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Lignin Deposition in Cotton Cells – Where is the lignin?

Colleen P Macmillan, Hannah Birke, Frank Bedon and Filomena A Pettolino*

CSIRO Plant Industry, PO Box 1600, Canberra, ACT, 2601, Australia

Introduction

Lignin is often described as one of the most abundant biopolymers on earth, second only to cellulose. Despite this, our understanding of lignin deposition, structure, function and the factors that control lignin biosynthesis and deposition lacks clarity. While model species such as *Arabidopsis thaliana* and poplar have been investigated, detailed examination of lignin deposition in agriculturally important crops such as cotton has been very limited.

Lignin is a complex polymeric structure of phenolic compounds containing monolignols (coniferyl alcohol (guaiacyl, G-lignin monomer), sinapyl alcohol (sinapyl, S-lignin monomer), p-coumaryl alcohol (hydroxyphenyl, H-lignin monomer) and 5-OH coniferyl alcohol (5-hydroxyguaiacyl, 5-OH G-lignin monomer)) [1]. The key genes involved in lignin biosynthesis have been identified and functionally characterized in model plants allowing the realisation of lignin manipulation for potential useful application in plants (reviewed by Bedon and Legay) [2]. The lignin monomers are generally associated with the plant secondary cell wall (SCW) typified by specialised cells such as xylem vessels, xylem fibres in angiosperms, and tracheids in gymnosperms. The SCWs in these cells function to allow water transport via xylem vessels and tracheids, to provide mechanical support via tracheids, xylem fibres and phloem fibres, and to protect against biotic attack and abiotic stresses. The physical properties of lignin that contribute to its roles in different cell types is highly dependent on its quantity and quality, the latter determined by the ratio of the monolignols and how they are covalently linked.

Cotton *(Gossypium hirsutum, G. barbadense)* is a major agricultural crop, mainly producing cotton seed fibre for textiles and then oil for food. The potential for value-adding as a feedstock for paper production and lignocellulosic biofuels using stem residue and cotton seed fibre waste is currently generating interest in the composition of cotton plant biomass [3]. Mature cotton seed fibres develop in about 50-60 days following the day of anthesis (DPA), and the fibre SCW develops around 20 DPA [4]. The cotton seed fibre SCW is composed predominantly of cellulose (~94%), and there is one report that it may also contain lignin at very low levels (0.4-1% of dried weight) [5]. G-lignin in cotton stems has been implicated with pathogen defence and recent analysis of lignin levels and types show that cotton stalk (stem) material contains G- and S-lignin with a G/S ratio of between 1 and 2.5, depending on the lignin fraction, and small amounts (~1 %) of H-lignin [6-8]. However there has been no focus on a cell-type specific analysis of cotton lignin, so it is unknown whether the different cell types and SCWs represented by the cotton stalk material or other cotton SCWs such as those of seed fibres, show differential lignin deposition. Given the complexity of SCW types through the whole plant, cotton may prove a useful system for the study of the control of lignin quantity and quality.

In this short communication we aim to show

i) How diverse lignification in cotton cell walls is, particularly in seed fibres, phloem fibres, xylem fibres and xylem vessels, and

ii) Provide *in silico* evidence for the presence of genes potentially involved in monolignol transport and lignin polymerization in stem tissue and seed fibres.

Experimental

Mature *Gossypium hirsutum* (Coker 315-11) plants were grown for 12 months in a glasshouse under natural daylight (Canberra, Australia) at an average of 28°C and watered twice a day. Stem, petiole and immature boll material was fixed in 70% (v/v) ethanol. Samples were sectioned with either a vibratome (stem), or by hand (seeds, seed fibres, leaf petioles). Sections were stained with Mäules stain or examined for autofluorescence. Sections were viewed and photographed with a light microscope (Zeiss Axioimager) [9]. A dissecting microscope (Leica MZFLIII) was used to view and photograph the cut seeds.

For *in silico* analysis the protein sequences of selected Arabidopsis proteins were used as query to identify expressed sequence tags (ESTs) from *G. hirsutum* mixed tissues, stem and seed fibres deposited at GenBank (tblastn). The corresponding full length protein sequence was retrieved from the only sequenced Gossypium genome, *G. raimondii*, (Phytozome; http://www.phytozome.net/) and used for pairwise sequence alignment using a Needleman-Wunsch alignment algorithm and a BLOSUM62 matrix to determine identity to the Arabidopsis
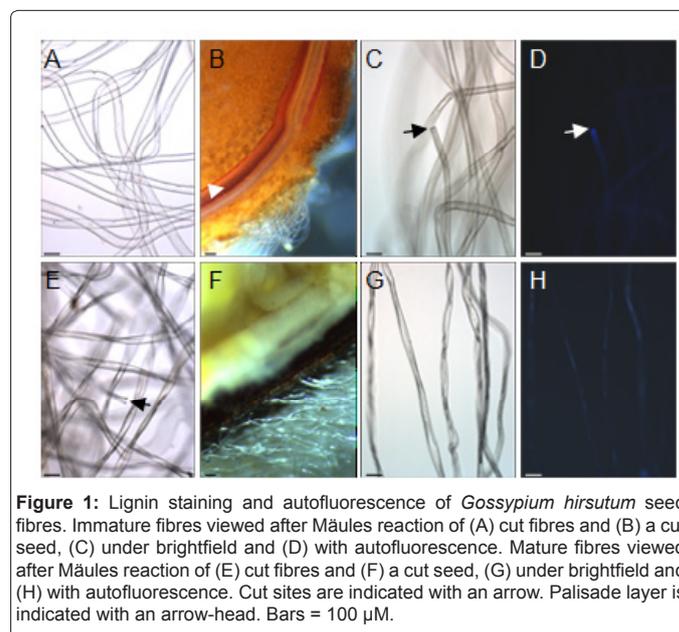
Figure 1: Lignin staining and autofluorescence of *Gossypium hirsutum* seed fibres. Immature fibres viewed after Mäules reaction of (A) cut fibres and (B) a cut seed, (C) under brightfield and (D) with autofluorescence. Mature fibres viewed after Mäules reaction of (E) cut fibres and (F) a cut seed, (G) under brightfield and (H) with autofluorescence. Cut sites are indicated with an arrow. Palisade layer is indicated with an arrow-head. Bars = 100 μM.

*Corresponding author: Filomena A. Pettolino, CSIRO Plant Industry, PO Box 1600, Canberra, ACT, 2601, Australia, Tel: +61 2 6246 4052; E-mail: Filomena.Pettolino@csiro.au

proteins. The *G. raimondii* genome is highly similar to the D genome present in allotetraploid *G. hirsutum* cotton.

Results and Discussion

The presence and localization of lignin in different cotton cell types was determined using Mäules staining – generally S-lignin stains brown and G-lignin stains red. Autofluorescence was also used as an indicator of phenolic compounds (lignins, flavins, certain amino acids etc.). No lignin was visible in cut seed fibres from immature (unopened; ~40 DPA) and mature (fully-opened) bolls, even at the very base of the seed fibre at the seed surface (Figures 1A, 1B, 1E-1G), while the palisade layer of immature seed coat showed clear lignin deposition (Figure 1B). Feint autofluorescence was visible in unstained seed fibres (mature and immature) (Figures 1D and 1H), and at the cut sites of immature seed fibres (Figures 1C and 1D). In contrast to the seed fibres, lignin was clearly visible in SCWs of stems and petioles: phloem fibres appear to be rich in S-lignin, xylem fibres in S-lignin, and xylem vessels in G-lignin (Figures 2A and 2C). Clear evidence for lignin was also found in pith cell walls of the stem and petiole (Figures 2A and 2C). Autofluorescence was visible most markedly in stem and pith phloem fibres, xylem fibres and xylem vessels (Figures 2B and 2D). The middle lamellae of phloem fibres and some xylem fibres in stems and petioles were particularly brightly autofluorescent, and some autofluorescence was also seen in pith cell walls (Figures 2B and 2D). Stem collenchyma cells were also strongly autofluorescent (Figure 2B), and this could be partially due to presence of G-lignin (as seen by brown Mäules staining; data not shown).

The apparent lack of lignin deposition, based on lignin-specific stains, in cotton seed fibre SCWs compared to phloem fibres (S-lignin), xylem fibres (S-lignin) and vessels (G-lignin) indicates that the cotton plant undergoes differential control of lignin deposition in cells with SCWs within the one plant. When measured chemically, lignin content in seed fibre was estimated to be very low - less than 1% although the polymeric nature of the phenolics isolated was not determined [5]. Flax

bast fibres also have low lignin which differs from the abundant lignin staining in cotton phloem fibres that is similar to that of Arabidopsis stem phloem fibres [10,11]. The pattern of lignin staining in cotton xylem fibres and xylem vessels appears similar to what is seen in other plants although the clear lignification of stem and petiole pith cell walls, typically considered primary cell walls (PCWs), is somewhat unexpected. Lignification of some PCWs has been reported and its function in pith cells may be to provide additional mechanical compressive strength to the cotton plant which can grow as a large perennial tree / bush [12,13]. The visualization of lignin deposition specifically in seed coat palisade cells supports similar findings using FTIR [14]. The specific autofluorescence observed at the cut sites in immature fibre was also reported by Fan et al. [5] and could be associated with wounding. Given that no clear lignin staining was visible at the cut sites or anywhere along the seed fibre, we speculate that the autofluorescence could be due to different phenolic-containing molecules. Indeed phenolic esters have been previously associated with suberin on the lint of a green cotton variety [15]. Moreover the autofluorescence of SCWs of some guard cells were attributed to the presence of phenolic esters of pectin [16].

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Little is known about the presence of the final steps of lignification in seed fibres and cotton in general. We therefore aimed to identify putative monolignol transporters and enzymes involved in lignin polymerization in an *in silico* approach. Using the protein sequence of the only known plant monolignol transporter, AtABCG29 [19], as query, we identified a total of five putative ABC-cassette transporters expressed in seed fibres and a cotton tissue mix containing stem tissue (Table 1).
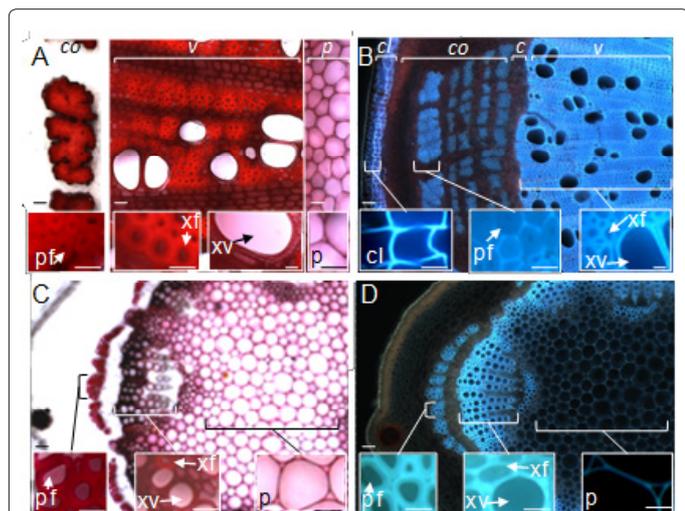
Figure 2: Lignin staining and autofluorescence of *Gossypium hirsutum* stems and petioles. Stem cross sections were examined (A) after Mäules reaction, and (B) with autofluorescence. Various tissue regions are visible in the stems such as cortex (*co*), cambium (*c*), vasculature (*v*), pith (*p*) and collenchyma (*cl*). Petiole cross sections were examined (C) after Mäules reaction, and (D) with autofluorescence. Inserts indicate higher magnification of specific cell types: phloem fibres (pf), xylem fibres (xf), xylem vessels (xv), pith (p), and collenchyma (cl). Bars = 50 µM.

Protein	*A. thaliana* Homolog	*G. raimondii* Homologs	Identity (%)	Tissue
Monolignol	AtABCG29	009G137200, 009G129000	57-72	m
Transporter	(At3g16340)	009G120300, 001G057200, 009G022400	55-57	sf
Peroxidase	AtPRX53	006G143400	74	m
	(At5g06720)	004G093200	56	sf
Peroxidase	AtPRX72	011G211300, 006G143400, 003G151700	44-49	m
	(At5g66390)	009G198700	57	sf
Laccase	AtLAC4	007G378200, 012G111900, 003G124600, 011G279600	64-79	m
	(At2g38080)	009G093800, 003G124600	75	st
		007G378200, 012G111900	77-79	sf
Laccase	AtLAC17	007G376600, 007G376800, 006G171500	68	m
	(At5g60020)	007G376600, 006G171500	68	st
		013G025500	78	sf

Table 1: Putative homologs of *Arabidopsis thaliana* proteins involved in monolignol transport and polymerization in cotton stem and seed fibres. *A. thaliana* protein sequences were used as queries to identify expressed sequence tags from *G. hirsutum* mixed tissues (m), stem (st) and seed fibres (sf) and these used to identify corresponding full length *G. raimondii* protein sequences.

Once transported to the cell wall, monolignols need to be dehydrogenated by peroxidases, laccases and other phenol oxidases before the activated monolignols polymerize in an enzyme-independent process [20]. Peroxidase-encoding genes, *AtPRX53* and *AtPRX72* were shown to be involved in lignin polymerization in Arabidopsis [21,22]. Our *in silico* approach revealed homologs of these proteins expressed in a tissue mix as well as in seed fibres of cotton (Table 1). The protein identity is not very high but attribution of specific functions to peroxidases based on the protein sequence is difficult due to high sequence similarities within the peroxidase family. Expression of group 3 superoxide dismutases and the concentration of their product H_2O_2 increase at the transition stage from elongation to SCW thickening in cotton seed fibres [23,24]. As H_2O_2 serves as substrate for peroxidases, these findings implicate the activity of peroxidases in cotton seed fibres in the SCW thickening stage. In contrast to the identified peroxidase homologs, functional homology can be expected for the laccases identified to be expressed in cotton stem and seed fibres, since they show very high sequence identities to the Arabidopsis proteins AtLAC4 and AtLAC17 (Table 1). Loss of LAC4 and LAC17 activity causes a 30 to 40 % reduction of lignin in the stem of Arabidopsis accompanied by the suppression of almost the whole monolignol synthesis pathway, pointing to an important function of these laccases for lignin polymerization [25].

The proteins encoded by the genes identified in the present study partially close the gap between the enzymes from the phenylpropanoid and monolignol synthesis pathway and the final product lignin, and build on the findings of Al-Ghazi et al. [17] and Fan et al. [5] Expression of a putative monolignol transporter as well as peroxidases and laccases in addition to the enzymes of the core biosynthetic pathway points to the presence of the whole lignification machinery in cotton seed fibres. It is therefore even more surprising that lignin

seems to be absent from these cells. However, it is not yet clear whether the observed mRNA expression reflects protein synthesis and persistence. Post-transcriptional regulation of lignin synthesis genes by microRNAs (miRNAs) might, for example, impact on lignin deposition. Expression of a *G. hirsutum* laccase is indeed regulated by GhmiR397 during seed fibre initiation and a number of other reports suggest the involvement of miRNAs in regulation of lignin synthesis in plants [26-29]. Additionally, Vanholme raised the hypothesis that G-type molecules (monomers, oligomers and/or polymers) regulate the mRNA abundance of genes from their own biosynthetic pathway in Arabidopsis [30]. Whether such a mechanism provides an explanation for the unusual lignin deposition in cotton tissues is speculative but warrants further investigation.

In conclusion, these findings flag cotton as a highly useful agricultural model with which to study lignin accumulation, its biosynthesis from genes to proteins, monolignol transport and polymerization, and biological functions. Indeed, it is clear that different cotton cells with SCWs deposit very different levels, and types, of lignin; for example seed fibres deposit virtually no lignin (Figure 1) while much of the biosynthetic machinery is expressed in seed fibres and stems (Table 1). Furthermore, cotton seed fibres are among the most elongated single plant cells [31] and yield near synchronous cell types as seed fibre initiation and development are highly coordinated. Compared to Arabidopsis, cotton seed fibres and other tissues such as stems produce large amounts of material for RNA and protein analyses, and for phenotyping e.g. yield/strength/elongation. Cotton is more physiologically relevant than other model species like Arabidopsis because it can be, and is, grown in the field, and unlike trees, matures within a season. Transformation methods for cotton are becoming more efficient, and the *G. raimondii* genome was recently released and characterized strengthening this system as a model [32-34]. However, use of cotton as a model could benefit from optimization of growth conditions to speed up flowering time and seed set (e.g. photoperiod / light quality, pruning-practices). From a functional perspective, the study of cotton lignin biology may answer how, and why, cotton seed fibres deposit virtually no lignin in their cell walls and provide further avenues of exploration for the clarification of lignin biosynthesis in all plant systems.

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