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REVIEW PAPER

# Secondary cell walls: biosynthesis and manipulation

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## Abstract

**Secondary cell walls (SCWs) are produced by specialized plant cell types, and are particularly important in those cells providing mechanical support or involved in water transport. As the main constituent of plant biomass, secondary cell walls are central to attempts to generate second-generation biofuels. Partly as a consequence of this renewed economic importance, excellent progress has been made in understanding how cell wall components are synthesized. SCWs are largely composed of three main polymers: cellulose, hemicellulose, and lignin. In this review, we will attempt to highlight the most recent progress in understanding the biosynthetic pathways for secondary cell wall components, how these pathways are regulated, and how this knowledge may be exploited to improve cell wall properties that facilitate breakdown without compromising plant growth and productivity. While knowledge of individual components in the pathway has improved dramatically, how they function together to make the final polymers and how these individual polymers are incorporated into the wall remain less well understood.**

**Key words:** Biofuels, cellulose, lignin, lignin engineering, regulation, SCW, xylan, xylan engineering.

## Introduction

Every plant cell is contained within the cell walls. Actively growing cells are surrounded by a thin primary cell wall that can be altered to allow for expansion. Once cells reach their final size and shape, many will produce a thicker secondary cell wall (SCW). While primary cell walls are formed by all plant cells, SCWs are produced only by some specialized cell types that are normally required either to support the plant or for water transport. The SCWs of some cell types, such as collenchyma, are cellulose rich and contain little or no lignin. Most SCWs, however, are associated with woody tissue. As the major component of wood and also grass biomass, these lignified cell walls are the major source of plant biomass, and it is these lignified SCWs that are the focus of this review. Typical SCWs are composed of cellulose (40–80%), hemicellulose (10–40%), lignin (5–25%), and cell wall proteins. Mechanically, the SCWs can be compared with reinforced concrete, with the cellulose microfibrils analogous to steel rods that are embedded in an amorphous matrix of lignin.

Since we reviewed this area in 2001 (Turner *et al.*, 2001), the field has grown enormously. Many of the individual processes that are integral to SCW formation have themselves been the focus of detailed reviews. In the interest of space, we cannot cover the entire topic in great detail, and we have necessarily been selective and aim to highlight recent work that we consider to be particularly significant. While we have tried to include sufficient background information, we have endeavoured to highlight other reviews where the reader may find more information.

## Models for studying secondary cell walls

A large proportion of our understanding of SCWs has come from three main model systems: Arabidopsis vascular bundles; developing xylem in woody stems of poplar; and SCW formation induced in cell culture systems (Turner *et al.*, 2007) (Fig. 1A–F). Arabidopsis has served as a model plant for understanding cell walls like so many other processes.

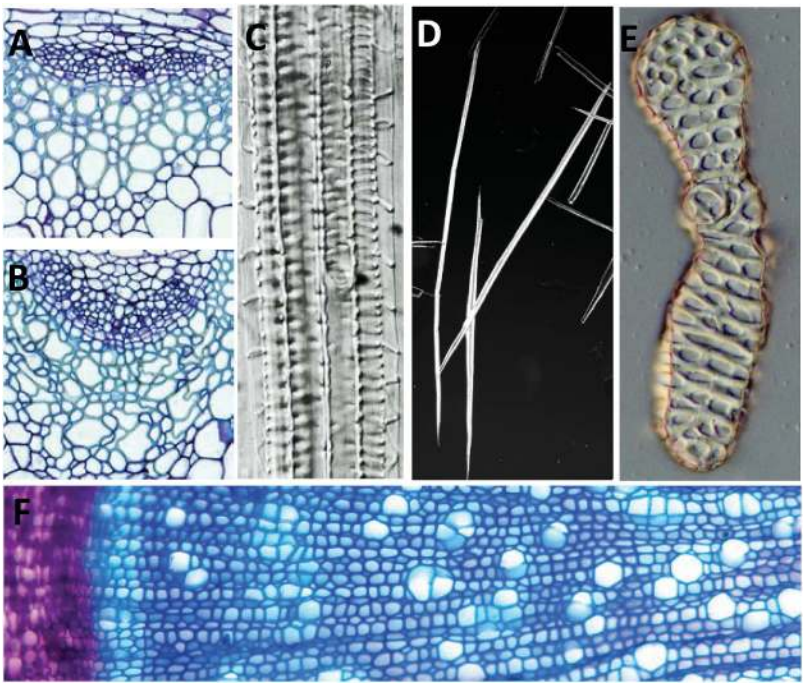


**Fig. 1.** Model systems for studying secondary cell walls. (A) Cross-section of an Arabidopsis stem. (B) Collapsed xylem of an Arabidopsis *irx* mutant. (A) and (B) reveal the gross anatomy of the cells which has been used for identifying mutants of SCW formation. Xylem cells in an intact cleared Arabidopsis root (C) are a good model for studying cell biology of the secondary cell walls. (D) Arabidopsis fibre cells. (E) TE induced from leaf cells of *Zinnia*. (F) Developing wood from poplar.

Arabidopsis vasculature has a developmental gradient in the inflorescence stem from top to bottom, with xylem cells having more pronounced secondary wall thickening at the base of the stem. Taking advantage of this gradient, researchers have identified candidate genes in SCW formation (Brown *et al.*, 2005; Ehlting *et al.*, 2005). Moreover, forward genetic screens for phenotypes such as *irregular xylem* (*irx*) and *fragile fibre* (*fra*) have identified important genes for cellulose and hemicellulose biosynthesis. Arabidopsis vascular tissues in developing roots have also proved ideal for studying SCW proteins using live cell imaging (Wightman and Turner, 2008; Wightman *et al.*, 2009). Trees are the biggest producers of SCWs. A cross-section of a tree stem reveals a highly ordered arrangement of cells that reflects the different stages of SCW deposition (Fig. 1F). A high-resolution sampling approach was developed to sample different cell types (Uggla *et al.*, 1996), and this approach has been used for large-scale transcriptomic, proteomic, and metabolomic studies. Finally, suspension-cultured cells of some species, *Zinnia* and Arabidopsis for example, can be induced to form SCWs (Fig. 1E). This can be done either using plant hormones (Fukuda, 1997) or by genetic means (Yamaguchi *et al.*, 2010). While initial studies have focused on the trans-differentiation of *Zinnia* cells, Arabidopsis and tobacco cultures have been used recently.

There is abundant evidence that Arabidopsis serves as an excellent model for many aspects of SCW formation and wood formation (Zhang *et al.*, 2011; Strabala and MacMillan, 2013). While trees, such as poplar, are excellent experimental systems in their own right (Mellerowicz *et al.*, 2001; Jansson and Douglas, 2007), they lack the molecular genetic tools available for Arabidopsis. These tools have been instrumental in defining many of the key players and biosynthetic pathways involved in SCW biosynthesis. Consequently, partly in the interests of space, SCW formation in Arabidopsis will be the main focus of this review.

## Biosynthesis of secondary cell walls

SCWs are composed mainly of the polysaccharides cellulose and hemicellulose, lignin, and cell wall proteins (Fig. 2). Building the complex cell wall structure involves a co-ordinated execution of several different biosynthetic and transport pathways (Fig. 2). These include biosynthesis and interconversion of precursor nucleotide sugars; polymerization of precursors into large polymers; transport of precursors or polymers to the cell wall; and final assembly of component polymers into the cell wall structure. A number of review articles discuss these in great details (Hao and Mohnen, 2014; Held *et al.*, 2015; Kumar and Turner, 2015a, *b*), and consequently the biosynthesis of cellulose, hemicellulose, lignin, and cell wall proteins is discussed relatively briefly here.

### *Cellulose*

Cellulose is made at the plasma membrane (PM) by a large rosette-shaped protein complex, the cellulose synthase complex (CSC). The CSC moves in the plane of the PM, extruding cellulose chains, which bind together to form cellulose microfibrils (Nishiyama, 2009). Three CESA proteins (CESA4, CESA7, and CESA8, also known as IRX5, IRX3, and IRX1, respectively) are the core components of the SCW CSC (Gardiner *et al.*, 2003; Taylor *et al.*, 2003;

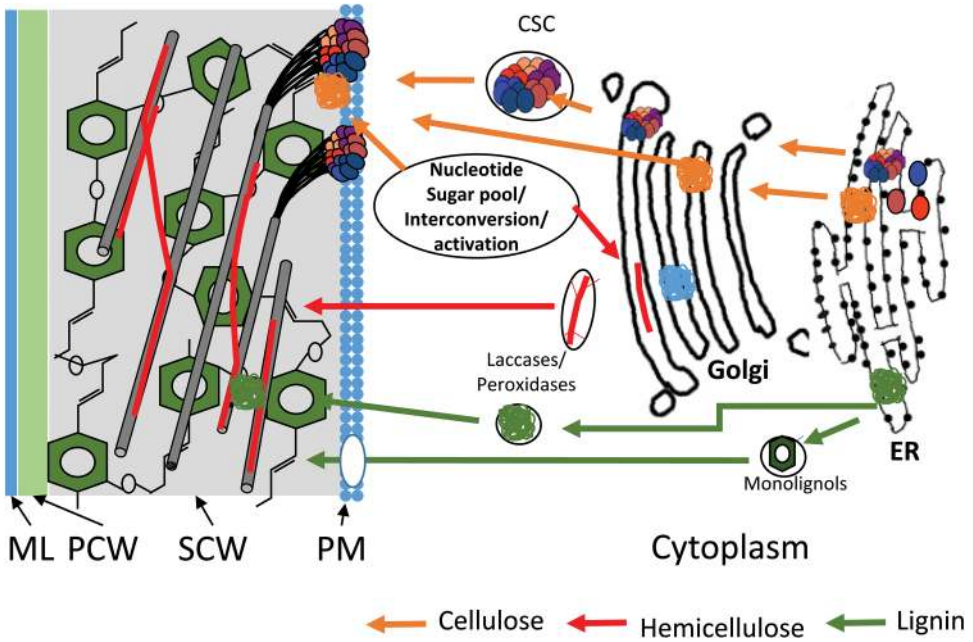


**Fig. 2.** Subcellular localization of the pathways for SCW synthesis and assembly. Cellulose is synthesized at the PM by the CSC. The components of the CSC are made on ER-bound ribosomes where assembly of the complex is also likely to take place. The assembled complexes are then transported to the PM via the Golgi. Actin plays a vital role in the trafficking of the CSC. Once inserted into the PM, the CSC moves around in the plane of the PM and extrudes glucan chains into cell walls which bind into cellulose microfibrils. The hemicelluloses are synthesized in the Golgi by resident GTs. The pre-synthesized hemicellulose molecules are transported to the cell wall, where they may be further modified. Monolignols of lignin are synthesized on the ER surface/cytoplasm and transported to the cell wall where they are activated and polymerized into complex lignin polymer. Finally the cell wall components are cross-linked to make the assembled cell wall.

Atanassov *et al.*, 2009). A similar triad of CESAs is responsible for cellulose synthesis in primary walls (Desprez *et al.*, 2007; Persson *et al.*, 2007b) where additional proteins including CELLULOSE SYNTHASE INTERACTING (CSI), KORRIGAN (KOR), and COMPANION OF CELLULOSE SYNTHASE (CC) are also associated with the CSC (Gu *et al.*, 2010; Vain *et al.*, 2014; Endler *et al.*, 2015). While mutations in KOR affect SCW cellulose biosynthesis, mutations in CSI appear to have little or no effect on SCW deposition (Szyjanowicz *et al.*, 2004; Gu *et al.*, 2010; Vain *et al.*, 2014). Xylem vessels offer certain advantages as a model system because during cellulose deposition, the CSCs are found at a very high density but restricted to specific regions of the PM (Herth, 1985; Schneider and Herth, 1986; Wightman and Turner, 2010; Watanabe *et al.*, 2015). Live cell imaging shows that yellow fluorescent protein (YFP)-labelled CESA proteins are localized to regions marked by thick bundles of microtubules. These microtubules are essential for SCW deposition because drug-induced depolymerization of the microtubules prevents proper localization of the CESA proteins (Wightman and Turner, 2008). As with primary cell wall cellulose synthesis, Golgi vesicles are central to delivery of the CSC to the PM. These exhibit a characteristic doughnut shape when labelled with CESA–YFP that may reflect a peripheral localization of the CSCs in the vesicle. While microtubules localize the CSC to regions of the SCW undergoing cell wall deposition, actin also plays an essential role in movement of the Golgi and hence delivery of the rosettes. Thick actin cables are required for rapid movement of the Golgi around the cell, while thin actin strands appear to move the Golgi close to sites of cell wall deposition (Wightman and Turner, 2008; Wightman and Turner, 2010). Live cell imaging in the SCW is challenging due to the fact that the cells undergoing SCW deposition are frequently buried within tissue, necessitating the use of a long working distance and the need to overcome distortions caused by air trapped within cell walls. A recent study has overcome some of the difficulties with imaging of SCW CESA proteins by using the inducible expression of the VND transcription factor (TF) to generate ectopic vessels in epidermal cells (Watanabe *et al.*, 2015). This has allowed resolution of individual CSCs or MASC/Smaccs, the small vesicles essential for delivery of the CSC to the PM in primary cell walls (Crowell *et al.*, 2009; Gutierrez *et al.*, 2009), and demonstrated that CSCs in the SCW move at a slightly faster rate than those recorded for the CSC in the primary cell wall.

Newly synthesized glucan chains are bonded together to form cellulose microfibrils, a process believed to be facilitated by members of the COBRA gene family. COBRA and COBL4 are involved in cellulose biosynthesis in primary and secondary cell walls, respectively (Brown *et al.*, 2005; Persson *et al.*, 2005). COBRA family members are glycosyl phosphatidylinositol (GPI)-anchored glycoproteins. The cellulose-deficient *britte-culm1* (*bc1*) mutant of rice is caused by a mutation in the rice homologue of COBL4. BC1 has a carbohydrate-binding domain and binds to cellulose to modify cellulose crystallinity (Liu *et al.*, 2013). Such a role is supported by studies of the primary wall COBRA protein that appears to act as a 'polysaccharide chaperone' to facilitate cellulose crystallization (Sorek *et al.*, 2014a).

*Xylan*

Xylan is the major hemicellulose in the SCW of both monocots and dicots. Unlike cellulose, it is synthesized inside the Golgi apparatus and transported to the PM where it becomes integrated into the growing cell wall (Rennie and Scheller, 2014). The backbone of xylans is a chain of β(1→4)-linked xylose residues with a variety of side chains (Fig. 3). The xylan backbone in dicot species is decorated with α(1→2)-linked glucuronic acid (GlcA) and 4-*O*-methyl-glucuronic acid (MeGlcA). This type of xylan is known as glucuronoxylan (GX) (Fig. 3B). Xylan in monocots on the other hand is glucuronoarabinoxylan (GAX), where the substituents are α(1→3)- and/or α(1→2)-linked arabinofuranosyl (Araf) and α(1→2)-linked GlcA/MeGlcA units (Fig. 3C).

Proteins catalysing the addition of many of the linkages in xylan have recently been identified. Xylan biosynthesis involves a terminal oligosaccharide at the reducing end which might act as a primer or terminator (York and O'Neill, 2008). This oligosaccharide is not found in xylan from all plant species and consequently it is unclear whether it is essential for xylan synthesis, or whether in some species the oligosaccharide could be removed from the mature xylan. This oligosaccharide is absent when members of the glycosyl transferase families GT47 (FRA8 and FRA8H) or GT8 (IRX8 and PARVUS) are mutated, implicating them in its synthesis (Brown *et al.*, 2007; Lee *et al.*, 2007; Pena *et al.*, 2007; Persson *et al.*, 2007a). Synthesis of the xylosyl backbone is impaired when any one of the three homologous pairs of proteins belonging to GT43 (IRX9, IRX9L, IRX14, IRX14L) and GT47 (IRX10, IRX10L) gene families are mutated. Xylan synthase activity could be detected for IRX9 or IRX14 heterologously expressed in tobacco. However, much higher levels of xylan synthase activity were found when both of these proteins were expressed together (Lee *et al.*, 2012). Overexpression of a rice IRX9 homologue in Arabidopsis gave plants with higher levels of xylan (Chiniquy *et al.*, 2013). Neither of these studies involved an analysis of purified proteins. More recent studies, however, have demonstrated xylosyl transferase activity for purified proteins following heterologous expression. IRX10L expressed in kidney cell lines (Urbanowicz *et al.*, 2014) and IRX10 and its homologues from *Physcomitrella* and *Plantago* expressed in *Pichia* (Jensen *et al.*, 2014) all exhibit activity following purification. Even though the rate of xylan chain elongation was relatively slow in these studies, they provided the clearest evidence of xylan synthase activity. This does not necessarily preclude that IRX9 and IRX14 also

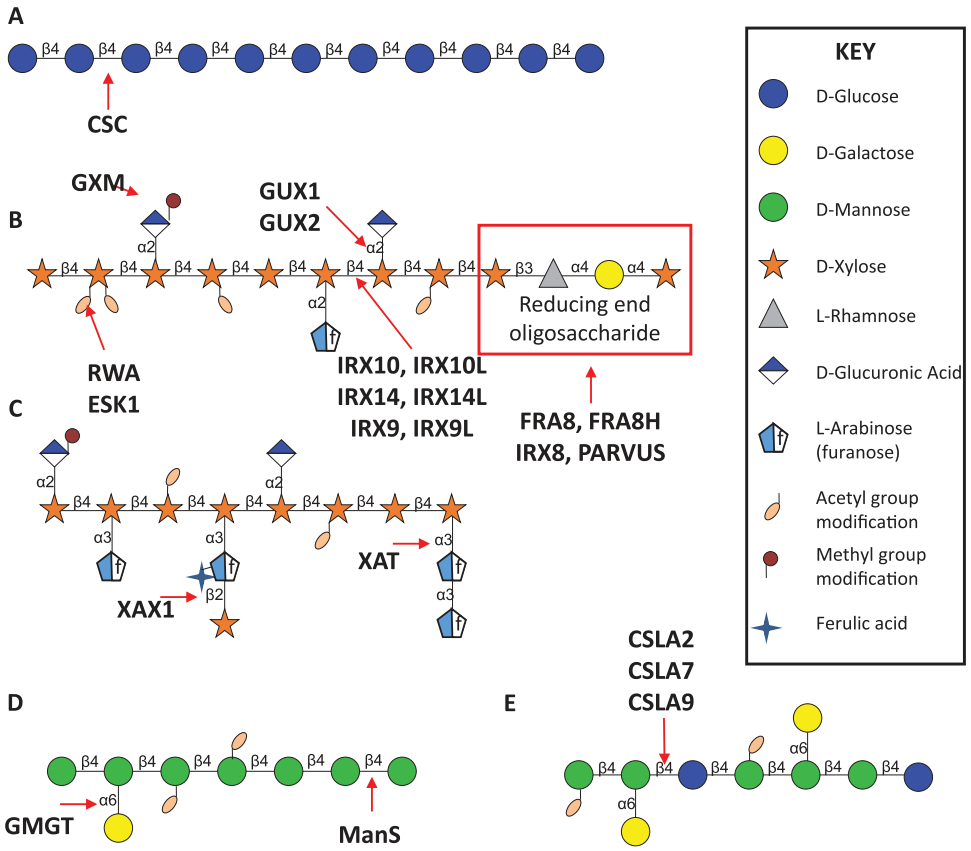


**Fig. 3.** SCW polysaccharide structure and biosynthesis. Simplified structures of major polysaccharides of SCWs—cellulose (A), glucurunoxylan (GX; B), glucuronoarabinoxylan (GAX; C), glucomannan (D), and galactoglucomannan (E)—are shown. Enzyme names are indicated by black text, and the position of linkages formed by red arrows. Only the enzymes discussed in the text are shown; for a more comprehensive list please refer to reviews such as Pauly *et al.* (2013), Scheller and Ulvskov (2010), Kumar and Turner (2015a), and Hao and Mohnen (2014). CSC, cellulose synthase complex; IRX, irregular xylem; FRA, fragile fiber; GUX, glucuronic acid substitution of xylan; RWA, reduced wall acetylation; ESK, eskimo; GXM, glucuronoxylan methyltransferase; XAT, xylan arabinosyltransferase; XAX, xylosyl arabinosyl substitution of xylan; CSL, cellulose synthase like; ManS, mannan synthase; GMGT, galactomannan galactosyl transferase.

exhibit this activity. It has been pointed out that IRX9 and its orthologues from poplar and rice all lack a conserved DxD motif that is normally associated with binding to the phosphate of the activated sugar (Urbanowicz *et al.*, 2014). This has led to the suggestion that IRX9 could be a non-catalytic component of a complex that works in a way analogous to GUAT1 and GUAT7 during pectin biosynthesis (Rennie and Scheller, 2014). GUAT1 and GUAT7 are both related members of the GT8 family, though the membrane-spanning protein GUAT7 is not itself catalytically active but functions by forming an association with the α-galacturonosyltransferase, GUAT1, thereby anchoring GUAT1 to the Golgi membrane (Atmodjo *et al.*, 2011). Indeed, using site-directed mutagenesis, Ren *et al.* (2014) have shown that the catalytic activity of IRX9 is not needed for its function in Arabidopsis.

The GlcA and MeGlcA side chains of xylan are abolished by mutation in two GT8 family members known as GUX1 and GUX2 (Mortimer *et al.*, 2010). Subsequent analysis has revealed that these two enzymes have different specificities and are responsible for generating two domains of xylan that exhibit different patterns of (Me)GlcA side chains (Bromley *et al.*, 2013). The possible significance of these domains in cell wall assembly are discussed below.

The enzymes that add the methyl group to GlcA, GXMTs, are members of a small family of genes that all contain a conserved DUF579 domain (Urbanowicz *et al.*, 2012). Interestingly, two other members of this family known as IRX15 and IRX15L affect xylan biosynthesis. An *irx15l irx15l* double mutant exhibits significantly reduced levels of xylan with shorter chain lengths (Brown *et al.*, 2011). While the presence of the DUF579 domain suggests that these enzymes are also likely to be methyltransferases, in common with all mutants with reduced total xylan, *irx15irx15l* double mutants actually have a large increase in the proportion of MeGlcA relative to (non-methylated) GlcA side chains. Two ideas have been put forward to explain these data: that IRX15 and IRX15L are non-catalytic regulatory components of the complex, analogous to that described for IRX9 above; or that they target uronic acids that are the predominant sugars of pectins and that altered pectin biosynthesis somehow results in decreased xylan content (Rennie and Scheller, 2014).

Xylan from Arabidopsis is modified by the addition of acetate groups to the O2 and O3 position of the xylan backbone. Members of a family of proteins known as REDUCED WALL ACETYLATION (RWA) appear to have a general role in plant polysaccharide acetylation. In contrast, mutations to the gene encoding ESKIMO1/TRICHOME BIREFRINGENCE-LIKE (ESK1/TBL29), which contains a DUF213 domain, appear to have a specific role in addition of acetate onto xylan. Two members of the family, TBL3 and TBL31, were recently shown to be involved in xylan monoacetylation (Yuan *et al.*, 2015). Heterologously expressed TBL29 can add acetate groups to xylan synthesized *in vitro* (Urbanowicz *et al.*, 2014). The critical role of xylan acetylation is demonstrated by the phenotype of *esk1* mutants that exhibit many of the classic phenotypes of xylan-deficient mutants including small plant stature and collapsed xylem vessels (Yuan *et al.*, 2013), probably reflecting the crucial role of acetylation in maintaining xylan solubility.

Grasses contain the GAX type of xylan which has side chains not found in other species. These include α(1→3)- and/or α(1→2)-linked Araf residues. The α(1→3)-linked residues may be further modified by the addition of ferulic acid at the O5 position and xylose at the O2 position (Fig. 3C). A comparative bioinformatics approach identified genes from grass species that were expressed during xylan biosynthesis, but appeared not to have a homologue in Arabidopsis (Mitchell *et al.*, 2007). Members of the GT61 family were identified as good candidates and were subsequently shown to exhibit α(1→3) arabinosyltransferase activity by expressing the gene in Arabidopsis, which resulted in a novel xylan in Arabidopsis with arabinose side chains (Anders *et al.*, 2012). A rice mutant that abolishes xylose addition on the arabinose is caused by a mutation in a gene encoding XYLOSYL ARABINOSYL SUBSTITUTION OF XYLAN 1 (XAX1) a member of the GT61 family. These mutants also exhibit reduced ferulic and coumaric acid levels, suggesting that xylose addition precedes the formation of ferulic ester (Chiniquy *et al.*, 2012).

### *Mannan*

Mannans are a major hemicellulose in conifer SCWs. Based on their backbone and side chain structure (Fig. 3D, E), mannans can be classed as mannan (mannose backbone), galactomannan (mannose backbone with galactose substituents), glucomannan (mannose and glucose backbone in a non-repeating manner), and galactoglucomannan (mannose and glucose backbone in a non-repeating manner with galactose substituents). Mannan synthase (Dhugga *et al.*, 2004; Liepman *et al.*, 2005) was first identified from guar bean and is a member of the cellulose synthase-like (CSL)A family. Other members of the family, CSLA2, CSLA7, and CSLA9, were found to be responsible for synthesis of glucomannan in Arabidopsis stems (Goubet *et al.*, 2009). The galactosyl transferase was identified from fenugreek (Edwards *et al.*, 1999). While mannans are largely associated with the SCWs, the *csla2*, *csla7*, *csla9* triple mutant of Arabidopsis exhibits severe embryonic defects. Clearly, since embryos have no SCWs, it suggests that there is a wider role for mannan in plant development (Goubet *et al.*, 2009).

### *Lignin*

Lignin is the phenolic component of woody cell walls and provides the compression strength to walls. Chemically, it is a heterogeneous and complex polymer largely derived from three hydroxycinnamyl alcohol precursors, *p*-coumaryl, coniferyl, and sinapyl alcohol. These precursors give rise to the *p*-hydroxylphenyl (H), guaiacyl (G), and syringyl (S) units of lignin, respectively. The exact proportion of the three lignin monomer units varies greatly depending on the biological source of the lignin. Lignin in SCWs of both monocots and dicots primarily contains G (~35–49%) and S (~40–61%) units. However, the monocot lignin also contains smaller but significant amounts (~4–15%) of H units, which are

only found in trace amounts in the lignin from dicot species (Vogel, 2008).

The pathway of lignin monomer biosynthesis has been extensively and frequently redrawn in the last two decades, but now appears well established (Boerjan *et al.*, 2003; Halpin, 2004; Bonawitz and Chapple, 2010; Vanholme *et al.*, 2010; Hao and Mohnen, 2014) (Fig. 4). The pathway starts with the committed step, deamination of phenylalanine by phenylalanine ammonia-lyase (PAL). Sequential action of cinnamic acid 4-hydroxylase (C4H) and 4-coumarate:CoA ligase (4CL) produces *p*-coumaroyl-CoA. This part of the pathway involving PAL, C4H, and 4CL is common to all phenylpropanoids and is sometimes referred to as the general phenylpropanoid pathway. Action of cinnamoyl-CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD) on *p*-coumaroyl-CoA leads to *p*-coumaryl alcohol and H units of lignin. Four enzymes, *p*-hydroxycinnamoyl-CoA:shikimate/quinate *p*-hydroxycinnamoyl transferase (HCT), *p*-coumarate 3-hydroxylase (C3H), CCR, and CAD, convert the *p*-coumaroyl-CoA into caffeoyl alcohol and G units of lignin. A side branch of this pathway leads to sinapyl alcohol and S units via the action of ferulate 5-hydroxylase (F5H), caffeic acid *O*-methyltransferase (COMT), and CAD (Fig. 4). While the pathway is relatively well established, the caffeoyl shikimate esterase (CSE) enzyme has only recently been added. CSE acts on caffeoyl shikimate, a product of C3H, to provide an alternative route to caffeoyl-CoA (Vanholme *et al.*, 2013).

In contrast to their biosynthesis, the transport and polymerization of monolignols remain poorly understood. In a comprehensive recent review of the cell biology of lignin biosynthesis, Barros *et al.* (2015) performed a meta-analysis of all previous studies on the subcellular localization of monolignol biosynthesis enzymes. There is a clear consensus that some enzymes such as C4H, C3H, and F5H localize to the endoplasmic reticulum (ER), whereas all the other lignin monomer biosynthetic enzymes, such as CCoAOMT, CCR, and COMT, are found in the cytoplasm. Some evidence suggests they may form a complex on the outer surface of the ER (reviewed in Barros *et al.*, 2015), but clearly once the monolignol units are synthesized, they need to be transported across the PM. Both passive diffusion and vesicle trafficking have been proposed as a mechanism, but recent attention has focused on the role of specific transporters. ATP-binding cassette (ABC) transporters were identified as good candidates based on expression data (Ehlting *et al.*, 2005). More recently, somewhat contradictory data have emerged from biochemical studies. Using membrane preparations from Arabidopsis roots and

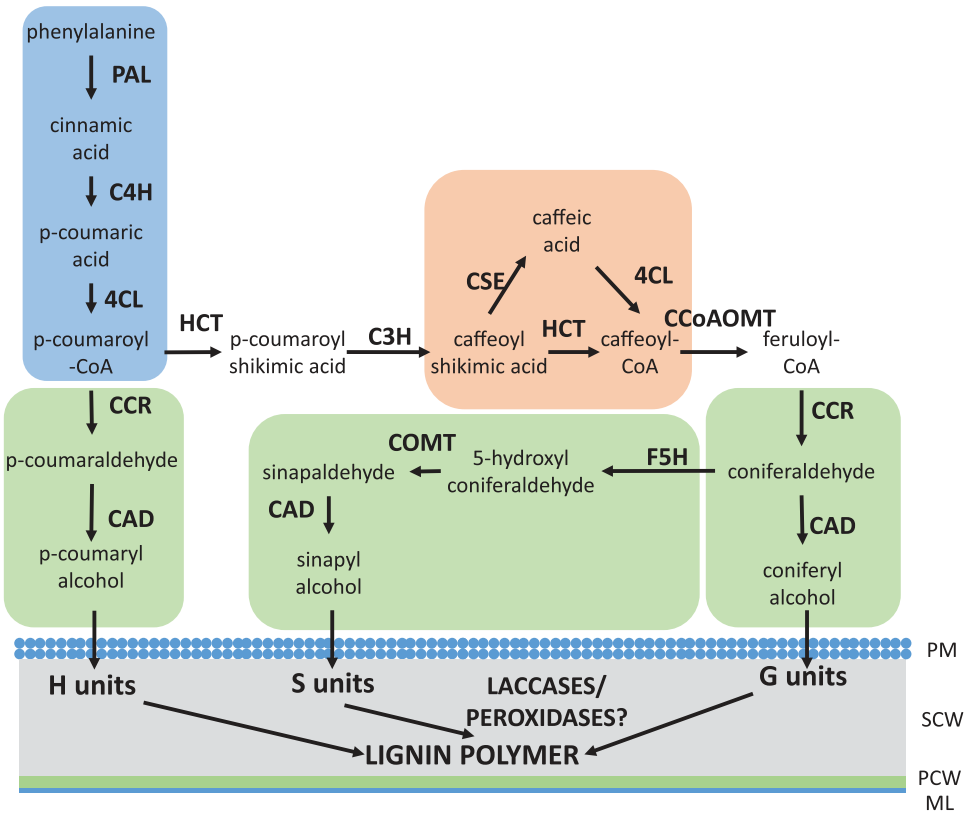


**Fig. 4.** Lignin biosynthesis. A simplified monolignol biosynthetic pathway representing the most common pathways for the biosynthesis of the three major lignin units. Many additional side pathways and alternative routes are known to exist. Please see reviews on lignin biosynthesis such as Boerjan *et al.* (2003), Hao and Mohnen (2014), and Barros *et al.* (2015) for more comprehensive pathways. The general phenylpropanoid part of the pathway is shown in a blue box while the reactions leading to the three lignin precursor alcohols are shown in green boxes. The most recent addition to the pathway involving CSE is shown inside a salmon pink box. PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase, 4CL, 4-coumarate:CoA ligase, HCT, *p*-hydroxycinnamoyl-CoA:quinate/shikimate *p*-hydroxycinnamoyltransferase; C3H, *p*-coumarate 3-hydroxylase; CSE, caffeoyl shikimate esterase; CCoAOMT, caffeoyl-CoA *O*-methyltransferase; CCR, cinnamoyl-CoA reductase; CAD, cinnamyl alcohol dehydrogenase; COMT, caffeic acid *O*-methyltransferase; F5H, ferulate 5-hydroxylase.

leaves and poplar leaves, Miao and Liu (2010) identified two distinct transporter activites that exhibited characteristics of ABC transporters: a PM transporter that was active with the monolignols, and a vacuolar transporter with a preference for the glycoconjugates, such as coniferin. This study was carried out with extracts of tissues containing only low levels of lignin deposition. However, a study on developing xylem from poplar and cypress could not find any evidence for the involvement of ABC transporters, but did identify a vacuole-localized transporter with high activity with coniferin that exhibited the characteristics of a proton antiporter (Tsuyama *et al.*, 2013). Support for the involvement of an ABC transporter, however, also comes from studies on AtABCG29, an Arabidopsis ABC transporter identified in endodermal cells where lignification of the Casparian strip occurs. Expression of ATABCG29 conferred the ability to transport *p*-coumaryl alcohol to yeast cells (Alejandro *et al.*, 2012). Arabidopsis mutants lacking AtABCG29 exhibited increased susceptibility to growth on *p*-coumaryl alcohol, but exhibited only modest decreases in lignin content and similar reductions in H, G, and S lignin. Having specific ABC transporters for lignin precursors is also hard to reconcile with the observation that various manipulations of the lignin biosynthesis pathway (see below) lead to novel precursors being generated intracellularly that become incorporated into lignin within the wall. If specific transporters were involved, it is unlikely they would be able to transport such 'designer monolignols'. Better loss-of-function data are needed to provide definitive evidence for a central role for specific transporters in lignin biosynthesis.

The work on transporters for coniferin leads into another area in which more definitive experiments are still required; that is, the role of glycoconjugates in lignin biosynthesis. These are abundant in many lignifying tissues; however, it is unclear whether they serve as lignin precursors, and it has been suggested they may function as storage compounds (Boerjan *et al.*, 2003). While these compounds are abundant in developing poplar xylem, they disappear upon lignification (Tsuyama *et al.*, 2013). Loss-of-function data from Arabidopsis do not support a direct role for these compounds in lignification. Mutants in two β-glucosidase genes highly expressed in lignifying cells of the stem do not exhibit any lignin-deficient phenotype despite having significantly lower coniferin levels (Chapelle *et al.*, 2012).

Polymerization of lignin occurs within the wall. Each monolignol loses one H atom (possibly to $H_2O_2$) to produce the free radical forms. These free radicals then can link together to form the complex lignin structure (Ralph *et al.*, 2004). Both laccases and peroxidases have been proposed to be involved in monolignol activation. However, the existence of a very large number of peroxidase genes has made it hard to address their function. Reports suggest that knockouts in AtPRX72 or double mutant combinations of AtPRX2, AtPRX25, and AtPRX71 exhibit lignin alteration, but the phenotypes are very mild compared with other lignin-deficient mutants (Herrero *et al.*, 2014; Shigeto *et al.*, 2015). In contrast, laccases, which are also encoded by a large gene family, have been demonstrated to have a very clear role in lignin biosynthesis via reverse genetic studies (Berthet *et al.*, 2011). Triple mutants with insertions in three different laccase genes result in plants that are severely dwarfed, and exhibit very low lignin levels and severely collapsed xylem cells (Zhao *et al.*, 2013). It remains possible that laccases and peroxidases work together in lignification and that higher order mutants that reduce potential redundancy among peroxidase genes may give phenotypes equally severe as those of the laccase multiple mutants. In protoxylem cells, laccases localize only to regions of the cell wall undergoing SCW deposition, making it likely that the cross-linking by laccases determines the sites of cell wall deposition (Schuetz *et al.*, 2014). This is supported by plants ectopically expressing laccases. When these plants are fed fluorescently labelled monolignols, they become incorporated into lignin in cells that would not normally synthesize lignin (Schuetz *et al.*, 2014). This experiment also highlights another aspect of lignin polymerization, that in some cell types lignification is not cell-autonomous. This was first demonstrated in differentiating *Zinnia* cell cultures where lignification of tracheary elements (TEs) continued after cell death and was dependent upon the presence of non-TE cells (Hosokawa *et al.*, 2001; Tokunaga *et al.*, 2005; Tsuyama *et al.*, 2013). This idea is also supported by experiments in Arabidopsis using cell-type-specific gene expression (Pesquet *et al.*, 2013; Smith *et al.*, 2013).

### *Cell wall proteins*

The protein component of SCWs is comparatively smaller than in primary walls. Most cell wall proteins are glycoproteins and can be placed into one of four classes based upon the abundance of certain amino acids or glycan motifs in their sequence. These are glycine-rich proteins (GRPs), proline-rich proteins (PRPs), arabinogalactan-rich proteins (AGPs), and hydroxyproline-rich glycoproteins (HRGPs or extensins) (Showalter, 1993). Additionally, there are the GPI-anchored proteins which are anchored into the PM forming a PM–cell wall continuum. These can be released into the cell wall when needed by the action of a phospholipase (Liu *et al.*, 2015).

The exact function has been ascertained for only a handful of cell wall proteins. However, many of them have been identified to be highly enriched in SCWs. A number of AGPs belonging to the fasciclin-like AGP (FLA) group and members of the COBRA family, for example, are highly enriched in SCWs of both Arabidopsis and poplar (Brown *et al.*, 2005; Andersson-Gunneras *et al.*, 2006). COBL4 has recently been shown to help crystallize cellulose in the wall (Liu *et al.*, 2013). The role of FLAs is currently unclear. The Arabidopsis FLA11 and FLA12 genes are strongly expressed during SCW biosynthesis, and *fla10 fla11* double mutants result in stems that are very brittle (MacMillan *et al.*, 2010). Although it is not clear how FLA11 and FLA12 function, defects in FLA3 affect cellulose deposition in pollen tubes (Li *et al.*, 2010) and a number of FLA proteins are very abundant during cellulose-rich tension wood formation (Andersson-Gunneras *et al.*, 2006). Given the GPI anchor on many FLAs, they may have a specific role in cellulose deposition, possibly during the early stages of microfibril formation.

### *Cell wall assembly*

Cell walls are supramolecular assemblies. Once all the components have been synthesized and exported into the extracellular space they must assemble into a cohesive unit. Moreover, it is an ordered and hierarchical process where the biosynthesis and transport of the components is controlled in a spatiotemporal manner.

Cellulose in SCWs, for example in wood, is composed of long microfibrils which contain both ordered and disordered components. Wood microfibrils from spruce were found to contain between 18 and 24 glucan chains (Fernandes *et al.*, 2011). Xylan, the major hemicellulose in dicot species, was found to contain two distinct domains (Bromley *et al.*, 2013). In the major domain, side chains occur on average every eight xylose residues and are only found on evenly spaced xylose residues, whereas in the minor domain they are much more closely spaced and exhibited no preference for evenly spaced residues. One explanation for this model is that xylan in the major domain binds to cellulose microfibrils and, if xylan exists as a 2-fold helical screw, this would allow all the side chain residues to face away from the cellulose microfibrils. The minor domains are found on the same molecule but cannot bind to cellulose microfibril and so might promote cross-bridges. Further evidence for the 2-fold helical screw structure of xylan comes from the distribution of acetyl groups that occur on the 2′ and 3′ position of the xylose. The acetates are predominantly found on evenly spaced xylose residues and consequently are predicted to face away from the cellulose microfibril and promote interactions with other cell wall components such as lignin (Busse-Wicher *et al.*, 2014). While the model is consistent, there is less evidence to identify the biological significance of this cellulose–xylan interaction. Mutants lacking any glucuronic side branches appear normal. Furthermore, in xylan-deficient mutants that lack or have reduced side branches, the walls are not expanded in any way (Brown *et al.*, 2007). In contrast, severe lignin-deficient mutants (Jones *et al.*, 2001) have dramatically expanded walls, suggesting that lignin is an essential component in cross-linking cellulose microfibrils in SCWs.

## Regulation of secondary cell wall biosynthesis

While a great deal of progress has been made in our understanding of cell wall biosynthetic enzymes and the genes that encode them, their precise regulation is poorly understood. There are four important regulatory steps during cell wall formation (Fig. 2): production of cell wall polymer precursors; production of cell wall polymer biosynthetic enzymes; trafficking of cell wall polymer precursors and enzymes; and the final assembly of components into a complex wall structure. At least 2000 genes are believed to be involved in the biosynthesis and assembly of cell walls (Carpita *et al.*, 2001). Of the cell wall components, only cell wall proteins are primary gene products. The remaining genes encode the enzymes responsible for the cell wall biosynthetic machinery. Spatial and temporal gene expression and enzyme activity of the cell wall biosynthetic machinery are under tight control at various stages of protein synthesis and activity.

### *Transcriptional regulation*

Transcriptional regulation of SCW deposition has been extensively studied. A large number of TFs have been identified which control the eventual expression of cell wall biosynthetic genes. The vast majority of TFs in the SCW regulatory network belong to one of two families—the NAC TFs (Ooka *et al.*, 2003; Zhong *et al.*, 2010a; Nakano *et al.*, 2015) and the MYB TFs (Stracke *et al.*, 2001; Wilkins *et al.*, 2009; Nakano *et al.*, 2015). A meta-analysis of the literature regarding transcriptional regulation in the SCW identified 435 documented interactions between different TFs (Hussey *et al.*, 2013). These can be broadly divided into three groups depending on where in the regulatory pathway they act (Fig. 5). Tier1 TFs bind directly to the *cis*-elements controlling the transcription of structural genes. Tier2 TFs can regulate expression of Tier1 TFs in addition to the structural genes, while Tier3 TFs can regulate either Tier2 or Tier1 TFs.

There are five Tier3 master regulators, namely NST1, SND1, NST2, VND6, and VND7 (Fig. 5). Overexpression

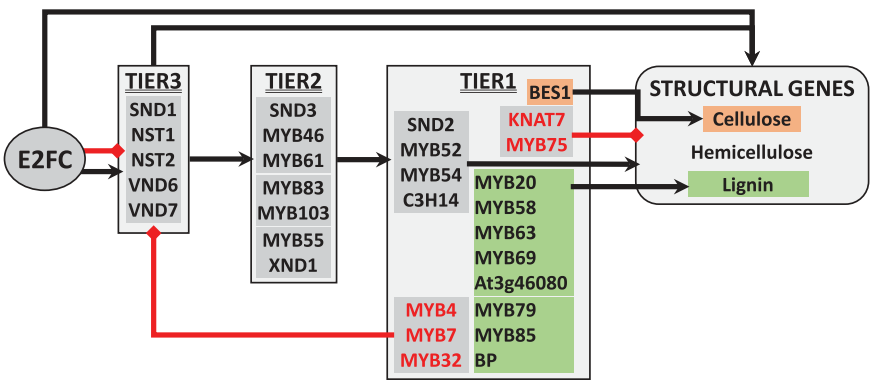


**Fig. 5.** Transcriptional regulation of secondary cell walls. Transcriptional regulation is co-ordinated by transcription factors (TFs). TFs involved in SCW biosynthesis can be broadly classified into three groups. Tier1 TFs bind directly to the structural genes responsible for biosynthesis of cell wall components. Tier2 TFs can regulate the Tier1 TFs in addition to the structural genes, while the Tier3 genes can regulate the Tier2 genes and the structural genes. Recently, a top level TF, E2Fc, was identified. Black arrows represent positive regulation while red lines indicate negative regulation. Groups of TFs are shaded: green, lignin; salmon pink, cellulose; others, grey.

of any of these genes leads to the ectopic deposition of SCW in a variety of cell types (Mitsuda *et al.*, 2005, 2007; Zhong *et al.*, 2006; Yamaguchi *et al.*, 2010). NST1 and SND1 control SCW formation in xylem fibres, whereas VND6 and VND7 act in the xylem vessels. Tier3 master regulators control the transcription of Tier2 TFs which include MYB family TFs (MYB46, MYB83, and MYB55) and NAC TFs (SND3 and XND1) (Zhong *et al.*, 2010b; Yamaguchi *et al.*, 2011). Tier1 TFs bind directly to the promoters of SCW biosynthetic genes and are themselves under the control of Tier2 and Tier3 regulators. BES1 was shown to control transcription of CESA genes (Xie *et al.*, 2011b), while a number of MYB TFs, namely MYB20, MYB69, MYB79, MYB85, MYB58, MYB63, and BP, control the lignin biosynthetic genes (Mele *et al.*, 2005; Yamaguchi *et al.*, 2010; Zhou *et al.*, 2009) (Fig. 5).

While it is convenient to represent these as a tiered structure, there are many documented examples where a TF from one tier activates a TF from a lower tier, but both of these TFs bind to the same structural genes. This is emphasized in a recent comprehensive yeast one-hybrid (Y1H) study where the promoters from 50 genes expressed during SCW biosynthesis were tested using a library of TFs (Taylor-Teeples *et al.*, 2015). The promoters tested included structural genes for cellulose, hemicellulose, and lignin, and many of the TFs regulating these structural genes. A total of 623 interactions between 45 promoters and 208 TFs were identified and demonstrated that transcriptional regulation of SCW biosynthesis is highly combinatorial, with multiple TFs binding to multiple promoters. Furthermore, Taylor-Teeples *et al.* (2015) were also able to identify E2Fc as a key upstream regulator of the Tier3 master regulators as well as the structural genes. E2Fc is a member of the E2F family of TFs (Mariconti *et al.*, 2002) and is known to act both as an activator (Kosugi and Ohashi, 2002; Heckmann *et al.*, 2011) and as a repressor (de Jager *et al.*, 2001; Del Pozo *et al.*, 2007). Interestingly, the E2Fc action on VND7 is dose dependent. Moderate levels of E2Fc resulted in activation of VND7. However, at extremely high or low concentrations, it led to repression of VND7 (Taylor-Teeples *et al.*, 2015).

We have re-analysed the Y1H data from Taylor-Teeples *et al.* (2015) and produced a Neighbor–Joining tree of promoters based on TF binding (Supplementary Fig. S1 available at *JXB* online), to determine whether the co-expression of genes is reflected in patterns of TF binding. This analysis makes it obvious that, based on TF binding patterns, various promoters tested in the study did not involve co-ordinated regulation of cellulose, xylan, and lignin biosynthesis. This point is illustrated by the SCW *CESA* genes that are very tightly co-expressed in Arabidopsis (Brown *et al.*, 2005; Persson *et al.*, 2005). While *CESA4* and *CESA7* exhibit similar patterns of TF binding, *CESA8* has very little overlap (Fig. 6; Supplementary Figs S1, S2). Two other features become obvious from this analysis. First there is enormous variation in the number of TFs binding to the promoters of genes that operate in the same pathway. For example, a total of 27 TFs bind to the promoter of the *C4H* gene, but only one binds to the promoter of *LAC4* even though they are both involved in synthesizing lignin in the SCW (Fig. 6). Secondly, the re-analysis confirms the central role of MYB TFs in lignin biosynthesis. Figure 6 illustrates that most MYBs are specific to lignin biosynthesis; only MYB73 binds to any of the genes involved in xylan biosynthesis, where ETHYLENE RESPONSE FACTOR (ERF) binding predominates.

### *Post-transcriptional regulation*

It is likely that many of the proteins involved in cell wall biosynthesis and regulation are modified post-translationally in a manner that would affect expression levels and/or activity. The top level regulator E2Fc is regulated via phosphorylation. E2Fc forms a heterodimer with DPB to regulate gene expression. The interaction takes place with the non-phosphorylated E2Fc but is lost upon phosphorylation (del Pozo et al., 2002, 2006, 2007). However, it is unclear if this regulation occurs or if it is important during SCW biosynthesis.

CESA7 has been shown to be phosphorylated (Taylor, 2007). It was suggested that phosphorylation of CESA7 targets it for degradation via a proteasome-dependent pathway. A study of SCW CESA proteins demonstrated that when one CESA was absent as a result of a mutation in the gene; the abundance of the two remaining secondary wall CESA proteins decreased dramatically (Hill *et al.*, 2014). The mutants used in this study are much smaller than the wild-type controls, making normalization of protein levels and accurate comparisons of equivalent developmental stages difficult. Furthermore, CESA1, the only control in these experiments, is involved in cellulose synthesis in the primary cell wall. Consequently it remains unclear to what extent the remaining SCW CESA proteins decrease relative to other SCW biosynthesis genes such as those involved in lignin or xylan biosynthesis. It is possible to obtain high levels of a single SCW protein such as CESA7 in leaves when ectopically expressed using the 35S promoter, so individual CESA proteins can accumulate in the absence of their normal binding partners.

### *Hormonal control*

The complex interplay now known to exist between different phytohormone classes makes it difficult to identify whether a putative role within SCW formation is due to direct or indirect effects. However, recent findings have identified specific SCW influences for a variety of hormones, with brassinosteroids (BRs), auxins, and cytokinins perhaps the most important. Here, we will mainly discuss the influence of hormones once cell fate has been decided, though it is important to mention that they also regulate the Tier3 master switches in earlier developmental stages. For example, the expression of VND6 and VND7 can become both up- and down-regulated by varying the combination of auxin, cytokinin, and BR application (Kubo *et al.*, 2005), whereas abscisic acid (ABA) can up-regulate SND1 (Jensen *et al.*, 2010).

#### *Brassinosteroids*

BR signalling is implicated in a variety of developmental and cellular processes in plants, demonstrated by the extreme growth defects of BR biosynthesis and signalling mutants.

**A**

| TFs | | Promoters | | | |
|---|---|---|---|---|---|
| GENE | Symbol | CESA4 | CESA7 | CESA8 | COBL4 |
| AT5G54680 | bHLH105 | ■ | ■ | ■ | ■ |
| AT5G10510 | AIL6 | ■ | ■ | | ■ |
| AT1G47870 | E2Fc | ■ | ■ | | ■ |
| AT1G61730 | | ■ | ■ | | ■ |
| AT2G44730 | | ■ | ■ | | ■ |
| AT3G28920 | HB34 | ■ | | ■ | |
| AT1G54060 | ASIL1 | | | ■ | ■ |
| AT2G21230 | | ■ | ■ | | |
| AT4G23980 | ARF9 | ■ | ■ | ■ | ■ |
| AT5G44210 | ERF9 | ■ | ■ | | ■ |
| AT1G21910 | DREB26 | | ■ | | ■ |
| AT1G12610 | DDF1 | ■ | | | ■ |
| AT4G37260 | MYB73 | | ■ | ■ | ■ |
| TOTAL | | 11 | 12 | 12 | 25 |

**B**

| TFs | | Promoters | | | | | |
|---|---|---|---|---|---|---|---|
| GENE | Symbol | FRA8 | GUX1 | GUX2 | IRX9 | IRX10 | GXM3 |
| AT1G61730 | | | ■ | ■ | | ■ | ■ |
| AT2G44730 | | ■ | | ■ | ■ | ■ | |
| AT1G76880 | | ■ | | ■ | ■ | | |
| AT5G54680 | bHLH105 | | | ■ | ■ | ■ | |
| AT1G47870 | E2Fc | ■ | | ■ | ■ | | |
| AT3G53340 | NF-YB10 | | | | ■ | ■ | |
| AT1G24625 | ZFP7 | | ■ | | ■ | | |
| AT1G54060 | ASIL1 | | | | ■ | ■ | |
| AT2G21230 | | | | ■ | ■ | | |
| AT5G15210 | HB30 | | ■ | | ■ | | |
| AT1G50640 | ERF3 | | | | ■ | | ■ |
| AT1G21910 | DREB26 | | ■ | | | ■ | |
| AT5G44210 | ERF9 | | | | ■ | | ■ |
| AT1G12610 | DDF1 | | | | ■ | ■ | |
| AT4G37260 | MYB73 | ■ | ■ | ■ | ■ | ■ | |
| AT5G63790 | ANAC102 | | | ■ | ■ | | |
| TOTAL | | 7 | 8 | 12 | 24 | 10 | 7 |

**C**

| TFs | | Promoters | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| GENE | Symbol | CCR1 | CCoAOMT7 | LAC17 | CAD4 | 4CL1 | HCT | C4H | CCoAOMT1 | LAC4 | PAL4 | OMT | PAL1 |
| AT2G44730 | | ■ | ■ | ■ | ■ | | | ■ | ■ | | | | |
| AT1G47870 | E2Fc | | ■ | ■ | ■ | | | ■ | | ■ | | | |
| AT1G54060 | ASIL1 | | | | ■ | | ■ | ■ | ■ | | | | |
| AT5G54680 | bHLH105 | | | ■ | ■ | | ■ | ■ | | | | | |
| AT1G61730 | | ■ | ■ | | | ■ | | ■ | | | | | |
| AT5G11260 | HY5 | | | | | | ■ | ■ | | | | | ■ |
| AT1G20910 | | | | | | | ■ | ■ | | | | ■ | |
| AT5G15210 | HB30 | | | ■ | | ■ | | | | | ■ | | |
| AT5G48150 | PAT1 | | | | | ■ | ■ | | | | | | |
| AT2G40950 | BZIP17 | | | | | | | ■ | | | | ■ | |
| AT5G60850 | OBP4 | | | | | ■ | ■ | | | | | | |
| AT1G76880 | | | | ■ | | | | ■ | | | | | |
| AT1G12610 | DDF1 | | | | ■ | ■ | ■ | | ■ | | | | |
| AT1G12630 | | | | | | | ■ | ■ | | | | ■ | |
| AT5G51990 | CBF4 | | | | ■ | | | ■ | | | ■ | | |
| AT5G47220 | ERF2 | ■ | | | | | | | | | ■ | | |
| AT1G21910 | DREB26 | | | | | ■ | | ■ | | | | | |
| AT5G44210 | ERF9 | | | | | | | ■ | | | ■ | | |
| AT1G30490 | PHV | | | | | ■ | | ■ | | | | | |
| AT5G26660 | MYB86 | | | | | ■ | ■ | ■ | ■ | | | | |
| AT1G16490 | MYB58 | | | | | ■ | ■ | ■ | ■ | | | | |
| AT1G79180 | MYB63 | | | | | ■ | ■ | ■ | ■ | | | | |
| AT4G22680 | MYB85 | | | | | ■ | ■ | ■ | ■ | | | | |
| AT4G37260 | MYB73 | | | ■ | ■ | ■ | ■ | | | | | | |
| AT1G66230 | MYB20 | | | | | ■ | ■ | ■ | | | | | |
| AT1G22640 | MYB3 | | | | | ■ | ■ | ■ | | | | | |
| AT3G08500 | MYB83 | | | | | ■ | ■ | ■ | | | | | |
| AT1G63910 | MYB103 | | | | | ■ | ■ | | | | | | |
| AT2G47460 | MYB12 | | | | | ■ | | ■ | | | | | |
| AT1G06180 | MYB13 | | | | | ■ | ■ | | | | | | |
| AT5G63790 | ANAC102 | | | | ■ | ■ | ■ | | ■ | | | | |
| AT5G18270 | ANAC087 | | | | | ■ | ■ | ■ | | | | | |
| AT1G77450 | anac032 | | | | | ■ | ■ | | | | | | |
| TOTAL | | 3 | 3 | 12 | 8 | 23 | 23 | 27 | 11 | 1 | 12 | 5 | 1 |

**Fig. 6.** A meta-analysis of Y1H data from Taylor-Teeples *et al.* (2015). A total of 623 interactions between 45 promoters and 208 TFs were identified. Here we have isolated interactions for promoters involved in cellulose (A), xylan (B), and lignin (C). A grey box indicates interaction, while the white box indicates no interaction. Only the TFs which bind to more than one promoter within the category are shown. The total number of TFs binding to each promoter (including the TFs binding to that promoter uniquely) are shown in red text at the bottom. The TFs involved in ethylene signalling and the MYB TFs are shaded light blue and salmon pink, respectively. The interactions shown here are a subset of the full list shown in Supplementary Fig. S2 at *JXB* online.

The dwarf phenotype of these mutants can be attributed to defects of cell elongation rather than cell number (Azpiroz *et al.*, 1998). Consistent with this are the large number of cell wall biosynthesis and remodelling genes that are transcriptionally up-regulated in response to BR (Sun *et al.*, 2010). Knockout mutants of the membrane-bound BR receptor kinase BRASSINOSTEROID INSENSITVE 1 (BRI1) have reduced cellulose content (Xie *et al.*, 2011a). The TF BRI1-EMS-SUPPRESSOR 1 (BES1) is located downstream of BRI1 in the BR signalling pathway. ChIP experiments suggest that BES1 binds to the promoters of various primary cell wall and SCW promoters via the CANNTG E-box motif (Xie *et al.*, 2011a). Accordingly, overexpression of BRI1 in *cesa1*, *cesa3*, and *cesa6* mutants could not fully rescue their dwarf phenotype, suggesting that CESAs function downstream of BR. BRI1 overexpression in *Gossypium* cotton fibres was recently shown to activate CESA gene expression and increase SCW thickness, while antisense BRI1 plants with reduced BRI1 expression produce the opposite phenotype (Sun *et al.*, 2015). However, the specific role of BRs in cell wall formation is still unclear. Hossain *et al.* (2012) characterized the SCW composition in gain- and loss-of-function DIM1 mutants, a protein involved in the early stages of BR biosynthesis. While RNAi silencing of DIM1 caused a 38% and 23% decrease in relative lignin and cellulose content, respectively, there was no corresponding decrease in the expression of CESA4, CESA7, and CESA8. DIM1 overexpression had no impact on cellulose or lignin levels, and the expression of SCW CESAs was no different from the wild type (Hossain *et al.*, 2012).

### *Auxin*

The xylary fibres produced by *walls are thin 1* (*wat1*) mutants have very thin SCWs with reduced lignin, xylan, and cellulose deposition, while vessels remain unaffected (Ranocha *et al.*,

2010). WAT1 has recently been identified as an auxin transporter that facilitates the movement of auxin across the tonoplast membrane (Ranocha *et al.*, 2013). Exogenous auxin application can restore the fibre phenotype, providing direct evidence of a role for the hormone in the production of fibre SCWs. Aside from cell wall defects, the otherwise normal appearance of fibres in *wat1* mutants (Ranocha *et al.*, 2010) suggests that auxin acts once the cell fate has been decided, although the specific point of involvement is unknown. Intriguingly, *wat1* mutants have enhanced resistance to vascular pathogens, but this enhanced defence response appears to be due to an elevated salicyclic acid (SA) content (Denancé *et al.*, 2013), highlighting the crosstalk that exists between different hormonal classes.

The lignification of anther endothecium SCWs is a crucial stage of the dehiscence process (Sanders *et al.*, 1999), with the TF MYB26 known to be required for the lignin deposition to occur (Yang *et al.*, 2007). Auxin-resistant *tir1 afb* mutant endothecium cells undergo the lignification process much earlier than in the wild type (Cecchetti *et al.*, 2013). Treatment with exogenous auxin was recently shown to down-regulate MYB26 expression and repress lignification of the endothecium SCWs (Cecchetti *et al.*, 2013), confirming the role of auxin as an inhibitor of the process. It is currently unknown whether auxin directly regulates other Tier1 or Tier2 SCW TFs outside of the anther dehiscence system. AUXIN BINDING PROTEIN 1 (ABP1), proposed as an auxin receptor, is also required for proper cell wall development, with ABP1 inactivation significantly altering the expression of >200 cell wall structural, biosynthesis, and remodelling genes, although these are mostly related to the primary rather than the secondary wall (Paque *et al.*, 2014). Furthermore, recent experiments also cast some doubt on the phenotype associated with ABP1 (Gao *et al.*, 2015).

### *Cytokinins*

The general role of cytokinin in the formation of SCWs is not yet clear. A 2012 meta-analysis of 28 publicly available microarray data sets found that amongst the genes identified as being cytokinin responsive, 42 were related to cell wall function (Brenner *et al.*, 2012). However, these were generally primary cell wall remodelling proteins rather than those involved in SCW biosynthesis, suggesting that any involvement of cytokinin is neither widespread nor central to the SCW developmental network. Despite this, there is evidence that cytokinin does have a major regulatory role in anther SCW formation. ARABIDOPSIS HISTIDINE PHOSOPHOTRANSTER 4 (AHP4) is a positive regulator of cytokinin signalling. Hutchison *et al.* (2006) reported that *ahp4* mutants undergo enhanced lignification of the endothecium, with AHP4-overexpressing (AHP4-OX) mutants displaying the opposite phenotype. Jung *et al.* (2008) later demonstrated that the impaired fertility of AHP4-OX lines is a result of reduced SCW thickening, and reported a decreased expression of many SCW biosynthesis genes, including IRX proteins. Thus, cytokinin appears to act as an inhibitor of SCW development in anthers through the action of AHP4. However, care must be taken to not extrapolate the involvement of cytokinin outside of anther SCW development without further studies. Tobacco suspension culture cells constitutively expressing the Tcyt gene contain high levels of cytokinin and yet produce very thick cell wall structures, with increased lignin and cellulose content (Blee *et al.*, 2001). This demonstrates the absence of a clear linear relationship between hormonal activity and SCW biosynthesis.

Recent evidence also points towards a specific role for cytokinin in the lignification process. Loss-of-function ATP-BINDING CASSETTE TRANSPORTER 14 (ABCG14) mutants have almost no acropetal cytokinin transport as well as a reduced SCW lignin content in xylary vessels and fibres (Ko *et al.*, 2014; Zhang *et al.*, 2014). Mutant scions, however, may be rescued by wild-type stocks, suggesting that disruption of lignification appears to be an indirect result of defective long-distance cytokinin signalling rather than any direct local role of ABCG14 in lignin deposition (Ko *et al.*, 2014). The downstream pathway that links cytokinin transport with lignification is not yet understood.

### *Abscisic acid and jasmonic acid*

The *leaf wilting2* (*lew2*) mutant, allelic to *irx1* that is caused by a mutation in *CESA8*, was found to have enhanced ABA content and stress tolerance, alongside the well-known cellulose defects (Chen *et al.*, 2005). Subsequently, a gene expression analysis of $CESA8^{irx1\text{-}6}$ and $CESA4^{irx5\text{-}5}$ mutants revealed a strong up-regulation of ABA-related genes compared with the wild type, including key biosynthetic enzymes and signalling components (Hernandez-Blanco *et al.*, 2007). This response to disruption of CESA4 or CESA8 was found was found to be independent of jasmonic acid (JA) and ethylene, hormones that are instead more involved in the maintenance of primary cell wall integrity (Lorenzo *et al.*, 2004). These findings suggest that the ABA pathway is activated upon disruption to the SCW and this probably acts as a pathogen response system that ultimately leads to the production of various antimicrobials and secondary metabolites (Hernandez-Blanco *et al.*, 2007). This is in keeping with the enhanced susceptibility to pathogens in ABA-deficient mutants (Audenaert *et al.*, 2002; Anderson *et al.*, 2004; AbuQamar *et al.*, 2006). Furthermore, the composition of the cell wall is altered in *aba1-6* biosynthetic mutants, as evidenced by Fourier transform infrared (FTIR) spectra and significantly decreased cellulose content (Sanchez-Vallet *et al.*, 2012).

Alongside auxin and cytokinin, there also appears to be a role for JA in anther dehiscence. Many JA mutants have a male-sterile phenotype, and this has recently been partly attributed to impairments in the SCW thickening process of the endothecial cells. JA application induces premature SCW thickening, with MYB26 and MYB85 becoming strongly up-regulated in response (Jung *et al.*, 2013). However, unlike auxin, JA is not thought to have a general role in SCW formation (Ishiguro *et al.*, 2001; von Malek *et al.*, 2002), with the anther dehiscence SCW thickening pathway seemingly very distinct from that elsewhere.

## Engineering secondary cell walls to improve biomass feedstock

The recent emphasis on renewable energy sources has made lignocellulose an attractive source of biofuels. Cellulose and,

to a certain extent, hemicelluloses can be broken down into simpler sugar molecules, a process known as saccharification, that can be fermented into bioethanol. Lignin, on the other hand, is highly resistant to degradation. Moreover, since the cellulose is embedded in a lignin matrix, the latter is an inhibitor of saccharification. The inhibition involves reduced access to the cellulose microfibrils and sequestration of degradation enzymes by adsorption to lignin. Biofuel production from lignocellulose has been reviewed in detail elsewhere (Himmel *et al.*, 2007; Carroll and Somerville, 2009; Pauly and Keegstra, 2010; Somerville *et al.*, 2010; Chundawat *et al.*, 2011; Burton and Fincher, 2014; Sorek *et al.*, 2014b; Vermerris and Abril, 2015). Here, we will summarize efforts to engineer cell wall components to improve SCW digestibility.

### *Lignin engineering*

Initial attempts to engineer lignocellulosic biomass focused on removal or reduction of lignin content. Chemical removal of lignin from the biomass pulp is impractical from both economic and environmental points of view. Early genetic attempts to modify lignin content involved down-regulation of lignin biosynthetic pathways (Baucher *et al.*, 1998; Boerjan *et al.*, 2003; Vanholme *et al.*, 2008). This is one of the few areas in which improvements have been tested in field trials. Field-grown poplar with down-regulated CCR yielded wood pulp that allowed increased saccharification and improved ethanol production (Van Acker *et al.*, 2014). A comprehensive study on the effects of down-regulating lignin biosynthesis has been carried out in alfalfa (Chen and Dixon, 2007). Down-regulation of HCT and C3H effectively removed the need for pre-treatment in saccharification as these lines released as much sugar from untreated material as was released from the pre-treated control. One problem with this approach is that these plants grew poorly. To overcome this problem, Yang *et al.* (2013) used the VND6 promoter to restore normal lignin specifically to vessels of the *c4h* mutants. This was sufficient to prevent xylem collapse and at least partially restore growth. Additionally, Yang *et al.* (2013) boosted SCW deposition in fibres by generating a positive feedback loop in which the SCW-specific IRX8 promoter was placed in front of the SND1 TF, driving SCW deposition in fibre and thereby generating fibre cells with thicker cell walls, but with reduced lignin content (Yang *et al.*, 2013).

A recent study has demonstrated the effectiveness of introducing novel lignin monomers (Wilkerson *et al.*, 2014). The idea of so-called 'zip' lignin is to introduce labile bonds into the three-dimensional structure of lignin, which are more easily broken and provide a means of opening up the lignin structure to facilitate further cell wall degradation. By introducing the feruloyl-CoA monolignol transferase (FMT) gene from Chinese angelica (*Angelica sinensis*), Wilkerson *et al.* (2014) were able to generate transgenic poplars containing monolignol ferulate conjugates that become incorporated into lignin. This introduces more labile ether linkages into the lignin and results in greater sugar release upon digestion. In another example of lignin engineering, Eudes *et al.* (2015) expressed a bacterial 3-dehydroshikimate dehydratase specifically targeted to plastids, leading to conversion of 3-dehydroshikimate into protocatechuate. The overall result was an increase of *p*-hydroxyphenyl units and a reduction of guaiacyl units in the lignin in the transgenic plants which led to an improved saccharification efficiency (Eudes *et al.*, 2015).

### *Xylan engineering*

While genetic manipulation of lignin has been pursued for more than two decades, xylan engineering is much more recent. Like lignin, xylans also make lignocellulosic material more difficult to deconstruct into fermentable sugars. All severe xylan-deficient mutants have severe growth phenotypes (Wu *et al.*, 2009, 2010; Brown *et al.*, 2011), so, while the xylan in some of the mutants has shorter chains and is more easily extractable, the plants are of little practical use. To overcome this problem, Petersen *et al.* (2012) used the VND6 and VND7 promoters to restore xylan biosynthesis specifically to the vessels of *irx7*, *irx8*, and *irx9* mutants and were able to rescue many of the growth defects. At the same time, it led to biomass with 42% better saccharification yields (Petersen *et al.*, 2012). Xylan from plants lacking GUX1 and GUX2, the enzymes that add the (methyl) glucuronic acid residues into the xylan molecule, is also easier to extract (Mortimer *et al.*, 2010). Double mutants (*irx15*/*irx15l*) in which two members of the DUF579 family are mutated have xylan-deficient phenotypes; however, the single mutants exhibit no obvious defects and grow normally. Furthermore, the *irx15* mutant exhibits dramatically improved saccharification that increases sugar release by 70% (Brown *et al.*, 2011).

Mutants with a large reduction in the acetylation of xylan exhibit enhanced saccharification, but also have severe growth defects (Xiong *et al.*, 2013). In contrast, plant expressing an acetyl esterase in the wall had reduced levels of xylan acetylation but were otherwise normal plants that showed increased resistance to pathogens (Pogorelko *et al.*, 2013). The severe growth defects of *tbl29* mutants which lack xylan acetylation can be overcome by expression of AtGUX1 (which adds GlcA subunits onto xylan) driven by the TBL29 promoter, thereby indicating that the GlcA units are functionally equivalent to the *O*-acetyl groups (Xiong *et al.*, 2015).

Xylan in the SCW of grasses is GAX. The arabinose residues in GAX are esterified with the phenolic compound ferulic acid. Feruloyl esters can undergo oxidative coupling with other phenylpropanoids in the vicinity, either on the GAX molecule itself or on lignin (Ralph, 2010). This results in GAX–lignin cross-linking. A number of recent studies have reported modification of enzymes that alter this cross-linking. The XAX1 enzyme adds the arabinosyl residues onto the xylan side chain. The arabinose can be further modified by the addition of ferulate. Rice plants lacking XAX1 released 62% more sugars compared with wild-type plants (Chiniquy *et al.*, 2012), probably as a result of these plants having 59% lower ferulate esters. Mutation of a XAX1 homologue from *Brachypodium*, SAC1, similarly improved sugar release (Marriott *et al.*, 2014). Overexpression of OsAT10, which is a putative *p*-coumaroyl-CoA transferase involved in GAX modification, also caused a 60% reduction in the ester

conjugates and improved saccharification yields (Bartley *et al.*, 2013).

## Concluding remarks

Enormous advances have been made in identifying individual enzymes required for synthesizing SCW components. However, many questions remain about how individual enzymes function together to make each component and how individual components are assembled into a functional SCW. Progress is still hampered by problems with generating functional enzymes and demonstrating their activity, and this problem is increased in magnitude where enzymes may work together as part of a complex. SCWs are the bulk of plant biomass, and our improved understanding has been put to good effect to generate feedstock with dramatically improved digestibility. Many of these improvements still need to be tested in crops plants and/or under field conditions, but there are many encouraging signs that feedstocks that are much more efficiently processed will soon be available. Further improvements in SCW properties are likely to come from stacking of multiple traits.

## Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. A meta-analysis of Y1H data from Taylor-Teeples *et al.* (2015). The tree was generated based upon similarities in transcription factor binding to each gene.

Figure S2. A meta-analysis of Y1H data from Taylor-Teeples *et al.* (2015). Interactions for promoters involved in cellulose, xylan, and lignin have been isolated.

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