Brachypodium distachyon as a Model Species to Understand Grass Cell Walls

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Abstract The utilization of *Brachypodium distachyon* as a model system has allowed for a synthesis of known aspects of grass cell wall biosynthesis and provided a platform to investigate new areas of the field. Compositional analysis of *B. distachyon* cell walls shows many similarities with the walls of major food and energy crop species. This chapter presents a summary of these comparisons, as well as a review of work done in *B. distachyon* characterizing genes involved with cell wall biogenesis. Aspects of lignin biosynthesis and polymerization, cellulose and hemicellulose synthesis, and transcriptional regulation of secondary walls have all been characterized in *B. distachyon*, with genetic, biochemical, and phenotypic data outlined herein. Finally, the use of *B. distachyon* in identifying saccharification and digestibility traits relatable to biofuel feedstock quality in grasses are discussed. Taken together, the reviewed material demonstrates the utility of *B. distachyon* as a model for grass cell wall research, highlighting known and novel facets of cell wall biosynthesis.

Keywords *Brachypodium distachyon* • Lignin • Cellulose • Mixed linkage glucan • Cell wall • Grasses • Biofuel feedstock • Digestibility • Saccharification

Introduction

The importance of grasses as food and forage crops, as well as their more recent appropriation as bioenergy feedstocks, is evident the world over. Thus, understanding the dynamics of growth in these plants is key to maximizing their use. One major aspect of these dynamics is the synthesis and deposition of cell walls. As in all plants, a primary wall surrounds grass cells, with certain cell types also developing a secondary wall. The composition and regulation of cell walls has been investigated in many plant species, most rigorously in the model eudicot *Arabidopsis thaliana*. However, because grasses belong to an evolutionarily distinct group, specific knowledge is needed to appropriately understand their cell walls. The prominent

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rise of *Brachypodium distachyon* as a model for the grasses has allowed for a unified system in which to discuss the known aspects of grass cell wall biology, and moreover has created a platform to expand this knowledge base. In this chapter we will review the use of *B. distachyon* as a model for grass cell walls. Compositional studies have characterized the contents of *B. distachyon* cell walls and compared them with those of other grass species. Significant work has been done in characterizing the enzymes involved in monolignol biosynthesis and incorporation, uncovering new aspects of secondary wall chemistry. Cellulose synthesis has also been investigated, as well as the formation of the distinctive wall polysaccharide, mixed-linkage glucans (MLG). Transcriptional regulation of secondary wall deposition is well defined in *A. thaliana*, and a similar network has begun to be understood in *B. distachyon*. Finally, screens for altered biomass digestibility and saccharification efficiency have been used to identify *B. distachyon* genetic variation for traits that may be applicable to biofuel feedstock engineering.

Brachypodium Cell Wall Composition

Grasses, as a subset of the commelinoid monocots, have salient differences in their cell wall composition from other angiosperms. Grass secondary cell walls are composed primarily of cellulose, hemicellulose, and lignin. Cellulose comprises the majority of grass secondary walls and is the major target of saccharification and fermentation efforts for biofuel production. The hemicelluloses in these species consist of glucuronoarabinoxylans, accompanied by high levels of mixed linkage glucans, as opposed to the xyloglucans common to non-commelinoid monocots, eudicots, and gymnosperms (Vogel 2008). Grasses also have much higher levels of hydroxycinnamates and significantly lower levels of pectins and structural proteins (Vogel 2008).

Grass cell wall composition varies across tissues and throughout development. For *B. distachyon*, these changes have been characterized in leaf, sheath, and stem tissue in plants at the seedling, elongating stem, and reproductive maturity stages. Lignin levels varied among the tissues examined, with the lowest levels in the leaf and sheath and significantly more in the stem. These levels increased with development (Rancour et al. 2012; Matos et al. 2013). Stem cross section analysis showed that lignification occurred early in the development of vascular bundles, concurrent with the presence of stem tissue, while the interfasicular fibers lignified later as the plants reached maturity (Matos et al. 2013). The levels of non-lignified hydroxycinnamates, comprising monomeric and dimeric ferulic acid as well as *p*-coumaric acid, also varied with tissue and development (Rancour et al. 2012). Dimeric ferulates accumulated at higher levels in the sheath and stem compared to leaves, where levels actually decreased from seedling to elongating stages. A similar pattern was observed for monomeric ferulic acid (Rancour et al. 2012). Ferulates are proposed nucleators of lignin polymerization, and as such the commensurate increase of ferulate levels with lignification in the maturing sheath and

stem is understandable, while the observed decrease in leaves remains to be explained. Much like ferulic acid, *p*-coumaric acid levels increased sequentially from leaf to sheath to stem, and likewise from the seedling to the mature plant (Rancour et al. 2012). Ferulic acid and *p*-coumaric acid form linkages between lignin and hemicelluloses through ester bonds. The accumulation of these hydroxycinnamates indicates increased cross linking between the lignin and hemicellulose polymers, a sign of greater secondary wall deposition and maturation.

The major hemicelluloses in grasses are arabinoxylan and MLGs (Vogel 2008). Xyloglucans may be present in low abundance, but their roles as seen in other plant groups seem to be performed by arabinoxylan and MLG in grasses (Vogel 2008). Mixed-linkage glucans are unsubstituted D-glucopyranosyl chains in which segments of 1,4-β-linkages are occasionally interrupted with a 1,3-β linkage. These polymers are deposited primarily during cell elongation, with a sharp decrease at the onset of secondary wall thickening, indicating that they may play a key structural role prior to cell maturation (Christensen et al. 2010; Fincher 2009).

Arabinoxylans consist of xylan chains highly substituted with α-L-arabinofuranosyl units at the O-2 and O-3 positions (Carpita 1996; Urahara et al. 2004). The xylan backbone can also be amended with α-D-glucuronopyranosyl acid and α-4-O-methyl-D-glucuronopyranosyl acid, yielding glucuronoarabinoxylans. Typically, glucuronoarabinoxylans are found in vegetative tissues, while arabinoxylans without glucuronic acids are typical of grain endosperm cell walls (Carpita 1996; Urahara et al. 2004). Higher degrees of substitution on the xylan backbone reduce bonding between arabinoxylans and other polymers, making the walls more soluble and fluid. As the cell matures, the degree of substitution decreases, allowing unsubstituted regions of the xylan backbone to crosslink with other polymers and stiffen the wall. Maturation of the cell wall is also tied to the esterification of O-5 arabinoxylan residues with the hydroxycinnamates ferulic acid and *p*-coumaric acid (Christensen et al. 2010; Grabber et al. 2004). The degree of this cross linking can be estimated over development by the ratios of arabinose:xylose compared to ferulic acid:xylose. Arabinose:xylose is higher in leaves and low in stems, while ferulic acid:xylose is high in stems and increases with maturity, commensurate with secondary wall deposition.

Chemical analysis of cell wall polysaccharides is often achieved by measurement of their constituent monomers. The saccharide components of these polymers are either uronic acids or neutral sugars. The uronic acids represent the oxidized forms of glucose and galactose as glucuronic and galacturonic acid, respectively. These compounds are present in the hemicellulose glucuronoarabinoxylan, as well as in pectin. Total uronic acid levels do not vary between tissue types or developmental stages.

Neutral sugars represent a wider range of compounds, including glucose, galactose, xylose, and arabinose, as well as the minor fractions of rhamnose, fucose, and mannose. The most abundant of these in both tissue types and developmental stages is glucose, derived from cellulose. Glucose levels in shoots at the elongating and mature stages were lowest in leaves and highest in stems; however, as a molar

percentage of total neutral sugar content in these tissues, leaves contained 62 %, sheaths 51 %, and stems 54 % (Rancour et al. 2012). Xylose, arabinose, and galactose rank as the second, third, and fourth most abundant monosaccharides, respectively, and are derived from arabinoxylan and xyloglucan hemicelluloses and arabinogalactan pectins. Xylose levels do not vary with developmental stage, but rather between tissue types in a manner inverse with glucose, with the highest levels of xylose in the leaves (Rancour et al. 2012). Arabinose in the elongating and mature plant reached its highest levels in sheaths and its lowest levels in leaves, but as a molar percentage of neutral sugars, arabinose does not vary between the organs. Galactose levels were highest in young seedlings, indicative of mostly primary walls where this sugar is derived from galactans and arabinogalactans. There was no difference in galactose levels between tissues. Finally, the minor neutral sugars rhamose, fucose, and mannose existed only at very low levels, less than 2 % of the molar percentage, and did not vary in their abundance (Rancour et al. 2012).

Comparison of *B. distachyon* with Other Grass Species

Aspects of *B. distachyon* stem anatomy are highly comparable to other grasses. Vascular bundles are arranged in two rings with the inner set being larger than the outer. A region of sclerenchyma cells known as the interfasicular fibers surrounds the vascular bundles (Matos et al. 2013). Interfasicular fibers and the vascular bundles make up the cortex, the region between the epidermis and inner pith parenchyma. As the plant matures, the stem thickens considerably. This gain in size can be attributed to thickening of the sclerenchyma secondary walls, which occurs from elongation to senescence, whereas vascular bundles are relatively unchanged following internode elongation (Matos et al. 2013). The xylem tends to have several large vessels separated by tracheids, and in some cases there is evidence of a lacuna, the remains of the protoxylem. Anatomically, these features of the *B. distachyon* stem are very similar to those of other related grass species. The vascular patterns of rice (*Oryza sativa*), wheat (*Triticum aestivum*), perennial rye-grass (*Lolium perenne*), and tall fescue (*Festuca arundinacea*) all exhibit two rings of bundles, although the specific arrangement of the bundles in these rings varies slightly between species (Chen et al. 2002; Tufarelli et al. 2003; Patrick 1972; Tu et al. 2010). Larger grasses such as maize (*Zea mays*) and sorghum (*Sorghum bicolor*) have significantly more vascular bundles distributed throughout their stems; however, the anatomy of the bundles themselves, as well as the sclerenchyma tissue surrounding them, is highly comparable to *B. distachyon* (Kiesselbach 1949; Wilson et al. 1993).

Cell wall composition has been compared between *B. distachyon* and several agricultural grasses to determine its suitability as a model for growth dynamics in related grass crops. In comparison with the cereals barley (*Hordeum vulgare*) and wheat at the seedling stage, *B. distachyon* showed similar levels of arabinose and

xylose, indicators of arabinoxylan content. The degree of arabinoxylan substitution decreased over the development of all three species, with the only difference being a generally lower arabinose:xylose ratio in *B. distachyon* (Christensen et al. 2010). Mixed-linkage glucan levels were also similar across the three species (Christensen et al. 2010). There was an increase in esterified hydroxycinnamates that correlated with the decreasing degree of substitutions on the arabinoxylans. While this trend was observed in all three species, *B. distachyon* had overall higher levels of hydroxycinnamates compared to barley and wheat. Interestingly, the percentage of ferulic acid remained constant in each species, with *B. distachyon* having the highest at 34 %, compared to about 27 % for the other two species (Christensen et al. 2010). The overall higher hydroxycinnamates levels in *B. distachyon*, which can be primarily attributed to its strikingly higher *p*-coumaric acid level, are correlated with the relatively lower degree of arabinoxylan substitution in the model grass.

B. distachyon was also compared to switchgrass (*Panicum virgatum*), a proposed energy crop. *B. distachyon* has an overall higher level of hydroxycinnamates, but lower relative lignin abundance in leaves, sheaths, and stems (Rancour et al. 2012). Both have similar levels of *p*-coumaric acid, but *B. distachyon* has significantly more ferulic acid, which may account for the overall difference in hydroxycinnamates.

Compared to several C3 forage grasses (tall fescue, bromegrass, orchardgrass, and reed canarygrass), *B. distachyon* has comparable composition, although there are some differences in the relative levels of neutral sugars as well as overall lignin content (Rancour et al. 2012; Hatfield et al. 2009). *B. distachyon* has more non-glucose sugars, as well as an overall greater glucose content and lower lignin levels. These differences in sugar and lignin content may be due to the small stature of *B. distachyon* compared to the aforementioned forage grasses, which may require greater lignin to support their size.

Comparison of senesced leaf and stem tissue from *B. distachyon*, wheat, *Miscanthus x giganteus*, and maize showed largely similar composition and saccharification (Gomez et al. 2008; Meineke et al. 2014). However, some differences were also observed that suggested phylogeny alone is not a sufficient indicator of relatable biomass productivity. Lower lignin was found in both leaves and stems of *B. distachyon* and wheat compared to *M. x giganteus* and maize. *B. distachyon* had lower cellulose and higher hemicellulose content compared to wheat (Meineke et al. 2014). After acid and enzyme pretreatment, saccharification and ethanol yield were highest in wheat and lowest in *B. distachyon*, with *M. x giganteus* and maize performing similarly to each other (Meineke et al. 2014). *B. distachyon* and wheat are more closely related, both being C3 grasses, while *M. x giganteus* and maize are C4. The differences in content and yield between the C3 grasses in this study suggest that phylogeny alone does not guarantee similar biofuel-related traits. Despite these differences, *B. distachyon* is far more relatable to the grasses than any eudicot models, making it useful model for grass cell wall traits.

Lignin

The term lignin refers broadly to 4-hydroxyphenylpropanoid polymers deposited in secondary plant cell walls. These aromatic polymers confer strength and hydrophobicity to structural and vascular tissues and are highly recalcitrant to mechanical and enzymatic degradation. Lignin polymers form from the combinatorial oxidative coupling of hydroxycinnamate subunits. Three hydroxycinnamyl alcohols, *p*-coumaryl, coniferyl, and sinapyl alcohols, give rise to *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) monolignol subunits respectively (Ralph 2010; Vanholme et al. 2010). The hydroxycinnamates *p*-coumaric acid and ferulic acid are also incorporated into secondary walls in linkages between lignin and hemicellulose polymers. The biochemistry and genetics of grass lignin are extensively reviewed in Harrington et al. (Harrington et al. 2012). Lignin biosynthesis has been defined largely in terms of monolignol biosynthesis, with extensive characterization of the enzymes and transcriptional regulators governing this branch of the phenylpropanoid pathway (Vanholme et al. 2010). Incorporation of these monomers has only recently been investigated, and the exact mechanisms controlling the varied linkages found within the lignin polymer remain to be uncovered. Many of the first lignin biosynthesis mutants were identified in grasses, and while the genetics and biochemistry of lignification have been extensively explored in *A. thaliana*, the understanding of the grasses has kept pace to some extent. *B. distachyon* provides an excellent system in which to further these studies.

The Lignin Methyltransferases, COMT and CCoAOMT

Two of the key steps in lignin biosynthesis involve methyl transferases: caffeoyl CoA 3-*O*-methyltransferase (CCoAOMT) and caffeic acid *O*-methyltransferase (COMT). CCoAOMT acts in the conversion of caffeoyl-CoA to feruloyl-CoA (Vanholme et al. 2010). COMT acts further downstream in the pathway, methylating 5-hydroxy-coniferaldehyde to sinapylaldehyde as part of the shift from coniferyl alcohol to sinapyl alcohol (Vanholme et al. 2010).

The first characterized *COMT* gene was identified in maize, and subsequent discovery in several other species solidified COMT as a key player in lignin biosynthesis (Collazo et al. 1992). In *B. distachyon*, eight genes were identified as putative COMTs numbered *BdCOMT1-8* (Dalmais et al. 2013). These genes were assigned numbers as they appear in the genome, *BdCOMT1-8*, respectively, by Dalmais et al. (Dalmais et al. 2013); however, other publications name *BdCOMT6* (Bradi3g16530) as *BdCOMT4* (Trabucco et al. 2013; Wu et al. 2013) or *BdCOMT1* (Lee et al. 2014). This difference in naming is due to the fact that Bradi3g16530 clusters with four other BdCOMTs as phylogenetically similar to characterized COMT proteins from *A. thaliana*, poplar, rice, maize, ryegrass, switchgrass,

sorghum, and fescue (Dalmais et al. 2013; Trabucco et al. 2013; Wu et al. 2013). BdCOMT1-3 (Bradi1g14870, Bradi2g02380, and Bradi2g02390) also fall into this clade, although they do not group as closely with characterized grass COMTs as Bradi3g16530 does (Dalmais et al. 2013; Trabucco et al. 2013; Wu et al. 2013). Ostensibly, these four genes form a lignin-biosynthesis-related subfamily within the *B. distachyon* COMTs, and analysis of their function by other researchers has resulted in naming these genes BdCOMT1-4. The remaining four *BdCOMT*s (Bradi2g19830, Bradi2g19850, Bradi3g55890, and Bradi4g20020) fall amongst clades containing *O*-methyltransferases in other species that are not known to be involved with lignin biosynthesis. The nomenclature used in this publication will follow Dalmais et al., with all genes and aliases listed in Table 1.

Analysis of the genomic regions surrounding *BdCOMT6* shows synteny to the genomic regions of other characterized grass COMTs (Wu et al. 2013). Beyond genomic collinearity with characterized *COMT* genes, *BdCOMT6* was identified as a COMT functioning in the *B. distachyon* lignin pathway by phylogeny with characterized COMTs and substantiated with enzymatic assays and genetics (Dalmais et al. 2013; Trabucco et al. 2013; Wu et al. 2013; Lee et al. 2014). From the crystal structure of alfalfa (*Medicago sativa*) COMT, catalytic and substrate binding sites have been identified (Zubieta et al. 2002), all of which are preserved in BdCOMT6 (Trabucco et al. 2013; Wu et al. 2013). Other members of the BdCOMT family contain the S adenosyl methyltransferase domain characteristic of OMTs, but vary in their conserved substrate binding residues.

In vitro enzymatic activity assays have shown that recombinant BdCOMT6 is highly active in the methylation of caffeic acid, as is expected in the canonical role of COMTs in lignin biosynthesis (Wu et al. 2013). However, some activity was also observed when BdCOMT6 was challenged with caffeoyl aldehyde and the flavonoid luteolin, indicating that COMT activity may not be strictly dedicated to one metabolic pathway. It has been suggested that single amino acid changes in the COMT substrate binding domains may dictate substrate specificity. For example, both BdCOMT6 and AtCOMT have a valine residue at comparable binding locations, and both show a preference for caffeic acid. Conversely, the alfalfa and *Vanilla planifolia* COMTs have a leucine and isoleucine, respectively, at this location and prefer caffeoyl aldehyde (Wu et al. 2013; Li et al. 2006). The influence of single amino acid changes on substrate specificity remains a topic for further investigation.

Genetic manipulation of the *BdCOMT6* allele revealed that mutations or down regulation resulted in reduced lignin content and modified lignin composition (Dalmais et al. 2013; Trabucco et al. 2013). *Bdcomt6* mutants had 24–31 % less total soluble lignin, with a compositional 40 % loss of S lignin (Dalmais et al. 2013). Also, plants with disrupted *BdCOMT6* activity were more digestible by the bacterium *Clostridium phytofermentans*, yielding more ethanol than control plants (Trabucco et al. 2013). These results solidify the importance of *BdCOMT6* in modulating S lignin levels in *B. distachyon*, and consequently influencing the availability of cell wall polysaccharides for conversion to ethanol.

Table 1 *Brachypodium distachyon* genes described as encoding cell wall related enzymes

Bradi ID#	Alias	Alternative aliases
Bradi1g14870	COMT1 (Dalmais et al. 2013)	
Bradi2g02380	COMT2 (Dalmais et al. 2013)	
Bradi2g02390	COMT3 (Dalmais et al. 2013)	
Bradi2g19830	COMT4 (Dalmais et al. 2013)	
Bradi2g19850	COMT5 (Dalmais et al. 2013)	
Bradi3g16530	**COMT6** (Dalmais et al. 2013)	COMT4 (Trabucco et al. 2013; Wu et al. 2013; Lee et al. 2014), COMT1 (Lee et al. 2014)
Bradi3g55890	COMT7 (Dalmais et al. 2013)	
Bradi4g20020	COMT8 (Dalmais et al. 2013)	
Bradi3g06480	**CAD1** (Trabucco et al. 2013; Bouvier et al. 2013)	CAD5 (Bukh et al. 2012)
Bradi3g17920	CAD2 (Trabucco et al. 2013; Bouvier et al. 2013)	CAD7 (Bukh et al. 2012)
Bradi3g22980	CAD3 (Trabucco et al. 2013; Bouvier et al. 2013)	CAD8 (Bukh et al. 2012)
Bradi4g29770	CAD4 (Trabucco et al. 2013; Bouvier et al. 2013)	CAD3 (Bukh et al. 2012)
Bradi4g29780	CAD5 (Trabucco et al. 2013; Bouvier et al. 2013)	CAD4 (Bukh et al. 2012)
Bradi5g04130	CAD6 (Trabucco et al. 2013; Bouvier et al. 2013)	CAD1 (Bukh et al. 2012)
Bradi5g21550	CAD7 (Trabucco et al. 2013; Bouvier et al. 2013)	CAD2 (Bukh et al. 2012)
Bradi5g15380	PAL1 (Lee et al. 2014)	
Bradi2g53470	C4H1 (Lee et al. 2014)	
Bradi4g08650	CCR3 (Lee et al. 2014)	
Bradi3g39420	CCoACOMT1 (Lee et al. 2014)	
Bradi3g39390	CCoACOMT2 (Lee et al. 2014)	
Bradi1g48370	CCoACOMT3 (Lee et al. 2014)	
Bradi2g36910	**PMT** (Petrik et al. 2014)	
Bradi1g66720	**LAC5** (Wang et al. 2015)	
Bradi1g74320	**LAC6** (Wang et al. 2015)	
Bradi2g23370	LAC8 (Wang et al. 2015)	
Bradi2g54740	LAC12 (Wang et al. 2015)	
Bradi2g34240	CESA1 (Handakumbura et al. 2013; Burton et al. 2008)	
Bradi1g04597	CESA2 (Handakumbura et al. 2013)	
Bradi1g54250	CESA3 (Handakumbura et al. 2013)	
Bradi3g28350	**CESA4** (Handakumbura et al. 2013)	
Bradi1g29060	CESA5 (Handakumbura et al. 2013)	
Bradi1g53207	CESA6 (Handakumbura et al. 2013)	
Bradi4g30540	**CESA7** (Handakumbura et al. 2013)	

(continued)

Table 1 (continued)

Bradi ID#	Alias	Alternative aliases
Bradi2g49912	CESA8 (Handakumbura et al. 2013)	CESA4 (Valdivia et al. 2013)
Bradi1g02510	CESA9 (Handakumbura et al. 2013)	
Bradi1g36740	CESA10 (Handakumbura et al. 2013)	
Bradi3g16310	**CSLF6** (Kim et al. 2015)	
Bradi1g76730	**SWN1** (Valdivia et al. 2013)	
Bradi5g16917	**SWN2** (Valdivia et al. 2013)	
Bradi3g50067	**SWN3** (Valdivia et al. 2013)	
Bradi1g52187	**SWN4** (Valdivia et al. 2013)	
Bradi5g27467	**SWN5** (Valdivia et al. 2013)	
Bradi3g13117	**SWN6** (Valdivia et al. 2013)	
Bradi1g50057	**SWN7** (Valdivia et al. 2013)	
Bradi3g13727	**SWN8** (Valdivia et al. 2013)	
Bradi2g39320	**BdXCP1** (Valdivia et al. 2013)	
Bradi4g06317	**BdMYB1** (Valdivia et al. 2013)	

Gene numbers and aliases are listed with references. Alternative aliases are also listed along with their respective references. Bolded genes have been functionally characterized

BdCAD Family Characterizations

The final step in monolignol biosynthesis is mediated by the cinnamyl alcohol dehydrogenase (CAD) enzyme. Some of the first mutants with lignin-associated defects were identified as having causative mutant *CAD* alleles, such as in the *brown midrib* mutants found in sorghum and maize (Saballos et al. 2009; Sattler et al. 2009; Fornalé et al. 2011; Vignols et al. 1995). In *B. distachyon*, the CAD gene family has seven members as determined by amino acid sequence alignment with known CAD enzymes from sorghum, rice, wheat, and tall fescue (Sattler et al. 2009; Chen et al. 2003; Hirano et al. 2012; Ma 2010). This family was numbered BdCAD1-7 by Trabucco et al. 2013 and Bouvier et al. 2013 (Trabucco et al. 2013; Bouvier et al. 2013); however, alternative numbering appears in publications by Guo et al. and Buhk et al. (Bukh et al. 2012; Guo et al. 2010). Refer to Table 1 for gene numbers and aliases used in this and other publications.

Amino acid sequence comparison between the BdCADs and characterized CAD proteins shows high similarity with the conserved binding and catalytic domains (Trabucco et al. 2013; Bouvier et al. 2013; Bukh et al. 2012). BdCAD1 was found to have the highest amino acid sequence similarity with characterized CAD proteins from other species, containing 10 of the 12 conserved amino acid residues known to be involved in substrate binding, including those that convey specificity for aromatic alcohol substrates, as well as NADP(H) binding (Trabucco et al. 2013; Bukh et al. 2012). *BdCAD1* is expressed primarily in stem tissue, with tenfold higher expression than other *BdCAD*s. Recombinant BdCAD1 and BdCAD4 enzyme were capable of reducing coniferyl aldehyde and sinapyl aldehyde to coniferyl and

sinapyl alcohol, respectively, with significantly higher activity seen in BdCAD1 (Bukh et al. 2012). Further genetic characterization of *BdCAD1* has reinforced the idea that this gene plays a prominent role in lignin biosynthesis. Similar to the analysis of *BdCOMT4*, knockdown lines expressing amiRNAs as well as TILLING mutant lines have been generated for the *BdCAD1* locus (Bouvier et al. 2013). One of the *Bdcad1* TILLING mutants has a change at amino acid 192 of glycine to aspartic acid; a substitution of glycine to serine at the identical site in sorghum was shown to be the causative mutation of the *bmr6* mutant (Saballos et al. 2009). This alteration may disrupt the ability of the CAD protein to bind NADPH as well as the substrate and may also influence the zinc-binding catalytic site. Two other lines with altered BdCAD1 protein structure also showed a mutant phenotype (Bouvier et al. 2013).

In mutant *Bdcad1* lines, the identifying visible phenotypes showed a range of brown to reddish discoloration of the spikelet, flowers, rachilla, nodes and lemma (Bouvier et al. 2013), as well as a classic brown leaf midrib (Trabucco et al. 2013). This type of discoloration is typical of lignin mutants with defects in CAD function, particularly the browning of the leaf midrib seen in C4 grass lignin mutants (Sattler et al. 2009; Vignols et al. 1995). In addition to the visible phenotypes, transverse cross-sections of the stem internode revealed a decrease in S lignin in the sclerenchyma, epidermis, and vascular bundle sheath (Trabucco et al. 2013; Bouvier et al. 2013). This was reflected in compositional analysis of the *Bdcad1* mutants, which had 20–26 % less total lignin and proportionally a 60 % reduction in S units (Trabucco et al. 2013; Bouvier et al. 2013). Consistent with the reduction of S lignin, there was also a reduction in the *p*-coumaric acid levels in *Bdcad1* mutants (Bouvier et al. 2013). *P*-coumaric acids are involved in the cross linking between hemicellulose arabinoxylans and lignin polymers, particularly through ester linkage to S lignin monomers (Ralph 2010; Vanholme et al. 2010; Harrington et al. 2012; Bouvier et al. 2013). Ferulic acid levels, which are ester-linked to hemicelluloses much like *p*-coumaric acid is, were not affected (Bouvier et al. 2013). The inter-unit bonds in the *Bdcad1* mutants were also affected, with higher levels of biphenyl ether (4-*O*-5) and biphenyl G-G dimer (5-5) linkages. Higher frequencies of these inter-unit bonds indicate a greater degree of branching within the polymer. Consistent with this observation, a greater frequency of terminal phenolic groups was observed through thioacidolysis in the *Bdcad1* mutants (Bouvier et al. 2013). The relative levels of phenolic terminal units on the lignin polymer are positively associated with the degree of reactivity to alkaline and oxidative degradation.

Interestingly, *Bdcad1* mutants also showed increased levels of β-*O*-4-coupled sinapyl aldehyde in the lignin polymer, while coniferyl aldehyde levels remained unchanged (Bouvier et al. 2013). Altered incorporation of sinapyl aldehyde alone has not been reported in *cad* mutants of other species, where sinapyl and coniferyl aldehyde levels accumulate similarly. This suggests that in *B. distachyon*, altered BdCAD1 activity not only reduces the incorporation of S units, but also promotes the β-*O*-4 coupling of sinapyl aldehyde. This is not altogether surprising considering that loss of BdCAD1 would interrupt the conversion of cinnamyl aldehydes to cinnamyl alcohol lignin monomers; however, the specific

accumulation of sinapyl aldehydes and not coniferyl aldehydes is thus far distinct to *B. distachyon*.

While there was no difference in biomass between the mutants and wild type lines, *Bdcad1* mutant saccharification levels were significantly higher than the wild type (Trabucco et al. 2013; Bouvier et al. 2013). This increase in available sugars was also reflected in a higher ethanol yield (9–17 %) from *C. phytofermentans* digestion of mutant lines (Trabucco et al. 2013). Reduction in total lignin content, altered cross linking with hemicellulose, and modified branching degrees within the lignin polymer are all likely contributors to the observed increase in saccharification and ethanol yield in these mutants, indicating that modulation of *BdCAD1* could play a major role in the engineering of bioenergy crops (Bouvier et al. 2013).

Hydroxycinnamate Cross Linking

The lignin polymer is cross-linked to hemicelluloses via non-lignified hydroxycinnamates. The addition of H lignin to the G/S backbone has been shown to result from acylation of the coniferyl and sinapyl monolignols with *p*-coumaric acid at the γ-OH position prior to their incorporation into the lignin polymer. Enzymatically, this is accomplished by *p*-coumaroyl-CoA:monolignol transferase (PMT), which was recently characterized in *B. distachyon* (Petrik et al. 2014). *BdPMT* RNAi and the sodium-azide-generated *Bdpmt-1* loss-of-function mutant had significantly reduced *p*-coumaric acid levels.

Since *p*-coumaric acid can acylate both lignin and hemicelluloses in grasses, an important distinction was made between the total *p*-coumaric acid released through mild alkaline hydrolysis and the *p*-coumaric acid specifically from lignin. In wild type plants, *p*-coumaric acid was found almost exclusively on S lignin subunits, with no detectable levels found in the *Bdpmt-1* mutant. No difference was found in the levels of *p*-coumaric acid or ferulic acid linked to arabinose between *Bdpmt-1* and the wild type, showing that *BdPMT* is exclusively responsible for monolignol acylation, with another unknown enzyme(s) decorating hemicelluloses with *p*-coumaric acid and ferulic acid (Petrik et al. 2014).

Over-expression of *BdPMT* resulted in reduced overall lignin, but almost two-fold increased *p*-coumaric acid levels compared to wild type plants (Petrik et al. 2014). The loss of lignin in the over-expression line is potentially due to a redirection of *p*-coumaroyl-CoA from the general monolignol synthesis pathway to the enhanced acylation activity of excess BdPMT. Reduced lignin content also implies a higher ratio of arabinoxylan to lignin, a metric which may explain the increased levels of ferulic acid in the over-expression lines. Over-expression lines were more digestible than wild type, while *Bdpmt-1* showed no difference in digestibility (Petrik et al. 2014). The increase in digestibility in these over-expression lines is likely due not only to modified lignin content, but also composition. As with *cad* mutants, lines over-expressing *BdPMT* with an abundance of phenolic terminal units on their polymer chains are more susceptible to alkaline pretreatment degradation.

Laccases

Incorporation of the monolignol subunits into polymer chains has long been known to be dependent on hydrogen peroxide radicals (Vanholme et al. 2010). Peroxidases and laccases have recently been characterized as apoplastic enzymes involved in catalyzing and mediating this polymerization. In *A. thaliana*, two laccases (AtLAC4 and AtLAC17) have been shown to directly regulate lignification in the protoxylem tracheary elements (Schuetz et al. 2014). Orthology with these *A. thaliana* laccases was used to identify 29 genes in *B. distachyon* with putative laccase activity, numbered *BdLAC1-29* based on sequential genome location (Wang et al. 2015). The BdLACs cluster into four large clades when compared with laccases involved with phenolic oxidation in other species, with clade IV including genes known to be involved with lignification. BdLAC6 (Bradi1g74320) is most similar to AtLAC4, while BdLAC5 (Bradi1g66720), BdLAC8 (Bradi2g23370), and BdLAC12 (Bradi2g54740) are most similar to AtLAC17. *BdLAC5* was also orthologous to sugarcane *SofLAC* which is implicated in lignification (Wang et al. 2015; Cesarino et al. 2013). As with characterized laccases in *A. thaliana, BdLAC5* and *BdLAC6* exhibit an expression pattern similar to other genes that encode secondary-wall-related enzymes. Both BdLAC5 and BdLAC6 were also found to localize in the apoplast adjacent to sclerenchyma cells (Schuetz et al. 2014).

Genetic evidence for *BdLAC5* and *BdLAC6* function was provided from the analysis of TILLING mutants with defects at the respective loci. *Bdlac* mutants were smaller in stem height and diameter than wild type, with a modest reduction in lignin levels. More specifically, the disruption of *BdLAC5* resulted in decreased G lignin. Ferulate levels were also affected in these lines, with more ferulic acid found in ester linkages to xylans and less ferulic acid ether linked to lignin (Wang et al. 2015). This shift towards ferulation of xylans is likely due to reduced availability of integrated lignin monomers on which to form ether bonds due to the loss of laccase activity.

Additional Lignin Biosynthetic Enzymes

While two enzymes in the monolignol biosynthetic pathways, CAD and COMT, and two involved with incorporation of monolignols into the polymer backbone, LAC and PMT, have been characterized in *B. distachyon*, many more genes related to lignin biosynthesis remain to be characterized. Several candidates have been identified by screening a population of gamma-irradiated mutants for reduced lignin content as determined by histochemical staining of internode cross sections (Lee et al. 2014). Twenty-five lines were analyzed for polymorphisms in loci of genes with homology to known lignin biosynthetic enzymes, including putative genes for *PAL1*, *C4H1*, *CCR3*, *CAD4*, *COMT1*, *CCoACOMT1*, *CCoACOMT2*, and *CCoACOMT3* (Table 1) (Lee et al. 2014). Multiple mutations were found

throughout these genes, with several predicted to be missense or nonsense mutations. Although these mutants were not characterized further, this study demonstrates the potential for using *B. distachyon* mutant populations for meaningful forward-genetic screens for cell wall traits.

Cellulose

Cellulose comprises the major fraction of cell wall polymers. It is synthesized from glucose monomers connected by β-1-4 glucan linkages to form long microfibrils that are extruded from the plasma membrane and aligned along microtubule tracks. The synthesis and deposition of the cellulose microfibrils is a long-studied area of plant cell biology that has been extensively elucidated in the model eudicot *A. thaliana* (Somerville 2006; McFarlane et al. 2014). The cellulose synthesis complex is comprised of CELLULOSE SYNTHASE A proteins arranged in a rosette formation on the plasma membrane. These processive glycosyltransferases typically represent a small gene family of about ten members in many plants. To an extent, these members have been shown to act differentially in the synthesis of either the primary or secondary cell wall.

The *B. distachyon* CESA family has ten members: *BdCESA1* (Bradi2g34240), *BdCESA2* (Bradi1g04597), *BdCESA3* (Bradi1g54250), *BdCESA4* (Bradi3g28350), *BdCESA5* (Bradi1g29060), *BdCESA6* (Bradi1g53207), *BdCESA7* (Bradi4g30540), *BdCESA8* (Bradi2g49912), *BdCESA9* (Bradi1g02510), and *BdCESA10* (Bradi1g36740) (Table 1) (Handakumbura et al. 2013). All ten members, with the exception of BdCESA10, contain the canonical eight transmembrane domains and the hyper-variable regions, as well as the UDP-glucose binding motif (D,D,D, QxxRW). Additionally, all the BdCESAs, with the exception of BdCESA5 and 10, have the RING-type zinc finger motif involved in protein dimerization. BdCESA1, 3, 6, and 9 are considered to be primary-wall-related CESAs, and exhibit high expression in both root and stem tissue (Handakumbura et al. 2013). The secondary-wall-associated CESAs are BdCESA4, 7, and 8, and these genes are expressed at higher levels in the stem compared to root tissue (Handakumbura et al. 2013).

The secondary CESAs in *B. distachyon* (BdCESA4, 7, 8) are numbered in accordance with their orthology to the *A. thaliana* secondary CESAs. BdCESA4 and 7 have been further characterized *in planta*. *In situ* hybridization was used to identify the expression patterns of these genes, and probe staining suggested high expression in vascular bundles, epidermis, and interfasicular fiber regions, with little expression detected in the pith parenchyma (Handakumbura et al. 2013). This expression pattern is consistent with tissue types that undergo secondary wall deposition. Knockdown of *BdCESA4* and *BdCESA7* resulted in delayed emergence of the inflorescence and smaller stature. Stem cross-section analysis showed thinner cell walls and reduced crystalline cellulose content in the knockdown lines (Handakumbura et al. 2013).

Mixed-Linkage Glucans

Mixed-linkage glucans are unbranched glucose chains with both β1,3- and β1,4 linkages. These polysaccharides are unique among land plants to the Poaceae, including the grasses, and the monilophyte *Equisetum* spp. These carbohydrates are also found in some pathogenic fungi and lichen-forming ascomycete species (Burton and Fincher 2009; Fry et al. 2008). The synthesis of these polymers is attributed in part to members of the cellulose synthase-like (CSL) families CSLF and CSLH. *CSLF4*, *CSLF6*, and *CSLH* from barley, as well as rice *CSLF2*, have been characterized *in planta* for the production of MLG (Burton et al. 2006, 2008, 2011; Doblin et al. 2009).

While manipulation of these *CSLF* and *CSLH* genes from barley and rice has been shown to modulate MLG content, parts of the mechanism behind MLG biosynthesis have been uncovered in *B. distachyon*. BdCSLF6 was shown to localize to the Golgi membrane, with the proposed catalytic domain on the cytosolic side (Kim et al. 2015). This is in line with the established paradigm of hemicelluloses and pectins being synthesized and modified at the Golgi before export to the cell wall; however, it also suggests that substrates do not need to be imported into the Golgi body, but rather synthesis occurs in the cytosol.

Furthermore, BdCSLF6 has been proposed to be capable of forming both β1,3 and β1,4 bonds. Transgenic yeast were found to have both bond types after expression of the single *BdCSLF6* transgene; previous studies in *A. thaliana* using rice and barley *CSLF6* orthologs also generated β1,3 and β1,4-containing MLG (Burton et al. 2006; Doblin et al. 2009; Kim et al. 2015). Although these observations do not rule out the role of endogenous genes in contributing to these multiple linkage types, similar dual functionality has been observed in the hyaluronic acid biosynthetic pathway where a related glycosyltransferase generates both linkages types (May et al. 2012; Weigel and DeAngelis 2007).

Transcriptional Regulation of the Secondary Cell Wall

The genetic control of secondary wall polymer synthesis has best been described through the elucidation of a transcription factor regulatory network. This network is most clearly defined in *A. thaliana*, with many transcription factors characterized as functional activators or repressors of secondary-wall-related genes. In this network there are a series of feed-forward loops containing NAC proteins that regulate a suite of other transcription factors, mostly MYB family proteins that also directly influence genes related to biosynthetic pathways (Taylor-Teeples et al. 2014; Zhong et al. 2008, 2010a). The cell wall regulatory network in grasses is far less well defined (Handakumbura and Hazen 2012). However, recent work in *B. distachyon* is beginning to assemble a framework similar to the *A. thaliana* network that is representative of cell wall transcriptional regulation in the grasses.

As in *A .thaliana*, several NAC proteins have been identified as regulators of secondary wall biosynthesis in *B. distachyon*. Eight such genes, the SECONDARY WALL NACs (SWNs), were identified by phylogenetic analysis (Valdivia et al. 2013). The first six members of the family, BdSWN1-6, are orthologs of the *A. thaliana* VASCULAR NAC DOMAIN (VND) transcription factors (Ohashi-Ito et al. 2010; Yamaguchi et al. 2011), while BdSWN7 and 8 are orthologous to the SND1, NST1, and NST2 transcription factors in *A. thaliana* (Mitsuda et al. 2005, 2007). All of the BdSWNs were found to be expressed in the stem internode, with particularly high expression of *BdSWN3*, *4*, *6*, and *7*. *BdSWN5* and *8* were found to be expressed in the leaves as well, while expression of all other *BdSWN* genes was very low in those organs. Likewise, with the exception of *BdSWN1* and *2*, expression in the roots was also low (Valdivia et al. 2013).

Transient expression of the *BdSWN*s in tobacco leaves via *Agrobacterium* infiltration resulted in ectopic deposition of secondary walls throughout the affected mesophyll and epidermal cells. This phenotype was particularly strong in the case of *BdSWN1*, *3*, *4*, and *7* (Valdivia et al. 2013). Furthermore, extensive cell death resulted from expression of *BdSWN1*, *2*, *3*, and *4*, sometimes even before secondary wall deposition began. Induced over-expression of *BdSWN5* resulted in ectopic cell wall deposition in the coleoptile parenchyma, as well as in the roots. In the roots, this deposition was only observed in cells close to the root tip, implying that only cells in the differentiation zone were responsive to *BdSWN5* (Valdivia et al. 2013).

Inducible expression of BdSWN5 in *B. distachyon* strongly upregulated *BdCESA4*, while the primary wall *BdCESA1* was not affected (Valdivia et al. 2013). Furthermore, *BdSWN5* expression was also shown to upregulate the representative secondary-wall-related genes encoding the xylem-specific cystine protease BdXCP1 and the MYB transcription factor BdMYB1. *BdXCP1* is the ortholog of *A. thaliana AtXCP1* and *2*, which are activated as part of the programmed cell death pathway that is associated with secondary wall deposition and senescence (Avci et al. 2008). Likewise, *BdMYB1* is the ortholog of *A. thaliana AtMYB46/83*, known transcriptional activators of secondary-wall-related genes (Zhong et al. 2007; Zhong and Ye 2012; Ko et al. 2012). The mechanism of BdSWN5-mediated activation was shown to be dependent on the presence of the highly conserved Secondary wall NAC Binding Element (SNBE) (Zhong et al. 2010a, b) in the promoters of *BdMYB1* and *BdXPC1*. Mutation of the SNBE site resulted in 93–95 % loss of BdSWN5 binding, indicating that this motif is necessary for activation by the BdSWNs (Valdivia et al. 2013).

Mutant Screens for Biofuel Feedstock Quality

With regard to the application of grasses as feedstocks for bioenergy production, the efficient extraction of fermentable sugars is paramount. Due to the recalcitrant nature of cell walls, these sugars are inaccessible without some measure of pretreatment. Force, heat, acid, enzymatic treatments, and other pretreatments

have been employed to extract cell wall polysaccharides for fermentation with varying degrees of success. In developing analytical assays for investigating differences in conversion, total sugar extraction is not always the goal, but rather sensitive detection of changes in saccharification potential. One such assay employs a modest acid treatment combined with heat followed by enzymatic digestion (Gomez et al. 2008). This was found to release approximately 25 % of the total wall polysaccharides in *B. distachyon*, particularly xylose and xylose oligomers along with arabinose, glucose and small amounts of mannose (Gomez et al. 2008). This is typical of grasses, where the more labile hemicelluloses require the least treatment to extract. Heat- and acid-based pretreatment of *B. distachyon* resulted in saccharification profiles similar to those of other grasses. Compared to wheat, *M. x giganteus*, maize, and barley, *B. distachyon* had a similar pretreatment-generated profile of arabinose and xylose (Gomez et al. 2008).

Feedstock quality has also been assessed with a simultaneous saccharification and fermentation assay using the forest soil bacterium *Clostridium phytofermentans*. This anaerobic bacterium can not only degrade a variety of cell wall polymers but also metabolize both five- and six-carbon sugars directly to ethanol. *C. phytofermentans* has been shown to efficiently ferment cellulose, hemicelluloses, pectins, and starch from a variety of lignocellulosic feedstocks (Lee et al. 2012; Warnick et al. 2002). Because of these properties, *C. phytofermentans* has been used to investigate natural genetic variation in *B. distachyon* feedstock conversion efficiency. Five lines representing diverse genetic backgrounds were tested. The highest ethanol yield came from the accessions Bd2-1 and Bd3-1, which are relatively similar to each other, while the least ethanol came from the Spanish Bd30-1 accession (Lee et al. 2012).

In addition, mutant *B. distachyon* populations have been used to identify candidate genes related to saccharification efficiency. Chemically mutagenized plants were screened for saccharide release following alkaline pretreatment and enzymatic digestion. Twelve lines with altered saccharification values were identified and named *saccharification1-12* (*sac1-12*) (Marriott et al. 2014). These mutants exhibited a range of cell-wall-related phenotypes, including reduced lignin, altered S:G lignin ratios, increased H lignin, reduced crystalline cellulose, and decreased esterified ferulates. The causative mutations in these lines were mapped to a 2.6 Mbp region containing 14 polymorphisms, two of which were in exons and resulted in altered amino acid sequences. The glycine-to-serine substitution in Bradi2g01480 is predicted to disrupt the function of glycosyltransferase 61 (GT61) (Marriott et al. 2014). Bradi2g01480 is a member of the grass-specific clade C of glycosytransferases. Members of this clade, such as the wheat *XYLAN ARABINOTRANSFERASES* (*XAT*s) and the rice *XYLOSYL ARABINOSYL SUBSTITUTION OF XYLAN* (*XAX*), have been shown to mediate arabinoxylan substitution (Anders et al. 2012; Chiniquy et al. 2012). Rice *xax1* and *B. distachyon sac1* have similar phenotypes, including increased saccharification and decreased ferulate content, indicating that *Bdsac1* phenotypes may also be the result of a glycosyltransferase defect, such as that detected in Bradi2g01480.

Mutant screens for altered saccharification also identified the *spaghetti1* (*spa1*) mutant, which has brittle stems and is twofold more digestible than wild type lines (Timpano et al. 2015). These mutant phenotypes are believed to be a result of not only altered polymer levels, but also compromised structure in the cells walls. The *spa1* mutant has thinner secondary cell walls with less cellulose but higher hemicellulose and lignin levels than wild type. The cellulose also exhibited reduced crystallinity. Arabinoxylan levels were increased, as was the level of *p*-coumaric acid ester linked to these hemicelluloses. Lignin from the *spa1* plants had a higher frequency of inter-unit resistant bonds, as well as increased H monomer content (Timpano et al. 2015). Together, these changes are reminiscent of stress lignin deposited in compression wood (Timell 1986), suggesting that *spa1* modulates some aspect of cell wall formation related to mechanical stress. Digestibility of *spa1* is attributed to the high friability of its biomass, giving rise to significantly smaller particles than wild type plants after grinding. Smaller particle size favors saccharification efficiency, and it is thought that the altered wall structure of *spa1* gives rise to brittle and thus more friable biomass (Timpano et al. 2015). The causal mutation behind the *spa1* phenotype has yet to be identified.

Conclusion

Our understanding of grass cell wall biosynthesis is rapidly growing, in part due to the expansion of available genetic resources and more relevant model systems such as *B. distachyon*. Characterizations of previously identified cell wall biogenesis components have established *B. distachyon* as a viable platform for cell wall research, while compositional studies have shown this species to be comparable to many grass crops in both content and anatomy. Outstanding questions regarding components of hemicellulose synthesis and deposition, lignin polymerization, expanded transcriptional regulatory networks, and saccharification traits all remain to be answered. Ongoing research in *B. distachyon* will further our knowledge in these areas and in doing so will inform our understanding of biomass accumulation in grasses.

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