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# Woody plant cell walls: Fundamentals and utilization

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## ABSTRACT

**Cell walls in plants, particularly forest trees, are the major carbon sink of the terrestrial ecosystem. Chemical and biosynthetic features of plant cell walls were revealed early on, focusing mostly on herbaceous model species. Recent developments in genomics, transcriptomics, epigenomics, transgenesis, and associated analytical techniques are enabling novel insights into formation of woody cell walls. Here, we review multilevel regulation of cell wall biosynthesis in forest tree species. We highlight current approaches to engineering cell walls as potential feedstock for materials and energy and survey reported field tests of such engineered transgenic trees. We outline opportunities and challenges in future research to better understand cell type biogenesis for more efficient wood cell wall modification and utilization for biomaterials or for enhanced carbon capture and storage.**

**Keywords:** regulation of cell wall formation, biosynthesis of cell wall components, lignin, cellulose, hemicellulose, cell wall utilization

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## INTRODUCTION

The land plants (embryophytes) are believed to have evolved about 500–600 million years ago from their ancestral charophyte, green algae, that were already terrestrial with an evolved cell wall for terrestrial habitats (Lewis and McCourt, 2004; Harholt et al., 2016; Cheng et al., 2019). However, land plants produce more advanced wall polymers (polysaccharides and lignin) than

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charophycean green algae to respond to specific terrestrial stressors, such as desiccation, gravitropism, rapid temperature changes, and high ultraviolet and photosynthetic irradiance (Delwiche et al., 1989; Popper et al., 2011; Sorensen et al., 2011; Harholt et al., 2016; Mikkelsen et al., 2021).

Gymnosperms and angiosperms are today the dominating plant group in most terrestrial biomes (Parkinson et al., 1999). One of the major factors contributing to their evolutionary success was the development of vascular tissues with thick-walled cells to form xylem or wood for mechanical support and water transportation. In these plants, the vascular cambium proliferates and differentiates into the secondary phloem (bark) and the secondary xylem (wood), allowing the stem to perennially increase in diameter. Wood, a well-organized composite of various mature cell types, plays an important role in the global carbon cycle as a carbon sink of the terrestrial ecosystem. The total amount of carbon sequestered in forest vegetation, mostly wood, is approximately 359 billion tons, which accounts for about 86% of the terrestrial above-ground carbon (Allen et al., 2010; Baccini et al., 2012; Harris et al., 2021). Here, we focus on the cell walls of forest tree species.

In this review, we first describe the three xylem cell types. We next highlight recent discoveries of causative genetic and epigenetic mechanisms and present factors, such as hormones and stress, regulating cell wall biosynthesis for wood formation. We then summarize the chemistry, biochemistry, and genetics of the biosynthesis of the three major cell wall components (lignin, cellulose, and hemicelluloses) and list key examples to illustrate how these regulatory and biosynthesis studies advance our knowledge and strategies for genetic modification of wood cell walls as feedstock for biomaterial/energy production. Finally, we recite all currently reported translational studies that link lab-based regulatory results and assumptions with field responses of the genetically modified tree species. We close with a discussion of opportunities and challenges for future research that should be driven by more holistic approaches for a more precise knowledge of formation and regulation of plant cell walls for potential industrial and ecological applications.

## BIOGENESIS AND FUNCTIONS OF CELL TYPES IN XYLEM

### Tracheary elements (TEs), libriform fibers, and ray parenchyma cells

To form xylem or wood in forest trees, two cell-producing systems (the stem vascular meristem [SVM] system) are needed: the axial and radial systems (Figure 1A), derived from fusiform and ray initials, respectively. These initials are believed to be the stem cells of the SVM for wood formation (Evert and Deshpande, 1970; Evert, 2006; Dai et al., 2023; Tung et al., 2023). The identity/location of the stem cells of the SVM remains to be ascertained. The axial system of gymnosperm wood consists almost entirely of tracheids and lacks vessel cells, which are present in the axial system of most angiosperms. Sanio (1873) demonstrated that, in Scots pine (*Pinus sylvestris*), a fusiform initial renews (Figure 1B, stage I) and divides into a mother cell (stage II), which divides into two daughter cells (stage III). These four cells are known as "Sanio's 4" (1 initial or stem cell, 1 xylem mother cell, and 2 daughter cells) (Figure 1B). After proliferation, the cells then undergo differentiation, including cell expansion and secondary cell wall (SCW) deposition. In Figure 1C, we take xylem cell differentiation in angiosperms as an example. The daughter cells then differentiate into different stages of precursor cells (e.g., early precursors and intermediate precursors) and then into the terminal differentiation stage (Figure 1C) (Tung et al., 2023) as vessel elements and libriform fibers in fusiform lineage and ray parenchyma cells in ray lineage (Figure 1A). Plants must constantly reprogram these cell progression processes to change cell types, wall thickening, and synthesis of wall components to tune to growth and recurrent stress cues.

All xylem cells initially develop a thin primary wall on their protoplasts, followed by thickening of an SCW inside it, and the progression, likely an evolutionary specialization, is accompanied by notable changes in cellular morphology and function. All primary walls contain cellulose and pectins (Timell, 1980; Arioli et al., 1998; Somerville, 2006; Mohnen, 2008; Pedersen et al., 2023). Vessel elements (Figure 1A) develop a thick SCW and are connected through perforation plates (open-ended walls between adjacent vessel elements aligned axially) and specialized primarily for water transportation (Evert, 2006; Brodersen et al., 2019) (Figure 1D). Tracheids are slender and longer than vessel elements and are connected to each other through bordered pits and specialized for water/nutrient conduction. But gymnosperm tracheids have a dual function of water transportation and mechanical support (Evert, 2006; Brodersen et al., 2019). Vessel elements and tracheids are called TEs, and their wall thickening and specializations are accompanied by the biosynthesis of specific types of lignin. Lignin biosynthesis is activated during secondary wall differentiation (Sarkanen and Ludwig, 1971; Higuchi, 1985; Terashima et al., 2009; Albersheim et al., 2010; Li et al., 2014b; Lin et al., 2014), where primary wall cellulose and pectin synthesis become insignificant (Arioli et al., 1998; Somerville, 2006; Mohnen, 2008; Albersheim et al., 2010). Lignin in gymnosperm TE walls consists almost entirely of coniferyl alcohol (G type) of monomeric subunits and in angiosperm vessel elements is a co-polymer of sinapyl alcohol (S type) and G-type subunits with the G type being more abundant (Fergus and Goring, 1970; Musha and Goring, 1975; Saka and Goring, 1988; Osakabe et al., 1999; Li et al., 2001, 2003).

Libriform fibers (Figure 1A), found mainly in angiosperms, are narrow and elongated cells with tapering ends. Lignin and wall thickening are activated at their precursor stage. The heavily lignified thick SCWs function mainly in mechanical support of the plant body (Dickison, 2000; Schweingruber and Poschlod, 2005). Libriform fiber lignin is also a G+S-type co-polymer but rich in S-type monomers. For example, the S/G lignin monomer ratios in libriform fiber and vessel element walls in birch (*Betula papyrifera*) wood are ~7 and ~0.14, respectively (Fergus and Goring, 1970; Musha and Goring, 1975; Higuchi, 1985; Saka and Goring, 1988).

Ray parenchyma cells (Figure 1A), the third type of wood cells in both gymnosperms and angiosperms, are oriented radially. They typically also have lignified SCWs and are biologically active for many years (Evert, 2006; Morris et al., 2016; Slupianek et al., 2021; Zimmer and Treu, 2021). Lignified ray parenchyma cells

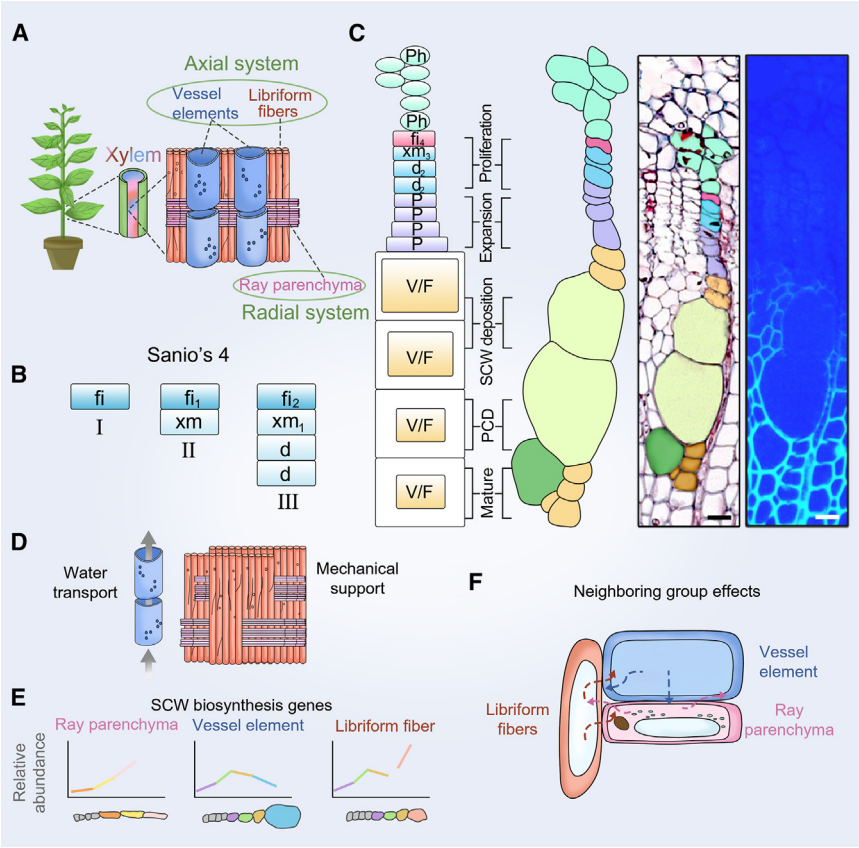


**Figure 1. Xylem cell development and expression of SCW biosynthesis genes.**

**(A)** Three major cell types in xylem, representing the axial (vessel elements and libriform fibers) and radial (ray parenchyma cells) systems.

**(B)** Stage I: one fusiform initial. Stage II: division of one fusiform initial into one renewed fusiform initial and one mother cell. Stage III: division of one mother cell into two daughter cells. The four cells (one initial, one mother cell, and two daughter cells) are named "Sanio's 4".

**(C)** Schematic of xylem axial system development from fusiform initial. After cell proliferation in Sanio's 4, the xylem cells undergo expansion, SCW deposition, and programmed cell death into mature xylem cells.

**(D)** The major function of vessel elements is water transportation. Libriform fibers and ray parenchyma cells contribute to axial and radial mechanical support.

**(E)** The expression of SCW biosynthesis genes in different xylem cells across the developmental lineages. The y axes represent relative transcript abundance of SCW biosynthesis genes. The x axes show the developmental lineages containing the precursor cells, ray parenchyma cells, vessel elements, and libriform fibers.

**(F)** The neighboring group effects. The dashed lines indicate the movement of genetic components.

Fi, fusiform initial; xm, xylem mother cell; d, daughter cell; p, precursor cell; V/F, vessel element or libriform fiber; ph, phloem; PCD, programmed cell death; SCW, secondary cell wall.

provide mechanical support for radial growth (Slupianek et al., 2021; Zimmer and Treu, 2021). Ray parenchyma cells are a water storage tissue and function in lateral transport and xylem embolism repair, allowing plants to withdraw water and help clear cavitation for survival under drought (Holbrook, 1995; Slupianek et al., 2021).

Using single-cell RNA sequencing for stem-differentiating xylem protoplasts, a recent study has revealed detailed SCW transcriptomes in different cell types during the developmental lineages in xylem of angiosperms (Tung et al., 2023). In general, transcript abundance of the SCW biosynthetic genes increases from fusiform or ray precursors to the cells closer to terminal differentiation (Figure 1C), consistent with the level of cell wall deposition and thickening (Figure 1C and 1E). A unique discovery in this study is that vessel elements are an exception in this increasing trend (Figure 1E) (Tung et al., 2023). The results suggest that xylem cells are reservoirs for mutually supplying genetic components to other cell types (Figure 1F) to maintain the homeostasis of cell wall deposition and maturation, supporting the neighboring group effects (Hosokawa et al., 2001; Tokunaga et al., 2005; De Meester et al., 2021) at the single-cell RNA level.

## REGULATION OF WOOD CELL WALL FORMATION

Numerous genetics, epigenetics, and hormonal signaling studies have revealed the upstream signaling and transcriptional regulation on xylem cell development and SCW biosynthesis (Figure 2). This regulation is usually integrated with various environmental stimuli to cooperatively govern cell development and wall deposition.

### Genetic regulation

Transcription factors (TFs) are essential to determine and maintain cell-type-specific transcription profiles. Epigenetic modifications (discussed in the next section) initiate and maintain the activity of these TFs, imposing cell type transcriptional memory on cell differentiation. Cell-type-determining TFs are high-level developmental regulators. Some of these high-level regulators are also involved in cell wall component biosynthesis, suggesting a transcriptional hierarchy with multifunctional TF interactions directing fusiform/stem cell development and cell wall biosynthesis in wood formation. TFs that regulate the fusiform/stem cell transition to dividing cells and enlarging cells (P cells; Figure 1), where cellulose starts to form, may therefore also regulate cell wall cellulose biosynthesis. TFs turning on cell-type-specific differentiation may also specify the biosynthesis of specific monolignol types (G or S monolignols; see lignin). Regulators other than TFs are likely to be involved in this transcriptional hierarchy, such as microRNAs (Lu et al., 2005, 2008, 2013) (Figure 2; Supplemental Table 1).

Members of a subfamily of NAC (for NAM, ATAF1/2, and CUC2) TFs, the VNS (VND [vascular-related NAC domain protein], NST [NAC secondary wall thickening promoting factor]/SND

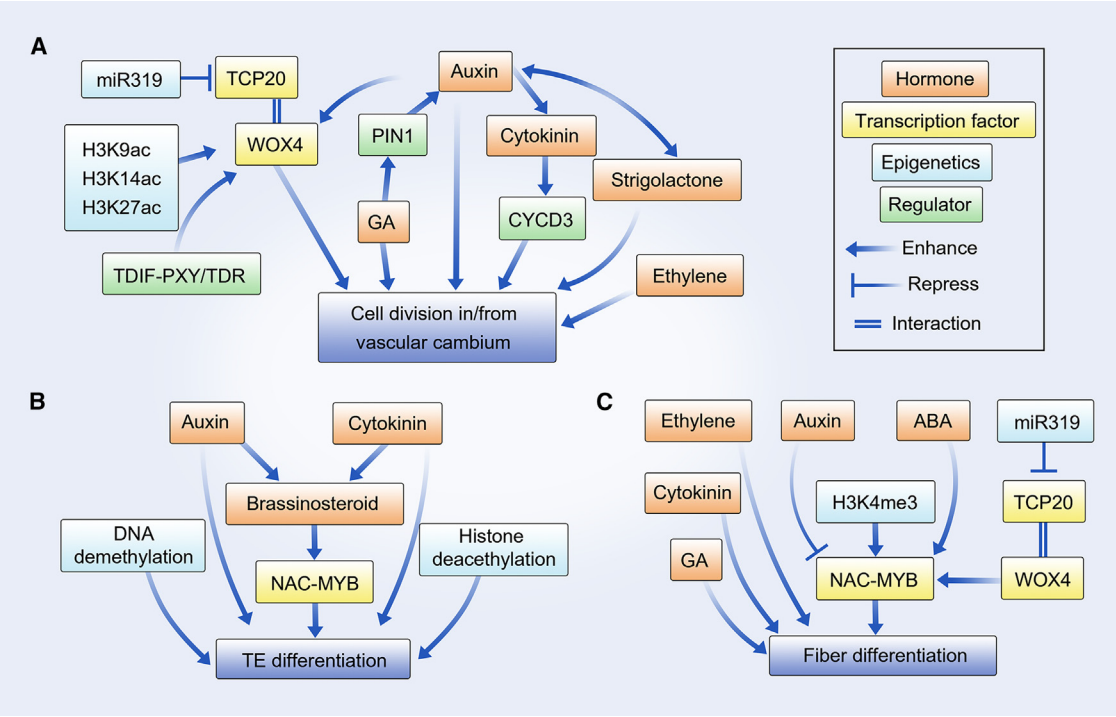


**Figure 2. Regulation of xylem cell development and SCW biosynthesis genes.**

**(A)** Cambial cell division is regulated by many internal cues, such as small RNAs, histone acetylation, small peptides, TFs, and phytohormones, including auxin, cytokinin, SLs, GAs, and ethylene.

**(B)** TE differentiation is coordinately regulated by multiple players, including DNA demethylation, histone acetylation, phytohormones, and TFs.

**(C)** Fiber differentiation is also regulated by a wide variety of factors, including small RNAs, histone methylation, phytohormones, and TFs.

miR319, micro RNA319; TCP20, teosinte branched 1/cycloidea/pcf20; WOX4, WUSCHEL-related homeobox 4; H3K9ac, H3K14ac, and H3K27ac, acetylated lysine residues 9, 14, and 27 of histone H3, respectively; TDIF, tracheary element differentiation inhibitory factor; PXY, phloem intercalated with xylem; TDR, TDIF receptor; PIN1, PIN-formed 1; CYCD3, CYCLIN D3; TE, tracheary element; GA, gibberellin; ABA, abscisic acid; H3K4me3, trimethylation of histone H3 lysine 4.

[secondary wall-associated NAC domain protein], and Sombrero) subfamily (Ohtani et al., 2011), initiate the differentiation of SCWs for vessel elements (VND6 and VND7) (Kubo et al., 2005) as well as fibers (NST1 and NST3/SND1) (Mitsuda et al., 2007; Zhong et al., 2007). The model tree *Populus trichocarpa* has 16 NAC TFs belonging to the VNS subfamily, where homologs of VND6/VND7 and SND1 are transcriptional switches for xylem vessel and fiber cell differentiation in *Populus* spp. (Zhong et al., 2011; Li et al., 2012; Lin et al., 2013; Zhao et al., 2014; Takata et al., 2019). A VND homolog, PdeNAC2, in *Pinus densiflora* was identified as a key regulator of tracheid formation (Kim et al., 2023).

Several v-myb avian myeloblastosis viral oncogene homolog (MYB) TFs, such as MYB46 and MYB83, in *Arabidopsis* are direct targets of VND6, VND7, and SND1 for vessel and fiber differentiation (McCarthy et al., 2009; Zhong and Ye, 2012; Ko et al., 2014b; Ohtani and Demura, 2019). MYB46 is a direct activator of most secondary cell wall cellulose and hemicellulose synthase genes and of 9 of the 20 monolignol pathway genes in *Arabidopsis* (Ko et al., 2014b). In *P. trichocarpa*, an SND1 homolog, PtrSND1-B1, directly activates PtrMYB021 (a homolog of *Arabidopsis* MYB46) and PtrMYB074 (a woody dicot-specific MYB), forming a 4-layered NAC-MYB mediated transcriptional regulatory network (TRN) that mediates 57 TF-DNA direct interactions through 17 TFs to *trans*-regulate 27 wood cell wall genes (Lin et al., 2013; Chen et al., 2019; Wang et al., 2020). This layered NAC-MYB TRN and its specific hierarchy were validated in transgenic *P. trichocarpa*. NAC-MYB networks were also believed to regulate tracheid formation in gymnosperms (Akiyoshi et al., 2020; Kim et al., 2023). The NAC-MYB-based TRN has been considered a nexus of regulation behind cell differentiation and cell wall synthesis for production of xylem in plants and wood in forest trees (Lin et al., 2013; Ko et al., 2014b; Chen et al., 2019; Ohtani and Demura, 2019). However, the NAC-MYB TRN is species specific, regulating different cell types, cell wall components, and functions needed in different species (Chen et al., 2019).

Studies of TF and network regulatory effects on cell wall biosynthesis in wood formation are extensive (Supplemental Table 2). Most of these studies, however, lack knowledge of the hierarchy and specificity of how the regulation is transduced through direct *trans*-regulatory (TF-DNA) interactions to activate cell wall genes. Therefore, manipulating these TFs often leads to pleiotropic phenotypes, whose underlying mechanisms are difficult to elucidate. We summarized these TFs and networks and their end effects on wood cell wall formation, highlighting TFs/networks with validated direct TF-DNA interactions that may help elucidate the pleiotropic effects (Table 1 and Supplemental Table 2). In addition, well-defined pairwise TF-DNA interactions may reveal coregulation of the same targets

| Species | TFs (ID) | Direct target genes | | | | | Regulatory effects | References |
|---|---|---|---|---|---|---|---|---|
| | | Quantification of regulatory effects | | Assays for validating direct TF-DNA interactions | | | | |
| | | Transgenics or mutants coupled with sequencing or qRT–PCR | *Trans*-activation assays | Electrophoretic mobility shift assay | Chromatin immunoprecipitation/ Chromatin immunoprecipitation sequencing | Yeast one-hybrid | | |
| *Betula platyphylla* | BplMYB46 (KP711284) | *BplPAL*, *BplCCoAOMT*, *Bpl4CL*, *BplCCR*, *BplLAC*, *BplCesA1*, *BplCesA2*, *BplCesA3*, *BplFRA*, *BplIRXs* | *BplPAL*, *Bpl4CL*, *BplCCoAOMT*, *BplLAC*, *BplCesA1*, *BplCesA2*, *BplCesA3* | | *BplPAL*, *BplCCoAOMT*, *Bpl4CL*, *BplLAC*, *BplCesA1*, *BplCesA2*, *BplCesA3* | | *BplMYB46*-overexpression (OE) birch: higher lignin and cellulose content, lower hemicellulose content | Guo et al. (2017) |
| *B. platyphylla* | BpNAC012 (KT344119) | *MYB46*, *MYB54*, *MYB63*, *MYB85*, *KNAT7*, *CCR1*, *4CL1*, *CCoAOMT*, *CESA1*, *CESA2*, *CESA3*, *FRA*, *IRX* | *MYB46*, *MYB54*, *MYB63*, *MYB85*, *KNAT7*, *CCR1*, *4CL1*, *CCoAOMT*, *CESA1*, *CESA2*, *CESA3*, *FRA*, *IRX* | | *MYB46*, *MYB54*, *MYB63*, *MYB85*, *KNAT7*, *CCR1*, *4CL1*, *CCoAOMT*, *CESA1*, *CESA2*, *CESA3*, *FRA*, *IRX* | | OE-*BpNAC012* birch: ectopic secondary wall deposition in stem epidermis | Hu et al. (2019) |
| *Pinus pinaster* | PpNAC1 (KY451900) | *C3H*, *HCT*, *CCoAOMT*, *CAD*, *CESA4* | *PpMYB4* | *PpMYB4* | | | silencing of *PpNAC1*: alteration of stem vascular radial patterning | Pascual et al. (2018) |
| *P. trichocarpa* | PtrMYB074 (Potri.015G082700) | *PtrbHLH186* | *PtrbHLH186* | *PtrbHLH186* | *PtrbHLH186* | | PtrMYB074-PtrWRKY19 dimers are required to transactive *PtrbHLH186*; overexpressing *PtrbHLH186*: retarded plant growth, | Liu et al. (2022) |
| *P. trichocarpa* | PtrWRKY19 (Potri.014G050000) | *PtrbHLH186* | *PtrbHLH186* | *PtrbHLH186* | *PtrbHLH186* | | | |

**Table 1. Direct TF-target DNA interactions that affect wood formation in some forest trees.**

*(Continued on next page)*

| Species | TFs (ID) | Direct target genes | | | | | Regulatory effects | References |
|---|---|---|---|---|---|---|---|---|
| | | Quantification of regulatory effects | | Assays for validating direct TF-DNA interactions | | | | |
| | | Transgenics or mutants coupled with sequencing or qRT–PCR | *Trans*-activation assays | Electrophoretic mobility shift assay | Chromatin immunoprecipitation/ Chromatin immunoprecipitation sequencing | Yeast one-hybrid | | |
| | | | | | | | increased G lignin, more smaller stem vessels and strong drought tolerance | |
| *P. trichocarpa* | PtrVCS2 (Potri.004G126600) | *PtrWOX4a* | *PtrWOX4a* | *PtrWOX4a* | *PtrWOX4a* | | OE-*PtrVCS2*: 4–6 less cambium cell layers; double-knockout *ptrvcs2*/*ptrvcs2-h*: 2–4 more cambium cell layers | Dai et al. (2023) |
| *P. tomentosa* | ARF7 (OK544549) | *WOX4a*, *WOX4b* | *WOX4a* | *WOX4a* | *WOX4a* | *WOX4a* | overexpressing *ARF7*: strongly improved cambial activity | Hu et al. (2022) |
| *P. alba* × *P. glandulosa* clone 84K | PagERF81 (Potri.019G131300) | *PagPAL*, *PagCOMT1*, *PagCCR1*, *PagCAD6*, *Pag4CLL9* | *PagCCR1*, *PagCAD6*, *Pag4CLL9* | *PagCCR1* | | *PagCCR1* | *Pagerf81* mutants: smaller vessel cells and longer fiber cells | Zhao et al. (2023b) |

**Table 1.** ***Continued***

by different but interactive TFs. Such coregulation has been identified among TFs in the PtrSND1-B1 directed hierarchical TRN, leading to the discovery of numerous TF protein complexes (dimers and trimers) implicated in regulating the biosynthesis of specific types of stem cell wall lignins (Chen et al., 2019). Other than TF-TF protein complexes, higher-level regulation, such as that mediated by epigenetic modifications and microRNAs, are also involved in the transcriptional control of cell wall and wood formation (Supplemental Tables 1 and 3). Current knowledge of high-level regulation is limited but critical for a holistic understanding of the control.

## Epigenetic regulation

DNA methylation and histone modifications are important aspects of epigenetic regulation of gene expression associated with plant growth and adaptation. DNA methylation is an epigenetic modification for gene transcriptional regulation (Jaenisch and Bird, 2003). In general, DNA methylation in promoters represses gene transcription, whereas DNA methylation in gene bodies induces constitutive gene expression (Jaenisch and Bird, 2003; Zhang et al., 2018a). In plants, DNA methylation is found on cytosine in the context of symmetric CG and CHG (where H is A, C, or T), and asymmetric CHH (Law and Jacobsen, 2010). Maintenance of normal DNA methylation patterns (through methylation and demethylation) allows normal growth and development in plants (Lloyd and Lister, 2022). For instance, the establishment of cell-type-specific demethylation signatures has been observed during xylem TE differentiation in *Arabidopsis*, where active DNA demethylation in the CH and CHG sequence contexts in SCW-related *CCoAOMT1* and *PRX25* was also discovered (Lin et al., 2020). Two TF genes, *LBD15* and *MYB20*, are also regulated by active DNA demethylation to affect TE differentiation (Lin et al., 2020). In *Populus tomentosa*, the expression of genes involved in cell wall biosynthesis during the dormant-active transition stage is potentially regulated by DNA hypomethylation of the promoter regions of these genes (Chen et al., 2021a).

Histone modification is another epigenetic mechanism that influences the chromatin structure and, hence, gene transcription (Pfluger and Wagner, 2007). Acetylation and methylation at histone lysine residues are two of the most studied epigenetic marks. Histone acetylation is generally associated with active chromatin and transcription (Bannister and Kouzarides, 2011). A recent study demonstrated that an epigenetic modification apparatus of PtrVCS2–PtrGCN5-1–PtrADA2b-3–PtrWOX13a regulates the *PtrWOX4a* transcript levels by controlling histone acetylation intensity on its promoter for the maintenance of normal cambial development in *P. trichocarpa* (Dai et al., 2023). Studies have illustrated that stress-induced cellular responses trigger epigenetic regulation of cell type- and cell wall biosynthesis-related genes. For instance, in response to salt stress in maize, cell wall genes encoding xyloglucan (XyG) endotransglucosylase and expansins were upregulated, and the upregulation corelated with elevated H3K9 acetylation levels on the promoter and coding regions of *ZmEXPB2* and *ZmXET1*, respectively, (Li et al., 2014a). In *P. trichocarpa*, elevated H3K9 acetylation of *PtrNAC006*, *PtrNAC007*, and *PtrNAC120* gene promoters by an AREB1–ADA2b–GCN5 ternary protein complex has been found to activate the expression of these *NAC* genes to promote xylem vessel cell differentiation (Li et al., 2019b). In *P. trichocarpa*, histone deacetylation encoded by putative PtrHDT3 histone deacetylases has been implicated in retarding the differentiation of xylem fibers but promoting that of vessel elements (Wang et al., 2020).

Histone methylation in plants represent both repressive and active marks (Liu et al., 2010). In general, trimethylated lysine 4 of histone H3 (H3K4me3) and trimethylation of H3 lysine-27 (H3K27me3) are associated with gene activation and repression, respectively (Xiao et al., 2016b). *Arabidopsis* homolog of trithorax 1, a H3K4 histone methyltransferase, positively regulates interfascicular SCW fiber deposition in *Arabidopsis* by activating the expression of *SND1* and *NST1* through increasing H3K4me3 levels at these gene loci (Wang et al., 2021b). In the developing xylem of *Eucalyptus grandis*, highly expressed SCW-related genes have high enrichment of H3K4 trimethylation at these gene loci (Hussey et al., 2015). Also in *E. grandis*, genomic regions of H3K4me3 and H3K27me3 deposition are distinctly associated with xylogenesis-related processes, with lignification being frequently found in regions co-occupied by H3K4me3 and H3K27me3 but not SCW polysaccharide biosynthesis (Hussey et al., 2017). For example, lignin biosynthesis pathway genes, such as *CAD2* and *CAD3*, were enriched with the H3K4me3 activation mark, contributing to high expression levels of these genes for SCW deposition (Hussey et al., 2017). So far, very few epigenetic regulators, particularly for DNA methylation, have been functionally characterized during cell type differentiation and cell wall biosynthesis in plants, obscuring the epigenetic regulation involved in these processes.

## Hormonal regulation

Auxin, a pivotal plant hormone, triggers developmental pathways and influences cambium cell proliferation in plants (Uggla et al., 1998; Smetana et al., 2019; Bagdassarian et al., 2020). Other phytohormones, such as gibberellins (GAs), cytokinins, and strigolactones (SLs), also influence cambial cell proliferation crosstalk with auxin signaling. GA increases auxin levels in the stem of *Populus* by stimulating polar auxin transport (Björklund et al., 2007). Consistent with this, PIN1-dependent polar auxin transport is promoted by GA treatment in *Arabidopsis* roots (Mäkilä et al., 2023). Cytokinin also functions as a key regulator of cambium activity (Matsumoto-Kitano et al., 2008); for example, by mediating the cell cycle regulator CYCLIN D3, to regulate cambial cell proliferation (Collins et al., 2015; Randall et al., 2015). Different but partially overlapping distribution of cytokinin, auxin, and GA has been found across stem wood-forming tissues of *P. trichocarpa*, with maximum levels of cytokinin, auxin, and bioactive GA (GA4) being detected in cambium and SCW-forming regions, respectively (Immanen et al., 2016). The elevated levels of cytokinin in transgenic *P. tremula* × *Populus tremuloides* overexpressing a key *Arabidopsis* cytokinin biosynthetic gene *AtIPT7* displayed enhanced cell division in cambium, concomitant with the increase of auxin concentration and the expression of auxin-responsive genes, indicating that cytokinin and auxin co-regulate cambial cell division (Immanen et al., 2016). SLs positively regulate cambial activity in *Arabidopsis* inflorescence stems, and SL signaling in the cambium interacts with the auxin signaling pathway (Agusti et al., 2011). Ethylene stimulates cambial cell division by acting

through ethylene receptors in *P. tremula* × *P. tremuloides* (Love et al., 2009). In *Arabidopsis*, the WOX4–TE differentiation inhibitory factor (TDIF)–phloem intercalated with xylem regulatory module acts as a hub of auxin and ethylene signaling paths to activate the transcriptional network for cambial cell proliferation (Brackmann et al., 2018; Zhang et al., 2019a; Yang et al., 2020). This ethylene/auxin-mediated regulation of cambial activity is believed to be conserved in tree species (Etchells et al., 2015; Kucukoglu et al., 2017).

Auxin also plays an important role in regulating differentiation of vessel elements and libriform fibers, especially during SCW deposition. In *Populus*, auxin orchestrates the development of specialized secondary xylem to form ''tension wood'' under mechanical stress, altering libriform fiber SCW composition via PIN3-mediated polar auxin transport (Gerttula et al., 2015). In rice, the auxin-responsive factors OsARF6 and OsARF17 activate SCW biosynthesis and control flag leaf angle (Huang et al., 2021). In cotton, crosstalk between auxin and ethylene signaling paths may modulate the activation of fiber SCW formation, including SCW deposition, wall thickening, and cellulose biosynthesis (Wang et al., 2023).

Other phytohormones, such as GAs, cytokinins, brassinosteroids (BRs), and abscisic acids (ABAs) are also involved in regulating SCW development for wood formation and modulating cell wall features and chemical composition. GAs counteract DELLA-mediated inhibition of SCW formation, promoting downstream lignin and cellulose biosynthetic genes and stimulating SCW deposition in cotton (*Gossypium hirsutum*) and rice (Huang et al., 2015; Ye et al., 2015; Wang et al., 2021c). Disruption of cytokinin transport in an *ABCG14 Arabidopsis* knockout leads to reduced endogenous cytokinin levels, resulting in delayed lignification of fibers and vessels (Ko et al., 2014a; Zhang et al., 2014). In *Arabidopsis*, the BR signaling pathway mediates *CesA* gene expression, influencing cellulose biosynthesis for both PCWs and SCWs (Xie et al., 2011). Disrupted BR signaling pathways in *Arabidopsis* result in drastic reduction of cellulose and lignin in stem SCWs and the production of smaller xylem vessels (Hossain et al., 2012). ABA activates SCW-related NAC genes in *Arabidopsis* (Jensen et al., 2010; Liu et al., 2021; Ramachandran et al., 2021). The core ABA signaling component SNRK2 kinase phosphorylates NST1, a NAC subfamily member, for *trans*-activation of downstream SCW-related genes for SCW thickening and lignin biosynthesis (Liu et al., 2021). A regulatory system through interlinking ABA signaling pathways and NAC-modulated *trans*-regulatory networks is implicated in xylem differentiation, particularly for vessels, and SCW thickening for drought stress response and tolerance in *Populus* (Li et al., 2019b).

### Regulation by stress

Vessel differentiation establishes the xylem conduction system for water uptake and adaptation to drought stress. In response to drought, plants tend to develop more adaptive features, such as smaller xylem vessels, for survival (Arend and Fromm, 2007; Fichot et al., 2009). Multiple hormone-induced signaling cascades are involved in such adaptive responses (Eckert et al., 2019). For example, in *Populus*, a stress-induced decrease in auxin levels causes a reduction in vessel diameter (Junghans et al., 2006), and drought-elevated ABA levels cause the formation of more and smaller stem xylem vessels (Popko et al., 2010; Yu et al., 2021). Under drought, an antagonistic interaction between jasmonic acid and cytokinin has been suggested to promote differentiation of xylem from procambial cells in *Arabidopsis* roots for resilience (Jang and Choi, 2018).

Phytohormone signaling pathways/levels affected by stress induction often coordinate with specific networks of gene *trans*-regulation to regulate cambial activity and cell type specification/differentiation to prevent xylem cavitation in wood development for adaptation. The ethylene response factor (ERF) family is one of the largest gene families in plants, and 170 ERFs have been identified in the *P. trichocarpa* genome (Vahala et al., 2013). Many active ERFs interlink with hormonal signals for function (Vahala et al., 2013). The expression of ERFs 18, 21, 30, 85, and 139 is strongly induced by ethylene, leading to modified wood chemistry (Vahala et al., 2013). Overexpression of *ERF139* in *P. tremula* × *P. tremuloides* or *P. tremula* × *Populus alba* regulates xylem cell expansion and SCW formation for reduced vessel size with accompanying drought tolerance (Wessels et al., 2019; Wang et al., 2022b; Huan et al., 2023). These changes are believed to be caused by ERF194-mediated activation of ABA signaling pathways. ERF85, an ERF member belonging to the cytokinin response factor subfamily, has been implicated in coregulation with cytokinin pathways for a negative role in xylem cell wall morphology and chemical composition (Seyfferth et al., 2021). ERFs and perhaps many other TF families likely work together with multiple hormone-induced signaling pathways to mediate cell expansion and specialization for xylem wood formation in trees for abiotic as well as biotic stress adaptation.

## BIOSYNTHESIS OF CELL-WALL COMPONENTS

### Lignin

#### *Biosynthesis and structure*

Payen (1839) discovered lignin in wood as a unique organic polymer distinct from cellulose (Schulze, 1879). Lignin and cellulose constitute roughly 20%–30% and 40%–50% of the wood biomass, respectively, and hemicelluloses make up the remaining wood. Lignin is a heterogeneous phenylpropanoid polymer that is synthesized through the oxidative radical coupling of three canonical monolignols, *p*-coumaryl, coniferyl, and sinapyl alcohols, the *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units, respectively (Figure 3A) (Sarkanen and Ludwig, 1971; Higuchi, 1985). Other minor phenolic compounds are also found in lignin (Figure 3A) (Boerjan et al., 2003; del Río et al., 2020; del Río et al., 2022; Vanholme et al., 2019). The basic features of the lignin chemical structure vary considerably among the major phylogenetic divisions of the plant kingdom (i.e., gymnosperms, angiosperms, and grasses). The composition of lignin units as well as the amount of lignin also varies greatly among organs and cell types, within cell wall layers, and under different development stages and growth environments (Boerjan et al., 2003; Rinaldi et al., 2016; Meents et al., 2018; Cesarino, 2019). For example, the herbaceous *Arabidopsis* stem, grass culm, and secondary xylem of trees

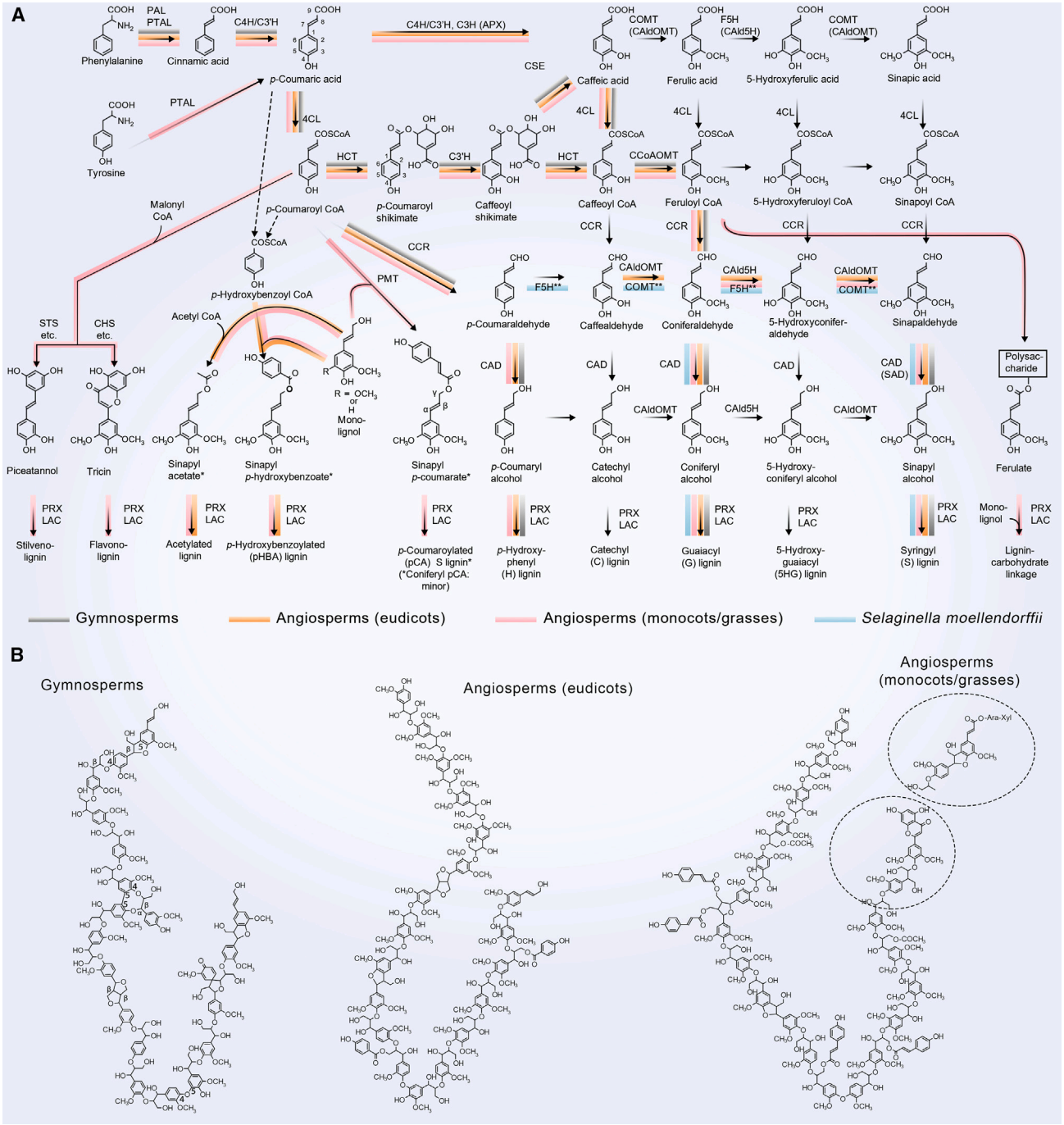


**Figure 3. Lignin biosynthesis and structures.**

**(A)** Biosynthetic pathways for various lignin units. C4H, cinnamate 4-hydroxylase; C3H, *p*-coumarate 3-hydroxylase; CAldOMT, 5-hydroxyconiferaldehyde *O*-methyltransferase; 4CL, 4-hydroxycinnamate CoA ligase; HCT, hydroxycinnamoyl CoA:shikimate/quinate hydroxycinnamoyl transferase; CCoAOMT, caffeoyl CoA *O*-methyltransferase; CCR, cinnamoyl CoA reductase; CAld5H, coniferaldehyde 5-hydroxylase; CAD, cinnamyl alcohol dehydrogenase; SAD, sinapyl alcohol dehydrogenase; F5H, ferulate 5-hydroxylase; COMT, caffeic acid *O*-methyltransferase; AEOMT, hydroxycinnamic acids/hydroxycinnamoyl CoA ester *O*-methyltransferase; C3′H, *p*-coumaroyl shikimate/quinate 3-hydroxylase; PAL, phenylalanine ammonia lyase; PTAL, phenylalanine/tyrosine ammonia lyase; CSE, caffeoyl shikimate esterase; PRX, peroxidase; LAC, laccase; CHS, chalcone synthase; STS, stilbene synthase; PMT, *p*-coumaroyl-CoA:monolignol transferase; APX, ascorbate peroxidase. *Only the structure of *p*-coumaroylated sinapyl alcohol is shown. ***S. moellendorffii* enzymes.

**(B)** Proposed structures of lignins in different species. The structures are adapted from Ralph et al. (2019) with a slight modification. The two alternative terminal structures are enclosed with a dashed line.

| Species | Tissues | Lignin content (%) | H/G/S ratio | References |
|---|---|---|---|---|
| *Arabidopsis thaliana* | stem | 14.4[a] | 2.8/77.1/20.1[b] | [a]Goujon et al. (2003); [b]Mansfield et al. (2012) |
| *P. trichocarpa* | young shoot | ~14 | 6/32/62 | Hori et al. (2020) |
| | mature xylem | ~21 | 1/34/65 | |
| *E. grandis* | whole stem of 1-month-old plants | 13 | 9.4/38.5/52.1 | Rencoret et al. (2011) |
| | debarked stem of 9-year-old plants | 19.8 | 1.9/20.4/77.7 | |
| *O. sativa* | mature culm | 15.4[a] | 4.2/43.0/37.9[b] | [a]Takeda et al. (2019); [b]Miyamoto et al. (2019) |
| *P. taeda* | mature wood | 30.1[a] | 1.7/0/98.3[b] | [a]Yeh et al. (2004); [b]Mansfield et al. (2012) |

**Table 2. Lignin content and aromatic composition in different species.**

differ greatly in the amount and structure of lignin (Table 2), although *Arabidopsis* and trees such as poplar and eucalyptus belong to the same angiosperm lineage. Thus, no single species can be a model for the others. The diverse complexity of monolignol biosynthesis among species described below explains the species-dependent nature of lignin properties.

In early studies, angiosperm lignin was thought to be formed almost entirely by the polymerization of G and S monolignols with a trace amount of the H unit and grass lignin from G and S monolignols with a slightly larger amounts of the H unit (Higuchi, 1985) and esterified significantly with *p*-coumaric acid (Shimada et al., 1971; Nakamura and Higuchi, 1976; Ralph, 2010). Gymnosperm lignin was thought to be derived exclusively from the G monolignol (Higuchi, 1985). These studies revealed the involvement of specific pathway metabolites and enzymes.

Studies since the late 1990s have demonstrated that many more lignin units and enzymes are involved in the biosynthetic pathways than previously thought (Figure 3A) (Umezawa, 2010; Weng and Chapple, 2010; Mottiar et al., 2016; Rinaldi et al., 2016; Wang et al., 2019a, 2019b; Dixon and Barros, 2019; Vanholme et al., 2019; Martarello et al., 2023). In the currently proposed pathways (Figure 3A), phenylalanine ammonia lyase converts phenylalanine into cinnamic acid, whereas phenylalanine/tyrosine ammonia lyase deaminates both phenylalanine and tyrosine to generate cinnamic acid and *p*-coumaric acid, respectively. Cinnamic acid is then hydroxylated by the cytochrome P450 monooxygenase, cinnamate 4-hydroxylase (C4H), to produce *p*-coumaric acid. Next, the carboxyl group of *p*-coumaric acid is activated to afford *p*-coumaroyl coenzyme A (CoA), which then serves as a common precursor to most phenylpropanoid compounds known to date, including flavonoids.

Gymnosperms produce G lignin (Figure 3A and 3B) from *p*-coumaroyl CoA via *p*-coumaroyl shikimate, caffeoyl shikimate, caffeoyl CoA, feruloyl CoA, coniferaldehyde, and coniferyl alcohol. These reactions are catalyzed by hydroxycinnamoyl CoA:shikimate/quinate hydroxycinnamoyl transferase (HCT), *p*-coumaroyl shikimate/quinate 3-hydroxylase (C3′H), HCT, caffeoyl CoA *O*-methyltransferase (CCoAOMT), cinnamoyl CoA reductase (CCR), and cinnamyl alcohol dehydrogenase (CAD), respectively. Gymnosperms also produce trace amounts of H units from *p*-coumaryl CoA via *p*-coumaraldehyde and *p*-coumaryl alcohol, but its content reaches significant levels in compression wood tissues. In addition to CCoAOMT, *O*-methylation of caffeoyl CoA to yield feruloyl CoA can also be catalyzed by hydroxycinnamic acid/hydroxycinnamoyl CoA ester *O*-methyltransferase (AEOMT) in *Pinus taeda* (Li et al., 1997) and pinosylvin *O*-methyltransferase 1 (POMT1) in *P. sylvestris* (Paasela et al., 2017). *CCoAOMT*, *AEOMT*, and *POMT1* cDNAs exhibit a similar expression pattern in *P. sylvestris*, suggesting their involvement in lignification in gymnosperms (Paasela et al., 2017).

Angiosperm eudicots have retained gymnosperm pathways for the production of H and G lignin units (Table 2; Figure 3A and 3B). C3′H, which catalyzes the 3-hydroxylation of *p*-coumaroyl shikimate to afford caffeoyl shikimate in the shikimate shunt, was first discovered in angiosperms (Schoch et al., 2001; Hoffmann et al., 2003). In *P. trichocarpa*, C3′H/C4H complex has been found to exhibit C3H activity as well as C3′H and C4H activity (Chen et al., 2011). In addition to the G monolignol pathways leading to *p*-coumaryl and coniferyl alcohols, a branch pathway from coniferaldehyde and/or coniferyl alcohol to produce sinapyl alcohol and S units functions in angiosperms (Figure 3A). In the branch pathway, coniferaldehyde is hydroxylated by coniferaldehyde 5-hydroxylate (CAld5H) to yield 5-hydroxyconiferaldehyde (Humphreys et al., 1999; Osakabe et al., 1999), which is then *O*-methylated to sinapaldehyde by 5-hydroxyconiferaldehyde *O-methyltransferase* (CAldOMT) (Li et al., 2000). CAld5H and CAldOMT are also named ferulate 5-hydroxylase (F5H) and caffeic acid *O-methyltransferase* (COMT), respectively. In *Selaginella moellendorffii*, a lycophyte plant, S unit pathways have also been found, where dual hydroxylation and *O*-methylation at the ring 3 and 5 positions are catalyzed by SmF5H and SmCOMT, which have low sequence identity to angiosperm CAld5Hs (F5Hs) and CAldOMTs (COMTs), respectively (Figure 3A) (Weng et al., 2010). The results suggest a convergent evolution of S monolignol biosynthesis.

In angiosperms, the aldehydes are then reduced by CAD. Among CADs from various plant species, a *P. tremuloides* CAD that exhibits a strong preference for sinapaldehyde was named sinapyl alcohol dehydrogenase (Li et al., 2001). A parallel pathway on the cinnamyl alcohol level from coniferyl alcohol to sinapyl alcohol via 5-hydroxyconiferyl alcohol has also been proposed (Matsui et al., 2000). Kinetics analysis of the CAldOMT- and CAld5H-catalyzed reactions indicated that coniferaldehyde and 5-hydroxyconiferaldehyde are better substrates than their respective corresponding alcohols and acids (Osakabe et al.,

1999; Li et al., 2000; Nakatsubo et al., 2007, 2008; Koshiba et al., 2013), while comprehensive metabolic flow analysis emphasized the alcohol-level conversion (Wang et al., 2014, 2019b). The caffeoyl shikimate esterase (CSE)/4-hydroxycinnamate CoA ligase (4CL) bypass (Figure 3A) was first reported for *Arabidopsis* (Vanholme et al., 2013), and later CSEs were identified in several angiosperms as well as gymnosperms, such as *Picea sitchensi* and *Larix kaempferi* (Ha et al., 2016; Saleme et al., 2017; Li et al., 2019a; Wang et al., 2019d, 2021a; de Vries et al., 2021). However, *CSE* genes were not detected in all plant species analyzed, suggesting that CSE may not be essential for lignification in all plants (Ha et al., 2016; Saleme et al., 2017; Wang et al., 2021a).

Among angiosperms, grass family (Poaceae/Gramineae) plants contain G and S lignins with a slightly higher H lignin than eudicots (Table 2; Figure 3A and 3B) (Mansfield et al., 2012; Ralph et al., 2019), while lignin of commelinid monocotyledons, including grasses, is acylated significantly by *p*-coumaric acid and, to a lesser extent, by ferulic acid mainly on S units (Figure 3B) (Ralph, 2010; Karlen et al., 2016, 2018). *p*-Coumaroylated lignin units are produced by the incorporation of *p*-coumaroyl monolignol conjugates (Ralph, 2010), formed by *p*-coumaroyl-CoA:monolignol transferase (PMT), which couples *p*-coumaroyl CoA and canonical monolignols (Chandrakanth et al., 2022; Mottiar et al., 2023). Likewise, feruloylated lignin units, albeit with lesser content, are formed by the action of feruryol-CoA:monolignol transferase (not shown in Figure 3) (Chandrakanth et al., 2022; Mottiar et al., 2023). In addition, the flavone tricin is incorporated into lignin in grasses as a lignin monomer to produce another grass-characteristic lignin structure, the tricin-lignin or flavonolignin unit (del Río et al., 2012; Lam et al., 2021; Lan et al., 2015; Lan et al., 2016). CAldOMT is also involved in the dual methylation of flavone precursors to produce tricin in *Oryza sativa* (Lam et al., 2019, 2021).

Several acylated lignin units have also been detected in various angiosperm plants (Figure 3A and 3B). *p*-Hydroxybenzoyl and benzoyl monolignols are incorporated into the lignin of some palms (monocots) and *Populus* (eudicot) (del Río et al., 2020; Kim et al., 2020a). Acyltransferase to produce the *p*-hydroxybenzoyl monolignols from *p*-hydroxybenzoyl CoA and monolignols (*p*-hydroxybenzoyl CoA monolignol transferase [PHBMT1]) have been identified in *P. trichocarpa* and *P. alba* × *P. grandidentata* (Zhao et al., 2021b), while the C4H activity of the C3′H/C4H complex is probably involved in *p*-hydroxybenzoic acid biosynthesis via 4-hydroxylation of cinnamic acid (Kim et al., 2020a). Furthermore, acetates, which are mainly present on S lignin units, are also common in both monocot and some dicot lignins but likely not in gymnosperm lignins (Ralph et al., 2019).

Traditionally, studies of monolignol biosynthetic pathways focus on single enzymes, but many protein complexes, through protein-protein interactions for more effective catalysis, have been discovered recently, mainly in *P. trichocarpa* (Wang et al., 2019b). A multienzyme complex of three membrane-bound P450 monooxygenases, C3H3–C4H1–C4H2, has been found to enhance the catalytic efficiency of 4- and 3-hydroxylation of cinnamic acid derivatives (Figure 3A) (Chen et al., 2011). Furthermore, the formation of a heterotetrametric 4CL3–4CL5 complex (Chen et al., 2014) and its interaction with HCTs (Lin et al., 2015) exhibits unique enzymatic specificity for CoA ligation of hydroxycinnamic acids (Figure 3A). Physical association between CCR and CAD is crucial for maintaining their respective catalytic activities *in planta* (Yan et al., 2019). Suppression of CAD reduced CCR activity in stem xylem of *P. trichocarpa* while suppressing CCR decreased CAD activity. CCR also interacts with Rac1, a signaling protein for defense response, in a guanosine triphosphate-dependent manner to activate CCR functions to increase lignin accumulation in *O. sativa* (Kawasaki et al., 2006). CCoAOMT and HCT form a multiprotein complex with Rp1, a plant disease resistance protein (Wang and Balint-Kurti, 2016). Downregulating CCoAOMT or HCT in tobacco disrupts this complex, re-activating Rp1 and intensifying the defense response to *Agrobacterium tumefaciens* infection. Interactions between C3H, C4H, and CAld5H with cytochrome P450 reductases are necessary for electron transfer to maintain their catalytic activity in monolignol biosynthesis (Sundin et al., 2014; Zhao et al., 2023a). Two membrane steroid binding proteins (MSBP1 and MSBP2) interact with C4H, C3H, and F5H, conferring stability and functional activity to these P450s (Gou et al., 2018). These discoveries provide new insights at the systems level into the metabolic flux for lignification and functions.

Following synthesis, monolignols and other units are believed to diffuse from cytoplasm through the plasma membrane to the cell wall for polymerization (Vermaas et al., 2019; Perkins et al., 2022), while an early study suggested that monolignols are transported by plant membrane transporters, such as ATP-binding cassette transporters (Miao and Liu, 2010). For polymerization, the weight of the evidence supports a combinatorial mode of the process, where the lignin polymer is generated by laccase- and peroxidase-mediated oxidative free radical-based coupling of monolignols and other units that add to an expanding polymer (Lin et al., 2016; Zhuo et al., 2022), creating the final polymer with enormous variation in the inter-unit linkages.

The structures of the most prominent lignin inter-unit linkages have been identified (Figure 3B). β-Ethers (β-O-4) are the predominant type in both gymnosperms and angiosperms, accounting for ∼40%–65% of the total linkages in wood lignins. Other linkages include 4-O-5 and C-C types, such as β-β, β-5, β-1, and 5-5 (Ralph et al., 2019; Yoshioka et al., 2023). The combinatorial formation of the highly variable composition and linkage structures readily explains the lack of a defined primary lignin sequence (Figure 3B). The highly variable structure of lignin also explains its resistance to enzymatic degradation, because no single enzyme can efficiently degrade it.

Lignin deposition is not limited to cell walls of plant xylem tissues, but also occurs in many other cell types in various plant tissues/organs for specific cellular functions. These tissues/organs include Casparian strip (Hosmani et al., 2013; Kamiya et al., 2015), seed pod, silique (Liljegren et al., 2000; Dong et al., 2014; Funatsuki et al., 2014; Yu et al., 2020), tendril coil (Gerbode et al., 2012), and anther (Mitsuda et al., 2005; Zhao et al., 2010). While vast amounts of lignin are deposited in cell walls of plant xylem tissues, the lignin deposition in these other cell types occurs in some narrow spaces, which requires strict and precise spatiotemporal control mechanisms for

biosynthesis. Dirigent or dirigent-like proteins (Ralph et al., 2006) have been noted (Meng et al., 2023) for their involvement in such control mechanisms. Dirigent protein was first reported as a protein that mediates asymmetric induction in the biosynthesis of coniferyl alcohol-derived lignans (Davin et al., 1997; Gang et al., 1999; Meng et al., 2023). Later, dirigent or dirigent-like proteins were found to be involved in Casparian strip formation (Hosmani et al., 2013) and pod dehiscence (Funatsuki et al., 2014; Parker et al., 2020). In addition, this class of proteins has been reported to have various roles in plant defense, stereoselective biosynthesis of gossypol and pterocarpans, and formation of neolignans (Meng et al., 2023). Moreover, Gao et al. (2023) demonstrated that a dirigent protein complex is essential for the localized polymerization of coniferyl alcohol for Casparian strip formation. In Casparian strip lignification, peroxidases but not laccases are required (Rojas-Murcia et al., 2020). On the other hand, both laccases and peroxidases are involved in lignification in xylem cell walls at different developmental stages and in specific subdomains of cell walls (Meents et al., 2018; Dixon and Barros 2019; Somssich 2020; Hoffmann et al., 2022).

## Cellulose

### *Cellulose properties in primary cells walls (PCWs) and SCWs*

Cellulose is an unbranched glucan composed of β-1,4-linked D-glucosyl residues. It is a major component of both PCWs and SCWs. Although the primary structure of cellulose is simple, its ultimate structure is complicated and involves an intricate formation process. The renascent glucans are usually bundled into protofibrils via intra- and intermolecular hydrogen bonds and van der Waals forces, thereby coalescing into multiscale cellulose microfibrils (CMFs). The CMFs can be further aggregated into higher-order structures (Tai et al., 2023). Celluloses in PCWs and SCWs display different characteristics, such as the degree of polymerization, CMF organization, and CMF crystalline structure, which match the functions of the corresponding plant cells and affect the utilization of cellulose (Mellerowicz and Sundberg, 2008).

The CMF dimensions vary across different cell types and species of vascular plants. CMFs in plant cell walls are typically 10–20 nm in diameter and contain 18 chains, characterized by wide-angle X-ray scattering and NMR (Yang and Kubicki, 2020; Deligey et al., 2022). Most wood CMFs in the cell walls of gymnosperm and angiosperm trees contain 24 glucan chains, with a crystalline-ordered core of ∼2.2-nm diameter and a semi-disordered shell of ∼0.5-nm thickness (Tai et al., 2023). The crystal structure of natural celluloses is complex. Cellulose in PCWs is predominantly in the Iα crystalline form (triclinic), whereas that in SCWs is rich in the Iβ crystalline form (monoclinic); both forms have parallel glucan chains in a flat ribbon conformation but with different hydrogen bonding patterns (Šturcová et al., 2004). Because of technical limitations, our understanding of cellulose crystalline structure is still rather superficial.

### *Cellulose biosynthesis and deposition*

Cellulose properties depend on cellulose biosynthesis and deposition, which involve a great number of proteins and regulatory factors. Cellulose synthases (CESAs), the enzymes directly catalyzing glucan chain elongation, determine the degree of polymerization of cellulose. Their active forms are CESA complexes (CSCs) composed of multimeric CESA, which function at the plasma membrane (Doblin et al., 2002). CESA proteins belong to a glycosyltransferase 2 (GT2) family and their sequences are highly similar in higher plants, suggesting that the mechanisms underlying cellulose biosynthesis may be conserved across plant species. Over the past decades, numerous CESA proteins have been functionally characterized from different species (Zhang and Zhou, 2011). *P. trichocarpa* has 17 CESA members (Suzuki et al., 2006). Proteomic analyses suggest that PtrCESA4, PtrCESA7A/B and PtrCESA8A/B, and PtrCESA1A/B, PtrCESA3C/D and PtrCESA6E/F are the components consisting of SCW and PCW CSCs, respectively (Xi et al., 2017). The suggestion has been corroborated by the 90% cellulose reduction in mutants deficient in PtrCESA4, PtrCESA7A/B, or PtrCESA8A/B (Xu et al., 2021). The functional characteristics of plant CESAs have been comprehensively reviewed in many reviews (Wilson et al., 2021; Pedersen et al., 2023).

CSCs are some of the most complicated protein complexes in plants. The exact combination of CESA subunits in each CSC remains to be resolved. Stoichiometry analysis and computational modeling demonstrated that CSCs likely consist of 18 or 24 CESAs per CSC that can organize CMFs with 3-fold glucan chains (Hill et al., 2014; Nixon et al., 2016). Three *Arabidopsis* SCW CESAs are thought to be assembled into a six-fold of trimers at an equimolar, which best conforms to the CSC rosette morphology. However, as revealed by domain swapping experiments, the contribution of each CESA in the rosette complexes to the overall rate of cellulose synthesis may differ due to variation in catalytic activity (Hill et al., 2018). In addition, not all species follow the equimolar subunit law to assemble CSCs. Stoichiometry analysis revealed that the SCW CSC in *Populus* developing xylem consists of PtrCESA8a/b, PtrCESA4, and PtrCESA7a/b CSCs with a 3:2:1 ratio in normal and 8:3:1 in tension wood (Zhang et al., 2018b). Understanding the exact assembly of CSCs requires cryoelectron microscopy, which has clarified the atomic structure of bacterial CESA BcsA (Morgan et al., 2013, 2016). Using cryoelectron microscopy, a *Populus* CESA homotrimer was determined at angstrom resolution, which resolved several controversial issues related to plant CSC assembly and function (Purushotham et al., 2020), bringing us one step closer to clarifying cellulose synthesis.

CSCs are thought to be assembled in the Golgi apparatus and translocated to the plasma membrane via vesicle trafficking. Many proteins involved in cytoskeleton-mediated membrane trafficking have been verified to govern cellulose biosynthesis through affecting CSC assembly and transport (Lei et al., 2015; Schneider et al., 2017). Most advances in our knowledge of mechanisms underpinning CSC trafficking and movement are made in *Arabidopsis* because the observations required of large amounts of living cells can be achieved using this species (Gu and Rasmussen, 2022; Zhu and McFarlane, 2022). However, whether the mechanisms are conserved in other plant species remains unclear. CESA phosphorylation and *S*-acylation have also been reported to regulate CSC movement and docking at the plasma membrane (Chen et al., 2010; Kumar et al., 2022).

The plant cell wall is a fibrillary network composed of multiscale CMFs that form load-bearing structures to support the functions

of plant cells. CMF organization and deposition require apoplast-targeting proteins, such as COBRA-like proteins, which anchor to the glycosylphosphatidylinositol (GPI) tail to mediate CMF assembly and crystallization (Liu et al., 2013). Recently, a GPI lipid remodeling protein have been identified in rice, which regulates the target of GPI anchor proteins, like the COBRA-like protein BC1, to the plasma membrane, thereby affecting CMF organization and mechanical strength (Xu et al., 2022). Because CMFs are generally bound by matrix polysaccharides, disruption in xylan, pectins, and XyG could alter the orientation of CMFs, which may affect biomass recalcitrance to conversion (Xiao et al., 2016a; Zhang et al., 2019b; Du et al., 2020). Unraveling the mechanisms by which CMFs are organized and deposited will lead to some novel aspects of plant cell wall functions as well as novel strategies for more effective utilization of cell wall polysaccharides. Such knowledge is particularly scarce for wood cell walls.

## Hemicelluloses

Hemicelluloses represent roughly one-third of the plant cell wall biomass and encompass xylans, mannans, XyG, and mixed-linkage glucans. While their backbones share β-1,4 glycosidic linkage similar to cellulose, their monosaccharide composition and linkage pattern vary. Most of the major enzymes involved in backbone synthesis, side-chain addition, and modifications have been identified. In this review, we focused on the progress made in xylans and mannans in SCWs first and then the PCW XyG.

### *Xylan structure and biosynthesis*

Xylans have universal β-1,4-linked xylosyl backbone and heterogeneous substituents. Xylans are usually substituted with 3-*O*-linked arabinofuranosyl (Ara*f*) or 2,3-di-*O*-linked Ara*f* residues (Figure 4A), called arabinoxylan (AX). AX with infrequent 2-*O*-linked glucuronic acid (GlcA) or methyl GlcA (MeGlcA) side chains is termed glucuronoarabinoxylan (GAX) or arabinoglucuronoxylan (Figure 4A) (Smith et al., 2017). AX and GAX are the major hemicelluloses in monocot grasses (Pauly et al., 2013; Wang et al., 2019c; Tryfona et al., 2019; Pellny et al., 2020). In eudicots, xylans are often substituted with GlcA with varied methylesterification, called glucuronoxylan (GX) (Figure 4B), which is the main type of hemicellulose for up to 15%–30% of the wood, while mannan only makes up 2%–5% of the wood (Jacobs et al., 2001; Scheller and Ulvskov, 2010; Yang et al., 2022). In gymnosperm tree species, arabinoglucuronoxylan and galactoglucomannan (GGM; Figure 4C; Mannans) account for 5%–15% and 15%–30% of the wood, respectively (Scheller and Ulvskov, 2010; Oinonen et al., 2015; Park and Cosgrove, 2015; Zheng et al., 2023). One important feature of xylans in gymnosperms, dicots, and some monocots is the presence of a reducing end sequence (RES): β-D-xylose-(1→3)-α-L-rhamnose-(1→2)-α-D-galacturonic acid-(1→4)-D-xylose (Figure 4B) (Zeng et al., 2010; Rennie and Scheller, 2014). This unique RES structure was postulated as an initiator for xylan synthesis or a signal to stop xylan elongation (Pena et al., 2007; York and O'Neill, 2008).

Xylans are important interlocking polysaccharides in several plant SCWs (Simmons et al., 2016; Zhao et al., 2021a; Duan et al., 2021). Xylans often display a two-fold helical screw conformation to bind cellulose and fold into a three-fold or distorted two-fold helical screw conformation to interact with lignin (Simmons et al., 2016; Kang et al., 2019). But some of the two-fold xylan and cellulose domains are forced to contact lignin in multiple hardwood and softwood species, probably due to a molecular crowding effect (Kirui et al., 2022). Exceptionally, mainly three-fold screw xylans are anchored to amorphous cellulose in sorghum (Gao et al., 2020). Xylan substitution/modification patterns are the major determinants of xylan folding (Busse-Wicher et al., 2016; Grantham et al., 2017; Zhang et al., 2019b). In particular, the acetylation patterns on the xylan side chain facilitate the formation of two- and three-fold conformers that bind to varied cell wall polymers, affecting the integrity of the SCW matrix and therefore the wall's mechanical strength (Zhang et al., 2019b). More importantly, novel xylan-based nanocompartments have been found in xylem vessel walls in rice and *Arabidopsis*, needed for sustaining cell wall cohesion and water/nutrient conduction throughout the plant body (Wang et al., 2022a). These wall integrity and physiological functions mediated by xylans are key factors affecting plant growth and development as well as wall characteristics for biomass conversion efficiency. These xylan functions need to be explored for forest tree species.

Shaping xylan structure requires the activities of tens of proteins (Smith et al., 2017; Ye and Zhong, 2022). The machinery underpinning the precise biosynthesis of diverse xylan structures has yet to be fully elucidated. Xylan is proposed to be synthesized by protein complexes, termed xylan synthase complexes, which may harbor the core component, like irregular xylem 10 (IRX10) for backbone polymerization, and other synergistic proteins for substitutions and modifications. It has also been proposed that IRX10 protein interacts with the membrane-bound GT43, IRX9, and IRX14 to form a membrane-bound complex for xylan backbone synthesis in the Golgi apparatus, where IRX9 and IRX14 may synergistically stimulate the activity of IRX10 (Zeng et al., 2016).

Xylan backbone biosynthesis in *Populus* also relies on GT members, as GT43 member genes can mediate alterations in xylan content and the ratio of xylose residues to RES, with the largest effect by the *IRX9* and *IRX14* members (Zhou et al., 2007; Ratke et al., 2018). Similarly, in *Populus*, GT47 and GT8 members and PARVUS also regulate xylan content (Zhou et al., 2007; Lee et al., 2009; Biswal et al., 2015, 2018).

Xylans are also highly acetylated, with *O*-acetyl groups often located at the *O*-2/*O*-3 or both of the xylosyl backbone and *O*-2 of the Ara*f* side chains, resulting in unique patterns of substituents and modifications (Figure 4A and 4B) (Ishii, 1991; Pauly et al., 2013). In *Arabidopsis*, acetyl modification and GlcA substitution are usually positioned on a xylosyl backbone with even residue spaces in most cases (Bromley et al., 2013). The even spaced acetyl decoration also exists in gymnosperms xylans (Busse-Wicher et al., 2016), suggesting that such acetylation is conserved in plants. Nine TBL (trichome birefringence-like) proteins have been demonstrated to be acetyltransferases catalyzing the regiospecific acetylation of xylan in *Arabidopsis* (Yuan et al., 2016; Zhong et al., 2017). *TBL29*, also called Xylan *O*-acetyltransferase 1, was the first gene reported and confirmed to encode acetyltransferase function in xylan acetylation (Xiong et al., 2013; Yuan et al., 2013; Urbanowicz et al., 2014; Lunin et al., 2020). In *Populus*, 12 PtrXOATs are

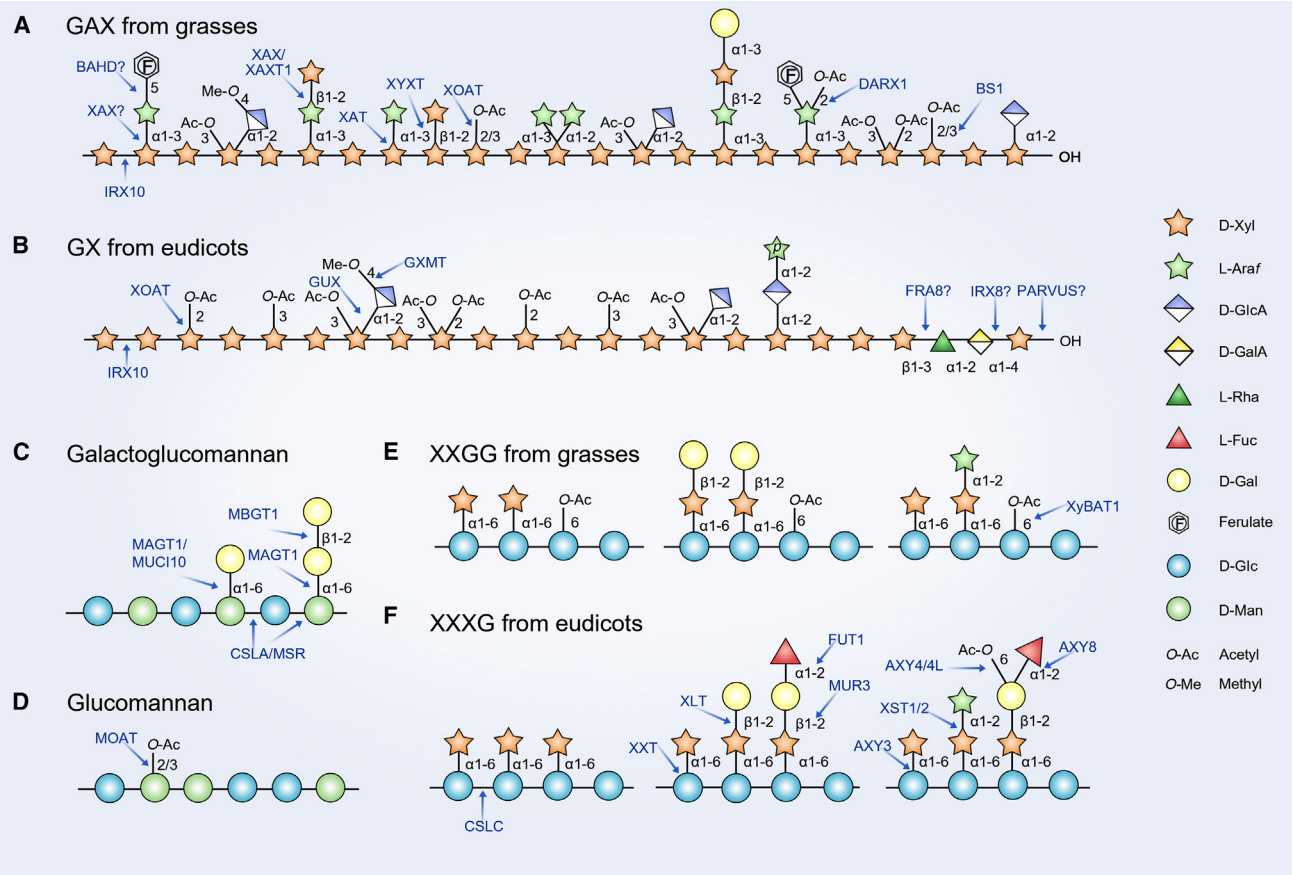


**Figure 4. Structural features of hemicelluloses.**
**(A)** GAX from grasses.
**(B)** GX from eudicots.
**(C)** GGM.
**(D)** Glucomannan.
**(E)** XXGG from grasses.
**(F)** XXXG from eudicots. Enzymes involved in these polysaccharides synthesis or hydrolysis are indicated. Question marks indicate enzymes that have yet to be identified or biochemically verified.
Xyl, xylose; Ara*f*, arabinofuranose; GlcA, glucuronic acid; GalA, galacturonic acid; Rha, rhamnose; Fuc, fucose; Gal, galactose; XOAT, xylan *O*-acetyltransferase; IRX, irregular xylem; XSC, xylan synthase complex; GUX, glucuronic acid substitution of xylan; GXMT, glucuronoxylan methyltransferase; BAHD, acyltransferase; XAX1, xylosyl arabinosyl substitution of xylan 1; XAXT1, xylan arabinosyl 2-*O*-xylosyltransferase 1; FRA8, fragile fiber 8; XAT, xylan arabinosyltransferase; XYXT, xylan β-1,2-xylosyltransferase. DARX1, deacetylase on arabinosyl side chain of xylan 1; BS1, brittle leaf sheath 1; CSLC/A, cellulose synthase like; XXT, xyloglucan xylosyltransferase; XLT, XyG β-glycosyltransferases; FUT, fucosyl transferase; AXY, altered xyloglucan; XST, Xyloglucan "S" side chain transferase 1; MOAT, mannan *O*-acetyltransferase; XyBAT, MSR, mannan synthesis related; GMGT, galactomannan GalT; MAGT, mannan α galactosyl transferase 1; MBGT, mannan β-galactosyltransferase.

putative enzymes involved in xylan acetylation (Figure 4B) (Zhong et al., 2018b). Research on acetylation of xylan in woody plants is still in its initial stage.

Five GlcA substitution of xylan (GUX) proteins catalyze the attachment of GlcA to the xylan backbone in *Arabidopsis* (Figure 4B) (Mortimer et al., 2010; Lee et al., 2012a; Rennie et al., 2012; Bromley et al., 2013). PgGUX proteins were identified in Norway spruce (*Picea glauca*), catalyzing different patterns of GlcA branching (Lyczakowski et al., 2017, 2021). In gymnosperms, three GX methyltransferases (GXM1/2/3) were found to mediate the formation of MeGlcA (Lee et al., 2012b; Kulkarni et al., 2012), and the frequency of MeGlcA is high in Norway spruce, larch, and two cypress species, with a space of six xylosyl units or two consecutive xylosyl residues (Yamasaki et al., 2011; Martinez-Abad et al., 2017). In *Populus*, the majority of the xylan GlcA side chains are 4-*O* methylated, and PtrGXM1–PtrGXM4 are responsible for this process (Lee et al., 2012b; Yuan et al., 2014; Song et al., 2016). A xylan backbone with α-1,3 arabinosyl substitution was found in grasses (Anders et al., 2012), but it has not been reported for woody plants.

### *Mannan structure and biosynthesis*

Mannans, including homomannan, glucomannan, galactomannan, and GGM, are the other types of SCW hemicelluloses. Homomannan's backbone contains only β-1,4-linked mannosyl (Man) residues and is the major hemicellulose in lower plants, such as certain algae, mosses, and ferns. In vascular plants, the mannan backbone is heterogeneous and composed of β-1,4-linked Glc and Man residues, with the Glc-to-Man ratio

ranging from 1:2 to 1:4 (Figure 4C and 4D) (Yu et al., 2018). Gymnosperm and angiosperm SCWs contain about 12%–18% and 3%–4% mannans, respectively. Being matrix polysaccharides, GGMs and glucomannans are believed to act as the cross-linking tethers between CMFs like xylans do. Solid-state NMR analysis of the Norway spruce further revealed that GGM and xylan can coat the same CMFs (Terrett et al., 2019). GGMs in gymnosperm wood SCWs may have similar functions as xylans (Wang et al., 2022a).

The backbone of mannans is catalyzed by CESA-like A (CslA) members (Liepman et al., 2005). Different CslA members have varied enzymatic activities and expression profiles, as modular domain swaps of CslAs fine-tune the activities of β-mannan synthase (Robert et al., 2021). Mannan synthesis-related (MSR) proteins (Figure 4C) can also facilitate mannan biosynthesis (Wang et al., 2013a; 2013b; Voiniciuc et al., 2019). Four *Arabidopsis AtCslA2/3/7/9* genes encode β-mannan synthases (Liepman et al., 2005; Goubet et al., 2009; Voiniciuc et al., 2019). Homologous proteins have been identified in loblolly pine (*P. taeda*) (Liepman et al., 2007). In *P. trichocarpa*, *PtCslA1/3/5* have been found specifically expressed in xylem, with *PtCslA5* being the highest expressed *CSL* gene in wood formation (Suzuki et al., 2006). *PtCslA1* and *PtCslA3* displayed mannan synthase activities, and *PtCslA1* further encodes a glucomannan synthase for the synthesis of (1→4)-β-D-glucomannan, but PtCslA5 had no *in vitro* activity with the substrates for xylan or glucomannan (Suzuki et al., 2006). Perhaps PtCslA5 may require MSR proteins for mannan and glucomannan synthesis, as CslA activities and glucomannan content are significantly reduced in *msr1* and *msr1msr2* mutants in *Arabidopsis* (Wang et al., 2013a; 2013b).

Mannan branches (Figure 4C) can be added by α-1,6-galactosyltransferases (mannan α-galactosyltransferases) from the GT34 family using uridine diphosphate glucose disodium salt as a donor sugar (Edwards et al., 1999). Mannans are often acetylated (Figure 4D), and several acetyltransferases (DUF231 members TBL23/24/25/26 or the mannan *O*-acetyltransferases MOAT1/2/3/4, respectively) could transfer acetyl groups to the *O*-2 and *O*-3 positions of mannose in *Amorphophallus konjac* (Zhong et al., 2018a). Several MOATs of the DUF231 family in *Selaginella*, loblolly pine (*P. taeda*), Norway spruce, and *P. trichocarpa* are thought to be mannan *O*-2 and *O*-3-acetyltransferases (Zhong et al., 2019). Knowledge of mannan biosynthesis is more advanced than for other hemicelluloses because most related biosynthetic enzymes have been identified, and mannan and glucomannan have been successfully made in *Pichia pastoris* (Suzuki et al., 2006; Voiniciuc et al., 2019).

#### *XyG structures and biosynthesis*

XyG, a major hemicellulose in PCWs, is composed of β-1,4-linked glucosyl backbone, with more than half of the glucosyl residues being substituted with xylosyl residues via α-linkage at the *O*-6 site. Many of these xylosyl residues are further substituted with a β-1,2-D-galactose and α-1,2-L-fucose to form di- or trisaccharide side chains. According to the frequency of xylosyl substituents (X) on the backbone, XyG is divided into XXGG (Figure 4E) and XXXG (Figure 4F) types. XXGG generally exists in grasses and Solanaceae, seedless vascular plants, and bryophytes, whereas XXXG is mainly present in most vascular plants and hornworts (Pauly and Keegstra, 2016). In addition, mono- or di-acetylation of the galactosyl residues on side chains of XXXG have been detected. However, in grass XXGG, acetylation usually occurs at *O*-6 site of glucosyl backbone, in addition to that at the *O*-6 site of the terminal galactose and *O*-5 site of the terminal arabinose on side chains.

XyG was once considered to tether CMFs for strengthening the PCW. However, this model has been questioned because loss of XyG xylosyltransferases (XXTs) in *Arabidopsis* results in the complete absence of XyG but has no obvious effect on cell wall architecture and plant growth (Cavalier et al., 2008). Current studies proposed that the tethering may occur in limited regions, referred to as "biomechanical hotspots," where XyG is trapped in a uniquely structured region of CMFs with expansin proteins closely packed to this "hotspot" (Park and Cosgrove, 2012; Wang et al., 2013a; 2013b). Moreover, the non-covalent cellulose-cellulose interactions, including the sliding between CMFs, have been found recently to be the key factors in regulating the mechanical properties of PCWs, supporting the updated role of XyG in cell wall assembly (Zhang et al., 2021).

The structures of XyG are shaped in the Golgi apparatus through the activities of a series of enzymes. The β-1,4-glucan chain is made by CslC family members, and XXTs are responsible for addition of xylosyl residues onto the backbone, forming the major framework of XyG (Cocuron et al., 2007; Cavalier et al., 2008; Kim et al., 2020b). Then, the GTs add β-D-galactosyl residues and the terminal α-L-fucosyl residues onto xylosyl substituents to form di- or trisaccharide side chains (Perrin et al., 1999; Vanzin et al., 2002). Altered XyG 4 (AXY4) and AXY4L specifically acetylate XyG, while AXY9 and reduced wall acetylation (RWA) proteins may work upstream of AXY4 (Manabe et al., 2013; Schultink et al., 2015). Homologs of these acetyltransferases have also been found in *Populus* (Zhong et al., 2018a). All of those proteins are proposed to form protein complexes, although the evidence is still limited.

## UTILIZATIONS AND FIELD TRIALS

### Cell wall modifications/utilizations and CRISPR-CAS9 technology

Lignocellulosic recalcitrance limits biomass conversion efficiency to materials and biofuel. Its main component, cellulose, is found mostly in the form of microfibrils in wood cell walls and used traditionally for paper products (Sarkanen, 1976; Chiang, 2002). Wood contains approximately 15% recalcitrant cellulose that has high crystallinity and about 30% amorphous cellulose. The highly crystalline cellulose has little effect on wood pulping but is a key barrier to bioconversion and processes to produce high-yield/quality cellulose nanomaterials (CNMs; including cellulose nanofibrils and cellulose nanocrystals) (D'Acierno et al., 2023). CNMs have all of the unique "green" properties to potentially replace most of the synthetic nanomaterials in various fields, including medical applications, in the near future (D'Acierno et al., 2023). However, work on tailoring cellulose biosynthesis to facilitate bioconversion and CNM production has been far less extensive than that for other cell wall polysaccharides.

Xylan modifications have been the focus for tailoring cell wall polysaccharides to improve biomass conversion efficiency. This is because pentoses, the main component of xylans, are less

fermentable than hexoses, xylan's acetyl groups are toxic to microbes, and ferulate esters reduce cell wall accessibility (Young et al., 2010). Xylan content reduction in many *Populus* species, through downregulating members of the GT8, GT43, and GT47 families, involved in xylan backbone biosynthesis, improved the efficiency of chemical or biochemical wood saccharification (Lee et al., 2011; Li et al., 2011; Biswal et al., 2015; Ratke et al., 2018). In a hybrid *Populus*, silencing RWA family genes or overexpressing fungal acetyl xylan esterase (AXEs) generated acetyl-reduced wood, a potential feedstock that may confer improved saccharification efficiency (Derba-Maceluch et al., 2020). Increasing xylan-bound *p*-coumarate in *Sorghum bicolor* by overexpressing a rice gene from the BAHD (acyl-CoA dependent acyltransferase) family also improved wall saccharification efficiency (Tian et al., 2021). In rice, CRISPR-Cas9-mediated loss of function in uridine diphosphate-xylose epimerase and xylan arabinosyl-transferase largely reduced xylan arabinose side chains, resulting in increased cell wall saccharification efficiency of the transgenics (Chen et al., 2021b).

Lignin is the key limiting factor for cell wall conversion efficiency because of its chemical nature and compositing effects on other wall polymers. Its modification to improve efficiency for many plant species has been extensively studied and reviewed recently for forest tree species (De Meester et al., 2022a). Here, we briefly outline recent studies of cell wall engineering of lignin in tree species using the CRISPR-Cas technology.

CRISPR-Cas technologies leverage the repurposing of programmable Cas nucleases (Cas9, Cas12, Cas13, and other CRISPR effector proteins) derived from CRISPR-Cas adaptive immune systems (Barrangou et al., 2007) to cleave genes of interest, thus prompting the endogenous DNA repair machinery to generate precise edits at the target sequence. CRISPR-Cas genome editing has been broadly adopted across ~120 plant species (Cardi et al., 2023), but for forest tree species, editing monolignol biosynthetic pathway genes has only been reported for *Populus* spp. Only the CRISPR-Cas9 system was adapted, and the editing was targeted on the *4CL1* (Tsai et al., 2020), *CSE1/2* (de Vries et al., 2021; Jang et al., 2021), and *CCR2* (De Meester et al., 2020) genes, leading to 10%–35% lignin content and lignin S/G ratio reductions. Other than monolignol pathway genes, CRISPR-Cas9 editing in *P. tomentosa* of a laccase gene, *PtoLAC14*, which oxidizes monolignols to initiate their coupling to lignin, also leads to a 7% reduction in lignin (Qin et al., 2020). Loss of function in PHBMT1, which mediates *p*-hydroxybenzoylation of monolignols, improves the acetyl bromide solvent solubility of lignin in *P. tremula* × *P. alba*, whereas overexpression of *PHBMT1* shows the opposite effect (Zhao et al., 2021b). Editing *CSE*, *CCR*, or *LAC*, described above, leads to improved wood saccharification efficiency, but their pulping response is unknown.

Studies of cell wall lignin modification have predominantly centered on the engineering of one or two genes, often neglecting the multigenics-driven metabolic complexity of monolignol biosynthesis. The metabolic complexity of the pathway was investigated using enzyme kinetics and proteomics for 21 enzymes and 24 metabolites in the monolignol biosynthetic pathway of *P. trichocarpa* (Chen et al., 2011, 2014; Wang et al., 2014, 2018; Lin et al., 2015; Yan et al., 2019). These studies revealed quantitatively the intricacies of metabolic regulation within the pathway and underscores the importance of systems-level coordination of enzyme activities in determining the overall metabolic flux and final lignin content and composition, offering a more holistic approach to plant improvement and utilization. Based on these studies, a recent study systematically evaluated 69 123 multigenic editing strategies for 21 lignin biosynthetic genes in *P. trichocarpa* (Sulis et al., 2023). Multiplex editing of up to 6 genes produced 174 edited variants that harbored diverse combinations of genome editing outcomes. A comprehensive technoeconomic and carbon life cycle analysis of CRISPR-edited wood revealed that such wood markedly elevates the kraft pulp yield and production efficiency (Sulis et al., 2023).

Multiplex CRISPR-editing of lignin genes could improve the economic viability and environmental sustainability of cellulosic fiber production. The approach could augment fiber productivity by as much as 40% with reduced use of harmful pulping chemicals as well as pulp mill energy consumption by up to 20% in many unit operations, particularly that for the recovery boiler (Sulis et al., 2023). Despite the promising results, plants engineered for cell wall or other trait modifications must be verified for their application readiness by field tests for growth and environmental adaptation and for large-scale conversion evaluations if used as material and energy feedstock.

## Field trials of cell-wall-engineered forest trees

In this review, we define a field trial as a trial of a single transgenic or mutant line evaluated from a single harvest and a single location. For instance, when a given line was planted at two different locations and evaluated at two different rotations (e.g., 1 and 2 years after planting), then we consider that four field trials were made with that line. To date, 191 field trials (durations from 1 to 8 years) with cell-wall-engineered plants have been reported that include mainly *Populus* spp. and their hybrids and a few cases for eucalyptus and loblolly pine (*cad* mutant) (Figure 5; Supplemental Table 4).

Approximately 80% of the field trials were conducted with lignin-engineered trees (De Meester et al., 2022b), and approximately half of these were trials (75 cases) with *4CL*-downregulated lines (see gene names in the Figure 3 legend) (Figure 5; Supplemental Table 4). The other 77 field trials with lignin-engineered transgenics were generated through downregulation or CRISPR-Cas knockout of other monolignol pathway genes (*C4H*, *C3′H*, *CSE*, *CCoAOMT*, *CCR*, *COMT*, and *CAD*), a TF (*LFT1*), and a microRNA (*miR408*) (Supplemental Table 4). Overall, these transgenics had reduced lignin, with some also having modified S/G ratios, incorporation of monolignol derivatives, or improved response to specific chemical treatments. Field trials revealed that lignin reduction in greenhouse-grown transgenics is generally more pronounced than in the corresponding transgenics grown in the field. In addition, consecutive harvests demonstrated that lignin reduction became less pronounced when trees become older (Stout et al., 2014; De Meester et al., 2022b). CRISPR-based gene manipulation has been suggested as a strategy for maintaining the stability of lignin properties in field-grown trees (Anders et al., 2023). Factors underlying the discrepancies between field- and greenhouse-grown transgenics are numerous; however, field-grown trees are the ultimate objective, whereas greenhouse-grown plants are efficient proof-of-concept tools, particularly for tree species.

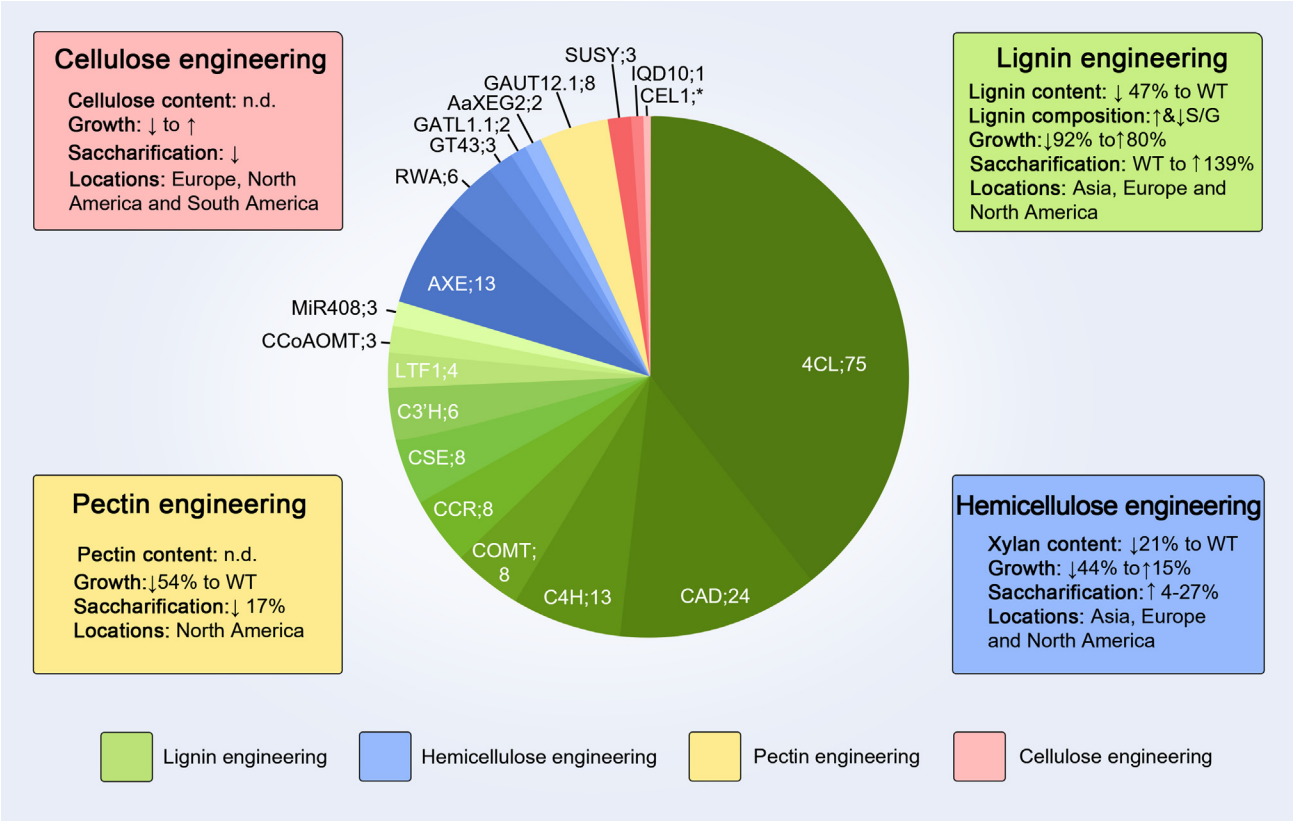


**Figure 5. Overview of field trials of cell-wall-engineered forest trees and a summary of the main findings.**
The graph depicts four groups, each representing a cell wall component. Within these groups, field trials are segmented per gene, with the corresponding number of field trial specified. *, number of field trials unknown; green, lignin-engineered trees w field trials); blue, hemicellulose-engineered trees (26 field trials); yellow, pectin-engineered trees (8 field trials); red, cellulose-engineered trees (5 field trials); C4H, cinnamate 4-hydroxylase; C3′H, coumarate 3-hydroxylase; CSE, caffeoyl shikimate esterase; CCR, cinnamoyl-CoA reductase; CCoAOMT, caffeoyl-CoA *O*-methyltransferase; COMT, caffeic acid *O*-methyltransferase; CAD, cinnamyl alcohol dehydrogenase; LFT1, lignin biosynthesis-related transcription factor 1; miR408, microRNA; AXE, acetyl xylan esterase; RWA, reduced wall acetylation; GT43, xylosyltransferase; GATL1.1, glycosyltransferase; AaXEG2, *Aspergillus* xyloglucanase; GAUT12.1, galacturonosyltransferase 12; SuSy, sucrose synthase; IQD, isoleucine/glutamine 67 domain 10; CEL1, β-endoglucanase. The values in the boxes represent the most extreme modifications achieved.

Trees with altered hemicellulose content and/or composition account for 13.5% of the trials (Figure 5; Supplemental Table 4), dominated by xylan acetylation modifications for the reasons explained above. These modifications included downregulating *RWA* (see Xylan structure and biosynthesis) or overexpressing fungal AXEs. Xylan deacetylation improved wood saccharification efficiency with or without retarded plant growth (Supplemental Table 4). A modified xylan backbone and its RES (Figure 4B) were also tested and found effective in improving biomass saccharification without compromising biomass yield. Chain length reduction in XyG, a major hemicellulose in PCW, resulted in reduced plant growth, but data on cell wall composition and saccharification are not yet available.

About 2.5% of field trials were trees with modified expression of genes associated with cellulose biosynthesis (Figure 5; Supplemental Table 4). The approaches resulted in either increased wood production or enhanced enzymatic saccharification, sometimes causing a dwarfed phenotype. Trees with modified pectin, a minor wood component, constituted about 4% of the field trials (Figure 5; Supplemental Table 4). Wood from field-grown trees overexpressing the pectin biosynthesis gene *GAUT* had a reduced saccharification efficiency, but tree growth was retarded.

In general, these field trials validated that lignin content reduction would improve biomass processing efficiency, but a strong lignin reduction often retards plant growth with less biomass production. Tuning the extent of downregulation is one strategy to minimize the yield penalty. Data on the adaptation of these field-grown transgenics to biotic and abiotic stresses are lacking.

## CONCLUDING REMARKS AND PERSPECTIVES

Progress in plant cell wall research has revealed much about the differentiation of cell types, their morphology/organization, wall deposition, and component biosynthesis and the complexity of the underlying regulations converging on molecular interactions at the levels of genetic, epigenetic, protein/enzyme, hormonal, and environmental signaling pathways. Despite these insights, a key question remains. What is the SVM system, and how does it work to allow continued lateral growth to form wood in

forest trees? The system, the identity or location of the system's central players, the wood stem cells, and regulatory genes controlling stem cell self-renewal and differentiation remain speculative. Finding answers to these questions may now become feasible using advanced technologies, such as single-cell and spatial multiomics (also known as multimodal omics) (Vandereyken et al., 2023), to integrate information from genome-wide CRISPR mutagenesis and activation of putative SVM system genes in a forest species with a well-annotated genome. These insights are necessary to better understand which regulatory factors determine and maintain cell fate (e.g., vessel vs. fiber) and the deposition of specific wall components across different cell types. Single-cell multimodal omics (Vandereyken et al., 2023) for forest tree species are tenable because of the recent establishment of single-cell transcriptomics for *Populus* spp. (Chen et al., 2021c; Li et al., 2021, 2023; Xie et al., 2022; Tung et al., 2023).

CRISPR technologies for gene editing in forestry species have been expanded to transcriptional and epigenetic alterations using deactivated nucleases like dCas9 fused with activators, repressors, or epigenetic modifiers (Nakamura et al., 2021; Pan et al., 2021). However, the transgenesis efficiency of all these CRISPR technologies in forestry species needs to be improved to enable effective gene perturbation, particularly at large scale for multilevel regulatory functions. Transgenesis efficiency has been a major obstacle to advancing our knowledge of tree biology. No genetic function of any gymnosperm gene has ever been demonstrated *in vivo* because a robust transformation and whole-plant regeneration are still lacking for any gymnosperm species. The absence of the transformation system greatly limits our understanding of evolutionary control in speciation and other genetic and biological processes for adaptive traits. Genetic engineering of gymnosperm wood would revolutionize the feedstock properties for more novel bioproducts or the design of sink attribution for carbon sequestration.

Despite trees being recalcitrant to genetic transformation, knowledge of cell wall formation and engineered modifications in forest trees has advanced substantially since the first successful case in 1987 in *Populus* (Fillatti et al., 1987). Much of the knowledge was derived from greenhouse results of manipulating single genes or gene families, revealing that cell wall or wood trait modifications may result in pleiotropic phenotypes, such as compromised growth. Wide-ranging pleiotropic effects were observed when modifications were made through perturbing TFs with unknown regulatory specificity (Table 1 and Supplemental Tables 1–3). Computational models have been developed to predict how one trait can be genetically improved without adversely affecting others or how specific advantageous traits can be improved simultaneously (Wang et al., 2018, 2019b; Matthews et al., 2021; Sulis et al., 2023). These models were developed using lab or greenhouse data. However, evidence from increasing numbers of field trails of transgenic trees (Figure 5; Supplemental Table 4) led to the conclusion that greenhouse data could not be precisely reproduced by field experiments. The early lab-based predictive models need to be reassessed using data from field-grown trees. Lab and greenhouse data are necessary for fundamental research that relies on defined experimental settings and are useful for guiding the selection of promising tree lines for field tests for commercialization or forestation.

Field tests of genetically engineered trees (Supplemental Table 4) are a tremendously valuable and perpetual source of knowledge ranging from fundamental aspects of tree biology to cell wall and wood utilization. Nonetheless, for utilization, the currently reported field trials for cell-wall biomass conversion focus mainly on chemical or enzymatic saccharification for biofuel production (Supplemental Table 4). Wood as feedstock for biofuels has not yet been commercialized anywhere in the world. Modified wood should be tested more for existing industrial production, such as pulp/paper, timber, and construction materials. Field trials of transgenic trees have demonstrated the existence of a complex tradeoff between cell wall conversion process efficiency, cell wall component content/properties, and plant growth and adaptation, a complex tradeoff that is not possible to be discerned by greenhouse experiments. These field-grown transgenics are a unique biological system for establishing a predictive model, as done previously (Wang et al., 2018, 2019b; Matthews et al., 2021; Sulis et al., 2023), integrating multidimensional molecular insights with technoeconomic analysis of the conversion process to generate new genetic engineering strategies that would help decide the extent and direction of the complex tradeoff.

## SUPPLEMENTAL INFORMATION

Supplemental information is available at *Molecular Plant Online*.

## FUNDING

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## AUTHOR CONTRIBUTIONS

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