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Review

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Grass secondary cell walls, *Brachypodium distachyon* as a model for discovery

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Summary

A key aspect of plant growth is the synthesis and deposition of cell walls. In specific tissues and cell types including xylem and fibre, a thick secondary wall comprised of cellulose, hemicellulose and lignin is deposited. Secondary cell walls provide a physical barrier that protects plants from pathogens, promotes tolerance to abiotic stresses and fortifies cells to withstand the forces associated with water transport and the physical weight of plant structures. Grasses have numerous cell wall features that are distinct from eudicots and other plants. Study of the model species *Brachypodium distachyon* as well as other grasses has revealed numerous features of the grass cell wall. These include the characterisation of xylosyl and arabinosyltransferases, a mixed-linkage glucan synthase and hydroxycinnamate acyltransferases. Perhaps the most fertile area for discovery has been the formation of lignins, including the identification of novel substrates and enzyme activities towards the synthesis of monolignols. Other enzymes function as polymerising agents or transferases that modify lignins and facilitate interactions with polysaccharides. The regulatory aspects of cell wall biosynthesis are largely overlapping with those of eudicots, but salient differences among species have been resolved that begin to identify the determinants that define grass cell walls.

*These authors contributed equally to this work.

I. Introduction to the secondary cell wall

The secondary plant cell wall provides mechanical strength that allows plants to stand upright, resist pest and pathogen invasion, and transport water over long distances. Both plants and humans have found this abundant matrix of cross-linked polymers useful as durable building material, with timber featuring in human construction around the world for generations. The secondary wall is distinct from other cell wall types in composition as well as the developmental timing and tissue types where it is deposited. Secondary walls form in thick layers, rich in cellulose, hemicelluloses and lignin. Cellulose microfibrils have a tensile strength rivalling steel, and form crystalline structures. Hemicelluloses include a variety of polysaccharides but, in grass secondary walls, these are mostly mixed-linkage glucans and heteroxylans, a defining aspect of this plant lineage. Finally, lignin is a recalcitrant and heterogeneous mixture of randomly polymerised phenolic monolignols that is interspersed and cross-linked with wall polysaccharide polymers. Lignification is a hallmark of secondary walls, and unique chemistry and synthesis of this polymer continues to be uncovered (Fig. 1).

Following cell expansion of cells surrounded by a primary wall, secondary walls are deposited in a highly specific spatio-temporal manner in certain cell types over development. Unlike eudicots, grass stem growth is a result of iterative division and elongation events from stacked intercalary meristems called nodes (Esau, 1977; Langer, 1979). New cells generated from the node elongate, pushing up the nodes above with the final node transitioning to the flowering meristem. Thus, the internode regions are most mature at the bottom of the stem, while cells within an internode are most mature at the top of that region, just before the next node (Langer, 1979). Secondary wall deposition occurs between cell elongation and senescence, with cellulose, lignin and hemicellulose content increasing with maturity (Rancour *et al.*, 2012; Matos *et al.*, 2013; Kapp *et al.*, 2015). Grass stems account for the majority of secondary wall-forming sclerenchyma tissues. The interfascicular fibres develop thick secondary walls and provide mechanical strength for the upright stem. Grasses form discrete vasculature with the xylem and phloem contained by bundle sheath cells, unlike eudicots in which a cambium separates colateral xylem and phloem (Fig. 2). Depending on the species, stem vascular bundles can be arranged in peripheral rings or dispersed throughout the stem (Esau, 1977). Xylem develops strong secondary walls that can tolerate the high pressures caused by evapotranspiration. Phloem cells do not have secondary walls. Besides phloem, grass stem

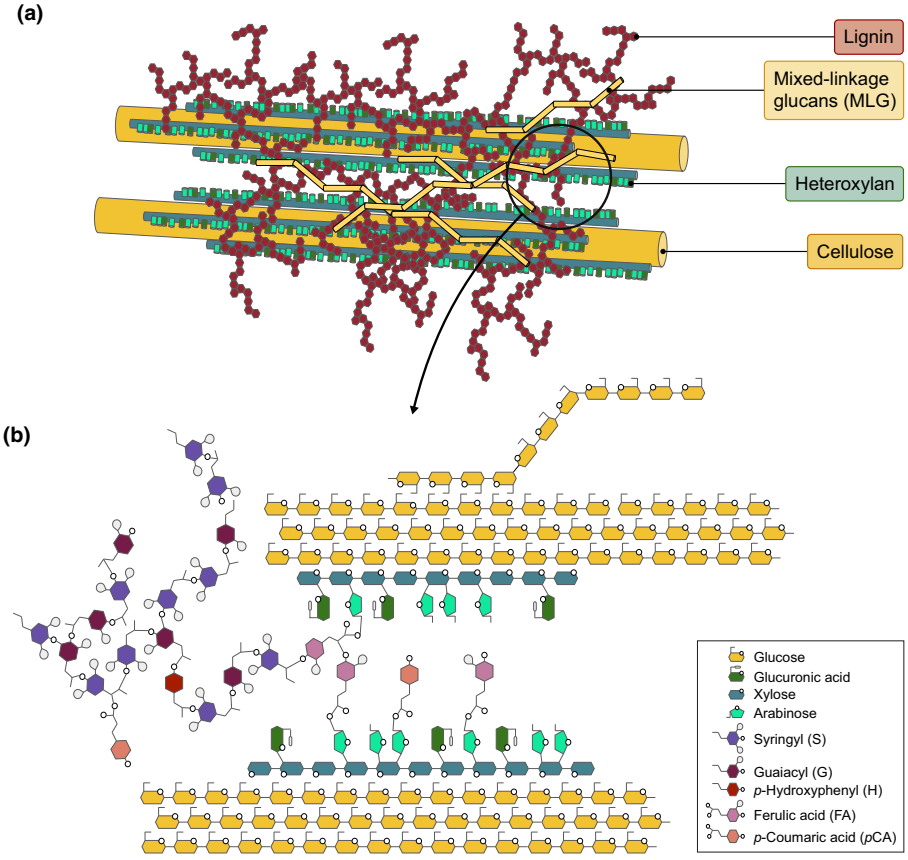

Fig. 1 General schematic of grass secondary cell wall matrix. The grass secondary cell wall matrix is made up of cellulose microfibrils, mixed-linkage glucans, heteroxylans and lignins. (a) Generalised cartoon of grass secondary wall polymer interactions. (b) Schematic fine structure of the circled region in (a). Cellulose microfibrils consist of multiple, organised, β(1,4)-linked glucose chains. Mixed-linkage glucans are also glucose chains, but include β(1,3) linkages. Heteroxylan has a xylose backbone that is decorated with sugar and phenolic side chains of xylose, arabinose, glucuronic acid, and hydroxycinnamates (FA and *p*CA). These polysaccharides can be interwoven with lignins, branched phenolic polymers made of three main lignin units, syringyl (S), guaiacyl (G), and *p*-hydroxyphenyl (H). Lignins can also contain ferulic and *p*-coumaric acids.

parenchyma tissue can be found in the pith and in cortex pockets, which have been shown to function as carbon storage tissues during development (Jensen & Wilkerson, 2017). While this review focuses on secondary cell walls in *Brachypodium distachyon* and other grasses, wall synthesis has also been investigated using *B. distachyon* as a model system for callus tissue, young vegetative growth, and endosperm development (Christensen *et al.*, 2010; Guillon *et al.*, 2011; Liu *et al.*, 2016; Betekhtin *et al.*, 2018; Francin-Allami *et al.*, 2019).

II. *Brachypodium distachyon*, a model grass system

Brachypodium distachyon is a model for cereal crops and temperate grasses because of its small stature, simple growth requirements, short life cycle, relatively small and sequenced genome, and close phylogenetic relation to those species (Scholthof *et al.*, 2018). *Brachypodium distachyon* has a 'finished' reference genome with the only ambiguity being the placement of some centromeric repeats (https://phytozome-next.jgi.doe.gov/info/Bdistachyon_v3_1). In addition, there is a growing atlas of gene expression profiles (Trabucco *et al.*, 2013; Sibout *et al.*, 2017; MacKinnon *et al.*, 2020). It is also remarkable in terms of the resources available for experimental molecular genetics. Genetic transformation protocols are well developed; current efficiency makes *B. distachyon* a grass highly amenable to transformation (Bragg *et al.*, 2012). Mutant resources consist of 23 000 T-DNA mutants and 1200 sequenced chemical mutants (Bragg *et al.*, 2012; Granier *et al.*, 2015). Given that these mutations are more-or-less randomly distributed across the genome and chemical mutagenesis typically induces multiple mutations per mutant line, this large collection is likely to include loss-of-function mutations in the majority of *B. distachyon* genes and multiple nonsynonymous mutations in virtually every gene (Dalmais *et al.*, 2013). This latter category of mutations may be particularly interesting because it can help to elucidate the function of cell wall genes, as well as the importance of specific amino acids and protein domains, information that cannot be inferred from knockout mutants. A large natural variation population exists for *B. distachyon*, with sequenced genomes for many accessions. These resources have been applied in several studies on growth- and biomass-related traits (Lee *et al.*, 2012; Tyler *et al.*, 2014; Kapp *et al.*, 2015; Gordon *et al.*, 2017) Thus, *B. distachyon* is well positioned for gaining fundamental insights into cell wall biosynthesis (Coomey & Hazen, 2015). This knowledge can then be leveraged for agronomic gains in more experimentally recalcitrant grass species.

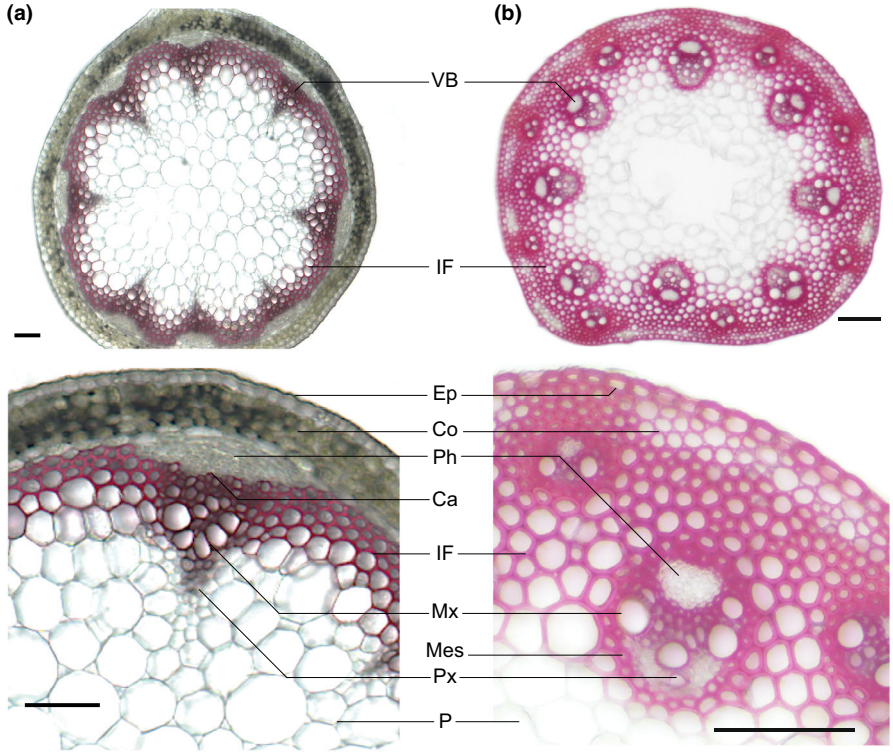

Fig. 2 Transverse section of *Brachypodium distachyon* and *Arabidopsis thaliana* stems. Transverse stem cross-sections of *A. thaliana* (a) and *B. distachyon* (b) stained with phloroglucinol-HCl, a general stain for lignified tissues. Most eudicots, such as *A. thaliana*, have vascular bundles of xylem separated from phloem by cambium layers, and flanked by interfascicular fibres. In *B. distachyon*, the stem vascular bundles also contain xylem and phloem, but there is no cambial layer, and the vasculature is encased by a lignified bundle sheath layer of mestome cells and surrounded by interfascicular fibre cells. In both species, a cortex region of less lignified cells separates the interfascicular region from the epidermis. VB, vascular bundles; Ep, epidermis; Co, cortex; Ca, cambium area; IF, interfascicular fibres; Ph, phloem; Mes, mestome; Mx, metaxylem; Px, protoxylem; P, parenchyma. Bars, 100 μm.

III. Cellulose

Cellulose is perhaps the most abundant polymer in the world, found in the walls of every plant cell. It is made of (1,4)-β-linked glucose monomers, and these glucan chains are synthesised at the plasma membrane by the cellulose synthase complex (Fig. 1). Extruded cellulose chains form organised microfibrils with crystalline structure; the degree of this organisation impacts wall mechanics, with greater crystallinity resulting in stiffer walls.

The cellulose synthase complex consists of multiple Cellulose Synthase A (CesA) subunits and associated proteins (Pear *et al.*, 1996; Polko & Kieber, 2019). *CesA* genes are a subclade of the cellulose synthase superfamily, along with the *Cellulose Synthase-like* (*Csl*) clades. Across plant species, seven major lineages have been identified in the *CesA* genes, which separate into the *CesA*s associated with primary or secondary wall synthesis (Little *et al.*, 2018). This distinction between primary and secondary wall synthesis is conserved across most vascular plants. In *B. distachyon*, *BdCesA4*, *7*, and *8* have been shown to function in secondary wall synthesis, and these proteins are highly similar to those characterised in other species for secondary wall function, such as *Arabidopsis thaliana* and rice (*Oryza sativa*) (Handakumbura *et al.*, 2013). In *B. distachyon*, loss-of-function in the secondary CesAs results in reduced crystalline cellulose content, compromised wall integrity, and reduced plant growth (Handakumbura *et al.*, 2013; Petrik *et al.*, 2016). Interestingly, the secondary *CesA* lineage contains a Poacea-specific clade, which in *B. distachyon* is represented by *BdCesA10*. This CesA10 group does not contain the canonical UDP-glucose binding motif (D,D,D,QxxRW) found in glucosyltransferases (Handakumbura *et al.*, 2013). While phylogenetic analysis clearly places these proteins in the CesA clade, it is not clear what role they play, if any, in cell wall synthesis.

Mutants in maize (*Zea mays*), barley (*Hordeum vulgare*), and rice with defects in cellulose synthesis have been identified through brittle stem phenotypes, aptly named brittle stalk, fragile stem, and brittle culm respectively (Tanaka *et al.*, 2003; Sindhu *et al.*, 2007; Burton *et al.*, 2010b; Kotake *et al.*, 2011). These mutants have been mapped both to genes encoding CesAs and other associated proteins, such as the COBRA-like family of glycosylphosphatidylinositol anchored proteins. While the precise function of these anchored proteins is not fully understood, they may play a role in properly orienting cellulose synthesis.

Cellulose synthase complex dynamics have been studied primarily in *A. thaliana*, but recent work in *B. distachyon* has added to our understanding of the conserved functions of this system. The complex moves along cortical microtubules, depositing cellulose microfibrils perpendicular to the axis of elongation (Paredez *et al.*, 2006). This has been observed in real time for primary CesAs in both *A. thaliana* and *B. distachyon*, which showed similar speeds in *B. distachyon* mesocotyl and root, as in *A. thaliana* hypocotyl. This motility was not affected by latrunculin B treatment, which destabilized actin filaments, but was dampened in both species when microtubules were disrupted (Liu *et al.*, 2017). Missense mutation in *Bdcesa1*, a primary wall cellulose synthase, showed reduced cellulose content and crystallinity, as do *A. thaliana AtcesA1* mutants (Arioli *et al.*, 1998; Persson *et al.*, 2007; Brabham *et al.*, 2019). Unlike *Atcesa1* mutants, *Bdcesa1* did not show reduced plant height. Rather, the *Bdcesa1* mutant had more internodes, giving rise to a plant with normal height despite reduced cellular elongation from compromised cellulose synthesis (Brabham *et al.*, 2019). Overall, the process of cellulose biosynthesis appears to be somewhat conserved between eudicots and grasses.

IV. Mixed-linkage glucans

One of the salient differences that defines grass secondary cell walls is the composition and utilization of noncellulosic polysaccharides. These can generally be thought of as pectins and hemicellulose, but discussion of these polymers is often better suited to classification by backbone structure (Scheller & Ulvskov, 2010; Atmodjo *et al.*, 2013). In eudicots, the predominant polysaccharide polymer after cellulose is xyloglucans, (1,4)-β-linked glucose chains that contain numerous 1–6 xylose substitutions. The xylose side chains can be further decorated with other sugars such as galactose or fucose (Bauer *et al.*, 1973; Fry, 1989; Scheller & Ulvskov, 2010). In grasses, the role of xyloglucans is largely replaced by mixed-linkage glucans (MLGs) and heteroxylans.

Mixed-linkage glucans are, as their name suggests, (1,4)-β-linked glucose chains that are interrupted with (1,3)-β linkages (Figs 1, 3). (1,3)-β-glucans are typically separated either by two or three (1,4)-β-glucans, forming oligosaccharide units of β-cellotriosyl or β-cellotetraosyl (Fig. 3), although longer chains of (1,4)-β-glucans are also observed (Bulone *et al.*, 2019). Almost no evidence of adjacent (1,3)-β-glucan bonds has been found (Buliga *et al.*, 1986). These altered linkages result in the polymer having kinks and bends, unlike the linear glucan chains that form cellulose. As a result, MLG does not form crystalline structures. The relative amounts of β-cellotriosyl and β-cellotetraosyl units strongly relate to the solubility of the overall polymer and are expressed as ratios of degrees of polymerization of trisaccharides and tetrasaccharides (DP3 : DP4). Solubility of the polymer decreases at either end of the ratio spectrum. Longer stretches of either β-cellotriosyl or β-cellotetraosyl units increases the overall order of the polymer with more undisturbed regions of (1,4)-β-glucan linkages, and thus decreases solubility. Greater solubility occurs with DP3 : DP4 ratios that range from 1 : 1 to 2.5 : 1 (Lazaridou & Biliaderis, 2007; Burton *et al.*, 2010a).

Mixed-linkage glucans were once thought to be unique to grass cell walls, but several examples have now been observed outside of the commelinid monocots, and indeed outside of green plants. Polysaccharides containing (1,3;1,4)-β-glucans have been observed in green, red and brown algae, lichens, fungi, bryophytes, and the monophyletic genus *Equisetum* (Bulone *et al.*, 2019). Genomic data further support the idea that MLGs are not specific to the Poaceae, with enzymes capable of synthesizing (1,3;1,4)-β-glucan linkages identified across monocots and in isolated cases in other species. MLG has been shown to be synthesized by members of the *CslF*, *CslH* and *CslJ* families (Bulone *et al.*, 2019). All three of these groups have co-evolved independently in monocots from sister Csl clades (Little *et al.*, 2018). Members of *CslF/H/J* clades have been

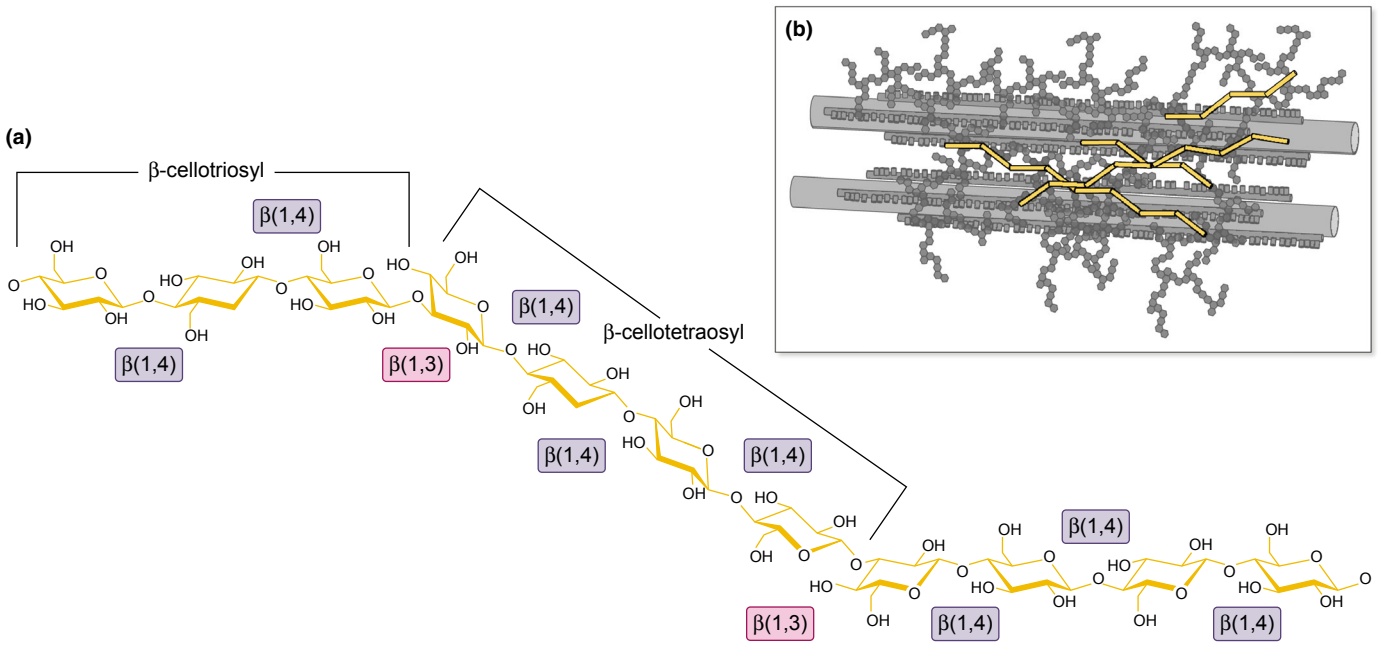

Fig. 3 Mixed-linkage glucan structure. (a) Fine structure of mixed-linkage glucan. Glucose monomers (yellow) linked by β(1,4) bonds (purple) are occasionally interrupted by β(1,3) linkages (pink). The β(1,3) bonds do not occur sequentially, but rather separate (1,4)-β-glucans into β-cellotriosyl or β-cellotetraosyl segments. The relative degree of β-cellotriosyl to β-cellotetraosyl units relates to the solubility of the overall polymer. (1,3;1,4)-β-Glucans are synthesised by Cellulose synthase-like F6, a Golgi membrane-bound protein with cytoplasmically active catalytic sites. (b) Miniature of Fig. 1(a) cell wall schematic highlighting the mixed-linkage glucan component.

shown to be capable of synthesizing (1,3;1,4)-β-glucan when heterologously expressed, but it is not clear whether all of these groups are responsible for native MLG synthesis. By far the best characterized enzyme in MLG synthesis is CslF6, which has been studied in barley, wheat (*Triticum aestivum*), rice, maize and B. *distachyon* (Nemeth *et al.*, 2010; Vega-Sanchez *et al.*, 2012; Kim *et al.*, 2015, 2018). BdCslF6 protein is localized to the Golgi membrane, with an external catalytic domain (Kim *et al.*, 2015, 2018). Antibody detection of (1,3;1,4)-β-glucan in maize also supports a Golgi-localized synthesis of MLG (Carpita & McCann, 2010). However, evidence in other grasses suggests that MLG synthesis occurs at the plasma membrane. In barley and wheat, antibody detection of MLG showed localization at the plasma membrane and cell wall, as did antibody detection of HvCslF6 and TaCslF6 (Trethewey & Harris, 2002; Trethewey *et al.*, 2005; Wilson *et al.*, 2006, 2015). The N-terminal region of the CslF6 protein in barley, maize, and sorghum (*Sorghum bicolor*) influences total MLG synthesis activity and the C-terminal region appears to influence the ratio of DP3 : DP4 linkages (Jobling, 2015; Dimitroff *et al.*, 2016).

The evolution of MLG appears to have been followed by the evolution of hydrolytic enzymes specific to (1,3;1,4)-β-glucan polymers (Høj & Fincher, 1995; Fincher, 2009). Both (1,4)-β-glucan and (1,3)-β-glucan endohydrolases exist across land plant lineages, capable of cleaving (1,4)-β-glucan bonds in both cellulose and MLG. Specific (1,3;1,4)-β-glucan endohydrolases have been well characterized in the metabolism of MLG, and analysis of their amino acid sequence and crystal structure shows strong similarity with barley (1,3)-β-glucan endohydrolases, indicating that the ability to cleave (1,3;1,4)-β-glucan polymers was achieved through a modification of (1,3)-β-glucan endohydrolase function (Varghese *et al.*, 1994).

The utility of increased MLG as a bioenergy source and the effect of increased MLG on wall content and plant health has been explored in studies on synthesis in barley and *A. thaliana* in which MLG was overexpressed. Excess MLG synthesis under constitutive promoters was detrimental to plant health, but tissue or developmentally specific promoters driving MLG synthesis resulted in plants with higher MLG content in grain or stem without such deleterious effects (Burton *et al.*, 2011; Vega-Sánchez *et al.*, 2015) In barley, MLG and starch levels have been shown to be inversely related in the developing coleoptile (Roulin *et al.*, 2002), and MLG levels have been shown to rise dynamically and fall over the course of development in vegetative tissue (Gibeaut *et al.*, 2005). The grain cell walls of *B. distachyon* differ from those of cultivated cereals with exceptionally high levels of MLG and relatively lower starch levels (Guillon *et al.*, 2011; Opanowicz *et al.*, 2011; Trafford *et al.*, 2013; Burton & Fincher, 2014). This shift in carbon storage suggests that *B. distachyon* may rely on MLG to a greater extent than starch for endosperm carbon storage (Trafford *et al.*, 2013; Burton & Fincher, 2014). It has been suggested that MLG metabolism is enzymatically simpler than starch metabolism, requiring fewer enzymes in more available cellular spaces than the multistep, amyloplast specific process of starch metabolism (Roulin *et al.*, 2002; Burton & Fincher, 2012; Trafford *et al.*, 2013; Bulone *et al.*, 2019). While this has yet to be explored experimentally, it has been noted that a fast, alternative glucose storage pathway from (1,3;1,4)-β-glucan metabolism may confer an advantage to the grasses, as evidenced by the development of this mechanism in a group with such widespread success.

V. Grass heteroxylans

After glucans, xylans are the most abundant polysaccharide in plants. Although present across angiosperms, heteroxylans play a more prominent role in the grasses as the major hemicellulose (Scheller & Ulvskov, 2010). This class of polysaccharide is based on a (1,4)-β-D-xylopyranosyl backbone, with side chains of arabinose, xylose, glucuronic acid, and hydroxycinnamates (Figs 1, 4). The nature and patterning of these side chains have major impacts on cell wall integrity, mediating xylan–cellulose and xylan–lignin polymer interactions (Simmons *et al.*, 2016; Martínez-Abad *et al.*, 2017). The β-(1,4)-xylan backbone has been shown to be synthesised in both eudicots and monocots by members of glycosyltransferase 43 (GT43) and GT47 family proteins. The *A. thaliana* irregular xylem mutants (*irx*) were some of the first identified xylan synthesis mutants, including *irx9*, *irx14* and *irx10*, all encoding GT43 and GT47 enzymes in wild-type plants (Brown *et al.*, 2005, 2009; Lee *et al.*, 2007; Peña *et al.*, 2007). In *B. distachyon*, recent work has shown that a member of the GT43 family is, in part, responsible for heteroxylan backbone synthesis. Genetic linkage mapping of saccharification rate in a recombinant inbred population identified a quantitative trait locus interval containing a *BdGT43A* orthologue of *A. thaliana IRX14* (Whitehead *et al.*, 2018). Allelic variation in *BdGT34A* between parental accessions Bd3-1 and Bd21 showed that the Bd3-1 allele encodes an alanine to threonine (A80T) shift that was associated with reduced Bd3-1 saccharification. Knockdown of *BdGT43A* resulted in reduced xylose, arabinose and ferulic acid deposition in stem tissue. Rice GT43 proteins have similarly been shown to mediate xylan synthesis, with *OsGT43A* and *OsGT43E* complimenting *A. thaliana irx9* mutant phenotypes, and *OsGT34J* complimenting *irx14* (Lee *et al.*, 2014).

The addition of side chains to the xylan backbone differentiates the various types of heteroxylans. In eudicots, glucuronoxylan is the most prevalent form, in which the side chain is formed by the addition of α-(1,2)-GlcA side chains, sometimes amended with 4-O-Me groups (Scheller & Ulvskov, 2010). Grass cell walls differ from those of eudicots in their abundance of arabinoxylans and glucuronoarabinoxylan. Arabinoxylans have monomer side chains of α-(1,3)-Ara*f* and β-(1,2)-Xyl*p*, or dimer side chains of α-(1,3)-Ara*f*-α-(1,2)-Ara*f*, α-(1,3)-Ara*f*-β-(1,2)-Xyl*p*, or α-(1,3)-Ara*f*-ferulic acid. Glucuronoarabinoxylans contain the same side chains as arabinoxylans, but also include α-(1,2-)GlcA-4-O-Me additions. Arabinoxylans are the more prevalent form found in endosperm cell walls, while glucuronoarabinoxylan is more common in vegetative tissue. The addition of these sugar side chains to heteroxylans is mediated by xylan arabinosyltransferases (XAT), which are members of the GT61 family. They function in the Golgi to add α-(1,3)-Ara*f* substitutions to the xylan backbone. Two XATs in wheat (*TaXAT1*, *TaXAT2*) and rice (*OsXAT2*, *OsXAT3*) have been characterised both natively and in heterologous systems for arabinosyltransferase activity (Anders *et al.*, 2012; Zhong *et al.*, 2018b). Other GT61 members possess xylosyltransferase activity. Rice xylosyl arabinosyl substitution of xylan 1 (OsXAX1) mediates the addition of xylose to arabinose units (Xyl*p*-1,2-β-Ara*f*) (Chiniquy *et al.*, 2012), while rice xylan xylosyltransferase 1 (OsXYXT1) adds xylose sidechains to the xylan backbone (Xyl*p*-1,2-β-Xyl*p*) (Zhong *et al.*, 2018b). While much of our understanding of heteroxylan synthesis comes from rice, some *B. distachyon* saccharification mutants identified from a sodium azide mutant population are candidates for characterising heteroxylan synthesis (Dalmais *et al.*, 2013). The *sac1* GT61 mutant in *B. distachyon* has a phenotype similar to the rice mutant *OsXAX1* (Marriott *et al.*, 2014). In *sac1*, plants have reduced xylose content, suggesting that the GT61 candidate, like *OsXAX*, mediates the incorporation of this saccharide component into the wall.

The presence of glucuronic acid (GlcA) side chains differentiates heteroxylans into glucuronoarabinoxylans and arabinoxylans. In *A. thaliana*, GlucUronic acid substitution of Xylan (AtGUX)-1 adds GlcA at evenly spaced intervals of 8–10 xylose residues, although greater spacing has been observed. AtGUX2 appears to preferentially

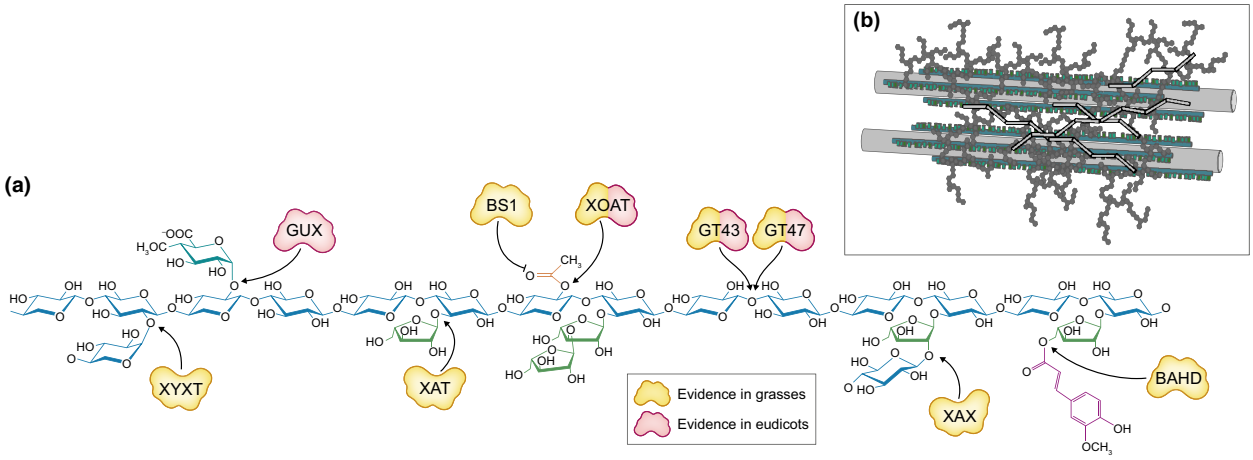

Fig. 4 Grass heteroxylan structure. (a) Fine structure of heteroxylan and biosynthetic enzymes. The major noncellulosic polysaccharides in grasses are xylans. A xylose (blue) backbone is decorated with side chains of xylose, arabinose (green), glucuronic acid (teal), and ferulic acid (purple). The enzymes responsible for forming certain linkages on the heteroxylan polymer are depicted in either yellow, pink or both, having been characterised respectively in grass systems, eudicots or showing conserved function. (b) Miniature of Fig. 1(a) cell wall schematic highlighting the heteroxylan component.

add GlcA more frequently, at 5–7 residue intervals without regard for even spacing (Bromley *et al.*, 2013). The evenly spaced xylan regions form the major xylan domain, and the less organised GlcA spacing populates the minor domain. The major domain has been shown to interact with cellulose microfibrils, an interaction that is also mediated by xylan acetylation. Similar GUX function has yet to be observed in grasses, but presumably a mechanism for adding GlcA to heteroxylan exists. Additionally, 4-*O*-methylation of GlcA by AtGXMT (glucuronoxylan methyltransferase), a DUF579 protein, has been characterised in *A. thaliana*, but not in any grasses to date (Urbanowicz *et al.*, 2012). The addition of GlcA and its methylation have been implicated in eudicots in mediating xylan interaction with other wall polymers, and this phenomenon is ripe for investigation in grasses.

VI. Xylan acetylation

Xylan acetylation has long been observed, but only recently has the role of these modifications been revealed. In *A. thaliana*, recent work has shown that acetylation pattern influences xylans–cellulose interaction. Regularly spaced acetylation on every other xylose monomer in regions of the xylan backbone results in the polymer forming a two-fold helix that closely bonds with the hydrophilic side of cellulose microfibrils (Busse-Wicher *et al.*, 2014). The modification of xylan with acetate has strong implications for the solubility of the polymer, as well as the strength of xylan–cellulose interactions. Xylan-*O*-acetyltransferases (XOATs) are DUF231 family proteins, and carry out 2-*O*- and 3-*O*-monoacetylation and 2,3-di-*O*-acetylation (Fig. 4). In *A. thaliana*, nine XOATs have been identified and genetically characterised, including the Trichome Birefringence protein, TBR-like proteins and ESKIMO1 (Zhong *et al.*, 2017). In grasses, there has been an expansion of the DUF231 XOATs, with rice containing 14 members. *OsXOAT1* and *OsXOAT7* complement the *A. thaliana esk1* xylan acetylation mutant, and all 14 rice XOATs can acetylate xylohexose *in vitro* (Zhong *et al.*, 2018a).

While the degree of xylan acetylation has been shown to play a critical role in wall integrity, evidence of deacetylation activity has not yet been shown in eudicots. However, *rice brittle sheath 1* (*OsBS1*) encodes a GDSL lipase/esterase that functions as an acetylesterase in the Golgi, removing acetyl groups from xylans (Zhang *et al.*, 2017). Mutation in *OsBS1* results in greater 2-*O*- and 3-*O*-acetylation, which compromises secondary wall patterning and integrity.

VII. Lignins

Lignins are large phenolic polymers mainly deposited in the primary and the secondary cell wall of xylem and sclerenchyma cells. These polymers provide the hydrophobicity and mechanical properties necessary for the development of land plant vasculature. Lignins embed polysaccharides in the cell wall and are a major barrier for biomass usage such as saccharification for biofuel production (Marriott *et al.*, 2014). Unlike other wall polymers, lignins contain many types of interunit bonds (aryl beta-aryl ether, phenyl coumaran, resinol, biphenyl) randomly formed during polymerisation, some being more (C–C) or less (C–O–C) resistant to degradation (Mnich *et al.*, 2020). Consequently, lignin structure is not predictable, although the abundance of each monomer seems to influence the occurrence of certain linkages (Stewart *et al.*, 2009).

Lignins are synthesised from three monolignols, *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol, that differ by their degree of methoxylation. Once incorporated into lignin polymers, these phenolics give rise to *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units, respectively. In *B. distachyon* stems, lignin content accounts for 18–25% of the dry cell wall residue and, in the wild-type Bd21-3 accession, stem lignin is comprised of about 62% S, 34% G, and 4% H units (Bouvier d'Yvoire *et al.*, 2013; Trabucco *et al.*, 2013). Within the grasses, *B. distachyon* has one of the highest proportions of S units reported (Clarke *et al.*, 1933; Méchin *et al.*, 2014; Herbaut *et al.*, 2018).

Lignin biosynthesis results from a branch of the phenylpropanoid pathway and has long been thought to rely on the aromatic amino acid L-phenylalanine (L-Phe) as a starting substrate (Fig. 5). The standard convention in most studied plant systems has been that L-Phe is first deaminated by phenylalanine ammonia lyase (PAL), yielding cinnamate, which is then C4-hydroxylated by coumarate-4-hydroxylase (C4H) to make coumarate. Coumarate is a common branch point for all three main monolignols. However, this conventional pathway has recently been challenged by work in *B. distachyon* demonstrating that L-tyrosine (L-Tyr) can also serve as an initial substrate for lignin synthesis as it already contains a C4 hydroxylation. Indeed, tyrosine ammonia lyase (TAL) activity in grasses (Higuchi *et al.*, 1967) suggests that C4H activity can be bypassed to produce coumarate (Fig. 5). In grasses, PAL and TAL activities are controlled by the same protein, but clear evidence for a genuine phenylalanine tyrosine ammonia lyase (PTAL) activity in the phenolic pathway was poorly documented until recently. In plants that expressed a *BdPAL* RNAi hairpin construct to knock down expression of multiple *BdPAL* genes, both PAL and TAL activities were affected and plants contained 43% less lignin (Cass *et al.*, 2015; Barros *et al.*, 2016). Only one predicted PTAL (PTAL1) was identified in this family and nearly half of the total lignin deposited in *B. distachyon* occurs via TAL activity (Barros *et al.*, 2016). Interestingly, *BdPTAL1* is mainly involved in the biosynthesis of S units and cell wall linked coumarates, with less effect on G units as revealed by plants fed with C13-labelled L-Phe or L-Tyr. A biological role for PTAL has only been shown in *B. distachyon* to date, but putative orthologues to *BdPTAL1* have been identified in several other grasses (Barros *et al.*, 2016). Further characterisation is needed to confirm whether this alternate initiation of lignin synthesis is shared broadly amongst grasses, or indeed present in other groups.

Other recent discoveries are further changing our understanding of lignin biosynthesis in grasses. Very recently, (Barros *et al.*, 2019) proposed that a cytosolic ascorbate peroxidase with genuine 4-coumarate 3-hydroxylase (C3H) activity oxidises coumarate into caffeate in the phenylpropanoid pathway. Decreased expression of this novel C3H in *B. distachyon* results in significantly reduced lignin content and structure. This 'acid' route to caffeic acid and thus to caffeoyl CoA through the activity of 4-hydroxycinnamate

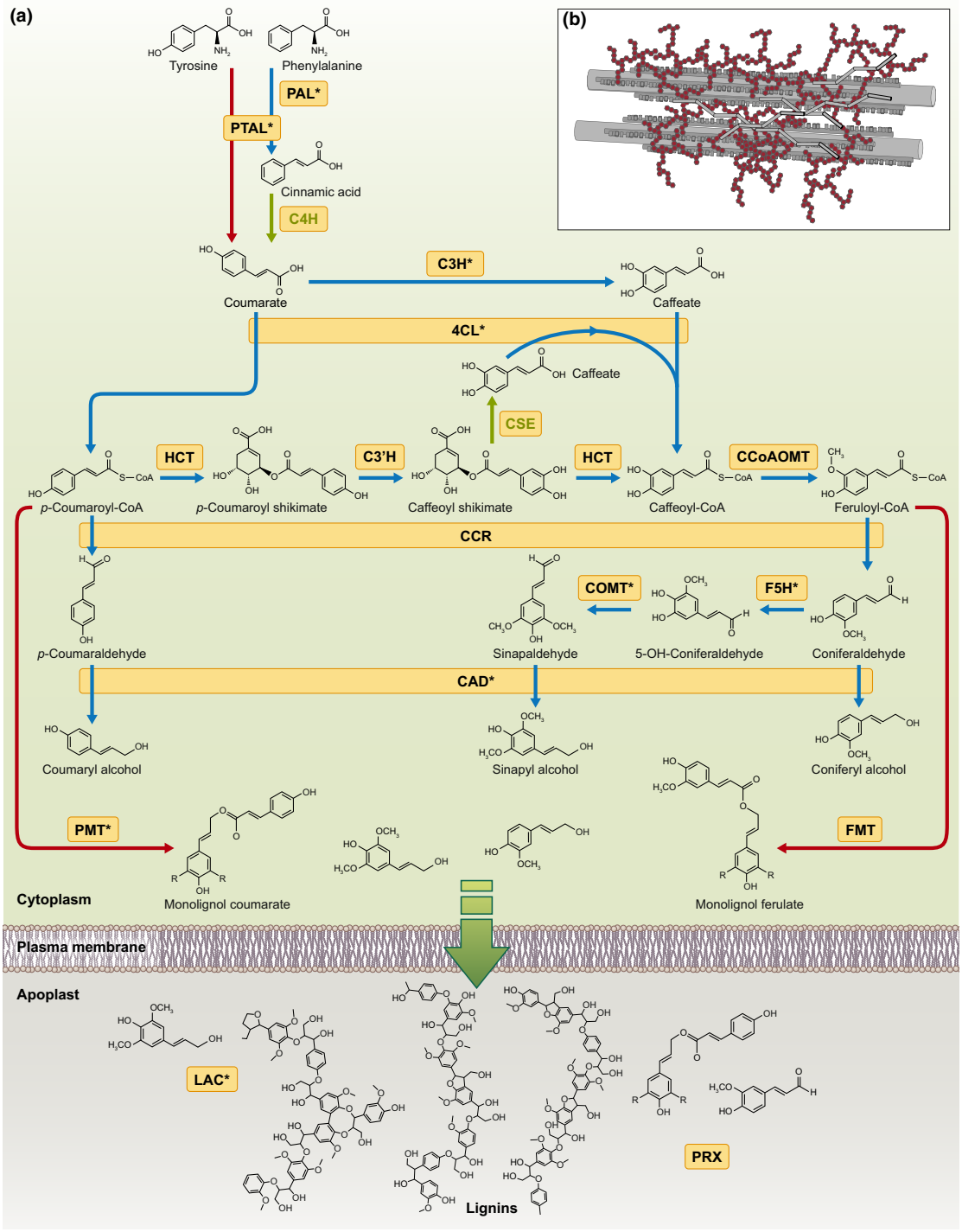

Fig. 5 Alternative routes to lignin biosynthesis. (a) In some grasses, phenylalanine tyrosine ammonia lyase (PTAL) can bypass cinnamic acid 4-hydroxylase (C4H) activity and thus directly produce *p*-coumarate from tyrosine. An ascorbate peroxidase-like gene with coumarate 3-hydroxylase (C3H) activity can directly synthesise caffeate from coumarate. Red arrows illustrate a grass-specific lignin pathway unique from other lignin pathways identified in most studied eudicots. Green arrows show routes potentially bypassed or absent in *Brachypodium distachyon*, such as caffeoyl shikimate esterase (CSE). Blue arrows are paths believed to be common to eudicots and grasses. Asterisks highlight enzymes characterised in *B. distachyon*. The mechanism for monolignol export across the plasma membrane remains unclear. Phenylalanine ammonia lyase (PAL), 4-hydroxycinnamoyl CoA ligase (4CL), hydroxycinnamoyl CoA:shikimate hydroxycinnamoyl transferase (HCT), *p*-coumaroyl shikimate 3′-hydroxylase (C3′H), caffeoyl CoA *O*-methyltransferase (CCoAOMT), hydroxycinnamoyl CoA reductase (CCR), ferulic acid 5-hydroxylase (F5H), caffeic acid/5-hydroxyferulic acid *O*-methyltransferase (COMT), cinnamyl alcohol dehydrogenase (CAD), laccase (LAC), peroxidase (PRX). R- in the monolignol hydroxycinnamics could be substituted by a hydrogen or methyl group. (b) Miniature of Fig. 1(a) cell wall schematic highlighting the lignin component.

CoA ligase (4CL) would be complementary to the C3′H pathway in which 4CL, 4-hydroxycinnamoyl CoA:shikimate/quinate hydroxycinnamoyltransferase (HCT), 4-coumaroyl shikimate/quinate 3′-hydroxylase (C3′H), function sequentially to convert coumarate to caffeoyl CoA (Fig. 5). Feruloyl-CoA produced by the methoxylation of caffeoyl coA by caffeoyl CoA *O*-methyl transferase (CCoAOMT) is a substrate of cinnamoyl-CoA reductase (CCR). The proposition of an alternate 'acid' route to monolignol synthesis is not new, but the discovery of a cytosolic ascorbate peroxidase with 4-coumarate 3-hydroxylase activity *in planta* was lacking until now. Interestingly, Barros *et al.* (2019) showed this is not unique to grasses, as the null allele of the *A. thaliana* C3H orthologue is lethal. The lignin pathway that involves the membrane-bound C3′H also plays a critical role in grass lignin synthesis. Indeed, C3′H-knockout rice mutants were severely affected in their development and displayed typical C3′H phenotypes with lignins largely enriched in H units at the expense of G and S units (Takeda *et al.*, 2018). Interestingly, caffeoyl shikimate esterase (CSE) activity was not detected in *B. distachyon* stem crude extract and this result is supported by the absence of close orthologues of *AtCSE* in *B. distachyon* (Ha *et al.*, 2016). CCR is a cornerstone step to monolignol biosynthesis. CCR activity converts CoA-conjugated intermediaries into the aldehyde precursors of monolignols. While *ccr* mutants with decreased lignin content and increased monolignol conjugates were studied in maize there are no reports on cell wall properties of CCR-deficient lines in *B. distachyon* (Tamasloukht *et al.*, 2011; Cass *et al.*, 2015).

The last enzyme in the monolignol pathway, cinnamyl alcohol dehydrogenase (CAD), reduces cinnamaldehyde into alcohols. Mutants and transgenics lines affected in CAD have been well characterised in *B. distachyon* (Bouvier d'Yvoire *et al.*, 2013; Trabucco *et al.*, 2013). Lignin content of *Bdcad1* mutants was drastically enriched in aryl β-aryl ether and diaryl ether-coupled S units, as well as resistant interunit bonds and free phenolic groups, a result previously observed in maize and sorghum *brown-midrib* mutants (Pillonel *et al.*, 1991; Barriere *et al.*, 2004). By contrast, there was little increase in coniferaldehyde-end groups in the *Bdcad1*, suggesting that another CAD gene specific to coniferyl alcohol is involved in lignification. As observed in CAD-deficient eudicot plants, sinapic acid esters linked to the cell wall were detected in *Bdcad1* (Bouvier d'Yvoire *et al.*, 2013).

As stated at an earlier point, *B. distachyon* lignin is relatively enriched in S units. Their precursor, sinapyl alcohol, is produced through the C5 hydroxylation of coniferaldehyde by the P450 enzyme ferulate-5-hydroxylase (F5H) and methoxylation by caffeyl-*O*-methyl transferase (COMT). When *F5H* was overexpressed in *B. distachyon*, cell wall analysis revealed an average increase of 25% in the content of S units in these lines, leading to an increase in the S/G ratio from 2.3 in wild-type to 8.1, with a modest increase of 5-hydroxy-guaiacyl units (5-OH-G UNIT) and 30% higher saccharification yield (Sibout *et al.*, 2017). Several *B. distachyon* mutants affected in COMT activity were identified in a sodium azide-induced mutant collection by TILLING (Dalmais *et al.*, 2013). As observed in maize *comt* mutants, *B. distachyon* mutants showed the accumulation of 5-OH-G units in their lignin and significantly altered lignin content (Piquemal *et al.*, 2002; Bouvier d'Yvoire *et al.*, 2013; Dalmais *et al.*, 2013; Trabucco *et al.*, 2013).

Once exported into the apoplast by an unknown mechanism, monolignols are oxidised by peroxidases and/or laccases (Vanholme *et al.*, 2012; Wang *et al.*, 2013; Perkins *et al.*, 2019; Vermaas *et al.*, 2019). Laccases are multicopper oxidases that use oxygen as an electron acceptor, while peroxidases use H_2O_2. Once oxidised, the monolignols radically polymerise into the branched lignin polymer with multiple bond types resulting from the various positions of the oxygen radical on the monolignol subunit. There are 17 laccases in *A. thaliana* and 29 in *B. distachyon* (Berthet *et al.*, 2011; Le Bris *et al.*, 2019). *Brachypodium distachyon LACCASE 5* and *8* were identified as orthologues of *AtLAC17* and were shown to be responsible for lignification in interfascicular fibres (Wang *et al.*, 2015; Le Bris *et al.*, 2019). A laccase gene from sugarcane (*SofLAC*) also genetically complemented an *A. thaliana lac17* mutant (Cesarino *et al.*, 2013). Lignin content decreased by 30% in the double *lac5lac8* mutant, and saccharification increased by 140% compared with the wild-type. Lignin deposition was less affected in vascular bundles compared with fibres, suggesting that different laccases or peroxidases are recruited for lignification of these tissues.

VIII. Hydroxycinnamic acids

The presence of hydroxycinnamic acids, namely ferulic acid (FA) and *p*-coumaric acid (*p*CA), in the cell wall is a defining feature of grass secondary cell walls (Ralph *et al.*, 1994; Hatfield *et al.*, 2009). FA is predominantly linked to heteroxylan through an ester bond. The oxidation of FA in the cell wall, probably by peroxidases, generates esterified dehydrodiferulates which serve as linkages between two arabinoxylan polymers. In lignified tissues, xylan-esterified ferulates can be etherified to G units of lignin and thus serve as a covalent linkage between hemicelluloses and lignins (Hatfield *et al.*, 2016; Lapierre *et al.*, 2019). An esterified ferulate on arabinoxylan is considered as a nucleation site of lignification in grasses and thus an important mechanism for cell wall reinforcement (Ralph *et al.*, 1995, 1998). *p*CA is esterified on arabinoxylan to a lesser extent than FA and tends to be found esterified to S units in *B. distachyon* lignins. Plant-specific acyl-CoA-dependent acyltransferases of the BAHD (BEAT, AHCT, HCBT, and DAT) family are the enzymes responsible for the acylation of the arabinose side chains of heteroxylans and monolignols with hydroxycinnamates (D'Auria, 2006; Mitchell *et al.*, 2007). An expanded grass-specific BAHD clade (also called the 'Mitchell clade') was identified by bioinformatic analysis in rice as candidates for hydroxycinnamate transfer (D'Auria, 2006; Mitchell *et al.*, 2007; Bartley *et al.*, 2013). Consequently, BAHD enzymes with feruloyl transferase activity were first explored in rice and have also been investigated in *B. distachyon* (Piston *et al.*, 2010; Bartley *et al.*, 2013). BAHD01 in *B. distachyon* and *Setaria viridis* appear to be involved in feruloylation of arabinoxylans (de Souza *et al.*, 2018). Downregulation of *SvBAHD01* significantly reduced FA on arabinoxylan, with an increase in *p*CA-Ara*f* acylation and no substantial change in lignin content while in *B. distachyon* only a moderate decrease in FA-arabinoxylan was observed (de Souza *et al.*, 2018). Interestingly,

BdBAHD01 downregulation lines showed increased saccharification efficiency, despite unchanged lignin content, highlighting the role of FA in maintaining cell wall integrity. Overexpression of *BdBAHD05* (also called *BdAT1*) caused a moderate increase in FA content and downregulation showed a moderate decrease (Buanafina *et al.*, 2016; de Souza *et al.*, 2018). Analysis in sugarcane revealed six *BAHD* genes, one of which is homologous to *SvBAHD01*, and downregulation of *SacBAHD01* similarly reduced stem FA content and increased biomass digestibility (de Souza *et al.*, 2019).

FA acylated monolignols were detected in several species, including willow (*Salix* sp.) and poplar (*Populus trichocarpa*), although in much lower amounts than *p*CA acylated monolignols in grasses (Karlen *et al.*, 2016). In rice, feruloyl monolignol transferase (OsFMT) was identified through homology with other BAHD acyltransferases that act on monolignols (Wilkerson *et al.*, 2014; Karlen *et al.*, 2016). *OsFMT* overexpression resulted in higher levels of FA on lignin. FA from heteroxylan, released through mild alkaline hydrolysis, was unchanged by altered *OsFMT* expression. Furthermore, there was no change in the levels of *p*CA acylated monolignols, suggesting specificity of this enzyme for monolignol feruloylation.

In *B. distachyon*, *p*-coumaryl-CoA:monolignol transferase (PMT) acylates lignin with *p*CA, but not heteroxylan (Petrik *et al.*, 2014). While OsPMT has a high affinity for coumaryl alcohol *in vitro*, BdPMT preferentially acylates sinapyl alcohol with *p*CA *in planta* (Withers *et al.*, 2012; Sibout *et al.*, 2016). Lines overexpressing *BdPMT* showed lower total lignin despite an increase of *p*CA content (Petrik *et al.*, 2014). This may be a consequence of redirecting *p*-coumaric acid CoA for acylation rather than monolignol synthesis, or the inhibition of the monolignol polymerisation by excessive *p*-coumaroylation (Sibout *et al.*, 2016). Interestingly, when *BdPMT* was overexpressed in *A. thaliana*, which does not natively produce *p*CA acylated lignin, a significant amount of *p*CA was found on lignins (Sibout *et al.*, 2016). More surprising, when *BdPMT* was expressed under a specific *C4H* promoter in a *ccr*-deficient *A. thaliana* mutant background, lignin was esterified with both *p*CA and FA. Mutants in *CCR* accumulate high levels of feruloyl-CoA, and BdPMT activity in this mutant suggests that not only is BdPMT functional in eudicots, but it is also able to use feruloyl-CoA as a substrate when available in sufficient quantities (Withers *et al.*, 2012; Sibout *et al.*, 2016). In maize, *ZmPMT* loss-of-function lines had less *p*CA and modified lignin structure, but not reduced total lignin content (Marita *et al.*, 2014). Overall, BAHD proteins have a related set of functions in decorating cell wall components; feruloylation of arabinoxylan (BAHD01), feruloylation of lignins (FMT), and coumaroylation of lignins (PMT). An enzyme responsible for the coumaroylation of arabinoxylan remains to be discovered.

IX. Tricin

As evidenced by their *p*CA and FA content, grasses are remarkable in their capacity to incorporate phenolic compounds other than the typical coumaryl, coniferyl and sinapyl alcohols into lignin. Tricin, an *O*-methylated flavone, was first characterised in wheat straw lignin (del Río *et al.*, 2012). Tricin is incorporated into grass lignin in varying amounts across grass species, with oat (*Avena sativa*), wheat and B. *distachyon* straw being particularly enriched in this compound (Lan *et al.*, 2016). Tricin is incorporated in lignin polymers via 4′-O-β coupling (Lan *et al.*, 2018). Biomimetic radical coupling reactions give evidence that tricin may serve as a possible nucleation site for lignification, as has been suggested for ferulate (Ralph *et al.*, 1995, 1998; Lan *et al.*, 2015). Tricin and monolignols come from two different branches of the phenylpropanoid pathway, and consequently their synthesis shares some common enzymes. This is particularly true for enzymes involved in the metabolic flux upstream of *p*-coumaric acid synthesis. CHALCONE SYNTHASE, a pivotal enzyme for flavonoids production, uses malonyl-CoA and *p*-coumaryl-CoA as substrates. Silencing this enzyme in maize resulted in strongly reduced levels of apigenin-related and tricin-related flavonoids, and also strongly reduced incorporation of tricin into the lignin polymer (Eloy *et al.*, 2017). The effect of the flavonoid pathway on the production of cell wall tricin content was also demonstrated in rice (Lam *et al.*, 2017, 2019). It is also possible that some of the cell wall changes observed in *BdPMT* overexpression lines may stem from the depletion of *p*-coumaroyl-CoA pool, as both chalcone synthase and PMT act on this substrate. *O*-methyltransferases involved in the *O*-methylation of 5-hydroxy-coniferaldehyde to produce sinapyl alcohol were also shown to be involved in the methylation of tricin in rice, maize, and sorghum (Eudes *et al.*, 2017; Fornalé *et al.*, 2017; Lam *et al.*, 2019). The bi-functionality of COMT in the lignin and flavonoid pathways is not unexpected, as a COMT involved in lignification of *A. thaliana* stems also *O*-methylates isorhamnetin, a flavonoid structurally similar to tricin (Do *et al.*, 2007). There is now abundant evidence that other molecules, called 'nontraditional monomers' like tricin or hydroxycinnamic acids, can be incorporated into the lignin polymer (del Río *et al.*, 2018; Vanholme *et al.*, 2019). The biological role of these novel lignin components remains to be determined.

X. Silicon

Poaceae accumulate high quantities of silicon in the cell wall of their shoots. This phenomenon is particularly marked in rice (Ma & Yamaji, 2006). The main role of silicon is to provide plant resistance to many biotic and abiotic stresses (Hattori *et al.*, 2005; Deshmukh *et al.*, 2017). However, silicon may interact with polysaccharides, which consequently affect plant biomass processing in biorefineries (Perry & Lu, 1992; Kido *et al.*, 2015). For biofuel production, there is a trade-off between soil amendment with silicon that can increase polysaccharide yield with a negative effect on the conversion of biomass into biofuels (Głazowska *et al.*, 2018b). Silicon content in rice and maize can be modulated by changing the expression of silicon transporters (Ma *et al.*, 2007; Mitani-Ueno *et al.*, 2016; Bokor *et al.*, 2017). The analysis of different silicon transporter mutants showed that silicon availability may impact the morphology and patterning of stem and leaf macrohairs (Głazowska *et al.*, 2018b). The *Bd low silicon 1* (*Bdlsi1*) mutant is impaired in silicon transporter function and has reduced

silicon uptake, with 93% less silicon present in the shoot. Mixed-linkage glucan content is drastically modified in *Bdlsi1* (Kido *et al.*, 2015; Głazowska *et al.*, 2018a). This result is in agreement with previous studies suggesting that (1,3;1,4)-β-glucan is involved in silicon-dependent strengthening of the rice cell wall (Kido *et al.*, 2015). The *Bdlsi1* mutant also displayed an altered degree and pattern of homogalacturonan methyl esterification. Despite the relatively low amount of pectins found in grasses, this change in homogalacturonan represents a significant alteration to the wall matrix. Lastly, *Bdlsi1* mutant FA extrability was lower with only minor changes in lignin content (Kido *et al.*, 2015; Głazowska *et al.*, 2018a). These data highlight the important role silicon plays in cell wall integrity in *B. distachyon* and grasses in general, and presents interesting avenues for further study.

XI. Transcriptional regulation of secondary cell wall thickening

Canonical transcription factors that directly bind DNA play a prominent role in the regulation of plant secondary cell wall thickening. The *cis*-regulatory regions of genes associated with cellulose, hemicellulose and lignin biosynthesis interact directly with numerous MYB and NAC family transcription factors (Fig. 6; Nakano *et al.*, 2015). Many of the R2R3-MYB protein family subgroups appear to bind a similar sequence motif, the AC element, also known as the M46RE (MYB46 responsive *cis*-regulatory element) and the SMRE (secondary wall MYB-responsive element) (Kim *et al.*, 2012; Zhong & Ye, 2012; Zhao & Bartley, 2014; Handakumbura *et al.*, 2018). In *A. thaliana*, *AtMYB46* and the close paralogue *AtMYB86* activate the expression of cellulose, hemicellulose and lignin biosynthetic genes, as well as other MYBs capable of activating secondary cell wall-related genes (Zhong *et al.*, 2007; Zhong & Ye, 2007). Some of the downstream MYB activators, among them *AtMYB58/63* and *AtMYB42/85*, activate only lignin genes (Rao & Dixon, 2018; Zhang J *et al.*, 2018). However, in sorghum, rice, and switchgrass (*Panicum virgatum*), ectopic expression of *OsMYB58/63*, *PvMYB58/63*, and the sorghum orthologue *SbMYB60* results in the activation of cellulose and hemicellulose genes as well as lignin (Noda *et al.*, 2015; Scully *et al.*, 2016; Rao *et al.*, 2019). A potential orthologue to *OsMYB42/85*, *ZmMYB167*, was overexpressed in maize and heterologously in *B. distachyon* to similar effect (Bhatia *et al.*, 2019). Similar functions have been resolved for the *A. thaliana* and rice orthologues *AtMYB61* and *OsMYB61* as well as *AtMYB103* and *OsMYB103* (Hirano *et al.*, 2013; Huang *et al.*, 2015; O'Malley *et al.*, 2016; Zhao *et al.*, 2019). These downstream MYBs bind the AC element and activate both lignin and wall polysaccharide biosynthesis genes. Overall, there are few distinctions in the transcription factor targets for these genes between grasses and *A. thaliana*. Those that have been observed may be the outcome of low-resolution experimental designs that sample one tissue type at one time point for a limited number of outputs.

The expression of cell wall-associated genes is often highly correlated (Brown *et al.*, 2005; Persson *et al.*, 2005). Coexpression analysis of a *B. distachyon* gene expression atlas resolved a cluster of 96 genes that is enriched for cell wall biosynthetic processes with numerous cellulose-, hemicellulose-, and lignin-associated genes (Sibout *et al.*, 2017). Among the identified genes, there are two primary and two secondary wall *CESA*s, as well as *COBRA*, *KORRIGAN*, *CSI1*, *CSLF2*, numerous glycosyltransferases and glycosylhydrolases, fasciclin-like family and numerous lignin-associated genes. The MYB transcription factor *SECONDARY WALL ASSOCIATED MYB 1* (*SWAM1*) is one of two canonical transcription factors that are part of the wall gene enriched cluster, making it a candidate for a regulator of genes in the cluster (Fig. 6). Similarly, analysis of *B. distachyon* leaf, root, and stem microarray gene expression data identified *SWAM1/2/3* and MYBs that are part of six other prominent subgroups orthologous to *AtMYB46/83*, *AtMYB103*, *AtMYB58/63*, *AtMYB52/54*, *AtMYB42/85*, and *AtMYB4/32* that are highly correlated with secondary *CESA* and lignin biosynthetic gene transcriptional targets (Supporting Information Table S1; Handakumbura *et al.*, 2018). Interestingly, the *SWAM1* gene and its two closest homologues, *SWAM2* and *SWAM3*, are conspicuously absent from genomes in the *A. thaliana* family Brassicaceae but present in other eudicots and monocots (Handakumbura *et al.*, 2018). Like the other described secondary cell wall regulating R2R3-MYBs, *SWAM1* interacts with the AC element and is an activator of secondary cell wall genes. Based on amino acid similarity with characterised genes in other systems and their expression pattern, all of the *B. distachyon* identified MYBs are excellent candidates for a role in cell wall biosynthesis.

The same promoters that interact with the secondary cell wall regulating MYB transcription factors often interact with NAC transcription factors, collectively referred to as the *SECONDARY WALL NAC*s (*SWN*) or the *VND*, *NST/SND*, *SMB* related (*VNS*) (Ohtani *et al.*, 2011; Zhong *et al.*, 2011). This group of proteins is generally classified into four clades, all binding the similarly named VNS element in *in vitro* assays (O'Malley *et al.*, 2016; Olins *et al.*, 2018), which is consistent with independently identified TERE and SNBE binding sites for the same proteins (Pyo *et al.*, 2007; Valdivia *et al.*, 2013). In *A. thaliana*, three of the clades that include the *VASCULAR-RELATED NAC-DOMAIN*s (*VND*s), activate cell wall thickening directly and by activating the previously described downstream MYBs (Kubo *et al.*, 2005). The VNDs can induce vascular cells differentiation, induce further thickening, and initiate programmed cell death (Kubo *et al.*, 2005; Zhong *et al.*, 2008). They function in xylem rather than fibres where thickening is activated by the clade IV NACs: *NAC SECONDARY WALL THICKENING FACTOR 1* (*NST1*), *NST2*, and *NST3* (also known as *SECONDARY WALL-ASSOCIATED NAC-DOMAIN PROTEIN 1* (*SND1*) (Zhong *et al.*, 2006; Mitsuda *et al.*, 2007). Uniquely, programmed cell death is not activated by clade IV NACs. Such cell type specific functions have not been resolved in grasses. The function of the SWNs is well conserved between *A. thaliana* and grasses where grass genes can complement mutants in *A. thaliana* (Zhong *et al.*, 2011, 2015; Rao *et al.*, 2019). In *B. distachyon*, members of all four clades induced the formation of secondary walls when ectopically expressed in tobacco leaves and the VND-type SWNs also activated programmed cell death (Valdivia *et al.*, 2013). Together with the MYBs, the NACs form feed-forward loops (Nakano *et al.*, 2015; Taylor-Teeples *et al.*, 2015). In general, all of the transcription factor proteins can bind to

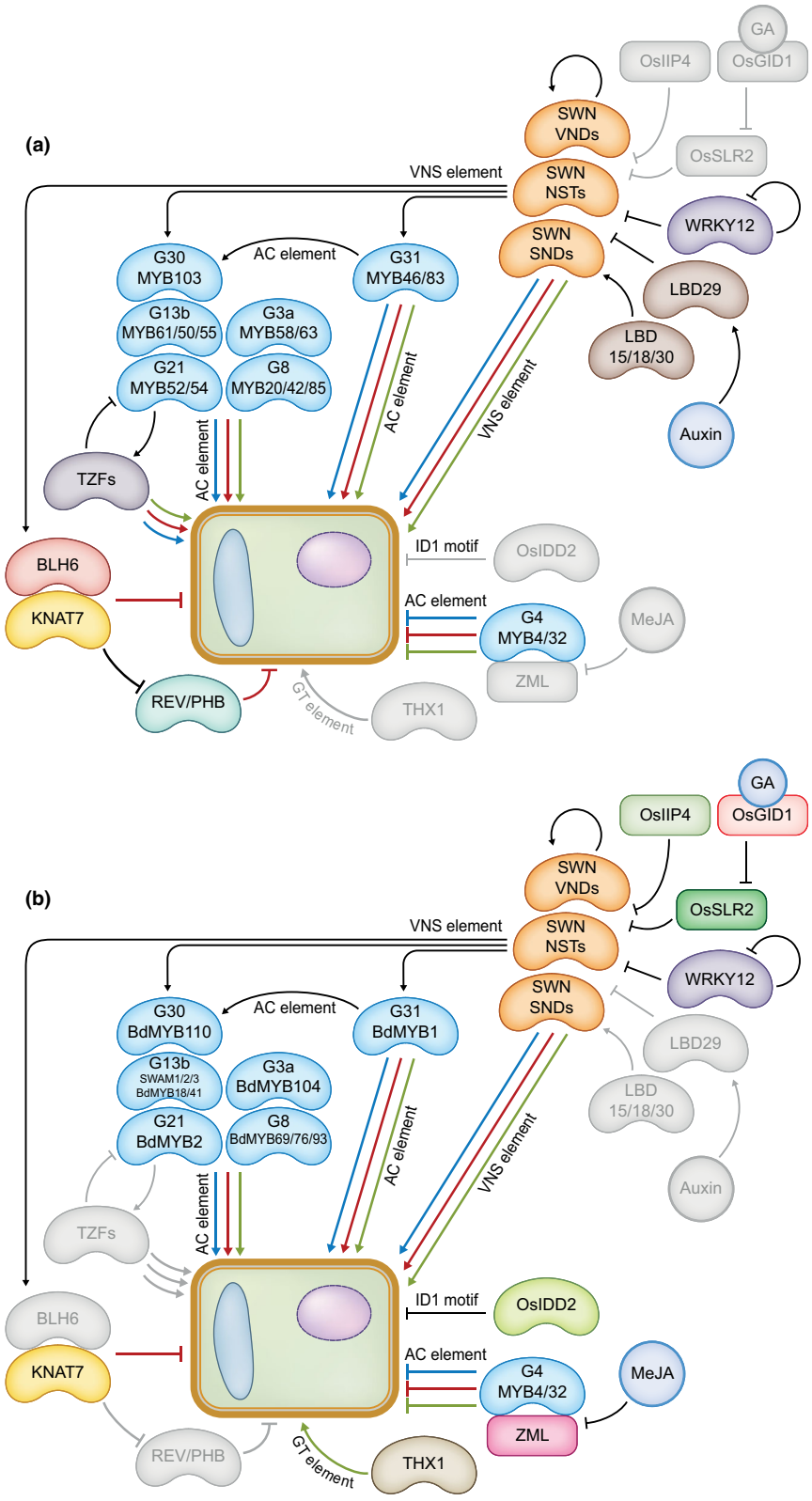

Fig. 6 Transcriptional regulation of secondary cell wall deposition. Secondary cell wall transcription regulatory network in *Arabidopsis thaliana* (a) and grasses (b). Bean shape indicates DNA binding transcription factors. Ovals indicate protein interactors. Circles are hormones. Orthology between *A. thaliana* and grasses is denoted by colour. Blue, red and green arrows indicate regulation of cellulose, lignin and hemicellulose, respectively. Arrows indicate activation and bars repression. Grey shaded items have not been described in both systems.

genes that encode cell wall structural enzymes and they also have the function of activating other activating transcription factors.

The finger-like protuberance formed by a zinc-finger protein domain can interact with DNA, RNA and proteins. Tandem CCCH zinc-finger (TZF) proteins modulate gene expression transcriptionally by interactions with DNA or posttranscriptionally by interactions with mRNA (Bogamuwa & Jang, 2014). *Arabidopsis thaliana C3H14* is a direct activator of lignin, cellulose and hemicellulose biosynthesis genes and may be a repressor of MYB cell wall activators (Ko *et al.*, 2009; Kim *et al.*, 2012). Conversely, the *INDETERMINATE* family C2H2-type zinc-finger transcription factor in rice, OsIDD2, interacts with the ID motif to repress the expression of lignin-associated genes (Huang *et al.*, 2018). Analysis of mutants and transgenic plants suggests that OsIDD2 directly represses lignin-associated gene expression and indirectly secondary wall *CESA* genes. By yet another possible mechanism of gene regulation, the rice TZF protein ILA1-interacting protein 4 (IIP4) functions as a repressor of secondary wall thickening through protein–protein interaction with OsSWN2 (NAC29) and SWN3 (NAC31) (Zhang D *et al.*, 2018). The association with the SWNs is attenuated by phosphorylation of IIP4 protein, which results in translocation to the cytosol. Thus, zinc-finger proteins influence the thickening of grass secondary walls through multiple mechanisms.

While the regulatory network is dominated in number by MYB and NAC transcription activators, several repressors have also been described, namely Three Amino acid Loop Extension (TALE), zinc-fingers, HD-ZIP III, WRKY, LATERAL ORGAN BOUNDARY (LBDs) and some MYBs. Among the many types of cells that do not have secondary cell walls are pith, which reside inside the stem and among the cells in the plant with the thickest walls. A WRKY transcription factor, *WRKY12*, is a repressor of wall thickening in pith and other cells. It can directly bind the promoters of *AtNST2* and poplar *C4H* and broadly repress wall thickening in *A. thaliana*, poplar, and switchgrass (Wang *et al.*, 2010; Yang *et al.*, 2016; Rao *et al.*, 2019). The five class III HD-ZIPs in *A. thaliana* (*REVOLUTA*, *PHABULOSA*, *PHAVOLUTA*, *CORONA*, and *HB8*) and some orthologues in poplar have been shown to play a role in cambium cell initiation and vascular bundle organisation (Floyd & Bowman, 2006). However, to our knowledge, there are no reports describing a function for this group of genes in grasses. Several LBD family transcription factors, *AtLBD15/18/30*, can activate the expression of *AtVND7* and induce wall thickening and differentiation into tracheary cells (Soyano *et al.*, 2008; Ohashi-Ito *et al.*, 2018). *AtLBD29*, conversely, is a repressor of stem secondary wall thickening and is activated by the phytohormone auxin (Lee *et al.*, 2019). Repression is also supplied by the MYB G4 clade and are the best characterised in grasses. These include ZmMYB11/31/42, PvMYB4/32, and OsMYB108, which are orthologous to AtMYB4/32 (Zhao & Bartley, 2014; Rao & Dixon, 2018; Miyamoto *et al.*, 2019). These were first described in a grass as direct repressors of lignin gene expression (Fornalé *et al.*, 2006; Sonbol *et al.*, 2009). In switchgrass, PvMYB4 is a direct repressor of lignin-associated genes (Shen *et al.*, 2012; Rao *et al.*, 2019). The MYB31/42 MYBs in sorghum, rice and maize directly bind to the *cis*-regulatory regions of various lignin biosynthetic gene, but there appears to be variation in phenylpropanoid gene expression and protein–DNA interactions across species (Agarwal *et al.*, 2016). Wounding-induced lignification occurs in maize by degradation of ZmMYB11/31/42 protein and a protein interacting partner ZML2 (Vélez-Bermúdez *et al.*, 2015). Thus, wounding and the subsequent activation of MeJA signalling will remove MYB G4 clade repression in maize and induce lignin gene expression. The repressing MYB G4 clade interacts with AC-like sequence motifs, similar to the wall activating MYBs (Fornalé *et al.*, 2010; Shen *et al.*, 2012; Agarwal *et al.*, 2016). The exact targets in the phenylpropanoid pathway vary across system and study, which suggests that there may be some transcription factor subfunctionalisation.

Members of two different classes of the TALE superfamily, KNOX and BEL, have been shown to regulate secondary wall synthesis. The class II KNOX gene *KNOTTED OF ARABIDOPSIS THALIANA 7* (*AtKNAT7*) was initially identified as an irregular xylem mutant (*irx11*) (Brown *et al.*, 2005). *KNAT7* orthologues are generally described in the literature as repressors and, while there is substantial evidence for this, there are also some outstanding issues raised by data indicating a role as an activator of wall deposition. *Atknat7* mutants have thicker interfascicular fibre walls, as expected for a repressor mutant, but this mutant also shows collapsed xylem (Brown *et al.*, 2005; Li *et al.*, 2012). *Atknat7* mutants have greater lignin content, but reduced xylan, suggesting that *AtKNAT7* may differentially regulate aspects of wall polymer synthesis. In conflicting reports, one group has shown xylan biosynthetic genes upregulated in *Atknat7* lines, while another shows downregulation (Li *et al.*, 2012; He *et al.*, 2018). AtKNAT7 protein can bind to the *AtIRX9* promoter, a gene responsible for xylan backbone synthesis.

The rice orthologue of *KNAT7*, *OsKNOR1* (also known as *OsKNAT7*), can negatively regulate cell wall synthesis in interfascicular fibre cells (Wang *et al.*, 2019; Zhao *et al.*, 2019). *Osknor1* mutants have thicker interfascicular fibre walls, with no reported xylem phenotype. However, *OsKNOR1* analysis revealed other functions unique to *AtKNAT7* (Wang *et al.*, 2019). OsKNOR1 protein interacts with OsSWN3 (also known as OsVND7 and OsNAC31) and OsGRF4 proteins and transient gene expression analysis showed that OsKNOR1–OsSWN3 jointly regulated *OsMYB61* and *OsMYB103* expression, with the addition of *OsKNOR1* reducing the positive regulation of OsSWN3 targets. Similarly, OsGRF4 is known to activate expression of expansin genes *OsEXPB3*, *OsEXPB17*, and *OsEXPA6* and addition of *OsKNOR1* also repressed that effect. This suggests that *OsKNOR1* regulates wall thickening and cell expansion by decreasing the transcriptional activation of *OsSWN3* and *OsGRF4*, respectively. This was validated by the observation of wall thickening in stem internodes and cell elongation along the panicle in relation to the expression of *OsKNOR1*, *OsSWN3* and *OsGRF4* (Wang *et al.*, 2019).

Among the genes co-expressed with *B. distachyon CSLF6*, a predominant MLG synthase, was a trihelix family transcription factor (*BdTHX1*) (Fan *et al.*, 2018; Kim *et al.*, 2018). This is the first THX protein associated with cell wall biosynthesis and the first shown to bind directly to a *CSLF* gene. *In planta* and *in vitro* assays showed that BdTHX1 protein binds to the GT element in the second intron of *BdCSLF6* and to the 3′ region of glycoside hydrolase family 61 endotransglucosylase/hydrolase 8 (*BdXTH8*), a grass-specific

enzyme that uses MLG as a substrate (Fan *et al.*, 2018). Attempts to recover viable transgenic plants were unsuccessful and suggest a strong selection against the perturbation of *BdTHX1*, thus it is uncertain if it is a transcriptional activator or repressor.

The presence of phytohormone gibberellin results in the induction of secondary wall CESA genes in rice, and *OsMYB103* is necessary for that activation (Ye *et al.*, 2015). Similarly, the function of OsSWN2 (also known as OsNAC29) and OsSWN3 protein can be activated by gibberellins. The mechanism for activation is to degrade a protein interaction with the rice DELLA protein SLENDER RICE1 (SLR1) (Huang *et al.*, 2015). SLR1 protein is degraded in the presence of gibberellins and, subsequently, wall gene expression is activated (Fig. 5). A similar mechanism for gibberellin signalling in eudicots has not been reported and the role of this hormone in the regulation of wall thickening is not well resolved.

There is nearly complete overlap between the regulatory network components between eudicots and grasses. The distinctions between grass and eudicot walls are difficult to assign to differences in varying functions or members of the regulatory network. While no LBD, BLH or HD-ZIP III has been described as regulators of cell wall biosynthesis in grasses, it is possible that they have simply not been studied or reported. Additionally, *THX1* is likely to be unique to grasses as it regulates a hemicellulose gene not present in eudicots. Meta-analysis of microarray gene expression data, to make a combined mutual ranked network for rice and *A. thaliana*, has revealed differences in the relative importance or each regulator (Zhao *et al.*, 2019). The degree of connectivity among genes, which is the number of edges for each network node, can suggest the importance of each transcription factor. Some highly connected genes in *A. thaliana*, including *VND1/2/6/7* and *AtMYB46/83* have a two-fold to five-fold decrease in connectivity in rice. Conversely, transcription factors with considerably more connections in rice than *A. thaliana* are *OsSND2/3*, the rice orthologue of *KNAT7*, *KNOR1* as well as *OsSWN1*, the orthologue of *AtNST1*.

XII. Conclusions

Much progress has been made in recent years to better understand grass cell wall composition and regulation, in large part thanks to the numerous genetic and genomic resources that have been developed. A case in point, *B. distachyon* as a model grass system has been central to these efforts, and provides fertile ground for future studies. The unique features of grass cell walls, such as MLG synthesis and the integration of hydroxycinnamates into lignin and xylan are beginning to be uncovered in detail. Elements that were thought to be more common between grasses and eudicots, such as lignin synthesis, continue to show evidence that there is yet unexplored diversity in plant cell wall chemistry, with alternate lignin biosynthetic pathways and atypical monomer components. Regulation remains an area of much overlap, but rather than playing catch-up with eudicots, grass networks now offer new insights that expand the cell wall network. Uncovering grass-specific functions, such as *BdTHX1* regulation of MLG, highlight the opportunities to advance this important area of plant biology.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Table S1 List of *B. distachyon* genes related to secondary cell wall synthesis.

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