of developing cotton fibers: a hypothesized relationship between motes and non-dyeing fibers. Field Crops Res 62:107–117

Zhang TZ, Pan JJ (1991) Genetic analysis of a fuzzless-lintless mutant in *Gossypium hirsutum* L. Jiangsu J Agric Sci 7:13–16