In situ analysis of cotton fibre cell wall polysaccharides

By

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The candidate confirms that the work submitted is his own, except where work which has formed part of jointly-authored publications has been included **(i)**. The contribution of the candidate and the other authors to this work has been explicitly indicated below **(ii)**. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

(i) Chapter 3 – *In* situ analysis of polysaccharides of mature cotton fibres:

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Chapter 5 - Processing and Crystallinity studies of mature cotton fibres:

Alenka kljun*, **Thomas A. S. Benians***, Florence Goubet, Frank Meulewaeter, J. Paul Knox, Richard S.Blackburn, 2011. Comparative analysis of crystallinity changes in cellulose I polymers using ATR-FTIR, X-ray diffraction, and carbohydrate-binding module probes. Biomacromolecules, 12(11), pp.4121-6. *Joint first author.

Jan Široký, **Thomas A. S. Benians**, Stephen J. Russell, Thomas Bechtold, J. Paul Knox, Richard S. Blackburn, 2012. Analysis of crystallinity changes in cellulose II polymers using carbohydrate-binding modules. Carbohydrate Polymers.

(ii) Kljun et al. 2011: Thomas Benians and Alenka Kljun shared the work load equally of the NaOH treatments, CBM labelling, FT-IR analysis, image analysis and X-ray diffraction.

Široký et al. 2012: Thomas Benians' contribution: carbohydrate-binding module labelling of lyocell fibre surfaces and cross sections. Jan Široký's contribution: Provided the lyocell fibre samples that had been treated with vary NaOH concentrations, temperatures and fabric tensions. Jan also did the FT-IR analysis of treated fibre samples, as well as the image analysis of the CBM fluorescence in the micrographs.

Marcus et al. 2010: Thomas Benians did the ELISA analysis of the impact of alkaline pH pre-treatments on the binding of four pectic homogalacturonan-directed monoclonal antibodies, JIM5, JIM7, LM19 and LM20 to a high methylester pectin.

“Do not seek to follow in the footsteps of the wise. Seek what they sought”

Matsuo Basho, 1644-1694

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Abstract

The cotton fibre is one of the most economically important cells in the world. Each year, over 25 million tonnes are harvested and the industry is responsible for 300 million jobs world-wide, with revenues of over $120 billion in the USA alone. For such an important cell there is little known about its cell wall composition as well as the functional roles of these polysaccharides during fibre development. Although much work has already been done on the cotton fibre transcriptome, a study of cell wall composition during development and maturation is crucial in linking these data to further understand fibre differentiation. This research explored the developmental biology of the cotton fibre in relation to key polysaccharide structures and architectural properties in the context of cell wall development. This has been achieved by the development of methodologies for the detection and imaging of low level polysaccharide epitopes of the cotton fibre using molecular probes known as monoclonal antibodies (mABs) and carbohydrate-binding modules (CBMs), directed *in-situ* to these glycans. Key polysaccharide changes were observed during fibre development, maturity and processing. Upon maturity, pectic homogalacturonan and xyloglucan were readily detectable at the surface of fibres after removal of the waxy outer layer. Other polysaccharides including arabinan, xylan and mannan, as well as cell wall glycoproteins were detected after treatments that removed the pectin-rich primary cell wall layer. This research shows that cell wall probes are powerful and useful tools to study cotton fibres throughout development, maturity and processing in the context of cell wall biology, though these polysaccharide changes need to be explored one by one to establish structure-function relationships. With the upcoming sequencing of the *G. hirsutum* genome, cotton fibre research will be an exciting field and the work presented here will provide a base for future studies, with potential for the manipulation of key developmental polysaccharides to alter the final fibre properties. The ultimate goal of improving cotton fibre properties will have significant economic, ecological and societal impacts for decades to come.

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° C	Degrees Celsius
AE	Acetyl-ester
AEC	Anion exchange chromatography
AceA	Aceric acid
AGI	Arabinogalactan I
AGII	Arabinogalactan II
AGP	Arabinogalactan proteins
Api	Apiose
Ara	Arabinose
BSA	Bovine serum albumin
CAPS	N-cyclohexyl-3-aminopropane sulfonic acid
CBMs	Carbohydrate-binding modules
CesA	Cellulose synthase
CDTA	Trans-1,2-cyclohexane diamine-N,N,N',N'-tetra acetic acid
CFML	Cotton fibre middle lamella
Cm	Length in Centimetres
Cm^3	Volume in cubic Centimetres
Ctrl	Control
dH_2O	De-ionised water
DPA	Days post-anthesis
DP	Degree of polymerisation
DW	De-waxed
ELISAs	Enzyme-linked immunosorbant assays
FITC	Fluorescein isothiocyante
Fuc	Fucose
Gal	Galactose
Glc	Glucose
GlcA	Galacturonic acid
GT	Glycosyltransferase
Ga	*Gossypium arboreum*
Gb	*Gossypium barbadense*
Ghb	*Gossypium herbaceum*
Gh	*Gossypium hirsutum*
g	Weight in grams
h	Time in hours
HG	Homogalacturonan
HPAEC-PAD	High performance anion exchange chromatography with pulsed amperometric detection
IDAs	Immuno dot assays
L	Volume in litres
mABs	Monoclonal antibodies
Man	Mannose

Me	Methyl-ester
Min	Time in minutes
MLG	Mixed linkage (1-3)(1-4)-β-D-glycans
MP	Milk powder
MP/PBS	3% milk powder solution in PBS
Mr	Relative molecular mass
NaOAc	Sodium Acetate
O/N	Overnight 16 h incubation
PAE	Pectin acetyl-esterase
PBS	Phosphate buffered saline solution
PCD	Programmed cell death
PL	Pectate lyase
PME	Pectin methyl-esterase
RG-I	Rhamnogalacturonan-I
RG-II	Rhamnogalacturonan-II
Rha	Rhamnose
ROS	Reactive oxygen species
RT	Room temp
SC	Sodium carbonate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TRIS	Tris(hydroxyl methyl)methamine
TS	Transverse section
Tyr	Tyrosine
Wt	Wild type
Xyg	Xyloglucan
XGA	Xylogalacturonan
XTH	Xyloglucan-endotransglycosylase/hydrolase
Xyl	Xylose

Chapter 1

Introduction

1.1 Cotton fibres

1.1.1 The importance of cotton

The cotton fibre has been cultivated for thousands of years and is now the dominant renewable textile fibre (Basra, 1999). With its unique properties cotton has become the world's most important fibre crop, extensively cultivated to make textiles, ropes and similar materials, as well as providing a useful source of seed oil and animal feed (Wendel and Cronn, 2003). Cotton belongs to the Malvaceae family of angiosperms and contains roughly fifty species that originated in arid and semi-arid regions around the world. Cotton is prized because of its long seed coat epidermal fibre cells and it is the only crop in which four different species were separately domesticated by civilisations from the old and new worlds in a convergent process (Chen et al., 2007). This domestication of spinnable cotton arose from four species; *Gossypium hirsutum* ('Upland cotton') and *Gossypium barbadense* ('Pima' or 'Egyptian' cotton) from the Americas, and *Gossypium arboreum* ('Tree cotton') and *Gossypium herbaceum* from Africa-Asia. While all four species are present around the world, it is *G. hirsutum* which has become the dominant model, accounting for 90% of all the cotton grown. Over ninety million bales are harvested globally each year, and in the US alone, the annual revenue generated from cotton exceeds $100 billion, with over 300,000 US citizens employed in the industry (Wilkins & Arpat 2005; Meinert & Delmer 1977; Singh et al. 2009). With the human population and demand on global resources increasing, the economic importance of *Gossypium hirsutum* cannot be overestimated, with its high production, low cost and range of desirable properties that give it such an important global market place. As a result, cotton biotechnology and development will continue to attract the interest and attention

of crop scientists and plant breeders for many years to come (Wendel & Cronn 2003; Gokani & Thaker 2000; Gokani & Thaker 2002).

1.2 Evolution of the spinnable fibre

Through several genomic polyploidy events and the intervention of old and new world civilisations the short, tightly adhered fibres of wild *Gossypium* underwent significant morphological and metabolic changes to produce long, spinnable fibres found in the cultivated species such as *G. hirsutum* (Hovav et al. 2008a). Why the epidermal cells have elongated and developed this way is still under debate, with several hypotheses in the cotton scientific community. Fryxell *et al*. (1979) speculated that they evolved to aid in avian dispersal, which was backed up with further evidence in which species of birds from geographically distinct and distance places had seeds from the *Gossypium* tribe present as part of their nest architecture. Other theories include the fibres acting as a biological inhibitor of seed germination unless the external conditions (e.g. moisture, temperature etc) are suitable for the survival of the plant. Whatever the reason, the outcome was that these functions paved the way for domestication by early human tribes many millions of years later (Fryxell 1979; Wendel & Cronn 2003; Hovav et al. 2008). After its origin, *Gossypium* branched out into 50 species, which resulted in genetic diversification and the formation of eight monophyletic groups, labelled A-K. Species from two of these monophyletic groups merged via an ancient allopolyploidization approximately 50 million years ago (Figure 1.1).

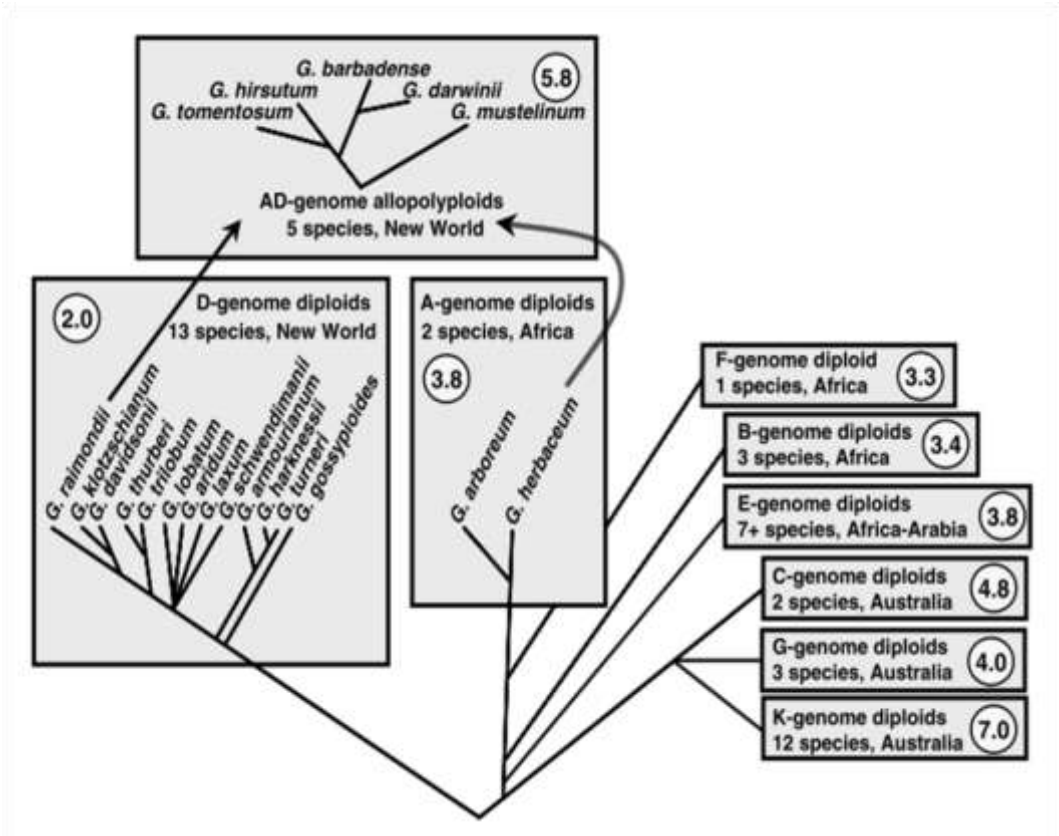

Figure 1.1: Evolution of the spinnable fibre. Phylogenetic analysis point towards all African-Arabian and Australian cotton lines comprising of a single clade that radiated early in the genus evolutionary history to produce the E, B, A and F genome lineages. 2C genome sizes are shown in circles (amount of DNA, in picograms, in the somatic nucleus) (Wendel and Cronn, 2003).

G. arboreum and *G. herbaceum* are A genome species, while *G. hirsutum* and *G. barbadense* were classed as AD genome species (Gross & Strasburg 2010). A-genomes were associated with the trait of a prolonged fibre development and elongation stage, which was consequently passed onto the allopolyploids and led to their gradual domestication and rising dominance of the AD genome species of *G. hirsutum* (Figure 1.1) (Hovav et al. 2008a; Hovav et al. 2008b). Microarray expression studies between the domesticated *G. hirsutum* cultivar and a sample from the population of its wild relative *G. hirsutum-yuctanense* showed that nearly a quarter of genes (9,465) are expressed differently (Gross & Strasburg

2010). Many gene groups that were significantly upregulated in the domesticated fibres included cytoskeleton control, sucrose transport and metabolism, as well as genes involved in the regulation of redox levels & reactive oxygen species (ROS) (Gross & Strasburg 2010; Rapp et al. 2010; Chaudhary et al. 2009; Hovav et al. 2008b). With this in mind it was predicted that the evolution of the long, spinnable fibre arose from a prolonged elongation stage which arose from the novel and differential gene regulation of H_2O_2 and ROS levels. Consequently, H_2O_2 levels have been hypothesised to play a direct role in controlling cotton fibre elongation. In other plant systems, H_2O_2 has been known to be important for cell elongation via wall loosening and expansion (Rodriguez et al., 2002). All species and genome-types of *Gossypium* produce H_2O_2 during their development and at high concentrations, it can become toxic, resulting in the induction of stress responses (Rodriguez et al., 2002). Short fibre development in F-genome species has shown to be linked with the premature expression of stress-related genes. The hypothesis is that the high levels of H_2O_2 generated are not regulated in F-genome species and as a result induce stress response which causes the early onset of secondary wall synthesis and cessation of elongation (Hovav et al. 2008a; Rodriguez et al. 2002). Conversely, genes which play a major role in controlling H_2O_2 and ROS were significantly upregulated in the long fibre A-genome species. In addition to these upregulated genes, A-genome fibres had several over-expressed genes involved in processes such as respiration, energy management, ribosome biogenesis and ROS/H_2O_2 control (Figure 1.2). Of these processes, three genes that have been implicated in other model plant systems to control cell elongation and stress responses were found to be upregulated in A-genomes, with only low/zero levels found in the F-genome species: *GAST1-like*, *Cop1/BONZA*

and *Pex1*. The H_2O_2 induced *GAST1-like* protein, which has been shown to be selectively expressed in fibre cells and trichomes. Inhibition of *GAST1-like* gene-homologs causes the cessation of cell elongation, whilst expression causes cell elongation and the scavenging of H_2O_2 via redox-sensitive cystein protein motifs (Wigoda et al., 2006; Chaudhary et al., 2009). The calcium dependent, membrane binding protein *BONZAI-1/Cpn-1*, which has been shown to inhibit stress-promoting R genes (plant resistance genes) and apoptosis pathways also plays a role in maintaining cell elongation (Yang et al. 2006). P*ex1*, which is involved in the biosynthesis of peroxisomes, the main organelle that deals with the toxic by-products within cells, was found to be significantly upregulated in A-genome, long-fibre species. The scavenging and removal of H_2O_2/ROS would allow further elongation while delaying the expression of stress-related pathways and ultimately secondary cell wall synthesis (Lopez-Huertas et al., 2000).

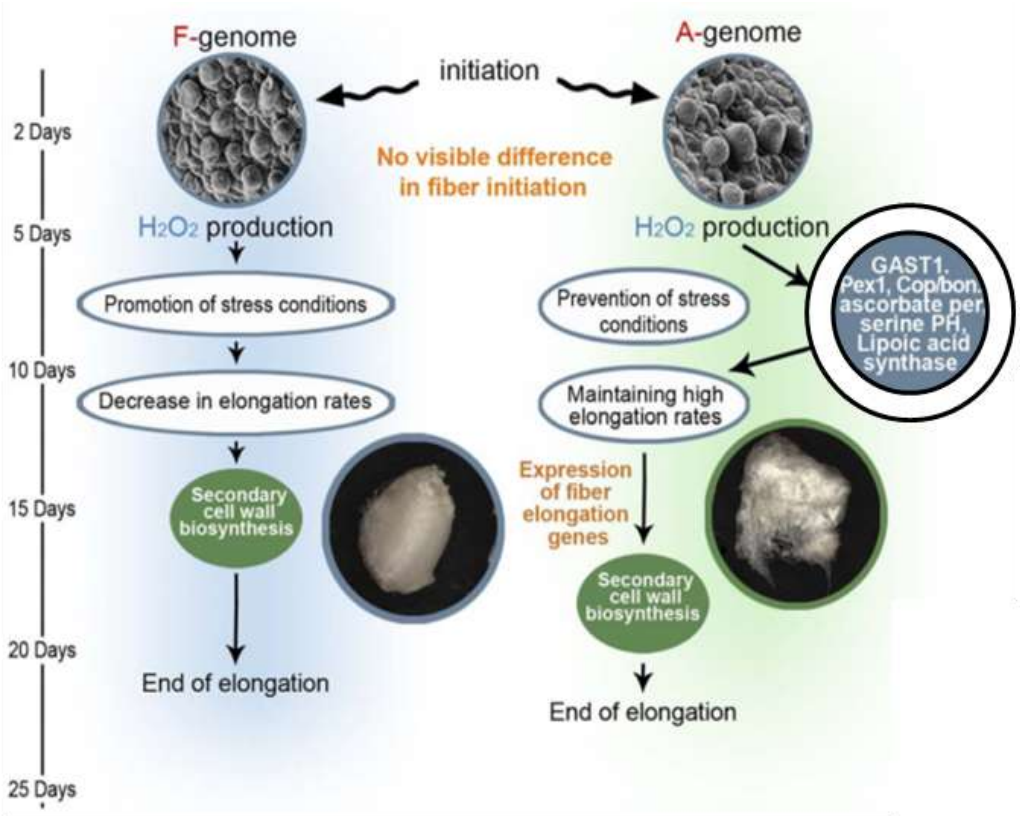

Figure 1.2: Evolutionary model highlighting metabolic differences between short fibre F- genomes and long fibre A-genomes and the processes that lead to the formation of a spinable fibre. (Hovav et al. 2008)

1.3 Cotton fibre development

The cotton fibre cell develops from an individual epidermal trichome cell on the seed coat and is one of the longest single cells known. It can reach lengths in excess of 60 mm which can be up to 3000 times its diameter. It is also regarded as a valuable research model due to the extreme elongation and formation of a relatively very thick secondary cell wall (Singh et al., 2009). The fibres grow in a boll fruit, which is made up of four to five individual carpels or locules, with eight to nine seeds in each one (Vaughn & Turley 2001). Each seed in the boll has on average 14,500 fibres, meaning there are approximately 120,000 fibres in a locule, thus over 500,000 fibres in a single boll (Gokani & Thaker 2002). The mature cotton fibre itself is a thick cellulose-rich cell wall with lumen, and has two parts;

the outer primary cell wall and a thick inner secondary cell wall (Weis et al., 1999). The primary cell wall is responsible for the final length of the cotton fibre, while the secondary cell wall determines the strength of it and is also important for dyeing processes. The market value of cotton depends upon a wide variety for factors including the fibre length, strength, uniformity, whiteness, micronaire (the relationship between linear density and cell wall maturity) and fineness (Xu et al. 2007). The development of the cotton fibre can be divided into four stages (Figure 1.3), and *days post anthesis* (DPA) is used to indicate how old the cotton fibres are since the flower first opens. The four stages are: Initiation (-2-3 dpa), elongation (3-24 dpa), secondary wall synthesis (18-50 dpa) and maturation (>50 dpa)(Gokani & Thaker 2000).

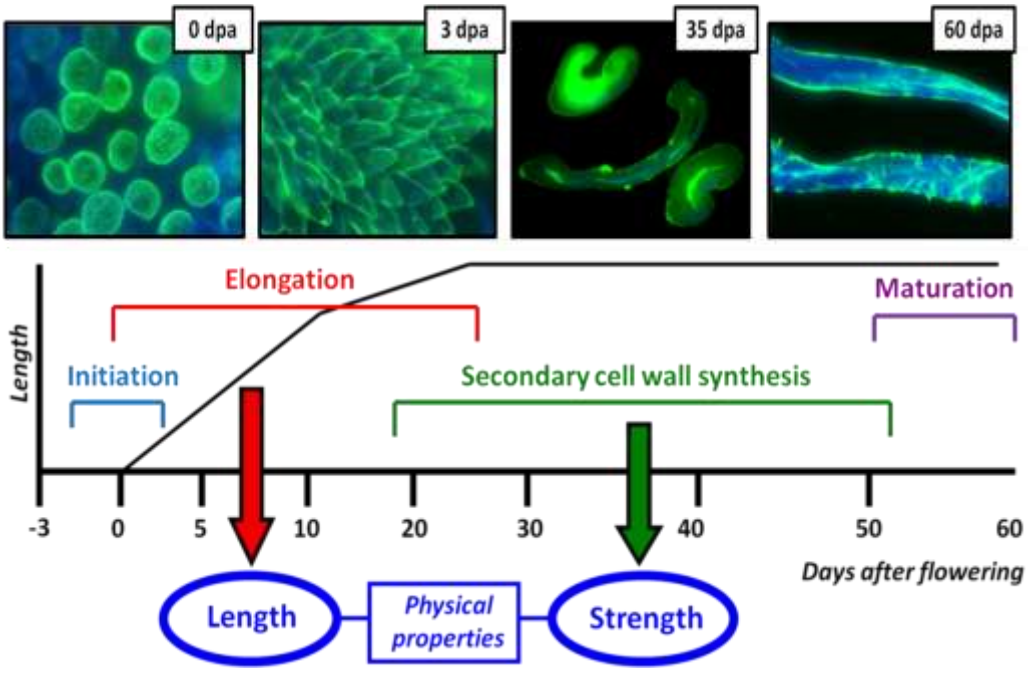

Figure 1.3: Four major stages of cotton fibre development: initiation, elongation, secondary wall synthesis and maturation. Fibre morphogenesis starts on the day of anthesis. Top row images are cell wall imunolabelling micrographs of developing cotton fibres. Green indicates FITC-tagged monoclonal antibodies binding to specific cell wall polysaccharides. Calcofluor white (in blue) was used as a counterstain for all beta-glucans (Timelines adapted from Wilkins & Arpat 2005).

1.3.1 Initiation

Initiation occurs at the start of anthesis, and the fibres grow as individual cells. The stage only lasts for three days and is followed by the elongation stage (Timpa & Triplett 1993). Approximately 25% of the seed coat epidermal cells differentiate into cotton fibre cells. However, the processes that determine which cells develop into fibres is still unknown. Fibres become visible at 0 DPA by the spherical expansion and bulging from the chalazal (opposite end to the micropyle) end of the seed coat surface. This is quickly followed by elongation, where the fibres continue to grow in a spiral manner. At -1 DPA, the cortical microtubules are re-orientated perpendicular to the seed surface in the fibre initials, but not in any epidermal or *fibreless* (*fls*) mutant cells (Basra, 1999). At 0 DPA, membrane bound sucrose synthase (SuSy) is upregulated in WT lines, however this is undetectable in *fls* mutants, indicating the role of SuSy in fibre initiation (Ruan & Chourey 1998). The initiation stage is also characterised by the massive increase in the numbers of Golgi complexes involved in the increased biosynthesis of cell wall polysaccharides and the upregulation of β-glycerophosphatase in fibre initials only. These characterisations were also only present in WT fibre lines and absent from the *fls* mutant (Basra, 1999). Fertilised cotton ovules are able to produce all the hormones necessary for fibre initiation and elongation. However through the addition of an inhibitor of gibberellin biosynthesis it was found that IAA auxins (indole-3-acetic acid), in the absence of gibberellic acid, are not able to cause fibre initiation (Basra, 1999). Fibre initiation can be inhibited by α-amanatin, a RNA polymerase II in-activator, and low temperature nights (<16°C). Additionally, the application of terpenoid lactones can counter the effects of low temperature and restore fibre initiation (Whittaker et al. 1999; Haigler et al. 2007).

1.3.2 Elongation

During this time the fibres elongate rapidly by diffuse growth for around 3 weeks at a rate of ~2 mm/day (dependant on species and lines) (Figure 1.4), expanding due to the turgor pressure exerted from the protoplast to the primary cell wall (Qin & Zhu 2011). Typically, plant cells elongate by either tip or diffuse growth with fast-elongating cells such as root-hairs, trichomes and pollen tubes following a tip-growth pattern at the apical cell end (Qin & Zhu 2011). The process of cotton fibre elongation, on the other hand, has not been determined and is still a topic under debate among academics in the cotton research field (Kim & Triplett 2001). Research indicates a diffuse *and* tip-growth model of elongation due to several key points of evidence. Firstly, diffuse-growth evidence includes the lack of organelle zonation or secretory vesicle accumulation in the fibre tips during elongation. This is a zonal characteristic normally found in tip-growing plant cells and is an indication that new cell wall material incorporation is not restricted to the apical end of the fibre (Tiwari & Wilkins 1995). Additionally, cellulose microfibrils are deposited transversely to the growth direction, which causes resistance to fibre radial expansion while promoting longitudinal elongation in the presence of turgor pressure. Tip-growing plant cells have a different microfibril arrangement, in which they are parallel to the direction of elongation and which is absent at the apical growing end (Seagull, 1993). While these points implicate the diffuse growth model in fibre elongation there is other evidence that also implicates the tip-growth model. Tip-growth requires the establishment of a high Ca^{2+} gradient at the apical end of the cell. A high level Ca^{2+}gradient was found in the tips of elongating cotton fibres by treating developing fibres with fluo-3-fluorescent calcium dye, indicating that a Ca^{2+}gradient influx around the fibre tips

is required for maintaining a rapid stage of cell elongation (Qin & Zhu 2011). As a result, Qin and Zhu (2011) proposed a linear cell-growth model that incorporated diffuse and tip-growth in the cotton fibre elongation (Qin & Zhu 2011). The major polysaccharides present at this time of fibre elongation are cellulose (650k molecular weight), xyloglucans and pectins (Timpa & Triplett 1993; Hayashi & Delmer 1988). During the middle of the elongation stage, the plasmodesmata are closed off for approximately 6-7 days until 16 dpa, which coincides with the peak of expression for sucrose and K+ transport-related membrane proteins. The combination of these two imports maintain high levels of turgor pressure, driving an extended period of fibre elongation (Ruan, 2007). During 9-27 dpa water content increases dramatically and declines at maturation where the fibre undergoes desiccation. The high uptake of water is required for elongation as it is assumed that only turgor pressure is the driving force behind fibre growth. The rate of elongation is determined by the fibre cell wall extensibility from the mechanical interactions of xyloglucan, which has a high turnover rate during elongation (Gokani & Thaker 2000). Additionally, calcium-signalling pathways (including calmodulin, glutamate decarboxylase and calcineurin proteins) are significantly upregulated during fibre elongation and absent in fibreless mutants, indicating that calcium-mediated signal transduction may be important in cotton fibre elongation (Gao et al. 2007). Furthermore, the roles of F-actin filaments in fibre elongation have recently been highlighted in research on *GhADF1,* a member of the actin depolymerising factor family. Using RNAi technology to induce down-regulation of *GhADF1* actin depolymerisation was reduced, resulting in higher amounts of filaments during development and longer, thicker cotton fibres. It is thought that the extra filaments allow more

tracks, and therefore more capacity, for intracellular vesicle trafficking of cell wall growth materials, resulting in the longer and thicker fibre cell walls (Wang et al. 2009). Typical elongating cotton primary cell walls are made up of approximately 20-25% cellulose, with the remainder comprising of hemicelluloses, pectins and waxes. There is also a significant increase in the biosynthesis pathways of ethylene production within the fibre during elongation (Xu et al. 2007; Shi et al. 2006).

It has long been assumed that fibres elongate separately as individual cells. However recent evidence has implicated some degree of fibre co-ordination during development. A cotton fibre middle lamella (CFML) was found to be adhering fibres together until around 24 dpa (Singh et al., 2009). This goes some way to explain how many hundreds of thousands of cotton fibres can grow to their vast length in a very confined cotton fruit boll. Fibre bundles are entrained by the fibre tips during elongation until the start of secondary wall synthesis, whereupon the CFML is broken down via cell wall hydrolysis to restore fibre independence (Singh et al., 2009). While much is still unknown about the CFML and how it is formed, there are several theories about its function within fibre development. Supplementary to encouraging fibre packing, the presence of the CFML may help developing fibres to withstand the turgor pressure during elongation (Ruan, 2007). There have been similar hypotheses proposed in other plant systems with adhesive cell wall parenchyma tissues (Niklas, 1992). In many other plant systems, examples of cell separation include: sloughing of root cells, dehiscence and fruit-ripening. Since cotton fibres develop in a fruit boll it is plausible to link this CFML degradation with the associated cell separation of fruit-ripening. It will also be of interest to look at the further implications for fibre cell wall

development when the CFML is broken down at the transition stage between elongation, secondary wall synthesis and the possible signalling actions of any small polysaccharides released during hydrolysis (Singh et al., 2009).

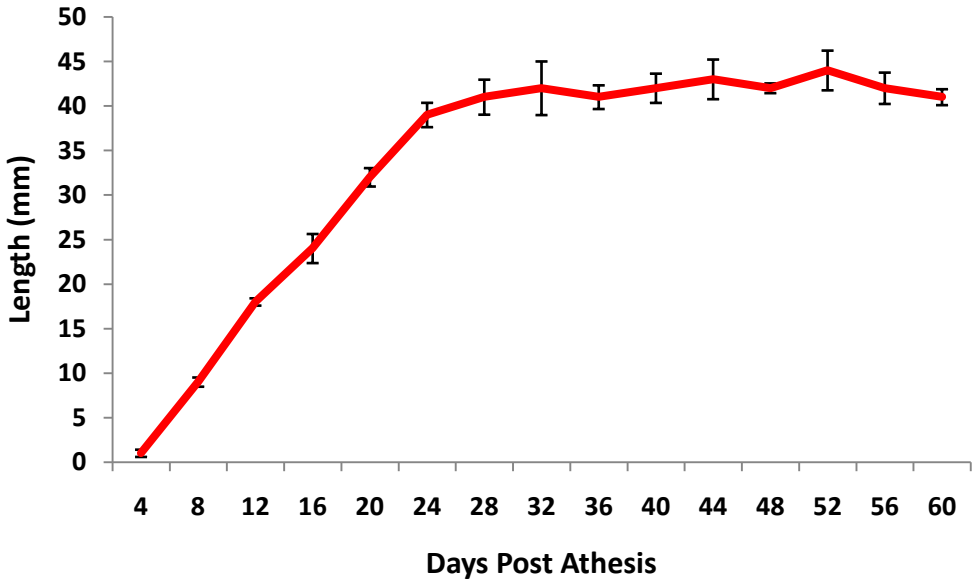

Figure 1.4: Increase in length of the *Gossypium hirsutum* FiberMax™ cotton fibre during development over a period of 60 days from the start of anthesis. (Unpublished data from Benians *et al*. 2012, with fibres measured under the microscope between days 0-5 dpa and with a ruler for >5 dpa).

1.3.3 Secondary wall synthesis

After nearly 3 weeks of elongation fibres synchronously enter the secondary wall synthesis stage in which the majority of cellulose biosynthesis occurs. The deposition of >97% type I cellulose in the secondary cell wall of the cotton fibre makes it a powerful model system to study cellulose biosynthesis, which is important in so many industries. The first cellulose biosynthesis genes were first characterised using cotton fibre research, and with that the role of cortical microtubules in controlling the orientation of deposition was also uncovered (Pear et al. 1996; Haigler et al. 2009). Cotton fibre research also illustrated the

role of sucrose synthase in providing UDP-Glc for secondary cell wall biosynthesis and how the CesA proteins have a very high turnover rate of <30 min (Kurek et al., 2002). After a detectable oxidative burst at the start of this stage, which is thought to initiate secondary wall synthesis through promotion of CesA interactions, successive layers of β-1-4-glucan chains are synthesised and deposited helically at an angle of 45-55° to the axis during the night, until the wall is 4-5 μm thick (Potikha et al. 1999; Murray et al. 2001). One of the first visible changes seen at the start of this stage is the changes in the orientation of cellulose microfibrils and microtubules. As secondary wall synthesis advances, cortical microtubules increase in length and number coupled with a steepening of the helix orientation to match that of the cellulose microfibrils, clearly indicating that ordered microtubule arrays play an important role in cotton secondary wall synthesis (Seagull 1993; Haigler et al. 2009). Regions of cellulose orientation changes, known as reversals, cause the mature fibre to twist upon desiccation, which is essential for these fibres to be spun into yarns (Kim & Triplett 2001). The formation of the secondary cell wall overlaps the previous elongation stage by around six to ten days, depending on specific cultivars. During this time, a thick cellulose cell wall is deposited on the inner surface of the primary wall, making up the majority of the fibre cell diameter. The major polysaccharides during this stage are cellulose (molecular weight increased to 2,200k) and callose (Timpa & Triplett 1993). In addition to cellulose synthesis there is evidence that β-glucuronyltransferases, involved in the elongation of glucuronoxylan (Glucuronoxylans, lignins, and cellulose are the three major components of secondary cell walls in plants) backbone, are upregulated during secondary wall synthesis (Peña et al., 2007). During this stage of development cotton fibres are

highly sensitive to cool temperatures, in that the rates of cellulose synthesis decrease to eventually cause a block in the sucrose synthase pathway (Roberts et al. 1992).

1.3.4 Maturation

Between 45 and 60 dpa, the cotton fruit bolls de-hisc (open up), causing the dense matt of fibres to desiccate and mature. Desiccation of the protoplast causes the cytoplasm to stick to the inner surface of the secondary wall, leaving a hollow lumen. This in turn causes the fibres to twist through the collapse of the hollow lumen and is the point where secondary wall thickness in relation to the overall diameter is important; if the wall is too thick then the fibre will not collapse and twist (Kim & Triplett 2001). The twists caused by drying are very important as they allow the fibres to be spun together to form yarns during the spinning process (Wilkins & Arpat 2005). Little information is known about the maturation stage of the cotton fibre development due to the minimal levels and difficulty in extracting proteins or nuclear material from the mature fibre cell (Kim & Triplett, 2001). While programmed cell death (PCD) has not been definitively characterised in cotton fibres it has been proposed that this controlled form of apoptosis is regulated and implemented in the cotton fibres around 42 dpa (Species dependant) (Kim & Triplett 2001; Potikha et al. 1999; Ji et al. 2003). PCD is a generally controlled and actively regulated form of cell death that occurs in many other plant developmental processes, often involving condensation of the nucleus, cytoplasmic vacuolisation and DNA fragmentation (Swidzinski et al. 2002). One example of PCD is xylem tracheary element differentiation, in which xylem vessels rapidly deposit cellulose to a thick secondary cell wall, followed by PCD. The maturation of cotton fibres has been proposed to be similar to this

tracheary element PCD, in that the end result of secondary wall thickening is followed by a coordinated cell death to produce a mature cell corpse that is almost entirely made of up cellulose (Groover & Jones, 1999; Kim & Triplett, 2001; Potikha et al. 1999). Much like the elongation stage, H_2O_2 has been detected during secondary cell wall synthesis in fibres (Up to 5 µM/mg of dry weight) (Potikha et al., 1999). In knockout experiments fibres lacking H_2O_2 were prevented from entering the secondary wall synthesis stage. Further to this, treating very young fibres with H_2O_2 caused a premature transition to the secondary cell wall synthesis stage (Potikha et al. 1999).

It was found that where the fibres attach to the epidermal seed coat the primary cell wall is much thicker and the secondary wall is conversely thinner. Despite these changes the thicker primary cell wall increases the strength of the fibre attachment, especially upon desiccation at maturation, when the primary wall material condenses (Vigil et al., 1996).

1.4 Post harvesting and industrial processes

During the cotton textile manufacturing process, the fibres undergo many pre-treatments to eliminate wax, pectins, hemicelluloses and proteins, allowing access to the cellulose structure. These treatments also "homogenise" the fibre differences to make samples that will react in the same way i.e. fibres with different maturities could react the same way after dyeing to make the process easier. The process of mercerisation involves treating the cotton with strong alkali, which reduces the crystallinity in parts by altering the hydrogen bonding of the cellulose. The treatments alter the initial α-cellulose into a more thermodynamically favourable β-cellulose cellulose structure. In layman terms, this causes the cellulose composition of the fibre to be more consistent and actually makes it stronger by changing the weaker areas and improving luster. An effective way to study these cellulosic crystallinity changes is to use Fourier Transform InfraRed spectroscopy (FT-IR). FT-IR can produce information about several factors including the chemical nature, isomerisation, conformational order and polymer orientation of samples. A crystallinity index value is determined from these factors and is a simple but effective method to study the changes in the crystallinity and supramolecular structure of cotton fibre cellulose (Blackburn and Burkinshaw, 2002; Abidi et al., 2007; Široký et al., 2009; Kljun et al., 2011).

1.5 Plant cell walls

Plant cell walls make up one of the most abundant sources of biomass, with their major component, cellulose, being the most abundant macromolecule on the planet. Plant cell walls are complex extracellular components which have many different important functions including cell adhesion in tissues, cell signalling, fruit development, cell growth and defence against pathogens (Willats et al. 1999; Ferrari et al. 2008; Cardoso et al. 2007; Ferreira et al. 2006; Li et al. 2003; Jarvis et al. 2003; Keegstra 2010). Their diversity and complexity is only matched by their wide range of uses and industrial applications in our everyday life, including textiles, paper, pharmaceuticals, construction material etc (Brecker et al. 2005; Nergard et al. 2005; Thude & Classen 2005; Dourado et al. 2006; O'Neill & York 2003). There is also much research carried out into unlocking the carbon of plant cell walls for the development of a new generation of bio fuels (Gomez et al., 2008; Pauly and Keegstra, 2008; Sims et al., 2010).

There are two main types of plant cell walls: primary cell walls and secondary cell walls (Figure 1.5). Primary cell walls are found in all plant cells and can be further divided into type I and type II walls. Type I cell walls are found in all dicotyledons and some monocotyledons. These type I cell walls consist of approximately 30% cellulose, 30% hemicelluloses and 40% pectins (O'Neill & York 2003). Type II cell walls are found in the monocotyledons and commelinids, which contain different ratios and polysaccharides. Hemicelluloses can make up to 70% of the primary walls, and also include monocotyledon-specific mixed–linkage glucans, which are not found in type I walls (O'Neill & York 2003; Carpita & Gibeaut 1993; Harris & Smith 2006). The secondary cell wall is a thick, cellulose and hemicellulose-rich component, and is a major part of the cotton fibre. Secondary cell walls are not present in all cells, but are often in cells that are dead at maturity (Harris & Smith 2006; Basra 1999).

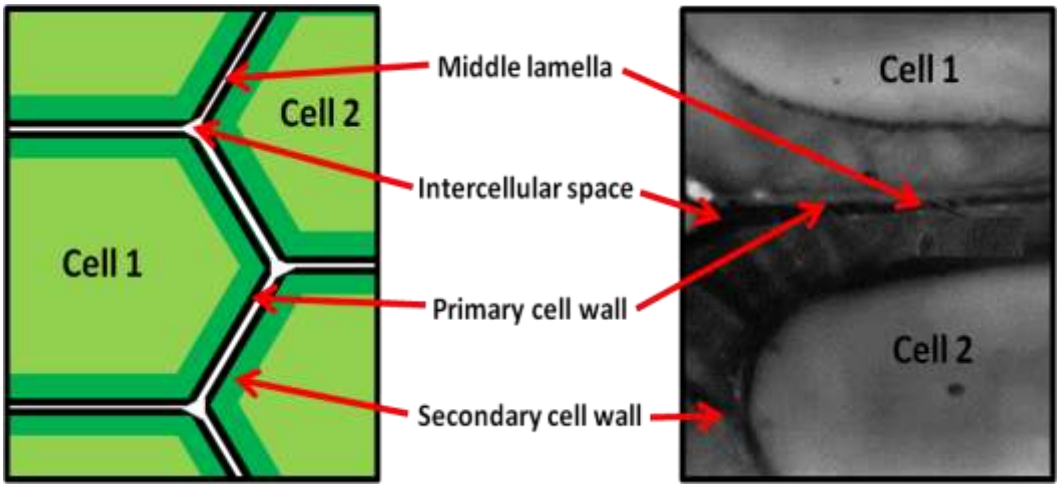

Figure 1.5: Plant cell walls. Left: Schematic representation of primary and secondary cell walls. Right: Electron microscopy of primary and secondary cell walls (Xu et al. 2006)

1.5.1 Cellulose

1.5.1.1 Structure

Cellulose is the most abundant biopolymer on Earth, primarily due to the fact that all land plants have cellulose in their cell walls (Lerouxel et al. 2006; Taylor 2008). Cellulose is a long, linear homopolymer of D-glucose sugar residues adjoined by (1→4)-β-glycosidic bonds, with each neighbouring glucose molecule inverted 180° (Figure 1.6).

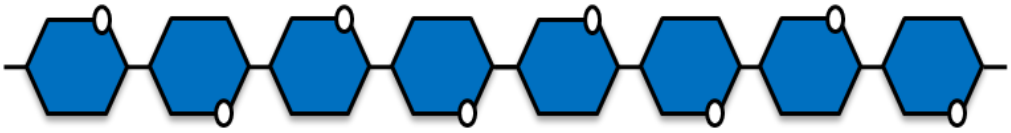

Figure 1.6: Cellulose chain schematic made up of (1→4)-β-D-glucose alternating subunits found in plant cell walls (Taylor, 2008).

This organisation allows parallel cellulose chains, to associate closely with anti-parallel chains and form hydrogen bonds, resulting in the formation of very strong cellulose microfibrils which are mechanically resistant to the turgor and stretching forces that the cell walls experience (Figure 1.7) (Taylor 2008). These microfibrils can consist of highly ordered crystalline regions as well as amorphous regions where the cellulose chains are less tightly packed (Figure 1.8) (Cosgrove 1997).

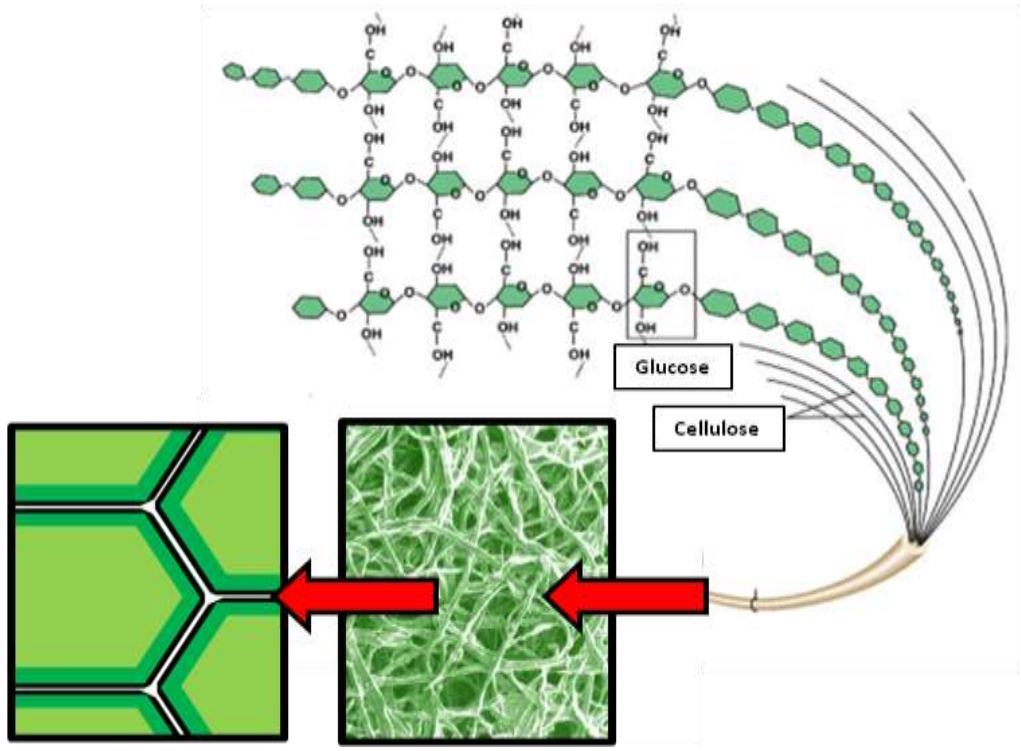

Figure 1.7: Schematic general representation of the structure of cellulose in plant cell walls (Taylor, 2008).

The degree of polymerisation (DP) of cellulose chains in primary cell walls are usually around ~ 8,000 residues (Brown, 2004), while in secondary walls, this is much higher at ~ 15,000 residues (Brett, 2000), although this difference is still not fully understood (Taylor 2008). It has been shown through structural and quantification analyses of cell wall fractions that cellulose microfibrils associate with hemicelluloses to create strong load-bearing networks. This organisation helps to resist the osmotic turgor pressure exerted on the cell wall during growth and development, with cellulose providing the majority of the tensile properties (Pauly et al. 1999; O'Neill & York 2003). Contrasting work has also indicated that hemicelluloses also regulate cell wall elasticity by acting as plasticisers through enzymatic re-modelling of their localisation within the cell wall (Chanliaud et al. 2004; Carpita & Gibeaut 1993; Pauly et al. 1999; Ikushima et al. 2008; Thompson 2005; Cavalier et al. 2008).

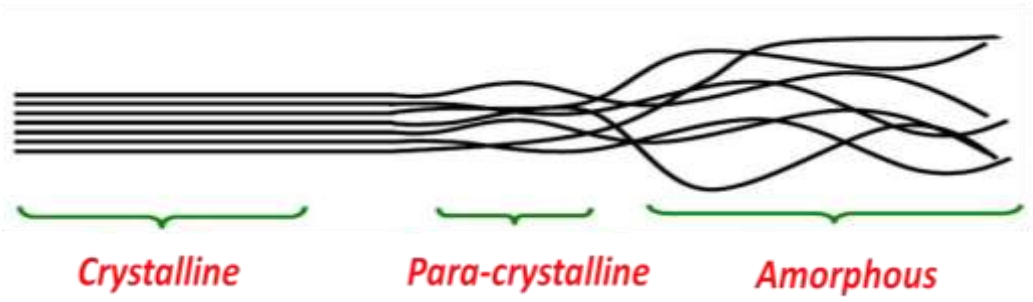

Figure 1.8: Organisation of cellulose chains and the nature of its crystalline, para-crystalline and amorphous regions (Segal et al., 1959).

1.5.1.2 Biosynthesis

Cellulose synthesis is a complex process in which soluble substrates in the cytoplasm are converted into insoluble polymers at the outer surface of the plasma membrane by protein complexes. The main family of cellulose synthesis proteins, CesA, was first discovered in 1998, and since then, there has been clear evidence indicating that these proteins form rosettes which are localised in the plasma membrane. Each CesA subunit is made up from three different isoforms (α1, α2 and β) to produce the rosette hexamer (Figure 1.9) (Doblin et al. 2002).

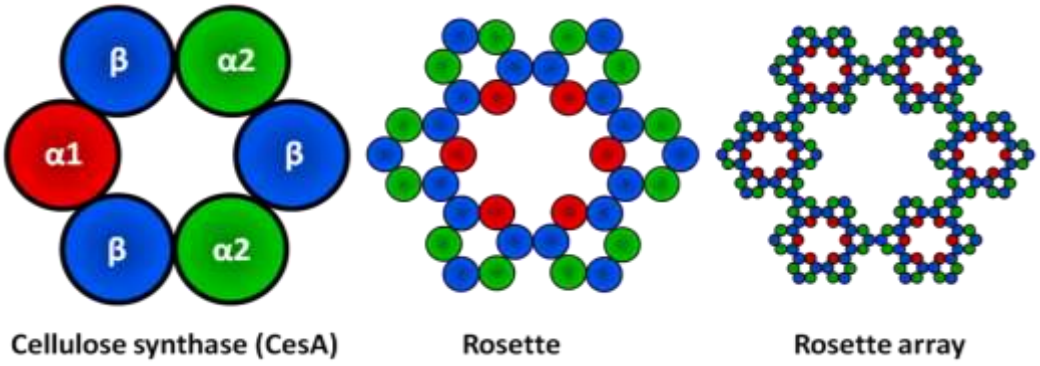

Figure 1.9: Cellulose synthase isoforms and rosette complexes in the plasma membrane. Adapted from (Ding and Himmel, 2006).

Each rosette array complex consists of six hexameric rosette proteins, each one generating a cellulose microfibril chain through glycosytransferase action. In total, thirty six chains are synthesised in parallel and their deposition is regulated, as the orientation can determine how the cell grows and the cell morphology upon maturation (Murray et al. 2001; Arioli 1998; Saxena & Brown 2005). Recent evidence has shown that different sets of proteins are involved for primary and secondary wall cellulose synthesis. In the primary plant cell walls of *Arabidopsis thaliana*, the three subunits are CesA1, CesA3 and CesA6; while the secondary wall cellulose is synthesised by a combination of CesA4, CesA7 and CesA8, which can partly explain the differences in the DP of the cellulose chains (Persson et al. 2007; Taylor et al. 2003). It is, however, still not entirely known how the rosettes complexes move along the plasma membrane. Recent evidence suggests a role of the cortical microtubules in regulating the transit of CesA complexes (Geisler et al., 2008); while contrasting evidence has suggested that the polymerisation of glucose into cellulose microfibrils provides the force necessary to push the complex along. Other proteins such as *Suzy* and *Korrigan* could also play an important role in cellulose synthesis but their functions are not yet completely known. (Paradez et al., 2006).

1.5.2 Callose

Callose is a polysaccharide composed of glucose residues connected by β-(1-3)-linkages, and is classed as a β-glucan. It is synthesised by callose synthases and is associated with cell plate during cytokinesis, pollen development and also in reponse to infections by pathogens and tissue damage (Carman et al., 1991). The presence of callose in plant cells has also been implicated as an indicator of the targets for PCD (Blackman & Overall 2001; Brodersen et al. 2002).

1.5.3 Pectins

Pectins are a large family of complex, galacturonic acid (GalA) rich and diverse polysaccharides which are present in all land plants and are also used as functional food ingredients (Marcus et al. 2008; Willats et al. 2001; Harholt et al. 2010). Structurally and functionally, pectins are the most complex macromolecules in the plant cell wall, and play major roles in morphology, growth and development. There are three major types of pectin; homogalacturonan (HG), rhamnogalacturonan-I (RG-I) and rhamnogalacturonan-II (RG-II) (Mohnen, 2008; Harholt et al., 2010).

1.5.4 Homogalacturonan (HG)

1.5.4.1 Structure

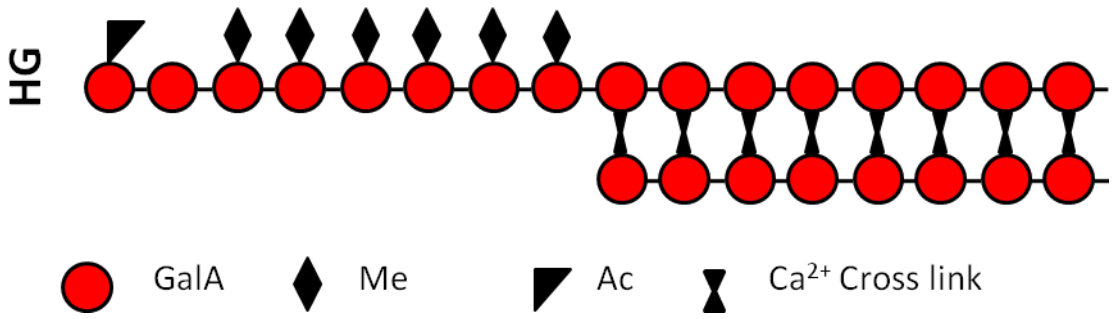

Figure 1.10: Structure and modifications of pectin homogalacturonan in plant cell walls. When first synthesised, HG chains are methylesterified (Me) by methyl-transferases and further modified by pectin methylesterases. HG chains longer than 9 DP can associate via calcium cross links to form rigid gels. HG chains can also be *O*-acetylated (Ac) to modify the cell wall pectin properties. Adapted from (Yapo et al., 2007).

Homogalacturonan (HG) is a helical homopolymer made up of (1→4)-α-D-GalA units covalently bound in a linear chain (Figure 1.10) and is often referred to as the "smooth" region as there are no branching side chains. HG may be *O*-acetlylated at the C-2 or C-3 carboxyl and can also be methyl-esterified at the C-6 carboxyl group (Ralet et al. 2005). The DP also varies between 70-250 GalA residues; and can be different between cells, tissues and species. However it has been shown that HG length is conserved between 80-117 residues (Hellín et al., 2005; Yapo et al., 2007).

1.5.4.2 Biosynthesis

HG is synthesised in the Golgi in two steps and has been the most studied and best understood form of pectin (Carpita & Gibeaut 1993; Mohnen 2008; Caffall & Mohnen 2009; Vincken et al. 2003). The GalA backbone is synthesised by a homogalacturonan (1→4)-α-D-galacturonosyltransferase (GAUT). HG can then be further modified at the C-6 carbon by HG methyltransferases (HG-MT) and HG acetyltransferases to produce methyl-esterified and acetylated HG respectively, which affect its properties within the cell wall (Somerville et al. 2004; Caffall & Mohnen 2009). There is evidence that HG is synthesised and deposited in the cell wall in a highly methyl-esterified form (unless it is highly acetylated), but is later modified by enzymes from the pectic methyl-esterase (PME) family to change the methyl-esterification pattern along the polysaccharide and ultimately affect its properties (Wolf et al., 2009). PMEs are enzymes which remove methyl-ester groups from pectin (Willats et al. 2001a; Willats et al. 2001b). The removal of these groups allows the HG chains to link to other HG chains via calcium, creating a matrix which traps water, solutes and also embeds the cellulose and hemicelluloses networks. As previously mentioned, several

pieces of the HG biosynthetic machinery have been isolated and characterised, including the type II membrane proteins GAUT1, GAUT7. GAUT1 is a (1→4)-α-D-galacturonosyltransferase that catalyses the elongation of HG chains by attaching to UDP-(1→4)-α-D-GalA (Sterling et al., 2006). GAUT7 does not have this same function, but is required to complex with GAUT1 to act as the catalytic unit of the HG biosynthesis in the Golgi apparatus (Sterling et al., 2006; Atmodjo et al., 2011). There is also much work on the Golgi localised putatative GalA transferase *QUA1* and HG methyltransferase *QUA2* proteins, which are thought to be part of the HG biosynthetic machinery by interacting with the GAUT1 and GAUT7 complex (Mouille et al. 2007; Ralet et al. 2008; Bouton et al. 2002; Atmodjo et al. 2011).

1.5.4.3 Function

The properties of HG and porosity of pectic gels in plant cell walls are determined by the degree and pattern of methyl-esterification along the HG chains. The degree of HG methyl-esterification can also limit cell wall growth in developing *Arabidopsis thaliana* hypocotyls (Derbyshire et al. 2007; Willats et al. 2001; Ross et al. 2011; Wolf et al. 2009). This regulation is controlled by the enzymatic family of PME proteins which remove the methyl-ester groups from the HG chains. The gibberellic acid *Arabidopsis thaliana* mutant *ga1-3* has reduced levels of methyl-esterification and reduced growth levels. The addition of gibberellic acid restored normal growth by restoring the normal levels of methyl-esterification (Derbyshire et al., 2007). This is further backed up by immunolocalisation studies which show that non-growing adhered cell walls have large regions of de-esterified HG chains (Liners et al., 1992). Non-esterified regions of HG chains allow them to form rigid gels which are ultimately

important in cell adhesion. This is also thought to regulate the supply of ions and water by trapping them in the rigid HG gel. This was verified studying the QUA1 knockout in *Arabidopsis thaliana*, which has up to 25% reduced levels of GalA and a phenotype of poor cell adhesion and dwarfism (Mouille et al. 2007; Bouton et al. 2002; Atmodjo et al. 2011). Alteration in HG methyl-esterification has also been seen in mutants where cellulose synthesis has been disrupted. A tobacco system with a silenced cellulose synthase CesA gene had a 25% reduced cell wall cellulose content. However, an increase in de-esterified HG was detected (Burton et al., 2000). An identical effect was seen in tobacco cells treated with the herbicide isoxaben which inhibits cellulose synthase. The formation of calcium cross-linking of non-esterified HG may act as a load-bearing substitute in response to the reduced levels of cellulose (Manfield et al., 2004). De-methyl-esterified HG has also been implicated in the opening and closure of stomatal cells on the leaf epidermis, with the hypothesis that RG-I arabinan side chains (see next section) can act as a physical barrier between HG chains to prevent the formation of calcium cross-links (Jones et al. 2003). Mechanical studies of low- or non-esterified HG show that it is much more rigid than RG-I, while compressive analysis shows that the presence of HG pectin increases the elasticity of cell walls (Chanliaud et al. 2002; Chanliaud et al. 2004; Ralet et al. 2008). It has also been recently shown that de-acetylation of HG at the C-3 and C-2 position of GalA, in tobacco, sugar beet and potato impairs cell elongation, pollen germination and plant reproduction. A decrease in pectic digestibility by fungal poly-galacturonase was also seen (Ishii, 1997; Gou et al., 2012).

Small endo-polygalacturonase-derived HG fragments with a DP between 2 and 20 GalA residues called oligogalacturonides (OGAs) have also been implicated in

regulating growth, active defence responses and fruit ripening (Redgwell & Fry 1993; Pilling & Höfte 2003; Ridley et al. 2001; Mathieu et al. 1991; Messiaen & Cutsem 1994; Simpson et al. 1998). With the exception of fruit ripening, OGA have an inhibitory effect on auxin induced responses, including root and shoot elongation, however, recent evidence has shown this relationship to be far more complex (Simpson et al., 1998). *Arabidopsis thaliana* transformants with over-expressed endo-polygalacturonase are hypersensitive to auxin responses and as a result produce a phenotype that exhibits dwarfism but with increased resistance to fungal pathogens (Capodicasa et al., 2004; Ferrari et al., 2008).

1.5.5 Rhamnoglacturonan II (RG-II)

1.5.5.1 Structure

Rhamnogalacturonan-II is by far the most complex polysaccharide in plant cell walls, being made up of (1→4)-α-D-GalA backbone (a HG backbone), with complex heteropolymeric side chains. RG-II is highly conserved among most land plants, with up to eleven different sugars found on the branching side chains, which can be methyl-esterified or *O*-acetylated (Table 1.1 & Figure 1.11). There is also little variation in its fine structure across all species (O'neill & York 2003; Ishii & Matsunaga 2001; Matsunaga et al. 2004; Kobayashi et al. 1996). RG-II chains can dimerize with each other at the apiose residue by the formation of a borate diester bond. This explains why most plants, including cotton, need boron as a nutrient, although too little causes deformations in the cell wall and too much is lethal (Johansen et al. 2006; Mohnen 2008; Kobayashi et al. 1996; Ishii 2001).

Table 1.1: Sugar residues in RG-II side chains.

Type	Sugar	Structure
Common sugars	Galactose	
	Arabinose	
	Rhamnose	
Methyl-esterified	Fucose	2-O-methyl fucose
	Xylose	2-O-methyl xylose
O-acetlyated	Fucose	3-O- or 4-O-acetyl fucose
Uncommon sugars	Apiose	3-O-apiose
	Aceric acid	3-C-carboxy-5-deoxy-L-xylose
	Dha	2-keto-3-deoxy-D-lyxo-heptulosaric acid
	Kdo	2-keto-3-deoxy-D-manno-octulosonic acid

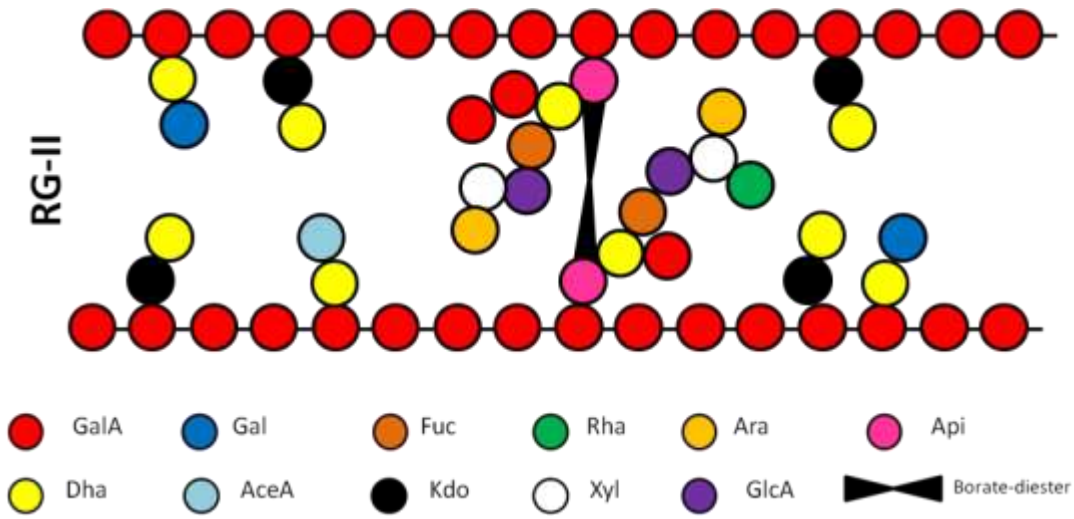

Figure 1.11: Structure and composition of pectin rhamnogalacturonan-II in plant cell walls

1.5.5.2 Function

Boron mediated cross-linking of RG-II is correlated with the pore size which affects the free permeation through the cell wall and changes during developmental stages such as the growth and stationary phases (Carpita & Gibeaut 1993; Fleischer et al. 1998). The ratio of RG-II borate dimers and monomeric RG-II can be affected by three main conditions within the cell wall: concentration of borate, cation activity and pH. These have all been shown to affect the dimerisation of RG-II and consequently have an effect on cell wall pore size. Acidic pH and CDTA treatments have been shown to increase the pore size while increasing the concentration of boric acid can prevent this decrease (Fleischer et al., 1998, 1999). Cells going through the growth phase were shown to have larger pore sizes than cells that had entered the stationary phase, where it is predicted that boron has a stabilising role. In contrast, borate deficient cells continue to expand and will eventually die through the cell wall rupturing. This led to the conclusion that RG-II cross linking is less important during

development, but becomes crucial once cell expansion has stopped (Fleischer et al., 1998).

Apiose residues in RG-II side chains act as the binding point of boron mediated cross-linking and are synthesised from a UDP-D-apiose/UDP-D-xylose synthase. Disruption of this biosynthetic pathway results in an altered side chain structure of RG-II, followed by cell rupture and death, which supports the crucial function of RG-II in plant cell walls of all species (Ahn et al., 2006). *Mur1* is another RG-II mutant of *Arabidopsis thaliana* in which there is a defective fucose biosynthetic pathway that ultimately results in a lack of fucose in the RG-II side chains. This lack of fucose in the side chains has an indirect effect on the rate of formation and stability of borate-diester cross-links. *Mur1* mutants were also shown to have a 50% decrease in cell wall tensile strength (O'Neill & Darvill 2001; Ryden et al. 2003). The *GUT1* mutant from the *nolac-H18* line had an absence of GlcA from the side chains, and resulted in RG-II being unable to form dimeric chains, and consequently little cell adhesion was also seen (Iwai et al., 2002). Iwai (2002) predicted that dimeric RG-II is important for cell adhesion, specifically affecting the adhesion of the plasmodesmata. This would prevent normal cell to cell communication which is essential for morphogenesis. Iwai et al. also went on to conclude that the whole structure of RG-II is crucial for the formation of borate cross-linking dimmers and would also explain the somewhat highly conserved nature of RG-II (Iwai et al., 2002). As RG-II can be directly attached to HG, it is thought that dimerisation can bring these HG chains closer together for calcium mediated cross-linking and the formation of more rigid gels (O'neill & York 2003; Yapo et al. 2007).

1.5.5.3 Xylogalacturonan (XGA)

XGA has a HG backbone of (1→4)-α-D-GalA, with between 40-90% of the residues substituted at the *O*-3 with either a monomeric or dimeric xylose residue (Figure 1.12) (Zandleven et al. 2007; Willats et al. 2004). Methyl-esterification or acetylation of the GalA backbone subunits may also be seen in XGA and is mostly seen in storage or reproductive tissues (Le Goff et al. 2001; Zandleven et al. 2007).

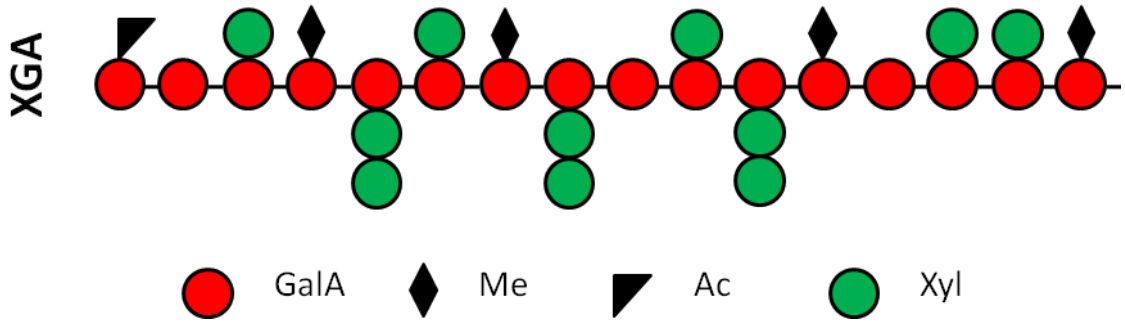

Figure 1.12: Structure and composition of xylogalacturonan in plant cell walls.

Although the exact function is still not fully understood, XGA has been implicated in cell detachment in pea seedling testa, where XGA is only found at the loosely attached inner parenchyma cells. It is also found to be associated with the root cap cells at the root apex during development (Willats et al. 2004). XGA has also been implicated in plant defence. *XGD1* was found to encode a xylogalacturonan-xylosyltransferase localized to the Golgi membrane which was significantly upregulated when the plant was infected with the necrotrophic fungus *Botrytis cinerea* (Jensen et al. 2008). Recent evidence also showed that de-acetylated XGA correlates with increased resistance to *B. cinerea* (Manabe et al., 2011). XGA is also highly resistant to enzymatic degradation by endo-

polygalacturonases, so it is thought to also play a role as cell wall scaffolding during cell wall remodelling stages such as ripening and elongation (Zheng & Mort 2008; Jensen et al. 2008).

1.5.6 Rhamnogalacturonan I (RG-I)

1.5.6.1 Structure

Rhamnogalacturonan-I, first characterised in 1933 (Schumaker 1933), is a disaccharide heteropolymer made up of (1→4)-α-D-GalA-(1-2)α-L-Rhamnose repeating units with mainly arabinan and galactan side chains linked to the Rha residues (Figure 1.13) (Renard et al. 1998; Nieman & Link 1933). RG-I is also known as the "hairy" region, due to many C-3 and C-4 branching side chains, which can occupy between 20% to 80% of the Rha residues (Carpita & Gibeaut 1993; Vincken et al. 2003; Oosterveld et al. 2000; Yapo 2011). Recently, however, small regions of HG GalA have been found intermittently along the RG-I backbone which has been shown to connect to XGA or HG (Coenen et al. 2007; Yapo et al., 2007). There is significant evidence to show that RG-I is *O*-acetylated at the C-2 and C-3 carbon (Ishii 1997), but it has not been definitively shown that RG-I can be methyl-esterified (Ridley et al., 2001). The majority of side chains are composed of a combination of arabinan, galactan or arabinogalactan chains (Vincken et al. 2003). However, the ratio and structure of these RG-I side chains vary quite significantly among different cell types, tissues and plant species (Yapo et al. 2007; Zheng & Mort 2008; Willats et al. 1999). While the function and biosynthesis of these variable side chains are not known, it is postulated to be a highly regulated process and has an impact on the properties of the cell wall (Western et al. 2001).

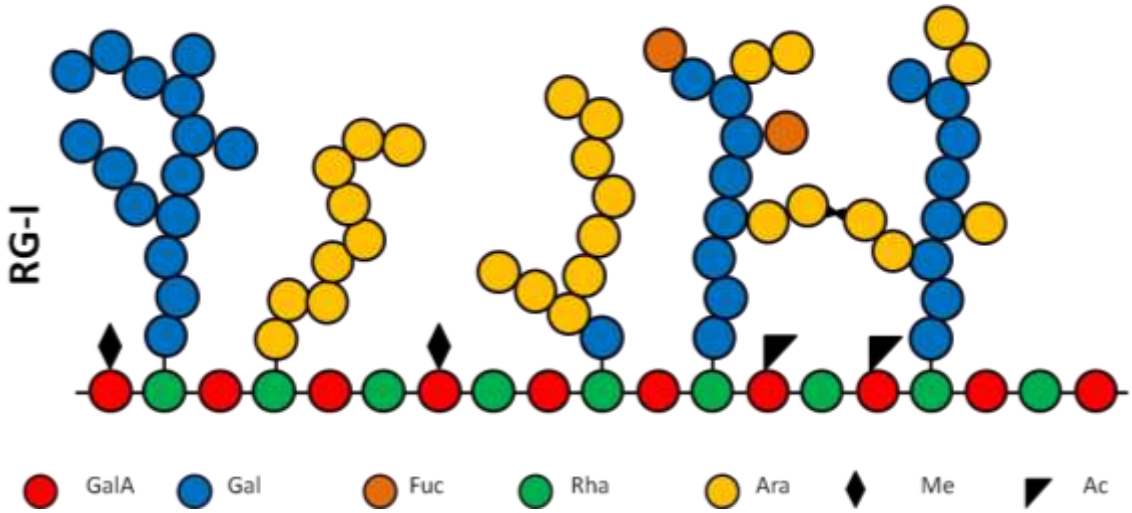

Figure 1.13: Structure and composition of pectin rhamnogalacturonan-I and its galactan and arabinan side chains in plant cell walls. The ability of RG-I side chains to form entanglements by hydrogen or diferulic bonds can affect the mobility of cell wall polysaccharides.

1.5.6.2 Arabinan side chains

Arabinans are highly mobile side chains in RG-I, which has been demonstrated with various experimental methods, including crystallography, NMR, mechanical test, viscometric and rheological assays (Cros et al. 1994; Renard & Jarvis 1999; Ha et al. 2005; Ralet et al. 2008). Linear arabinan chains are made up of a (1→5)-α-L-arabinofuranosyl backbone, which can be further substituted at the O-2 or O-3 by more arabinofuranosyl residues (Cros et al., 1994). The DP has been shown to be highly variable, even when the linear chains are less than 100 Ara residues (Oosterveld et al., 2000; Zykwinska et al. 2008). Substituted arabinans on branched (1→4)-β-D-galactan side chains have also been shown to form connections with arabinans from other galactan side chains, and can also form hydrogen and di-ferulic bonds (Figure 1.13) (Huisman et al. 2001; Levigne et al. 2004a; Levigne et al. 2004b). Linear arabinan side chains can also be created by the formation of O-3-H-O-2 bonds, a 2-fold helix structure within the cell wall

(Janaswamy and Chandrasekaran, 2005). This conformation can have a direct effect on the rigidity of pectin within plant cell walls by preventing non- or low-methyl-esterified HG chains from forming calcium cross-links (Figure 1.14) (Verhertbruggen et al. 2009; Jones et al. 2005). Furthermore, it has recently been shown that some arabinan side chains are cross-linked with cellulose which increases the rigidity of these RG-I side chains (Zykwinska et al. 2005; Zykwinska et al. 2006; Zykwinska et al. 2008).

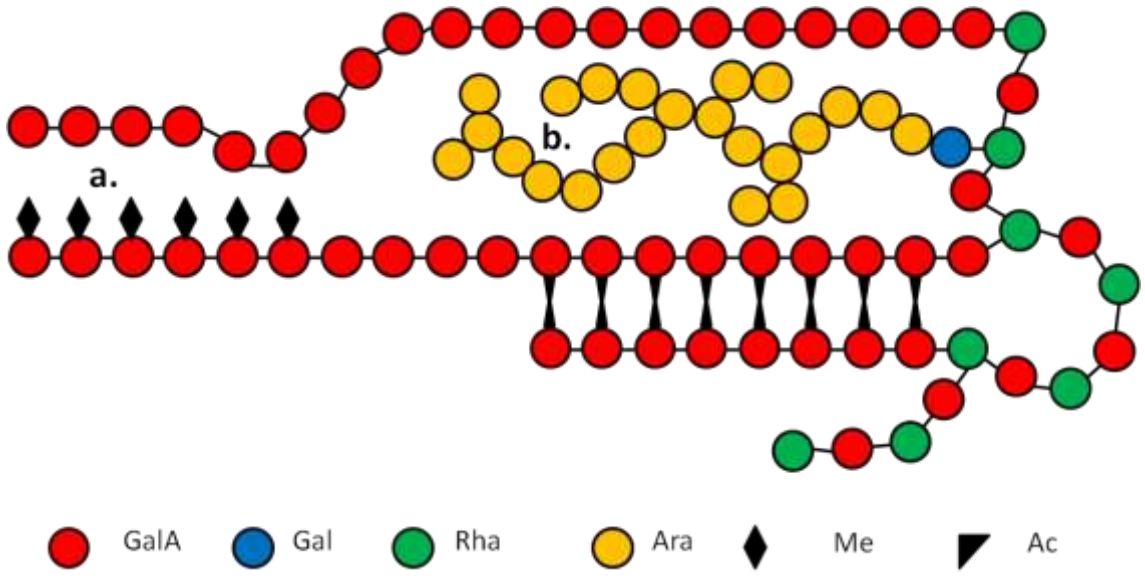

Figure 1.14: Factors that can affect pectic HG and its ability to form rigid gels in plant cell walls. a. methyl-esterification of GalA subunits in HG prevent calcium cross-linkings. b. Arabinan interfering with HG and preventing calcium cross-linkings from forming. In this figure, HG is portrayed as a side chain of RG-I, which is gradually becoming a more accepted model of pectic polysaccharides in plant cell walls (Vincken et al. 2003).

1.5.6.3 Galactan side chains

Although not possessing as high mobility as arabinans, the mobility of galactan side chains are also reduced in the same way when they cross-link with cellulose in the cell wall (Ha et al. 2005; Zykwinska et al. 2006). Galactan side chains are comprised of (1→4)-β-D-galactosyl and sometimes have (1→2)-α-L-fucosyl substitutions and (1→6)-β-D-galactosyl branches (Figure 1.13) (O'Neill & York 2003). Numerous NMR studies have also shown that galactans bestow tensile properties to the cell wall and can mark the separation between primary and secondary cell walls, which was first seen in flax fibre cells (Girault et al. 1997; Arend 2008; Zykwinska et al. 2005).

1.5.6.4 Arabinogalactan side chains

There are two types of arabinogalactans in plant cell walls which are characterised into two types depending on the branching of the backbone. Arabinogalactan-I has a (1→4)-β-D-galactan backbone with several arabinan chain substitutions at the *O*-2 and *O*-3 regions (Clarke et al. 1978; Vincken et al. 2003). Arabinogalactan-II has a (1→3)-β-D-galactan or (1→6)-β-D-galactan backbone with several complex side chains that vary significantly between cells, tissues and species. Arabinogalactans are also sometimes classified as hemicelluloses (Clarke et al. 1978; Seifert & Roberts 2007). The majority of side chains are comprised of (1→3)-α-L-arabinosyl and (1→6)-α-L-arabinosyl residues (Sun et al. 2005; Thude & Classen 2005). The presence of these arabinan side chains increases the overall mobility of AG-II within the cell wall. AG-II also makes up the carbohydrate component of AGPs (Thude and Classen, 2005).

1.5.6.5 Function

While much work has been done on uncovering the functions of HG and RG-II, there is much less known about the functions of RG-I within the cell wall. NMR has shown to be a very good approach to studying the physical and chemical properties of RG-I, specifically the arabinan and galactan side chains (Fenwick et al. 1999; Renard & Jarvis 1999; Ha et al. 2005). *In-muro* NMR studies have shown that pectic galactan and arabinan are some of the most highly mobile polysaccharides within the hydrated cell walls of dicotyledons (Ha et al., 2005). Consequently, arabinans have been predicted to act a cell wall plasticisers and do not contribute to cell wall tensile properties, as they have shown to not be able to form entanglements with other cell wall polysaccharides (Girault et al., 1997; Ha et al., 2005). Monoclonal antibodies have played an important role in many RG-I related experiments (Willats et al. 2000; Jones et al. 2003; Steffan et al. 1995; McCartney et al. 2000; Zykwinska et al. 2006; Verhertbruggen et al. 2009). Arabinans have been shown to be essential for normal stomata functionality. This was shown after arabinase treatments of leaf epidermal cells, in which the stomatal opening and closing was impeded (Jones et al. 2005), in this case the flexibility of the stomata, so the removal of arabinans resulted in stiffening of the cell walls (Jones et al. 2003). There is also evidence to suggest that RG-I polymers play a role in regulating the properties of the cell wall during hydration stress. Even during extreme desiccation, galactan and arabinan chains retain high mobility, and are often the first to be mobilised after the re-hydration of dry plant cells (Tang et al., 1999). This is also reported in *Arabidopsis thaliana* seeds which have high arabinan content (40% of polysaccharides) and usually undergo extreme desiccation. Arabinans may help in maintaining the stability of the seed

cell wall, while also acting as a storage polysaccharide, after enzymatic depletion caused a delay in seed germination and growth (Gomez et al., 2009). In pea cotyledons, mechanical studies were done before and after the arrival of a (1→4)-β-D-galactan-rich layer which appears at a defined stage in the late stages of development. The results indicated that the presence of (1→4)-β-D-galactan increased the cell wall stiffness by 100%. However, it is not known if there were other factors which affected this (McCartney et al. 2000). In *Arabidopsis thaliana* seedlings, (1→4)-β-D-galactan occurs in a restricted manner and marks the transition zone near the onset of rapid cell elongation. Mutations that affect the rate of root elongation also reduced the amount of (1→4)-β-D-galactan present in the transition zone, indicating a role in modulating cell wall properties and is hypothesised to be possibly acting downstream of an AGP-derived action (McCartney et al. 2003). Using a rheological approach (the study of the flow of solid matter), it has been shown that the elastic properties of the cell walls in WT and modified potato tubers (*Solanum tuberosum*) decreased with significant reductions in cell wall galactan and arabinan. This was the result of the cell walls stiffening in a similar way to the effects seen in stomatal cells. These results promote the idea that RG-I cell wall components are directly involved in cell wall rheology and the transmission of mechanical stress (Ulvskov et al. 2005; Jones et al. 2005; Oomen et al. 2002). Interestingly, in recent work in which the galactan and arabinan side chains were truncated within *Arabidopsis thaliana*, no severe effects were observed, indicating the adaptability of plant cell walls to the loss of specific polysaccharides (Øbro, Borkhardt, et al., 2009). The adhesive role of RG-I had also been elucidated in tobacco transformants with reduced arabinan chains. The tobacco cell calli showed a distinct loss of cell attachment, which was

correlated with the loss of arabinan (Iwai et al., 2002). Very few cell wall RG-I mutants have been generated due to the essential function that this class of polysaccharides plays in cell integrity and viability. *Rhm-2* is a mutant where GalA and Rha content are reduced 50% and 65% each, resulting in a disrupted seed mucilage phenotype with reduced integrity (Western et al., 2001). *Rhm-2* encodes an enzyme that catalyses the production of UDP-Rha of RG-I biosynthesis, with increased upregulation in reproductive tissues, implying a role in plant development (Usadel et al., 2004; Oka et al., 2007). *ARAD-1* is another RG-I mutant in which there is a 46% reduction in stem and a 75% reduction in leaf arabinan. However there is no visible phenotype and no alteration of cell structure, including fully functional stomata, which was the effect seen in Jones et al. 2005 (Harholt et al. 2006; Jones et al. 2005). *ARAD-1* encodes a Golgi localised arabinosyltransferase that forms homo-and heterodimers (Harholt et al., 2012). Disruption of RG-I related arabinan side chains have also been shown to indirectly cause alterations within the cell wall. In *Cnr* (colourless, non-ripening) tomatoes, reduced levels of (1→5)-α-L-arabinan were correlated with a HG gel with reduced calcium-binding ability, resulting in reduced cell adhesion and tissues that could not swell (Orfila et al. 2002).

1.5.6.6 RG-I in maturation and fruit ripening

During fruit ripening and maturation, RG-I has been shown to play a direct role. Loss of RG-I galactan side chains is correlated with fruit ripening and softening (Gros et al. 1985). In commercial apple lines (Gala, Red Delicious, Firm Gold and Gold Rush), the loss of (1→4)-β-D-galactan happens during the cell enlargements and maturation stage. Selective loss of highly branched (1→5)-α-L-arabinans was seen after maturation, and coincided with a significant loss of fruit firmness and

compressive resistance. Unbranched (1→5)-α-L-arabinans remained attached to the RG-I polymers within the cell wall (Pena & Carpita 2004). Loss of galactan in tomato cells during ripening was also seen and correlated with a reduction in the biosynthesis of this polymer and an increase in the synthesis of β-galactosidase(Lackey et al., 1980; Pressey, 1983). Recently, highly branched arabinans were only detected in the cell walls of mature black olives, but were absent in developing olive fruits, which elucidates the potential role of specifically branched arabinans in fruit maturation and ripening (Cardoso et al. 2007). It was also hypothesised that de-branching of RG-I arabinan side chains increases the enzymatic accessibility of other cell wall components, like HG, which has been shown to be involved in cell to cell adhesion. This could then lead to the degradation of HG and loss of cell adhesion, resulting in fruit softening (Pena & Carpita 2004; Pena et al. 2004).

1.5.7 Hemicelluloses

1.5.7.1 Structure

After cellulose and pectins, hemicelluloses are a major non-cellulosic constituent of primary cell walls. In all major dicotyledon plant cell walls, the main groups of hemicelluloses are: xyloglucans, heteromannans, arabinoxylans and heteroxylans (Harris & Smith 2006). Xyloglucan (Xyg) is found in all vascular plants, and can make up to 20% of the total cell wall dry weight (O'neill & York 2003). It is a heavily substituted β1-4-glucan backbone with α-1-6-xylosyl residues, sometimes including further (1→2)-β-D-galactose and (1→2)-α-fucose side chain residues in its most substituted form (Figure 1.15).

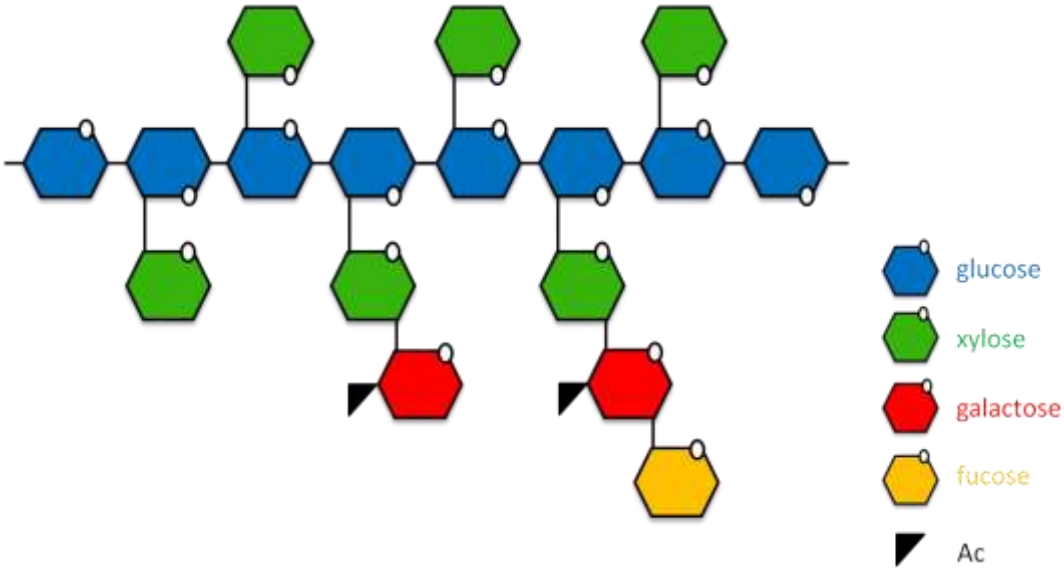

Figure 1.15: Structure and composition of xyloglucan in plant cell walls.

XLG substitution and structure however, varies between different cells, tissues and also species (Cavalier et al. 2008; Carpita & Gibeaut 1993; Reiter 2002; Pauly et al. 1999). These side chains can also affect the conformation of the overall polysaccharide and can help optimise the parallel binding of XLG chains to cellulose microfibrils through non-covalent linkages (Cosgrove, 2005; Pauly et al., 1999; Zykwinska et al., 2008). There is also recent evidence, using tobacco cells, that xyloglucan is also associated with homogalacturonan and that this pectin somewhat masks the hemicellulose in the outer primary cell wall of the fibre. The role of this xyloglucan:pectin interaction may contribute to the overall cell wall strength, but may also control hydrolysis in the primary and secondary cell walls in plants generally (Verhertbruggen et al. 2009; Marcus et al. 2008). It should be noted, however, that *Arabidopsis thaliana* mutants deficient in xyloglucan are still able to grow (Eckardt, 2008). During plant cell elongation, xyloglucan is modified so that the cellulose microfibrils can move apart by turgor pressure to allow the placement of new cell wall material (Carpita & Gibeaut 1993).

Mannans have a variable substitution of a β-1-4-mannose backbone with branching residues of heteromannans, with most found in seed endosperms to act as an energy reserve. Its role is not really known but it is clearly that the AtCslA7 (involved in the synthesis of mannan) mutation has an effect on pollen growth and embryo development. In *Arabidopsis thaliana*, other CesA mutants have less mannan in the overall plant but no visible phenotype has yet been observed (Reiter, 2002).

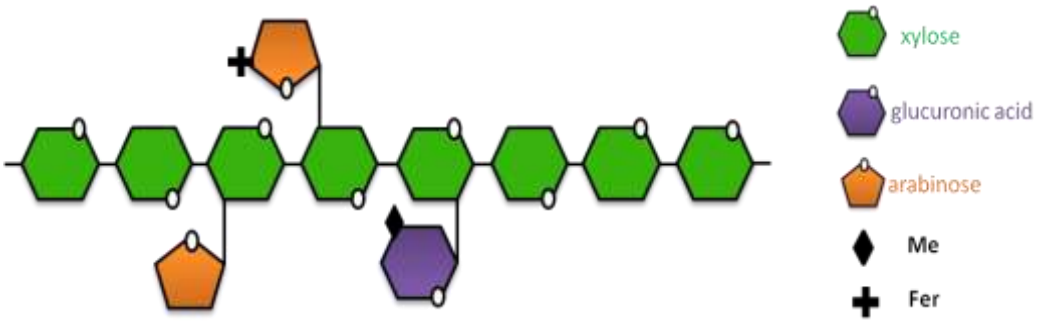

Figure 1.16: Structure and composition of (arabino)xylans in plant cell walls. Xylans are one of the most abundant hemicelluloses in nature, and are an extensive source of ferulyated (Fer) arabinose and methylated (Me) glucuronic acid.

Xylans consist of a (1→4)-β-D-xylose backbone which can often be substituted with a ferulyated (1→3)-α-L-arabinose and methylated (1→2)-α-D-glucuronic acid (Figure 1.16) (Anders et al., 2012). Xylan substitution and structure are not totally understood in relation to function. However recent evidence suggests a role for α-(1,3)-arabinosyltransferase activity in gain of function mutants in *Arabidopsis thaliana* (Anders et al. 2012; Harris & Smith 2006).

1.5.7.2 Biosynthesis

Unlike cellulose, hemicelluloses are synthesised in the *cis*-Golgi membranes before travelling through the *trans*-Golgi and eventually released into the cell wall via fusion of the vesicles with the plasma membrane (Northcote and Pickett-Heaps, 1966; Fry, 2004). Biosynthesis occurs by compartmentalisation of polysaccharide biosynthetic and modifying enzymes in separate Golgi stacks. For example, the XLG backbone is found in the *cis*-Golgi stacks, while fucose side chains were only detected in *trans*-Golgi stacks (Zhang & Staehelin 1992). GTP/UDP-monosaccharide subunits are incorporated into polysaccharides by membrane-bound glycosyltransferases, however the signals which initiate this are

still not fully understood (Caffall and Mohnen, 2009). REB-1/RHD-1 is an example of an enzyme involved in XLG biosynthesis, specifically the addition of D-Galactoses. Disruption of this enzyme results in the loss of galactosylated fucose side chains in xyloglucan (Nguema-ona et al., 2006). Another example is *AtFUT1*, which is a xyloglucan fucosyltransferase isolated from the *mur2* *Arabidopsis thaliana* mutant and recently localized to the *cis*-Golgi stacks (Vanzin et al., 2002; Chevalier et al., 2010), while *xxt1* and *xxt2* encoded xyloglucan xylosyltransferases (Reiter 2002; Cavalier et al. 2008; Park et al. 2012). However, detecting XLG glycosytransferase activity *in vitro* is difficult, indicating that they are a complex set of biosynthetic machinery (Cavalier et al., 2008).

1.5.7.3 Functions

It has long been established that xyloglucan hemicelluloses form non-covalent linkages with cellulose microfibrils to form a mechanically strong, load-bearing structure in plant cell walls. However, while several biological functions have been discovered through the use of hemicellulose-modifying enzymes, there have been contrasting results relating to the mechanical properties of XLG (Carpita & Gibeaut 1993; Ryden et al. 2004; Pauly et al. 1999; Park et al. 2012). For example, the cell walls in *Arabidopsis thaliana* xxt1 and *xxt2* mutants were found to be much more extensible than *Arabidopsis thaliana* wt cell walls, but less extensible to in the presence of α-expansins This supports the functional role of XLG as a load-bearing component (Park et al. 2012; Ryden et al. 2003). In contrast to this, work done by Chanliaud elucidates a role of XLG in cell wall elongation through the modification by XLG specific enzymes. Furthermore, the disruption of XLG in plant cell walls does not affect the general morphology of the plants, except in

the case of the xxt1 and *xxt2* mutants, which were found to have altered root hair structures. This in contrast suggests that XLG is not a crucial component on the load-bearing structure of the plant cell wall (Park et al. 2012; Nguema-ona et al. 2006; Eckardt 2008; Chanliaud et al. 2002; Chanliaud et al. 2004). In tomato pericarp parenchyma cells, xyloglucan was seen specifically at the cell adhesion planes/intercellular spaces from unripe fruit, however galactan and mannan epitopes were also present at these locations. It was concluded that these hemicelluloses, including xyloglucan, are likely to contribute to the tomato cell adhesion, as well as separation in pericarp parenchyma (Ordaz-Ortiz et al. 2009).

1.5.8 Hemicellulose modifying enzymes

1.5.8.1 Expansins

These wall-loosening proteins were first discovered in 1992 and were shown to contain a conserved sequence of ~ 250 amino acids (~ 20-25 KDa in size) with a similar binding domain to that of carbohydrate-binding modules. They can be classified according to their protein structure as α, β, γ or δ expansins (McQueen-Mason et al. 1992; Brummell & Harpster 2001; Sampedro et al. 2005). Expansins have been shown to be involved in many developmental stages, including cell expansion, root elongation, fruit ripening and softening, as well as plant defence (Pien et al. 2001; Cosgrove et al. 2002; Baumberger et al. 2003; Gao et al. 2007). δ expansins are the least understood of the four types, while recent evidence has shown that γ expansins are involved with controlling the osmotic balance of the plant cell (Li et al. 2003). α and β expansins are the two largest groups of proteins and have been implicated in cell wall extension and also acid growth by acting as a catalyst for the breaking of hydrogen bonds between cellulose and hemicellulose

to cause slippage and cell-loosening (Figure 1.17) (Sampedro et al. 2005; Sánchez et al. 2004; McQueen-Mason et al. 1995).

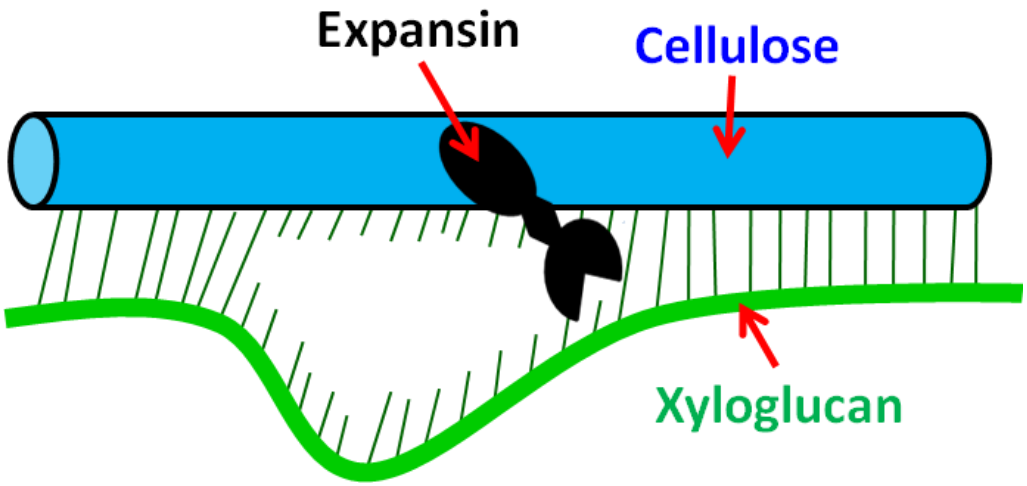

Figure 1.17: Model of expansin action on cellulose-xyloglucan networks in plant cell walls. Adapted from Cosgrove et al. 2002.

1.5.8.2 Xyloglucan endotransglycosylases/hydrolases

XTHs are another class of cell wall-loosening enzymes consisting of approximately ~300 amino acids (30-35 KDa) and are found in all land-based plants, indicating that XTHs play a fundamental role in cell wall biosynthesis and morphogenesis (Vissenberg et al., 2003). They were first discovered in 1992 by two independent research groups (Nishitani & Tominaga 1992; Fry et al. 1992). These enzymes have several functions by cleaving the internal linkages of the Xyg backbone and attaching them to the C4 end of a new Xyg chain, as well as acting as an endo-(1→4)-β-D-glucanase (Sulova et al., 1998; Brummell and Harpster, 2001). This could allow the incorporation of new cell wall material, such as newly synthesised Xyg, so that while the cell wall is expanding it keeps the same thickness (Van Sandt et al. 2007). By breaking the internal linkages of

the XLG backbone XTHs could affect the cellulose-hemicellulose interactions and cause cell wall-loosening following slippage of the microfibrils (Rose et al., 2002). Recent evidence also further validates this hypothesis, such as the localisation of XTHs is always in the proximity of cellulose microfibrils attached to XLG (Vissenberg et al. 2005; Kaku et al. 2004). XTH activity has also been shown to be increased in fruit ripening and cell responses to external mechanical stresses (Redgwell and Fry, 1993; Vissenberg et al., 2000, 2005; Lee et al., 2005; Miedes and Lorences, 2009).

1.6 Cell wall glycoproteins

Proteins make up approximately 10% of most dicotyledon cell walls. They can be heavily glycosylated, especially on the hydroxyproline and proline amino acids (Liepman et al., 2010). The exact roles of many cell wall proteins are still not fully understood. However they are classified based on the conserved amino acid sequences and are further classified as either structural or non-structural proteins (Liepman et al., 2010).

1.6.1 Extensins

Extensins are rod-like, structural glycoproteins associated with cell wall extensibility and rigidity, and with interactions between the plasma membrane and intracellular spaces. They fall into the main groups of hydroxyl proline-rich proteins (HPRPs) and proline-rich proteins (PRPs) and are rich in ser-$(hyp)_4$. They usually have one hydrophilic and once hydrophobic repetitive peptide motif with the potential for crosslinking (Smallwood et al., 1994). Extensins are also rich in tyr residues, which have been hypothesised to form isodityrosine crosslinks, that are thought to play a role in anchoring the proteins to the cell wall and prevent solubilisation (Keller, 1993). Extensin gene expression is also seen to be upregulated during pathogen infection, indicating a potential role in the defence/hypersensitivity mechanism (Keller, 1993).

1.6.2 Arabinogalactan proteins (AGPs)

Arabinogalactan proteins (AGPs) are a complex and diverse class of plant cell wall surface structural glycoproteins that are involved in a range of fundamental processes associated with the development of the plant cell, including: cell division, cell recognition, cell differentiation, growth and cell expansion. The

precise mode of action in these processes, however, is still not known (Yates et al. 1996; Majewska-sawka & Nothnagel 2000; Willats & Knox 1996; Seifert & Roberts 2007; Thude & Classen 2005). AGPs consist of a minor core-protein that makes up around 10% of the overall structure which is often *o*-glycosylated by complex AG-II polysaccharide chains. These carbohydrate chains make up the remaining 90% of the AGP structure. The protein core consists of alanine, proline, serine and hydroxyproline. The latter two are the sites of the previously mentioned *0*-glycosylation (Thude & Classen 2005; Seifert & Roberts 2007). AGPs are thought to play a role by marking specific cells that are targets for programmed cell death, based on the data that AGP expression is significantly upregulated during xylem tracheary element formation (Majewska-sawka and Nothnagel, 2000). AGP populations in developing cotton fibres have been shown to peak during the secondary wall synthesis compared to the elongation stage. In other systems, through the use of Yariv reagent, AGP precipitation caused the interference and inhibition of cell wall elongation, cellulose deposition and eventually cell death (Gao & Showalter, 1999; Timpa & Triplett, 1993; Vissenberg et al. 2001). As a result, this precipitation of AGPs and the subsequent release of arabinogalactan-II polymers have been predicted to cause the induction of PCD in cotton fibres after the secondary cell wall has formed. It is also predicted that AGPs have a role in helping elongation as they are then released from the membranes as small signal molecules to initiate the secondary cell wall and maturation stages (Showalter, 2001).

1.7 Water and apoplastic solutes

Plant cell walls contain approximately 60% water with primary walls being composed of up to 90% water, which acts as the main solvent for plant hormones,

signalling compounds, ions and small molecular weight organic polymers (O'neill and York, 2003). Water also plays a role in changing the mobility and conformation of some cell wall components, for example interfering with the formation of hydrogen bonds found in the wall (Cosgrove, 2005; Ha et al., 2005). Water and apoplastic solutes have also been shown to have an effect on cell elongation, with solutes also being shown to be found in an increased concentration during these growth stages (Meinzer and Moore, 1988; James et al., 2006).

1.8 The cotton fibre cell wall

The primary wall of the cotton fibre is very similar to that of many other dicots, and the fibres grow by diffuse and tip growth through the elongation stage (Meinert & Delmer 1977; Hayashi & Delmer 1988). The inner and outer layer of the fibre primary cell walls are different. The outer surface is mostly made up of pectin, specifically HG, which masks the majority of epitopes of the underlying cellulose and hemicelluloses (Vaughn & Turley 1999) (Figure 1.18). More recently, however, there was the discovery of an adhesive outer layer of the primary cell wall, called the cotton fibre middle lamella (CFML), which exists only during initiation and fibre elongation stages. This CFML holds many developing fibres together in tissue-like bundles, coordinating them as a group while they elongate (Singh et al., 2009). Further evidence has shown that towards the end of elongation, and around the time of the disappearance of the CFML, there was an upregulation of gene expression for genes related to cell wall hydrolysis. This indicates that this outer layer is broken down enzymatically by hydrolysis. Future work on the CFML should aim to uncover the regulation behind its synthesis and spatial localisation on the fibre. There is also a need to understand how this affects the final properties of the cotton and whether modification of this outer layer can produce beneficial changes in the fibre traits (Singh et al., 2009).

The secondary cell walls of cotton fibres are found deposited on the inside of the primary wall, of which the majority is composed of β1-4-glucosyl subunits which are synthesised into long chains of cellulose. These in turn aggregate to form cellulose microfibrils. The microfibrils are deposited in an ordered fashion within the secondary wall, as opposed to being randomly deposited in the primary cell

wall of the cotton fibre (Timpa & Triplett 1993). There is substantial evidence that the cellulose microfibrils in this secondary wall are synthesised and deposited at night due to the wall precursors being extracted in concentrations that follow diurnal variations (Murray et al. 2001). During elongation, glucose and fucose are stored, from where they are later used for the synthesis of secondary wall cellulose (Jaquet et al., 1982). The secondary cell wall is responsible for fibre dyebility. Immature fibres from unfertilized or aborted ovules dye poorly. These "motes" have normal primary cell wall but thin or deformed secondary walls which absorb dyes poorly, resulting in textile defects and decreasing its value (Smith, 1991; Weis et al., 1999).

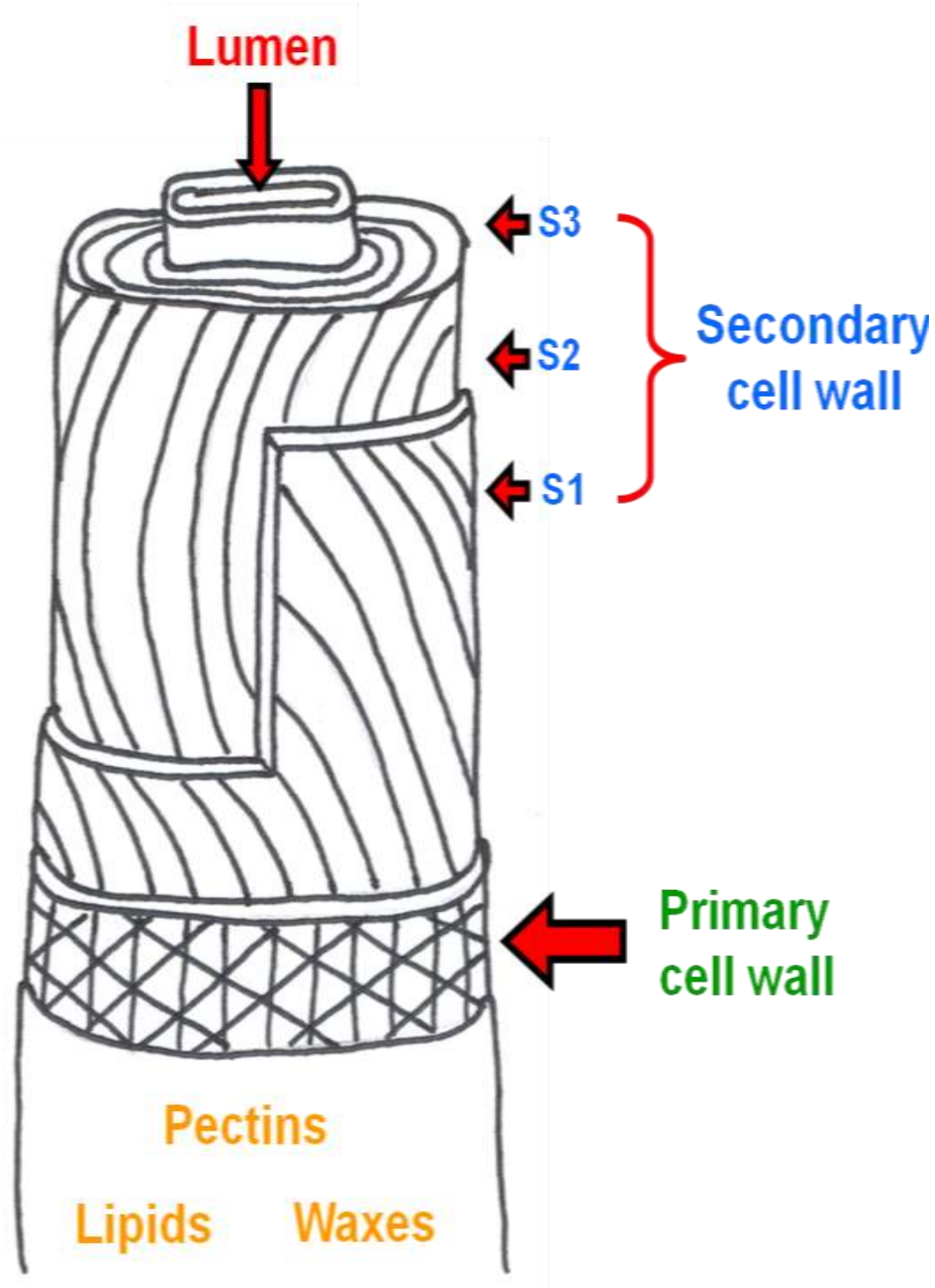

Figure 1.18: The mature cotton fibre structure. The outer cuticle layer consists of pectins, lipids and waxes, followed by a thin primary wall and a thick secondary cell wall. The secondary wall is composed of 3 layers; S1, S2 and S3, in which the cellulose orientation differs. Adapted from (Warwicker *et al*. Shirley Insitute pamphlet No93, 1996).

1.8.1 Cellulose

The mature fibre is mostly comprised of highly crystalline cellulose with some varying islands of semi-crystallinity (Heyn 1966). Highly crystalline microfibrils naturally occur in two forms, known as Iα and Iβ. The cellulose microfibrils in the primary wall of the fibre contain over ninety percent Iβ, and they are arranged in a two-fold screw axis orientation utilising glucosyl residues that are identical. Cellulose in the primary wall is made up of cellulose IV due to the disorder, while the secondary cell wall is mostly made up of type I cellulose (Haigler et al. 2009). This recent work was done using neutron crystallography. The orientation of cellulose microfibrils ultimately establishes the fibre shape, whilst non cellulosic polysaccharides in the matrix determine the rate of elongation during the second fibre stage (Nishiyama *et al.* 2008).

1.8.2 Callose

Callose or (1→3)-β-glucans are mostly found as a soluble polysaccharide on the innermost surface of the cotton fibre cell wall, with around 29% percent being in an insoluble form although this can be removed by treating it with (1→3)-β-glucanase. Callose is also associated with cell plates, plasmodesmata, pollen tubes and responses to tissue damage in higher plants, including cotton. The deposition of callose has been shown to occur at the same time as the onset of cellulose synthesis for the secondary wall formation (18 dpa). Previously it was thought that callose was the precursor to cellulose in the cotton fibre's development, however using radioactive incorporation studies (Maltby et al., 1979), the evidence produced showed that callose and cellulose are not synthesised from the same pool of substrates, nor that callose is the precursor of cellulose (Timpa & Triplett 1993). During secondary wall synthesis sucrose

synthase is specifically targeted to the cell wall of the cotton fibre to synthesise callose (Brill et al., 2011). Pulse chase experiments also rule out callose as a storage polysaccharide. This means that the high rate of callose synthesis during secondary wall deposition cannot be entirely due to any cotton fibre damage responses. In other systems callose is deposited after cell wounding and also at the plasmodesmata. Callose is synthesised in the cotton fibre during elongation and secondary wall synthesis, downstream of sucrose synthase which requires an influx of Ca^{2+}(Andrawis et al., 1993; Amor et al., 1995). Callose deposition occurs during the period of peak fibre elongation in which the plasmodesmata are closed between 10-16 dpa. This closure is attributed to callose deposition at the plasmodesmata. After 16 dpa, callose is degraded and later replaced at the onset of secondary wall synthesis, at which point cellulose synthesis occurs (Brill et al. 2011; Ruan & Chourey 1998; Ruan 2007; Amor et al. 1995; Salnikov et al. 2003). Between secondary wall synthesis and maturation, a thin callose layer at the cell wall-lumen interface is seen, making up approximately 0.1-0.3% of the final cell wall composition (Maltby et al., 1979; Waterkeyn, 1981; Salnikov et al., 2003). In cotton fibres, GhSusA1 encodes a putative sucrose synthase that is conserved among all land plant species. So far three types of sucrose synthase have been found in cotton (Jiang et al. 2011; Ruan 2005). When GhSusA1 was over-expressed in developing cotton fibres it resulted in an increase in fibre length and strength. Conversely, GhSusA1 antisense knockouts resulted in shorter, weaker fibres and smaller fruit bolls. It was postulated that this was due to a higher osmotic potential in the developing fibre causing stronger turgor driving forces and therefore more elongation. At the secondary wall stage the excess substrate generated by the GhSusA1 would keep cellulose deposition machinery saturated

(Ruan 2007; Y. Ruan & Chourey 1998). GhSusA1 would be an excellent candidate for biotechnological improvements of cotton fibres for their overall yield as well as their strength and length (Jiang et al., 2011). Thus, the role of (1→3)-β-glucan in fibre development is that it may determine the plasticity of the primary cell wall during the latter stages of elongation, as well as during that onset of secondary wall formation (Maltby et al. 1979; Timpa 1991).

1.8.3 Non-cellulosic polysaccharides

The presence of a variety of neutral sugars, other than glucose (Rha, Fuc, Ara, Xyl, Man and Gal), confirms the existence of non-cellulosic polysaccharides in the fibre cell walls. Non-cellulosic polysaccharides generally give the cell walls resistance to the stress caused by longitudinal forces by holding cellulose microfibrils together. Of the primary interlocking, non-cellulosic polysaccharides, hemicelluloses make up the majority. In the past it has been difficult to characterise the cell wall polymers in regard to individual molecular weights (Mr). However after studying development of the fibres it was shown that high molecular weight polymers decrease in quantity during 10-18 dpa, while small molecular weight polymers increase during that same time. This indicates a turnover by hydrolysis during the latter stages of elongation (Timpa & Triplett 1993).

1.8.4 Pectins

The presence of pectins in cotton has long been observed through biochemical and cytochemical experiments and they are a large family of complex and diverse polysaccharides (Meinert & Delmer 1977). They are a major component of the primary cell wall of the cotton fibre, making up about one-third of the wall and it is generally accepted. In other systems pectins have been shown to play a role in cell extensibility and expansion in ripening fruit and it would be plausible that a similar sort of regulation would control fibre elongation. Most pectin in the cotton fibre is actively turned over during development. Vaughn & Turley (1999) found that the primary cell wall of the fibre is made up of two distinct layers. Using electron microscopy and monoclonal antibody labelling it was found that the outer layer was significantly more pectin-rich and electron opaque than the inner layer of the primary wall (Vaughn & Turley 1999). This bi-layered wall is also seen in elongating cotton leaf trichomes from the *Gossypium* tribe (with a pectic-rich outer layer). However these cells and cotton fibres are the only cells that elongate with a pectic-rich outer layer. Elongating epidermal cells of pea stems, flax stems and maize coleoptiles also have this bi-layered wall, but the presence of an outer pectic-rich layer is negatively correlated with elongation (Vaughn & Turley 1999). This work also confirmed the presence of a very thin waxy cuticle layer on the very outside of this bi-layer. Further studies of the waxy cuticle revealed it was comprised mainly of 1-triacontanol, montanol, beta-sistanol and some unknown high molecular weight esters (Liu et al. 2008; Ma et al. 1995). The waxy layer acts as a hydrophobic barrier preventing the fibre from external wetting as well as contributing to protection against desiccation. Using monoclonal antibodies specific to HG (Verhertbruggen et al. 2009), HG pectin was localised to the outer

layer of the primary cell wall only and is largely de-esterified. This is also the case for developing cotton leaf trichome (Vaughn & Turley 1999). Fibre elongation is largely implemented by cell wall loosening coupled with turgor driving forces. In the primary cell wall HG pectin represents approximately 25% of the composition, which decreases with development towards secondary cell wall synthesis where it is largely absent (Weis et al. 1999; Vaughn & Turley 1999; Willats et al. 2001). GhPEL encodes a pectate lyase enzyme in developing cotton fibres which catalyses the degradation and depolymerisation of de-esterified pectin, specifically blocks of polygalacturonic acid (PGA) residues (Wang et al. 2010). Peak expression of GhPEL in cotton fibres occurs at 10 dpa, mid-way through the elongation stage. It is predicted that the GhPEL causes a loosening of the pectin rich outer sheath that allows an efficient and rapid elongation of the fibre (Vaughn & Turley 1999; Wang et al. 2010). Anti-sense knockouts of GhPEL in cotton plants cause a reduction in the degradation of de-esterified pectin in the fibre primary cell walls. This correlates with a decrease in cell wall loosening and remodelling which ultimately results in fibres that are much shorter than WT lines. A similar effect was seen in *Fragaria x ananassa* (Garden strawberry), in which a GhPEL homolog was knocked out using anti-sense technology resulting in a firmer, less extensible cell walls and reduced fruit softening post-harvesting (Wang et al. 2010; Jimenez-Bermudez et al. 2002). As a result, this GhPEL could be an interesting candidate for crop improvements in cotton biotechnology. If GhPEL knockouts cause shorter fibres then over-expression could cause the cell walls in elongating cotton fibres to become more extensible and less resistant to turgor driving forces, potentially resulting in longer fibres due to a higher rate of elongation (Wang et al. 2010; Jimenez-Bermudez et al. 2002).

Recently, CGR-3 (cotton Golgi-related-3) was shown to be a Golgi localized *S*-adenosylmethionine methyltransferase, and it is directly involved in controlling the levels of methyl-esterification of HG in cotton fibres (Held et al., 2011).

RG-II distribution has been strongly detected along the plasmalemma wall interface in the elongating cotton fibre (Vaughn & Turley 1999).

1.8.5 Hemicelluloses

Xyloglucan is the primary connective linkage between cellulose microfibrils via hydrogen bonds in the primary cell wall, tethering them in place and resisting cell tension forces, such as turgor pressure, during fibre elongation (Huwyler et al., 1979). Together, hemicellulose and cellulose form a matrix which is responsible for the overall strength, as well as the extensibility of the cell wall (Gokani & Thaker 2000). Furthermore, xyloglucan has a very high turnover and deposition rate during elongation (Huwyler et al., 1979). Xyloglucan deposition is restricted to the elongation phase of fibre development with 5% in a soluble form. Purified xyloglucan from the cotton fibre is homogenous with a molecular weight of approximately 80,000 and is identical to purified xyloglucans from *Rosa glauca* and *Pisum sativum* (Hayashi & Delmer 1988). These purified xyloglucans are comprised of at least 4 different oligosaccharide subunits which were found to be containing fucose and galactose (Joseleau & Chambat 1984). Xyloglucan is located on the inner layer of the primary cell wall of the cotton fibre and is associated strongly with cellulose microfibrils (Vaughn & Turley 1999). A similar effect was observed in recent evidence by Marcus (2008) using monoclonal antibodies for xyloglucan. It was shown that this polysaccharide is associated with HG to a degree that the pectin masks it, preventing any immunolabelling. Enzymatic treatment with pectate lyase removes the pectic HG and unmasks the

abundant xyloglucan. It should be noted, however, that this was carried out on tobacco plants (Marcus et al. 2008).

The xyloglucan hemicellulose network is constantly changing. One way involves xyloglucan endotransglycosylases/hydrolases which have a dual functionality of endotransglycosylation and hydrolysis. Several XTH genes from cotton species (GhXTH1-1, GhXTH1-2, GaXTH1 and GhXTH) have recently been shown to be directly involved with fibre growth and have shown to be significantly upregulated in the fibre elongation stage (3-24 dpa) but absent from other tissues (Kabel et al., 2007; Cavalier et al., 2008; Michailidis et al., 2009). It is thought that these levels of expression regulate the plasticity of the cotton fibre wall expansion during this developmental stage. The maximal rate of expression of XTH genes takes place during 6-12 dpa, which coincides with the maximal rate of fibre elongation. It then decreases sharply at the onset of secondary cell wall formation (Meinert & Delmer 1977; Timpa & Triplett 1993). In long fibre lines, XTH was significantly upregulated during elongation compared to short fibre lines, implicating XTH as a positive regulator of cotton fibre density. Further evidence predicts that XTH could potentially be manipulated in low yield fibre lines to improve the overall properties and increase the overall length (Michailidis et al., 2009). (Glucuronoarabino)-xylans and mixed linked β-glucans, however, so far have not been found in any cotton fibres (Lerouxel et al. 2006).

1.8.6 Glycoproteins

During development, the cell wall undergoes dramatic changes in size and structure. As a result it is highly metabolically active comprising of around 12% protein of which 50% of this are hydrophobic as indicated by amino acid composition studies (Gokani & Thaker 2000). During elongation the genes for

proline rich proteins, AGPs and expansins are upregulated. By the time the cotton is harvested the percentage of proteins present goes down to one percent (Huwyler et al. 1979).

Recently fasciclin-like AGPs, rich in Ala, Ser, Thr, Pro and Hyp, were found in developing cotton fibres (GhAGP2, GhAGP3, GhAGP4 and GhFLA1). Fasciclin-like proteins are found in animal cells and are thought to act as adhesion proteins (Liu et al., 2008). It is thought that in developing cotton fibres they have a suggested role in cell-cell communication during fibre elongation and the transition stage to secondary cell wall synthesis. Peak expression of GhFLA1 and GhAGP2 was seen during early elongation, implicating them in the rapid elongation of fibres. GhAGP3 and GhAGP4 were upregulated at the transition stage at the end of elongation before the secondary cell wall synthesis stage occurred, implicating them in the signal transduction leading to this cellulose deposition stage (Liu et al., 2008). Further to this, it was found that RNAi suppression of GhAGP4 caused the inhibition of fibre initiation and elongation. In addition to this, it also had a knock-on effect of causing a down regulation of GhAGP2, GhAGP3 and GhFLA1. As a result, GhAGP4 was postulated to be involved in regulating cellulose and F-actin deposition (Liu et al., 2008; Li et al., 2010).

1.9 Structural development linked to fibre properties

1.9.1 Pectins

With regards to mechanical properties, when all pectin was removed from the fibre with strong acid, there was no change in tensile strength observed. However, when there was only partial removal of pectin with weaker acid a decrease in tensile strength and fibre viscosity was observed. From these results, in 1940, it was believed that a pectin:cellulose complex did not exist, whilst Gokani *et al.* (2000) states that pectin *does* bind to cellulose (Gokani & Thaker 2000). This does, however, open up the possibility of trying to manipulate pectic polysaccharides *in-planta* which would, in theory, have a major impact on the plant cell wall (Whistler et al. 1940; Willats et al. 2006).

1.9.2 Cellulose

Cellulose, determines several key properties of fibre for industrial usage. Future cotton fibre biotechnology may produce fibre with special properties for specific high-value traits. However, before this is possible a complete understanding of all the mechanisms controlling fibre cell wall differentiation and development are needed (Haigler et al. 2009). Cotton cellulose determines the thickness of the fibre relative to the fibre diameter. Upon harvesting, fibres need to be neither overly mature nor too immature as the hollow lumen space needs to be big enough to collapse during desiccation which results in the essential fibre twisting (convolutions) (Figure 1.19). Immature fibres will not be strong enough or dye properly, while overly mature fibres with much thicker cell walls will remain cylindrical (Hutchinson et al. 1945). Most modern-day fibres have between 4 and 7 convolutions per mm, with the higher number of twists positively correlated

with increasing fibre strength, and this has been one of the main factors for human domestication of cotton for textile production in the new world (Hsieh et al. 1996). Secondary wall thickness also positively correlates with strength, dyeing ability and water absorption, which was shown with transgenic lines of cotton fibres over-expressing a membrane-bound spinach sucrose synthase (Haigler et al. 2007).

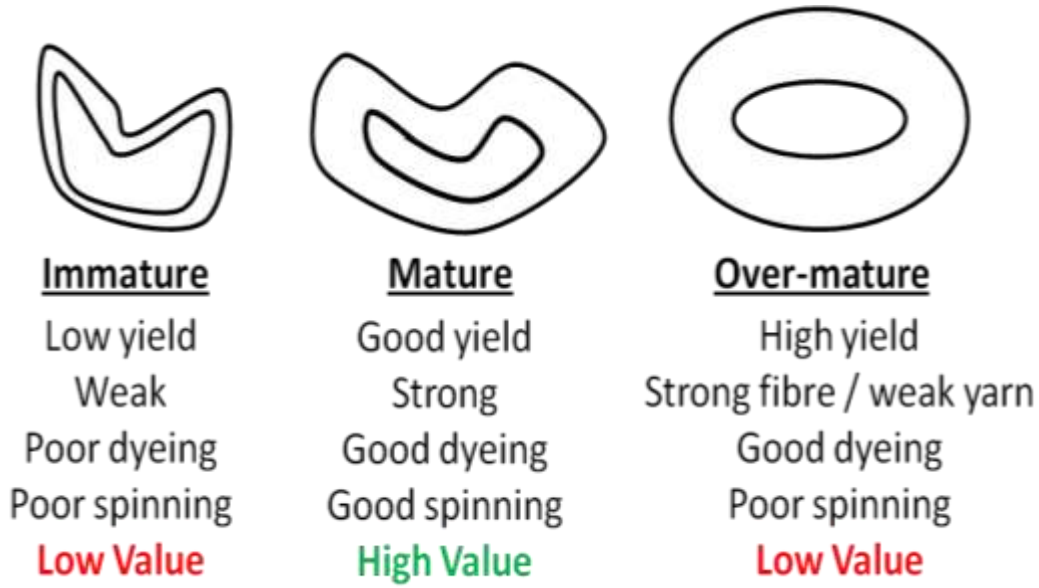

Figure 1.19: Fibre quality and value in relation to levels of secondary wall synthesis. Adapted from Haigler et al. 2005.

The molecular properties of cellulose also affect the quality and characteristics of most modern cotton fibre lines and these include percentage crystallinity, crystallite size, cellulose orientation and the degree of polymerisation of the cellulose (Haigler et al. 2009). Percentage crystallinity is the percentage of crystalline/ordered cellulose against disordered states and there have been conflicting results in the correlation of fibre properties. It has been positively correlated with individual and bundle fibre strength (Hindeleh et al. 1980; Hsieh

et al. 1996), but also negatively correlated in other reports (Timpa & Ramey, 1994). These relationships could be explained by differences in variety and conditions, which make experimental comparisons especially difficult. Cellulose crystallite size is determined by the self assembly of cellulose microfibrils which is more likely to happen in cellulose I than IV due to the more ordered structure. Hence the secondary cell wall has a larger crystallite size than the primary wall (Hu & Hsieh, 1996; Ryser et al. 1979). Cellulose crystallite size is positively correlated with the fibre-breaking force. However, there is a negative correlation for dyeing capability and water absorption, which is caused by the highly crystalline microfibril cores in which liquids cannot easily penetrate (Haigler et al. 2009). The degrees of glucose polymerisation (DP) in the primary walls (<4,000) are lower than secondary fibre walls (<11,000) (Timpa & Ramey, 1994) and are correlated with fibre bundle strength. Finally, more highly-orientated microfibrils (i.e. smaller angles relative to the longitudinal axis) are correlated with strength and lower extensibility (stretching). During development, the orientation changes depending on the stage: primary wall (70-90°), transition (45-55°) and the secondary wall synthesis (20°). However the exact mechanisms by which these changes occur is still yet to be uncovered or elucidated (Haigler et al. 2009).

1.10 Probes for plant cell walls

Despite the fact that most plant cell wall polysaccharides have been well characterised, it is still not properly understood how these polymers dynamically interact with each other and what biological functions they confer to the cell wall biology (Cosgrove 2005; Caffall & Mohnen 2009; Taylor 2008; Wolf & Greiner 2012). An important methodology to study these polymers and their interactions is by the *in situ* microscopy detection of monoclonal antibodies (MAbs) and carbohydrate-binding modules (CBMs) of plant cell walls. For MAbs, there is currently a large selection of rodent derived probes that have increased over the past few years. They do not, however, cover all the structural aspects of plant cell walls, such as RG-II. CBMs are nature's cell wall binders and are derived from the non-catalytic subunits of cell wall degrading enzymes. There is a smaller selection of CBMs, which can bind to cellulose of vary crystallinities and the hemicelluloses (Blake et al. 2006; McCartney et al. 2006; Boraston et al. 2002).

1.11 Conclusion

All plant cells are surrounded by a cell wall, and these organelles have a major impact on our daily life with their extensive use in food, cosmetics, textiles, paper, timber-based industries and as an increasing source of new pharmaceutical products. The structural complexity of the cell wall and the heterogeneity of its regions make the study of its function difficult. Our understanding of spatial and temporal regulation of pectin and hemicelluloses as well as cell specific expression of glycoproteins has been helped greatly through the generation of a large range of monoclonal antibody probes and carbohydrate-binding modules to polysaccharide epitopes present in plant cell walls (Knox, 2008; Smallwood M., Yates, Willats, Martin, & Knox, 1996; Willats et al., 2000). The use of these probes has allowed the investigation into cell wall polysaccharide domains in relation to plant growth, development and function. Used in conjunction with genetic studies, they will ultimately provide very useful information into understanding cell walls. The key question in cell wall and fibre research is what the exact functions are for specific polysaccharide structures within the wall, including what mechanical properties they bestow. This is especially the case for cotton fibre cell wall research. The mechanical and industrial properties of many cotton fibre species have been comprehensively characterised, which is why *G. hirsutum* and *G. barbadense* make up 97% of all harvested cotton in the world each year (Chen et al., 2007). The discovery of the CFML in developing fibres has shown that they elongate in adhesive bundles between 3-18 dpa, potentially explaining how hundreds of thousands of fibres can achieve their great length in a very confined boll space (Singh et al., 2009). This discovery opens up exciting opportunities to uncover the mechanistic basis of cotton fibre properties and

quality, such as length by modifying CFML-related pathways. Engineering a delay of CFML degradation could cause the elongation stage to be drawn out to produce longer fibres, as seen in the valuable long fibre species *G. barbadense* (Singh et al., 2009). Compounding this obstacle in cotton fibre cell wall research is the lack of a fully sequenced genome resource. The current sequencing of the *G. raimondii* genome will act as a fundamental base for the sequencing of the main AD genome species of *G. hirsutum* and *G. barbadense* , which should be completed within the next few years(Chen et al., 2007). The release of these data will not only shed light on cotton fibre cell wall development, but may also help to gain a better understanding of plant cell wall structure and function in other elongating cell types. Before this can be done, a comprehensive analysis of the structural composition of the fibre cell wall is needed throughout development. Our better understanding of the fibre cell wall, as well as cell walls in general, will allow for the manipulation of these key polysaccharides during development to improve the final properties.

The cotton industry may be an important global business, but, for example, by 2025 the world population will be over 8 billon people needing to be fed with crops grown on arable land which, at present, is used to grow non-food crops such as cotton. This will lead to more demands on limited arable land to feed or house this expanding population and ways must be found to produce these cotton crops in a more efficient, greener and economical way. The research into the improvement and development of the cotton plant as well as gaining more fundamental understanding of cell walls in general must therefore be continued to meet these increasing demands.

1.12 Outline of the thesis

For such an important cell wall, the localisation of compounds along the fibre and the functional roles of these compounds during fibre development are still not fully understood. Although much work has already been done on the cotton fibre transcriptome, a study of cell wall composition during development and maturation is crucial in linking the transcriptome data and to understanding fibre differentiation (Wilkins & Arpat 2005). The aim of this research is to explore the developmental biology of the cotton fibre in relation to key polysaccharide structures and architectural properties in the context of cell wall development. This research also investigates at the relationship of these compounds to its properties when it is harvested and processed as a textile fibre. Despite the cotton fibre being up to 97% cellulose (Haigler et al. 1999). This has been achieved by the development of methodologies for the detection and imaging of low level polysaccharide glycan epitopes at the fibre surfaces and interiors. The aim of the research is to provide a clear understanding of the role and function of key polysaccharides in relation to cell wall development. It has been achieved so far using highly specific molecular probes known as monoclonal antibodies (mABs) and carbohydrate-binding modules (CBMs) which are directed to these polysaccharide glycan epitopes.

The objectives were:

- Characterisation of polysaccharide epitopes of the mature cotton fibre cell surface and transverse sections using antibodies and carbohydrate-binding modules with specificity to a variety of cell wall components.
- Characterisation of the developmental polysaccharide epitopes and crystallinity changes during the four growth stages: initiation, elongation, secondary cell wall synthesis and maturation.
- Observation of the changes in cellulose crystallinity in the fibre cell wall after undergoing industrial pre-treatments of increasing alkali concentrations using fourier transform infrared spectroscopy (ATR-FTIR) and carbohydrate-binding modules. Furthermore, using these techniques to investigate similar approaches in reconstituted cellulose fibres.

Chapter 2

Materials and Methods

2.1 Plant materials

Cotton fibre lines, presented in Table 2.1, were supplied by Bayer BioScience NV, Ghent, Belgium. Plants were grown in 5 L pots containing Perlite soil mixture, in a greenhouse with 16 h days and maintained between 25° C and 30° C. Flowers were tagged as soon as they appeared, and harvested at the relevant days post anthesis. Plain woven and scoured lyocell fibres (Tensel, 140 g m^{-2}) were kindly supplied by Lenzig, AG, Austria.

Table 2.1: Different fibre lines and species used in this research.

Number	**Name**	**Species**
1	JFW10	*G. arboreum*
2	Obtusifolium hirsutum	*G. arboreum*
3	Russia #393	*G. herbaceum*
4	Tanguis LMW1736-60	*G. barbadense*
5	Red Tashent	*G. barbadense*
6	Pima S7	*G. barbadense*
7	Acala GC-362	*G. hirsutum*
8	FM966 FiberMax	*G. hirsutum*
9	Acala SJ1	*G. hirsutum*
10	AK-Djura Green Lint	*G. hirsutum*

2.2 Monoclonal antibodies

Monoclonal antibodies directed to cell wall polymers that were used in this project are presented in table 2.2 below.

Table 2.2: Monoclonal antibodies to plant cell walls

Anti-mannan	**Epitope**	**Reference**
LM21	Galacto-gluco-mannan	(Marcus et al. 2010)
LM22	Galacto-gluco-mannan	(Marcus et al. 2010)
Anti-xyloglucan		
LM15	XXXG motif of xyloglucan	(Marcus et al. 2008)
LM24		
LM25		
CCRC-M1	Xyloglucan, RG-I	(Puhlmann et al., 1994)
Anti-xylan		
LM10	(1→4)-β-D-xylan	(McCartney et al., 2005)
LM11	(1→4)-β-D-xylans / arabinoxylans	(McCartney et al., 2005)
Anti-pectin		
Anti-HG		
LM18	Partially Me-HG / no ester	(Verhertbruggen, et al. 2009)
LM19	Partially Me-HG / no ester	
LM20	Partially Me-HG	
LM7	Partially Me-HG / non-blockwise	(Willats et al. 2001; Clausen et al. 2003)
LM8	Xylogalacturonan	(Willats et al. 2004)(Clausen et al., 2003)
JIM5	Partially Me-HG / no ester	(Clausen et al., 2003)(Manfield et al., 2004)(Willats et al. 2000)
JIM7	Partially Me-HG	(Clausen et al., 2003)(Manfield et al., 2004)(Willats et al. 2000)
PAM1	Blockwise de-esterified HG	(Manfield et al. 2005; Willats et al. 2001)
Anti-RG-I		
LM5	(1→4)-β-D-galactan	(Jones et al. 1997; Willats et al. 1999)
LM6	(1→5)-α-L-arabinan	(Willats et al. 1998)(Jones et al., 1997)(Willats et al. 1999)
LM9	Feruloylated (1→4)-β-D-galactan	(Clausen et al., 2004)
LM13	Linearised (1→5)-α-L-arabinan	(Moller et al. 2008; Verhertbruggen et al.

		2009)
LM16	Processed arabinan	(Verhertbruggen et al. 2009)
LM17	(1→5)-α-L-arabinan	(Verhertbruggen et al. 2009)
INRA-RU1	Unbranched RG-I	(Ralet et al. 2010)
INRA-RU2	Unbranched RG-I	(Ralet et al. 2010)
Anti-AGP		
LM14	AGP glycan	(Moller et al., 2008)
LM2	β-linked-GlcA in AGP glycan	(Yates et al. 1996; Smallwood et al. 1996)
JIM4	AGP glycan	(Yates et al. 1996)
JIM13-16	AGP glycan	(Yates et al. 1996)
MAC207	AGP glycan	(Pennell et al. 1989; Yates et al. 1996)
Anti-extensin		
LM1	Extensin	(Smallwood et al., 1995)
JIM11	Extensin	(Smallwood et al., 1994)
JIM12	Extensin	(Smallwood et al., 1994)
JIM19	Extensin, ABA action	(Smallwood et al., 1994, 1995)
JIM20	Extensin	(Smallwood et al., 1994, 1995)
Other		
BG1	(1→3)(1→4)-β-glucan	(Meikle et al., 1994)
LAMP2H12H7	(1→3)-β-glucan	(Meikle et al., 1991)

2.3 Carbohydrate-binding modules

Carbohydrate-binding modulesdirected to cell wall polymers that were used in this project are presented in table 2.2 below.

Table 2.3: Carbohydrate-binding modules used in this research

CBM	Epitope	Reference
CBM3a	Crystalline cellulose	(Blake et al., 2006)
CBM2a	Crystalline cellulose	(Bolam et al. 1998; Boraston et al. 2004)
CBM10	Crystalline cellulose	(Raghothama et al., 2000)
CBM4-1	Amorphous cellulose	(Tomme et al., 1996)
CBM17	Amorphous cellulose	(Boraston et al. 2000)
CBM28	Amorphous cellulose	(Boraston et al. 2002)

2.4 Dewaxing of cotton fibres

Fibres were carded with hand cards and then were rolled into a thin flat structure to reduce the capture of air. They were then put into glass jars. The samples were added to technical ethanol (1 g of cotton was used for every 75 ml of solvent, liquor ratio 75:1) and vacuum infiltrated using a strong vacuum pump for 90 min. The solvent was removed and ethanol added again for 90 min. This was followed by 60 min with acetone, then 60 min with ether and allowed to air dry overnight. All steps were carried out at room temperature and under a fume hood.

2.5 Pectate lyase treatment of cotton fibres

Enzymatic pre-treatments of fibres were carried out with recombinant *Cellvibrio japonicus* pectate lyase from Megazyme used at 10 µg/ml for 2 h with shaking in 2 mM $CaCl_2$ and 50 mM N-cyclohexyl-3-aminopropane sulfonic acid (CAPS) buffer set to pH 10.0. In some cases, sodium carbonate pre-treatments were carried out using 0.1 M Na_2CO_3 (pH 11.4), incubated at room temperature for 2 h at 45 rpm. Samples were washed three times in dH_2O. The high pH of the chemical de-esterification treatment removed the methyl ester groups found on homogalacturonan, allowing the enzyme to work more efficiently (Brown et al., 2001).

2.6 NaOH treatments of cotton fibres

Mature cotton fibres were treated with dH_2O or one of a series of aqueous sodium hydroxide solutions between 1 M to 8 M (Liquor ratio 100:1) and samples were incubated on a Grant OLS 200 oscillating water bath at 25°C for 30 min at 45 rpm. Samples were then washed in dH_2O, air dried and conditioned accordingly. In the

case of cellulose II lyocell fibres, treatments were done with varied temperatures and fabric tensions (25°C/40°C and 49 N m^{-1}/147 N m^{-1}) (Široký et al., 2009).

2.7 Industrial scouring of cotton fibres

Fibres were incubated at 60°C for 30 min in a scouring solution (Liquor ratio 100:1) made up with 0.1 M Na_2CO_3 and 2 gL^{-3} of non-ionic detergent.

2.8 Mercerization of cotton fibres

Scoured cotton fibres were treated with a solution of 23% aqueous sodium hydroxide (Liquor ratio 100:1) at RT for 15 min and then washed excessively in dH_2O. Samples were then soaked in a 5% solution of acetic acid for 1 min, washed excessively in dH_2O and then air dried.

2.9 Dyeing treatments of cotton fibres

2.9.1 Goldthwait maturity test

A maturity test stock solution containing 0.5 g/L of Chlorantine Fast Green BLL™ and 0.5 g/L of Diphenyl Fast Red 5BL Supra™ was added to 1 g of cotton fibres at a dilution of 1/50 and incubated for 45 min at 90° C before rinsing once in cold water, then dipped in boiling water for 30 s and then air dried. The cuticle and primary wall dyed green and with increasing maturity the samples were stained red (Goldthwait et al., 1947). Dye effluent was removed and the absorbance read at 600 nm using a Thermo-Scientific MultiScan FC micro plate reader.

2.9.2 Direct Red™ 4B (Scarlet pontamine) dyeing

A stock solution containing 0.5 g/L of scarlet pontamine was added to 1 g of cotton fibres at a dilution of 1/10 and incubated for 1 hr at RT before rinsing three

times in cold water and then air dried. Scarlet pontamine stains cellulose chains, but has a preference for regions of lower crystallinity (Hoch et al., 2005). Dye effluent was removed and the absorbance read at 600 nm using a Thermo-Scientific MultiScan FC micro plate reader.

2.10 Analysis of cell wall material

2.10.1 Mechanical preparation of fibre cell wall material

Fibres were extracted from cotton plants as detailed in 2.1, frozen in liquid nitrogen and stored at -80°C until needed. 20 mg of frozen fibres were added to 2 ml Eppendorf tubes containing steel beads (1x 7mm, 2x 5mm, 10x 3mm beads) and placed in liquid nitrogen for 5 min. The Eppendorf tubes were placed in the chilled holders of a Retsch Mixer Mill MM400, and crushed for 5 min at 30 Hz. Ethanol was added to the tubes, and removed along with the suspensions into clean tubes. The samples were incubated for 30 min at 65°C, and then centrifuged at 13,000 rpm for 10 min. The ethanol supernatant was discarded and the pellets washed twice in ethanol. The pellets were allowed to dry in a 65°C incubator, before being stored at 4°C.

2.10.2 Chemical extraction of fibre cell wall polysaccharides

2.10.2.1 Water extraction

2.5 g of homogenised cotton fibres were added to a 50 ml falcon tubes with 25 ml of dH_2O and incubated at RT for 1 hr on a rocker at 50 RPM. After 1 hr the tubes were spun down for 10 min at 5000 RPM and the supernatant was extracted and stored as frozen aliquots until needed. The pellet was allowed to dry.

2.10.2.2 1,2-Cyclohexylenedi nitrilo-tetraacetic Acid (CDTA) extraction

The pellet from the previous water extraction was resuspended in 25 ml of 0.1 M CDTA and incubated for 2 hr on a rocker at 50 RPM. After 2 hr the tubes were spun down for 10 min at 5000 RPM and the supernatant was extracted and stored as frozen aliquots until needed. The pellet was allowed to dry.

2.10.2.3 Na_2CO_3 extraction

The pellet from the previous CDTA extraction was resuspended in 25 ml of 0.1 M Na_2CO_3 and incubated for 2 hr on a rocker at 50 RPM. After 2 hr the tubes were spun down for 10 min at 5000 RPM and the supernatant extracted, neutralised and stored as frozen aliquots until needed. The pellet was allowed to dry.

2.10.2.4 KOH extraction

The pellet from the previous Na_2CO_3 extraction was resuspended in 25 ml of 4 M KOH and incubated O/N on a rocker at 50 RPM. After the incubation the tubes were spun down for 10 min at 5000 RPM and the supernatant extracted, neutralised and stored as frozen aliquots until needed and then the pellet was discarded.

2.10.3 Immunodot assay (IDA)

Using the extraction supernatants, 1ul of antigen solutions were dotted onto nitrocellulose paper and allowed to dry. They were then blocked with 5% milk powder in PBS/azide for 1 hour before washing six times in water and then three times for 5 min in PBS. Primary antibody was added at a 1 in 5 dilution in 1% Milk Powder in PBS for 1 hour at room temperature with shaking and then washed three times for 5 min each with tap water and PBS. The secondary antibody (anti-rat HRP) was added at a 1 in 1000 dilution in 1% milk powder in

PBS for 1 hour at room temperature with shaking before washing again 3x 5 min with PBS. To detect antibody binding, samples were washed with a colour reagent made up of 5ml of chloronapthol solution (5mg/ml in ethanol) and 30 µl of 6% hydrogen peroxide made up to 25 ml with dH_2O. Positive blots were stained purple.

2.10.4 Enzyme linked immunoabsorbant assay (ELISA)

100ul per well of 5-50ug/ml samples were coated o/n at 4°C with in PBS before being washed nine times in tap water. Plates were then blocked with 200ul per well with 3% MP/PBS for at least 1h at room temperature and then washed vigorously nine times using tap water. Primary antibodies were used at a 1 in 10 dilution in 3% MP/PBS, with 100ul per well and incubated for 1 h before washing vigorously nine times in tap water, and then shaken dry. The secondary antibody of anti-rat IgG-HRP was used at a concentration of 1 in 1000 in 3% MP/PBS for 1 h, with 100ul per well, and incubated at room temperature for 1 h before extensively washing 9 times in tap water and shaken dry. 150 µl of substrate was added to each well which was made up of 18ml water, 2ml of 1 M sodium acetate buffer (pH 6.0), 200 µl of tetramethyl benzidine (3,3,5’5’-TetramethylBenzidine, Sigma T-2885, 10mg/ml in DMSO) and 20ul 6% hydrogen peroxide. The reaction colour developed within 5 min and was stopped by the addition of 30 µl per well of 2.5 M sulphuric acid before being read at 450 nm on a Thermo-Scientific MultiScan FC micro plate reader.

2.10.5 Attenuated Total Reflectance Fourier transform infrared (ATR FT-IR) spectroscopy for fibre crystallinity analysis

In preparation, samples were conditioned FT-IR spectra 4000 cm^{-1} to 600 cm^{-1} were recorded on fibre samples using a Perkin-Elmer Spectrum BX spectrophotometer with a diamond ATR attachment. A resolution of 4 cm^{-1} and an interval scanning of 2 cm^{-1} were used with 64 scans per sample and a total range of 4000 to 6000 cm^{-1}. Before taking measurements, fibres were conditioned in a controlled atmosphere of 65% ± 2% relative humidity and 20 ± 2°C for 48 h and then stored in a P_2O_5 desiccator to maintain the atmospheric conditions. Obtained spectra data values were normalized to the absorbance of the O–H band at 1336 cm^{-1} due to any obtained changes in this band among all examined samples. LOI ($\alpha_{1429/893}$) and HBI ($\alpha_{3336/1336}$) were calculated as described in (Široký et al., 2009).

2.10.6 X-ray diffraction of intact cotton fibres

Before taking measurements, fibres were conditioned in a controlled atmosphere of 65% ± 2% relative humidity and 20 ± 2°C for 48 h and then stored in a P_2O_5 desiccator to maintain the atmospheric conditions. Cotton fibres were aligned in the form of compressed layers laid in parallel to one another. In total, the layer was 3 mm deep and 16 mm in diameter, which was kept constant using pressure sensitive tape laid over the fibre sample holder. A Panalytical MPD diffractometer was used employing using a voltage of 40 kV, 40 mA current and Cu Kα radiation (λ = 1.5406 Å). Difractograms were made using continuous scanning over a range of 5° to 30° for 7 minutes. The three principal planes of reflection (Miller indices) for cellulose I (110), (110) and (200); and cellulose II (110), (110) and (020) were used. The crystallinity index was calculated for each sample, equation 1 for cellulose I and equation 2 for cellulose II (Figure 2.1) (Segal et al., 1959).

Cellulose I	Cellulose II
$\mathrm{CrI} = \frac{I_{200} - I_{am}}{I_{200}} \times 100$	$\mathrm{CrI} = \frac{I_{1\bar{1}0} - I_{am}}{I_{1\bar{1}0}} \times 100$

Figure 2.10: Equations for calculating crystallinity index for cellulose I and cellulose II from x-ray diffraction.

2.10.7 Analysis of cell wall glycoproteins

2.10.7.1 Protein preparation

Mechanically prepared cotton fibre powder was suspended in an extraction buffer at 10 mg/ml of 0.1 M TRIS at pH 8.0 with 1% SDS and incubated at 95° C for 30 min. Samples were then spun at 3000 RPM for 10 min at 4° C and the supernatant set aside and frozen until needed. Protein contents of 20 µl fibre extract samples, diluted 1 in 10, were measured using the Bradford assay (BioRad, UK) which was adapted from the original Bradford assay (Bradford 1976; Zor & Selinger 1996). Ten BSA standard solutions were made up containing between 0 to 2 mg/ml of the protein. The Bradford reagent was added at a 1 in 5 dilution and then read at 595 nm using a Thermo-Scientific MultiScan FC micro plate reader.

2.10.7.2 SDS-PAGE

The separation of fibre extracted proteins was carried out using SDS-PAGE (Laemmli, 1970). A 4% stacking and a 9% separating gel were used with a Mini-PROTEAN II BioRad system. They were placed in a gel tank containing a running buffer (0.025 M TRIS pH 8.3, 0.2 M Glycine). For each sample, 40 µl of fibre extract solution was added to 10 µl of 5x sample buffer (25% v/v 0.5 M TRIS pH 6.8, 20% v/v glycerol, 4% m/v SDS, 2.5 % v/v Bromophenol Blue, 10% v/v β-mercaptoethanol, 20% v/v dH_2O). The samples were incubated for 2 min at

95° C then 50 µl of each sample was then loaded into each well of a TRIS polyacrylamide gel, with 10 µl of ColourBurst protein ladder (Sigma) in the first and last wells. Gels were run at approximately 80 V, 45 mA for 1 h. Gels were then either used in Western blotting or stained with Coomassie blue protein dye (next section).

2.10.7.3 Coomassie brilliant blue protein gel staining

SDS-PAGE gels were incubated in the staining solution (0.1 % v/v Coomassie brilliant blue (Sigma), 40% v/v methanol, 10% v/v acetic acid and 49.9% v/v dH_2O) for 4 hours which was replaced every 1 h until satisfactory protein staining was seen and the approximate molecular weights recorded.

2.10.7.4 Western blotting

Post-electrophoresis, SDS-PAGE gels were applied to nitrocellulose blotting paper and reinforced with filter paper and nylon scour pads. Pads were then secured in a Bio-Rad blotting cassette. Proteins were vertically transferred to the nitrocellulose paper in a Bio-Rad tank with TRIS transfer buffer (7.5 g TRIS, 36 g glycine, 250 ml methanol, 2250 ml dH_2O) by running at 100 V for 2 h, at 4° C. After the protein transfer had been completed the nitrocellulose paper was washed several times in PBS and then blocked overnight in a solution of 3% MP/PBS. Nitrocellulose membranes were incubated with the primary monoclonal antibody used at a 1/20 dilution in 3% MP/PBS for 1 h at RT. Samples were then washed three times in PBS and incubated with a secondary antibody, an anti-rat IgG coupled horse radish peroxidase (Sigma), at 1/1000 for 1 h at RT then washed three times in PBS. Antibody binding was detected by chemiluminesence. Samples were washed with a colour reagent made up of 5ml of chloronapthol

solution (5mg/ml in ethanol) and 30 µl 6% hydrogen peroxide made up to 25 ml with distilled water. Positive blots were stained purple.

2.11 Microscopy and immunolocalisation

2.11.1 Preparation of plant material for light microscopy

Cotton bolls were opened and the fibres separated from the seeds by hand within 1 h of harvesting. The fibres were then fixed in 4% formaldehyde PEM fixation medium made up of 50 mM PIPES, 5 mM magnesium sulphate and 5 mM EGTA. Samples were incubated at 4°C for 16 h to ensure thorough penetration. The fibres were washed in dH_2O thee times and allowed to air dry before being stored in screw cap Falcon tubes.

2.11.2 Low temperature LR resin embedding and sectioning for light microscopy

Formaldehyde-fixed cotton fibres were dehydrated with graded resin:ethanol series (10% resin, 20% resin, 30% resin, 50% resin, 70% resin, 90% resin, 100% resin, and again 100%) at -20°C for 1 h at each stage. Hard LR white resin (London Resin Company) was infiltrated three times at room temperature for 60 min each time, with the final infiltration left overnight in polymerisation capsules. Polymerisation of the resin embedded fibre was completed at 36°C for 5 days. Sections were cut using glass knives, and cut to a thickness of 0.5 µm and placed on Vectabond coated multi-well microscope slides.

2.11.3 Indirect immunofluorescence labelling and cytochemical staining of fibres for light microscopy

Using a 500 µl tube, fibre samples were incubated in 5% (w/v) milk protein/PBS (Phosphate Buffered Saline solution) for 30 min and then washed once with 1x

PBS. Primary monoclonal antibodies were added at 5 fold dilutions and incubated for 90 min at room temperature with 45 rpm shaking. Samples were washed three times with 1x PBS. The secondary antibody (anti-rat IgG FITC) was added, diluted 1 in 100 in 5% milk protein/PBS and incubated for 90 min in the dark. Samples were washed again three times with 1x PBS. Calcofluor counter-stain (Sigma-Aldrich, Cat.No.F3543) was added for 10 min in the dark, at a 1 in 10 dilution. This stains all β-glycan chains (callose and cellulose) in the fibre. Fibres were mounted on microscope slides and anti-fade reagent Citifluor (Agar Scientific, Cat.No.R1320) was added to reduce fluorescence fading. After adding the cover slip slides were stored at 4°C in the dark. In some cases, samples were pre-treated with cell wall-degrading enzymes, specifically pectate lyases. Samples were treated with 10 µg/ml of *Cellvibrio japonicas* pectate lyase for 60 min and then washed thoroughly in PBS before immunofluorescence labelling.

2.11.4 Indirect immunofluorescence labelling of fibres using recombinant CBMs.

Using a 500 µl tube, fibre samples were incubated in 5% (w/v) milk protein/PBS (Phosphate Buffered Saline solution) for 30 min and then washed once with 1x PBS. Primary CBMs (Carbohydrate Binding Modules) were added at a concentration of 10 µg/ml and incubated with shaking for 90 min at room temperature. Samples were washed three times with 1x PBS. The secondary antibody (mouse IgG anti-his) was added, diluted 1 in 1000 in 5% milk protein/PBS and incubated for 90 min. Samples were washed again three times with 1x PBS. The tertiary antibody (anti-mouse IgG FITC) was added at a 1 in 50 dilution in 5% milk protein/PBS and incubated for at least 90 min in the dark. Samples were washed three times with 1x PBS. Calcofluor counter-stain was

added for 10 min in the dark, at a 1 in 10 dilution, followed by mounting on glass slides with Citifluor and stored in the dark at 4°C as before.

2.11.5 Quantitative fluorescence imaging using Image J

Quantitative fluorescence imaging was completed using Image J software. CBM fluorescence micrographs were run through the software and intensity measurements were taken on regions of the fibre using a standard ellipse shape template. Three intensity measurements were taken for each micrograph for each treatment set, with each treatment done in triplicate. Values were normalised normalised between 0 and 1 through dividing the intensity values by the max value for each carbohydrate-binding module.

Chapter 3

In situ analysis of cell wall polysaccharides in mature cotton fibres

3.1 Introduction

The general protein and polysaccharide components of plant cell walls are generally conserved across all plant species, however there a specific variances in the structures of these polysaccharides between different species, tissues and cell types (Huisman et al., 2001; Burton et al., 2010; Keegstra, 2010). This variability is postulated to be related to the cell functions and is also shown to be linked with taxonomic relationships. As a result, plant cell walls are ideal systems to study the functional evolution of plants, with the cotton fibre being a prime example of an ideal model to study cell wall development, elongation and maturation (Basra, 1999; Bush and Mccann, 1999; Naran et al., 2007; Popper, 2008; Popper et al., 2011). There is much evidence indicating that HG and RG-II are conserved in all land plants, including the degree of polymerisation for HG (Popper et al., 2011; Yapo, 2011). In contrast, RG-I is highly complex and variable among species (Zheng & Mort 2008). In addition to this, there is recent evidence that the hemicellulose constituents of plant cell walls can be masked by pectic homogalacturonan (Marcus et al., 2008). In immunochemistry studies of plant cell wall, it is assumed that cross sections of organs/cells and therefore across cell wall layers from the middle lamella to the plasma membrane would expose all polysaccharides that are present in the cell walls. Masking is when an polysaccharide, such as xyloglucan is prevented from being detected by the presence of another polysaccharide in cell walls sections, in this case, HG. The primary wall composition of the cotton fibre is very similar to that of many other dicots (Meinert & Delmer 1977; Hayashi & Delmer 1988). The outer surface is mostly made up of pectin, specifically HG, which masks the majority of epitopes of the underlying cellulose and hemicelluloses (Vaughn & Turley 1999). The

secondary cell walls of cotton fibres are found deposited on the inside of the primary wall, of which the majority is composed of β1-4-glucosyl subunits which are synthesised into long chains of cellulose. These in turn aggregate to form cellulose microfibrils. The microfibrils are deposited in an ordered fashion within the secondary wall, as opposed to being randomly deposited in the primary cell wall of the cotton fibre (Timpa & Triplett 1993). The secondary cell wall is responsible for fibre dyebility (Smith, 1991; Weis et al., 1999). In this work, the capacity of sets of molecular probes to recognise cell wall polysaccharides using intact and transverse sections of mature cotton fibres are used to study the composition and localisation of these polysaccharides in the cell walls.

3.2 In vitro assay of pectic homogalacturonan binding antibodies

JIM5 and JIM7, the widely used pectic HG monoclonal antibodies (Knox et al. 1990; Clausen et al., 2003) were analysed alongside the recently generated pectic HG monoclonal antibodies LM18, LM19 and LM20 to study the specificity to HG with different levels of methyl-esterification (Verhertbruggen et al. 2009). ELISA plates were coated with E96 pectin, which has a degree of methyl-esterification of 96% (http://www.danisco.com), and was used to coat plates at 50 $\mu g/ml^{-1}$ for analysis of the impact of pre-treatment at alkaline pH (adjusted between 7 and 12 using N-cyclohexyl-3- aminopropanesulfonic acid (CAPS)-based buffers on pectic HG antibody binding. LM20 and JIM7 epitopes (both need some methyl-esterified regions of HG for recognition) were shown to be lost when the pectin samples were pre-treated with a pH of 11 or above. The high pH removes the methyl-ester groups of the pectic HG. LM19 was shown to be most effective probe for binding to de-esterified pectic HG, with maximised binding seen after identical pre-treatments of pH 11 or above. LM18 and JIM5 have similar binding profiles to one another, with maximised binding seen at pH 11.0 and pH 10.0 respectively, and a decrease after pre-treatments of pH 11 and above (Figure 3.1).

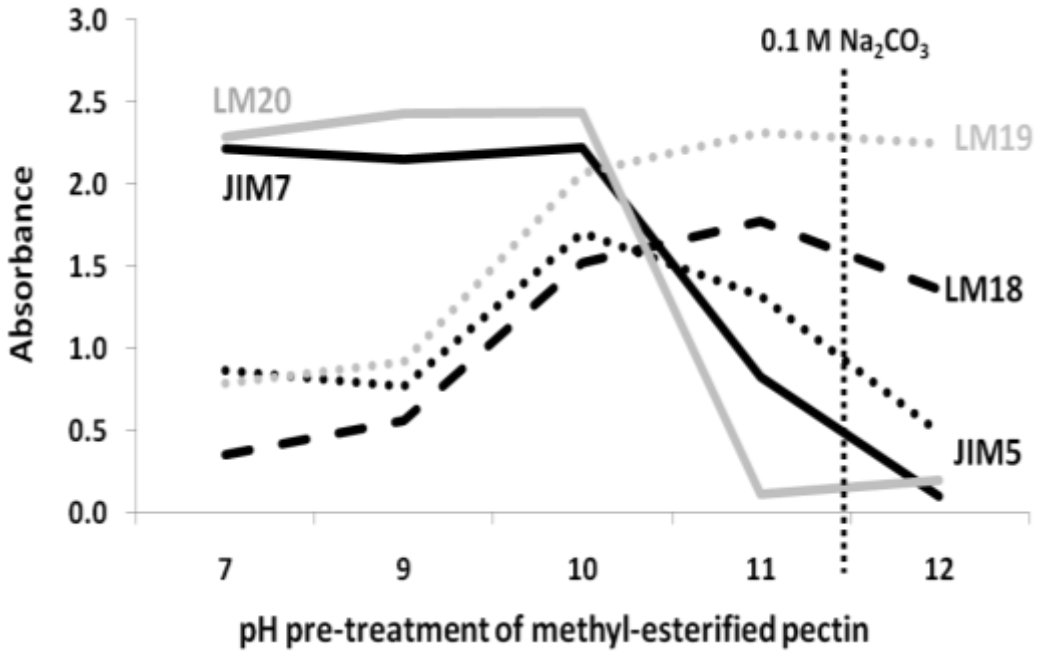

Figure 3.1: ELISA analysis of the effect of alkaline pH pre-treatments on the *in vitro* binding of five pectic homogalacturonan directed monoclonal antibodies, JIM5, JIM7, LM18, LM19 and LM20 to a highly methyl-esterified E96 pectin. E96 pectin was coated onto the plate at 50 µg/ml^{-1}, and all antibodies used at 10-fold dilution. E96 pectin has a degree of methyl-esterification of 96% (http://www.danisco.com), and was used to coat plates at for analysis of the impact of pre-treatment at alkaline pH on pectic HG antibody binding. Pre-treatments using CAPS (N-cyclohexyl-3-aminopropane sulfonic acid) buffered solutions of pH 7–12 for 2 h before immunolabelling were performed. The pH of a 0.1 M sodium carbonate solution is shown for comparison as a vertical dotted line.

3.3 A waxy outer layer and homogalacturonan sheath stops immunolabelling of fibres

Intact cotton fibre surfaces were screened with the monoclonal antibodies LM18 and LM19 (Verhertbruggen, Marcus, Haeger, Ordaz-Ortiz, et al., 2009) for homogalacturonan (HG), LM6 (Willats et al. 1998) and INRA-RU2 (Ralet et al. 2010) for rhamnogalacturonan (RG-I). To explore the effect of pectate lyase (PL) on carbohydrate-binding module and monoclonal antibody recognition of polysaccharides in cotton fibre cell walls, samples were pre-treated with 0.1 M of sodium carbonate (pH 11.4) for 2 h. This was done to maximise the pectic HG degradation from the oncoming enzymatic PL treatments. In all cases, the enzymatic effects of PL were verified by studying the samples after sodium carbonate pre-treatment alone. CBM3a was used to detect crystalline cellulose. The xyloglucan, xylan, and RG-I antibodies, as well as CBM3a showed little or no binding to the cotton fibre surfaces. This was due to a waxy layer on the surface of the fibres, most likely acting as a hydrophobic barrier to buffers and probes that were used. The solutions used for dewaxing were kept aside to be evaporated and analysed with IDA to check if any polysaccharides were being extracted during this process. CCRC-M1, LM15 and LM25 for xyloglucans and JIM13 for AGPs were detected strongly in the ethanol and acetone solutions. Furthermore, LM25 had the strongest signal in all cases and was also detected in the ether dewaxing solution (Figure 3.2). Treating the cotton fibres with 90 min sequential incubations of ethanol, acetone and ether increased the detection of the HG epitope (LM18) significantly along the fibre surfaces, with a minor increase in the detection of the RG-I and hemicellulose probes. Detection of pectic HG epitopes was lost after pectate lyase treatments. Cellulose comprises of over 97% of the fibre (Haigler et al., 2009) and is only detectable after the total removal of

HG using pectate lyase. Low levels of (1-5)-α-L-arabinan and the rhamnogalacturonan-I backbone were detected after dewaxing, however they were abundantly detected after the removal of HG using pectate lyase (Figure 3.3). Immunolabelling of LR White embedded sections cut to a thickness of 0.5 µm showed that HG is localised to the primary cell wall of the fibre. Cellulose localises throughout the fibre cross section, independent of pectate lyase treatment. LM6 and INRA-RU2 also bound to the primary cell wall sporadically, and these epitopes were clearly revealed by enzymatic treatment with pectate lyase (Figure 3.4).

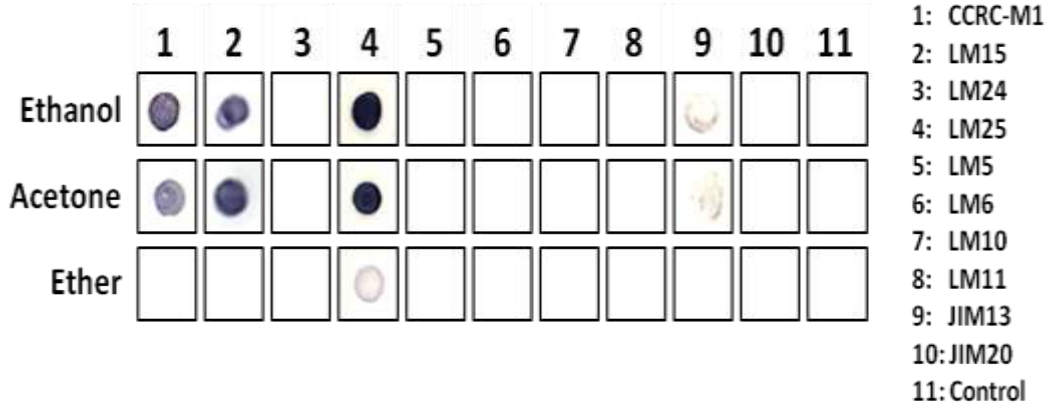

Figure 3.2: Immunodot assay detection of polysaccharides extracted from the mature cotton fibre cell wall during the dewaxing process. Monoclonal antibodies CCRC-M1, LM15, LM24, LM25 (Xyloglucans), LM5 (Galactan), LM6 (Arabinan), LM10 (Xylan), LM11 (Xylan/arabinoxylans), JIM13 (AGP) and JIM20 (Extensins) to material solubilised from cotton fibre dewaxing solutions. Polysaccharides were detected with probes 1 (CCRC-M1), 2 (LM15) and 4 (LM25) and 9 (JIM13).

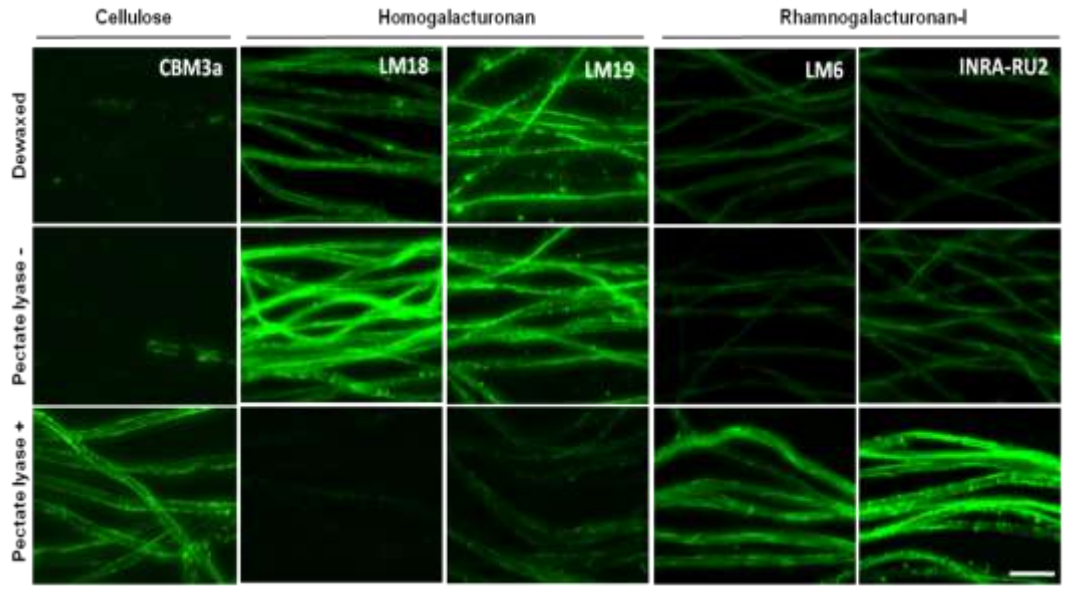

Figure 3.3: Indirect immunofluorescence detection of cellulose, homogalacturonan and rhamnogalacturonan-I epitopes on the surfaces of FM966 fibres after dewaxing and pectate lyase treatments. Monoclonal antibodies LM18 and LM19 bind specifically to the outer layer of the fibre cell walls before enzymatic pre-treatment. The binding of these homogalacturonan antibodies were used to verify the pectate lyase action. Binding of the carbohydrate-binding module (CBM) 3a and rhamnogalacturonan antibodies LM6 and INRA-RU2 are revealed fully after pectate lyase treatment. Scale bar, 100 µm.

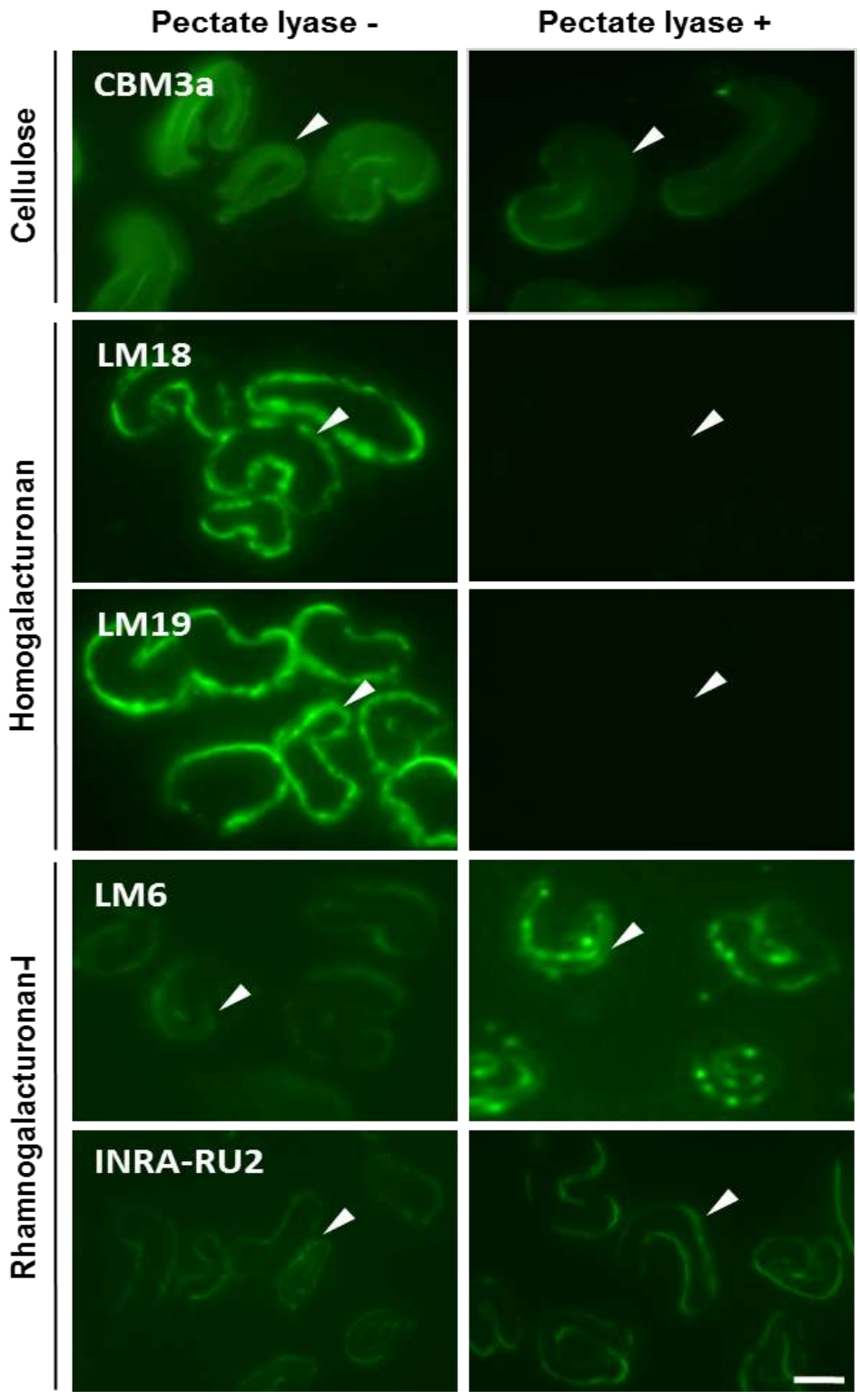

Figure 3.4: Indirect immunofluorescence detection of cellulose, homogalacturonan and rhamnogalacturonan-I epitopes in transverse mature cotton fibre sections with pectate lyase. Homogalacturonan antibodies LM18 and LM19 were used to verify the effectiveness of the pectate lyase treatment. CBM3a detected cellulose throughout the primary and secondary cell walls, while LM18, LM19, LM6 and INRA-RU2 are localised to the primary cell wall. Scale bar, 10 µm.

3.4 Localisation and composition of xyloglucan and xylan polysaccharides in the cotton fibre cell walls

The hemicelluloses make up a large component of plant cell walls. Xyloglucans are a major component of dicots, while xylans are the major hemicellulose in monocots (Carpita and McCann, 2000). These polysaccharides were studied in the mature cotton fibre. Initial screens of intact fibres with LM15, a monoclonal antibody that bind to XXXG motif xyloglucan (Marcus *et al.* 2008) and CCRC-M1, which binds to α-L-fucosylated xyloglucans (Pullman *et al*. 1994), showed very weak or no binding. Xyloglucan and xylan epitopes were revealed and detected in abundance after the removal of pectic HG. Xyloglucan epitopes were detected as smooth, consistent layer all along the fibre, while the LM11 xylan epitope was more sporadic (Figure 3.5). Transverse section analysis showed very weak detection of all hemicellulose probes for untreated transverse fibre sections. The enzymatic removal of homogalacturonan resulted in increased signal intensity of the xyloglucan probes LM15 and CCRC-M1; however the PL+ pre-treatment revealed the LM11 xylan epitope to be more diffuse and localised to the primary cell wall and the inner lumen of the secondary cell wall (Figure 3.5).

To further characterise the cotton fibre hemicelluloses, fibre cell wall polysaccharides were extracted with water, trans-1,2-cyclohexane diamine-N,N,N',N'-tetra acetic acid (CDTA), sodium carbonate (SC) and potassium hydroxide (KOH), followed by ELISA and Immunodot blot analysis (Figure 3.6). Two recently developed monoclonal antibodies for xyloglucan, LM24 and LM25 (unpublished) were used in the *in vitro* analysis of fibre cell wall extracts. The ELISA binding analysis showed that three of the xyloglucan-directed monoclonal antibodies, CCRC-M1, LM15 and LM25 were abundantly detected in the KOH cell wall extractions, with reduced levels detected in the SC extraction (Figure

3.6a). LM24, which is thought to bind to glycosylated xyloglucan epitopes, was not detected in any of the cell wall extractions, nor was it seen when used *in situ* against the intact surfaces of cotton fibres. Small amounts of the LM6 (arabinan) epitope were detected in the CDTA, SC and KOH fractions, while the LM11 (xylans/arabinoxylans) epitope was detected in the KOH fraction only. LM5 and LM10 were not detected in the ELISA analysis. Immunodot assay (IDA) complimented the ELISA results for the xyloglucan-directed antibodies CCRC-M1, LM15, LM24 and LM25. Polysaccharide immunoprofiling rings (Willats and Knox, 1999) were seen on the IDA for the KOH extractions indicate the potential presence of at least two populations of xyloglucans in the fibre extracts (Figure 3.6b). As previously mentioned LM5 and LM10 were not detected in ELISAs, IDAs or *in situ* immunofluorescence fibre surface studies. LM6 and LM11 were also not detected in the ELISA or IDA analysis; however they were seen with *in situ* immunofluorescence fibre surface studies (Figure 3.4 and Figure 3.5). The abundance of xyloglucans was expected, as *Gossypium hirsutum* is a dicot, while the presence of xylans in the mature cotton fibre will need to be further explored.

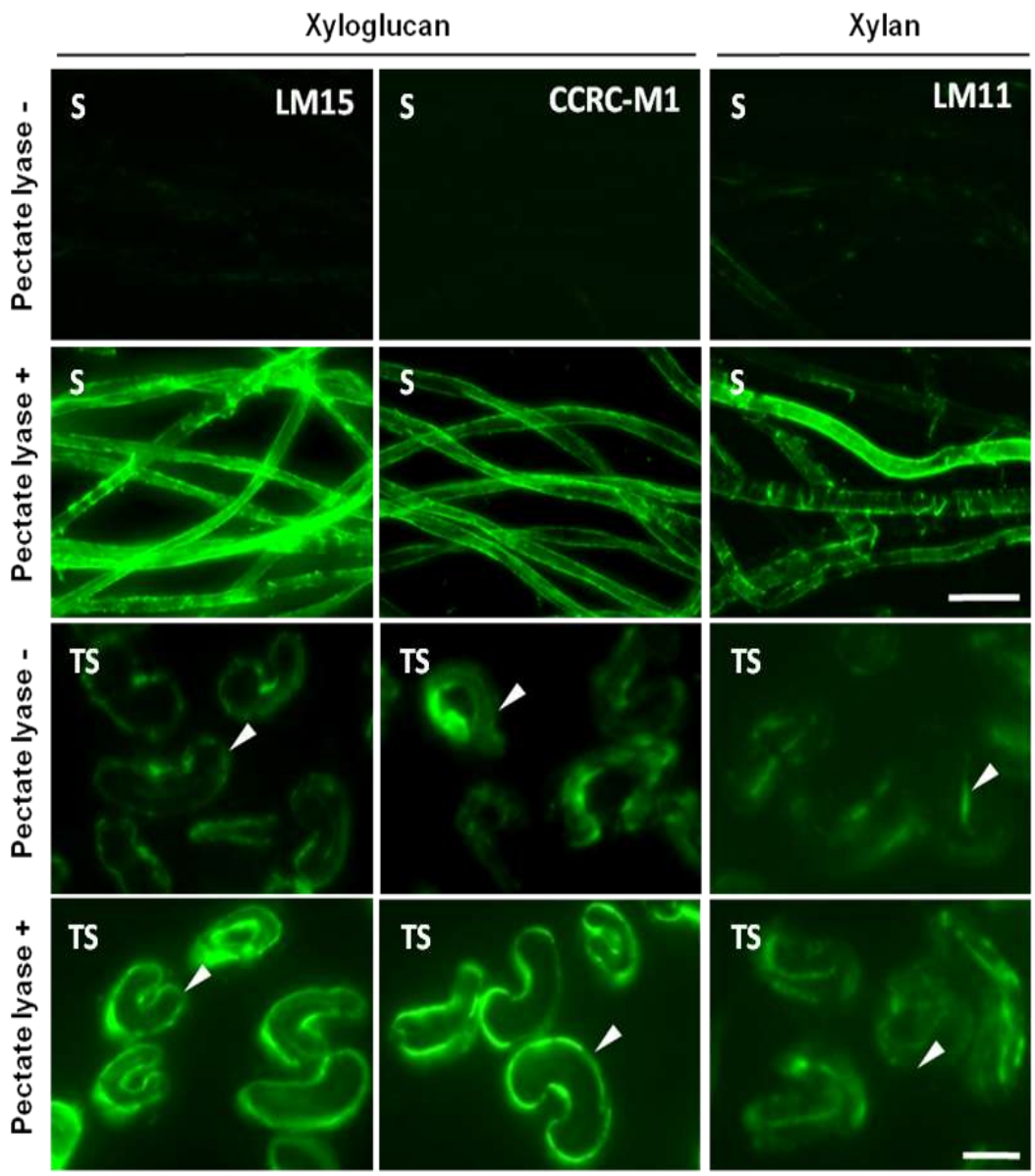

Figure 3.5: Indirect immunofluorescence detection of xyloglucan and xylan epitopes on the surfaces and transverse sections of FM966 fibres after pectate lyase treatment. Monoclonal antibodies LM15, CCRC-M1 and LM11 were detected on the surfaces (S) only after pectate lyase treatment; however weak detection was seen in untreated transverse sections (TS). After pectate lyase treatments, increased signal intensity was seen at the primary walls (arrows). Surface scale bar, 100 µm; transverse sections, 10 µm.

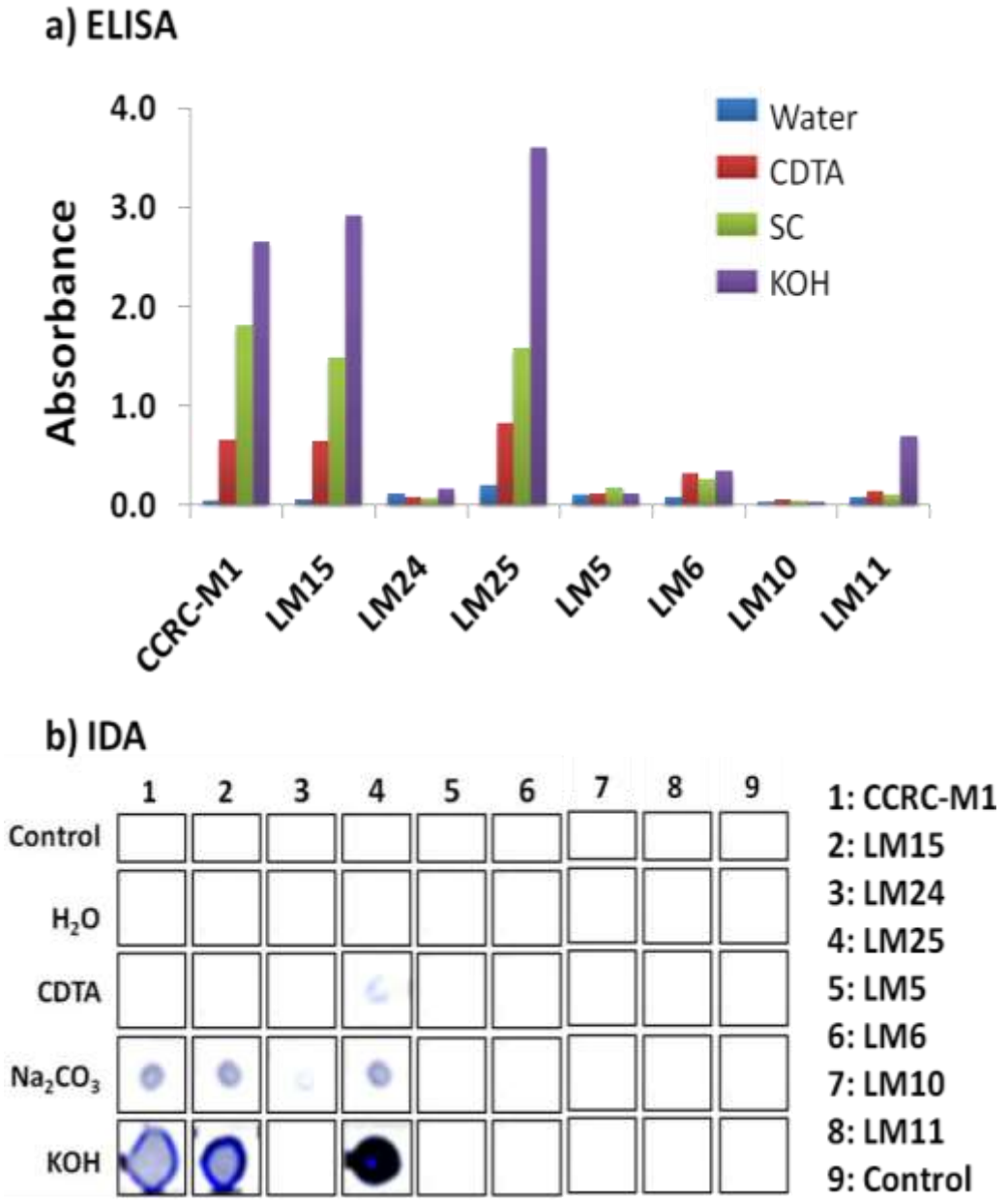

Figure 3.6: a) ELISA binding analysis of monoclonal antibodies CCRC-M1, LM15, LM24, LM25, LM5, LM6, LM10 and LM11 to fibre cell wall material solubilised by four solutions: water, CDTA, SC and KOH. 1 g of homogenised cotton fibre material was used for every 10 ml of extraction buffers, and plates coated using a 10-fold dilution and monoclonal antibodies used at a 5-fold dilution. **b)** Immunodot assays of monoclonal antibodies CCRC-M1, LM15, LM24, LM25, LM5, LM6, LM10 and LM11 to material solubilised from 10 mg/ml homogenised cotton fibre cell wall. Polysaccharide profiling was seen in with probes 1 (CCRC-M1), 2 (LM15) and 3 (LM25).

3.5 Extensin and AGP epitopes revealed after removal of pectic homogalacturonan

Extensins are rod-like, structural glycoproteins associated with cell wall extensibility and rigidity, and with interactions between the plasma membrane and intracellular spaces. They fall into the main groups of hydroxyl proline-rich proteins (HPRPs) and proline-rich proteins (PRPs) and are rich in ser-$(hyp)_4$. Extensins usually have one hydrophilic and once hydrophobic repetitive peptide motif with the potential for crosslinking (Smallwood et al., 1994). Arabinogalactan proteins (AGPs) are a complex and diverse class of plant cell wall surface glycoproteins that are involved in a range of fundamental processes associated with the development of plant cells (Seifert & Roberts 2007). Using an *in situ* immunolabelling approach, the glycoprotein composition of the mature cotton fibre was studied. LM1, JIM11, JIM12, JIM19 and JIM20 antibodies for arabinosylated HRGPs extensin epitopes were not detected on the dewaxed fibres under the PL- conditions. After treatment with PL+, the JIM11 and JIM20 epitopes were abundantly detected, indicating that the enzymatic treatment does reveal these extensin glycoproteins and that the pectic HG sheath blocks access to these epitopes (Figure 3.7). JIM19 however, was not detected, indicating that this glycoprotein epitope is not present on the fibre surface. The binding of JIM11 and JIM20 to the fibre surface after incubation with PL+ led to their recognition, indicating With regard to the epitopes of JIM11 and JIM19, there is little information available on the nature of the epitopes and they are just called extensin epitopes and may recognize different glycosylation and arabinosylation patterns (Smallwood *et al*. 1994). For AGP epitopes, no fibre surface detection was seen under PL- conditions for the anti-AGP antibodies LM2, JIM4 and JIM15 (Not shown). After PL+ treatment, LM2, JIM4 and JIM15 were still not detected,

giving an indication that these AGP epitopes are not present on the cell wall surface (Not shown). Low levels of the JIM13 epitope was detected on the fibre surface after enzymatic treatment with PL+ (Not shown). Transverse section analysis demonstrated that the JIM20 and JIM11 epitopes are revealed after the removal of pectic HG, indicating that this pectin acts as more than just a physical barrier. JIM13, however, the epitope was clearly detected, independent of pectate lyase treatment, and strongly localised to the lumen of the secondary cell wall of the fibre, at the face of where the cell membrane and cytoplasm would have been during development. This indicates HG is not associated with the JIM13 AGP epitope. Weak detection of JIM13 was also seen at the primary wall (Figure 3.7).

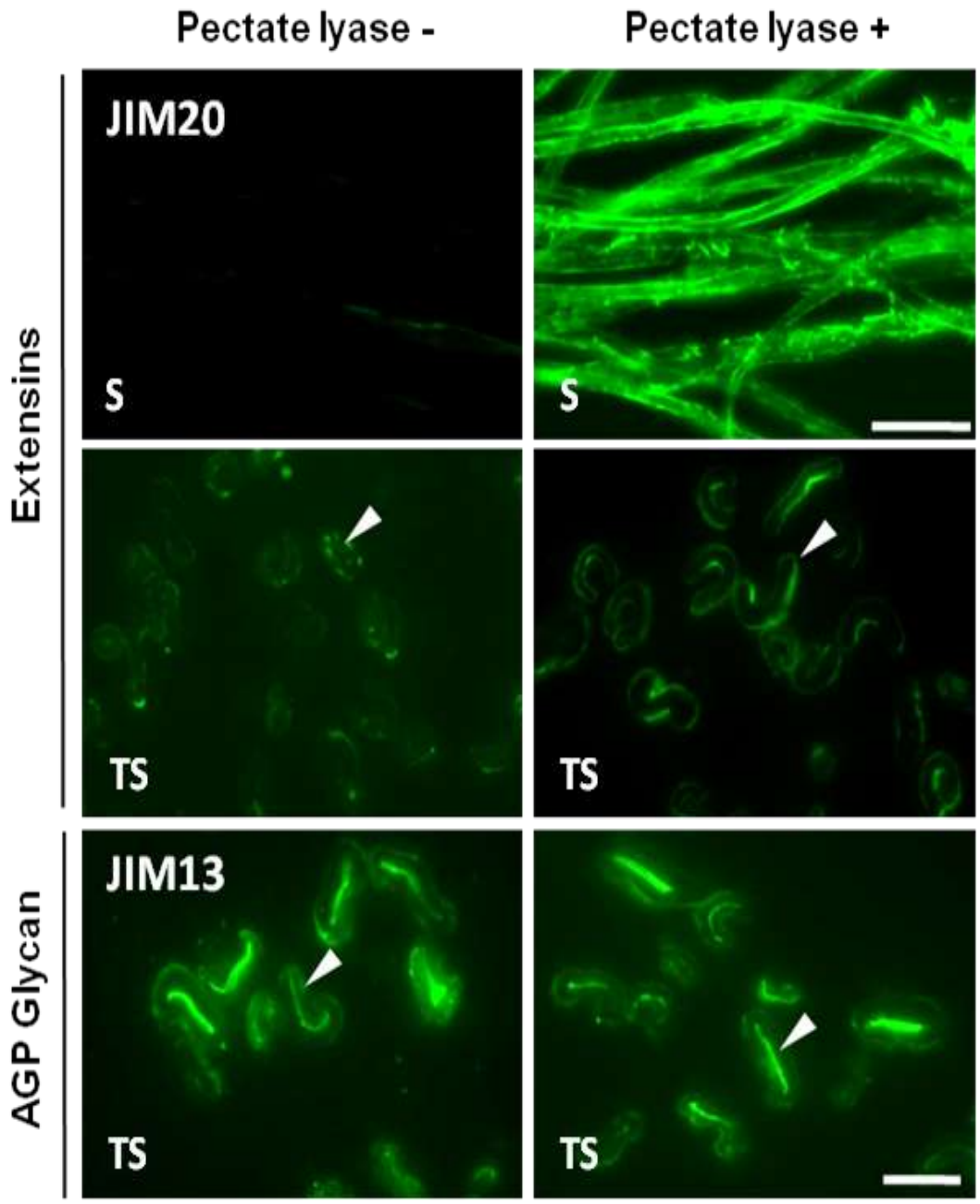

Figure 3.7: Extensin and AGP epitopes revealed on fibres after pectate lyase action. The detection of the AGP epitope using JIM20 for surface detection was revealed after pectate lyase treatment. In transverse sections, JIM20 and JIM13 bind without enzymatic treatment to the primary cell wall (arrow), however stronger detection is seen after pectate lyase treatment in the case of JIM20. Surface (S) scale bar, 100 µm; transverse sections (TS) scale bar, 10 µm.

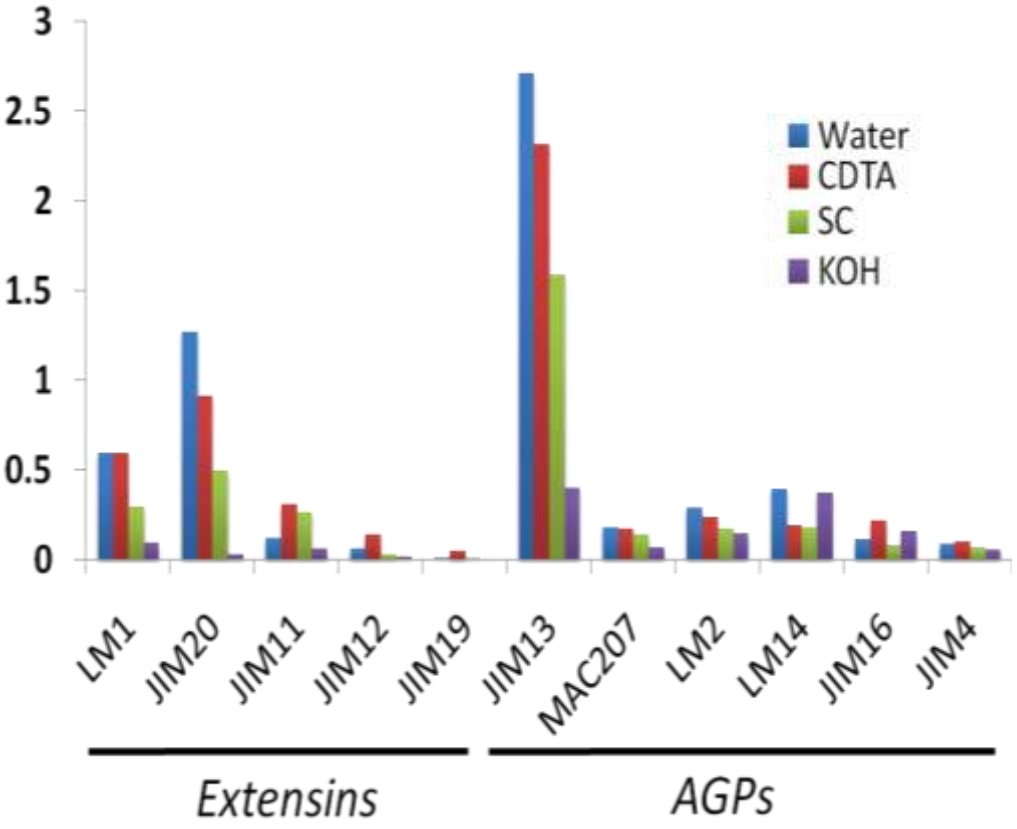

Figure 3.8: *In vitro* ELISA binding analysis of glycoprotein-directed monoclonal antibodies LM1, JIM20, JIM11, JIM12, JIM19 (extensins), JIM13, MAC207, LM2, LM14, JIM16 and JIM4 (AGPs) to material solubilised by four cell wall extraction solutions: water, CDTA, SC and KOH. 1 g of homogenised cotton fibre was used for every 10 ml of extraction buffers, and plates coated using a 10-fold dilution.

To further characterise the cotton fibre cell wall glycoproteins, cell wall fractions were extracted with water, CDTA, sodium carbonate and KOH, followed by ELISA analysis (Figure 3.8). LM1 and JIM20 were the main extensin epitopes detected in the cell wall extractions, specifically the water and CDTA fractions. JIM13 gave the strongest signal out of all the cell wall glycoprotein probes, with the highest levels of the epitope seen in the water, CDTA and SC extractions. JIM11, JIM12, JIM19 (extensin epitopes), MAC207, LM2 LM14, JIM16 and JIM4 (AGP epitopes) were not detected strongly in the ELISAs, and this is supported with the *in situ* immunolabelling of intact cotton fibre surfaces.

3.6 Detection of heteromannans in the cotton fibre primary cell wall

There is currently no evidence on mannans being present in cotton fibres. In tobacco and *A. thaliana* it has been previously demonstrated that pectic HG masking of mannan epitopes are a cell wall phenomenon of biological significance (Marcus et al., 2010). Mature FM966 fibres were treated with sodium carbonate to see if it would solubilise sets of polysaccharides that may expose hidden epitopes (Figure 3.9). As none were detected, pectate lyase was used to remove the pectic HG layer, after which mannans were detected, but only if the samples had been treated with Na_2CO_3 before. To determine whether these mannans had any acetyl groups or masking, pectate lyase was used in conjunction with Na_2CO_3 before and after (Na_2CO_3 / PL+ and PL+ / Na_2CO_3). The sample was treated with pectate lyase, along with treatments of Na_2CO_3 before or after the degradation of HG. No mannan was detected for any of the PL- samples, however fluorescence was seen for the Na_2CO_3-PL+ and PL+-Na_2CO_3 treated samples, potentially indicating that the mannans present in the fibre are blocked and masked by HG, as well as acetylated. The Na_2CO_3 treatments would remove these acetyl groups, allowing the mAb LM21 to bind to the de-acetylated mannans. In transverse sections, no mannans were detected in untreated samples; however, strong LM21 detection was seen after only Na_2CO_3, independent of pectate lyase treatment. This is an indication that the pectic HG sheath acts as a physical barrier to LM21 epitope *in situ* surface binding, and that these mannans are not intimately associated with HG as they were detected in transverse sections. To verify whether it was actually mannan that the antibodies were binding to, a further assay was done, in which Na_2CO_3-PL+ treated fibres were incubated with

Thermotoga maritima endo-β-mannanase (Figure 3.9). This enzymatic treatment was effective at removing the LM21 mannan epitope.

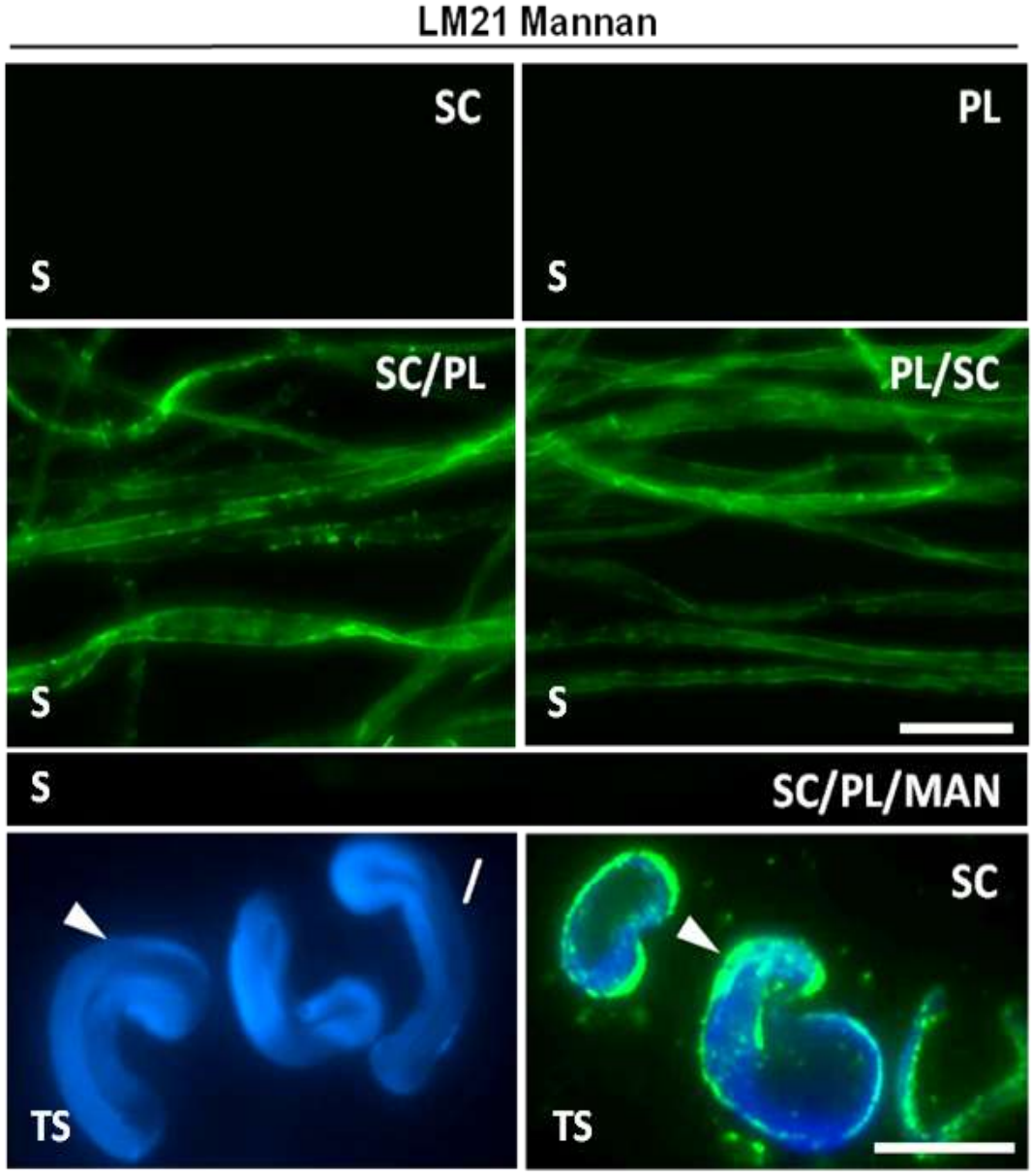

Figure 3.9: Detection of heteromannans in the outer fibre primary cell wall. LM21 bound effectively to the fibre surface (S) after both Na_2CO_3 (SC) and pectate lyase (PL) treatment, independent of which was used first. *T. maritima* endo-β-mannanase (MAN) was used to verify the presence of heteromannans. LM21 was detected in the primary cell walls (arrow) of the fibre transverse sections (TS) after sodium carbonate treatment. Surface (S) scale bar, 100 µm; transverse section (TS) scale bar, 10 µm.

3.7 Comparative analysis of the occurrence of polysaccharides in mature cell walls of fibres across different *Gossypium* species and lines

A comparative analysis was done on 10 fibre lines over 4 species with a range of different properties to characterise the key differences in the mature cotton fibre cell walls (Figure 3.10), LM18 for partially/no methyl-esterified pectic HG, was strongly seen in all fibre lines except no#7 and no#9 (Acala GC-362 and Acala SJ1, *G. hirsutum*). LM19 which binds to a subtly different form of partially/no methyl-esterified pectic HG (Figure 3.1) was detected in all lines, including no#7 and no#9. Both LM18 and LM19 epitopes are both lost after pectate lyase treatments. Low levels of the LM15 epitope (XXXG xyloglucan) were detected in untreated fibre lines no#2 (Obtusifolium, *G. arboreum*), no#3 (#393, *G. herbaceum*) and no#10 (AK-Djura green lint, *G. hirsutum*). After removal of the pectic HG, abundant levels of the XXXG xyloglucan epitopes were seen in all the species lines. The LM5 epitope (1-4-β-D-galactan) was very weak in all lines after PL+ treatments, except line no#3 (#393, *G. herbaceum*), potentially implication an evolutionary difference between this line and the other 9. The LM6 epitope (1-5)-α-L-arabinan was detected in all lines and species, however, much like LM5, the strongest fluorescence was seen in line no#3 (#393, *G. herbaceum*). The LM21 mannan epitope was only detectable after both sodium carbonate and pectate lyase treatments, and only visible in lines no#4 through to no#10, covering the species *G. barbadense* and *G. hirsutum*. Fibre lines no#1 to no#3 (*G. arboreum* and *G. herbaceum*), however, had no detectable mannan epitopes, which could again be related to the evolutionary developments before the AD-genome species *G. hirsutum* and *G. barbadense* evolved from A-genome species

G. arboreum and *G. herbaceum* (Figure 1.1, Evolution of the spinnable fibre, Introduction).

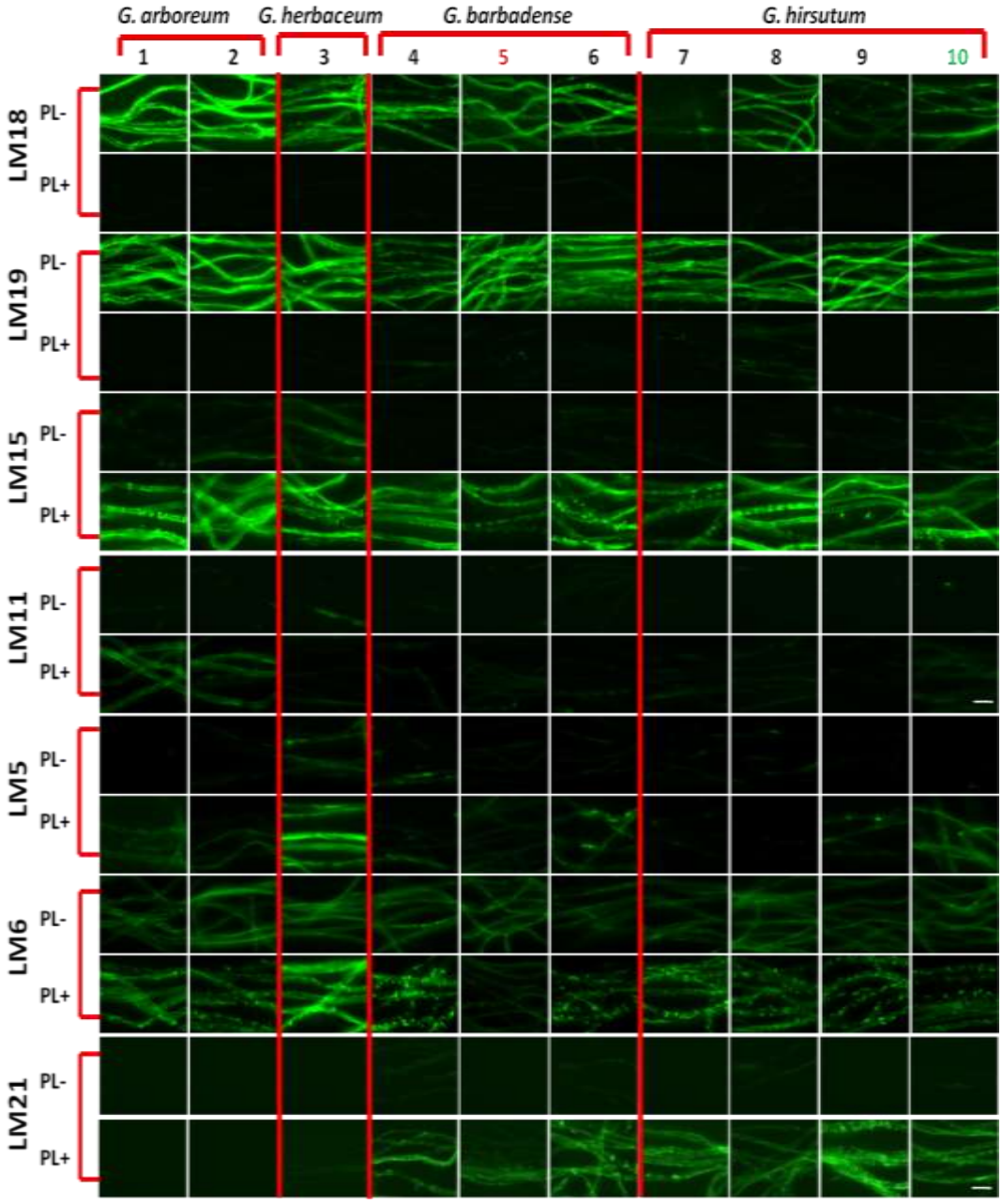

Figure 3.10: Comparative analysis across ten lines through four *Gossypium* species of cell wall epitopes via indirect immunofluorescence on the fibre surfaces before (PL-) and after (PL+) pectate lyase treatments. Ten lines used: 1 – Obtusifolium hirsutum, 2 - #393, 4 – Tangius LMW1736-60, 5 – G. barbadense tashent (Red fibres), 6 – Pima S7, 7 – Acala GC-362, 8 – FM966, 9 – Acala SJ1 and 10 – AK-Djura Green Lint (Green fibres). Scale bar, 100 µm.

3.8 Summary

In summary,

- The new range of pectic HG antibodies, LM18, LM19 and LM20 were demonstrated to have similarities and differences to the existing JIM5 and JIM7 antibodies. These new HG antibodies are a welcome addition to the antibody tool kit due to their varying binding sensitivities under different pH and pectate lyase treatments.
- The mature cotton fibre has an outer waxy layer that acts as a hydrophobic barrier and prevents efficient immunolabelling with mAbs. Additionally, steps taken to remove this barrier through the use of solvents also removes some xyloglucans, shown by the effective detection of LM15, LM25 and CCRC-M1 epitopes in this treatment elutes. After removal of the waxy layer, more pectic HG can be detected on the fibre surfaces.
- The cotton fibre has a pectic HG rich outer layer that acts as a physical barrier to *in situ* surface immunolabelling of non-HG epitopes, as well as masking hemicellulose epitopes in transverse cross sections of mature cotton fibres. Removal of this pectic HG using pectate lyase allows detection of xyloglucan, xylan, extensin and cellulose epitopes.
- The presence of the waxy cuticle and the pectic HG rich outer layer of the mature cotton fibre necessitated a careful approach for the detection and imaging of low level cell wall glycan epitopes. This removal of the wax will be needed in order to detect these polysaccharides during development, which is the subject of the next chapter. This research could be taken further by utilising a wider range of recombinant cell wall degrading enzymes in combination with visualisation of polysaccharide epitopes via immunofluorescence detection.
- From a functional perspective, the presence of the pectic HG rich outer layer is obviously a biologically important remnant from the earlier fibre developmental stages, and the varying degree of methyl esterification can

affect the rigidity of the cell wall in relation to elongation. The occurrence of HG during development will be elucidated in chapter 4.

- In fibre cell wall extraction, the LM15, CCRC-M1 and LM25 epitopes were detected strongly in the Na_2CO_3 and KOH fractions. The JIM13 AGP epitope, LM1 and JIM20 epitopes for extensins were detected in abundance in the water and CDTA fractions.
- The LM21 mannan epitope was detected in pectate lyase- and Na_2CO_3-treated fibre surfaces, independent of which treatment was done first. In transverse cross sections, only Na_2CO_3 treatment was needed to reveal the LM21 mannan epitope.
- While the mature cotton fibre is 97% cellulose (Haigler, Singh, Wang, & Zhang, 2009), a diverse range of non-cellulosic polysaccharides were detected in the fibre cell wall. Despite forming less than 3% of the cell wall at maturity, these polysaccharides are undoubtedly vital to the final form of the mature fibre, which is determined during the earlier developmental stages of initiation, elongation and secondary wall synthesis. These factors will be relevant to work presented in chapter 4.
- The comparative analysis of 10 lines of mature cotton fibres, across 4 different species highlighted several similarities and subtle differences between them, especially for LM11 (xylan/arabinoxylans epitope), LM5 (galactan epitope) and LM6 (arabinan epitope).
- The comparative analysis of 10 different fibre samples indicated the subtle differences between these lines and species. For these low level glycan epitope differences to be most efficiently understood in functional terms, a comprehensive analysis of each fibre lines mechanical properties will need to be done to see if there are any correlations between specific polysaccharides and these mechanical traits. Furthermore, these analyses should be extended to looking at the fibres throughout the various stages of

the industrial processing (for example; mercerised cotton, spun cotton yarn/fabric) to see how the polysaccharide profile changes and if any further correlations can be found between cell wall composition and the mechanical properties.

- These observations also indicate that a more careful approach is needed in regards to cell wall immunocytochemistry of pectins, xyloglucans, xylans and mannans in the context of potentially cryptic epitopes. While certain polysaccharides have been identified in the mature cotton fibre cell wall, these epitopes need to be explored one by one in future work to establish the structure-function relationship, if any, to final fibre properties.

Chapter 4

In situ cell wall analysis of developing cotton fibres

4.1 Introduction

Although plant cell wall development is well characterised among some species, the changes in polysaccharide composition of cotton fibres during development and how these changes are related to cell biology still remain uncertain. During development, the cotton fibre grows up to 60 mm from an individual epidermal trichome on the seed coat surface. This extreme elongation and synthesis of a thick secondary cell wall make the cotton fibre a useful research model (Singh et al., 2009). The primary wall is mostly responsible for the length of the fibre, while the secondary cell wall is largely responsible for fibre strength and dyeing ability (Weis et al., 1999). As previously mentioned, the cotton fibre development process can be divided up into four main processes: Initiation (-2-3 dpa), elongation (3-24 dpa), secondary wall synthesis (18-50 dpa) and maturation (>50 dpa) (Gokani & Thaker 2000). Initiation occurs at the start of anthesis and lasts for three days, in which approximately 25% of the seed coat epidermal cells differentiate into fibre cells. The exact process by which these 25% of cells differentiate is still not fully understood (Timpa & Triplett 1993). During the elongation stage, fibres elongate rapidly by diffuse growth for around 3 weeks (Qin & Zhu 2011). Of all the polysaccharides, xyloglucan has the highest turnover during elongation, where the rate of elongation is determined by cell wall extensibility, which itself is controlled by the mechanical interactions of xyloglucan with other cell wall polysaccharides (Gokani & Thaker 2000). It has long been assumed that fibres elongate separately as individual cells. However recent evidence has implicated the presence of a pectic rich cotton fibre middle lamella (CFML), which was found to adhere fibres together until around 24 dpa (Singh et al., 2009). Fibre bundles are entrained by the fibre tips during elongation

until the start of secondary wall synthesis, whereupon the CFML is broken down via hydrolysis to restore fibre independence (Singh et al., 2009). While much is still unknown about the CFML and how it is formed, there are several theories about its function within fibre development. Supplementary to encouraging fibre packing, the presence of the CFML may help developing fibres to withstand the turgor pressure during elongation (Ruan, 2007). There have been similar hypotheses proposed in other plant systems with adhesive cell wall parenchyma tissues (Niklas, 1992). In many other plant systems, examples of cell separation include: sloughing of root cells, dehiscence and fruit-ripening. Since cotton fibres develop in a fruit boll it is plausible to link this CFML degradation with the associated cell separation of fruit-ripening. After nearly 3 weeks of elongation fibres synchronously enter the secondary wall synthesis stage via an oxidative burst in which the majority of cellulose biosynthesis is initiated. The deposition of >97% type I cellulose in the secondary cell wall of the cotton fibre makes it a powerful model system to study cellulose biosynthesis, which is important in so many industries. Between 45 and 60 dpa, the cotton fruit bolls dehisce (open up), causing the dense matt of fibres to desiccate and mature. Desiccation of the protoplast causes the cytoplasm to stick to the inner surface of the secondary wall, leaving a hollow lumen. This in turn causes the fibres to twist through the collapse of the hollow lumen and is the point where secondary wall thickness in relation to the overall diameter is important; if the wall is too thick then the fibre will not collapse and twist (Kim & Triplett 2001). It was also found that where the fibres attach to the epidermal seed coat the primary cell wall is much thicker and the secondary wall is conversely thinner. Despite these changes the thicker primary cell wall increases the strength of the fibre attachment, especially upon

desiccation at maturation, when the primary wall material condenses (Vigil et al. 1996).

In this chapter, developing fibres were characterized using *in situ* immunolabelling with monoclonal antibodies (mABs) and carbohydrate-binding modules (CBMs). CBMs have the advantage of being generally smaller than mAbs, potentially allowing access to the more confined regions of the cell wall. Their limitations are however that there is a smaller range of CBMs available and the fact that these proteins have a wider specificity to cell wall polysaccharides when compared to the highly specific mAbs.

Additionally, polysaccharide extracts from developing fibres were used with immune dot assays (IDAs), enzyme-linked immunosorbent assays (ELISAs) and Western blots were also carried out to study the cell wall proteins, including extensins and arabinogalactan proteins (AGPs) present during fibre development.

4.2 Detection and changes of β-glycans in the developing cotton fibre

Intact and untreated surfaces of developing cotton fibres were stained with Calcofluor White (Sigma) to detect all the β-glycans present (Hoch et al., 2005), which includes cellulose and callose (Figure 4.1a). Image-J software was used to quantify the micrographs (Figure 4.1b). Through micrograph quantification, the fluorescence intensity starts to increase at 15 dpa, continuing through 20-25 dpa, before slowing down between 30-40 dpa. Maximal fluorescence intensity was achieved at 40 dpa, potentially indicating that cellulose deposition stopped and fibre development shifted into the maturity stage. Between 8-20 dpa, developing cotton fibres are adhered to one another via the CFML (Singh et al., 2009), so after Calcofluor staining or *in situ* immunolabelling, fibre bundles were teased apart using fine tweezers.

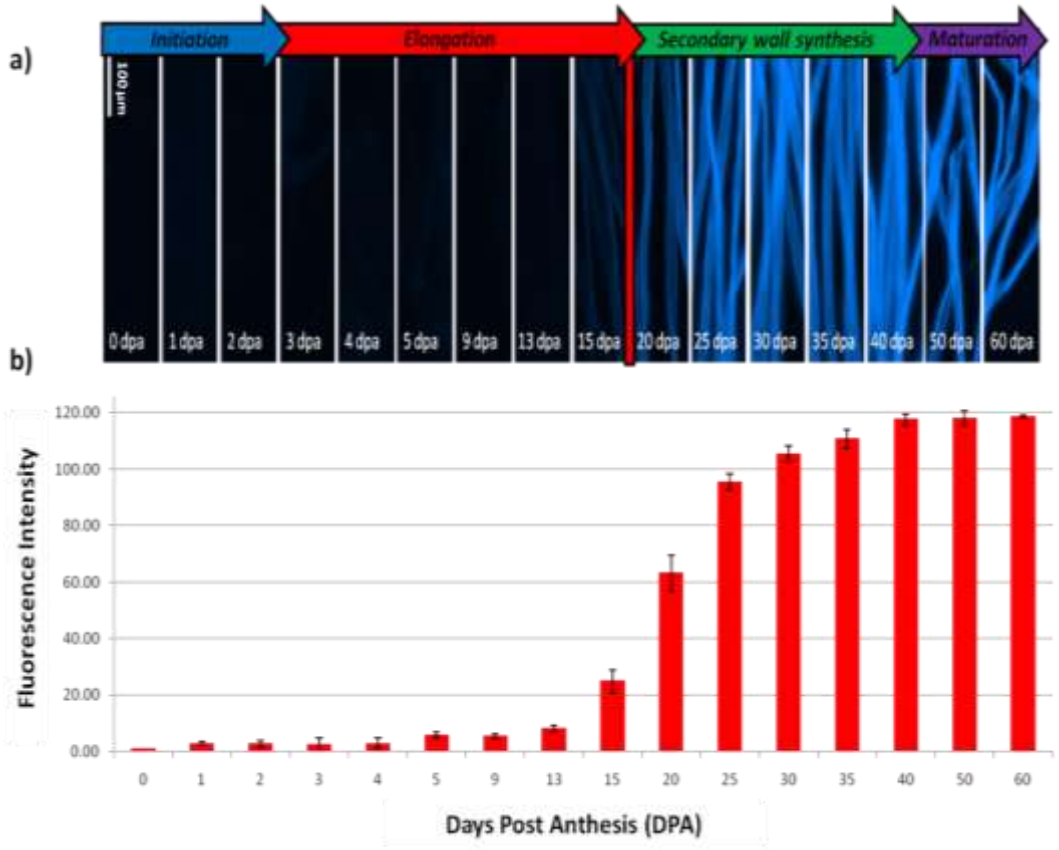

Figure 4.1: Calcofluor White staining to β-glycans of the cotton fibre during development. a) Cotton fibres were stained with Calcofluor White for 10 min with shaking, and micrographs captured under UV fluorescence for 0.5 ms. Equivalent exposure times and areas used. Banners at the top indicate the time points for developmental stages. In samples 0-1 dpa, fibres are not developed, so ovules are also present in the micrographs b) Image J quantification of Calcofluor White fluorescence. Micrograph fibre quantification was done in triplicate as described in the methods chapter. Scale bar: 100 µm.

Developing cotton fibres were labelled *in situ* with CBM3a to detect regions of crystalline cellulose (Figure 4.2). Crystalline cellulose was detectable from untreated 1 dpa fibres through till 15 dpa. By 20 dpa, the detection of cellulose decreases dramatically in untreated fibres, and from 25-40 dpa, it cannot be seen at all on the surfaces of untreated fibres. From 20 dpa, crystalline cellulose is fully revealed by pectate lyase treatment. This leads up to the hypothesis that the structure of the cell wall changes during cotton fibre development between 15-20 dpa.

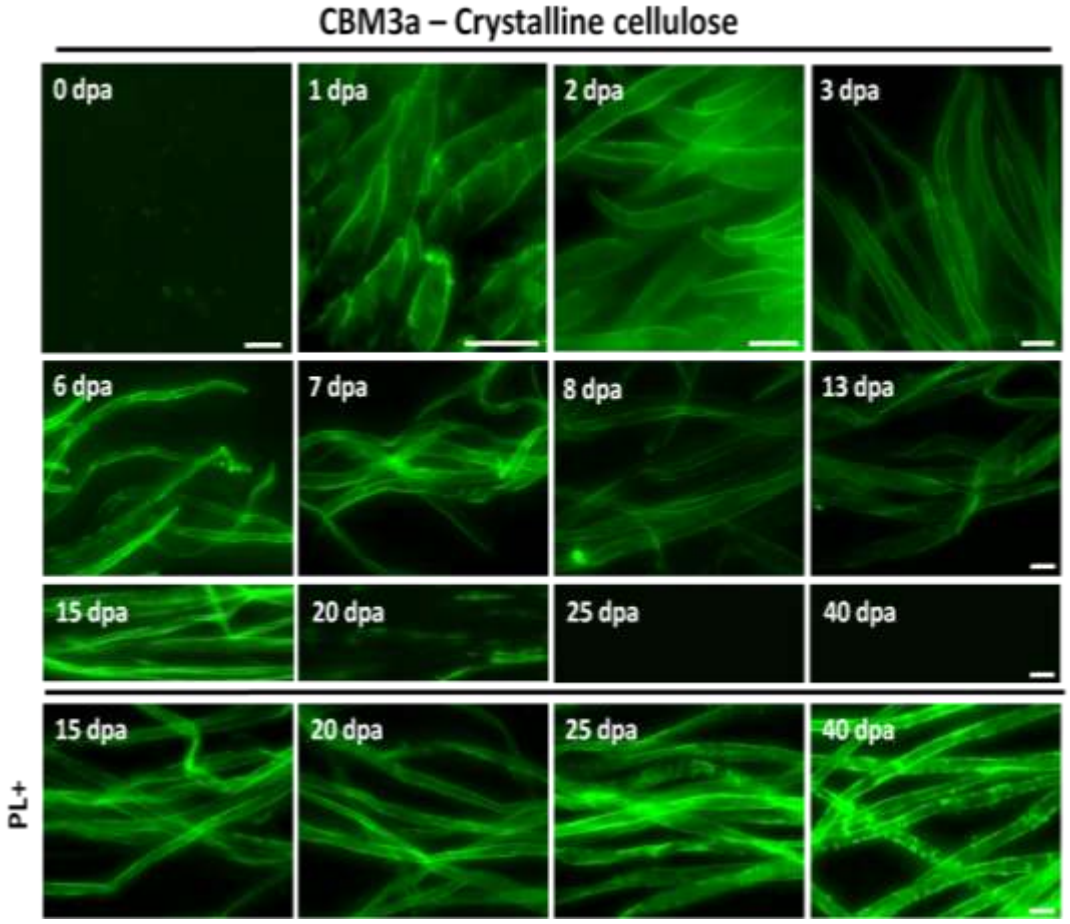

Figure 4.2: Indirect fluorescence detection of crystalline cellulose on the surfaces of untreated and pectate lyase-treated FM966 developing fibres. CBM3a detection was revealed fully after pectate lyase treatment from 20 dpa onwards. Pectate lyase action had no effect on cellulose detection from 0 dpa to 15 dpa. Scale bar: 20 µm.

In addition to cellulose, developing cotton fibres were also tested for the presence for callose (β-(1→3)glucans) and mixed-linkage glycans (β(1→3)(1→4)-glycans) on the surfaces and through transverse cell wall cross sections (Figure 4.3). In 20 dpa untreated cotton fibres, callose was detected along the surfaces and across whole transverse sections, while in 60 dpa mature fibres, there was very little detection at all, with sporadic labelling across the fibre cross section. After pectate lyase treatments, higher fluorescence intensity was seen on 20-60 dpa fibre surfaces, although the strongest detection of callose was seen in 20 dpa fibres. Pectate lyase treatment had no effect upon the fluorescence intensity in fibre cross sections throughout development. Mixed-linkage glucans, which are largely isolated to the flowering mono-cotyledon Poales group, were not detected at all during development on the surfaces or transverse cross sections, independent of pectate lyase treatment, which makes the MLG mAB an interesting and useful negative control in cotton fibres.

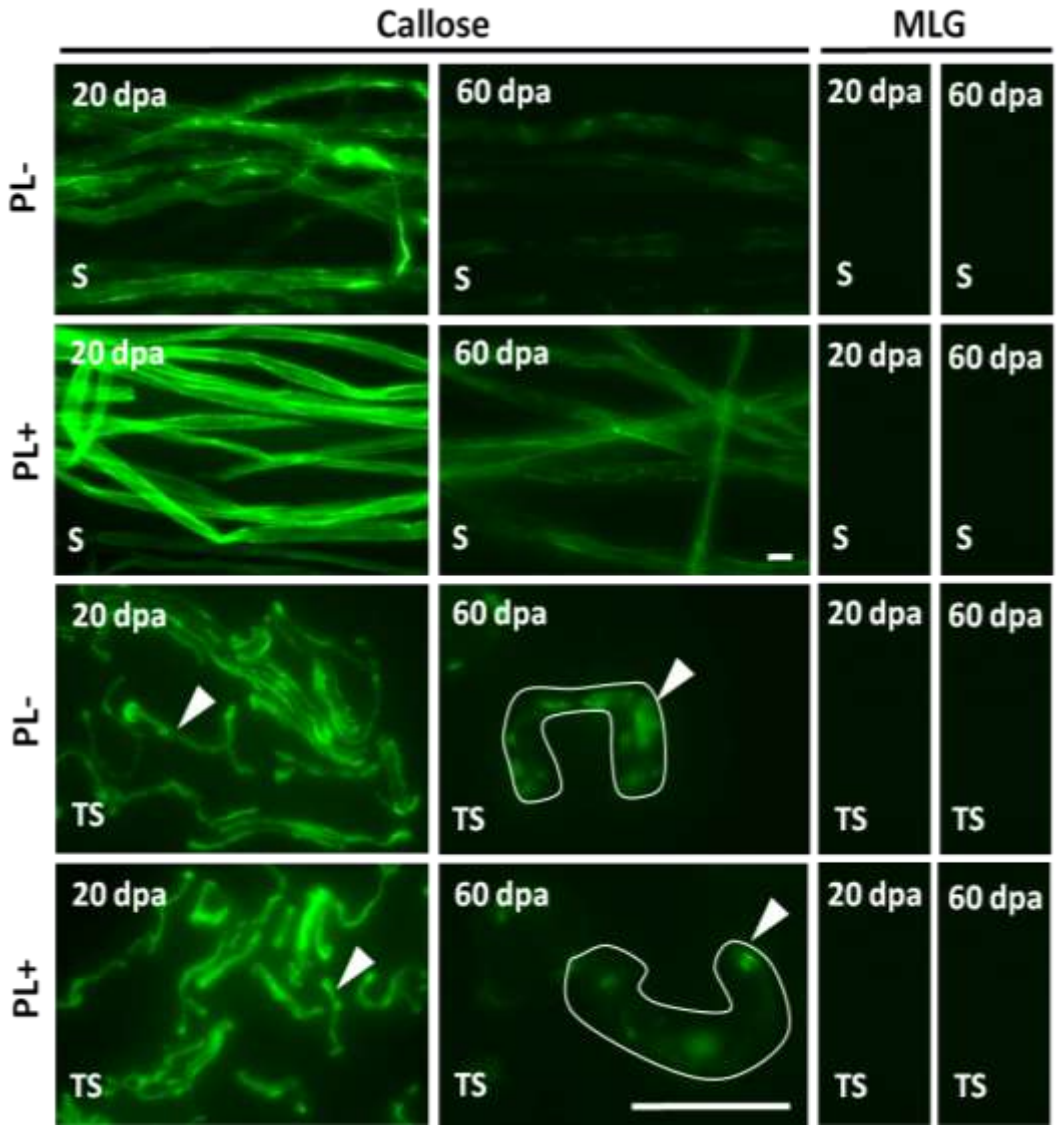

Figure 4.3: Indirect immunofluorescence detection of callose epitopes on the surfaces (S) and transverse sections (TS) of untreated and pectate lyase treated FM966 developing fibres. Increased fluorescence was seen after pectate lyase treatment for callose surface samples. Arrows indicate primary wall of the cotton fibre. Callose epitope cross section areas are highlighted with a white line to show the extent of the fibre. Mixed-linkage glucans were not detected in surface or transverse sections with any treatments. Scale bars: 20 µm.

4.3 Localisation, composition and changes in pectic HG in the developing cotton fibre

Intact and untreated developing cotton fibre surfaces between 0-60 dpa were screened with the monoclonal antibodies LM18, LM19 and LM20 (Verhertbruggen, et al., 2009) for pectic homogalacturonan (HG) (Figure 4.4). LM18 and LM19 epitopes are both present at 0 dpa until maturity at 60 dpa, with the latter showing stronger detection during the early stages of initiation and elongation (LM19, 0-3 dpa). The LM20 epitope however was not detected until 1 dpa, with peak intensity of epitope detection seen at 3 dpa. Using high magnification, the LM19 epitope labelling of 2 dpa developing cotton fibres showed the presence of small circular structures on the fibre surface, indicated with arrows in the inset 2 dpa image of Figure 4.4. By 25 dpa, where secondary wall synthesis will be in taking place, was when the peak detection of the LM18 and LM19 epitopes were detected, which remained this way till maturity at 60 dpa. The LM20 epitope however was no longer detected after 20 dpa. Intact and untreated developing cotton fibre surfaces between 0-60 dpa were also screened with the well characterised monoclonal antibodies JIM5 and JIM7 (Figure 4.5). For JIM5, low levels of epitope detection were seen throughout development, with peak intensity of fluorescence seen at 2 dpa and 17 dpa. The JIM5 epitope is very similar to LM18 in that they both bind to largely de-esterified regions on pectic homogalacturonan, albeit with very subtle differences in the binding profile, as highlighted in Chapter 3 (Figure 3.1). These subtle differences were seen in the fact that while low levels of the LM18 epitope are detected in early development like JIM5, a higher fluorescence intensity of the LM18 epitope is seen from 3 dpa through to maturity at 60 dpa. JIM7 is similar to LM20 and this is seen in the fluorescence intensity of the epitope detection. JIM7 fluorescence intensity is

generally weak throughout initiation and elongation and is very weak from 17 dpa. A similar result is seen with LM20, in which the fluorescence is relatively weak when it is present and disappears by 25 dpa (Figure 4.5). The surface detection of these epitopes with these five mABs reveal subtle information about the structure and composition of HG. From the strong epitope detection of LM18, LM19 and JIM5 throughout development, coupled with decreasing detection of JIM20 and JIM7 indicate that the mature fibre is largely made up of de-esterified pectin, which is initially in an esterified form during the early days of development.

High magnification micrographs of JIM5 and JIM7 epitopes between 9-17 dpa show the presence of longitudinal stripes along the fibre surfaces. These patterns were also seen with LM18, LM19 and LM20 epitopes under high magnification, and will be covered later in this chapter.

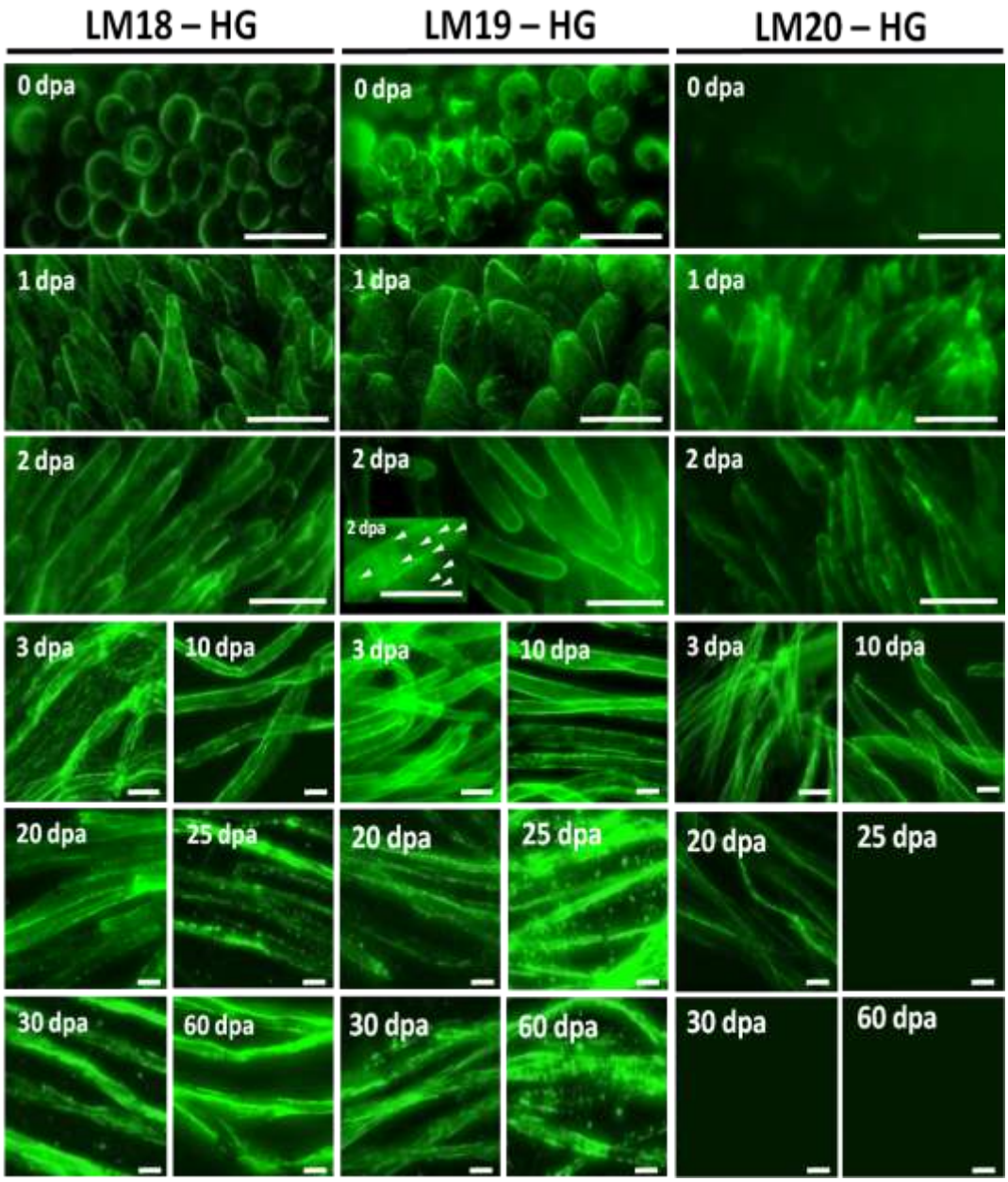

Figure 4.4: Indirect immunofluorescence detection of LM18, LM19 and LM20 pectic homogalacturonan (HG) epitopes on the surfaces of untreated FM966 developing fibres. Peak intensities of fluorescence was seen post-30 dpa for LM18, post-25 dpa for LM19 and 3 dpa for LM20. Individual fibres at 0-1 dpa are marked with an arrow. LM19 labelling 2 dpa fibres (inset) high magnification micrographs of individual ring structures (arrows). Scale bars: 20 µm.

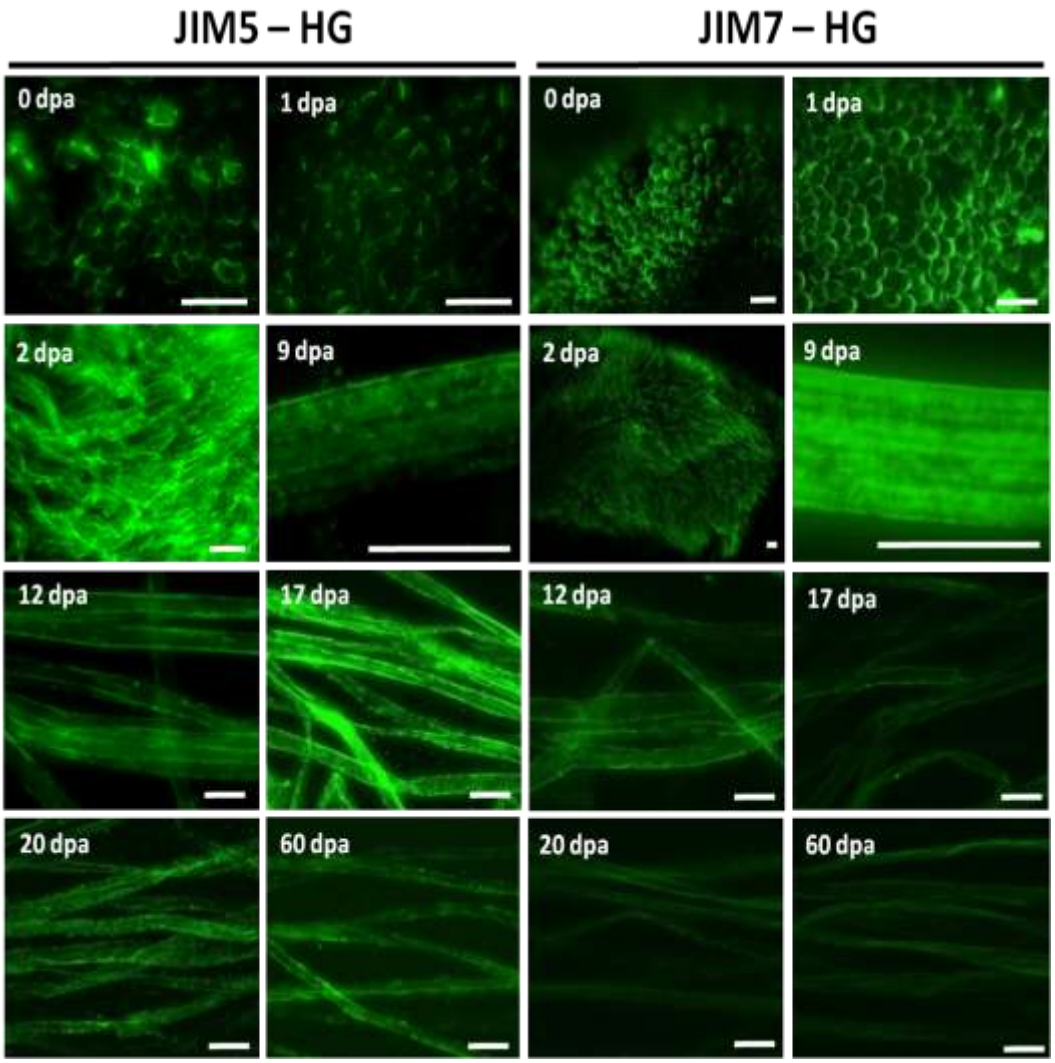

Figure 4.5: Indirect immunofluorescence detection of JIM5 and JIM7 pectic homogalacturonan (HG) on the surfaces of untreated FM966 developing fibres. Peak intensities of fluorescence were seen at 2 and 17 dpa for JIM5. JIM7 peak intensities were seen at 9 dpa. In both bases, fluorescence remains constant between 20 dpa and 60 dpa. Scale bars: 20 µm.

4.4 Location, composition and changes of RG-I related polysaccharides in developing cotton fibres

Developing cotton fibres were immunolabelled *in situ* with mAbs LM5 for the (1-4)-β-D-galactan epitope and LM6 for the (1-5)-α-L-arabinan epitope (Figure 4.6). In untreated fibre samples, the LM5 epitope was detected sporadically in the early developmental stages. From 4 dpa, when the developing fibres were long enough for detachment from the ovule, the localisation of the LM5 epitope in untreated samples was only detected at the base of these fibres. Pectate lyase treatment of the fibres revealed the LM5 galactan epitope along the whole fibre. After 20 dpa, however LM5 was not detected at all in untreated or pectate lyase treated developing cotton fibres. The LM6 arabinan epitope was also sporadic along all the fibres in the untreated, early stages of 0 dpa. Between 6 and 15 dpa, the LM6 epitope was detected in a very similar way to that of the LM15 xyloglucan epitope, in the form of thin, longitudinal bands along the fibre length. Pectate lyase treatments revealed the LM6 epitope along all of the fibre, with a punctate appearance at 25 dpa and an overall smooth detection in 60 dpa.

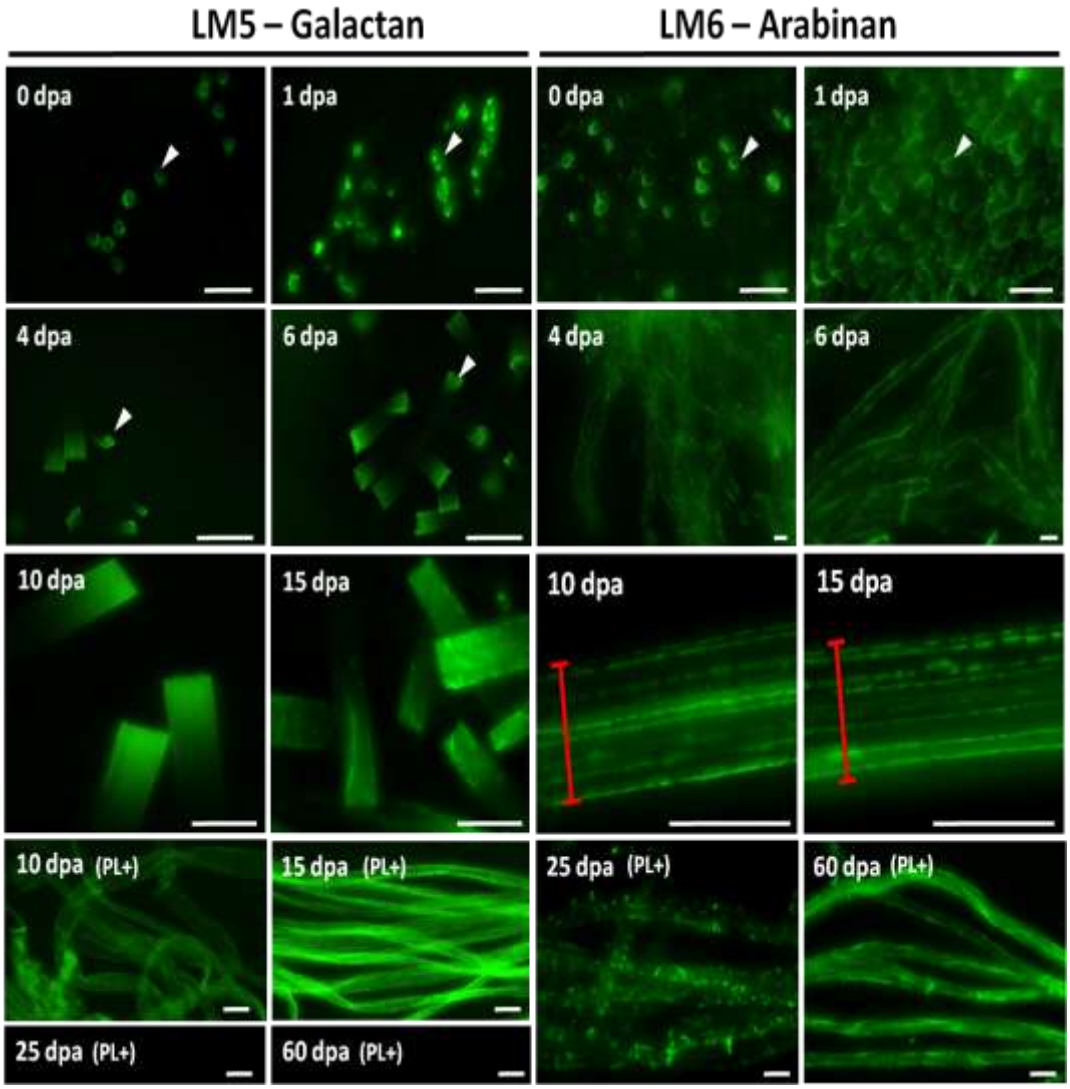

Figure 4.6: Indirect fluorescence detection of galactan and arabinan epitopes on the surfaces of untreated and pectate lyase-treated FM966 developing fibres. . The LM5 galactan epitope detection was revealed fully after pectate lyase treatment throughout development until 15 dpa. The LM6 arabinan epitope was present throughout development, but after 15 dpa, pectate lyase was needed to detect the epitope. Red bars indicate the diameter of one fibre. Scale bar: 20 µm.

4.5 Location, composition and changes of xyloglucan in developing cotton fibres

Intact fibres labelled *in situ* with LM15, a monoclonal antibody that bind to XXXG motif xyloglucan (Marcus *et al.* 2008) and CCRC-M1 (Not shown) (Pullman *et al*. 1994), showed very weak or no binding. Xyloglucan epitopes were revealed and detected in abundance after the removal of pectic HG. Xyloglucan is one of the most abundant non-cellulosic polysaccharides in cotton fibres (Hayashi and Delmer, 1988), and much like in mature 60 dpa FM966 cotton fibres, pectate lyase is required for full epitope detection during development (Figure 4.7). Without pectate lyase treatment, the occurrence of the LM15 XXXG xyloglucan epitope was weak and sporadic along the whole fibre, however between 12-17 dpa, the untreated developing fibres exhibited clearly defined, thin longitudinal bands of the LM15 epitope fluorescence. After pectate lyase treatment, the days for peak LM15 XXXG xyloglucan epitope detection were 25 dpa and 35-60 dpa (Figure 4.7). For most cases of pectate lyase treated developing FM966 cotton fibres, the epitopes were detected in a even, consistent layer all along the fibre, with some areas of sporadic detection along the fibre. In addition to *in situ* surface labelling, xyloglucan epitopes were systematically mapped with the development of two new monoclonal antibodies, called LM24 and LM25. Due to time constraints, developing fibre polysaccharides were extracted with CDTA and NaOH and analysed using the comprehensive microarray polymer profiling (CoMPP) technique (Moller et al., 2007). Initial *in* vitro screens of CDTA and NaOH extracts from developing cotton fibre cell walls were carried out with the xyloglucan probes LM15, LM24 and LM25 (Figure 4.8). The LM25 epitope was the only xyloglucan mAb detected in the CDTA fraction, albeit at very low levels, with peak detection occurring between 25-30 dpa. In the NaOH extracts, the

LM25 and LM15 xyloglucan epitopes were abundantly detected throughout development, reaching a peak between 20-25 dpa. The LM15 epitope however was not detected as strongly during the early days of development of 15-20 dpa when compared to the LM25 epitope. Very low levels of the LM24 epitope were detected in the NaOH extracted material between 15-20 dpa, after which no signal was detected.

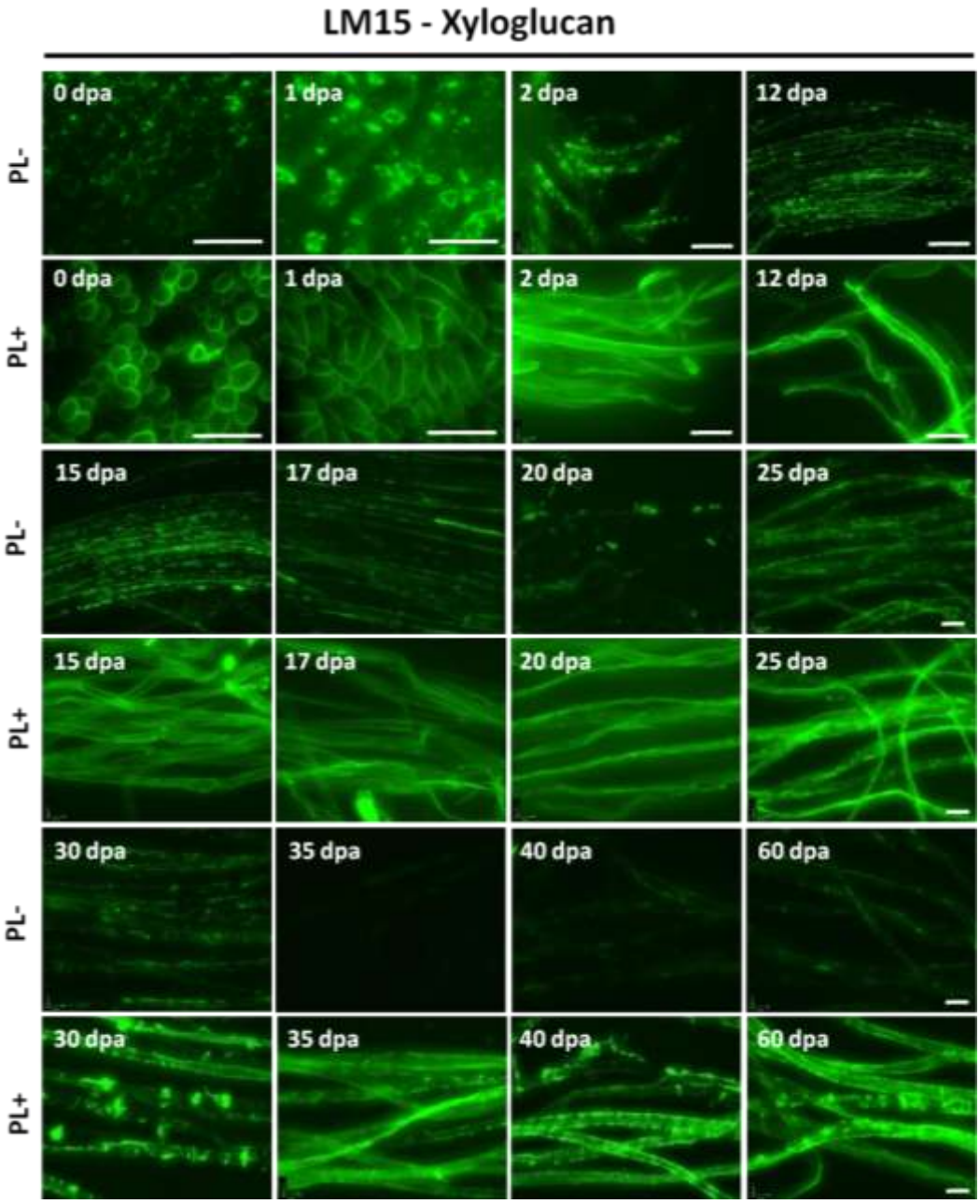

Figure 4.7: Indirect fluorescence detection of XXXG xyloglucan on the surfaces of untreated and pectate lyase-treated FM966 developing fibres. LM15 epitope detection was revealed fully after pectate lyase treatment throughout development. Due to time restrictions, the two new xyloglucan mAbs LM24 and LM25 were not used with *in situ* surface labelling, but instead were used with the CoMPP assay. Scale bar: 20 µm.

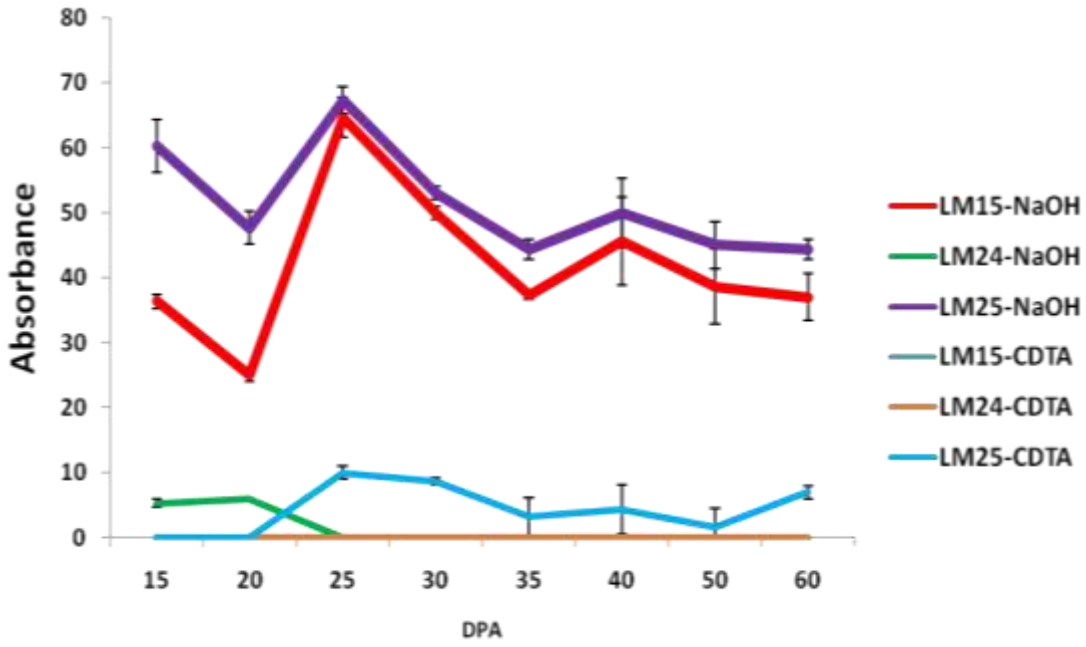

Figure 4.8: Graphical representation of a comprehensive microarray polymer profiling (CoMPP) heat map for xyloglucans during cotton fibre development. CDTA and NaOH extracts from developing fibre cell wall were used. References for probe specificity are listed Chapter 2. DPA starts at 15 due to limited sample material of very young developing cotton fibres. Extraction and CoMPP performed by Dr Julia Schückel.

4.6 Detection of homogalacturonan, xyloglucan and arabinan epitopes correlate with the presence of the CFML

Antibodies binding to pectic homogalacturonan, xyloglucan and arabinan highlighted the presence of unique longitudinal structures all the way along developing cotton fibres that were midway through the elongation stage of fibre development. All these fibre samples were bound together in bundles when harvested from the fruit bolls, so before immunolabelling, individual fibres were teased apart for *in situ* mAB binding. Pectic homogalacturonan probes LM19 (not shown), LM18, LM20, JIM5 and JIM7 were detected in thick longitudinal bands approximately 2-3 μm in diameter (Figure 4.9a;i-iv). Additionally, LM15 for the XXXG conformation of xyloglucan and LM6 for arabinan were detected in the form of thin longitudinal bands along the fibre surfaces, approximately 1 μm in diameter. Pectate lyase treatment revealed the LM15 and LM6 epitopes to be all over the fibre. This indicated that the thick black regions in-between the LM15 and LM6 epitopes must be composed of pectic homogalacturonan. To elucidate the structure-function relationship of these longitudinal pectic HG bands in the elongation stage of cotton fibre development, samples were treated with the calcium ion-chelator CDTA for up to 12 hours (Figure 4.9b). Untreated 14 dpa fibre bundles remain aggregated after extraction from the boll fruit, illustrated by the brightfield schematic in Figure 4.9b. Fibre bundles treated with 500 mM of CDTA for 1 h and 12 h exhibited cell wall separation with more individual and non-adhered developing cotton fibres visible.

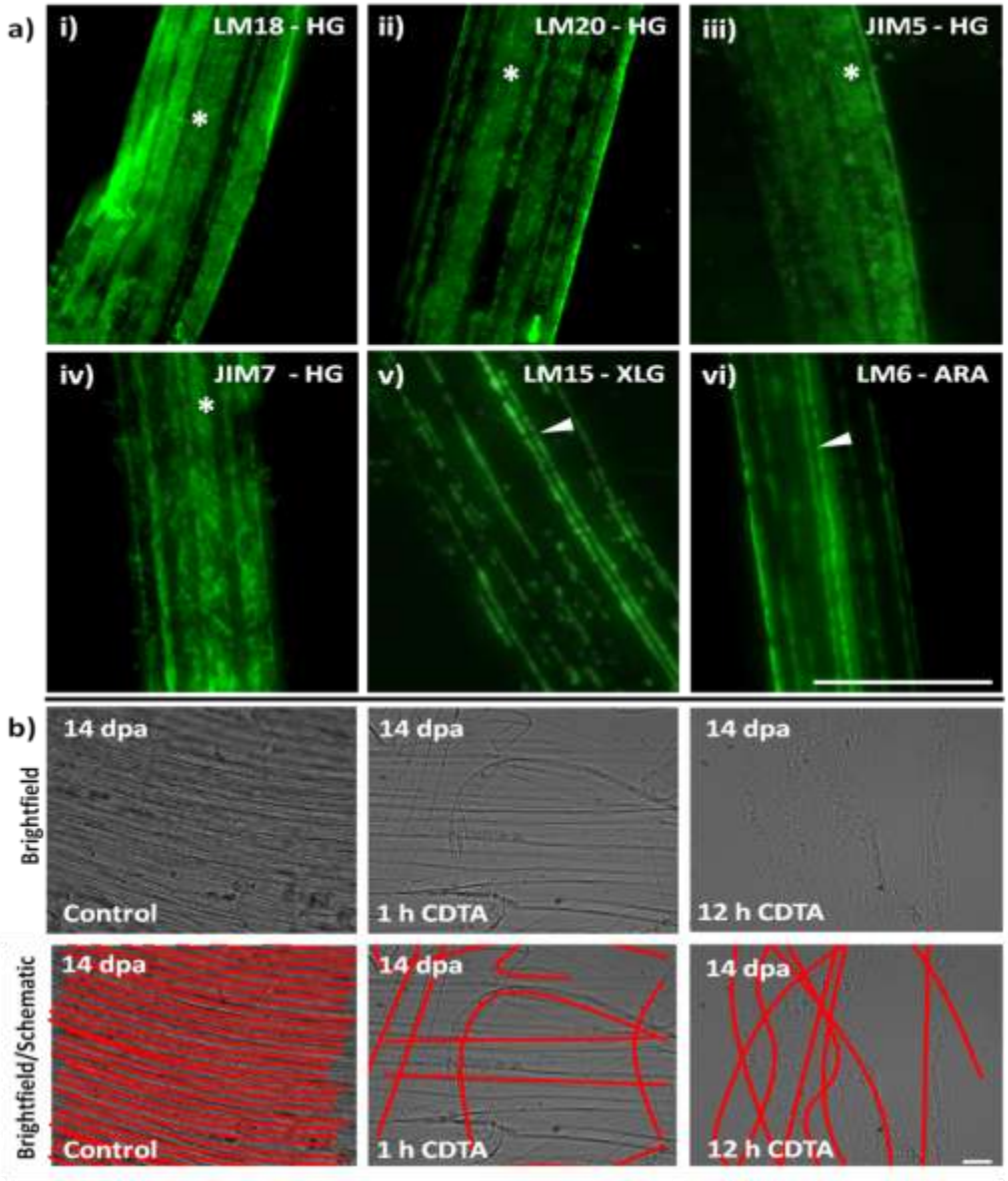

Figure 4.9: a) Indirect immunofluorescence detection of pectic HG , xyloglucan (XLG) and arabinan (ARA) epitopes on the surfaces of developing FM966 cotton fibres. Monoclonal antibodies for pectic HG: JIM5, JIM7, LM20 and LM18 localise in thick banded regions (*) along the developing cotton fibre. Xyloglucan (LM15) and arabinan (LM6) epitopes localised in thin bands (arrows) along the developing cotton fibre. Samples were 15 dpa cotton fibres. **b)** Analysis of developing fibre adhesion cell wall. 14 dpa fibres were treated with CDTA for 0, 1 and 12 h analyse the nature of developing fibre cell wall adhesion. Brightfield micrographs were captured for untreated and CDTA treated samples (top row), with brightfield micrograph schematics of fibres shown in the bottom row of images. Scale bars: 20 µm.

4.7 Detection, localisation and changes in heteromannans during cotton fibre development

As mentioned previously, there was currently no evidence on mannans being present in cotton fibres, and in the previous chapter it was shown that in the mature 60 dpa cotton fibre, mannans are present in a potentially putatively acetylated form that is enveloped in a pectic sheath that does not covalently mask the LM21 epitope. To study this further, developing cotton fibres were labelled in *situ* with LM21 (Figure 4.10). Additionally, fibres were treated with sodium carbonate and pectate lyase. Detection of the LM21 epitope was only possible after the use of both sodium carbonate and pectate lyase throughout all stages of development, suggesting that these mannans are also acetylated and sheathed by the presence of a pectic layer. The LM21 epitope is present from 0 dpa to 20 dpa, after which the fluorescence intensity decreases until maturity at 60 dpa. Cell wall material was extracted from developing cotton fibres with CDTA and NaOH treatments and then analysed *in vitro* with a CoMPP (Moller et al., 2007) assay (Figure 4.11). An overall decrease in heteromannan content was seen as development progressed, with the exception of the 25 dpa sample, in which the peak detection of LM21 was seen.

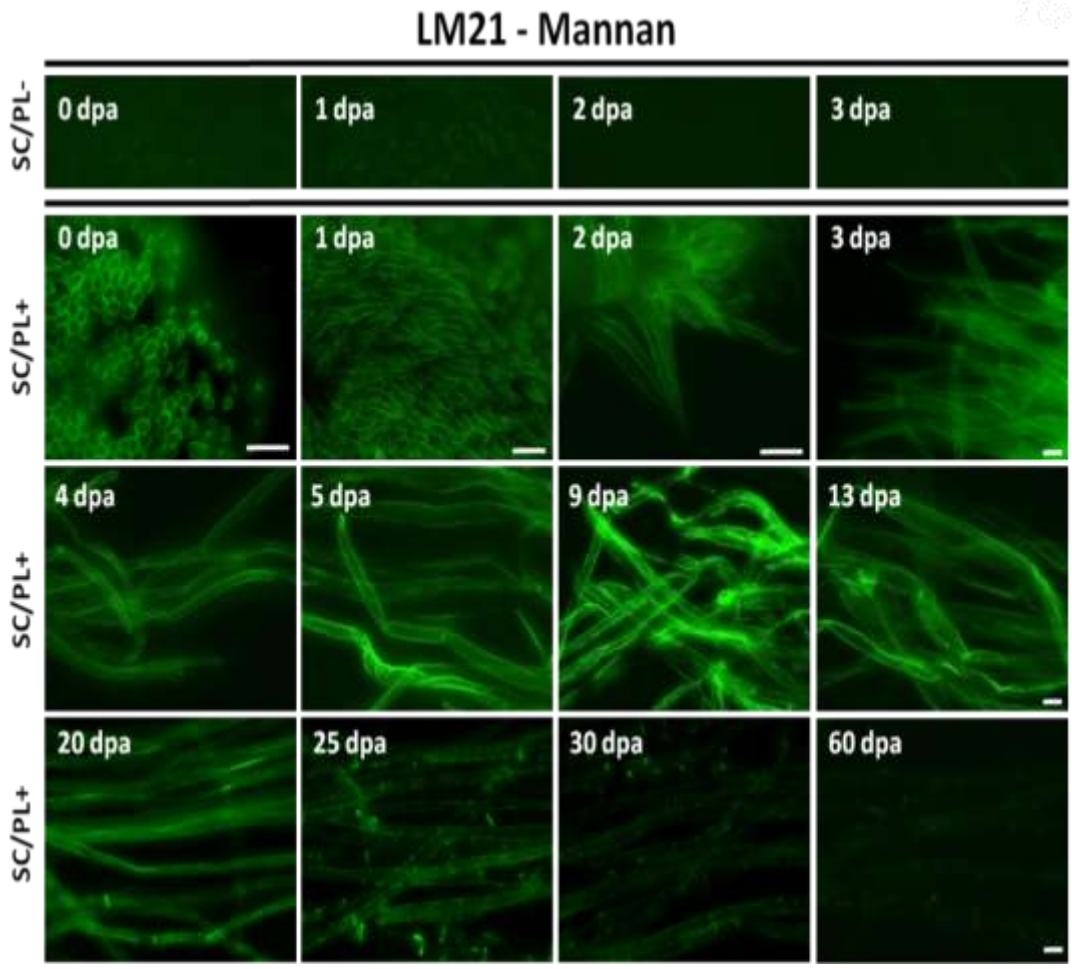

Figure 4.10: Indirect immunofluorescence detection of LM21 hetero-mannan on the surfaces of untreated and pectate lyase treated FM966 developing fibres. The LM21 epitope was fully revealed after pectate lyase (PL+) and sodium carbonate (SC) treatment for all stages of development. Peak intensities of fluorescence were seen between 5-9 dpa. Fluorescence decreased from 20 dpa for LM21. NB heteromannans were detected in 60 dpa fibres at 100 ms exposure in Chapter 3. However for mannans in development, for comparison a 20 ms exposure time was used, hence the appearance of very little mannan in 60 dpa fibre samples in this chapter. Scale bars: 20 µm.

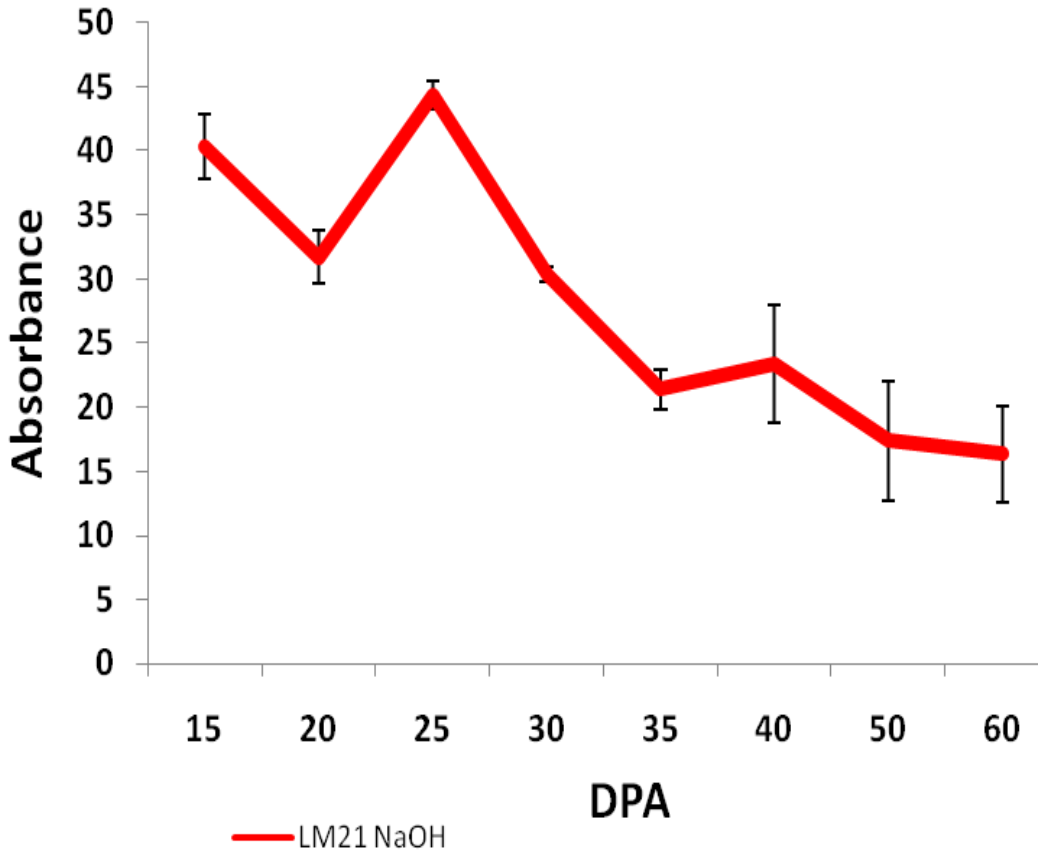

Figure 4.11: Graphical representation of a comprehensive microarray polymer profiling (CoMPP) heat map for heteromannan during cotton fibre development. CDTA and NaOH extracts from developing fibre cell wall were used. References for probe specificity are listed Chapter 2. DPA starts at 15 due to limited sample material of very young developing cotton fibres. Extraction and CoMPP performed by Dr Julia Schückel.

4.8 Analysis of AGP and extensin glycoprotein epitopes in developing cotton fibres

In addition to the white FiberMax cotton line, two different coloured lines of cotton fibres were initially analysed, covering naturally green and red varieties (Figure 4.12). These were grown and samples harvested during fibre development to study the cell wall glycoproteins, comprising of AGPs and extensins. Proteins were extracted using SDS buffer from homogenised fibre samples and the approximate concentrations calculated using the Bradford assay (Bradford, 1976). White FM966 and green *G. hirsutum* cotton fibres had abundant levels of Bradford assay-detectable proteins at 10 dpa. In FM966 white fibres, the protein concentrations gradually decreased throughout development, while green *G. hirsutum* protein concentrations dropped sharply by 15 dpa. In *G. barbadense* red fibres however, relatively low levels of proteins were detected throughout development, even with repeated extractions.

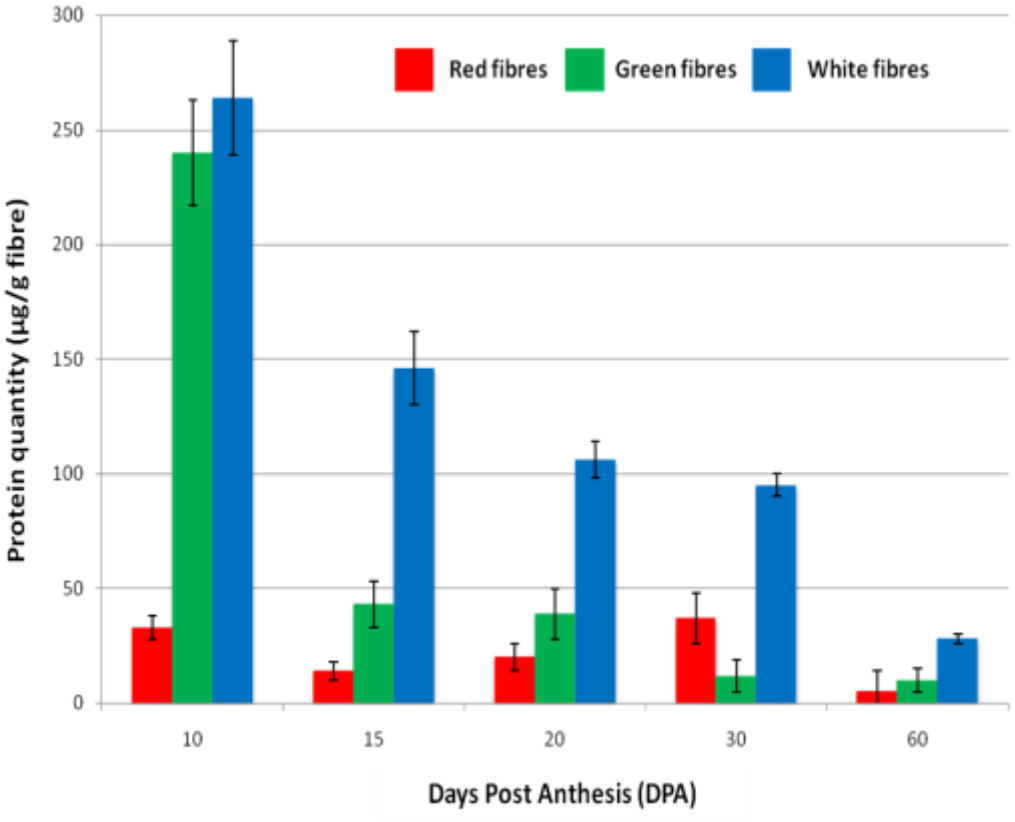

Figure 4.12: Bradford protein analysis of three lines of developing cotton fibres. Bradford assay of *G. barbadense* (Red fibre line), *G. hirsutum* (Green fibre line) and *G. hirsutum* (White fibre line, FM966). Samples were done in quadruplicate from 1 g fibre samples, extracted with 1ml of buffer.

The Bradford assays above show the approximate concentration of all proteins extracted from the developing fibres. Using just FM966 *G. hirsutum* fibres, monoclonal antibodies directed to extensin and AGP epitopes were used in the ELISA *in vitro* analysis of developing fibre cell wall extracts. The ELISA binding analysis showed for all probes that cell wall glycoprotein content decreases as fibre development occurs, with more AGP epitopes being detected with a stronger signal overall compared to extensin epitopes (Figure 4.13). For extensins, LM1, JIM20 and JIM11 were the main epitopes detected, while for AGPs, JIM13, MAC207, LM2, LM14 and JIM16 had the strongest signals. For LM1 and JIM20,

there was a sharp drop in epitope detection between 15 and 30 dpa (end of elongation and start of the secondary wall synthesis stage), while LM1 almost disappears upon fibre maturity at 60 dpa and low levels of JIM20 detected at 60 dpa. The JIM11 epitope has a relatively sharp drop between 9 and 15 dpa (elongation stage), and then again between 15 and 30 dpa. JIM12 and JIM19 were detected in very low levels. For AGP epitopes, the most abundantly detected epitope was for JIM13, with the biggest decrease seen between 15-30 dpa and 30-60 dpa. For the other AGP mAB MAC207, LM2, LM14 and JIM16 were all detected quite strongly, but had a noticeable decline between 9-15 dpa only. These mAbs for extensin and AGP epitopes were also directed against developing cotton fibre protein extracts on nitrocellulose dot blots (Figure 4.14). Using the previously mentioned mAbs, the blot results were very similar to the ELISA data in Figure 4.13, however, the dot blots have the advantage of showing polysaccharide immunoprofiling (Willats & Knox 1999) with several probes, potentially indicating the presence of multiple glycoprotein epitope populations. Much like the ELISA, JIM12 and JIM19 for extensin epitopes and JIM4 for AGP epitopes were not detected at all in the dot blots. Polysaccharide immunoprofiling was seen for LM1, JIM20, JIM11, JIM13, LM2, LM14 and JIM16 indicating the presence of multiple versions of the glycoprotein epitope populations. Developing 9 dpa cotton fibres give the strongest signal, however by 15 dpa, only small amounts of the LM1, JIM20, JIM11, JIM13, MAC207, LM2 and LM14 epitopes were detected. By 30 dpa, there was no epitopes detection on the dot blots at all. From this it can be seen that the ELISA is more sensitive to the detection of extensin and AGP epitopes, as even at 60 dpa, ELISAs can detect the developing fibre cell wall glycoprotein extracts.

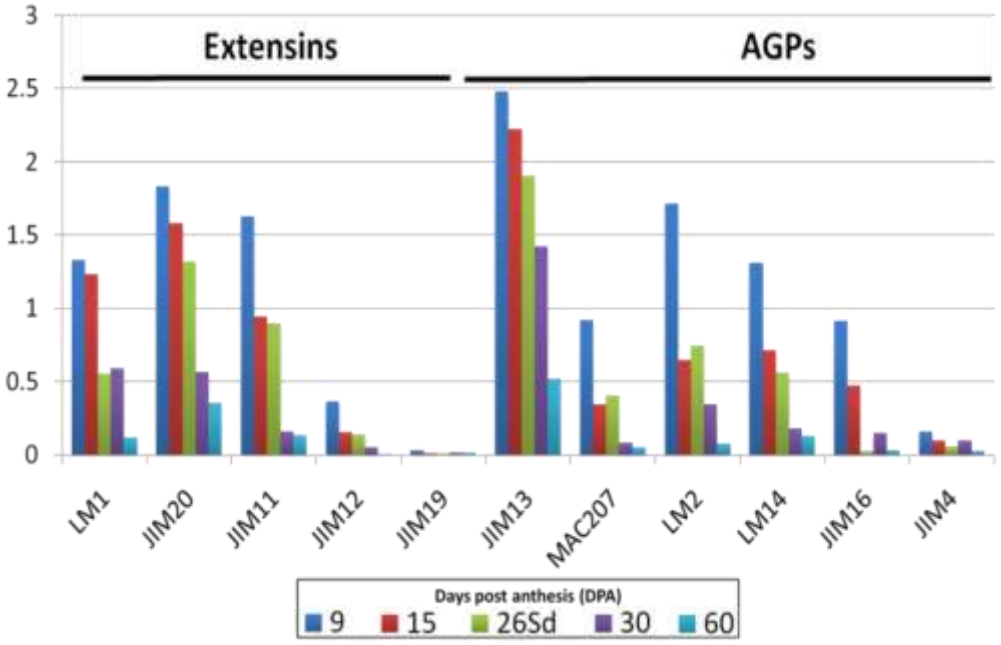

Figure 4.13: ELISA analysis of glycoprotein-directed monoclonal antibodies to cell wall extracts from developing cotton fibres. LM1, JIM20, JIM11, JIM12, JIM19 (Extensins), JIM13, MAC207, LM2, LM14, JIM16, JIM4 (AGPs) to solubilised fibre cell wall material. 1 g of homogenised FM966 cotton fibre material was used per 10 ml of buffer. Plates coated using a 10-fold dilution of extraction buffers and monoclonal antibodies used at a 5-fold dilution.

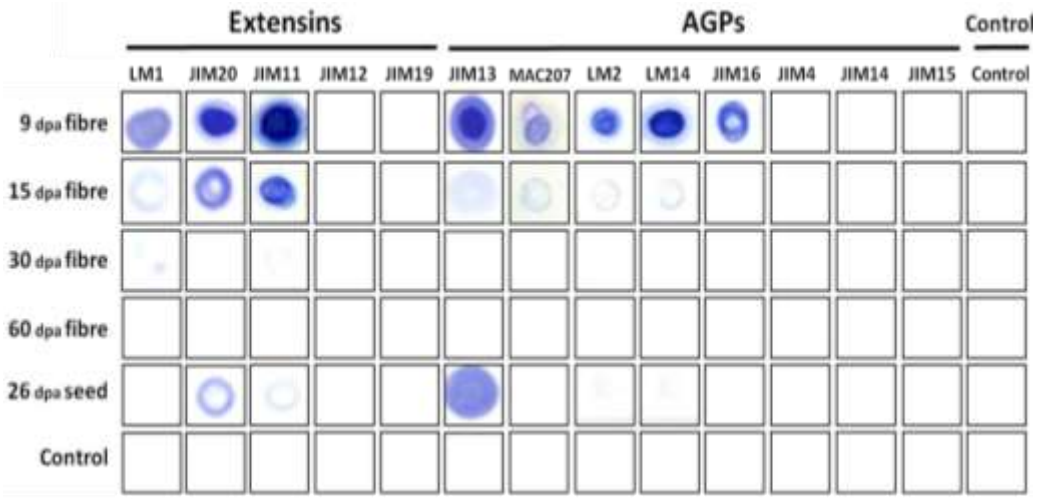

Figure 4.14: Immunodot assay detection of glycoprotein epitopes extracted from the developing cotton fibre cell walls. Immunodot assays of monoclonal antibodies LM1, JIM20, JIM11, JIM12, JIM19 (Extensins), JIM13, MAC207, LM2, LM14, JIM16, JIM4, JIM14 and JIM15 (AGPs) to material solubilised from 10 mg/ml homogenised developing cotton fibre cell walls. Polysaccharide immunoprofiling was seen in with probes 2 (JIM20 - Extensins), 3 (JIM11 - Extensins), 6 (JIM13 - AGPs) and 9 (LM14 - AGPs).

The protein extracts from the developing cotton fibres were used with SDS-PAGE and western blot analysis, using the mAbs LM1 and JIM20 for the extensin epitopes as well as the JIM13 AGP epitope (Figure 4.15). For the SDS-PAGE analysis (Figure 4.15a), the 9 dpa developing fibre cell wall sample was stained the most with Coomassie blue, gradually getting weaker through 15 dpa to 60 dpa. Taking a closer look at the 9 dpa sample, many individual protein bands can be seen from the Coomassie blue staining of the smear. The mAbs LM1 and JIM20 for extensin epitopes and JIM13 for AGP epitopes were selected for western blot analysis of the SDS gels (Figure 4.15b). For all epitopes, the largest amounts of glycoproteins were seen in the 9 dpa developing fibre cell wall extracts. LM1 for the extensin epitope detected the presence of two distinct bands that gradually got weaker through 15-30 dpa. Four bands of the JIM20 extensin epitope were detected in 9 dpa developing fibre cell wall extracts, which decreased in quantity and intensity to three bands by 15 dpa. A further decrease in quantity and intensity to two bands was also seen by 30 dpa. For the JIM13 AGP epitope, five bands were seen in the smear at 9 dpa. Much like JIM20, a further decrease in quantity and intensity to four bands was also seen by 15 dpa, and then again to three bands by 30 dpa. To further analyse the cell wall glycoprotein profiles of developing cotton fibres, FM966 samples harvested *in planta* were immunolabelled *in situ* with mAbs LM1 and JIM20 for extensin epitopes. In untreated fibre samples, the JIM20 extensin epitope was not detected at all throughout fibre development (Figure 4.16). After treating developing fibres with pectate lyase, the JIM20 extensin epitope was detected throughout development. Although at 15 dpa the fluorescence is weak overall, the JIM20 extensin epitope was strongly detected and localised to approximately 30% of the fibre tips. The fluorescence intensity,

however, reaches its peak at 30-60 dpa; with a more punctuate appearance from 35 dpa. For the LM1 extensin epitope, much like JIM20, was not detected at all in untreated developing FM966 cotton fibres (Figure 4.17). After the enzymatic degradation of pectic homogalacturonan by pectate lyase, the LM1 epitope was detected strongly in 0-3dpa and extremely weak for 5-60dpa in developing FM966 cotton fibre samples. Additionally, by 60 dpa the LM1 epitope while still very weak, developed a punctate appearance.

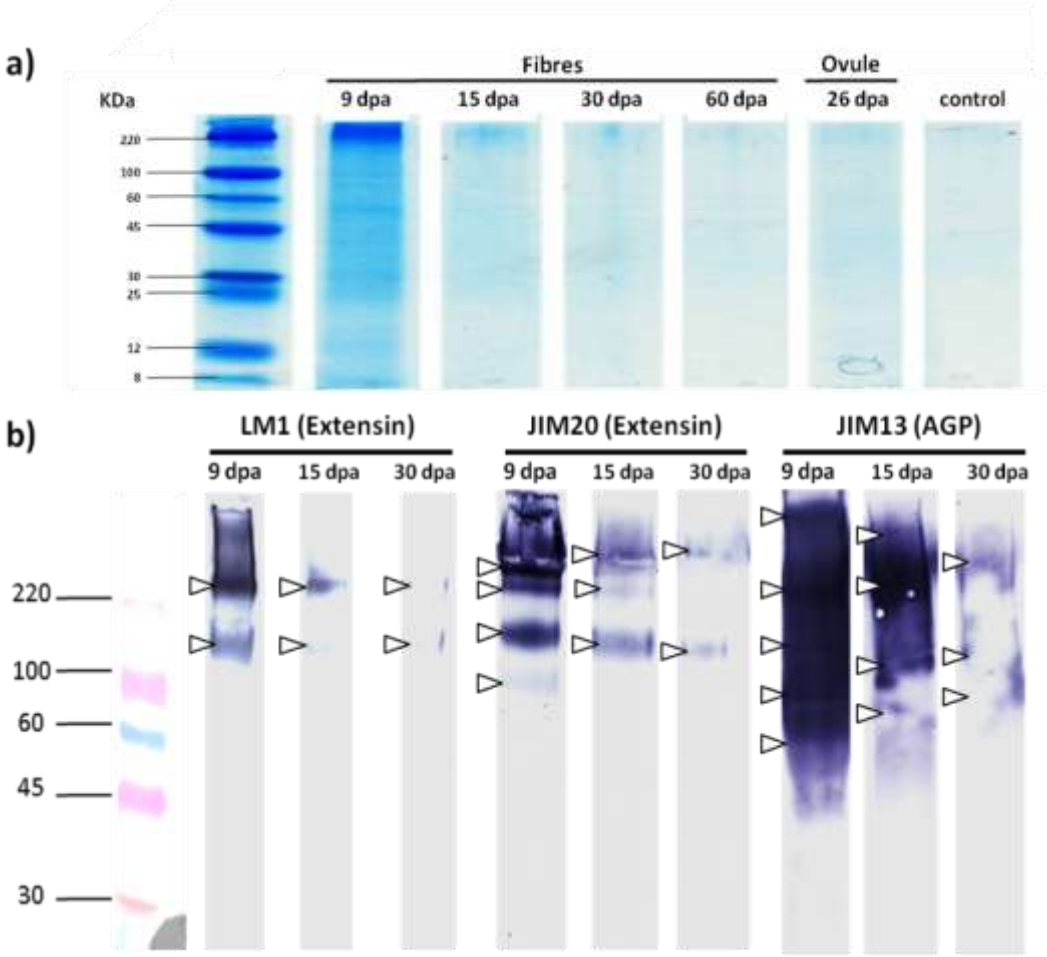

Figure 4.15: SDS-PAGE and Western blot analysis of protein extracts from developing cotton fibres. a) SDS-PAGE analysis of protein extracts from developing cotton fibres. The gel was stained with Coomassie Brilliant Blue. b) Extensin-directed monoclonal antibodies LM1 and JIM20, as well as the AGP-directed monoclonal antibody JIM13 were selected from the IDA assay to be analysed with western blotting. Samples 9, 15 and 30 dpa were used. Bands annotated with arrows.

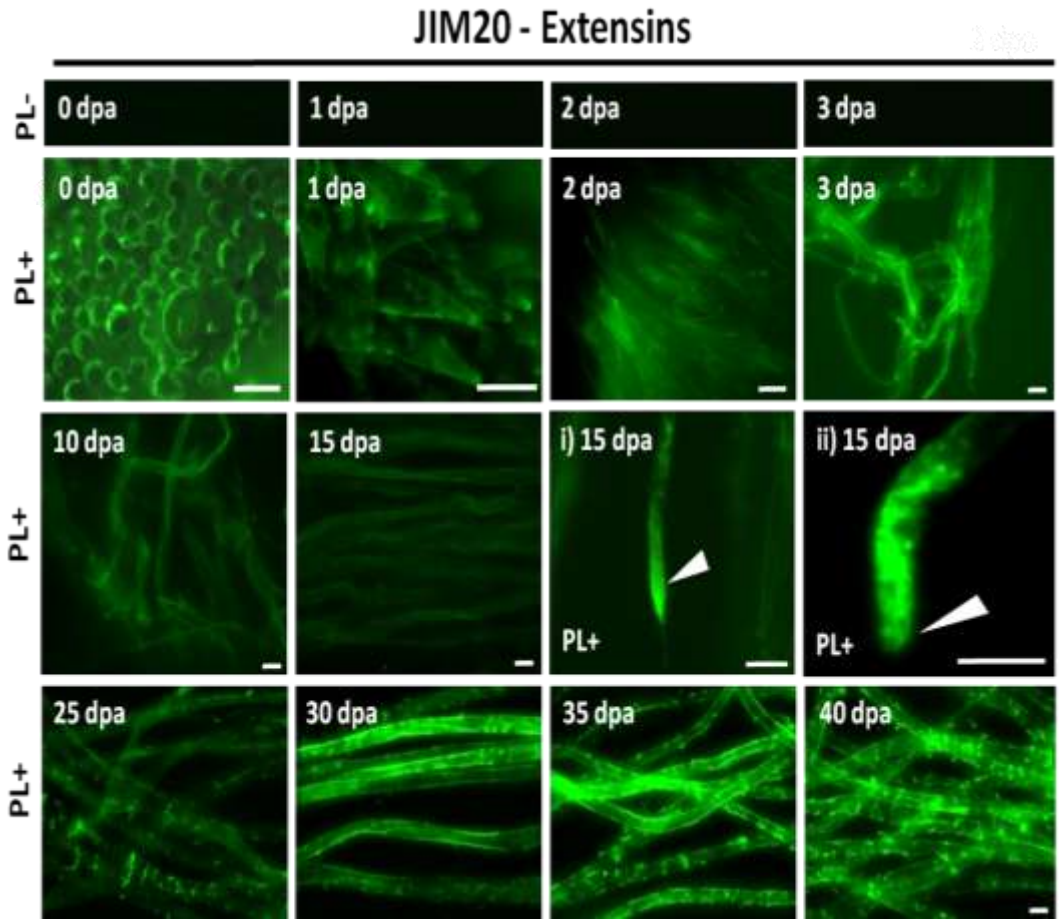

Figure 4.16: Indirect immunofluorescence detection of JIM20 AGP epitopes on the surfaces of untreated and pectate lyase treated FM966 developing fibres. The JIM20 epitope was fully revealed after pectate lyase treatment for all stages of development. Small equivalent areas are shown for PL- in early days of fibre development. Peak intensities of fluorescence were seen post 35 dpa. Scale bars: 20 µm.

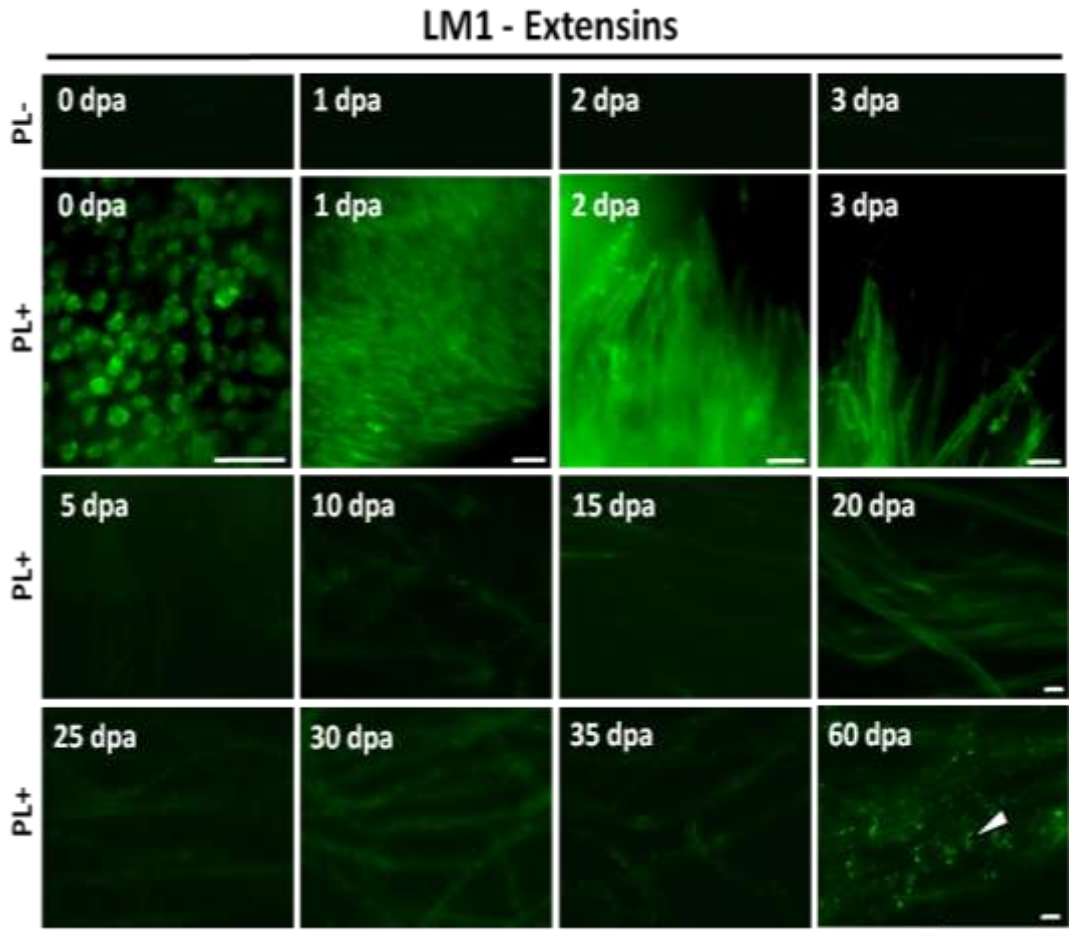

Figure 4.17: Indirect immunofluorescence detection of the LM1 extensin epitopes on the surfaces of untreated and pectate lyase treated FM966 developing fibres. The LM1 epitope was fully revealed after pectate lyase treatment between 0-3 dpa, with weak fluorescence detection with PL+ post 5 dpa. Arrow indicates the punctuate appearance of the LM1 epitope in 60 dpa mature cotton fibres. Peak intensities of fluorescence were seen post 35 dpa. Scale bars: 20 µm.

Additionally, preliminary SDS-PAGE protein gels and western blots were carried out with non-glycoprotein related monoclonal antibodies to determine whether any polysaccharides are associated with the extensin and AGP components in the form of AGP polysaccharide complexes (APCs) (Tan et al., 2012). Figure 4.18 suggests that the LM6 epitope of short chain arabinans and the INRA-RU2 RG-I backbone epitope are associated with several populations of (glyco)proteins in the cotton fibre cell wall. The LM6 epitope was detected in two main bands, which got weaker as development progressed, disappearing by maturity. The INRA-RU2 RG-I backbone epitope was detected very strongly, with several distinct bands, however the signal increased at 15 dpa, suggesting that there are larger amounts of the RG-I backbone intimately associated with (glyco)proteins at this stage of development.

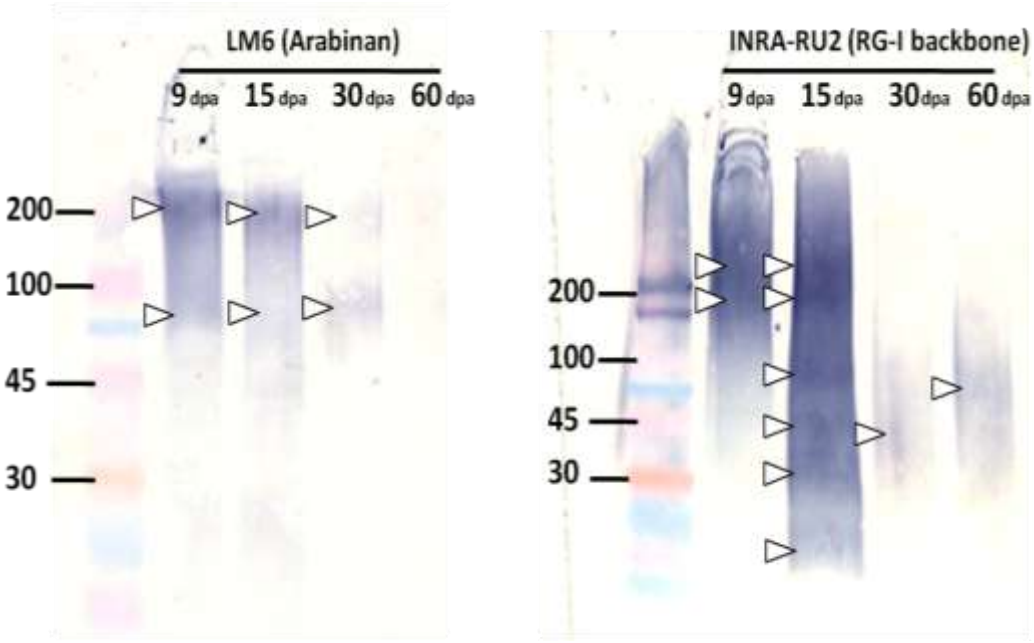

Figure 4.18: SDS-PAGE and Western blot analysis of protein extracts from developing cotton fibres using non-glycoprotein monoclonal antibodies. Positive blots were found using LM6 for arabinan and INRA-RU2 for the RG-I backbone. Negative blots were seen with LM5, LM10, LM11, LM15, LM21, LM18, LM19 and LM20. Samples 9, 15, 30 and 60 dpa were used. Bands annotated with arrows.

4.9 Preliminary studies into high-throughput approaches for developing cotton fibre cell walls

Figure 4.19 below gives a preliminary analysis of what this approach would show, and suggests that a high throughput technique of many species and developmental stages of cotton fibres using monoclonal antibodies can be realised (Pedersen et al., 2012). While only CDTA and NaOH were used to extract the cell wall polysaccharides from developing cotton fibres, this technique could be expanded with other chemical as well as enzymatic extractions. In the CDTA extraction, mostly pectic homogalacturonan and glycoprotein epitopes were detected, although interestingly arabinans, galactans and xyloglucans were detected in the green fibre CDTA samples. In the NaOH extraction, xyloglucan was abundantly detected, especially in the green fibre samples. Additionally, heteromannans were also detected, which decrease in content as development progressed.

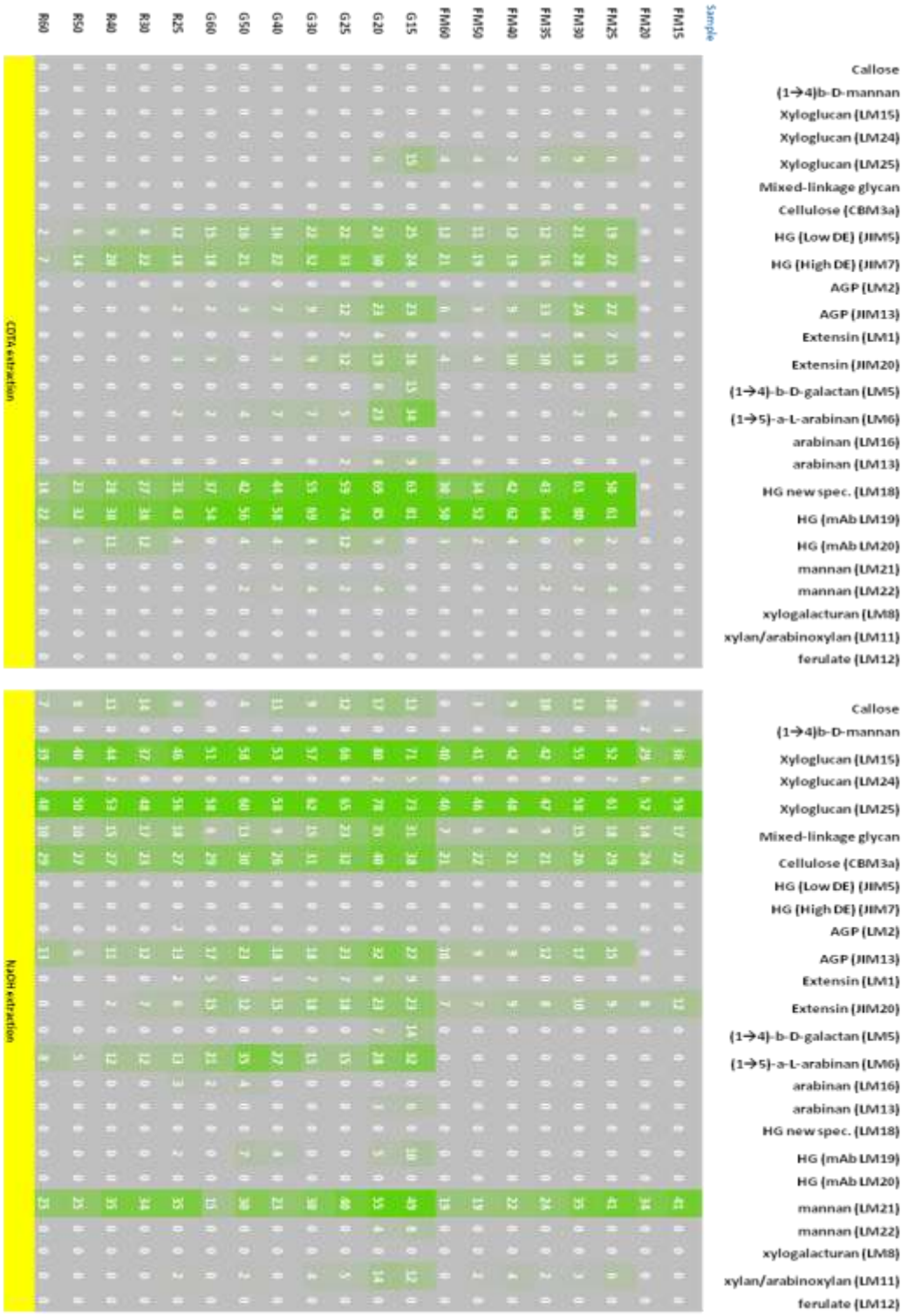

Figure 4.19: Comprehensive microarray polymer profiling (CoMPP) analysis of three lines of cotton throughout development. Cell wall polysaccharides were extracted using CDTA and NaOH. FM – Fibermax, G – green fibres of G. hirsutum, R – red fibres of G. barbadense. The number next to the species code represents the fibre sample DPA. Extraction and CoMPP performed by Dr Julia Schückel.

4.10 Summary

In summary, the differential occurrences and changes of these cell wall polysaccharides and glycoproteins during cotton fibre development suggest that they have important and diverse roles in cell wall growth.

- Using CBMs and cellulose staining dyes, it was shown that cellulose content increases as development progresses, especially during the secondary cell wall synthesis stage. During the early stages of development (0-18 dpa), cellulose makes up to 40% of the elongating fibre cell wall (Haigler et al., 2009). Between 15-20 dpa is the transition stage of elongation to secondary wall synthesis, and a rapid cellulose deposition was seen.
- Callose is detected in fibres during early-mid stages of the developmental elongation, with some remnants observed in the fibre cell walls upon maturity. During the peak of the elongation rate, callose was detected with a high intensity. It has been documented that during elongation, callose is synthesised and deposited in the plasmodesmata pores, preventing the movement of water and ions, while several membrane transporter genes are upregulated to import osmotically active solutes. This provides the constant turgor pressure needed to drive the high rate of fibre cell wall elongation (Brill et al., 2011; Ruan, 2005).
- Of the five pectic HG mABs available, subtle differences were seen between them, and suggest that HG is synthesised in a methyl-esterified form during the early stages of development, which is de-esterified by fibre maturity. One result that was seen with all these probes was the presence of thick, longitudinal HG pectic rich bands along the fibre surfaces. The levels

and patterns of methylesterification in the HG of plant cell walls can distinctively affect the mechanical properties and porosity of elongating cells (Willats et al., 2001). Further studies should aim to characterise the methylesterification patterns in detail during development using a wider range of pectin degrading enzymes as well as enzymatic removal of these ester groups. Furthermore, pectic acetylation in the developing cotton fibre cell wall should not be overlooked, as in other plant cell systems, the de-acetylation of pectin has been implicated in the cessation of cell elongation (Gou et al., 2012).

- Xyloglucan is a major non-cellulosic polysaccharide found in the cell walls of developing cotton fibres, the occurrence of which seems to peak at several stages during development, indicating a prominent turnover of this polysaccharide during the elongation stage (Verlag, Tokumoto, Wakabayashi, Kamisaka, & Hoson, 2003). Xyloglucan was also detected in thin, longitudinal bands along untreated fibres around 15 dpa. Co-localisation immunolabelling studies will be useful in comparing the presence of pectic HG, xyloglucans as well as arabinans in this distinct banding pattern in the developing cotton fibre.
- Due to time and material constraints, the occurrence of xylans during cotton fibre development was not covered in detail. Due to their structural role in other plant cell wall systems, they will be an interesting aspect to study further, as arabinoxylans have been detected in the developing cotton fibre using NMR (Jaquet, Buchala, & Meier, 1982).
- Heteromannans were only detected in developing cotton fibres after the application of both sodium carbonate and pectate lyase to remove acetyl

groups and the pectic sheath. The overall mannan content appears to decrease during fibre development between initiation and maturity. While the role of heteromannans in developing cotton fibres is still unclear, it could be postulated that they may have a role as a storage polysaccharide, a role which has been demonstrated in other systems. More interestingly, hetero mannans have also been implicated in cell wall signalling (Carpita & McCann, 2000).

- The LM5 galactan epitope for RG-I side chains, was only detected at the fibre bases in untreated fibres during development and absent by 25 dpa. Galactan has been extensively implicated in providing tensile properties to plant cell walls, so it is conceivable that the localisation of this polysaccharide in the developing cotton fibre may play a role in adhering it to the seed coat epidermis (Girault et al. 1997; Arend 2008; Zykwinska et al. 2005). Additionally, it has been shown that the secondary wall is much thinner at the distal end of the fibre, which is compensated by a thicker primary cell wall (Vigil, Anthony, Columbus, Erbe, & William, 1996). Further studies should aim to look at the transverse cross sections of these fibre bases to correlate any polysaccharide epitopes with functionality.
- The LM6 epitope for arabinan side chains was present throughout development, but between 6-15 dpa it exhibited a profile similar to that of the LM15 xyloglucan epitope, in which it was detected in thin, longitudinal bands along the fibre. Arabinan is a highly mobile side chain of RG-I, and have been proposed to add flexibility to plant cell walls (Ha, Vie, Jardine, Apperley, & Jarvis, 2005). Additionally, arabinans have been implicated in cell wall adhesion (Iwai, Masaoka, Ishii, & Satoh, 2002), which would be an

important factor while fibres are elongating in adhered bundles via the CFML (Singh et al., 2009).

- The protein content of developing cotton fibres decreases between 0 and 60 dpa, and a diversity of extensin and AGP epitopes were detected. *In vitro* studies suggest that AGPs were the most abundant glycoprotein component in the developing cotton fibre with five distinct bands on SDS-PAGE at 9 dpa, which decreases with time. Extensin epitopes were also detected in developing cotton fibres, with the most abundant epitopes being LM1 and JIM20.
- Co-localisation of extensin and AGP epitopes with LM6 arabinan and INRA-RU2 RG-I backbone epitopes in SDS-PAGE studies suggest that they may be interacting partners that could play a coordinated role in cell wall assembly and architecture. It will be interesting to explore this aspect of developmental biology further by looking for polysaccharide associations with cell wall protein components to try an elucidate the complexity of cell wall construction and growth. By comparing different lines of cotton during development, it would be possible to correlate these polysaccharide-glycoprotein interactions with structural and mechanical fibre properties. Additionally, these interactions could be explored further in other model plant systems (Tan et al., 2012).
- CoMPP assays of cell wall polysaccharides extracted with CDTA and NaOH suggests that a sensitive, high throughput technique of many species and growth stages of developing cotton fibres using monoclonal antibodies can be realised. While so far the development and maturity of cotton fibres have been covered, it will also be important to study the cell wall

polysaccharide profiles of the various stages after the harvest where fibres are spun and treated to improve their overall quality. The CoMPP analysis will be a powerful tool in detecting and quantifying these changes during the industrial processes.

- Overall, the differential occurrences and changes of these cell wall polysaccharides and glycoproteins during cotton fibre development suggest that they have important and diverse roles in cell wall during fibre growth.

Chapter 5

Processing and crystallinity studies of mature cotton fibres

5.1 Introduction

The cotton fibre is one of the purest sources of cellulose on the planet, as well as being one of the most important industrial plant fibres. Before the textile manufacturing processes, cotton fibres undergo several intensive treatments to eliminate wax, pectins, hemicelluloses and proteins, to allow access to the cellulose structure. Large amounts of water are used in several processing stages including scouring, mercerisation and dyeing treatments. It can take up to 20,000 L of water to produce just 1 Kg of processed cotton (WWF 'Thirsty crops' report 2003). These post-harvest treatments will also 'homogenise' the fibre differences to make samples that will react in the same way i.e. fibres with different maturities will react the same way after dyeing, mostly to make the dyeing process easier (Colom and Carrillo, 2002).

The supramolecular organisation of cellulosic based fibres can be interpreted by a two-phase model consisting of regions of highly ordered (crystalline) and low order (amorphous) levels of cellulose (Krassig, 1993; Bredereck and Hermanutz, 2005; Hsieh, 2007). Industrial treatments of cotton fibres with strong alkali cause significant changes to the molecular, supramolecular and mechanical properties. It is widely known that in the presence of strong alkali solutions, cotton fibre cellulosics are altered to form modified crystal structures composed of cellulose II (Colom and Carrillo, 2002; Bredereck and Hermanutz, 2005). The transition of parallel cellulose I chains to anti-parallel cellulose II chains causes the cellulose populations in the fibre to become more energetically stable. In the textile industry these cellulose changes, after strong alkali treatments, are significantly exploited in the pre-treatment process known as 'mercerisation' (Krassig, 1993; Colom and Carrillo, 2002). The rates and types of changes in the cellulose

supramolecular structure can be affected by alkali concentration, degree of cellulose polymerisation, temperature and tension (Jaturapiree et al., 2006). At certain concentrations, crystalline lattices of cellulose can be penetrated by NaOH to produce crystal complexes of cellulose containing sodium and water ions (Porro et al., 2007). As a result, it was predicted that five alkali-celluloses could be produced, dependent on the concentration of NaOH used (Figure 5.1). These isoforms were named: cellulose I, cellulose IIA, cellulose IIB, cellulose III and cellulose IV as outlined in Figure 5.1 (Okano and Sarko, 1984). Methods used to study cellulose crystallinity and morphology have significantly increased over the past twenty years. Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) has been used extensively to study crystallinity in natural cotton fibres and regenerated forms of cellulose such as lyocell (O'Connor et al., 1958). From ATR-FTIR spectra readings, three values relating cellulose crystallinity can be extracted. The crystallinity index (TCI) was the first to be developed. However it was shown to be linearly dependent on the degree of mercerisation. A more appropriate value is the lateral order index (LOI), as it is positively correlated with the levels of mercerisation and crystallinity. Finally, the hydrogen bond intensity (HBI) was developed which relates to the degree of intermolecular regularity in cellulose and the amount of bound water (Nelson and Connor, 1964). Changes in cellulose crystallinity can be quantitatively interpreted using the LOI readings from ATR-FTIR spectra. This value is calculated by looking at the absorbance ratios of specific wavelengths. A rule of thumb is that as crystallinity decreases, so does the LOI (Colom and Carrillo, 2002). In the ATR-FTIR spectra the 893 cm^{-1} and 1429 cm^{-1} absorbance bands can be used to interpret the crystallinity changes and cellulose class, due to the fact that cellulose

I (generally crystalline cellulose) absorbance readings significantly differ from cellulose II (generally more amorphous cellulose) (O'Connor et al., 1958; Colom and Carrillo, 2002). Cotton fibres rich in crystalline cellulose I have a band that leans towards the 1429 cm^{-1} absorbance peak, and conversely, cotton fibres rich in amorphous cellulose II have a peak closer to the 893 cm^{-1} band. The band at 1336 cm^{-1}, however, was shown to have the greatest difference between crystalline and amorphous cellulose (Colom and Carrillo, 2002). Alternatively, hydrogen bond intensity (HBI) can also be used to chart the quantitative changes of amorphous cellulose. An increase in HBI is generally correlated with a decrease in cellulose crystallinity. Increasing hydrogen bond intensity is associated with an increase in the amount of hydrogen bonding between specific regions in the cellulose, which is a typical trait in the characterisation of the conversion of crystalline cellulose I into amorphous cellulose II. Interestingly, while the hydrogen bonding increases, the overall percentage of crystallinity in the cellulose decreases (O'Connor et al., 1958; Nelson and Connor, 1964; Colom and Carrillo, 2002).

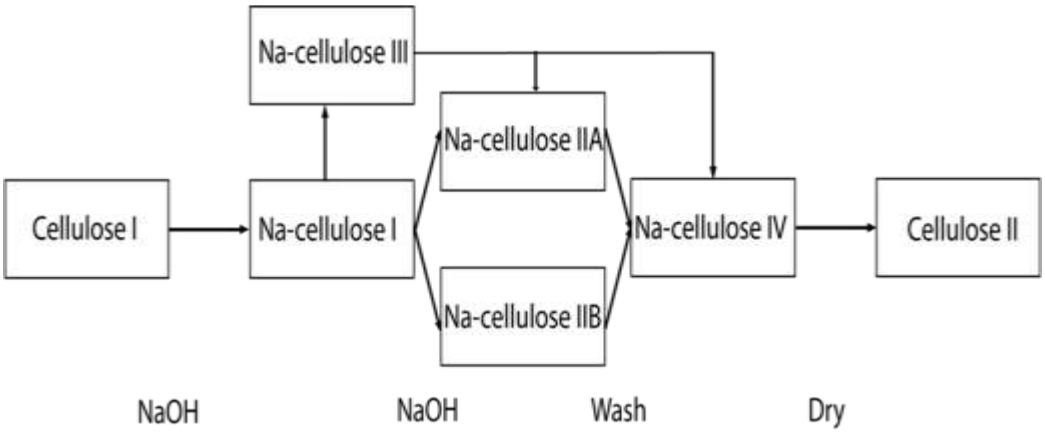

Figure 5.1: Inter-conversion of Na-cellulose structure during the mercerization processes. Adapted from (Okano and Sarko, 1984). Under alkali treatments, cellulose I form Na-cellulose I, which can convert into either Na-cellulose III, Na-cellulose IIA or Na-cellulose IIB. These three forms ultimately turn into Na-cellulose IV which forms cellulose II after washing and drying.

While, X-ray diffraction has been used for over 100 years to study cellulose crystallinity and its supramolecular structure, there has been little comparative work done on cotton fibres using these techniques. Using X-ray diffraction with cotton fibres usually creates a dilemma, the intact fibres are not suitable for quantitative analysis so they have to be homogenised into a fine powder. This process however causes changes to the crystallinity, thus defeating the object of doing it in the first place (Chidambareswaran et al., 1987). X-ray diffraction is a non-destructive analytical technique that can reveal information about the crystallinity, chemical composition and physical properties of thin films and materials. The process is based on measuring the scattering of X-ray beams after they hit a sample material, taking into account the scatter angle, wave polarization and energy (Segal et al., 1959). From both techniques, a crystallinity index value is determined from these factors, and it is a simple but effective method for measuring cellulose crystallinity in the mature cotton fibres (Segal et al., 1959;

Blackburn and Burkinshaw, 2002; Abidi et al., 2007; Široký et al., 2009; Kljun et al., 2011). In addition to FT-IR and X-ray diffraction, crystallinity changes have been demonstrated through the development of methodologies for the detection and imaging of low level cellulose polymers at the fibre surfaces and interiors using molecular probes. It has been achieved so far using highly specific proteins known as carbohydrate-binding modules (CBMs), which are directed *in-situ* to these cellulose glycan epitopes. CBMs are nature's cell wall binders and are the non-catalytic subunits of microbial cell wall degrading enzymes which have been modified to have a his-tag tail to allow detection (Table 5.1) (Blake et al., 2006; McCartney et al., 2006; Obembe et al., 2007). These techniques are also transferable to other cellulose textile systems, such as lyocell, a form of reconstituted cellulose from birch wood fibres (Peng et al., 2003; Široký et al., 2012). CBMs have been previously shown to be powerful tools to investigate the structural aspects of cellulose in a cell-wall context (Blake et al., 2006; Obembe et al., 2007). In this chapter, the indirect *in-situ* labelling of cotton with CBMs was used in conjunction with ATR-FTIR and X-ray diffraction to chart the changes that occur in cellulose crystallinity during the mercerisation process of these cellulose fibres.

Table 5.1: Cellulose directed CBMs used in this chapter

CBM	Epitope	Reference
CBM3a	Crystalline cellulose	
CBM2a	Crystalline cellulose	Bolam et al. 1998; Boraston et al. 2004
CBM10	Crystalline cellulose	
CBM4-1	Amorphous cellulose	
CBM17	Amorphous cellulose	Boraston et al. 2000
CBM28	Amorphous cellulose	Boraston et al. 2002

5.2 Comparative analysis of crystallinity changes in cellulose I polymers

Changes in cellulose crystallinity can be quantitatively interpreted using the lateral order index readings from ATR-FTIR spectra and X-ray diffraction. In addition to this, carbohydrate-binding modules have specific recognition capabilities for cellulose and other polysaccharides in plant cell walls, but have yet to be used in a comparative and quantitative manner to interpret the changes in cell wall crystallinity after the industrial mercerization treatments. Intact *Gossypium hirsumtum* lines FM966 and Cooker were treated with increasing NaOH concentrations and then labelled with CBMs for cellulose of varying crystallinities. These his-tagged proteins bind to cellulose, and can then be further tagged with a fluorochrome probe to allow detection under UV excitation.

The micrographs shown in figure 5.1 shows the *in situ* fluorescence imaging of CBMs directed to the intact surfaces of FM966 and Cooker lines of cotton fibres after NaOH mercerization treatments. Four specific CBMs were used to visualise the crystallinity changes seen with the increasing NaOH concentrations (0.0-8.0 mol dm^{-3}) for both fibre lines, shown in Figure 5.2. With increasing alkali treatments, the binding intensities of CBM2a and CBM3a, which bind to crystalline cellulose, were shown to decrease. This can be correlated with a decrease in the cellulose crystallinity of the cotton fibres. Additionally, the significant decrease in binding intensity of CBM2a and CBM3a in both cooker and FM966 lines were seen occurring between 1.0 mol dm^{-3} and 2.0 mol dm^{-3} NaOH. In contrast, CBM17 and CBM4-1, which are directed to amorphous cellulose on intact fibres showed an opposite effect. With increasing NaOH

mercerization treatments, the binding intensity of CBM17 and CBM4-1 also increases, correlating with a decrease in the fibre cellulose crystallinity.

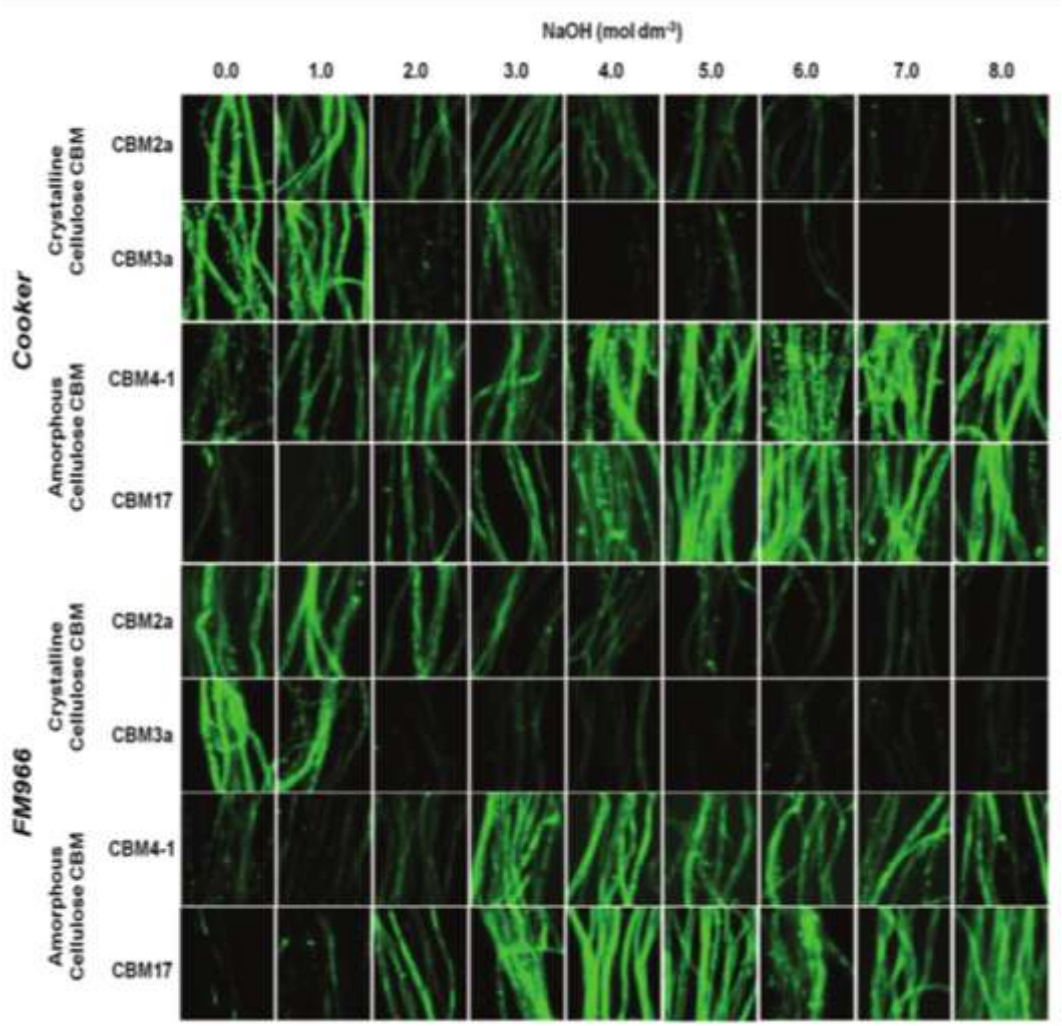

Figure 5.2: In situ fluorescence imaging of four cellulose-directed CBMs (CBM2a, CBM3a, CBM4-1 and CBM17) to FM966 and Cooker cotton fibres after pre-treatments with a series of NaOH concentrations (0.0−8.0 mol dm^{-3}).

The observations shown in figure 5.2 are qualitative, so using Image J; quantitative data sets were generated from the CBM micrographs for comparison with the ATR-FTIR data. Figure 5.3 shows the quantitative changes in CBM binding intensities after increasing NaOH concentrations for both fibre lines; cooker and FM966. In Figure 5.3a and Figure 5.3b, the changes in the lateral order index of cotton fibres using ATR-FTIR were shown after increasing NaOH concentrations. The most significant changes in the LOI occurred between 2.0 mol dm^{-3} and 4.0 mol dm^{-3} NaOH treatments, which correlate with the cellulose I transformation to cellulose II. Post 4.0 mol dm^{-3} NaOH, no further changes in the cotton cellulose were seen, most likely because cellulose II is the most stable and energetically favourable class of cellulose. For the crystalline cellulose-binding CBM3a and CBM2a (Figure 5.3a & Figure 5.3b) the observed changes were seen up to 2.0 mol dm^{-3} NaOH less than was seen with ATR-FTIR. With CBMs, a steeper decrease in crystallinity was also seen. In Figure 5.3c and Figure 5.3d, the HBI changes seen with increasing NaOH concentrations were compared with the intensity readings for CBM17 and CBM4-1. The significant changes in cellulose HBI were observed between 3.0 mol dm^{-3} and 4.0 mol dm^{-3} NaOH. This is correlated with the increase in hydrogen bonds, yet a decrease in crystallinity is seen through the conversion of cellulose I to cellulose II. In comparison, amorphous cellulose directed CBM17 and CBM4-1 (Figure 5.3c & Figure 5.3d) had very similar binding intensities for both FM966 and cooker fibre lines with the increasing NaOH treatments. The binding intensities steadily increased along with the NaOH concentration, especially between 2.0 mol dm^{-3} and 6.0 mol dm^{-3} NaOH. In comparison to the ATR-FTIR readings, this data shows that using

CBMs visualises a less sudden transition in the generation of amorphous cellulose II.

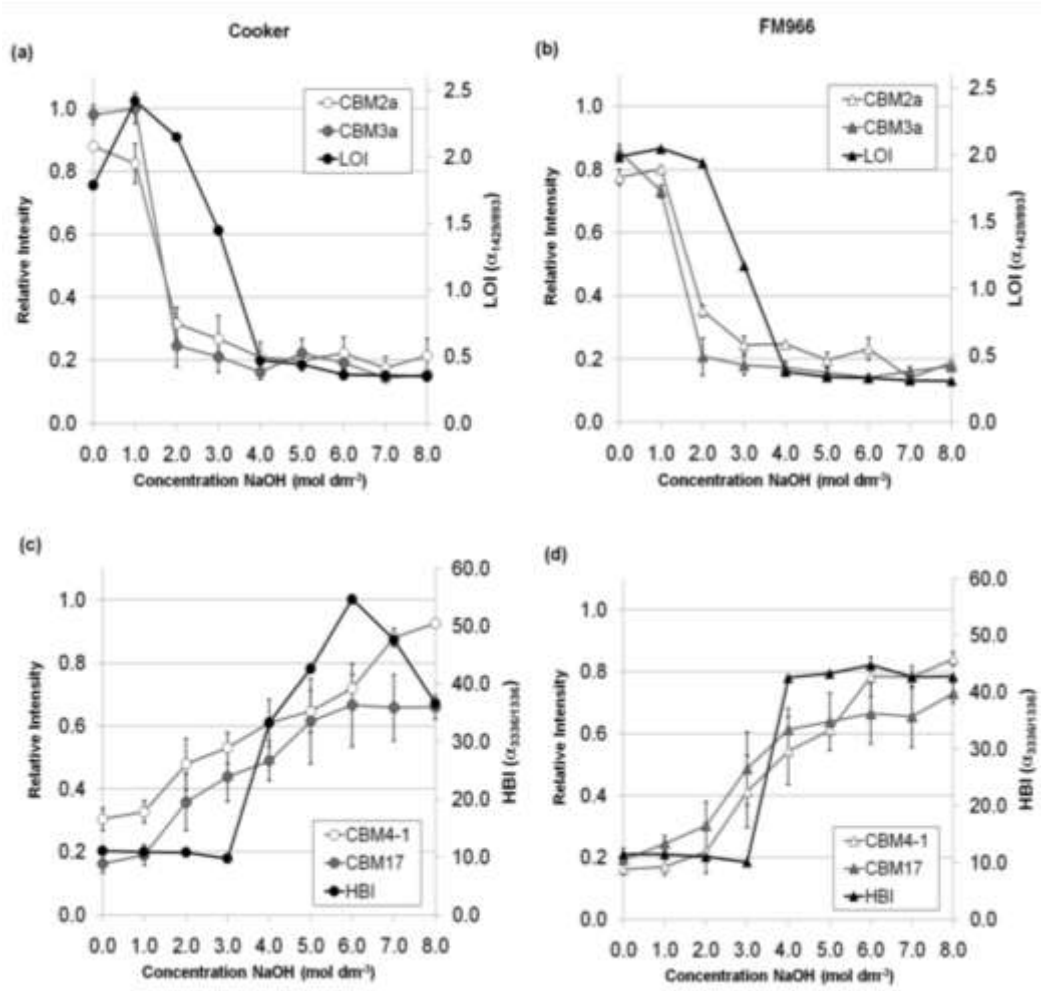

Figure 5.3: Quantification of fluorescence micrographs and ATR-FTIR analyses in response to NaOH treatments. (a) CBM2a and CBM3a directed to crystalline regions of Cooker cotton fibres with increasing NaOH concentration in comparison with LOI (ATR-FTIR). (b) CBM2a and CBM3a directed to crystalline regions of FM966 cotton fibres with increasing NaOH concentration in comparison with LOI (ATR-FTIR). (c) CBM4-1 and CBM17 directed to amorphous regions of Cooker cotton fibres with increasing NaOH concentration in comparison with HBI (ATR-FTIR). (d) CBM4-1 and CBM17 directed to amorphous regions of Cooker cotton fibres with increasing NaOH concentration in comparison with HBI (ATR-FTIR). Error bars show plus and minus of standard deviation of fluorescence quantification. Produced in collaboration with Alenka Kljun (Kljun et al., 2011).

As previously mentioned, X-ray diffraction is a non-destructive analytical technique that can reveal information about the crystallinity, chemical composition and physical properties of cellulose rich materials, such as cotton fibres. The process is based on measuring the scattering of X-ray beams after they hit a fibre sample, taking into account the scatter angle, wave polarization and energy (Segal et al., 1959).

The binding of CBM3a and CBM2a directed to crystalline cellulose, was compared with the CrI values calculated from the X-ray diffraction readings, shown in Figure 5.4. The CrI changes observed with increasing NaOH mercerisation treatments roughly followed the results from the ATR-FTIR crystallinity analysis.

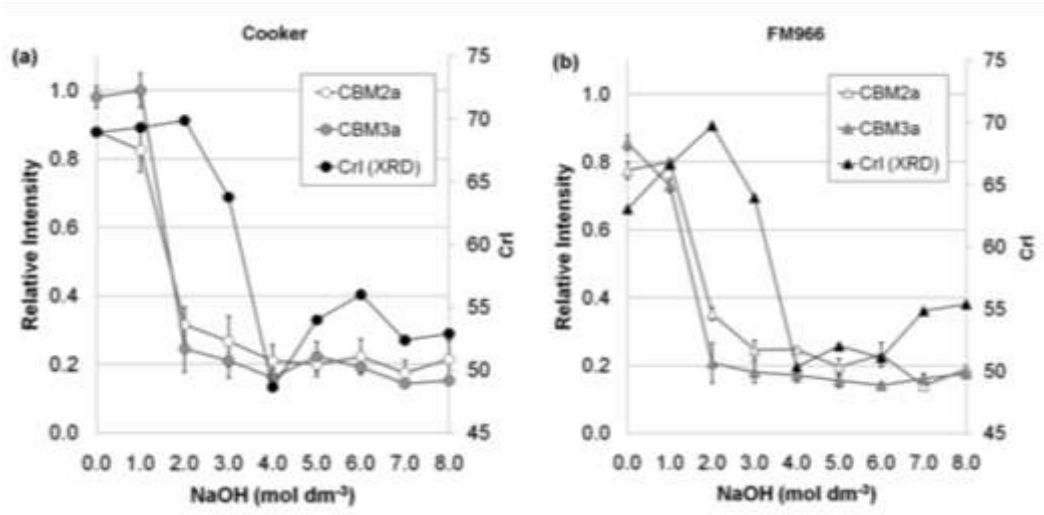

Figure 5.4: Quantification of cellulose crystallinity using carbohydrate-binding module labelling to cotton fibres and X-Ray diffraction analyses in response to NaOH treatments: (a) CBM2a and CBM3a directed to crystalline regions of Cooker cotton fibres with increasing NaOH concentration in comparison with X-Ray diffraction. (b) CBM2a and CBM3a directed to crystalline regions of FM966 cotton fibres with increasing NaOH concentration in comparison with X-Ray diffraction. Produced in collaboration with Alenka Kljun (Kljun et al., 2011)

5.3 Industrial pre-treatments on cotton fibre

During processing, cotton fibres undergo scouring and mercerisation treatments to homogenise the structural properties and reactivity to dyeing processes. In Figure 5.5, mature scoured and mercerized FM966 fibres were stained with the β-glycan binding stain, Calcofluor white (Hughes and Mccully, 1975). As previously mentioned, the mature fibre primary cell wall is divided into two layers which differ in their composition on each surface. The outer surface is mostly made up of waxes and pectins, specifically HG, which masks the majority of epitopes of the underlying cellulose and hemicelluloses (Vaughn & Turley 1999). This can be seen in Figure 5.5 (UNTR) in which the fibre is untreated and as a result dyes very poorly with Calcofluor white. The scouring process (Figure 5.5 SCR) removes most of the waxes and pectins of the primary cell wall which allows a greater amount of access to the cellulose by the dye, hence a more intense binding was seen. Finally, the mercerized mature fibres (Figure 5.5 MERC) have a lower overall crystallinity and more cellulose II than the untreated fibre, as shown by the previous ATR-FTIR, CBM and X-ray diffraction results in this chapter.

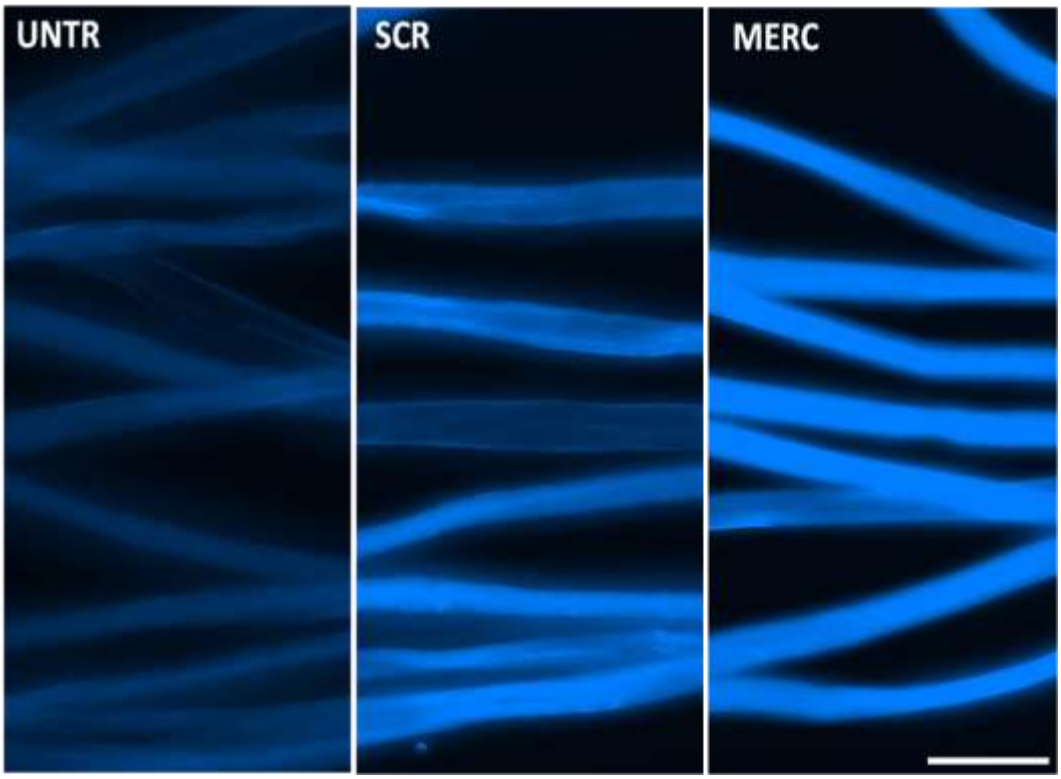

Figure 5.5: Industrial pre-treatments and the effect on Calcofluor fluorescence cellulose staining. Scale 50 µm.

5.4 Comparative analysis of crystallinity changes in cellulose II polymers of lyocell fibres

In addition to natural cellulosic I rich fibres such as cotton, man-made lyocell fibres were also examined. Lyocell is a cellulose II rich fibre made from reconstituted cellulose from eucalyptus wood pulp. Lyocell fibres are made up of highly ordered linear chains of β-(1-4)-glucan polymers and as a result the degree of crystallinity is very high (≤80%). These high degrees of crystallinity are generated during the manufacturing process, in which the extraction and stretching of the reconstituted fibres cause the microfibrils to more likely orientate in a parallel fashion. One characteristic of lyocell fibres is the microfibrillar structures where a portion of the cellulose chains aggregate to form micro-crystals, while the remainder of the chains exist in an amorphous phase. Like natural cellulose fibres, such as cotton, alkali treatments have a significant impact on the supramolecular, molecular and morphological properties of the cellulose II polymers. This translates as changes in the properties of crystallinity, microfibre orientation, lyocell pore structure and accessibility (Crawshaw and Cameron, 2000; Široký et al., 2009). As mentioned previously, X-ray diffraction and ATR-FTIR only give overall measurements of the changes in a large sample size. Carbohydrate-binding modules can effectively define changes seen in crystalline and amorphous cellulose at a spatial context (Blake et al., 2006; Kljun et al., 2011).

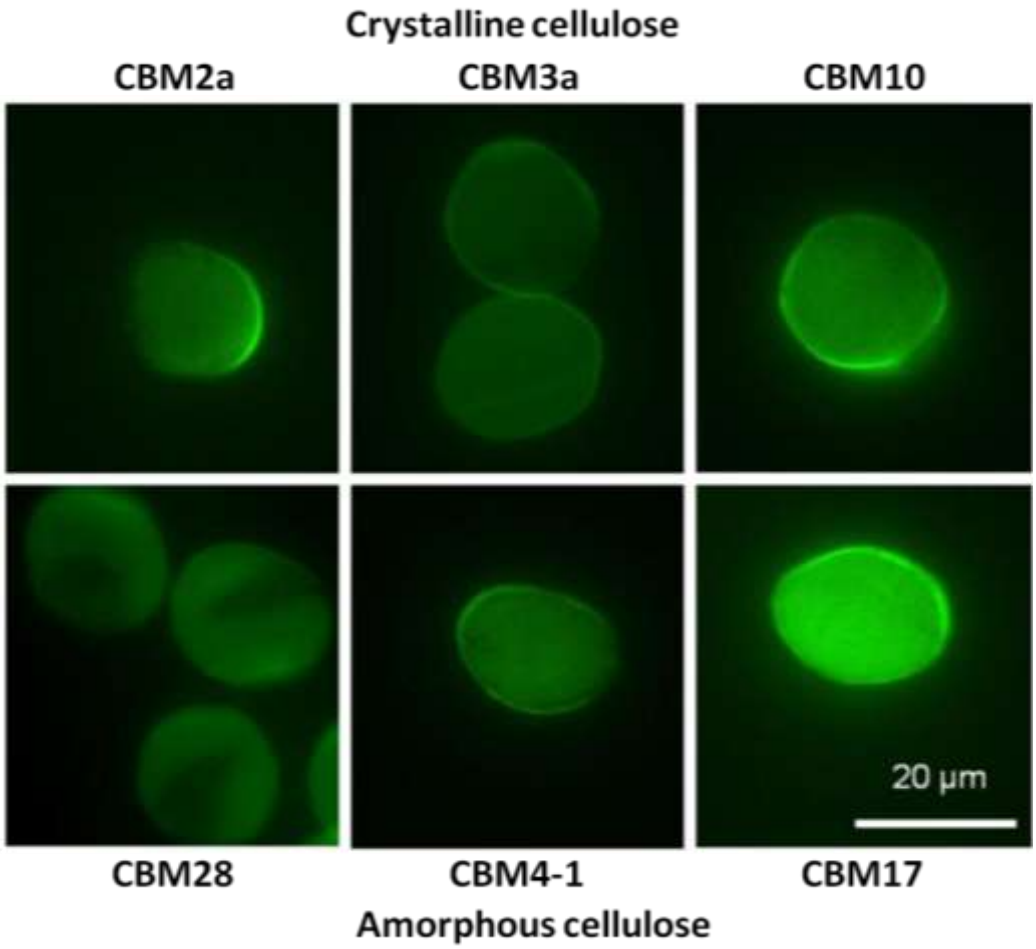

Figure 5.6: Indirect immunofluorescence detection of amorphous and crystalline cellulose carbohydrate-binding modules labelling transverse-sections (thickness of 0.5 µm) of resin-embedded lyocell fibres to highlight the different proposed areas through the fibre.

CBMs were used on resin-embedded cross sections of untreated lyocell fibres in Figure 5.6. From the CBM labelling, it can be seen that the lyocell semi-permeable fibre skin is present through the very strong binding of all CBMs at the outer surface layer. The presence of a 'skin', approximately 100 nm in thickness and composed of mostly amorphous cellulose II, is discussed in Bredereck 2000 (Bredereck and Hermanutz, 2005). In Biganska (2002), there are three proposed regions of cellulose in lyocell fibres. However, using CBMs only two regions (outer skin and inside the fibres) were visibly detected. Additionally, CBM17 binds very well to the inside areas of the untreated lyocell fibres, where high levels of amorphous cellulose are expected. In contrast, CBM28 binds to most of the transverse section with the exception of a segment area in which the binding of CBM28 is very weak. This may support the hypothesis of the dominance of detection of amorphous, non-ordered regions over crystalline regions with the lyocell fibre and its skin-core differences (Biganska et al., 2002; Široký et al., 2012).

CBM3a and CBM28 (Figure 5.7 and Figure 5.8) are shown binding to crystalline and amorphous cellulose after varying treatment conditions of NaOH, temperature and fabric tension. Of all the regenerated cellulosic fibres, lyocell is known to have high degrees of crystallinity. This can be seen with the stronger levels of binding of CBM3a, which binds to crystalline cellulose, compared to CBM28, which is much weaker. Further work carried out by Široký (2012), showed that in addition to NaOH, temperature and fabric tension also have an effect on the crystalline and amorphous supramolecular structures of the lyocell cellulose skin (Široký et al., 2012). For CBM3a (Figure 5.7), most crystalline cellulose was detected under the conditions: 3.33 mol dm^{-3} NaOH, 49 Nm^{-1} fabric tension at

25°C and 4.48 mol dm^{-3} NaOH, 147 Nm^{-1} fabric tension at 40°C (Lyocell tension, temperature and alkali treatments varied out by Dr Jan Široký, The University of Leeds). In the case of CBM28 binding (Figure 5.8) there were significant variations seen, which can potentially indicate that amorphous regions of cellulose are more sensitive and dependent upon thermal, mechanical and chemical treatments when compared to crystalline regions. These results from CBM28 on lyocell can be linked to the data shown in Boraston (2003), in which chains of amorphous or 'shapeless' cellulose are predicted to exist in at least two or more physical substructures. As a result, CBM28 may have strong affinity or perhaps discrimination for these different physical forms of amorphous cellulose (Boraston et al., 2003).

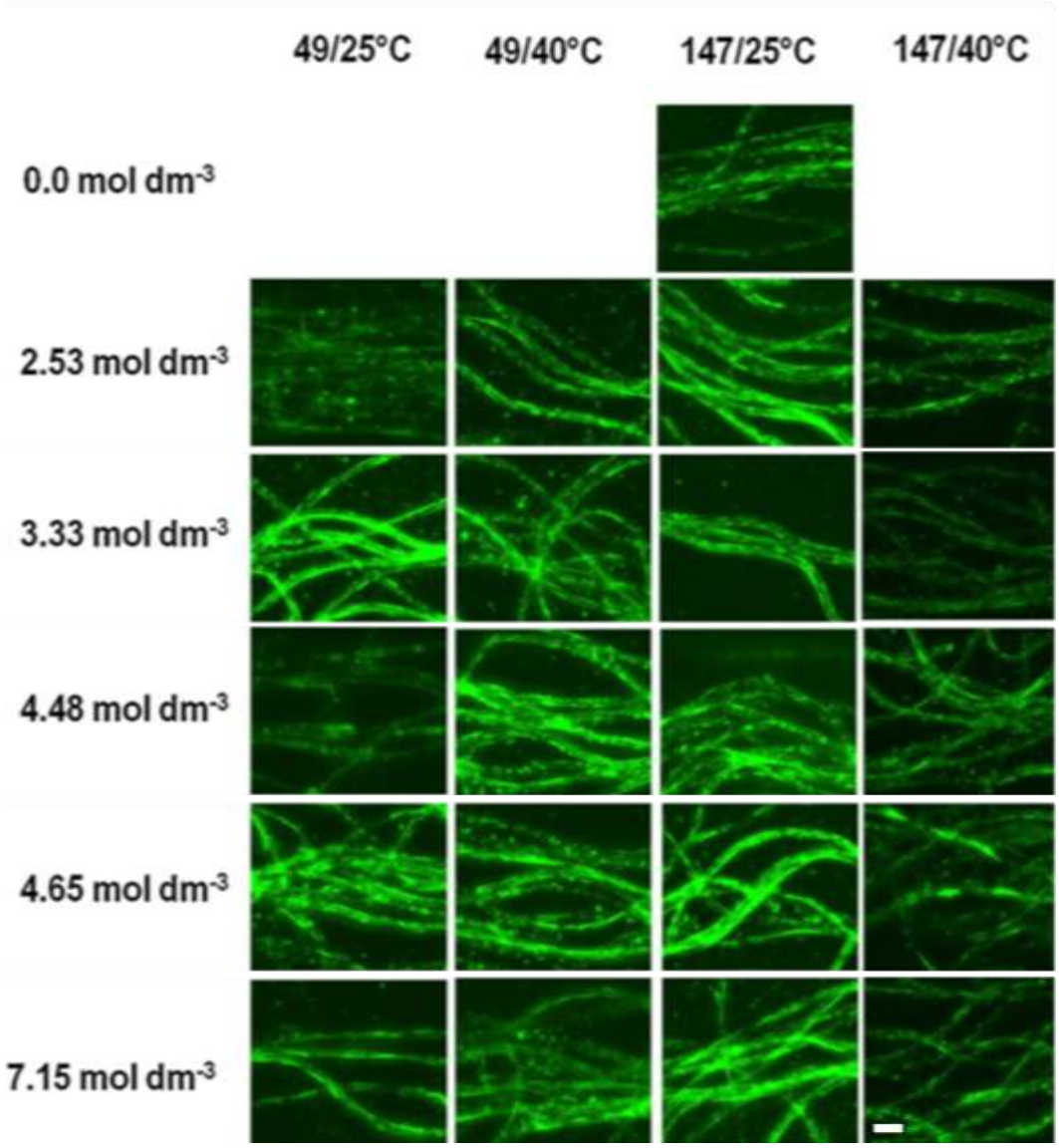

Figure 5.7: *In situ* fluorescence analysis of CBM3a labelling to lyocell fibres in response to NaOH under varying temperatures (25°C and 40°C) and fabric tensions (49 Nm^{-1} and 147 Nm^{-1}). Samples provided by Dr Jan Široký (Široký et al., 2012). Scale: 10 µm.

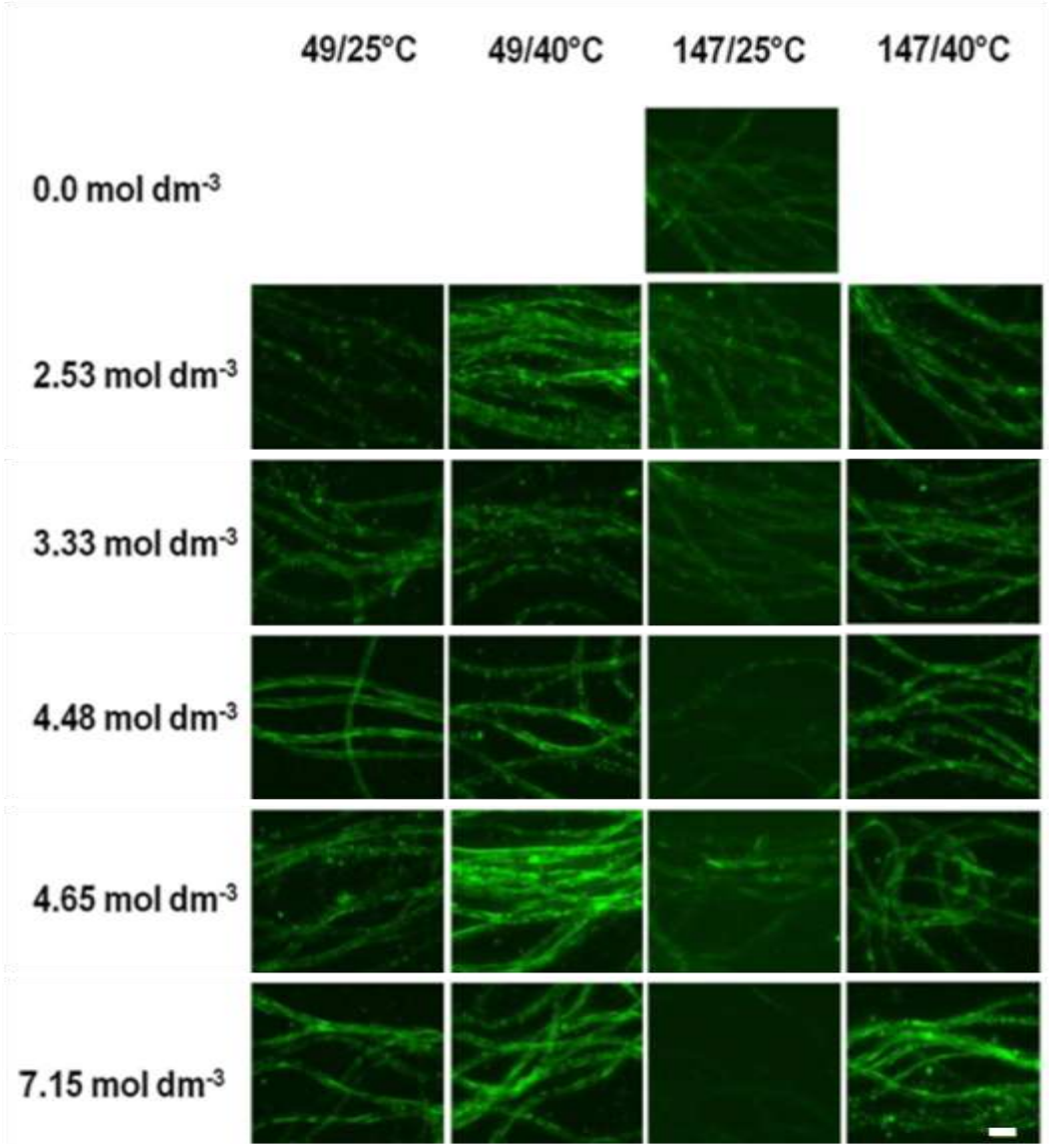

Figure 5.8: *In situ* fluorescence analysis of CBM28 labelling to lyocell fibres in response to NaOH under varying temperatures (25°C and 40°C) and fabric tensions (49 Nm^{-1} and 147 Nm^{-1}). Samples provided by Dr Jan Široký (Široký et al., 2012). Scale: 10 µm.

After quantifying the fluorescence in CBM3a and CBM28, Široký *et al*. (2012) compared the values with ATR-FTIR LOI data (Široký et al., 2009, 2012). Generally the image analysis of CBM3a binding correlates closely with the ATR-FTIR LOI and HBI readings (Figure 5.9) respectively. Tension of the fabric under 49 Nm^{-1} had a stronger effect on the supramolecular crystallinity than 147 Nm^{-1}, while the effects of temperature were seen to be more pronounced on amorphous cellulose than crystalline. With CBM28 for amorphous cellulose, the binding also generally follows in that as HBI decreases, so does the intensity of the immunofluorescence (Figure 5.10).

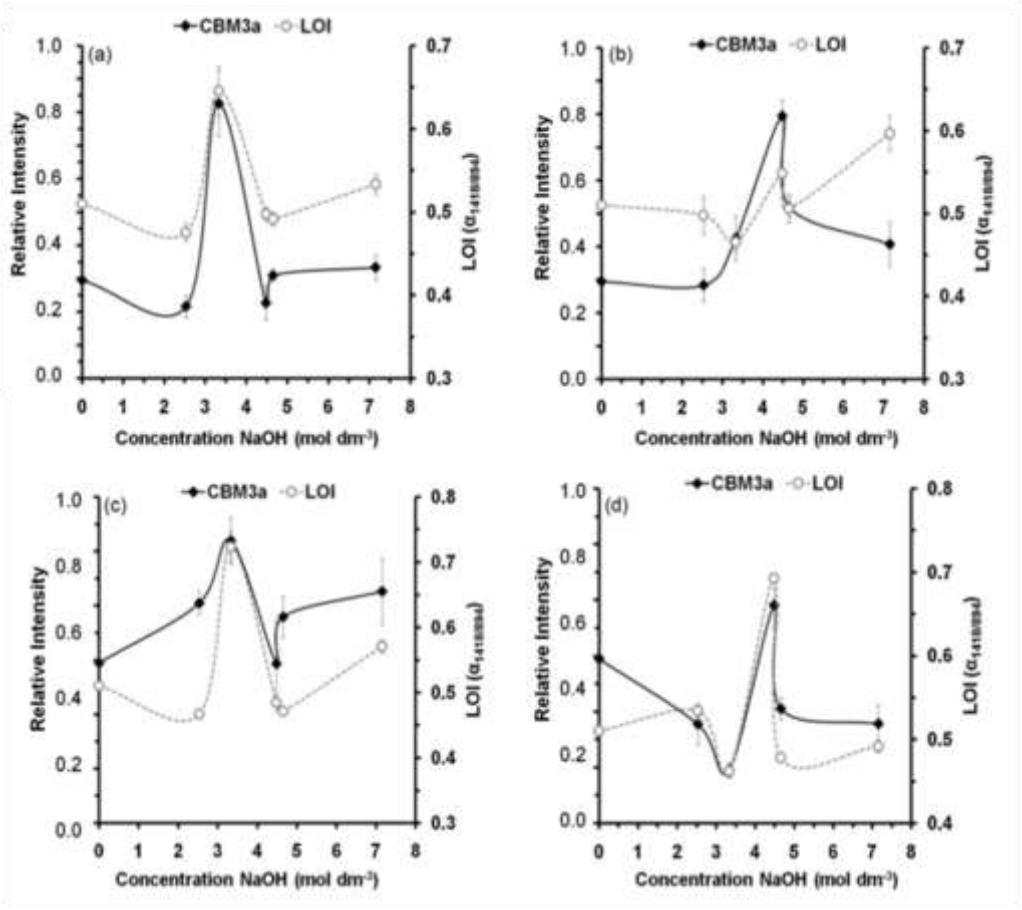

Figure 5.9: Relative intensity of CBM3a fluorescence with lateral order index readings (Široký et al., 2009) with increasing NaOH concentrations under varying tension and temperatures (49-147 N m^{-1} and 25-40°C respectively). (a) 49 N m^{-1}/25°C; (b) 49 N m^{-1}/40°C; (c) 147 N m^{-1}/25°C; (d) 147 N m^{-1}/40°C. Figure provided by Dr Jan Široký (Široký et al., 2012).

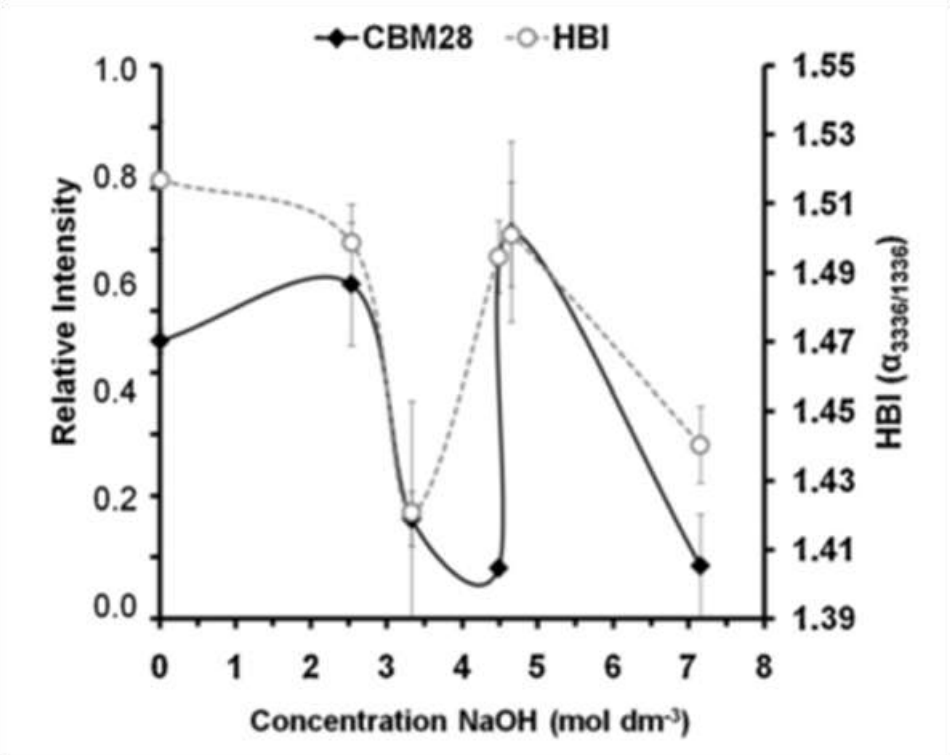

Figure 5.10: Relative intensity of CBM28 fluorescence compared with hydrogen bond intensity readings (Široký et al., 2009) with increasing NaOH concentrations, under an applied tension strength of 147 N m-1 at 25°C. Figure provided by Dr Jan Široký (Široký et al., 2012).

5.5 Preliminary studies of fibre cell wall crystallinity and processing during development

Figure 5.11 and Figure 5.12 below give a preliminary glimpse of merging these techniques with a comparative analysis of the different stages of cotton fibre development in relation to studying the cellulose crystallinity, hydrogen bond intensity and dyebility. In Figure 5.11, developing cotton fibres were studied with ATR FT-IR to determine the LOI, TCI and HBI. Between 10-25 dpa, the lateral order index (Figure 5.11a) was highly variable, but this variation decreased post 25 dpa. The total crystallinity index (Figure 5.11b) was also variable, but overall gradually increased, suggesting that the fibre cell wall cellulose crystallinity increases throughout development, however there were several peaks and troughs at all stages. The hydrogen bond intensity (Figure 5.11c) started off high at 10 dpa, which was also highly variable until 15 dpa, after which reduced and stayed minimal, with a few small variations at 26, 34, 42 and 52 dpa.

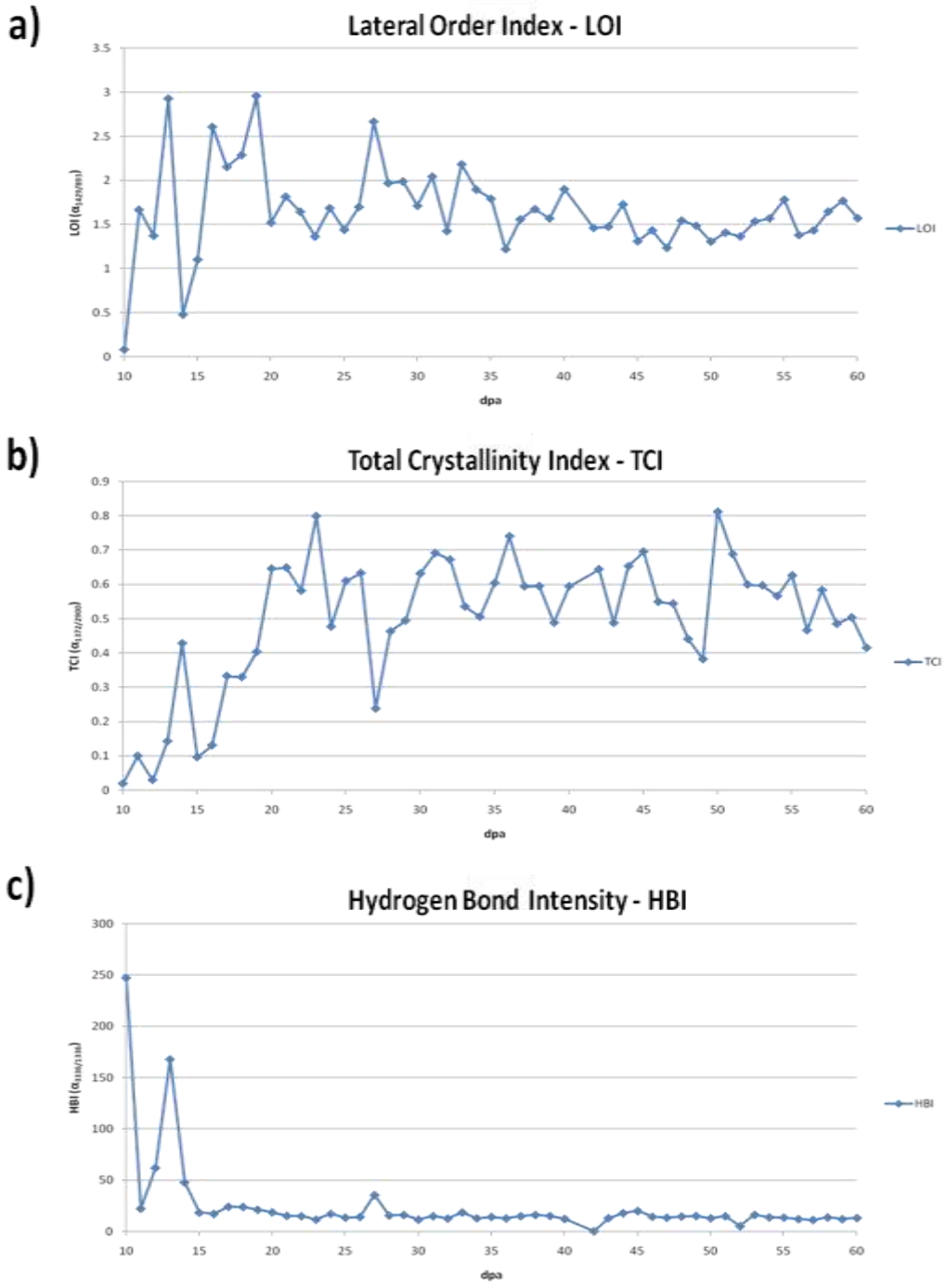

Figure 5.11: ATR-FTIR analysis of untreated, developing cotton fibre samples. a) Lateral Order Index (LOI) analysis of FT-IR data. LOI is positively correlated with crystallinity (and levels of mercerisation). b) Total crystallinity Index (TCI) analysis of FT-IR data. TCI is linearly dependant on crystallinity (and mercerisation). c) Hydrogen Bond Intensity (HBI) analysis of FT-IR data. HBI relates to the degree of intermolecular regularity in cellulose and the amount of bound water in the developing cotton fibres. Data analysed as covered in (Kljun et al. 2011) and produced in collaboration with A. Kljun.

In addition to ATR FT-IR, developing fibres with different treatments were also dyed with Direct Red 81 (Figure 5.12), a cellulose binding disazo dye. Between 10-27 dpa, there is a high level of variability between samples, with scoured samples having a low dye-efficiency, fixed samples having a gradual increase in dye-efficiency, and non-scoured samples exhibiting a large peak between 18-23 dpa. After 30 dpa, the degree of variability decreases massively, and all samples have a high dye-efficiency around 80-95% that remains stable through to maturity.

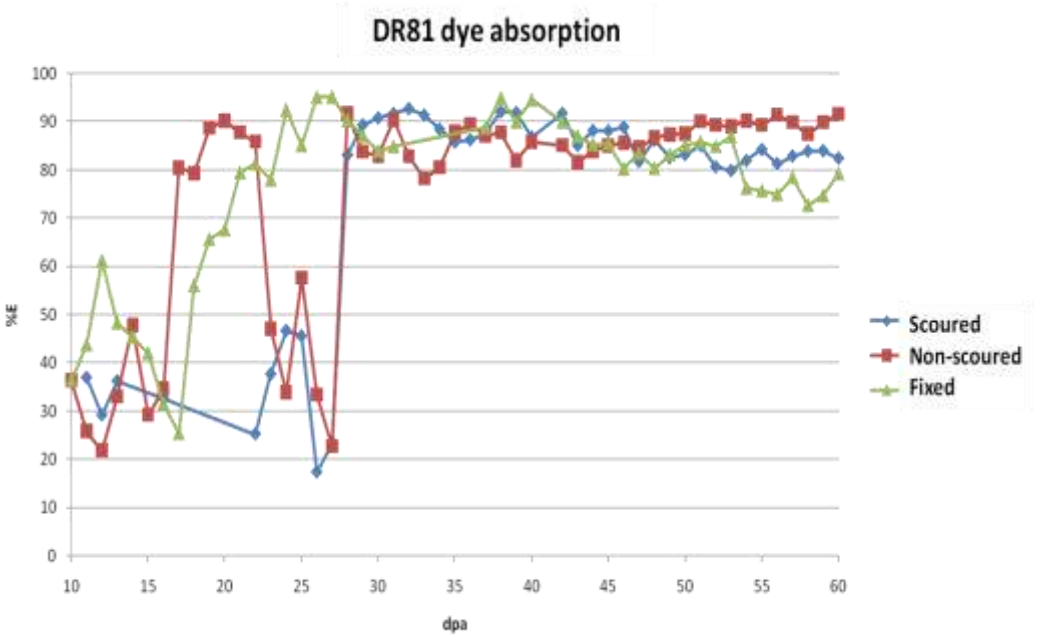

Figure 5.12: Direct Red 81 (DR81) dye efficiency absorption to developing fibres. Fibres were either scoured, non-scoured (untreated) or fixed in formaldehyde before the dyeing process. Dye treatments were done in triplicate for each dpa sample and each treatment. Produced in collaboration with A. Kljun.

5.6 Summary

In summary,

- Understanding cotton fibre maturity and development is only part of the story for fibre improvement. While these stages are important issues, if we are to improve cotton fibres at every stage, it is vital to fully understand the changes in cell wall polysaccharides during the industrial processing of cotton fibres. One crucial aspect of post harvesting modifications is to that of the cellulose crystallinity in the thick secondary cell wall, as this affects many properties including dyebility, tensile strength and uniformity (Blackburn & Burkinshaw, 2002).
- Whilst X-ray diffraction and ATR-FTIR related techniques are taking measurements throughout the samples, CBMs are only binding to the outer surfaces of the fibres. An interesting aspect of the CBMs directed to crystalline cellulose (CBM3a and CBM2a) is that they are especially sensitive to modifications in the surface supramolecular structure of cotton fibre cellulose after NaOH treatments at 2.0 mol dm^{-3}. This most likely reflects the cellulose rearrangement at the fibre surfaces – the first point of contact for the alkali treatments.
- Additionally, the CBMs directed to amorphous cellulose also show sensitivity to the low concentrations of NaOH, although they have a more gradual increase in binding with the increasing alkali treatments.
- These two differential recognition profiles of cellulose crystallinity are therefore significantly distinct and do not just recognise the two forms of cellulose (cellulose I and cellulose II). They have capabilities to detect a wide range of cellulose states at the surfaces of cotton fibres and man-made fibres.
- Additionally, these techniques can be used to study the cellulose crystallinity and dyebility during fibre development. The peaks and troughs of developing fibre dyebility will need to be correlated with quantitatively analysed polysaccharide and glycoprotein profile changes which in turn can be used to relate to the overall fibre dyebility. One noticeable change detailed in chapter 4, was the decrease in AGP and

extensin detection during development, which coincides with an increase in immature fibre dyebility by Direct Red 81. From this result, it could be proposed that the reduction in cell wall glycoproteins during development frees up hydroxyl groups, which in turn allow more Direct Red 81 to bind to the cotton fibre (Zollinger et al. 1991).

Chapter 6

Discussion

The aim of this work was to provide knowledge and a better understanding of the localisation, structure and function of cell wall polysaccharides in the cotton fibre during development, maturity and processing. To achieve this,

- Polysaccharide epitopes of the mature cotton fibre cell wall were characterised on the surfaces and through transverse cross sections using antibodies and carbohydrate-binding modules with specificity to a variety of cell wall components.
- Polysaccharide profiles were charted throughout the developmental process using monoclonal antibodies, carbohydrate-binding modules and *in vitro* studies of cell wall polysaccharides of the cotton fibre. This included the four growth stages of initiation, elongation, secondary wall synthesis and maturation.
- The changes in cellulose crystallinity in the fibre cell wall were charted after undergoing industrial pre-treatments of increasing alkali concentrations using Fourier transform infrared spectroscopy (ATR-FTIR), X-ray diffraction and carbohydrate-binding modules. Additionally, these techniques were used to investigate the changes in reconstituted cellulose.

6.1 Characterisation of polysaccharide profiles in mature cotton fibres

6.1.1 Epitope accessibility and masking of polysaccharides in mature cotton fibres

Cotton fibres have a thin waxy layer on the outside, which appears to act as a hydrophobic barrier against immunolabelling buffers and probes that can however be removed using organic solvents. This research demonstrates that polysaccharides are not always readily accessible to CBM probes and monoclonal antibodies in intact fibre cell walls and that recognition of its structural heterogeneity can be greatly increased by enzymatic removal of pectic homogalacturonan using pectate lyase. In some cases, acetylation additionally prevents the recognition of mannan epitopes. When cotton fibres were immunolabelled with various probes, there was little or no binding in most cases, however using a dewaxing protocol on the fibres resulted in increased detection of epitopes. For other polysaccharides, such as xyloglucan, detection was still reduced, indicating that an outer pectin layer could be blocking further detection. Additionally, some xyloglucan was readily soluble with ethanol during the dewaxing process. LM20, on the other hand, did not bind to the cotton fibres in either of the treatments, even after repeated experiments; indicating that the LM20 epitope of methyl-esterified HG is not present on the mature fibre surface, and could be a measure of the level of methyl-esterfication in the mature sample fibre cell wall, as this epitope is more sensitive to pH of the alkali treatment buffers than LM18 or LM19. After the removal of pectic HG using pectate lyase, antibodies LM6 (arabinan) and LM11 (xylan/arabinoxylans), JIM11 (Extensin), JIM13 (AGP), JIM14 (AGP), JIM16 (AGP) and JIM20 (Extensin) were shown to

bind strongly to the fibres, whereas under PL- conditions little or none immunofluorescence was seen. Additionally, the JIM13 for AGP epitope was not surface-detectable and instead located at the inner face of the cell wall, where the plasma membrane would reside during development. This suggests that these JIM13 AGP epitope may be involved in signalling to allow communication between the cytoplasm and the cell wall. The LM6 arabinan epitope has been demonstrated to play a role in cell adhesion as well as cell wall flexibility, which may also be part of cell wall glycoproteins, in the form of LM6-reactive AGP epitopes with cell wall plasticising abilities (Pena & Carpita 2004; Verhertbruggen et al. 2009; Jones et al. 2003; Orfila et al. 2001; Ha et al. 2005; Harholt et al. 2012). For CBM3a, there was little or no binding in the PL- conditions, but after the enzymatic removal of pectin, there were greater levels of binding to the fibres. However, for the case of xyloglucan and the LM11 xylan epitope, pectic HG was shown to be masking these polysaccharides in transverse section analysis, indicating that they are intimately associated with homogalaturonan and not just covered by a physical barrier. For all these polysaccharides that are detected in the mature cotton fibre, they are obviously remnants of the developmental stages, yet the question remains how they relate to the developmental process as well as how they impact on the fibre structure and properties.

6.1.2 Heteromannans and cryptic epitopes in the cotton fibre

While there is little evidence on the localisation and type of heteromannans in cotton fibres, the results do give evidence to support the hypothesis that they are masked and possibly acetylated. In other systems, there is a strong indication from genetic studies that hetero mannans play a role in embryogenesis (Goubet et al. 2009). Pretreating fibre surface samples with KOH or Na_2CO_3 does not allow detection of heteromannans, however if the samples is then treated with pectate lyase to remove the ensheathing pectic HG layer, then there is detection only with Na_2CO_3. Further analysis went on to show that it does not matter which order the pre-treatments of Na_2CO_3 or pectate lyase are done, as it has an identical effect. This means that the Na_2CO_3 potentially de-acetylates the heteromannans, while pectate lyase removes the masking by HG, allowing LM21 antibody access and binding to the epitope. After mannanase treatment, there was no detection, verifying the presence of heteromannan after the use of alkali and pectate lyase. Recent evidence highlights that heteromannans are intimately associated with pectic homogalacturonan in *Arabidopsis thaliana Pisum sativum* and *Nicotiana tabacum* (Marcus et al. 2010). Although there is currently no evidence that suggests mannans are connected to pectins, a recent study found that treating tomato pericarp cells with mannanase resulted in a loss of cell adhesion of the fruit tissues (Ordaz-Ortiz et al. 2009), which potentiaaly indicate that hetero mannans may be part of a mechanism that play a role in cross-linking pectins to primary cell walls. Alternatively, the ensheathing of hemicellulosic polysaccharides, such as heteromannans, may regulate the accessibility of cell wall remodelling proteins (in addition to monoclonal antibodies) and thus control the structure and evelopment of primary cell walls. The acetylation status of the

heteromannans in the cotton fibre is a speculation, and further studies using acetyl-esterases will verify this.

6.1.3 Comparative analysis of the occurrence of mature cotton fibre polysaccharide epitopes across several species

Currently only one species of cotton, *Gossypium raimondii*, has had its genome sequenced, which began as part of a public initiative in 2007 (Chen et al. 2007). In this initiative, *G. raimondii* (a D-genome progenitor species of cotton), was planned to be sequenced first, followed by *G. arboreum* (a A-genome progenitor species) and finally the globally important *G. barbadense* and *G. hirsutum* (AD-genome species representing 99% of cotton grown each year) (Al-Ghazi et al., 2009). In 2010, *G. raimondii was* sequenced in a joint partnership between Monsanto and Illumis, the results of which were made freely available to the scientific community (Lin et al., 2010). While *G. raimondii* was not available for the research carried out in this chapter, the A-genome progenitor *G. arboreum* was used along with three other species for a comparative study of the mature cotton fibre cell wall. Ten lines of mature cotton fibres were chosen, encompassing four species: the A-genome progenitor species of *G. arboreum*, *G. herbaceum* and the AD-genome species of *G. barbadense* and *G. hirsutum*. Seven monoclonal antibodies were used, covering the main non-cellulosic polysaccharides present in the mature cotton fibre. Low-methylesterified pectic HG and xyloglucan were detected abundantly in the mature fibre across all the species. Heteromannans were not detected in the A-genome species *G. arboreum* or *G. herbaceum* at all, while the LM21 epitope was detected in the AD-genome species of *G. barbadense* and *G. hirsutum*. A-genome species are evolutionary much older than AD-genome species, which were formed from a hybridisation

event of which all the major commercial cotton crops are descended from (Wendel and Cronn, 2003). This absence of heteromannan could mean AD-genome species got their heteromannan synthesis genes from D-genome species and not A-genome species. Additionally, the LM5 and LM6 epitopes for galactan and arabinan, respectively, were abundantly detected in the A-genome species of *G. herbaceum* when compared to the nine other lines used in this study. In respect of cell wall evolution of the cotton fibre, this research shows that monoclonal antibodies can be useful and powerful tools to study the structural epitopes and features of the cell wall. Ultimately, the sequencing of the AD-genomes of *G. barbadense* and *G. hirsutum* over the next decade will bring a much needed stimulation to the rudimental research of genome evolution, polyploidisation, fibre gene expression, cellulose biosynthesis, cell wall differentiation and development and cotton epigenetics. Through further research on these fundamental aspects will lead to practical cell wall consequences such as improvements in fibre length, yield, qualities, fertiliser/pesticide usage, water usage and stress tolerance.

6.1.4 Significance for the future understanding of cotton fibre cell walls.

This observation adds to our understanding of the distinctive cell wall structural heterogeneity of the mature cotton fibre and cell walls in general. However, what is the significance of the association of xyloglucan, xylan and mannan polysaccharides with pectic homogalacturonan in terms of fibre cell wall assembly, functions and properties? Cell wall polymers need to be interlinked to generate insoluble and rigid cell walls. Links between pectins and hemicelluloses, including mannans may have a structural role to maintain primary cell wall integrity during development and stay as polysaccharide remnants in the mature fibre cell wall. In summary, the observations from this research goes towards confirming that masking of certain hemicellulose polymers, made up of xyloglucans, xylans and heteromannans, by pectic HG is a real biological phenomenon with potential significance for understanding the cotton fibre cell wall development, structure and properties. This suggests an important role for homogalacturonan in the fibre primary cell wall and how it may generate conditions that regulate the access of cell wall modifying enzymes or other proteins to regions of action. These observations also indicate that a more careful approach is needed in regards to cell wall immunocytochemistry of xyloglucans, xylans and mannans in the context of potentially cryptic epitopes. While certain polysaccharides have been identified in the mature cotton fibre cell wall, these epitopes need to be explored one by one in future work to establish the structure-function relationship to final fibre properties.

6.2 Characterisation of polysaccharide profile changes in the developing cotton fibre

6.2.1 β-glycans in the developing cotton fibre

The cotton fibre cell wall is one of the purest forms of cellulose in nature, making up to 97% of the fibre at maturity, and determines several key properties of fibre for industrial usage after harvesting (Barber and Hassid, 1965; Zeronian and Ryu, 1987; Abidi et al., 2009; Morais Teixeira et al., 2010). Using the cellulose staining dye in developing cotton fibre microscopy and image J quantification analysis, the majority of cellulose deposition occurs between 15 and 35 dpa, and after 40 dpa, there would appear to be little change in the cellulose content of the fibre. In terms of the cellulose structure, using CBM3a to detect regions of crystalline cellulose shows that overall; the fibre cell wall cellulose appears to become more crystalline with development. One interesting aspect during development is that after 15 dpa, pectate lyase action is required to detect cellulose, indicating that perhaps this pectic homogalacturonan sheath of the cell wall undergoes a dynamic change in its structure that causes a reduction in the accessibility of CBMs to the cellulose regions compared to other non-CBM detected epitopes. For callose (β-(1→3)glucans), the peak detection of this epitope was seen around 20 dpa, which coincides with the transition of the elongation phase to that of the secondary wall synthesis stage, which has been reported several times in cotton fibre callose research (Maltby et al., 1979; Waterkeyn, 1981; Brill et al., 2011). Additionally, callose remains present in the developing fibre until the later stages of secondary wall synthesis and by maturation, the callose content is less than 2% (Brill et al. 2011; Pillonel & Meier 1985; Pillonel et al. 1980). These previously reported findings complement the *in situ* immunolabelling of 60 dpa fibre surfaces and

transverse sections, as well as preliminary CoMPP assays (**Error! Reference ource not found.**), where a decrease in callose content is seen through development and the detection of this polysaccharide epitope is very low in mature fibres by 60 dpa. Mixed linkage glycans were not detected at all in the cotton fibre during development, which should be expected as they are mostly confined to the poales, however the use of MLG monoclonal antibodies in cotton fibre research are still useful and can function as negative controls.

6.2.2 Pectins in the developing cotton fibre

JIM5, JIM7 and the newer LM18, LM19 and LM20 monoclonal antibodies are a useful set of probes which bind to subtly different epitope of pectic homogalacturonan of different levels of methyl-esterification (Verhertbruggen, Marcus, Haeger, Ordaz-Ortiz, et al., 2009). JIM5 and JIM7 have been extensively characterised and studied using plant cell walls (Clausen et al., 2003). The LM19 epitope is similar to that of JIM5, which bind to regions of pectic homogalacturonan with some regions of pectic methyl-esterification. JIM7 on the other hand, detects an epitope that is similar to that of LM20; however JIM7 can detect a lower level of methylesterification of the pectic homogalacturonan (Verhertbruggen, Marcus, Haeger, Ordaz-Ortiz, et al., 2009). The LM18, LM19 and JIM5 epitopes are all detected with increasing intensity as development progresses. JIM5 is more selective than LM19; hence there is a much stronger intensity with the newer monoclonal antibody. LM20 and JIM7, however decrease throughout development, to the point where LM20 is not detectable at all by 25 dpa and very weak detection of the JIM7 epitope upon maturity. These finding suggest that the pectic homogalacturonan is initially found in the developing cotton fibre in a form that highly methyl-esterified, which is gradually lost as

development progresses. Additionally, previous investigations suggest that the synthesis of the pectic sheath begins at 1 dpa, which rapidly covers the fibre cell by 2dpa, shown using a wide range of pectic homogalacturonan binding probes (Bowling et al., 2011). As homogalacturonan can modified in varying degrees by pectin methyl-esterases to alter its structural properties (Wolf and Greiner, 2012), such as cell elongation and adhesion and in other plant systems, areas of non/low-methylesterified homogalacturonan have been found specifically in the regions of adhered/adhesive cell walls as well as the ability for cells to elongate (Liners et al., 1992; Derbyshire et al., 2007). Additionally, the pattern as well as the degree of methylesterification has an effect on the structural and mechanical properties of pectic homogalacturonan (Willats et al. 2001). The *Arabidopsis thaliana* mutant, *gal-3,* had reduced amounts of methyl-esterified homogalacturonan in the leaf hypocotyls cell walls and as a result, had reduced cell elongation when compared to the wild type phenotype (Derbyshire et al., 2007). Pectic homogalacturonan chains that have low levels of methyl-esterification have been shown to be able to cross link with each other in the presence of calcium ions, which cause the cell walls to become more rigid and in turn reduce cell elongation (Willats et al. 2001). It is widely accepted that pectic homogalacturonan is synthesised and deposited in a highly methylesterified form, which allows this polysaccharide to be modified in varying degrees by pectin methyl esterases (PME) (Øbro et al. 2009; Willats et al. 2001; Wolf et al. 2009; Wolf & Greiner 2012). In elongating cotton fibres, this property would be important, and would explain why the methyl-esterification is lost after the elongation phase ceases and the secondary wall synthesis stage begins, where a more rigid fibre cell wall would be advantageous. On top of this,

the role of pectin methyl-esterases during early development must be an important factor in cotton fibre cell wall growth.

6.2.3 Hemicelluloses in the developing cotton fibre

Xyloglucan is one of the most abundant non-cellulosic polysaccharides in the cotton fibre (Hayashi & Delmer 1988; Verlag et al. 2003; Michailidis et al. 2009), and this work has highlighted that this polysaccharide is masked by pectic homogalaturonan. The monoclonal antibodies LM15, CCRC-M1 and well as the newly developed LM25, all detected large amounts of the xyloglucan epitope in developing cotton fibres, however only after the enzymatic treatment using pectate lyase. The LM24 epitope, which is thought to be in the form of partially galactosylated xyloglucans, was only detected in very small amounts in the early stages of development. Overall, xyloglucan content in the developing fibre increased with the fibre age, especially during the stages of elongation and secondary wall synthesis, with an increase seen between 20-25 dpa, and the strongest detection signal seen between 40-60 dpa. In previous investigations, peak levels of xyloglucan were detected in the developing cell walls at 15 dpa, which gradually decreased (Shimizu et al., 1997; Verlag et al., 2003; Al-Ghazi et al., 2009). The elongation stage stops around 20 dpa, and the cell length stops increasing while the secondary cell wall is rapidly synthesised and deposited. Xyloglucan has been extensively shown to bind to cellulose microfibrils though non-covalent associations and stabilise the cell wall by acting as a load bearing structure (Pauly et al. 1999; Carpita & Gibeaut 1993; Ryden et al. 2003). In previous work, xyloglucan-cellulose components were found more in the areas close to the plasma membrane (Bowling et al., 2011). In the hypocotyls of the *arabidopsis thaliana* mutants *mur2, mur3, xxt1, xxt2 and reb1-1* had reduced

amounts of xyloglucan that could cross-link with cellulose, and as a result, the overall tensile strength of the tissue was found to be lower than that of the wild type (Ryden et al., 2003; Cavalier et al., 2008). Xyloglucan endotransglycosylases/hydrolases (XTHs) in other systems have been extensively shown to catalyse the breakdown and reattachment of xyloglucan chains and in turn cause cell wall loosening, especially in elongating cell walls (Fry et al. 1992; Vissenberg et al. 2003; Miedes & Lorences 2009; Vissenberg et al. 2000). In developing cotton fibres, these XTHs have been repeatedly shown to be upregulated during the early stages of the fibre elongation stage, as opposed to the latter stages around 20 dpa (Michailidis et al., 2009), where they have also been show *in vitro* to preferentially degrade fucosylated xyloglucans (Verlag et al., 2003). These findings suggest that cotton fibre XTHs are positively correlated with fibre cell wall elongation (Michailidis et al., 2009). During fibre elongation, the wall thickness does not decrease, so XTHs may incorporate new cell wall material as it is biosynthesised. Additionally, the wall loosening ability of XTHs, which has been shown in other systems to be localised to regions of xyloglucan-cellulose, and through the rearrangement of these xyloglucan chains would allow the cell to expand through turgor driven forces (Vissenberg et al. 2005; Van Sandt et al. 2007).Furthermore, it is suggested that over expression of the genes encoding these fibre XTHs could result in longer fibres, highlighting the importance of linking fibre cell wall immunocytochemistry with transcriptome data (Michailidis et al., 2009). Whilst XTHs have been extensively studied, there are also another group of proteins called expansins which are involved in cell wall loosening and expansion (McQueen-Mason et al. 1992; Sampedro & Cosgrove 2005; McQueen-Mason & Cosgrove 1995). While no hydrolytic or

transglycosylase activity has been detected, repeated evidence suggests that these proteins function by acting at the interface of cellulose-hemicellulose chains and catalyse the breakage of non-covalent associations t allow cell wall loosening and expansion (McQueen-Mason & Cosgrove 1995). In previous work, mRNA transcripts for expansins and endoxyloglucan transferases were detected in abundance between 9-16 dpa, followed by a sharp decrease (Shimizu et al., 1997). The presence of expansins would aid in the cell wall expansion, while the endoxyloglucan transferases would help maintain the cell wall architecture during the elongation stage, which would explain the increased detection of xyloglucans using *in situ* labelling during this fibre development stage. In terms of other properties, the side chains of xyloglucans in plant cell walls have been suggested to optimise the non-covalent binding to cellulose, as well as stabilise the overall conformation (Cosgrove, 1997). This would have an effect of strengthening the cell wall and reduce the ability of elongation, which would explain why xyloglucan content appears to increase after 20 dpa, when the fibre ceases to elongate and develop the thick secondary cell wall.

6.2.4 The presence of a cotton fibre middle lamella (CFML)

During development, specifically around 10-18 dpa, thick bands of pectic homogalacturonan and thin bands of xyloglucan and arabinan epitopes were detected along the fibre surfaces. This time period of development coincides with the majority of the elongation stage, in which the fibres have previously been shown to elongate in adhesive bundles via a pectin rich cotton fibre middle lamella (CFML) during 3-18 dpa (Vaughn and Turley, 1999; Singh et al., 2009). In this work by Singh 2009, the presence of the CFML is predicted to explain how hundreds of thousands of fibres can achieve their great length in a very confined boll space. The distinct xyloglucan and arabinan polysaccharide profiles seen in Chapter 4, is similar to cell wall adhesion planes in other plant systems , consequently it could be speculated with these preliminary results these polysaccharides, in addition to pectic homogalacturonan, play a key role in keeping the fibres together in the CFML. Pectic homogalacturonan has been extensively studied in plant cell walls, and there is significant evidence for the roles it plays in cell wall development and assembly (Miao et al. 2011; Capodicasa et al. 2004; M.-C. Ralet et al. 2008; Willats et al. 2001; Wolf et al. 2009; Verhertbruggen et al. 2009; Wolf & Greiner 2012). As previously mentioned, homogalacturonan can modified in varying degrees by methyl-esterification to alter its structural properties, such as cell elongation and adhesion. In other plant systems, areas of non/low-methylesterified homogalacturonan have been found specifically in the regions of adhered/adhesive cell walls (Liners et al., 1992). Additionally, the pattern as well as the degree of methylesterification has an effect on the structural and mechanical properties of pectic homogalacturonan (Willats et al. 2001). Despite these factors, which are widely accepted, there is

still a wide consensus that homogalacturonan is not the only polysaccharide involved in adhesion, with evidence pointing towards xyloglucans, arabinans and galactans also speculated to be important (Jarvis et al. 2003; Ordaz-Ortiz et al. 2009). In a tobacco mutant system with reduced arabinose, a reduction in cell adhesion was seen, further suggesting that arabinan has a key role in adhesion (Iwai et al., 2002). Additionally, while arabinans are suspected to be highly mobile polysaccharides (Ha et al. 2005; Ralet et al. 2008), there is also evidence that suggests they can form cross linkages with cellulose (Zykwinska et al., 2005; Ferreira et al., 2006). Arabinan has also been shown to be positively correlated with maintaining the cell wall flexibility by interfering with the formation for calcium mediated homogalacturonan interactions (Jones et al., 2003). In other studies, arabinan was detected strongly in younger fibres, and galactan strongly detected in fibres that were undergoing rapid elongation (Bowling et al., 2011). As for the role of these polysaccharides in cotton fibre cell wall adhesion and development, two simplified models of how they are organised is presented below (Figure 6.1, Figure 6.2 & Figure 6.3). In Figure 6.1, the fibre cells are adhered to one another, with the formation of middle lamellas and intercellular junctions at the adhesion planes. Using pectic homogalacturonan mAbs in other systems, high levels of epitope detection were often seen at the regions of cell junctions and corners, which suggested the important role of pectic homogalacturonan in cell wall adhesion (Christiaens et al. 2011; Sila et al. 2009; Willats et al. 2001). When the fibres are teased apart, it would leave regions of pectic homogalacturonan which would appear in the form of thick longitudinal bands. Pectic homogalacturonan has been extensively studied and reported to be present at the planes of cell wall adhesion and intercellular spaces (Orfila et al. 2002; Jarvis et al.

2003; Verhertbruggen et al. 2009). There would appear, however, to be too many homogalacturonan bands on the surface of developing fibres for it to be part of the intercellular spaces, so two more models are presented below. In Figure 6.2, during elongation, the pectic homogalacturonan interlocks, adhering to other polysaccharides, namely xyloglucan and arabinan. In Figure 6.3 however, the HG could bind to HG, and xyloglucan/arabinan could adhere to the xyloglucan/arabinan polymers on the adhering fibre. It could even be a combination of both models. Overall, this opens up exciting opportunities and future work should aim to uncover the effects this CFML on cotton fibre quality, such as length, by modifying CFML-related pathways. Additionally, they suggest that the de-methylesterification of pectic homogalacturonan plays a role in fibre cell adhesion and elongation.

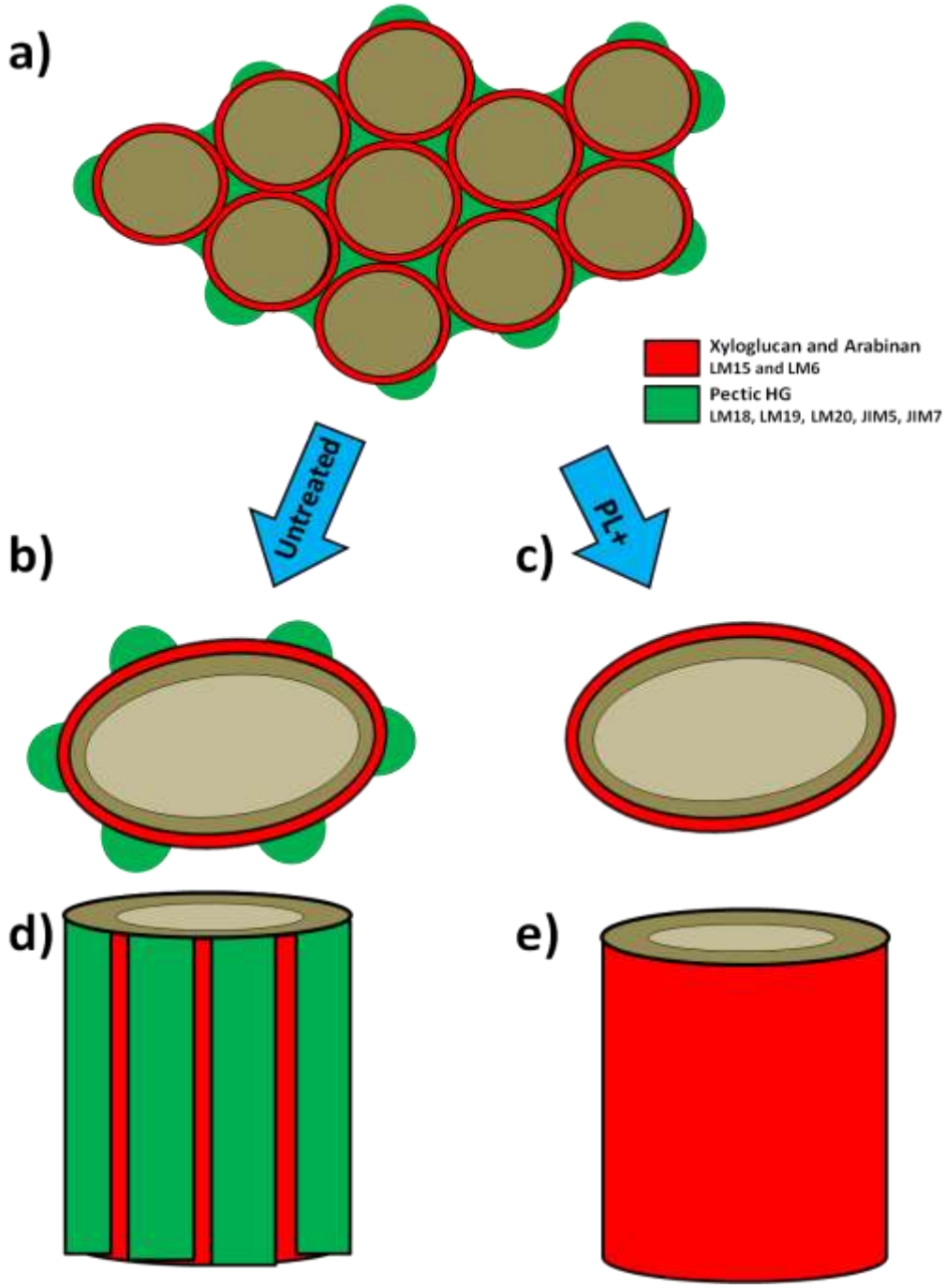

Figure 6.1: Simplified middle lamella model of the CFML in relation to pectic HG, arabinan and xyloglucan. a) Developing fibres elongate in bundles, adhered by a middle lamella that is rich in homogalacturonan. b) d) Untreated fibres that are teased apart suggest that HG epitopes are in thick bands, while arabinan and xyloglucan epitopes are detected in thin bands. c) e) Pectate lyase treatment removed the pectic HG epitope, while fully revealing arabinan and xyloglucan epitopes. Brown – cotton fibre, green – HG, red – arabinan and xyloglucan.

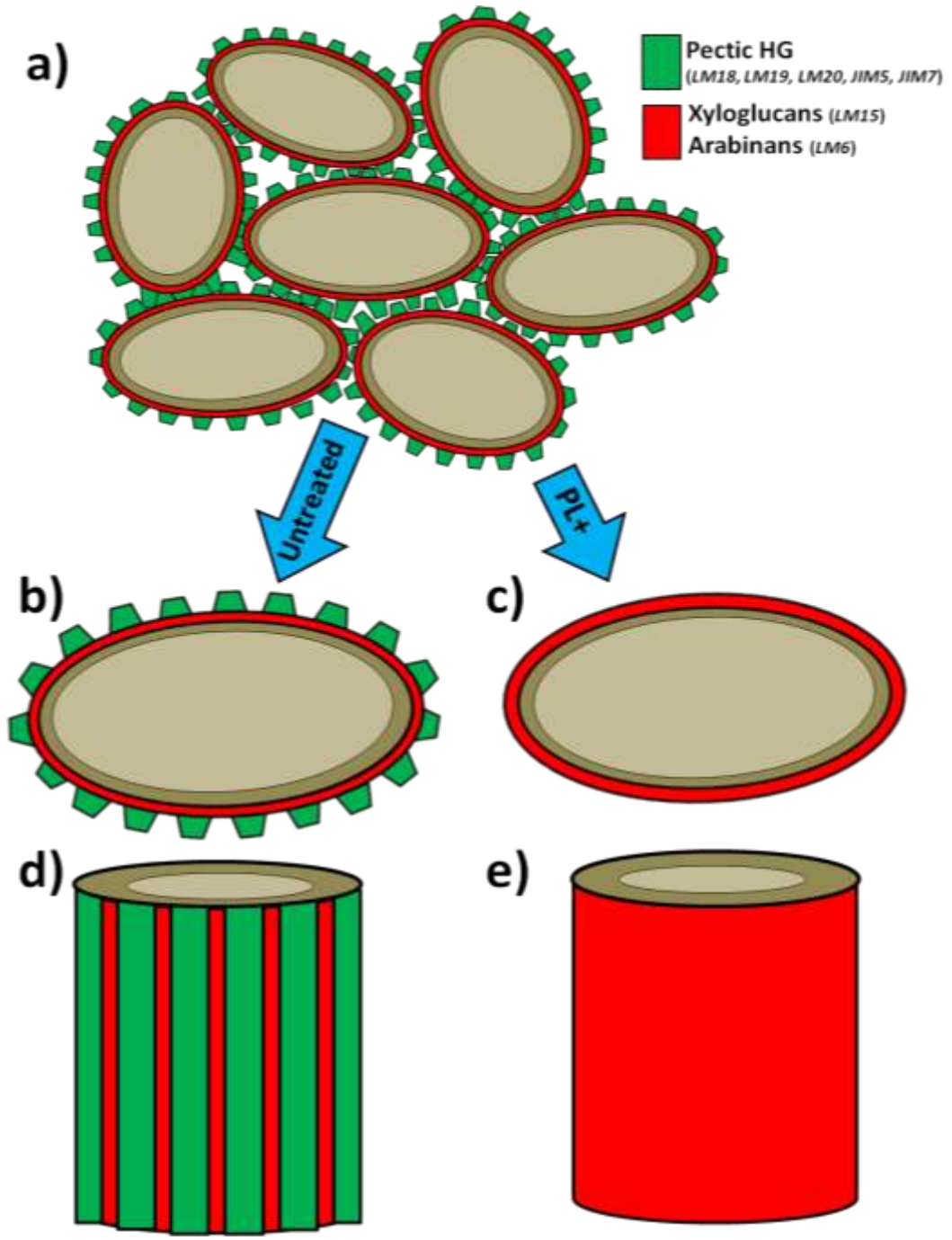

Figure 6.2: Simplified interlocking model of the CFML in relation to pectic HG, arabinan and xyloglucan. a) Developing fibres elongate in bundles, adhered by an interlocking CFML that is rich in homogalacturonan that forms intimate associations with xyloglucan/arabinan polysaccharides in the primary cell wall. b) d) Untreated fibres that are teased apart suggest that HG epitopes are in thick bands, while arabinan and xyloglucan epitopes are detected in thin bands. c) e) Pectate lyase treatment removed the pectic HG epitope, while fully revealing arabinan and xyloglucan epitopes. Brown – cotton fibre, green – HG, red – arabinan and xyloglucan.

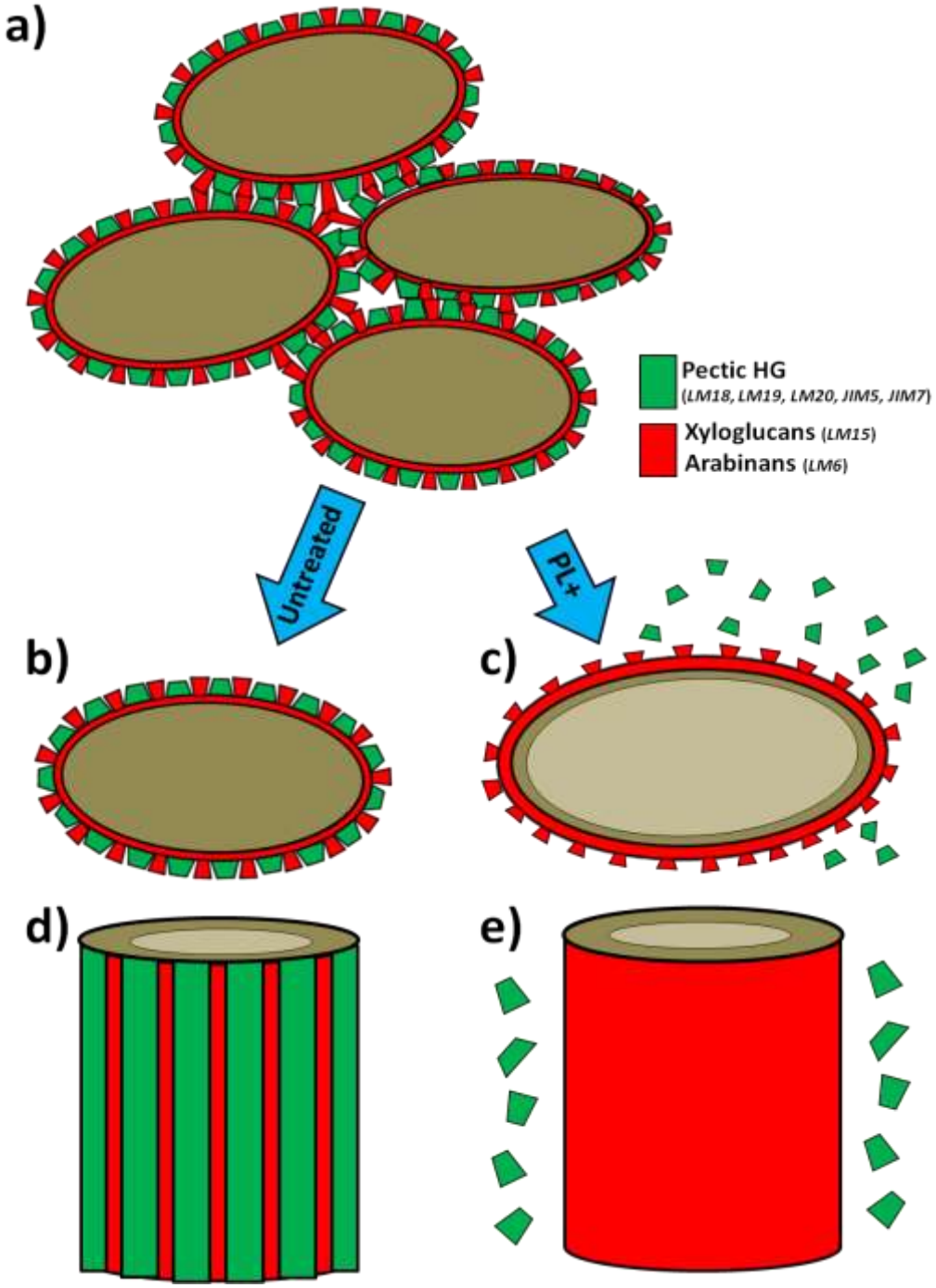

Figure 6.3: Simplified adhesion model #2 of the CFML in relation to pectic HG, arabinan and xyloglucan. a) Developing fibres elongate in bundles, adhered by a CFML that is rich in homogalacturonan, but adhered by the interactions of HG with HG, and xyloglucan/arabinan with xyloglucan/arabinan polysaccharides. b) d) Untreated fibres that are teased apart suggest that HG epitopes are in thick bands, while arabinan and xyloglucan epitopes are detected in thin bands. c) e) Pectate lyase treatment removed the pectic HG epitope, while fully revealing arabinan and xyloglucan epitopes. Brown – cotton fibre, green – HG, red – arabinan and xyloglucan.

6.2.5 Proximal detection of (1-4)-β-D-galactan in elongating cotton fibres

The presence of (1-4)-β-D-galactan, which was detected by the monoclonal antibody LM5, was found in developing cotton fibres. The LM5 galactan epitope was found localised to all the proximal ends of the fibres (bases) where it is attached to the seed coat epidermis (Figure 6.4a-b). Pectate lyase revealed the epitope all along the fibre (Figure 6.4c); however this disappeared by 20-25 dpa. In terms of the fibre developmental stages, the galactan epitope is only found at the fibre bases during the initiation and elongation stages. By 20 dpa, the galactan epitope is not detectable in the fibre – a point in development that coincides with the transition of the fibre elongation stage to the fibre secondary wall biosynthesis stage, in which high levels of cellulose deposition occur, as well as the point at which fibres no longer adhere to one another via the degradation of the CFML (Haigler et al., 2009; Singh et al., 2009). In terms of physical properties, the regions where the fibre cell bases attach to the seed coat epidermis have previously been described to have a thicker primary wall than the distal regions of the fibre (Vigil et al., 1996), and this may actually be in the form of a (1-4)-β-D-galactan rich region that allows the fibre to form a strong attachment to the ovule. In other plant systems, galactan has been shown to be present in cells undergoing rapid cell elongation while also positively correlated with contributing to wall tensile strength (Jones et al., 2003; McCartney et al., 2003). These factors potentially implicates galactan in fibre elongation and as well as the degradation of the CFML, such as acting as a load bearing substitute polymer scaffold at the fibre bases until the majority of load bearing cellulose is synthesised. It could be speculated that the galactan turnover at 20 dpa actually produces downstream signalling polysaccharides, in the form of possible small galactan polymers

released by the degradation of the CFML or perhaps playing a role in fibre cell wall adhesion (Singh et al., 2009). It will be worthwhile to further explore the sharp decrease in galactan at the transition stage, as there may be a possible role for it in fibre elongation and adhesion.

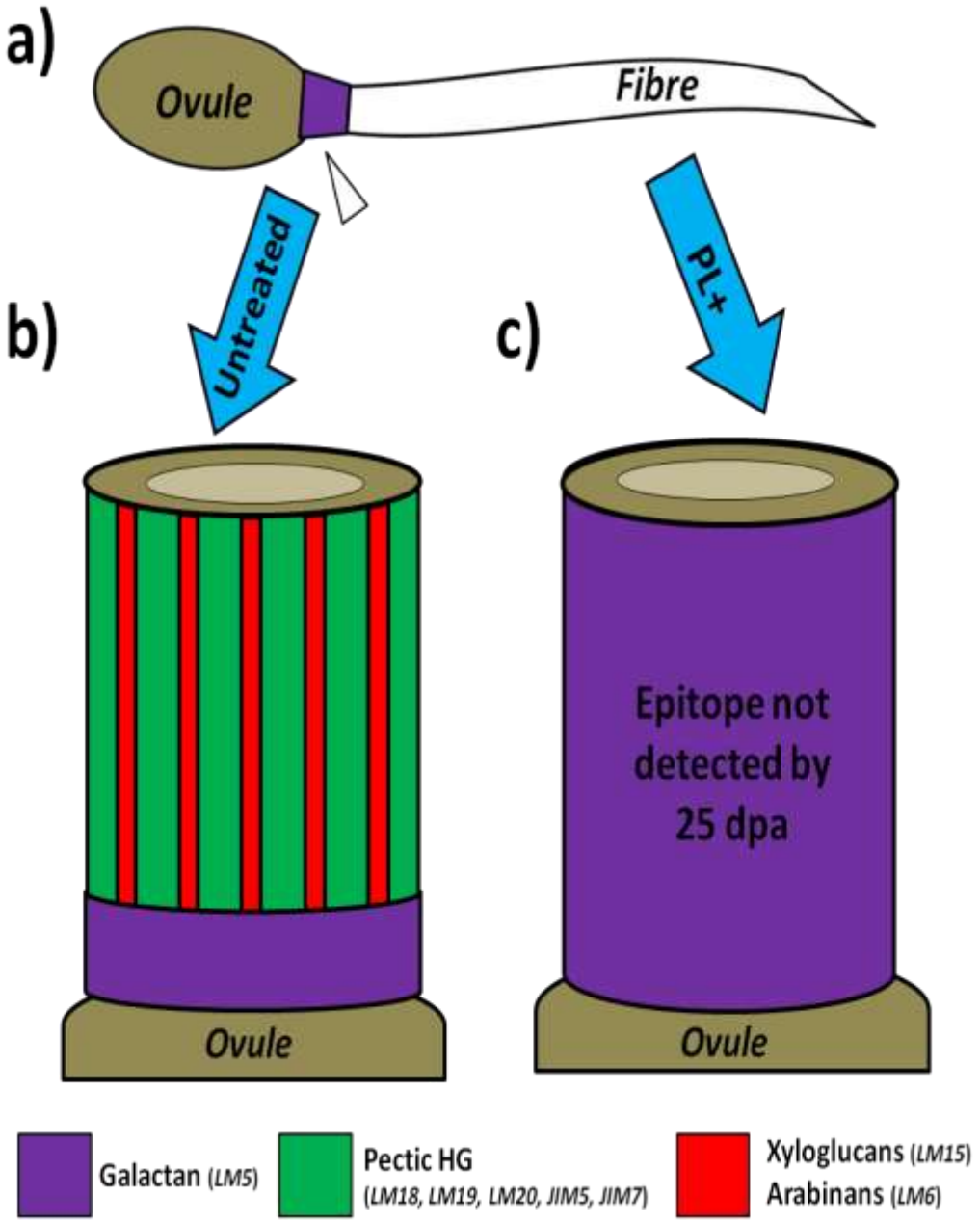

Figure 6.4: Simplified model schematic of the galactan epitope localisation during elongation. a) b) The galactan epitope in untreated fibres was localised to the fibre developing fibre bases where the fibre was adhered to the seed coat epidermis of the ovule. c) Pectate lyase treatment reveals the LM5 epitope to be all over the fibre, which disappears by 25 dpa.

6.2.6 Cell wall glycoproteins and the developing cotton fibre

This research has demonstrated that cotton fibre cell wall have detectable AGPs present through the use of AGP binding monoclonal antibodies. Through *in vitro* studies and *in situ* analyses, suggest that the developing cotton fibre cell wall protein content decreases throughout development (Figure 4.12, Figure 4.13, Figure 4.14, Figure 4.15 and Figure 4.16) and in previous work, this reduction has been shown several times (Huwyler et al., 1979; Gokani and Thaker, 2000). A diversity of extensin and AGP epitopes were detected, especially during the early stages of development, suggesting that these glycoproteins have a crucial role in the cell wall assembly and architecture of the developing cotton fibre. Studying developing cotton fibre cell extracts using SDS-PAGE and Western blotting highlighted the presence of multiple populations of AGPs and extensins, ranging from 200 kDa to 45 kDA in the form of smears with distinct bands. As mentioned, the JIM13 AGP epitope was not only found at the cell wall-plasma membrane interface in mature fibres, and was detected very strongly in developing cotton fibres. This again suggests that these JIM13 AGP epitope may be involved in cell wall signalling to the cytoplasm. Additionally, preliminary work on looking at the intimate associations between these glycoproteins and polysaccharide chains of the RG-I backbone and arabinan will hopefully shed further light on how they are involved in cotton fibre cell wall assembly. While the LM6 epitope is arabinan, it may also detect arabinan-reactive AGPs in the cell wall, suggesting a plasticising role in cell wall flexibility during developing. These interesting AGP polysaccharide complexes (Tan et al., 2012) may also be involved in cell wall assembly/architecture and further studies utilising a combination of monoclonal

antibody immunolabelling and SDS-PAGE gels/western blots will be excellent tools to characterise these APCs throughout the fibre developmental stages.

6.2.7 The developing cotton fibre and future work

As mentioned, this research shows that monoclonal antibodies and carbohydrate-binding modules can be useful and powerful tools to study the structural epitopes and features of cell wall assembly. The preparation of sample material for microscopy, whether developing or mature fibres, is however a time consuming process. This means that immuno-microscopy is not ideal for high throughput approaches towards characterising the cell wall profiles across multiple lines and species of cotton at different developmental stages. An attractive approach to using monoclonal antibodies for high throughput studies is the comprehensive microarray polymer profiling (CoMPP) technique, which produces quantifiable data on the extracted polysaccharides of plant cell walls (Moller et al., 2007).

6.3 Processing of mature cotton fibres

Using a series of carbohydrate-binding modules alongside ATR-FTIR and X-ray diffraction can be a powerful combination of tools that can supply some useful interpretations of the crystallinity changes in the cellulose of cotton fibres (Kljun et al., 2011; Široký et al., 2012). Using two CBMs directed to crystalline cellulose and amorphous cellulose, the crystallinity changes were rapidly detected through quantitative immunolabelling. As CBMs are directed and bind to the surfaces of cotton fibres, this is the first point of contact for the alkali treatments and the variability of probe binding is more sensitive to crystallinity changes at the lower alkali treatment concentrations. As a result, CBMs are more sensitive to detecting alkali induced cellulose crystallinity changes, when compared to the crystallinity changes detected from ATR-FTIR and X-ray diffraction. While ATR-FTIR and X-ray diffraction have been used in the study of crystallinity changes in cotton fibre cellulose, using molecular probes such as CBMs can offer a novel method of quantitative and qualitative analysis of the changes that occur with alkali treatments. Additionally, CBMs have advantages over ATR-FTIR and X-ray diffraction, primarily by not changing the crystallinity of cellulose samples during preparation. CBMs have also shown to be more sensitive with the added ability to analyse the spatial crystallinity changes along individual fibres rather than relying on averages of bulk samples. Future work should apply these techniques to determine the changes in cellulose crystallinity during cotton fibre development and relate these changes to its structural and functional relationships. Additionally, more reconstituted cellulose fibres can be analysed in relation to their crystallinity changes. Reconstituted cellulose fibres were also studied using these techniques, and the results demonstrate that it is also possible to use these CBM

immunolabelling techniques in a versatile way to study regenerated cellulosic fibres such as lyocell. The CBMs also show sensitivity to surface supramolecular changes in cellulose crystallinity after mechanical, chemical and thermal treatments. Additionally, the uses of CBMs also visually dictate the presence of a semi-permeable skin layer, as well as a differential lyocell fibre core (Široký et al., 2012), which will certainly be in interesting area for further research. As the global population grows and the available land for cotton crops decreases, coupled with oil supplies running out for petroleum based fibres (e.g. Nylon), textiles made from reconstituted cellulose and other polysaccharides will become increasingly dominant. Therefore a thorough understanding of the structural properties in response to industrial processing will be very important.

6.4 Concluding remarks

Our understanding of polysaccharide function and cell wall assembly in general is still poor. In terms of the actual cell wall, little detail is known about the spatial localisation, distribution and interaction of these polysaccharides. Due to this, the understanding of the functions of these differential occurrences during cell wall development, represent the current major challenge in plant cell wall biology. Specifically, the cotton fibre is an excellent model to study the spatial localisation, distribution and functionality of cell wall polysaccharides and glycoproteins during fibre development, maturity and processing. This study shows that monoclonal antibodies and carbohydrate-binding modules are powerful and useful tools to study cotton fibres throughout development, maturity and processing. These results indicate that a diversity of polysaccharide structures other than cellulose are present in cotton fibres. Certain polysaccharides have been identified in the mature and developing cotton fibre cell wall; and these epitopes need to be explored one by one in future work to establish the structure-function relationship to final fibre properties. Using this research as a base, future studies should aim to further explore several key points:

- The role of the galactan side chains of RG-I in fibre elongation and cell wall adhesion in the CFML during development.
- The role of xyloglucan and arabinan, as well as pectic HG in the CFML form and function. Furthermore, a detailed analysis of the CFML composition and changes during development is required.
- Acetylated heteromannans and their role in cotton fibres development will need to be studied further. In other systems, the deacetylation of pectic HG causes a cessation of cell elongation (Ishii et al. 1997).

- The lack of understanding of cotton fibre AGP and glycoproteins are still a major issue in this field, and the structure-function relations need to be clarified. This can be done with genetic studies and manipulation of cell wall gene expression.
- Further exploitation of the CoMPP glycan microarrays, as well as the use of chromatography, used in conjunction with immunolabelling and microscopy will be a very useful strategy to highlight all the structural, functional compositions of the cotton fibre cell wall (and cell walls in general). This will allow the discovery of important polysaccharide fibre property links that will define the future path of cotton fibre cell wall research.

This work will provide a base for further cotton fibre cell wall developmental studies. With this there will be potential for the manipulation of these key polysaccharides during development to alter the final fibre properties. With the predicted sequencing of the *G. barbadense* and *G. hirsutum* genomes over the next few years, the area of cotton fibre research will be an exciting field. While some aims are more achievable than others, the ultimate goal of improving cotton fibres and the efficiency of associated industrial practices will have significant economic, ecological and societal impacts of both national and international scales.

Chapter 7

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