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ANALYSIS OF PLANT LIGNIN FORMATION AND ENGINEERING FOR BIOFUELS PRODUCTION

Deborah L. Petrik

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Grasses such as switchgrass and Miscanthus are attractive feedstock candidates for second generation biofuels because they are perennials that can grow on less fertile or "marginal" lands not used for food crops, require little fertilizer and modest pest and disease management, and are not a human food source. Releasing the energy potential contained in plant-based cellulosic material requires either conversion to a liquid biofuel such as ethanol or burning. Thus, much of the current research focuses not only on understanding grass development as it relates to yield, but on how to best enhance traits affecting the quality of biomass for its efficient conversion to biofuels. In order to efficiently extract the sugars making up the cellulose and hemicelluloses components of the plant cell wall, the phenolic polymer lignin, in which they are embedded and to which they are crosslinked, must be removed. To lower the cost of biomass conversion, approaches to decrease lignin content or alter its structure to allow ease of removal may be employed. This work aims to study genes involved in lignin biosynthesis and modification, in order to identify points in the pathway that can be targeted to ease lignin recalcitrance. Efforts to modify plant cell walls must be done without harming plant

fitness, thus cell wall related gene promoters hold value as switches for transgenic modification in specific cells and at specific times in development. Therefore, the second goal of this work was to clone three secondary cell wall gene promoters into monocot transformation vectors and thoroughly characterize their expression patterns. These utility vectors will be made available to the scientific community for use in driving genes of interest specifically in cells producing secondary cell walls.

ANALYSIS OF PLANT LIGNIN FORMATION AND ENGINEERING FOR
BIOFUELS PRODUCTION

DEBORAH L. PETRIK

A Dissertation Submitted in Partial
Fulfillment of the Requirements
for the Degree of

DOCTOR OF PHILOSOPHY

School of Biological Sciences

ILLINOIS STATE UNIVERSITY

2015

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ANALYSIS OF PLANT LIGNIN FORMATION AND ENGINEERING FOR
BIOFUELS PRODUCTION

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D.L.P.

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CHAPTER I

LIGNOCELLULOSIC DERIVED BIOFUEL: BACKGROUND

Current and Future Energy Demands

In 2008, oil, natural gas, and coal provided a total of 81.2% of the world's energy sources (Source: The USA Energy Information Administration). In the United States, military leaders and politicians recommend decreasing the nation's dependence on foreign oil as a means to improving political and economic stability. Yet, the level of fossil fuel consumption has lessened only slightly, despite the known environmental consequences due to the extraction methods used and the emission of greenhouse gases. The production of ethanol derived via fermentation of sugars harvested from corn endosperm shows the potential of using chemical compounds produced by plants as a renewable fuel resource. Currently, the only large-scale commercial production of bioethanol has been from corn seed in the US, and sugar cane in Brazil. In the US, the majority of corn produced is used as animal feed, and for the production of corn syrup used as sweeteners in many processed foods. This has led to debate over land usage for "fuel versus food" application, which has made the public wary to the expansion of highly productive crop land to grow corn purely for bioethanol production.

Ability of Lignocellulosic Derived Biofuels to Partially Fill the Energy Supply Gap

In order to provide alternative feedstocks for biofuel production that will not compete with the food supply, scientists are exploring the feasibility of developing renewable and sustainable sources of energy, including generating biofuels from plant biomass. Plant biomass refers to the vegetative tissue of the plant, namely the leaves and stems. Most likely, plants would be harvested once senesced; therefore, the sugars in the plant biomass material would be extracted from plant cell walls.

Components of the Plant Cell Wall

Cell walls serve multiple functions to the plant including providing mechanical strength, a physical barrier against pathogens, and encasing the plasma membrane of the cell to aid in maintenance of turgor pressure. Plant cell walls consist of up to three specialized layers: the middle lamella, the primary cell wall, and the secondary cell wall. Understanding the makeup of each of these layers will allow the design of efficient methods to extract sugars contained in plant cell wall polymers. Further, knowledge of how the components of each layer are synthesized will foster innovative approaches to transgenic modification of the cell wall to permit efficient sugar extraction without weakening the health of the plant.

The outermost layer of the plant cell wall, the middle lamella, is located at the junction of two adjacent plant cells and the pectin it contains serves to glue adjacent cells together. Internal to the middle lamella, the primary cell wall consists of a network of linked cellulose and hemicellulose fibers embedded in a pectin matrix. The primary cell wall has the ability to expand during plant cell elongation via the movement of cellulose

microfibrils within the loose hemicellulose/pectin matrix. Upon cessation of cell elongation and differentiation, certain plant cell walls synthesize a third internal layer, the secondary cell wall, which is made of cellulose, xylan containing hemicellulose (substituted with arabinoxylan in the case of grasses), and lignin. Cross-linking of the lignin polymer to hemicelluloses that coat the cellulose microfibrils makes the secondary cell wall mechanically strong to support a taller growth habit. The phenolic rings within lignin provide hydrophobicity within mature xylem vessels to allow for effective water movement across greater distances via the mechanism of transpiration.

Cellulose Biosynthesis

Extensive research to characterize the mechanisms of cellulose, hemicellulose, pectin, and lignin biosynthesis has reached a moderate level of understanding of the substrates, products, and enzymatic reactions involved. For example, it has been well characterized that cellulose consists of linear chains of B-1,4 D-glucose subunits that are linked together into parallel chains to form a crystalline structure. These parallel cellulose chains spontaneously bundle together to form cellulose microfibrils (Kumar and Turner, 2015; Carroll and Sommerville, 2009). Each cellulose microfibril is synthesized at the plasma membrane from its glucose subunits by a rosette shaped protein complex containing six cellulose synthase enzymes (Mueller and Brown, 1980). Verification of this rosette shaped structure of the cellulose synthase complex (CSC) came in 1999 when Kimura et al. (1999) successfully visualized CSCs in freeze fractured plasma membranes using immune-gold localization. Upon sequencing of the *Arabidopsis thaliana* genome, functional genomics was employed to identify ten different *CESA* genes (Richmond, 2000; Richmond and Somerville, 2000).

In the primary cell wall of most tissues of *Arabidopsis thaliana* (the first plant species in which the CESA genes were characterized), the three enzymatic subunits of the cellulose synthase complex are encoded by the *CESA1*, *CESA3*, and *CESA6* genes (with *CESA2* and *CESA5* able to substitute for *CESA6*) in the case of cellulose made in the primary cell wall (Li et al, 2014). Yet, expression of other *CESA* genes appears to occur in specialized tissues. For example, *CESA1*, *CESA2*, *CESA3*, and *CESA9* are the only *CESAs* highly expressed in the *Arabidopsis thaliana* embryo. Interestingly a putative null mutation in the *CESA1* gene allowed primary cell walls to still be made in the embryo, but they were thin and fragmented (Beekman et al, 2002). This implies that another gene was able to substitute for *CESA1*, but its encoded enzyme subunit may not function as well as *CESA1* in the CSC. In contrast to possible overlap of function of *CESAs* involved in making primary cell walls depending on tissue type, identification of *Arabidopsis thaliana* *IRX* mutants having irregular xylem phenotypes in the inflorescence, followed by the mapping, sequencing, and characterization of the responsible genes, led to identification of *CESA4*, *CESA7*, and *CESA8* as the genes necessary and sufficient to encode the three subunits of the cellulose synthase complex in the secondary cell wall (Taylor et al, 1999, 2000; Turner and Somerville, 1997). Knockout of one of these three genes led to the inability to produce cellulose in the secondary cell wall, although cellulose could still be synthesized in the primary cell wall, leading to the conclusion that other members of the *CESA* family were unable to substitute for *BdCESA4*, *BdCESA7*, or *BdCESA8* to constitute a functional CSC at the secondary cell wall.

Hemicellulose Biosynthesis

Hemicellulose consists of a more complex polysaccharide structure compared to cellulose. In contrast to cellulose's singular makeup of linear glucose subunits, hemicellulose is a branched structure made from several different polysaccharides including xylan, arabinoxylan, glucuronoxylan, xyloglucan, and glucomannan linked in an equatorial configuration (Scheller and Ulvskov, 2010). These polysaccharides are in turn made from several sugar monomers including mannose, apiose, arabinose, galactose, rhamnose, and xylose, many of which are derived from UDP-GlcUA (Tenhaken and Thulke, 1996). The synthesis of hemicellulose from its sugar subunits occurs at the membrane of the Golgi, and is performed by transmembrane proteins. Upon synthesis, hemicellulose is transported via vesicular trafficking to the plasma membrane. Once there, it can be further transported across the apoplast to the primary or secondary cell wall. There are likely mechanistic discoveries still to be made regarding hemicellulose biosynthesis, but in the application of easing recalcitrance of digestion of plant cell walls to harvest cellulose for fermentation to bioethanol, the presence of hemicellulose is not a substantial problem because its random, amorphous structure makes it easily hydrolyzed (Tong et al, 2012).

Pectin Biosynthesis

Pectin is present in the middle lamella, primary cell wall, and in low abundance in the secondary cell walls of grasses, and consists of a complex heteropolysaccharide made of galacturonic acid, rhamnogalacturonan I, rhamnogalacturonan II, D-galactose, L-arabinose, and D-xylose. The carboxyl groups of galacturonic acid are typically highly

methyl esterified (Harholt et al, 2010). Those galacturonic acids which are not esterified can exist either as free acids or as calcium, potassium, or sodium salts. Pectins dissolve under high heat and low pH conditions. Furthermore, they are typically highly digestible by bacterial enzymes present in the gut of ruminants. This suggests that pectin does not pose a significant problem in the digestion of vegetative feedstocks by hydrolytic enzymes in biomass conversion processes. In fact, the sugars pectin contains could provide an additional food source to yeast or other microbes used for subsequent fermentation, as long as their gelling temperature is below that used during the fermentation. Pectins provide additional benefits to the plant besides helping to provide mechanical strength and a physical barrier against the outside environment. Upon pectin degradation due to plant pathogenesis, oligogalacturonides are produced, which serve as a trigger in a signaling cascade involving the production of radical oxidative species. These reactive oxygen species activate transcription factors of plant defense related genes (Ferrari et al, 2013).

Lignin Biosynthesis

In the area of lignin biosynthesis, prior work has shown that phenylalanine is the starting substrate, which is first deaminated by the enzyme PHENYLALANINE AMMONIA LYASE (PAL) to form cinnamic acid. This intermediate undergoes repeated rounds of hydroxylation of its aromatic ring catalyzed by multiple enzymes, followed by phenolic *O*-methylation performed by the CAFFEIC ACID *O*-METHYLTRANSFERASE (COMT) enzyme, and finally, reduction of the side-chain carboxyl on the cinnamaldehydes to an alcohol group catalyzed by the CINNAMYL ALCOHOL DEHYDROGENASE (CAD) enzyme (Boerjan et al, 2003). Depending on the flux of

intermediates through the lignin biosynthetic pathway, which is affected by feedback modulation, three different monolignol alcohols are synthesized, namely p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol. Side groups on these monolignol alcohols can be modified by acylation prior to their assisted transport to the apoplast, where polymerization occurs by radical oxidative coupling (Petrik et al, 2014). The addition of side groups via acylation provides alternate points for cross-linking to occur within lignin subunits, as well as a means for lignin to cross-link to hemicellulose.

Lignin Caused Recalcitrance of Vegetative Biomass

In the area of easing vegetative biomass recalcitrance to conversion to bioethanol, a major contributor to this recalcitrance is the presence of lignin in the secondary cell walls of plants. If this bottleneck to the use of lignocellulosic feedstocks can be overcome, conversion of senesced plant vegetative material to biofuel has the potential to contribute significantly to global transport fuel demand. The International Energy Agency projects that if 10% of available agricultural and forestry residues could be converted to biofuel in the year 2030, this could provide approximately 5% of the projected total transport fuel demand at that time. Greater allocation of global residues to biofuel production, 25% rather than 10%, could provide 10-15% of transportation fuel demand by the same year (Eisentraut, 2010).

Research Aims

Towards the goal of producing plant feedstocks with improved lignin characteristics, we proposed generating and characterizing transgenic *Brachypodium distachyon* plants as a grass model, using RNAi technology to knock down expression of

key monolignol biosynthesis enzymes. Given that *Brachypodium* is a new model system for monocots, this work is novel and will contribute to understanding important aspects of bioenergy crop grass cell walls. As a second research goal, three secondary cell wall specific gene promoters were cloned and thoroughly characterized for their patterns of gene expression in multiple tissues and developmental stages. These promoters, or gene switches, were subcloned into utility monocot transformation vectors. These utility vectors will be made available to the scientific community for use in driving genes of interest in a secondary cell wall-specific manner, specifically in such monocot bioenergy crops as corn, switchgrass, and Miscanthus.

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CHAPTER II

P-COUMAROYL-COA:MONOLIGNOL TRANSFERASE (PMT) ACTS
SPECIFICALLY IN THE LIGNIN BIOSYNTHETIC PATHWAY IN
BRACHYPODIUM DISTACHYON

Summary

Grass lignins contain substantial amounts of *p*-coumarate (*p*CA) acylating the sidechains of the phenylpropanoid polymer backbone. An acyltransferase, named *p*-coumaroyl-CoA:monolignol transferase (OsPMT), that could acylate monolignols with *p*CA *in vitro* was recently identified from rice. *In planta*, such monolignol-*p*CA conjugates become incorporated into lignin via oxidative radical coupling, thereby generating the observed *p*CA appendages. However, *p*-coumarates also acylate arabinoxylans in grasses. To test the authenticity of PMT as a lignin biosynthetic pathway enzyme, we examined *Brachypodium distachyon* plants misregulated in the putative *BdPMT* gene. Using newly developed cell wall analytical methods, we determined that the transferase was specifically involved in lignin acylation. A sodium azide-generated *Bdpmt-1* missense mutant had no (<0.5%) residual *p*CA on lignin, and *BdPMT* RNAi plants had levels as low as 10% of wild-type, whereas the amounts of *p*CA acylating arabinosyl units on arabinoxylans in these *PMT*-misregulated plants remained unchanged. *p*CA acylation of lignin from *BdPMT* overexpressing plants was found to be more than

three-fold higher than that of wild-type, but again the level on arabinosyl units remained unchanged. Taken together, these data are consistent with a defined role for grass *PMT* genes in encoding BAHD acyltransferases that specifically acylate monolignols with *pCA*, producing monolignol *p*-coumarate conjugates that are used for lignification *in planta*.

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Introduction

Lignocellulosic biomass from grasses could serve as a major feedstock for the generation of liquid biofuels, contributing more than half of the envisioned one billion tons of biomass available in the United States each year (Perlack et al., 2005; Carroll and Somerville, 2009; U.S. Department of Energy, 2011). Lignocellulosic biomass typically refers to the harvestable plant portions not consumed by humans (e.g., for grasses, the stems and leaves) and is primarily composed of secondary cell walls consisting of the polysaccharides cellulose and hemicelluloses embedded within the heterogeneous phenolic polymer lignin (Pauly and Keegstra, 2008). Lignin polymer “backbones” in grasses are composed of guaiacyl (G) and syringyl (S) units (derived from the monolignols coniferyl alcohol and sinapyl alcohol, respectively), together with a lesser amount of *p*-hydroxyphenyl (H) units (derived from *p*-coumaryl alcohol), interconnected by β -O-4-aryl ether and other inter-unit bonds, and cross-linked to hemicelluloses by ferulate (Ralph et al., 1995; Boerjan et al., 2003; Ralph et al., 2004; Zhang et al., 2009; Lapierre, 2010).

C3 grasses such as rice, wheat, rye, oat, and bamboo, and C4 grasses such as corn, switchgrass, and sorghum, all have lignin polymers that are partially acylated by *p*-coumarate “appendages” and, as more recently shown, by acetate (Ralph, 2010; Ralph and Landucci, 2010; del Río *et al.*, 2012). It is now well established that such lignin acylation results from *in planta* lignification using pre-acylated monolignols (Ralph *et al.*, 1994; Lu and Ralph, 1999; 2002; 2008; Hatfield *et al.*, 2009; Ralph, 2010; Withers *et al.*, 2012); in the case of *p*-coumarate, this is via coniferyl and sinapyl *p*-coumarate ester

conjugates **3** (Figure 1). As the *p*-coumarate acylates the monolignol γ -OH, the resulting lignin is partially γ -*p*-coumaroylated (c.f., **5**, Figure 1) (Ralph *et al.*, 1994; Ralph, 2010).

Although the role for these conjugated monolignols remains somewhat mysterious, evidence is that they are synthesized in the cell and exported to the wall along with the traditional monolignols **1**, coniferyl and sinapyl alcohols (along with smaller amounts of *p*-coumaryl alcohol) where they undergo the radical coupling and cross-coupling reactions, mainly by so-called endwise polymerization in which the monomer radical couples to the growing polymer radical (Ralph, 2010). It was initially puzzling but is now clear why the *p*-coumarate moieties, as phenolic entities, remain as free-phenolic pendant units on the polymer and do not themselves undergo radical coupling reactions. Although they readily form radicals, such radicals preferentially undergo radical transfer reactions with phenols that produce more stable phenolic radicals, such as the monolignols themselves and the guaiacyl and syringyl phenolic end-units on the growing polymer, i.e., they undergo radical transfer reactions rather than participating in radical coupling (Takahama *et al.*, 1996; Ralph *et al.*, 2004; Hatfield *et al.*, 2008; Ralph, 2010). This has been demonstrated in a model system involving the typically slow peroxidase-H₂O₂-mediated dehydrodimerization of sinapyl alcohol that is significantly sped up in the presence of catalytic levels of a *p*-coumarate ester (Ralph *et al.*, 2004; Hatfield *et al.*, 2008). Therefore, the polymerization whether from the monolignols **1** or from the *p*-coumaroylated monolignols **3**, is solely via the monolignol moiety.

Current preliminary evidence, and more extensive evidence with monolignol acetates, shows that acylation does not greatly affect the coupling propensity of the

monolignol moiety, i.e., radical coupling produces the same array of coupling products in approximately the same ratios whether the monolignols are acylated or not (Lu and Ralph, 2008). The one difference is that, for the product of monolignol β - β -coupling or dehydrodimerization, the post-coupling rearomatization pathways are necessarily different because the γ -OH is no longer available for internal trapping of the quinone methide intermediate.

Recently, we identified from rice the first putative monolignol transferase gene, *OsPMT*. Heterologously expressed OsPMT had all the properties anticipated for a *p*-coumaroyl-CoA:monolignol transferase (PMT) enzyme responsible for *p*-coumaroylation of monolignols (Figure 1) (Withers *et al.*, 2012). The enzyme produced, with favorable kinetics, monolignol *p*-coumarate conjugates **3** from *p*-coumaroyl-CoA **2** and *p*-coumaryl, coniferyl, and sinapyl alcohols **1H**, **1G**, and **1S** (Hatfield *et al.*, 2009; Withers *et al.*, 2012). In this paper, we demonstrate the veracity of the hypothesized *in planta* action by misregulation of the closest gene homolog in Brachypodium, and furthermore demonstrate its selectivity for *p*-coumaroylation of monolignols, and thereby lignin, over the arabinoxylans that are also partially *p*-coumaroylated in grasses.

Results and Discussion

It was recently shown that bacterially expressed and purified OsPMT protein could acylate monolignols with *p*-coumarate (*p*CA), producing monolignol-*p*CA ester conjugates (Withers *et al.*, 2012). We hypothesized that, *in planta*, this enzyme is therefore responsible for biosynthesizing the monolignol *p*-coumarate conjugates that become incorporated into the lignin polymer by oxidative radical coupling reactions

(Figure 1). To validate the *in planta* activity of PMT, we employed a reverse genetic approach, using the model grass *Brachypodium distachyon* (Brachypodium).

To identify the likely Brachypodium *PMT* gene, the OsPMT (LOC_Os01g18744.1) amino acid sequence was used as a query in a BLASTP search of the Brachypodium proteome (<http://www.gramene.org>; <http://mips.helmholtz-muenchen.de/plant/brachypodium/>). The predicted Bradi2g36910 protein was found to have the highest sequence homology to OsPMT, sharing 63% amino acid sequence identity and 75% similarity. Recently published phylogenetic trees containing both Brachypodium and rice BAHD acyltransferases show that Bradi2g36910 is the most closely related Brachypodium protein to OsPMT (Bartley et al., 2013; Molinari et al., 2013).

As the Bradi2g36910 coding sequence was only partially supported by published expressed sequence tag sequences (Vogel *et al.*, 2006), we generated and sequenced Bradi2g36910 cDNA clones (GenBank accession no. HG421450), from Brachypodium inbred line Bd21-3 stem tissue transcripts, finding the ORF sequence to be 100% identical to the annotated gene model Bradi2g36910.1.

qRT-PCR analysis was performed on transcripts isolated from various plant tissues, revealing that Bradi2g36910 (hereafter referred to as *BdPMT*) was expressed in all organs tested (spikelet, stem internodes, leaves, and root), with highest expression levels in the spikelet (Figure 2a). Co-expression analysis using PlaNet (<http://aranet.mpimp-golm.mpg.de/index.html>), a web-based platform that predicts transcriptomic co-expression networks based on temporal and spatial gene expression data (Mutwil *et al.*, 2011; Harrington *et al.*, 2012), revealed that *BdPMT* expression

clustered with those of other predicted lignin biosynthetic genes including *Phenylalanine Ammonia Lyase* (*PAL*; Bradi3g49250 and Bradi3g49260), *Cinnamoyl-CoA Reductase* (*CCR*; Bradi3g36887), and *Caffeic acid O-Methyltransferase* (*COMT*; Bradi3g16530) (Table S1).

To study how altered *BdPMT* expression and function affects cell wall composition and structure as well as plant growth, we generated Brachypodium *BdPMT* RNA interference (RNAi) as well as overexpression (OX) lines. We also identified a Brachypodium *Bdpmt* mutant in a sodium azide-mutagenized population generated by the Institut National de la Recherche Agronomique (INRA; Dalmais et al., 2013).

For the RNAi approach, two different hairpin constructs were generated, using *BdPMT* coding sequences that were chosen based on their relatively low homologies to other Brachypodium genes (Figure S1). RNAi construct #1 (*BdPMT* RNAi #1) consisted of a 301 base pair fragment derived from the 3' UTR of the *BdPMT* coding sequence, whereas *BdPMT* RNAi #2 consisted of a 258 base pair fragment derived from coding sequence positioned just upstream of the translational termination site (Figure 2b). These constructs were transformed into embryo-derived callus using *Agrobacterium tumefaciens*, from which transgenic plants were regenerated on hygromycin selection media. qRT-PCR analysis was performed on transcripts isolated from the stems of T₁ and T₂ generation plants from four independent transgenic lines, revealing average *BdPMT* transcript level reductions of 53% to 92%, i.e., residual levels of 47% to just 8% (Table S2).

The Brachypodium *Bdpmt-1* mutant was isolated serendipitously during a Targeting Induced Local Lesions in Genomes (TILLING) screen for another gene

(Bouvier d'Yvoire et al., 2013; Dalmais et al., 2013). One of the lines isolated in that screen had cell wall compositional changes consistent with those expected for a *pmt* mutant (described below). Subsequent DNA sequencing of the *BdPMT* gene in that line (hereafter named *Bdpmt-1*) revealed a nucleotide base substitution at position 563 of the Bradi2g36910 ORF (guanine replaced by adenine), resulting in codon 188 of the 450-codon ORF changed to encode aspartic acid instead of glycine (G188D). Incidentally, this base substitution disrupts a *NarI* restriction enzyme site, which allows one to track the mutation with a Cleaved Amplified Polymorphic Sequence (CAPS) marker (Neff et al., 2002).

The mutated codon in *Bdpmt-1* is positioned three codons downstream of that encoding aspartic acid in the highly conserved HXXXD motif, which is considered the catalytic center of BAHD acyltransferases (Figure 2c) (St-Pierre and De Luca, 2000; D'Auria, 2006). Altering or deleting the HXXXD motif in BAHD acyltransferases was found to greatly reduce enzyme activities of those proteins (Suzuki *et al.*, 2003; Bayer *et al.*, 2004; D'Auria, 2006). A scan of the amino acid sequences of 20 known and putative BAHD acyltransferases that fall into five clades based on phylogenetic analysis (D'Auria, 2006) revealed that proteins belonging to Clade V (to which *BdPMT* belongs) all have a glycine at the position mutated in *Bdpmt-1*. Proteins in clades I through IV have a serine (S), glycine (G), or threonine (T) at that position (Figure 2c). The amino acids S, G, and T all have small and uncharged side-chains, whereas the aspartic acid (D) that substitutes for glycine in the *Bdpmt-1* mutant protein has a negatively charged and slightly larger side-chain (Betts and Russell, 2003).

Like *BdPMT* RNAi plants, *Bdpmt-1* mutant plants grew similar to WT and empty vector transgenic control plants grown side by side under our controlled growth chamber conditions, with no obvious morphological differences (Figure 3a, b; Figure S2).

To determine if cell wall biomass from the aforementioned *Bdpmt* mutants had reduced levels of ester-linked *pCA*, as was our hypothesis, pulverized senesced stems were solvent-extracted then subjected to mild alkaline hydrolysis. Figure 4a and Table S2 show that *Bdpmt-1* mutant plant cell walls had nearly an 80% reduction in the amount of released *pCA*. Stem cell walls from all four RNAi lines also had reduced amounts of released *pCA* compared to WT, with the relative levels of reduction coinciding with the relative reductions in *BdPMT* transcript levels. For example, Line 4B stems had the highest levels of reduction in *pCA* and *BdPMT* transcripts (51% and 92%, respectively) whereas Line 8A stems had the lowest levels of reduction (3% and 53%, respectively). The 3% *pCA* reduction from Line 8A plant cell walls was not significantly different from that of WT, whereas the 14% *pCA* reduction from Line 1A cell walls was significantly different; Line 1A stems had a 58% reduction in *BdPMT* transcripts. These data suggest that the 53% reduction in *BdPMT* transcript levels represents the approximate threshold at which significant cell wall compositional changes occur.

Analysis of *p*-coumarate levels is complicated by the fact that, unlike ferulate in *Brachypodium* mature stems, *p*-coumarate acylates both cell wall hemicelluloses (more specifically, arabinoxylans) and lignin (Mueller-Harvey et al., 1986). Although mild alkaline hydrolysis clearly detected reduced amounts of *pCA* liberated from alkali-treated *Bdpmt-1* and *BdPMT* RNAi cell wall biomass compared to WT (Figure 4a; Table S2), it remained unclear whether that reduction was due to a decrease of the *pCA* ester levels on

lignin and/or on arabinoxylan. To unambiguously parse apart these possibilities, we employed two methods: 1) DFRC (Derivatization Followed by Reductive Cleavage) (Lu and Ralph, 1997; Lu and Ralph, 1998c; Lu and Ralph, 1999), which cleaves β -aryl ether bonds in lignin while leaving ester bonds intact, thereby releasing diagnostic lignin-derived coniferyl and sinapyl dihydro-*p*-coumarate conjugates **8** (Figure 5a), and 2) a newly-developed mild acidolysis method that allows for the purification and quantification of *p*CA-arabinose **10** and FA-arabinose **11** ester moieties originating from arabinoxylans (Figure 5b).

The DFRC method releases monolignol units retaining their γ -acylation by *p*CA, (conjugates **8** in Figure 5a), products that are obviously exclusively from lignin. In WT *Brachypodium*, in which the lignin has a ~60:40 S:G ratio, the *p*CA was found on the syringyl units with no detectable amount of the *p*CA on guaiacyl units (Figure S3). Biomass stem tissue samples from *Bdpmt-1*, *BdPMT* RNAi Line 4B and Line 7A plants were found to have 0% (<0.5%), 8%, and 50% residual lignin *p*CA levels, respectively (Figure 4b; Table S3). The *Bdpmt-1* mutant allele appears to be essentially a null, yielding a negligible amount of lignin-derived *p*CA. These data strongly suggest that *BdPMT* is solely responsible for placing *p*CA on stem lignin in *Brachypodium*.

Using a mild acidolysis method (Figure 5b; Figure S4), we quantified *p*CA-arabinose **10** and FA-arabinose **11** released from stem tissue biomass, these moieties being derived exclusively from the hemicellulose arabinoxylan. The mild acidolysis method released essentially the same amounts of *p*CA-arabinose and FA-arabinose from *Bdpmt-1* and *BdPMT* RNAi Line 4B stem cell wall samples as was released from WT samples (Figure 4c; Table S3). This result signifies that another enzyme(s) and not

BdPMT acylates arabinosyl units with *p*CA and FA, one of those enzymes perhaps being the Brachypodium orthologue to the rice OsAT10 enzyme (Bartley et al., 2013). Taken together with the DFRC results, these data unambiguously establish that *BdPMT* is responsible for the acylation of lignin with *p*CA *in planta* and is not involved in the incorporation of *p*CA into hemicelluloses.

In order to obtain a more detailed picture of the cell wall compositional and structural differences between *Bdpmt-1*, *BdPMT* RNAi, and WT plants, whole cell walls of senesced stems were analyzed by gel-state 2D NMR (Kim and Ralph, 2010). Figure 6, top row, shows the spectral aromatic fingerprints obtained by this method, profiling S, G, and H lignin backbone units as well as pendent moieties, *p*CA, FA, and triclin, in the cell wall; the latter mobile units on the periphery of polymer chains are over-represented due to their slower spin relaxation than units in the polymer backbone. As with total saponified hydroxycinnamate measurements, these spectra, being from the whole wall material, reflect the total levels of *p*CA and FA in the wall, i.e., without distinction between those components on lignins vs polysaccharides.

Compared to WT, and consistent with the alkaline treatment analysis results detailed above, the correlation peaks for *p*CA are clearly reduced in *Bdpmt-1* and *BdPMT* RNAi Line 4B, whereas those of FA appear unchanged. In addition, the peaks for syringyl units are relatively lower in Line 4B and *Bdpmt-1* cell walls compared to WT, whereas those for guaiacyl units and *p*-hydroxyphenyl units appear relatively unchanged, denoting a relative decrease in S lignin in Line 4B cell walls and a reduced S/G units ratio. This altered S/G units ratio result was corroborated by thioacidolysis data, where

we found *Bdpmt-1* as well as *BdPMT* RNAi plant cell walls had reduced S/G units ratios compared to WT (Table 1).

The lignin-bound *p*CA (and FA moieties, if there were any) are more clearly delineated after enzymatic digestion of the wall polysaccharides using crude polysaccharidases (Chang *et al.*, 1975; Wagner *et al.*, 2007). The resulting ‘milled wood enzyme lignins’ (MWELs) are lignin-enriched (retaining essentially the entire lignin component), and are devoid of the hemicellulosic polysaccharides that are also acylated with *p*CA and FA, i.e., most or all of the hydroxycinnamates remaining are on the lignin component only. NMR spectra of these CELs compellingly confirm the conclusions made from examining the saponification, polysaccharide-specific, and lignin-specific analyses above. Consistent with ferulates’ solely acylating wall arabinoxylans, signals corresponding to ferulate are absent in both WT and mutant lines, with the possible exception of a trace in the overexpression line (*BdPMT* OX), which bears further scrutiny. *p*-Coumarates remain, but at a lower level, because those acylating arabinoxylans are no longer in this fraction and only those acylating lignin remain. Comparisons to the WT material indicated that 11%, and 0% residual levels (compared to WT) remain in *BdPMT* RNAi Line 4B and the *Bdpmt-1* mutant. Such data are in line with the reduced levels in these materials determined by the lignin-specific DFRC method. The data from both analysis methods therefore compellingly demonstrate that the PMT activity is associated solely with *p*-coumaroylation of lignin and not arabinoxylan.

To more fully explore how *PMT* influences lignin biosynthetic fluxes and cell wall composition, we generated transgenic plants overexpressing *BdPMT* in the form of

an EYFP:BdPMT fusion protein, with expression driven by the maize *UBIQUITIN* promoter and intron; these lines are hereafter referred to as *BdPMT* OX. qRT-PCR analysis of T₂ generation plants from two independent transformation events (initially chosen as having high expression based on EYFP fluorescence and Western blot analysis; Figure S5a) revealed *BdPMT* transcript levels to be, on average, 35-fold or more higher than in WT (Table S2). In our climate-controlled growth chambers, *BdPMT* OX plants grew like WT, attaining comparable culm heights and above-ground biomass weights (Figure 3a, b; Figure S5b, c). However, for five lines with the highest *BdPMT* expression including *BdPMT* OX lines 57A and 67B but not 23A, a small percentage of the plants were stunted. This stunted phenotype displayed high penetrance (was commonly observed) when *BdPMT* OX plants were grown in a greenhouse, where growth conditions were variable and perhaps more challenging to the plants (Figure S5d).

In growth chambers as well as in the greenhouse, *BdPMT* OX leaf collars commonly became blackened (never observed with WT) and leaf blades grew along the stems, whereas WT leaf blades grew at about 60° angles from the stems (Figure S5e, f). The blackened collar phenotype might be due to a pathogen infection, deposition of a phenolic compound, and/or spontaneous necrosis. We note that all of the gene expression experiments and cell wall analyses described herein were performed on *BdPMT* OX plants that grew to be the same sizes as WT plants grown side by side in climate-controlled chambers.

Whereas *BdPMT* OX and WT stem cross-sections revealed no morphological differences and no collapsed vascular bundle cells (Figure 7a-d), *BdPMT* OX stem section tissues stained considerably less with both phloroglucinol (Weisner test) and

Mäule reagent compared to WT (Figure 7a-d). Phloroglucinol stains cinnamaldehyde end groups of lignin, whereas Mäule reagent is used as a diagnostic of S lignin units (Lin and Dence, 1992). Consistent with these observed staining differences, Klason lignin analysis revealed that senesced *BdPMT* OX stems contained, on average, 23% less lignin than WT stems (Table 1). This reduced lignin was corroborated by gel-state 2D NMR analysis (Figure 6), and may be due to excessive *BdPMT* activity drawing *p*-coumaroyl-CoA away from monolignol biosynthesis, thereby lowering the amounts of monolignols available for polymerization. We speculate the above-noted stunted growth phenotype observed with the highest overexpressing *BdPMT* OX plants might be due to lignin amounts being reduced below levels necessary for normal plant growth.

Using the above-mentioned mild alkaline hydrolysis, cell wall *p*CA levels were found to be more than two-fold higher in *BdPMT* OX plants stem biomass compared to WT (Figure 4a; Table S2). As expected, the increased amounts of *p*CA were determined to be exclusively due to higher *p*CA levels on lignin (3.4-fold higher, Table S3; Figure S3) and not on polysaccharides (Figure 4b, c; Table S3), reinforcing our conclusion that *BdPMT* exclusively catalyzes the acylation of monolignols. With the increased level of *p*CA, a small but notable amount of acylation on the guaiacyl subunits was observed (Figure S3); *p*-coumaroylation was, however, found to favor S over G units (~99:1).

FA levels were found to be as much as 40% higher in *BdPMT* OX stem biomass compared to WT. These higher FA levels mirrored the slightly lower FA levels detected in the *Bdpm1* loss of function mutant (Figure 4a; Table S2). At least part of the relative cell wall FA content increase can be explained as being a consequence of the reduced amounts of lignin in *BdPMT* OX plant stems; ferulate in the cell wall is on

polysaccharides (arabinoxylans), so an increase in the ratio of arabinoxylan to lignin would lead to a relative FA level increase. It seems unlikely that these altered FA levels are directly due to altered *BdPMT* activity, given that *in vitro* studies of OsPMT substrate specificity revealed that the PMT enzyme utilizes the thioester feruloyl-CoA only poorly as a substrate (Withers et al., 2012). We cannot rule out the possibility that the expression and/or activity of a currently unknown transferase responsible for feruloylating arabinosyl units on arabinoxylans may be altered in the *BdPMT* mutants, or that *PMT*-related flux alterations in the phenylpropanoid biosynthetic pathway result in altered FA availability and/or incorporation.

To determine how the *BdPMT* misexpression-related cell wall alterations affect biomass digestibility, we first subjected the WT and *BdPMT* OX extract-free senesced stems to simple cellulolysis assays, as previously described (Berthet *et al.*, 2011). The susceptibility of stem cell walls to enzymatic hydrolysis was roughly evaluated by the induced weight loss. Comparing *BdPMT* OX to WT, we observed a 33% higher weight loss upon hydrolysis with a commercial cellulase preparation containing both cellulase and hemicellulase activities (mean weight loss from *BdPMT* OX samples 45.6 ± 1.9 , versus 34.3 ± 1.2 for WT samples; $p < 0.0001$; $n=5$). This result was consistent with an expectation that *BdPMT* OX biomass would have improved digestibility owing to a significant reduction in cell wall lignin (Chen and Dixon, 2007; Chapple et al., 2007; Fu et al., 2011).

As a more encompassing evaluation of cell wall degradability, we subjected pulverized stem biomass to a range of pretreatments and enzyme hydrolysis using a high-throughput lignocelluloses digestibility assay (Santoro et al., 2010). With the exception of

62mM NaOH, these methods were designed to be mild and incomplete so as to identify possible differences in rates of biomass digestion. We found *BdPMT* OX senesced stem tissue to consistently have higher cell wall polysaccharides sugar release compared to controls, in particular with base pretreatments (Figure 8; on average, 21% to 36% more glucose and 10% to 69% more pentose sugars released with 62 mM and 6.2 mM NaOH, respectively). By contrast, the amounts of sugars released from *BdPMT* RNAi and *Bdpmt-1* tissues were not consistently different from controls, suggesting that the abolishment of *pCA* on lignin has limited if any effects on stem biomass digestibility (Figure 8).

Conclusion

Diagnostic analytical methods provide compelling support for our contention that the *PMT* gene *BdPMT* identified in *Brachypodium*, a homolog of the *OsPMT* gene in rice, is specifically involved in the acylation of monolignols with *p*-coumarate. Its upregulation roughly doubles the total *pCA* and more than triples the *pCA* on lignin level. Conversely, RNAi downregulation results in *pCA* total levels down to as low as 50%, with ~10% on lignin, whereas a *PMT* mutant likely to be a null, with ~24% total *pCA*, has zero (or at least <0.5%) on lignin. In all cases, *pCA* levels on arabinoxylan remain statistically unchanged. Thus, we appear to have finally identified and authenticated the first transferase, *PMT*, involved in acylating monolignols. *BdPMT* OX plant biomass was found to have about 23% less lignin compared to WT and a higher S:G lignin ratio; *Bdpmt* loss of function mutants had a lower S:G lignin ratio, suggesting *PMT* integrally affects phenylpropanoid biosynthetic fluxes in grasses.

Experimental Procedures

Transgenic Plant Generation

Two *BdPMT* RNAi constructs were generated by PCR amplification of Bradi2g36910 coding sequences from Brachypodium accession Bd21-3 purified genomic DNA using the primer pairs listed in Table S4. The PCR products were restriction digested and cloned into both the BamHI-AscI and KpnI-SpeI sites of the pStarling vector (<http://www.plantindustry.csiro.au/RNAi/vectors.htm>). The resulting hairpin cassettes were removed as NotI fragments and ligated into the pWBVec8 binary vector backbone (Wang *et al.*, 1998).

The *BdPMT* OX construct (*Zea mays* Ubiquitin-1 promoter:*EYFP:BdPMT*) was generated by performing Reverse Transcription-PCR (RT-PCR) on Bd21-3 stem RNA using oligo(dT)₁₈, then amplifying the 1.35 kb Bradi2g36910 ORF. The PCR product was digested with BamHI-XhoI, cloned into pENTR2B, then Gateway (Invitrogen) cloned into a modified pH7WGY2 (Karimi *et al.*, 2002) binary vector that had the 35S promoter replaced with the *Zea mays* Ubiquitin-1 promoter plus intron 1 from pStarling (Christensen and Quail, 1996). All constructs were sequenced to validate accuracy.

Plants harboring the *BdPMT* RNAi or *BdPMT* OX construct were regenerated from Bd21-3 embryonic callus tissue transformed using *Agrobacterium tumefaciens* strain AGL-1, as described by Vogel and Hill (2008); media were supplemented with 40 U/ml hygromycin B (Phytotechnology Laboratories).

Transgenic *BdPMT* RNAi plants were identified and confirmed by both seed selection on hygromycin and PCR amplification of RNAi construct portions from leaf genomic DNA using the ExtractNAmp Plant PCR kit (Sigma-Aldrich) and the following

primers: pStarling_F1 + pStarling_R1 and pStarling_F2 + pStarling_R2 flanking *BdPMT* RNAi DNA in the sense and antisense orientations, respectively.

BdPMT OX plants were identified by tracking EYFP fluorescence from the EYFP:*BdPMT* fusion protein using a Leica MZ8 fluorescence dissecting scope. The EYFP:*BdPMT* protein in these plants was found to be the expected size based on Western blot analysis of stem tissue protein extracts detected with an anti-YFP antibody (1:2,500 dilution, Allele Biotech catalog # ABG-MP-MMGFP10; secondary antibody: 1:5,000 goat anti-mouse IgG-HRP, Thermofisher catalog # 31430).

Seed Sterilization and Plant Growth

Seeds were surface sterilized and plated on selective plates (1.5% agar/one half-strength Murashige and Skoog salts (Caisson Laboratories)/40 U/mL hygromycin B), stratified for three days (4 °C, dark), then moved to a growth chamber (22 °C, 16 h light) until hygromycin selection was apparent.

Plants were grown in a 50:50 mix of SunGro Rediearth and MetroMix 510 soil in 4 inch pots in growth chambers (20 h light:4 h dark photoperiod, 22 °C, 50% humidity). Control plants were either WT Bd21-3 seedlings plated on non-selective plates or planted directly in soil, or hygromycin-selected plants harboring a *Zea mays* Ubiquitin-1 promoter with intron driving GUSPlus (Cambia Labs) in pWBVec8. The UBIProm:GUS control was used for the Klason lignin analysis and the digestibility analyses.

Quantitative RT-PCR (qRT-PCR) and RNA-Seq

Total stem RNA was extracted from the top two internodes of transgenic and control plants by grinding in liquid nitrogen, followed by RNA extraction using the Plant RNeasy Extraction Kit (Qiagen Ltd.) following the manufacturer's protocol. 0.5 µg of DNaseI-treated (Fermentas) RNA was reverse transcribed using oligo(dT)₁₈ in a 20 µL MMLV (Promega) reaction volume.

BdPMT expression analysis was performed by qRT-PCR using a DyNAmo HS SYBR Green qPCR kit (Thermo-Fisher), cDNA samples diluted to 10 ng/µL and primers listed in Table S4. Thermal cycling conditions: 95 °C for 6 min; 40 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s; followed by a dissociation stage 95 °C for 15 s, 60 °C for 30 s, 95 °C for 15 s. PCR reactions were analyzed in triplicate by an Applied Biosystems 7300 Real Time PCR system. Relative expression ratios of target genes were determined by normalizing to wild-type samples using an efficiency calibrated model (Pfaffl, 2001; Equation 1 in Yuan *et al.*, 2006); *BdUBC18* (Bradi4g00660) or *EF1α* (Bradi1g06860) were used as reference genes. Primer efficiency (E), defined as $2^{-(1/\text{slope})}$, was calculated from log₂ cDNA concentration vs. Ct value plot slopes of serially-diluted cDNA samples across treatment groups (Yuan *et al.*, 2006).

For RNA-seq analysis, Poly(A) RNA and libraries were quality-checked before 2x100bp sequencing with an Illumina HiSeq2000 instrument. Gene expression data were computed using the CLC Genomics Workbench version 6.5. Reads were trimmed and filtered on quality with the Trim Sequences algorithm (Limit: 0.05, Maximum ambiguities: 2). RPKM was generated by aligning ESTs to Gene annotations using the RNA-Seq Analysis algorithm for annotated sequence. (Parameters: Similarity 0.8; Length

fraction 0.9). Genome sequence and annotations were downloaded from NCBI (<http://www.ncbi.nlm.nih.gov/bioproject/PRJNA74771>). Genome sequence, annotation, and expression data were stored in an Oracle relational database and displayed using a custom web application.

Stem Sectioning, Phloroglucinol and Mäule Reagent Staining

Hand-cut stem sections from the first internode (1 cm below first node below spikelet) of plants 39 days after planting were made with a Teflon-coated razor blade (GEM, Ted Pella). Sections were stained 15 minutes in acid phloroglucinol (2% (w/v) phloroglucinol/20% (v/v) ethanol/2.4 M HCl), mounted on microscope slides, and then photographed using a Leica DMRBE microscope with brightfield illumination. Care was taken to stain each section the same duration of time before imaging. Mäule method analysis was performed as in Bouvier d'Yvoire *et al.* (2013).

Digestibility Assays

Digestibility assays were performed as in Santoro *et al.*, (2010). Ball mill-pulverized senesced culms having spikelets and leaves at leaf collars removed were analyzed. Free glucose amounts in the undigested biomass was determined by extracting the glucose from ground biomass with distilled water and quantifying using an alditol acetate/gas chromatography method (Blakeney *et al.*, 1983). Free glucose values were subtracted from the glucose values obtained after the chemical and enzymatic treatments so as to have digestibility values reflecting sugars released exclusively from cell wall polysaccharides. Free glucose values did not need to be subtracted for NaOH treated

samples, since those treatments degraded any free glucose in the ground biomass before enzyme hydrolysis. No detected glucose originated from starch, since the hydrolytic enzyme mixes contained no amyloglucosidase or α -amylase activity.

The cellulolysis protocol was performed as described in Berthet *et al.* (2011).

Thioacidolysis Method

The thioacidolysis protocol was performed as described in Lapierre *et al.*, (1995). Lignin-derived thioacidolysis monomers were identified by gas chromatography–mass spectrometry as their trimethylsilylated derivatives.

Mild Alkaline Hydrolysis of Extract-Free Senesced Stems

The determination of ester-linked *p*CA and FA was via mild alkaline hydrolysis, following previously published procedures (Ralph *et al.*, 1994; Bouvier d'Yvoire *et al.*, 2013).

DFRC Method to Quantitate *p*CA Specifically Bound to Lignin

The quantification of lignin-bound *p*CA was performed using the DFRC method, which is capable of cleaving lignin ethers while retaining esters (Lu and Ralph, 1997; 1998b; a; Lu and Ralph, 1999). Dried extract-free whole cell walls (20-40 mg) were treated with acetyl bromide in acetic acid (5 mL, 1:4 v/v) at 50 °C for 3 h. The benzyl (α -carbon) brominated lignin solution was evaporated to dryness on a rotary evaporator (water bath at 50 °C). The dry film was suspended in 2 mL absolute ethanol and the

ethanol was evaporated. The dry sample was then immediately dissolved in a mixture of 1,4-dioxane:acetic acid:water (5 mL, 5:4:1 by volume), and a stir bar and zinc dust (150 mg) was added to the flask. The reaction was stirred for 1 h at room temperature and then quenched with saturated ammonium chloride. The sample was spiked with 50 μ g diethyl 5,5'-diferulate diacetate and the organics were extracted with dichloromethane (4 x 15 mL). The combined organic fraction was dried over sodium sulfate, filtered, and the solvent was removed under vacuum. The residue was treated with a mixture of acetic anhydride and pyridine (4 mL, 1:1 v:v) at room temperature overnight. Solvents were removed on a rotary evaporator. The crude product was loaded on a Supelco Supelclean LC-SI SPE tube (Sigma-Aldrich part #505048) with DCM (3 x 1 mL), eluted using a mixture of 1,4-dioxane:ethyl acetate (5 mL, 1:1 v:v), and concentrated to dryness. The dry film was dissolved in DCM (1 mL) and analyzed on a GC-MS (Shimadzu QP2010 Plus) using a 0.25 μ m x 0.25 mm x 30 m DB-1701 (Agilent) column with He as a carrier gas (0.85 mL/min, 10:1 split ratio). The column temperature was held at 150 $^{\circ}$ C for 1 min then increased at 20 $^{\circ}$ C min⁻¹ to 300 $^{\circ}$ C and held for 24 min. The injector was set at 250 $^{\circ}$ C, the ion source was set at 260 $^{\circ}$ C and the detector voltage was 0.99 kV. The detector was run in selective ion monitoring (SIM) mode, acquiring three masses per target compound: coniferyl dihydro-*p*-coumarate diacetate (the diacetate of **8G**, Figure 5a) m/z: 370, 163, 131; sinapyl dihydro-*p*-coumarate diacetate (the diacetate of **8S**, Figure 5a) m/z: 400, 193, 161; diethyl 5,5'-diferulate diacetate (DEDF) m/z: 484, 442, 350. Authentic standards of **8G**, **8S**, and DEDF were used to generate a 6-point calibration curve, with the DEDF used as an internal reference.

Analysis of Arabinoxylan-Bound *p*CA and FA

Mild acidolysis was carried out on 5-10 mg of extractive-free sample using 2 ml dioxane/water 0.2 M HCl containing 0.05 mg C21 internal standard in a Teflon-lined screwed cap tube overnight at 50 °C on a carousel. Then 2 ml water was added and samples were extracted with 3 x 4 ml EtOAc. The combined organic extracts were dried over Na₂SO₄ and then concentrated to ~0.5-1 ml. For GC-MS analyses, a 10 µl aliquot of the sample solution was silylated with 100 µl BSTFA + 10 µl pyridine for 1 h at 50 °C before injection into a GC-MS system (Supelco) methylsilicone 15 m x 0.32 mm x 0.25 µm film thickness column, using He (1.5 ml/min flow rate) as the carrier gas, and with the following temperature program: from 45 to 180 °C at 30 °C/min, then from 180 to 280 °C at +3 °C/min. Quantitative determinations were made from ion chromatograms reconstructed at *m/z* (57+71+85) for the internal standard C21 (heneicosane), at (308+293) for *p*CA-TMS, at (338+323) for FA-TMS, and at 219 for *p*CA-Ara-TMS, the per-TMS ether of 5-*O-p*-coumaroyl arabinose **10**, and at 249 for FA-Ara-TMS, the per-TMS ether of 5-*O*-feruloyl arabinose **11**.

2D HSQC NMR Analysis of Cell Wall Components

The preparation of the extract-free whole cell walls and the cellulolytic enzyme lignin was performed as described previously (Lu and Ralph, 2003; Wagner *et al.*, 2007; Kim and Ralph, 2010)). Ball milled, extract-free whole cell wall samples (40 mg) were prepared in a 5 mm NMR tube and suspended/swelled in DMSO-*d*₆/pyridine-*d*₅ '100%' (4:1, 500 µL).

The milled wood enzyme lignin (MWEL) was prepared from ball-milled material (50–70 mg) as described previously (Chang *et al.*, 1975; Wagner *et al.*, 2007). The material was digested at room temperature with 2 mg of crude cellulases (Cellulysin; Calbiochem), in pH 5.0 acetate buffer (30 mL) on a shaker for 3 days. The samples were then centrifuged (Sorval biofuge primo; 8,500 rpm/10,016 xG for 10 min), and decanted. The cellulase digestion was then repeated. After decanting the second batch of acetate buffer, the sample was rinsed with RO water (3 x 40 mL) and the sample lyophilized. The resulting brown powder (5-10 mg) was transferred to a 5 mm NMR tube and dissolved in DMSO- d_6 /pyridine- d_5 ‘100%’ (4:1, 500 μ L).

HSQC NMR spectroscopy was performed as previously (Kim and Ralph, 2010; Mansfield *et al.*, 2012) on a Bruker Biospin (Rheinstetten, Germany) Avance 700 MHz NMR spectrometer equipped with an inverse gradient 5mm TXI $^1\text{H}/^{13}\text{C}/^{15}\text{N}$ cryoprobe. The central DMSO solvent peak was used as internal reference (δ_c 39.5, δ_H 2.49 ppm). Peak assignments for syringyl (S), guaiacyl (G), and *p*-hydroxyphenyl (H) lignin units, as well as *p*-coumarate (*p*CA), ferulate (FA), and tricetin (T), were made by comparison with previously assigned spectra (Lu and Ralph, 2003; Marita *et al.*, 2003; Kim *et al.*, 2008; Kim and Ralph, 2010; Ralph and Landucci, 2010; del Río *et al.*, 2012; del Río *et al.*, 2012; Mansfield *et al.*, 2012; Rencoret *et al.*, 2013).

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Additional Supporting information may be found in the online version of this article.

<http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4282527/>

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Table 1. Lignin Analyses for Wild-Type (WT) and *BdPMT* Misregulated Brachypodium Plants. Lignin Content and Composition for Extract-Free Senesced Stems as Determined by Klason and Thioacidolysis Methods.

Genotype	Lignin content (%)	Relative frequency of lignin-derived thioacidolysis monomers		
		% H	%G	%S
WT	16.85 ± 0.07	3.5 ± 0.2	31.8 ± 0.0	64.8 ± 0.1
<i>Bdpmt-1</i>	16.71 ± 1.60	4.6 ± 0.2	36.7 ± 3.4	58.8 ± 3.2
WT	18.53 ± 0.16	4.6 ± 0.1	42.9 ± 1.1	52.5 ± 1.2
RNAi#2 4B	18.48 ± 0.39	4.9 ± 0.1	45.8 ± 1.2	49.4 ± 1.3
RNAi#2 7A	ND	6.0 ± 0.9	45.7 ± 1.6	48.3 ± 2.1
WT	17.12 ± 0.38	4.3 ± 0.2	35.8 ± 0.7	59.9 ± 0.9
<i>BdPMT</i> OX	13.20 ± 0.26*	3.4 ± 0.4*	24.9 ± 2.4*	71.8 ± 2.6*

Note: Values are means ± SD from individually analyzed plants (n≥3). Asterisks denote statistically significant differences (Student's test) compared to the WT values at p<0.0001. For the lignin content analysis (Klason method), UBIProm:GUS transgenic plants were used as WT.

CHAPTER II FIGURES

Figure 1. Pathway for the acylation of monolignols. Acylation of monolignols **1** by *p*-coumarate, via its CoA derivative **2**, to form monolignol *p*-coumarate conjugates **3**, and an example (using the favored β -O-4-coupling, following rearomatization of the quinone methide intermediate **4**) of how the monolignol moiety undergoes radical coupling reactions during lignification resulting in lignin units such as **5** in which *p*-coumarate acylates the γ -position of lignin unit side-chains; monolignol *p*-coumarate conjugates **3** can also (not shown) β -5-couple (with a **G** or **H** unit) or β - β -couple with a monolignol **1** or another monolignol *p*-coumarate **3**. Further lignification via coupling with additional monomers can etherify the phenolic unit of **5**, via either 5- β - (when the phenolic ring is **H** or **G**) or 4-O- β -coupling.

Figure 2. (a) *BdPMT* transcript levels in WT Brachypodium tissues, as determined by qRT-PCR. Columns represent means (n=3), bars, standard deviations. Means were normalized to the first internode mean transcript level value, which was set to one. (b) Diagram of the *BdPMT* gene Bradi2g36910 (drawn to scale; 300 bp scale bar). Solid rectangles represent the two exons, connected by a 4,009 bp intron. Labeled are relative locations of translational start/stop codons, *Bdpmt-1* transition mutation, and the RNAi constructs. (c) Alignment of *BdPMT* polypeptide sequences containing the HXXXD motif with sequences from BAHD acyltransferases representing each of the five clades as delineated by phylogenetic analysis (D'Auria, 2006). Consensus amino acids are boxed in black. The HXXXD motif is delineated along with the position of the G that was mutated in the *Bdpmt-1* mutant allele. References for the acyltransferases (delineated by accession numbers) are in D'Auria, 2006.

Figure 3. Comparisons of tallest culm heights and total above-ground biomass weights of senesced mutant (a) and WT (b) plants grown under long days. Columns represent means plus/minus standard deviations. Samples grouped together in the graphs represent plants grown side by side. Note that subtle growth chamber and/or epigenetic differences affect *Brachypodium* average plant growth, so only plants grown side by side and having similar seed histories should be compared to each other. $11 < n < 63$.

Figure 4. Graphs showing the amounts of *pCA*, *FA*, *pCA-Arabinose*, and *FA-Arabinose* released from senesced stem biomass lignin and hemicelluloses combined (a), lignin only (b), or arabinoxylan only (c), using the treatments denoted in the graph titles. Columns represent mean percents relative to WT, with mean WT amounts normalized to 100%. Bars denote standard deviations. Asterisks denote statistically significant differences compared to WT plants that were grown alongside, as determined by Student's t-tests, where * represents $p < 0.02$ and ** represents $p < 1 \times 10^{-4}$. PMT OX denotes plants from transgenic lines 23A, 57A, 64A, and 79A. See Tables S2 and S3 for actual values. $n \geq 3$.

Figure 5. Schematic for analytical methods used to delineate between hydroxycinnamates, *p*-coumarate and ferulate, on lignin versus on polysaccharides. a) The derivatization following by reductive cleavage (DFRC) method cleaves lignin ethers while leaving the γ -esters intact, releasing quantifiable, lignin-diagnostic acylated units, the monolignol dihydro-*p*-coumarate ester conjugates **8**. b) Mild hydrolysis cleaves arabinose units from arabinoxylans and, at least in large part, leaves esters intact, therefore releasing 5-*O*-*p*-coumaroyl arabinose **10** and 5-*O*-feruloyl arabinose **11**. Free *p*-coumaric and ferulic acids are also released and detected/quantitated; the *p*-coumarate may derive from either its ester on lignin or arabinoxylan, but compounds **10** and **11** are specifically derived from acylated arabinoxylan.

Figure 6. Solution-state HSQC NMR of ball-milled plant cell walls (top row) and cellulolytic enzyme lignin (CEL) treatment (bottom row) in DMSO- d_6 /pyridine- d_5 . From left to right are the *Bdpmt-1* mutant, *BdPMT* downregulated line 4B (*PMT* RNAi line 4B), wild-type and *BdPMT* overexpressed (*BdPMT* OX). Sample peaks have been color-coded according to the lignin substructures below the plots. The percentages and ratios reported for each spectrum are integrals relative to total H+G+S aromatic units.

Figure 7. BdPMT OX stem sections (b, d) have considerably lighter phloroglucinol (a, b) and Mäule Reagent (c, d) staining compared to WT (a, c), which is consistent with relatively less cell wall lignin. Scale bars represent 50 μ m.

Figure 8. Digestibility of stem biomass, comparing *BdPMT OX* and *BdPMT RNAi* Line 4B to transgenic control plants (UBIprom:GUS), and *Bdpm1-1* to wild-type. Shown are the average amounts of glucose (top two charts; non-cell wall glucose subtracted from totals) and pentose sugars (bottom two charts) released per mg of senesced ground biomass using the various pretreatments along with enzyme hydrolysis. Vertical bars represent standard deviations. Asterisks represent Student's t-test (*: p<0.03; **: p<0.005). n=5 biological reps, 3 technical reps.

CHAPTER III

BDCESA7, *BDCESA8*, AND *BDPMT* UTILITY PROMOTER CONSTRUCTS FOR TARGETING GENE-OF-INTEREST EXPRESSION IN SECONDARY CELL WALL FORMING CELLS OF GRASSES

Abstract

The spatial and temporal control of gene-of-interest expression is a useful tool for studying gene and cellular function and for industrial applications. For targeting gene-of-interest expression to plant biomass-generating secondary cell wall forming cells, upstream regulatory sequences of the *Brachypodium distachyon* lignin biosynthetic gene *BdPMT* and cellulose synthase genes *BdCESA7* and *BdCESA8* were isolated and cloned into binary vectors designed for *Agrobacterium*-mediated transformation of monocots. Expression patterns were assessed using the β -glucuronidase gene *GUSPLUS* and X-glucuronide staining. In stem cross-sections, expression of all three promoters was detected strongest in xylem vessels and tracheary elements of the vascular bundles and in interfascicular and epidermal tissues, with expression less pronounced in developmentally older tissues. In leaves, *BdCESA7* and *BdCESA8* promoter expression was strongest in leaf veins, leaf margins, and trichomes; relatively weaker and patchy expression was observed in regions of the epidermis. *BdPMT* promoter expression was similar to the *BdCESA* promoters' expression patterns, but was not detected in leaf epidermal cells. The intensity and extent of

GUS staining varied considerably between transgenic lines, suggesting that chromosomal location greatly affected the promoters' expression. In floral buds, all three promoter::*GUSPLUS* constructs expressed strongly in the outer glume, moderately to faintly in the palea, and moderately in the feathery styles, at the base of the ovary, and in pollen grains. The functionality of these constructs will be validated by demonstrating that *BdPMT* promoter::*BdPMT* cDNA and *BdCESA8* promoter::*BdCESA8* cDNA constructs can rescue the mutant phenotypes of *Brachypodium pmt* and *cesa8* mutants, respectively.

Keywords: Binary vectors; *Brachypodium distachyon*; cellulose; cellulose synthase; lignin; tissue-specific expression

Introduction

Efforts are underway to engineer plant vegetative biomass, such as stems and leaves of grasses, to be more easily deconstructed and converted to liquid biofuels via fermentation of secondary cell wall-derived polysaccharide sugars. In this process, the highly recalcitrant phenolic polymer lignin poses challenges in that it encapsulates and crosslinks cellulose and hemicelluloses thereby interfering with their chemical and enzymatic hydrolysis. One approach to improving biomass deconstructability is to engineer genetic changes that alter flux through the lignin biosynthetic pathway, e.g. by overexpressing or knocking down key monolignol biosynthetic pathway genes resulting in lower lignin quantities, altered lignin composition, and/or altered cell wall polymer crosslinking (Vanholme et al., 2012; Eudes et al., 2014). Plant biomass yields and/or deconstruction properties can also be improved by introducing genetic changes that increase deposition of secondary cell wall polysaccharides

and/or that result in the deposition of less tightly packed (amorphous) polysaccharides. However, plant phenotypic problems can arise when genes-of-interest are misexpressed in a non-targeted fashion. Those problems include tissue structures lacking proper strength resulting in collapsed xylem elements and/or lodging of stems, or developmental abnormalities or toxicity that negatively impacts plant growth and fitness (Yang et al., 2013). The creation of a “toolbox” of gene promoters that drive expression of transgenes in desired cells (e.g. only in cells involved in secondary cell wall formation) would be of benefit to molecular biologists aiming to improve plant biomass digestibility and yields while minimizing plant fitness costs.

Multiple approaches exist to identify genes whose promoters and cis-acting regulatory sequences drive expression with specific spatial and temporal patterns. For example, transcriptome profiling was recently employed to determine the cellular expression patterns of the ten members of the *Brachypodium distachyon* *CELLULOSE SYNTHASE A* (*CESA*) gene family (Handakumbura et al., 2013). In that study, *CESA4*, *CESA7*, and *CESA8* were determined to be expressed at high levels in plant organs known to be active in secondary cell wall biosynthesis. The expression patterns of *CESA4* and *CESA7* were characterized and validated by performing *in situ* hybridization in stem cross-sections, using labeled *CESA4* and *CESA7* antisense sequences as probes; expression was found to be highest in vascular bundles and surrounding interfascicular fibers as well as in nearby epidermal cells (Handakumbura et al., 2013). Wang et al. (2014) recently characterized the *Poplar trichocarpa* R2R3 MYB transcription factor *PtrMYB152*, finding that it activates genes involved in secondary cell wall biosynthesis. To verify that a *PtrMYB152* promoter fragment drove expression similarly to that of the validated secondary cell wall forming

Arabidopsis thaliana (*Arabidopsis*) *AtCESA8* gene promoter (Chen et al, 2005), the *PtrCESA8* promoter was fused to the β -*GLUCURONIDASE* (*GUS*) gene and transformed into *Arabidopsis*. X-Gluc staining verified expected expression in the veins of leaves and stele of seedling roots. Stem sections from the upper (developmentally younger) as well as lower (developmentally older) internodes of the inflorescence showed *PtrCESA8* expression of *GUS* in the vascular bundles and interfascicular fibers (Wang et al., 2014).

Bioinformatic resources that are now available for *Brachypodium distachyon* (*Brachypodium*) have allowed its transcriptome to be mined for promising gene promoter candidates. Recently, Coussens et al. (2012) searched the publically available Expressed Sequence Tag (EST) database available at PlantGDB for *Brachypodium* accession Bd21 genes that were either constitutively expressed across tissues and development, or uniquely expressed in leaf, stem and leaf sheath, roots, flowers, or callus. Putative candidates were then tested for levels of expression across multiple organs using quantitative real-time reverse-transcription PCR (qRT-PCR). In that publication, promoters that were predicted by *in silico* analysis of the EST database to be constitutive, and an endosperm-specific promoter, were cloned into a monocot transformation vector harbouring the *GUS* reporter gene. The corresponding maize transformants exhibited the expected expression patterns as viewed by X-Gluc staining and 4-methylumbelliferyl- β -D-glucuronide (MUG) fluorometry. That work showed that a combined approach of bioinformatic purveyance of all expressed genes and whittling down of promoter candidates based on qRT-PCR analysis was a useful means by which to identify gene promoters with desired expression patterns for a particular transgenic application.

An elegant example of the power of tissue-specific gene expression can be found in Yang et al. (2012). In an *Arabidopsis CINNAMATE 4-HYDROXYLASE (C4H)* mutant background, functional *C4H* expression was reintroduced under the control of the xylem vessel-specific *VASCULAR-RELATED NAC-DOMAIN6 (VND6)* gene promoter. These genome edits directed normal lignin biosynthesis to xylem vessels, while lignin reductions in interfascicular fiber cells allowed for easier extraction of polysaccharide sugars (Yang et al., 2012). This example provides evidence that promoter substitution can be successfully used to transgenically alter cell wall phenotypes including lignin deposition while avoiding negative growth effects.

For the present study, the expression patterns produced by *Brachypodium BdPMT*, *BdCESA7*, and *BdCESA8* gene promoter fragments were determined by introducing those fragments into a binary vector designed for monocots, upstream of the *GUSPLUS* reporter gene. *Brachypodium* plants transformed with each of these utility promoter vectors displayed GUS staining patterns that one would expect for promoters involved in secondary cell wall formation. In addition, to validate the functionality of these utility promoter vectors, the *GUSPLUS* gene in the *BdPMT* and *BdCESA8* vectors was replaced by the *BdPMT* cDNA and *BdCESA8* cDNA, respectively, and the constructs transformed into the *Bdpmt-1* loss-of-function mutant (Petrik et al., 2014) and a Bradi2g49912 *BdCESA8* loss-of-function T-DNA mutant that we identified. Given the observed *GUSPLUS* reporter gene expression patterns, it is expected that the *BdPMT* promoter::*BdPMT* cDNA construct will rescue the reduced cell wall *para*-coumarate (*pCA*) and ferulate (*FA*) phenotypes of the *Bdpmt-1* mutant and that the *BdCESA8* promoter::*BdCESA8* cDNA construct will rescue the reduced cellulose and stunted growth phenotypes that we identified in the *Bdcesa8* mutant.

Materials and Methods

PCR Amplification of Promoters from *Brachypodium distachyon* Genomic DNA

DNA sequence beginning (-) 2385 bp upstream and continuing to (-) 1 of the Bradi4g30540 ATG start site was PCR amplified using accession Bd21-3 genomic DNA as template and the primers listed in Table 1. The *BdCESA7* promoter PCR product was sequenced then cloned into the transformation vector *SwaI/AscI* restriction sites, followed by re-sequencing in the purified plasmid clone. For the *BdCESA8* promoter, DNA sequence beginning at (-) 1890 bp upstream of the Bradi2g49912 gene translational start site and extending to (-) 1 was PCR amplified, sequenced, subcloned into the *StuI/AscI* restriction sites in the transformation vector and re-sequenced in the purified plasmid clone. For the *BdPMT* promoter, 3000 bp was PCR amplified from (-) 3045 to (-) 45 of the Bradi2g36910 gene, sequenced, cloned using *SwaI/AscI* into the transformation vector, then re-sequenced in the purified plasmid clone. An exogenous pair of restriction enzyme sites was added to the 5' end of the sequence specific regions of the primers to allow subcloning of the PCR amplified promoter sequences into a modified pIPKb001cereal transformation vector (Himmelbach et al, 2008). Restriction enzymes were chosen based on the sites present upstream of the recombination sites in pIPKb001, and the lack of the restriction enzyme site internally within the PCR amplified promoter sequence. The DNA sequences of the *BdPMT*, *BdCESA7*, and *BdCESA8* cloned promoter sequences can be found in Supplemental Figures S1, S2, S3, respectively.

Modification of pIPKb001 Cereal Transformation Vector for Optimal Expression of *GUSPLUS* Reporter Gene

To create the base vector to which the gene promoters would be inserted, two modifications of the cereal transformation vector pIPKb001 (Himmelbach et al, 2008) were made. The unmodified pIPKb001 vector was provided by the Leibniz Institute of Plant Genetics and Crop Plant Research (Himmelbach et al, 2008). First, the maize ubiquitin intron 1 (*ZmUbiIN1*) was inserted in the HindIII site of the multiple cloning site, then screened for proper orientation by PCR. It has been reported that the addition of an intron sequence improves exogenous gene expression in grasses (Mann et al, 2011; Sivamani, 2006; Mascarenhas et al, 1990; Plesse et al, 2001). Second, the *ccdB* gene was then replaced by the *GUSPLUS* (Figure 2.1C) reporter gene using LR recombination based cloning (Invitrogen). This construct was then ready for promoter insertion upstream of the *ZmUbiIN1* using either the *SwaI/AscI* or *StuI/AscI* restriction enzyme sites.

Following creation of the modified Pipkb001 transformation vectors housing the *GUSPLUS* reporter gene, the *BdPMT* (Bradi2g36910), *BdCESA7* (Bradi4g30540), *BdCESA8* (Bradi2g49912) gene promoters were cloned in upstream of the reporter genes, as shown in Supplemental Figure S4. *SwaI/AscI* were used in the case of the *BdPMT* and *BdCESA7* promoters, while *StuI/AscI* was used in the case of the *BdCESA8* promoter due to the presence of an internal *SwaI* site in the promoter sequence.

Transformation of Promoter Characterization Vectors into *Brachypodium distachyon* via *Agrobacterium tumefaciens* Transformation

Plants harboring the *BdPMT promoter: ZmUbi IN1: GUSPLUS*, *BdCESA7promoter: ZmUbi IN1: GUSPLUS*, or *BdCESA8 promoter: ZmUbi IN1: GUSPLUS* constructs were regenerated from Bd21-3 embryonic callus tissue transformed using *Agrobacterium tumefaciens* strain AGL-1, as described by Vogel and Hill (2008); media were supplemented with 40 U/ml hygromycin B (Phytotechnology Laboratories).

Seed Sterilization and Plant Growth

Seeds were surface sterilized and plated on selective plates (1.5% agar/one half-strength Murashige and Skoog salts (Caisson Laboratories)/40 U/mL hygromycin B), stratified for three days (4 °C, dark), then moved to a growth chamber (22 °C, 16 h light) until hygromycin selection was apparent.

Plants were grown in a 50:50 mix of SunGro Rediearth and MetroMix 510 soil in 4 inch pots in growth chambers (20 h light:4 h dark photoperiod, 22 °C, 50% humidity). Control plants were either WT Bd21-3 seedlings plated on non-selective plates or planted directly in soil, or hygromycin-selected plants harboring a *Zea mays* Ubiquitin-1 promoter with intron driving *GUSPLUS* (Cambia Labs) in pWBVec8.

Tissue Collection for X-Gluc Staining

A one centimeter section of the leaf tip was taken for X-Gluc colorimetric detection of *GUS* expression in the leaf. For juvenile leaf sections, the uppermost leaf

was chosen for sectioning, 18 days after planting the seedling germinated on ½ MS + 40 U/ml hygromycin plates.

In the case of adult leaf sections, the lowest fully expanded leaf with no cell death at the leaf tip, typically the first or second leaf from the crown, was chosen for sectioning. Again, a one centimeter section of the leaf tip was used for X-Gluc staining.

Additionally, a small experiment was performed to compare *GUS* expression of 1.0 cm leaf tips taken from both upper and lower leaves sampled from adult plants 32 days after transplantation from hygromycin selection plates.

Free-hand cross sections were taken from both juvenile and adult *Brachypodium distachyon* stems. Because *GUSPLUS* would be localized in the cytoplasm (no localization signal was added to the *GUSPLUS* gene), hand sections were taken to be several cell layers thick to allow blue colored product from the X-Glucuronidase assay to be maintained in the cytoplasm of internal cell layers.

Juvenile stage stem sections were taken from the bottom 0.5 cm of the first internode of plants 18 days after transplanting from hygromycin selection plates. To make sectioning easier of this juvenile, less lignified stem tissue, the leaf sheath enwrapping the internode stem was removed prior to stem sectioning. This allowed less crushing of the soft stem inside of the leaf sheath.

Adult stage stem sections were taken from the bottom 1.0 cm of the uppermost internode of the inflorescence stem. These were sampled 32 days after transplantation of hygromycin selected seedlings to soil.

To examine *GUSPLUS* expression along every internode of the adult plant, sections were taken at the median of every internode; at the apical internode, sections were taken within the basal 2 cm, the median, and the apical 2 cm.

Transgenic seeds were germinated on ½ MS plates minus sucrose plates with 40 U/ml hygromycin B for three days at 22C in the light. After 3 days, seedling roots approximately 2 cm in length were excised from the seed and immediately placed in X-Gluc staining solution with 10 mM L-ascorbic acid added. Incubations were performed overnight at room temperature, then rinsed in water prior to visualization.

All florets from spikelets which included 1-3 filling seeds were separated and X-Gluc stained overnight at room temperature overnight. This allowed a full range of floret development from 2 spikelets per plant to be tested for *GUS* expression patterns. Staining was performed at room temperature because preliminary tests showed intensive blue staining of the outer glume, actually allowing blue precipitate to form and collect between trichome hairs of the glume and in solution when incubation was performed overnight at 37C. The strongest expressing transgenic lines were tested overnight at room temperature both with and without potassium ferri- and ferrocyanide addition. Addition of these compounds both lessens intensity of *GUS* staining, allowing difference across floral organs to be visualized, and lessens leakage of blue product from cell to neighboring cells.

X-Gluc Staining for Detection of *GUS* Expression

Leaf, stem section, and floral buds were immersed in an X-Gluc staining solution consisting of 0.1M NaPO₄ buffer PH 7.0, 10 mM EDTA, 0.1% Triton X-114, and 0.1 M

X-Gluc dissolved in N,N-DMF (Jefferson, R.. (1987) Plant Mol. Biol. Reporter, 5:387-405). Roots were stained similarly, except for the addition of ascorbic acid to the X-Gluc staining solution to a final concentration of 10 mM, which prevented oxidative browning of the tissue. No vacuum infiltration was performed; incubations were typically done overnight at 37C, followed by removal of chlorophyll by 2 incubations in 70% EtOH until the wash solution was clear.

In the case of very strongly *GUSPLUS* expressing transgenic lines, 1.0 mM potassium ferricyanide and 1.0 mM potassium ferrocyanide was added to the staining solution to limit “bleeding” of blue product into neighboring cells. Additionally, when ascertaining cell specificity in these strongly expressing transgenic plant lines, incubation was performed overnight at room temperature rather than 37C. The chlorophyll removal using 70% EtOH was identical.

Rescue of *Bdpmt* and *Bdcesa8* Mutant Phenotypes

The cDNA sequence (+1 to stop codon) of *BdPMT* Bradi2g36910 was LR cloned into the empty pIPKb001 vector housing the cloned *BdPMT* promoter sequence:: *ZmUbi* IN1:: *ccdB*:: NOS TER cassette. The *BdPMT* promoter::*ZmUbi* IN1::*BdPMT* cDNA::NOS TER vector was then transformed into embryogenic callus derived from the homozygous *Bdpmt-1* mutant using *Agrobacterium tumefaciens* mediated transformation.

To perform the phenotypic rescue of *BdCESA8*, a T-DNA mutant inserted into Bradi2g49912 was used as the genetic background. This mutant JJ18282 was obtained from the *Brachypodium distachyon* T-DNA mutant library created by John Vogel (Bragg et al, 2012).

Similar to *BdPMT*, the *BdCESA8* cDNA was cloned from the +1 to the stop codon initially into the pENTR2B entry vector (Invitrogen), prior to LR cloning into the transformation vector. Due to the presence of the hygromycin resistance gene in the T-DNA inserted into the *BdCESA8* Bradi2g49912 gene, a transformation vector using a different selectable marker needed to be used to select for the presence of the *BdCESA8* promoter sequence::*ZmUbi* IN1::*BdCESA8* cDNA::*NOS* TER rescue cassette. Therefore, the B258 p9ioACT vector available from DNA Cloning Service (<http://www.dna-cloning.com/>) was used, which allowed for paromomycin selection. Empty vector and phenotypic rescue constructs are shown in Supplemental Figures S5 and S6, respectively. The *BdCESA8* promoter sequence::*ZmUbi* IN1::*BdCESA8* cDNA::*NOS* TER transformation vector was transformed into embryogenic callus heterozygous for the T-DNA insertion in the Bradi2g49912 gene. This was done because homozygotes grown and screened by genomic DNA PCR showed a fertility problem which caused seeds to abort, thereby preventing harvest of homozygous embryos for callus creation.

Upon senescence of *BdPMT* phenotypic rescue T0 regenerants, dry stem tissue was cut into 2 mm long pieces, bead milled, and tested for pCA and FA content in total secondary cell wall extracts by mild hydrolysis followed by GC-FID (Petrik et al, 2014).

Bdpmt-1 and WT plants, grown together with the rescue plants, were used as controls.

For the *BdCESA8* phenotypic rescue experiment, initial regenerants obtained were segregating for both the T-DNA insertion in the Bradi2g49912 gene which rendered them *BdCESA8* mutants, and the *BdCESA8* cloned promoter::*ZmUbi* IN1::*BdCESA8* cDNA::*NOS* TER rescue cassette which was inserted by *Agrobacterium tumefaciens* mediated transformation. Therefore, these initial regenerants were allowed to self-pollinate, and

progeny were selected using paromomycin resistance. Approximately $\frac{1}{4}$ of the progeny obtained were homozygous for the T-DNA insertion in the *BdCESA8* Bradi2g49912 gene, and $\frac{3}{4}$ of these would be expected to be homozygous or heterozygous for the *BdCESA8* rescue T-DNA insertion, inserted randomly into the genome. Zygosity of the T-DNA insertion into the *BdCESA8* Bradi2g49912 gene was confirmed by genomic DNA PCR, with one primer in the T-DNA, and one primer in the 3' flanking genomic sequence. Inheritance of at least one allele of the *BdCESA8* rescue cassette was selected for by paromomycin resistance and confirmed by genomic DNA PCR for the *BdCESA8* rescue T-DNA insertion. This was done using a forward primer in *BdCESA8* exon 13, and a reverse primer in the NOS terminator. Plants which were homozygous for the T-DNA insertion in the *BdCESA8* Bradi2g49912 gene were normally stunted; thus, *BdCESA8* T-DNA insertion homozygotes that obtained a height similar to WT would be suggestive of a successful phenotypic rescue if they had also inherited at least one integrated allele of the *BdCESA8* rescue cassette somewhere in their genome. Cellulose was quantitated in the *BdCESA8* rescued progeny, as well as the *BdCESA8* T-DNA mutants, and WT controls using the Updegraff method (Updegraff, 1969).

Results

Selection of Promoter Candidates

A targeted approach was used to choose gene promoters for isolation and subsequent expression specificity characterization. Due to the previous characterization of the Bradi2g36910 *BdPMT* gene necessary for *p*-coumarylation of monolignol alcohols prior to polymerization to form lignin in the secondary cell wall (Petric et al, 2014), it was deemed a

prudent candidate to further study its expression patterns. Real-time PCR data of the *BdPMT* gene across multiple wildtype tissues 34 days after planting showed expression in multiple internodes of the stem, as well as in the spikelet. Also chosen were the *BdCESA7* (Bradi4g30540) and *BdCESA8* (Bradi2g49912) gene promoters, as these genes have been identified to be involved in cellulose synthesis in the secondary cell wall (Handakumbura et al, 2013; Chen et al, 2005; Wang et al, 2014). The sequences of the *BdPMT*, *BdCESA7*, and *BdCESA8* promoters are shown in Supplemental Figures S1, S2, and S3. Included 5' UTR sequence of the downstream gene was marked in boldface when this information was available via Phytozome or Gramene websites (which was not the case for the *BdCESA7* promoter). The motifs in the promoter sequences which were identified as over-represented in primary and secondary cell wall related genes (Cell-wall Related Promoter Elements, CRPEs) by Creux et al (2008) were queried for in each promoter sequence in both the 5' to 3' direction on the sense strand and the 3' to 5' direction on the antisense strand (in relation to the downstream respective gene). Due to the abundance of these motifs in each promoter, they were not marked on the promoter sequences themselves, but rather annotation tables citing each motif found, the number of instances it was identified in the forward or reverse direction, and its putative function, are provided in Tables 2, 3, and 4 for the *BdPMT*, *BdCESA7*, and *BdCESA8* promoters, respectively. Also included in these tables are vascular expression, cell-wall related, and responsive element motifs (allowing the plant to respond to biotic and abiotic conditions such as cold temperature, pathogen attack, changes in light intensity, etc.) identified by querying the Plant Cis-acting DNA regulatory Elements (PLACE) database (<http://www.dna.affrc.go.jp/PLACE/>). Due to the lengthy nature of the gene co-expression tables for *BdPMT*, *BdCESA7*, and *BdCESA8*, these can be referred to

using the PlaNet platform of web-tools dedicated to the visualization and analysis of plant co-function networks. The *BdPMT* Bradi2g36910 gene expression profile and its table of co-expressed genes can be found at <http://aranet.mpimp-golm.mpg.de/responder.py?name=gene!bdi!15780>. The *BdCESA7* Bradi4g30540 gene expression profile and table of co-expressed genes can be found at <http://aranet.mpimp-golm.mpg.de/responder.py?name=gene!bdi!13203>. Lastly, the *BdCESA8* Bradi2g49912 gene expression profile and table of co-expressed genes can be found at <http://aranet.mpimp-golm.mpg.de/responder.py?name=gene!bdi!18830>.

A phylogenetic tree of the *CesA* gene family based on amino acid similarity of the proteins is provided in Figure 10. As shown, the closest *Brachypodium* ortholog to *AtCESA7* (At5g17420) was identified as Bradi4g30540, while the closest *Brachypodium* ortholog to *AtCESA8* (At4g18780) was identified as Bradi2g49912.

***BdPMT* Promoter:*GUSPLUS* Transgenic Lines**

The *BdPMT* promoter, consisting of a 3 kb fragment of DNA spanning from (-) 3,045 to (-) 45 upstream of the Bradi2g36910 gene translational start site was cloned upstream of the maize *UBIQUITIN1* intron fused to the *GUSPLUS* reporter gene in the pIPKb001 monocot transformation vector (Himmelbach et al., 2007). The construct was transformed into *Brachypodium* embryo-derived callus, and four independent T0-generation transgenic plants were regenerated. T1- and T2-generation plants were analyzed for X-Gluc (GUS) staining, at juvenile and adult developmental stages.

***BdPMT* Promoter:*GUSPLUS* Expression in Stems**

In stem sections sampled from juvenile (18 days after planting) plants, taken from the first internode, *BdPMT* promoter:*GUSPLUS*-related GUS staining was observed in vascular bundles, particularly in the xylem vessels and tracheids (Supplemental Figure S7). Only occasionally was staining observed in sclerenchyma cells in the ring surrounding the vascular bundles. GUS staining was also evident in epidermal cells, including trichomes.

Examination of apical internodes of adult (32 days after planting) *BdPMT* promoter:*GUSPLUS* plants displayed GUS staining along the outer epidermis of the stem in the microhair trichomes, with indigo product extending into underlying cells (Figures 2B and 2C). Potassium ferri- and ferro-cyanide were added to the X-Gluc solution when staining sample (2C), in order to reduce colorimetric product diffusion (Vitha et al, 1995) in this strongly expressing line, yet in both 2B and 2C, staining was seen in the epidermis subdermal to the trichomes. Since similar results were obtained with or without potassium ferri- and ferro-cyanide addition, this indicates that the staining observed in epidermal cells subdermal to the trichomes is due to true *GUSPLUS* expression in these epidermal cells when driven by the *BdPMT* promoter.

X-Gluc staining was also detected in sclerenchyma fiber cells (Figure 2, SFs) located in a ring surrounding the vascular bundles. Sclerenchyma-specific staining appeared sporadically in the lower (developmentally mature) internode cells, and was more pronounced in the upper internode cells where secondary cell wall biosynthesis is more active (Figure 1).

Vascular bundle staining was detected in both xylem vessels and tracheid elements and was particularly evident under high magnification (Figure 2, VBs). With respect to

vascular bundles exhibiting relatively light GUS staining, indigo coloring was not visible in the cell walls of the phloem or in xylary parenchyma cells. The stem section staining pattern correlated well with the pattern of lignin deposition as detected by phloroglucinol staining of stem sections at the same adult developmental stage (Figure 2A).

***BdPMT* Promoter:*GUSPLUS* Expression in Leaves**

In *BdPMT* promoter:*GUSPLUS* T1 generation plants' leaves, production of colorimetric product was strongest at the leaf margins, the leaf tip, and in major leaf veins. Strong staining was also apparent in trichome macrohairs (mh), whereas lighter staining occurred in developing and mature bicellular microhairs (bm) and in prickly hair cells (ph) at the leaf margin (Figure 3, Supplemental Figure S8). Trichome macrohairs, microhairs, and prickly hair cells are described and documented by scanning electron microscopy (SEM) in maize by Moose et al, 2004. Note that *BdPMT* expression in these trichome subtypes was as expected given that lignification has been documented in the walls of these cell types (Marks et al, 2008). *BdPMT* promoter-driven *GUS* expression was not detected in non-trichome epidermal cells except at the leaf margin, or in the guard cells of stomata.

***BdPMT* Promoter:*GUSPLUS* Expression in Roots**

GUSPLUS expression driven by the *BdPMT* promoter was assayed in 3 day old roots of seedlings which had been germinated on vertically-oriented ½ MS minus sucrose plus 40U/ml Hygromycin agar media plates. In all transgenic lines, GUS staining was visible in the vasculature and weakly in the root cap (Figure 4, Supplemental Figure S9). In the strongest expressing lines, staining was also detected weakly in the root hairs (Figure

4H). Overall, staining was stronger in older tissues proximal to the seedling shoot compared to younger tissues closer to the root tip. This suggests that *BdPMT* promoter-driven expression of *GUSPLUS* begins in the elongation zone and continues into the mature zone.

***BdPMT* Promoter:*GUSPLUS* Expression in Floral Organs**

Immature florets were removed from spikelets of adult plants approximately 35 days after seedling planting into soil. Microscopic examination of whole florets after overnight X-Gluc staining revealed strong GUS staining in the lemma (le), often to the degree that indigo precipitate became deposited in the solution between trichomes (Figure 5). Lesser staining was observed in the palea (pa). For more exact view of wildtype, nonstained palea, reference (Garvin, 2009, <http://www.ars.usda.gov/SP2UserFiles/person/1931/BrachypodiumCrossing.pdf>). The lemma and palea were carefully dissected away, and the internal floral organs removed and immediately photographed. *BdPMT* promoter::*GUSPLUS*-related staining was visible at the base of the ovary, in the feathery stigma, and lightly in pollen grains (Figure 6).

***BdCESA7* and *BdCESA8* Promoter:*GUSPLUS* Transgenic Lines**

As with the *BdPMT* promoter fragment, a 2,385 bp *BdCESA7* promoter fragment and a 1,890 bp *BdCESA8* promoter fragment (both of which extended to the genes' translational start sites) were PCR amplified and cloned into the pIPKb001 vector upstream of the maize *UBIQUITIN1* intron and *GUSPLUS* gene. The resultant constructs were used to generate transgenic *Brachypodium* plants. T1 and T2-generation plants from eight independent lines each were analyzed. Overall, the GUS staining patterns observed in plants

from these lines were very similar. However, plants from the *BdCESA7* promoter:*GUSPLUS* transgenic lines tended to stain more strongly.

***BdCESA7* and *BdCESA8*:*GUSPLUS* Expression in Stems**

In *BdCESA7* and *BdCESA8* promoter:*GUSPLUS* plants' first internode stem sections, strong GUS staining was observed in epidermal cells and vascular bundles, whereas moderate GUS staining was visible in sclerenchyma cells. Staining appeared more ubiquitous in comparison to the staining observed in *BdPMT* promoter:*GUSPLUS* plants' first internode stem sections (Supplemental Figure S7).

In *BdCESA7* and *BdCESA8* promoter:*GUSPLUS* T1 adult stem sections, GUS staining was visible in sclerenchyma cells flanking the vascular bundles, within vascular bundles, and in epidermal cells (Figures 1, 7, 8). Strong expression in the vascular bundles occurred in internodes where secondary cell wall biosynthesis was actively occurring; thus, in adult plants just prior to seed fill, *GUSPLUS* expression driven by the *BdCESA7* and *BdCESA8* promoters occurred in the apical internode, while less expression occurred in more basal internodes. In internodes where *GUSPLUS* expression-derived staining was witnessed in the vascular bundles, staining was visible in the xylem vessels and tracheids (Figures 7 and 8). Staining in bundle sheath cells was sometimes visible in the strongest expressing lines, but not visible in moderately expressing lines. This corresponded to the weak expression of *BdCESA7* in bundle sheath cells visualized by *in situ* hybridization, as determined by Handakumbura et al. (Figure 4E, Handakumbura et al., 2013). GUS staining was not detected in phloem or xylary parenchyma cells of *BdCESA7* and *BdCESA8* promoter:*GUSPLUS* plants (Figures 7B and 8B).

***BdCESA7* and *BdCESA8:GUSPLUS* Expression in Leaves**

In leaves of T1-generation plants, significant differences in the intensity of staining were observed, likely due to positional effects of the transgene integration site. Specifically, regulatory sequences which were already present in the host plant's genomic DNA may have *cis*- or *trans*- regulatory effects on expression of the transgene. In moderately staining *BdCESA7* and *BdCESA8* promoter lines, *GUS* expression-related staining was detected in leaf veins, macrohair trichomes, microhair trichomes, and faintly in the cell walls between adjacent epidermal cells (Figure 3, Supplemental Figures S11 & S12). This is consistent with *CESA7* and *CESA8* expression being involved in cellulose biosynthesis in the cell wall, and cellulose deposition being noted to occur in trichomes.

***BdCESA7* and *BdCESA8:GUSPLUS* Expression in Roots**

GUSPLUS expression driven by the *BdCESA7* and *BdCESA8* promoters was assayed in 3 day old roots of seedlings which had been germinated on ½ MS minus sucrose plus 40U/ml Hygromycin agar plates. In all transgenic lines, *GUS* staining was detected in the vasculature and weakly in the root cap (Figure 4, Supplemental Figure S9). Weak root hair staining was also detected in the strongest expressing lines (Figures 4F, 4G). Overall, expression was stronger in older tissues proximal to the seedling shoot compared to younger tissue closer to the root tip. This suggests that *BdCESA7* and *BdCESA8* promoter driven expression of *GUSPLUS* begins to occur in the elongation zone and continues strongly into the mature zone.

***BdCESA7* and *BdCESA8* :*GUSPLUS* Expression in Floral Organs**

BdCESA7 and *BdCESA8* promoter driven expression of the *GUSPLUS* reporter gene was detected in multiple tissues of immature florets. Strong expression was observed in the lemma (le), to the extreme of having blue precipitate deposited in the solution between the trichomes after overnight incubation (Figure 5). Staining was weak on the palea (dorsal side of palea shown in Figure 5, (pa)), For exact view of wildtype, non-stained palea, reference (Garvin, 2009,

<http://www.ars.usda.gov/SP2UserFiles/person/1931/BrachypodiumCrossing.pdf>, page 3).

Although weak palea staining could be dismissed as lesser exposure to the X-Gluc staining solution, this is contraindicated by efficient penetration of the stain to the internal floral organs. Upon dissection of the lemma and palea, the floral organs were removed, placed in a thin film of water to prevent the style from dessicating, and immediately photographed.

BdCESA7 and *BdCESA8* promoter driven expression of *GUSPLUS* resulted similar patterns of staining, with strong staining visible at the base of the ovary, moderate staining in the stigma and style, and weak staining in the pollen grains (Figures 6 and 9).

***Bdpmt* Phenotypic Rescue**

In order to validate that the *BdPMT* promoter sequence cloned was complete and sufficient to drive expression of any gene inserted its corresponding empty vector construct, a phenotypic rescue experiment was performed in which the *BdPMT* cloned promoter sequence was used to drive expression of the *BdPMT* cDNA in a *Bdpmt* mutant genetic background. The *BdPMT* phenotypic rescue construct (Supplemental Figure S5) was transformed using *Agrobacterium tumefaciens* mediated transformation into a *Bdpmt* mutant

Brachypodium distachyon line obtained from Richard Sibout and described previously (Petrik et al, 2014). Regeneration was performed under hygromycin B selection, to obtain only regenerants inheriting the *BdPMT* rescue T-DNA insert. The *Bdpmt* mutant was received as a homozygous line, and thus, once the *BdPMT* rescue construct was transformed into this genetic background, the initial regenerants could be analyzed for restoration of *p*-coumarate (*pCA*) levels in the cell wall back to wildtype levels. Senesced stem tissue from fifteen independent transgenic lines were quantitated for average $\mu\text{g } p\text{CA}/\text{mg sample}$, in comparison to six wildtype non-transgenic controls and three *Bdpmt-1* mutant lines serving as positive and negative controls, respectively. Of the fifteen transgenic line analyzed, two lines successfully showed restoration of the wildtype level of *p*-coumarate (Supplemental Figure S15). This indicates that although positional effects of the transgene insertion site were likely affecting expression levels of the *BdPMT* rescue construct, that the sequence cloned of the *BdPMT* promoter was sufficient to drive *BdPMT* expression similar to expression of the endogenous *BdPMT* gene by its promoter sequence at its native locus.

***Bdcesa8* Phenotypic Rescue**

In order to validate that the *BdCESA8* promoter sequence cloned was complete and sufficient to drive expression of any gene inserted into its corresponding empty vector construct, a phenotypic rescue experiment was performed in which the *BdCESA8* cloned promoter sequence was used to drive expression of the *BdCESA8* cDNA in a *Bdcesa8* mutant genetic background. The *BdCESA8* phenotypic rescue construct (Supplemental Figure S5) was transformed using *Agrobacterium tumefaciens* mediated transformation into a *Bdcesa8* mutant genetic background. This *Bdcesa8* mutant was obtained from a library of

T-DNA insertational mutants created by Dr. John Vogel (Bragg et al, 2012). Because the *Bdcesa8* mutant failed to form seed when the T-DNA insertion was homozygously inherited, the homozygous mutant could not be used to derive embryos for callus generation to create host tissue for the phenotypic rescue *Agrobacterium* mediated transformation. Instead, embryos were harvested from heterozygous *Bdcesa8* mutants for callus generation. This meant that in order for phenotypic rescue to be validated, the initial transformed regenerants would need to be allowed to self-pollinate, so that progeny which were homozygous for the presence of the T-DNA insertion in the *BdCESA8* gene and also had at least one copy of the phenotypic rescue T-DNA inherited to be identified prior to biochemical analysis for cellulose content in comparison to *Bdcesa8* homozygous and heterozygous mutants, the B258p910ACT empty vector control, and wildtype plants. Homozygous *Bdcesa8* mutants were both stunted in height and exhibited the expected decreased cellulose content, whereas heterozygous *Bdcesa8* mutants exhibited a growth habit and cellulose content similar to wildtype (Supplemental Figures S13 and S14). Therefore, successful phenotypically rescued *Bdcesa8* mutants should exhibit both normal height and cellulose levels as determined by the Updegraff method (Updegraff, 1969). Although cellulose quantitation of rescued plants is not yet available due to plants are still senescing, genomic DNA PCR was performed to confirm inheritance of the *Bdcesa8* mutation and the successful integration of the *BdCESA8* phenotypic rescue T-DNA (Supplemental Figure S16). This analysis indicated that ten transgenic lines were obtained which are homozygous for inheritance of the *Bdcesa8* mutation, and they have integrated at least one allele of the *BdCESA8* phenotypic rescue T-DNA. These ten lines can therefore be directly analyzed upon their senescence, (rather than having to analyze the progeny of their

self-pollination) for cellulose content compared to the *Bdcesa8* mutant and wildtype plants as controls.

Discussion

For this study, we generated utility promoter vectors that can be used for transforming monocot plant species, for driving gene-of-interest expression in secondary cell wall forming cells. Histochemical assessment of *Brachypodium distachyon* plants transgenic for the *BdPMT*, *BdCESA7*, and *BdCESA8* promoter::*GUSPLUS* constructs showed expected expression in xylem vessels, tracheids, interfascicular fiber cells, and trichomes.

Although expression patterns were consistently similar between independent transgenic plants for each promoter tested, the strength of expression varied considerably between the transgenic lines (Supplemental Figures S8-S12). This was likely due to the effects of *cis*- and *trans*- regulatory elements in the surrounding native genomic DNA proximal to the integrated T-DNA insertion. This “positional effect” has been reported previously (Ziemienowicz, 2010). Further, the number of copies of the transgene inserted into the genome can certainly affect transgene expression levels in the plant, and copy number was not assessed in these transgenic lines. Although *Agrobacterium tumefaciens* typically inserts its T-DNA in a single copy or two copy fashion, copy number effect may certainly be causing expression level differences across transgenic lines of a given construct. Transgene zygosity differences in different progeny individuals within one transgenic line can also cause differences between them in strength of transgene expression. Gene expression differences between transgenic lines may be desirable in instances where

complete loss-of-function or too-high overexpression causes severe phenotypes or lethality. Efforts to mediate transgene expression variation across transgenic lines have included flanking the transgene with scaffold/matrix attachment regions (S/MARs; Breyne et al, 1992). However, although S/MAR inclusion often increased transgene expression levels overall, variation across transgenic lines still occurred (Nowak et al, 2001).

BdPMT, *BdCESA7*, and *BdCESA8* promoter::*GUSPLUS*-derived staining was also observed in floral tissues at the base of the ovary, in the internal tissue of the feathery style, and within pollen grains. *GUS* expression driven by the *BdCESA7* and *BdCESA8* promoters could indicate that the *BdCESA7* and *BdCESA8* genes are involved in cellulose biosynthesis within pollen grain walls (Ferguson et al, 1998). *BdPMT* expression may be related to biosynthesis of phenolics in the intine of the pollen grain, which is composed of pectin, cellulose, and hemicellulose (Quilichini et al, 2015). Previous work by Grienberger et al demonstrated the activity of a BAHD acyltransferase in the tapetum layer of the *Arabidopsis thaliana* anther, allowing synthesis of hydroxycinnamoyl spermidines (Grienberger et al, 2009). These compounds may become a component of the pollen grain exine, as the outerwall of the pollen grain lies adjacent to the tapetum layer of the anther during development. This indicates a role of BAHD expression in developing floral tissues, which may be an area of future study in regards to *BdPMT* expression and enzymatic function. It is important to note that the *GUS* enzyme produced by these constructs localizes cytoplasmically by default, given that it does not contain targeting sequences. As such, in the case of pollen grain *GUS* staining, no data can be gleaned as to whether the *BdCESA7*, *BdCESA8*, and *BdPMT* proteins are involved in intine formation or exine formation.

The use of spatiotemporal promoters to drive ectopic expression of genes of interest can, in some instances, circumvent phenotypic abnormalities such as altered plant growth, activation of defense pathways, and related fitness costs resulting from strong constitutive overexpression (Yang et al., 2012; Hernandez-Garcia and Finer, 2014). By definition, spatiotemporal gene promoters restrict gene expression to specific tissues at different stages of development throughout the life cycle of the plant. Although seed specific, fruit specific, root or tuber specific, and anther specific gene promoters have been identified (Potenza et al, 2004), few vascular specific promoters have been cloned and characterized for grass species. One example is the *Oshox1* gene from *Oryza sativa*, in which proximal and distal sequences within the 1,600 bp upstream sequence region were found to convey vascular-specific *GUS* expression patterns in multiple tissues and organs via modular combinations of repeated subsequences (Scarpella et al., 2000). The expression pattern driven by the *Oshox1* gene promoter fragment differs from those driven by the promoter fragments characterized in this study in that it is more specific to the vasculature and is modulated by sucrose and auxin (Scarpella et al., 2000).

Secondary cell wall-specific gene promoters are available and have been utilized in dicot species. Recently, the poplar *PtrCesA8* promoter was used to drive the poplar R2R3 MYB transcription factor *PtrMYB152* in *Arabidopsis thaliana* to demonstrate the ability of this transcription factor to upregulate lignin biosynthesis-related genes, leading to increased secondary cell wall thickness (Wang et al, 2014). Another example in transgenic poplar was the use of the *C4H* promoter to drive expression of the *FERULATE 5-HYDROXYLASE* (*F5H*) gene. This secondary cell wall-specific expression of *F5H* caused an increase in the syringyl/guaicyl (S/G) monolignol ratio in the transgenic poplar compared to

nontransformed controls, which led to wood more resistant to wood decaying fungi (Skyba et al, 2013). More promising to the application of easing vegetative biomass recalcitrance to conversion to bioethanol was the recent success in using the *Populus trichocarpa* *PtrCesA8* promoter to drive expression of a non-native *FERULOYL MONOLIGNOL TRANSFERASE* (*AsFMT*) gene from *Angelica sinensis* in poplar. The *AsFMT* enzyme functions to ester-conjugate ferulate to sinapyl or coniferyl monolignol alcohols. When these ferulated monolignols are incorporated by radical oxidative coupling into the backbone of the growing lignin polymer, the ester bond serves as a easily broken linkage between adjacent monolignols. Due to the facility of breaking this ester bond, lignin modified in this way has been termed “zip lignin”, and this technology holds promise as a means to lower the cost of biomass pretreatment due to a lesser requirement for high heat or chemical treatments (Wilkerson et al, 2014).

The creation of synthetic promoters is still in its infancy, due to the laborious study of regulatory elements within gene promoters either by promoter deletion or sequence synthesis followed by testing of function by fusion to reporter genes in a whole plant or cell culture system (Rushton et al, 2002). However, the fruits of these approaches would be the synthesis of designer promoters with combined spatiotemporal expression patterns.

Although the use of cell culture systems to study gene promoters offers the advantages of speed of growth compared to the life cycle of an entire plant, as well as ease of isolation of desired cell types, its main disadvantage is the removal of adjoining cells which may alter cell wall deposition in the desired cell type (as seen in the “good neighbor” hypothesis described by Smith et al, 2013). The bioinformatic comparison of multiple promoter sequences to identify common regulatory elements (Molina and Grotewold, 2005),

combined with thorough cell and developmental specificity assays of expression of their respective genes, may allow sufficient and necessary sequences to be identified for desired expression patterns.

In conclusion, it is anticipated that the utility promoter binary vector constructs characterized in this study could be used to drive gene-of-interest expression in secondary cell wall forming tissues of various grass species such as switchgrass, wheat, rice, or maize, given that other monocot promoter sequences have been shown to be functional in related species (Coussens et al., 2012). Therefore, these utility vectors should hold value in application in both model species, such as *Brachypodium distachyon*, and monocot crops of commercial interest.

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CHAPTER III TABLES

Table 1. PCR Primers Used to Amplify Promoter Sequences of *BdCESA7* Bradi4g30540, *BdCESA8* Bradi2g49912, *BdPMT* Bradi2g36910, and to Test Inheritance of *Bdcesa8* T-DNA Insertion and *BdCESA8* Phenotypic Rescue.

Sequence Name	Sequence 5' to 3'	Len	%GC Content	Tm
BdCESA7proFSwaI	GAGAGATTTAAATTGTCGTGGTACGGAGGAGCTAG	35	45.7	69.4
BdCESA7proRAscI	GAGAGGCGCGCCCGCCGGCCGGTCGATCTCCGC	33	81.8	83.3
BdCESA8proFSuI	GAGAGAGGCCTGCTGCCACGCTGGTGAAGG	30	66.7	75.6
BdCESA8proRAscI	GAGAGGCGCGCCGGCTCTCCCTCCGTGCG	29	79.3	80.2
BdPMTproFSwaI	GAGAGATTTAAATGATGCGGAGGAGATAG	29	41.4	64.6
BdPMTproRAscI	GAGAGGCGCGCCTAAGCTTGCATGCTCTAGC	31	61.3	73.9
NOST primer up	GCAAGACCGGCAACAGGATT	20	55	54.69
BdCESA8 EX13 F1	CCTGGTGCTCAACCTTGTCG	20	60	55.34
T3 T-DNA LB	AGCTGTTTCCTGTGTGAAATTG	22	41	52.42
GSP R Mandy	GAAGACTGCCCAAACGACTT	20	50	52.34
JJ18282 F1	TTGATGCTTGCTGATCCTTCCTAG	24	46	57.58
JJ18282 R1	CTGGTCCTTGGATGCCATCAAGC	23	57	58.42

Note: Tm calculated using Nearest Neighbor method.

Table 2. *BdPMT* Promoter Annotation of Cell Wall, Vascular Expression, and Responsive Element Related Motifs.

Motif Name	#	Sequence	Putative function
CACTFTPPCA1	50	YACT	Mesophyll-specific expression
ACGTATERD1	38	ACGT	Etiolation-induced expression; early response to dehydration
CURECORECR	30	GTAC	Copper-response element; Also involved in oxygen-response
WRKY71OS	30	TGAC	Gibberillin-responsive
MYCCONSUSUSAT	28	CANNTG	dehydration-responsive; cold-responsive
GATABOX	27	GATA	Required for high level, light regulated, and tissue specific expression
CAATBOX1	23	CCAAT	CCAAT box found in the promoter of heat shock proteins genes
ARR1AT	21	NGATT	"ARR1-binding element" found in Arabidopsis; ARR1 is a response regulator
POLLEN1ELAT52	21	AGAAA	Pollen specific activation
GTGANTG10	17	GTGA	May confer pollen specificity
WBOXNTERF3	14	TGACY	Wounding responsive
GT1CONSUSUS	12	GRWAAW	Light regulated
CBFHV	9	RYCGAC	Dehydration-responsive
P1BS	8	GNATATNC	Phosphate-responsive
WBOXATNPR1	8	TTGAC	W-box; recognized specifically by salicylic acid (SA)-induced WRKY DNA binding proteins
ABRELATERD1	7	ACGTG	Etiolation-induced expression; early response to dehydration
IBOXCORE	7	GATAA	Conserved sequence upstream of light-regulated genes
INRNTPSADB	7	YTCANTYY	Light-responsive transcription
TATABOX5	7	TTATT	TATA box
ASF1MOTIFCAMV	6	TGACG	Transcriptional activation by salicylic acid or auxin; Also may be light responsive
NTBBF1ARROLB	6	ACTTTA	Required for tissue-specific expression and auxin induction
WBOXNTCHN48	6	CTGACY	W box; NtWRKY1, NtWRKY2 and NtWRKY4 bound to W box; NtWRKYs possibly involved in elicitor-responsive transcription of defense genes
DPBFCOREDCDC3	5	ACACNNG	May confer embryo and ABA specificity
MYB1AT	5	WAACCA	Dehydration responsive
MYB2CONSUSUSAT	5	YAACKG	Dehydration responsive
OSE2ROOTNODULE	5	CTCTT	Organ-specific elements (OSE) activated in infected cells of root nodules
RHERPATEXPA7	5	KCACGW	Root Hair Cell-Specific cis-Element
ABRERATCAL	4	MACGYGB	Ca(2+)-responsive upregulation
CCAATBOX1	4	CCAAT	CCAAT box found in the promoter of heat shock proteins genes
RYREPEATBNNAPA	4	CATGCA	Required for seed specific expression
TBOXATGAPB	3	ACTTTG	Light-activation
ARFAT	2	TGTCTC	ARF (auxin response factor) binding site
CGCGBOXAT	2	VCGCGB	Ca++/calmodulin responsive
CRTDREHVCBF2	2	GTCGAC	Temperature-responsive
EECCRCAL1	2	GANTTNC	EEC; Consensus motif of the two enhancer elements, EE-1 and EE-2
ELRECOREPCR1	2	TTGACC	EIRE (Elicitor Responsive Element)
EMHVCHORD	2	TGTAAAGT	Endosperm motif (EM)
GT1GMSCAM4	2	GAAAAA	Pays a role in pathogen- and salt-induced
MYBATRD22	2	CTAACCA	Dehydration and ABA responsive

(Table Continues)

Motif Name	#	Sequence	Putative function
MYBPLANT	2	MACCWAMC	Plant MYB binding site; Consensus sequence related to box P in promoters of phenylpropanoid biosynthetic genes
MYCATERD1	2	CATGTG	Dehydration-responsive
OSE1ROOTNODULE	2	AAAGAT	organ-specific elements (OSEs); activated in infected cells of root nodules
PYRIMIDINEBOXOSRAMY1A	2	CCTTTT	Gibberillin-responsive
REALPHALGLHCB21	2	AACCAA	Required for phytochrome regulation
SORLIP1AT	2	GCCAC	Light-Induced
SORLIP2AT	2	GGGCC	Light-Induced
"T/GBOXATPIN2 "	1	AACGTG	Jasmonate (JA) induction
ABREATRD22	1	RYACGTGGYR	ABA and dehydration responsive
ABREOSRAB21	1	ACGTSSSC	ABA responsive element (ABRE)
ACGTABREMOTIFA2OSEM	1	ACGTGKC	ABA responsive element (ABRE)
ACGTOSGLUB1	1	GTACGTG	Required for endosperm-specific expression
BOXLCOREDCPAL	1	ACCWWCC	Consensus of the putative "core" sequences of box-L-like sequences in the carrot (D.c.) PAL1 promoter region
CANBNNAPA	1	CNAACAC	Seed specificity; activator and repressor
CIACADIANLELHC	1	CAANNNNATC	Region necessary for circadian expression
CMSRE1BSPOA	1	TGGACGG	CMSRE-1 (Carbohydrate Metabolite Signal Responsive Element 1)
CPBCSPOR	1	TATTAG	Cytokinin-responsive
GADOWNAT	1	ACGTGTC	Gibberillin-downregulated
GARE2OSREP1	1	TAACGTA	Gibberellin-responsive element (GARE)
GAREAT	1	TAACAAR	GARE (GA-responsive element)
L1BOXATPDF1	1	TAAATGYA	Involved in L1 layer-specific expression
LTRECOREATCOR15	1	CCGAC	Core of low temperature responsive element (LTRE)
MYB2AT	1	TAACTG	Water-stress responsive
PALBOXAPC	1	CCGTCC	Box A; Consensus; One of three putative cis-acting elements (boxes P, A, L) of the phenylalanine ammonia-lyase (PAL); These elements appear to be necessary but not sufficient for elicitor- or light-mediated PAL gene activation
SORLIP3AT	1	CTCAAGTGA	Light-Induced
SORLIP5AT	1	GAGTGAG	Light-Induced
SORLREP3AT	1	TGTATATAT	Light-Induced
SREATMSD	1	TTATCC	sugar-repressive element (SRE)
SV40COREENHAN	1	GTGGWWHG	SV40 core enhancer
TATABOX2	1	TATAAAT	Putative TATA box
TATABOX4	1	TATATAA	Putative TATA box
TATABOXOSPAL	1	TATTTAA	Binding site for OstBP2, found in the promoter of rice pal gene encoding phenylalanine ammonia-lyase.
TELOBOXATEEF1AA1	1	AAACCCTAA	Required for the activation of expression in root primordia
XYLAT	1	ACAAAGAA	Cis-element identified among the promoters of the "core xylem gene set"
CRPE5	1	GNCA(C/G)TGA	Primary cell wall associated CesA motif in A.t., P.t., and E.g.; Jasmonic acid responsive element
CRPE8	2	GGNGGTGG	Primary cell wall associated CesA motif in A.t., P.t., and E.g.; Anthocyanin regulatory element
CRPE9	9	CNCNNCNC	Primary cell wall associated CesA motif in A.t., P.t., and E.g.; Vascular-specific expression
CRPE12	6	ATN(A/T)ATTA	Primary cell wall associated CesA motif in A.t., P.t., and E.g.; Phosphate response domain.
CRPE13	25	GC(A/T)NGC	Primary cell wall associated CesA motif in A.t., P.t., and E.g.; Absciscic acid responsive element
CRPE14	3	NG(A/G)CNGTG	Primary cell wall associated CesA motif in A.t., P.t., and E.g.; Function not available.
CRPE16	2	GAGCG(A/C)	Primary cell wall associated CesA motif in A.t., P.t., and E.g.; Light repression element
CRPE17	2	GTC(G/T)GT	Primary cell wall associated CesA motif in A.t., P.t., and E.g.; Function not available.
CRPE18	5	ANNGA(C/T)AG	Primary cell wall associated CesA motif in A.t., P.t., and E.g.; Function not available.
CRPE19	3	ACAGNCNG	Primary cell wall associated CesA motif in A.t., P.t., and E.g.; Function not available.
CRPE 20	7	TTTTTT	Primary cell wall associated CesA motif in A.t., P.t., and E.g.; AT-rich element
CRPE 23	33	TNNCN(G/T)NC	Primary cell wall associated CesA motif in A.t., P.t., and E.g.; Function not available.
CRPE 28	4	NNGCATGC	Secondary cell wall associated CesA motif in A.t., P.t., and E.g.; Iron deficiency responsive element
CRPE 30	13	NNNT(C/G)AAG	Secondary cell wall associated CesA motif in A.t., P.t., and E.g.; Function not available.
CRPE31	3	GNGNAGNG	Secondary cell wall associated CesA motif in A.t., P.t., and E.g.; Function not available.
CRPE 32	13	(A/G)N(C/G)(C/T)T(A/G)(C/G)C	Secondary cell wall associated CesA motif in A.t., P.t., and E.g.; Function not available.

Note: Annotation of over-represented motifs in the promoters of primary and secondary cell wall-associated *CESA* genes of *Arabidopsis thaliana* (A.t.), *Populus trichocarpa* (P.t.), and *Eucalyptus grandis* (E.g.). These motifs are abbreviated CRPE (*CESA*-related promoter element). In addition, vascular, cell wall related, and responsive element motifs identified using the PLACE database have been included.

Table 3. *BdCESA7* Promoter Annotation of Cell Wall, Vascular Expression, and Responsive Element Related Motifs.

Motif Name	#	Sequence	Putative Function
MYCONSENSUSAT	22	CANNTG	Dehydration-responsive
GTGANTG10	21	GTGA	May confer pollen-specific expression
WRKY71OS	20	TGAC	A core of TGAC-containing W-box; Gibberillin-responsive
ACGTATERD1	18	ACGT	Etiolation-induced expression
CGCGBOXAT	18	VCGGB	Signal-responsive
CACTFTPPCA1	16	YACT	Mesophyll expression module 1
WBOXNTERF3	11	TGACY	May be wounding responsive
CBFHV	10	RYCGAC	dehydration-responsive element
GATABOX	10	GATA	Required for high level, light regulated, and tissue specific expression
GT1CONSENSUS	9	GRWAAW	Consensus GT-1 binding site in many light-regulated genes
LTRECOREATCOR15	8	CCGAC	Core of low temperature responsive element (LTRE)
SORLIP1AT	8	GCCAC	Light-Induced
DRECRTCOREAT	6	RCCGAC	Dehydration-responsive element
GT1GMSCAM4	6	GAAAAA	Pathogen- and salt-induced
OSE2ROOTNODULE	6	CTCTT	Organ-specific elements (OSE); activated in infected cells of root nodules
POLLEN1LELAT52	6	AGAAA	Pollen specificity
ABRELATERD1	5	ACGTG	etiolation-induced
INRNTPSADB	5	YTCANTYY	Light-responsive
SORLIP2AT	5	GGGCC	Light-Induced
WBOXATNPR1	5	TTGAC	Recognized specifically by salicylic acid (SA)-induced WRKY DNA binding proteins
"-300ELEMENT"	4	TGHAAARK	Endosperm-specific gene expression
DREZCOREZMRAB17	4	ACCGAC	May confer ABA-responsiveness
MYB1AT	4	WAACCA	Dehydration-responsive
MYB2CONSENSUSAT	4	YAACKG	Dehydration-responsive
WBOXNTCHN48	4	CTGACY	"W box"; NtWRKY1, NtWRKY2, and NtWRKY4 bound to W box; NtWRKYs possibly involved in elicitor-responsive transcription of defense genes
CAATBOX1	3	CAAT	CAAT promoter consensus sequence
MYBPLANT	3	MACCWAMC	Consensus sequence related to box P in promoters of phenylpropanoid biosynthetic genes
RHERPATXPA7	3	KCACGW	Right part of RHEs (Root Hair-specific cis-Elements)
RYREPEATBNNAPA	3	CATGCA	Required for seed specific expression
CANBNNAPA	2	CNAACAC	Embryo- and endosperm-specific transcription
CATATGGMSAUR	2	CATATG	Auxin responsiveness
DPBFCOREDCC3	2	ACACNNG	Embryo-specific, and also can be induced by ABA
EECCRCAH1	2	GANTTNC	Consensus motif of the two enhancer elements, EE-1 and EE-2
GLMHVCHORD	2	RTGASTCAT	Nitrogen responsive element
MYCATERD1	2	CATGTG	Early responsive to dehydration
MYCATRD22	2	CACATG	
PALBOXAPC	2	CCGTCC	Box A; Consensus; One of three putative cis-acting elements (boxes P, A, and L) of phenylalanine ammonia lyase (PAL); These elements appear to be necessary but not sufficient for elicitor- or light-mediated PAL gene activation
PROLAMINBOXOSGLUB1	2	TGCAAG	Involved in quantitative regulation
PYRIMIDINEBOXHVEPB1	2	TTTTTCC	Required for GA induction
RYREPEATLEGUMINBOX	2	CATGCAY	May confer seed-specific expression
SORLREP3AT	2	TGTATATAT	
"-300MOTIFZMZEIN"	1	RTGAGTCAT	May confer endosperm specificity
"T/GBOXATPIN2"	1	AACGTG	Involved in jasmonate (JA) induction
ABREOSRAB21	1	ACGTSSSC	
ACGTABREMOTIFA2OSEM	1	ACGTGKC	ABA-responsive
ACGTOSGLUB1	1	GTACGTG	Required for endosperm-specific expression
AMMORRESIVDCRNIA1	1	CGAACTT	Ammonium responsive
ARE1	1	RGTGACNNNGC	Antioxidant response element
BOXIIPCCHS	1	ACGTGGC	Essential for light regulation
CCAATBOX1	1	CCAAT	CCAAT box found in the promoter of heat shock proteins genes
CIACADIANLELHC	1	CAANNNNATC	Region necessary for circadian expression
GARE1OSREP1	1	TAACAGA	Gibberellin-responsive element (GARE)
GCCCORE	1	GCCGCC	Pathogen-responsive

(Table Continues)

Motif Name	#	Sequence	Putative Function
GCN4OSGLUB1	1	TGAGTCA	Required for endosperm-specific expression
LTRE1HVBTL49	1	CCGAAA	LTRE-1 (low-temperature-responsive element)
OSE1ROOTNODULE	1	AAAGAT	Organ-specific elements (OSE) characteristic of the promoters activated in infected cells of root nodules
POLLEN2LELAT52	1	TCCACCATA	pollen specific activation
QELEMENTZM2M13	1	AGGTCA	May be involved in up-regulation of genes in pollen
TATABOX3	1	TATTAAT	TATA box
TATABOX5	1	TTATTT	TATA box
TBOXATGAPB	1	ACTTTG	Light-activated gene transcription
XYLAT	1	ACAAAGAA	cis-element identified among the promoters of the "core xylem gene set"
CRPE 4	1	(A/C)TGTCGG	Primary cell wall associated CesA motif in A.t., P.t., and E.g.; Absciscic acid (ABA) responsive element
CRPE 8	1	GGNGGTGG	Primary cell wall associated CesA motif in A.t., P.t., and E.g.; Anthocyanin regulatory element
CRPE 9	30	CNCNCCNC	Over-represented in promoters of the primary cell wall-associated CesA genes in A.t., P.t., and E.g.; Function not available
CRPE 10	2	CCNC(A/C)CCC	Primary cell wall associated CesA motif in A.t., P.t., and E.g.; Vascular-specific expression
CRPE 13	17	GC(A/T)NGC	Primary cell wall associated CesA motif in A.t., P.t., and E.g.; Absciscic acid (ABA) responsive element
CRPE 15	1	G(C/T)GCTC	Primary cell wall associated CesA motif in A.t., P.t., and E.g.; Phloem-specific expression
CRPE 16	2	GAGCG(A/C)	Primary cell wall associated CesA motif in A.t., P.t., and E.g.; Light repression element
CRPE 17	6	GTC(G/T) GT	Over-represented in promoters of the primary cell wall-associated CesA genes in A.t., P.t., and E.g.; Function not available
CRPE 18	2	ANNGA(C/T)AG	Over-represented in promoters of the primary cell wall-associated CesA genes in A.t., P.t., and E.g.; Function not available
CRPE 19	1	ACAGNCNG	Over-represented in promoters of the primary cell wall-associated CesA genes in A.t., P.t., and E.g.; Function not available
CRPE 20	16	TTTTTT	Primary cell wall associated CesA motif in A.t., P.t., and E.g.; AT-rich element (AT1BOX)
CRPE 22	1	C(C/T)C(C/G)NCCC	Over-represented in promoters of the primary cell wall-associated CesA genes in A.t., P.t., and E.g.; Function not available
CRPE 23	35	TNNCN(G/T)NC	Over-represented in promoters of the primary cell wall-associated CesA genes in A.t., P.t., and E.g.; Function not available
CRPE 28	2	NNGCATGC	Secondary cell wall associated CesA motif in A.t., P.t., and E.g.; Iron deficiency responsive element
CRPE 30	29	NNNT(C/G)AAG	Secondary cell wall associated CesA motif in A.t., P.t., and E.g.; Function not available
CRPE 31	7	GNGNAGNG	Secondary cell wall associated CesA motif in A.t., P.t., and E.g.; Function not available
CRPE 32	10	(A/G)N(C/G)(C/T)T(A/G)(C/G)C	Secondary cell wall associated CesA motif in A.t., P.t., and E.g.; Function not available

Note: Annotation of over-represented motifs in the promoters of primary and secondary cell wall-associated *CESA* genes of *Arabidopsis thaliana* (A.t.), *Populus trichocarpa* (P.t.), and *Eucalyptus grandis* (E.g.). These motifs are abbreviated CRPE (*CESA*-related promoter element). In addition, vascular, cell wall related, and responsive element motifs identified using the PLACE database have been included.

Table 4. *BdCESA8* Promoter Annotation of Cell Wall, Vascular Expression, and Responsive Element Related Motifs.

Motif Name	#	Motif sequence	Putative Function
MYCCONSENSUSAT	20	CANNTG	Dehydration-responsive gene
GTGANTG10	19	GTGA	Might confer pollen specific gene expression.
WRKY71OS	12	TGAC	Binding site of rice WRKY71, a transcriptional repressor of the gibberellin signaling pathway
CURECORECR	10	GTAC	Copper-response element
OSE2ROOTNODULE	9	CTCTT	Organ-specific elements of root nodules
WBOXNTERF3	8	TGACY	May be involved in wounding-activated expression
GT1CONSENSUS	7	GRWAAW	Consensus GT-1 binding site in many light-regulated genes
INRNTPSADB	7	YTCANTYY	Light-responsive transcription
ACGTATERD1	6	ACGT	Etiolation-induced expression (early responsive to dehydration) in At
BS1EGCCR	5	AGCGGG	BS1 (binding site 1) found in E. gunnii Cinnamoyl-CoA reductase (CCR) gene promoter; Required for vascular expression
CBFHV	4	RYCGAC	Dehydration-responsive elements (DRE)
GATABOX	4	GATA	Required for high level, light regulated, and tissue specific expression
LTRCOREATCOR15	4	CCGAC	ABA responsiveness; Involved in cold induction; drought-induced gene expression.
MYB1AT	4	WAACCA	Dehydration-responsive
SORLIP1AT	4	GCCAC	One of "Sequences Over-Represented in Light-Induced Promoters (SORLIPs) in At
WBOXATNPR1	4	TTGAC	They were recognized specifically by salicylic acid (SA)-induced WRKY DNA binding proteins
ABRELATERD1	3	ACGTG	Etiolation-induced expression
ABRERATCAL	3	MACGYGB	Ca(2+)-responsive upregulated genes
GT1GMSCAM4	3	GA AAAA	Plays a role in pathogen- and salt-induced ScaM-4 gene expression
LTR1HVBTL49	3	CCGAAA	LTR-1 (low-temperature-responsive element) in barley (H.v.)
BOXLCOREDCPAL	2	ACCWGCC	Consensus of the putative "core" sequences of box-L-like sequences in the carrot (D.c.) PAL1 promoter region
CCAATBOX1	2	CCAAT	Common sequence found in the 5'-non-coding regions of eukaryotic genes; "CCAAT box" found in the promoter of heat shock proteins genes
ELRECOREPCRP1	2	TTGACC	EIRE (Elicitor Responsive Element) core of parsley (P.c.) PR1 genes; Required for elicitor responsiveness
OSE1ROOTNODULE	2	AAAGAT	Organ-specific elements (OSEs) characterisitc of the promoters activated in infected cells of root nodules
RHERPATEXPA7	2	KCACGW	Root Hair Cell-Specific cis-Element in Angiosperms
SORLIPSAT	2	GAGTGAG	One of "Sequences Over-Represented in Light-Induced Promoters (SORLIPs) in At
SURECOREATSULTR11	2	GAGAC	Core of sulfur-responsive element (SURE)
"T/GBOXATPIN2"	1	AACGTG	Involved in jasmonate (JA)
AACACOREOSGLUB1	1	AACAAC	Endosperm-specific expression
CIACADIANLELHC	1	CAANNNNATC	Circadian expression
ERELEE4	1	AWTTCAAA	ERE (ethylene responsive element)
GAREAT	1	TAACAAR	GARE (GA-responsive element)
GCCCORE	1	GCCGCC	Core of GCC-box found in many pathogen-responsive genes; Has been shown to funtion as an ethylene-responsive element; jasmonate-responsive gene expression
IBOX	1	GATAAG	Light-regulated
IBOXCORE	1	GATAA	Light-regulated
IRO2OS	1	CACGTGG	Induced by Fe deficiency
MYB2CONSENSUSAT	1	YAACKG	Dehydration-responsive
MYBGAHV	1	TAACAAA	Central element of gibberellin (GA) response complex
MYBPLANT	1	MACCWAMC	Consensus sequence related to box P in promoters of phenylpropanoid biosynthetic genes such as PAL, CHS, CHI, DR, CL, Bz1
MYCATERD1	1	CATGTG	Early responsive to dehydration
MYCATRD22	1	CACATG	Dehydration responsive
PALBOXAPC	1	CCGTCC	One of three putative cis-acting elements (boxes P, A, L) of the phenylalanine ammonia-lyase (PAL
PALBOXPPC	1	YTYMMCMAMCMMC	Box P; Consensus; One of three putative cis-acting elements (boxes P, A, L) of the phenylalanine ammonia-lyase (PAL) necessary but not sufficient for elicitor- or light-mediated PAL gene activation.
QARBNEXTA	1	AACGTGT	QAR (quantitative activator region)
RYREPEATBNNAPA	1	CATGCA	Required for seed specific expression
SORLIP2AT	1	GGGCC	Light-Induced Promoters (SORLIPs) in At
TATABOX5	1	TTATTT	TATA Box
TBOXATGAPB	1	ACTTTG	Light-activated gene transcription
WBOXNTCHN48	1	CTGACY	NtWRKY1, NtWRKY2 and NtWRKY4 bound to W box; NtWRKYs possibly involved in elicitor-responsive transcription of defense genes

(Table Continues)

Motif Name	#	Motif sequence	Putative Function
CRPE 7	2	GGGGC(A/G)NGNN	Primary and Secondary cell wall associated CesA motif in A.t., P.t., and E.g.; Ethylene Response Element
CRPE 8	2	GGNGGTGG	Primary cell wall associated CesA motif in A.t., P.t., and E.g.; Anthocyanin regulatory element
CRPE 9	32	CNCNNCNC	Primary cell wall associated CesA motif in A.t., P.t., and E.g.; Function not available.
CRPE 10	8	CCNC(A/C)CCC	Primary cell wall associated CesA motif in A.t., P.t., and E.g.; Vascular-specific expression
CRPE 13	5	GC(A/T)NGC	Primary cell wall associated CesA motif in A.t., P.t., and E.g.; Absciscic acid responsive element
CRPE 14	1	NG(A/G)CNGTG	Primary cell wall associated CesA motif in A.t., P.t., and E.g.; Function not available.
CRPE 15	3	G(C/T)GCTC	Primary cell wall associated CesA motif in A.t., P.t., and E.g.; Phloem-specific gene expression
CRPE 16	2	GAGCG(A/C)	Primary cell wall associated CesA motif in A.t., P.t., and E.g.; Light repression element
CRPE 17	1	GTC(G/T)GT	Primary cell wall associated CesA motif in A.t., P.t., and E.g.; Function not available.
CRPE 19	2	ACAGNCNG	Primary cell wall associated CesA motif in A.t., P.t., and E.g.; Over-represented motifs in the promoters of the primary and secondary cell wall-associated CESA genes; Function not available.
CRPE 20	5	TTTTTT	Primary cell wall associated CesA motif in A.t., P.t., and E.g.; AT-rich element
CRPE 22	9	C(C/T)C(C/G)NCCC	Primary cell wall associated CesA motif in A.t., P.t., and E.g.; Over-represented motifs in the promoters of the primary and secondary cell wall-associated CESA genes; Function not available.
CRPE 23	25	TNNCN(G/T)NC	Primary cell wall associated CesA motif in A.t., P.t., and E.g.; Over-represented motifs in the promoters of the primary and secondary cell wall-associated CESA genes; Function not available.
CRPE 24	5	GG(C/G)(A/T)(C/G)G(A/G)(G/C)	Primary cell wall associated CesA motif in A.t., P.t., and E.g.; Over-represented motifs in the promoters of the primary and secondary cell wall-associated CESA genes; Function not available.
CRPE 26	1	TCCTGC(C/T)G	Secondary cell wall associated CesA motif in A.t., P.t., and E.g.; Function not available.
CRPE 30	12	NNNT(C/G)AAG	Secondary cell wall associated CesA motif in A.t., P.t., and E.g.; Function not available.
CRPE 31	9	GNGNAGNG	Secondary cell wall associated CesA motif in A.t., P.t., and E.g.; Function not available.
CRPE 32	3	(A/G)N(C/G)(C/T)T(A/G)(C/G)C	Secondary cell wall associated CesA motif in A.t., P.t., and E.g.; Function not available.

Note: Annotation of over-represented motifs in the promoters of primary and secondary cell wall-associated *CESA* genes of *Arabidopsis thaliana* (A.t.), *Populus trichocarpa* (P.t.), and *Eucalyptus grandis* (E.g.). These motifs are abbreviated CRPE (*CESA*-related promoter element). In addition, vascular, cell wall related, and responsive element motifs identified using the PLACE database have been included.

CHAPTER III FIGURES

Figure 1. X-Gluc stained *Brachypodium distachyon* stem cross sections of plants transformed with the *BdCESA7*, *BdCESA8*, and *BdPMT* promoter sequences driving expression of the *GUSPLUS* reporter gene. A) One culm of a *Brachypodium distachyon* plant, 32 days after transfer of hygromycin selected seedlings to soil. Stem sections were taken from the longitudinal median of the first internode (I1M), second internode (I2M), third internode (I3M), fourth internode (I4M), and the bottom just above the leaf collar of the fifth internode (I5B). Sections were also taken from the middle and top of the apical internode (not shown), which were less intense staining than the bottom of the apical internode. Note that not all plants, whether transgenic or wild type had the same number of internodes. Therefore, the median of each internode, and the bottom, median and top of the apical internode were sampled and assayed for GUS activity. B) Stem sections of *BdCESA7* promoter, *BdCESA8* promoter, *BdPMT* promoter, and wildtype Bd21-3 first, second, third, fourth and fifth internodes. Sections shown are from the median of each internode, with the exception of the apical internode 5, in which sections just above the flag leaf are shown. Note that addition of potassium ferricyanide or ferro-cyanide was not performed, as lesser expression in more basal internodes was desired to be seen if present. (Addition of these compounds not only reduces leakage of blue product from the X-Gluc assay, but also decreases intensity of staining seen on a per cell basis). Scale bar for stem sections represents 170 um.

*Figure 2. BdPMT promoter::GUSPLUS expression in adult plant apical internode stem sections as compared to lignin deposition as evidenced by phloroglucinol staining. A) Wild-type *Brachypodium distachyon* Bd21-3 stem section stained with phloroglucinol for the presence of lignin, 200X. B) *BdPMT promoter::GUSPLUS* Line 2, plant 3, 200X. C) *BdPMT promoter::GUSPLUS* Line 2, plant 10, 200X. Potassium ferri- and ferro-cyanide was added to the X-Gluc staining solution for this plant to allow clear delineation of *GUSPLUS* expressing cells versus adjacent nonexpressing cells.*

Figure 3. Apical leaf snippet X-glucuronidase treated to monitor GUS activity driven by the *BdCESA7*, *BdCESA8*, and *BdPMT* promoters cloned. (A,C,E,G,I) Apical leaf snips taken 18 days after planting of *BdCESA7*, *BdCESA8*, *BdPMT*, *ZmUBQ*pro, and WT transgenic lines, respectively. (B, D, F, H, J) Leaf snips taken from the flag leaf of the same transgenic lines, sampled 32 days after planting. (K) *BdPMT* promoter::*GUSPLUS* Line 2, viewed at 64X magnification. Trichome macrohairs (mh), bicellular microhairs (bm), and prickly hair (ph) cells are noted. (L) *BdPMT* promoter::*GUSPLUS* Line 3, viewed under 400X magnification. Staining at the leaf margin (LM) can be seen of both prickly hair cells (ph) as well as epidermal cells (ec) at the leaf margin. Note that at this developmental stage, the peduncle of the sampled culm was fully elongated, and a young, unfilled spikelet was present. Scale bar represents 1.0 mm.

Figure 4. BdCESA7 promoter, BdCESA8 promoter, BdPMT promoter, ZmUBQ promoter driving GUSPLUS expression in seedling roots X-Gluc stained for GUS Activity 3 days after germination. Seedling roots sampled 3 days after germination on MS plates including 40 ug/ml hygromycin (except WT control, which was germinated without antibiotic selection). A) BdCESA7 promoter::GUSPLUS Line 156A. B) BdCESA8 promoter::GUSPLUS. Line 3A. C) BdPMT promoter::GUSPLUS. Line 2. D) ZmUBQpro::GUSPLUS Line 6A. E) WT #2. All samples viewed under 11.2X magnification. F) Strongly expressing BdCESA7 promoter::GUSPLUS Line 122A viewed under 20X magnification to see staining root hairs. G) Strongly expressing BdCESA8 promoter::GUSPLUS viewed under 20X magnification to see staining root hairs. H) Strongly expressing BdPMT promoter::GUSPLUS Line 2 viewed under 20X magnification to see staining root hairs. Scale bar indicates 1.0 mm.

Figure 5. Florets stained for GUS enzymatic activity, prior to seed fill. Unfilled florets were removed from spikelets and X-Gluc stained overnight at 37C. Chlorophyll was removed using 70% Ethanol prior to imaging. Potassium ferri- and ferro-cyanide was added to the X-Gluc staining solution when staining samples *BdCESA7* promoter::*GUSPLUS* Line 156A and *BdCESA8* promoter::*GUSPLUS* Line 41A to decrease the amount of leakage of blue product previously seen collecting between trichomes on the lemma when staining these lines overnight at 37C. Florets viewed at 11.2X; scale bar represents 1.0 mm.

Figure 6. GUS activity of internal floral organs. Scale bar indicates 0.1 mm. A) BdPMT promoter::GUSPLUS Line 3, 20X. B) BdCESA7 promoter::GUSPLUS Line 122A, 20X. C) BdCESA8 promoter::GUSPLUS Line 113A, 20X. D) WT, 20X.

Figure 7. Stem sections taken from the bottom region of the apical internode of *BdCESA7* promoter::*GUSPLUS* and WT adult plants. 200X magnification. A) WT Bd21-3 stained with phloroglucinol; (B-D) *BdCESA7* promoter driving *GUSPLUS* expression. B) *BdCESA7* promoter::*GUSPLUS* Line 38A. C) *BdCESA7* promoter::*GUSPLUS* Line 71B. D) *BdCESA7* promoter::*GUSPLUS* Line 153A. Addition of potassium ferri- and ferro-cyanide to the X-Gluc staining solution was performed for *BdCESA7* promoter::*GUSPLUS* Lines 38A and 71B to decrease leakage of blue product between adjacent cells and out into the staining solution previously seen in these very strongly expressing transgenic lines.

Figure 8. Stem sections taken from the bottom region of the apical internode of *BdCESA8* promoter::*GUSPLUS* and WT adult plants. 200X magnification A) WT stained with phloroglucinol, (B-D) *BdCESA8* promoter::*GUSPLUS* Line 89A (Potassium ferri- and ferro-cyanide added to X-Gluc solution to decrease leakage of blue product between cells).

Figure 9. X-Gluc staining to examine GUS activity driven by the *BdPMT*, *BdCESA7* and *BdCESA8* promoters in floral stigmas and anthers. Stigma and styles viewed at 400X; anthers of same plant viewed at 200X. (A) *BdPMT* promoter::*GUSPLUS* stigmas and styles. (B) *BdPMT* promoter::*GUSPLUS* anther with pollen grains. (C) *BdCESA7* promoter::*GUSPLUS* stigmas and styles. (D) *BdCESA7* promoter::*GUSPLUS* anther with pollen grains. (E) *BdCESA8* promoter::*GUSPLUS* stigmas and styles. (F) *BdCESA8* promoter::*GUSPLUS* anther with pollen grains. (G) Wildtype stigmas and styles. (H) Wildtype anthers with pollen grains.

Figure 10. Phylogenetic tree of the *Brachypodium distachyon* and *Arabidopsis thaliana* CESA genes based on amino acid similarity. Relatedness is based on amino acid similarity determined using ClustalW algorithm within the MegAlign program of LaserGene 8.

CHAPTER III SUPPLEMENTAL FIGURES

GATGCGGAGGAGATAGAAATGCAAAAGAGAAAAATAATTTACTTTTTGTGTC
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GAAACAAGTTTATAAATTAAAATTATATATACTCACTTCAATCCATAGTAAGT
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 AACTTCTCCACCGCCACTATGATGACACAAGCGTGCCGTGTACGGCCGTAGT
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 TACCATGAGTACAAGCAACGCACACCAAATAAACTTAATTAAGTGGCCCTA
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 CTAGCTGAGCTCCGAAGTCCTGAACTAATAACCCAGCCCGTCTATATATAC
ACAGAGCATATATATCCATACACTCATCGCAGCTAGAGCATGCAAGCTTA

Supplemental Figure S1. Sequence of 3,000 bp cloned *BdPMT* promoter fragment.
 Begins at (-) 3,045 and extends to (-) 45 of Bradi2g36910.1. Included 5' UTR
 sequence is marked in boldface.

TGTCGTGGTACGGAGGAGCTAGATTTCGCGAGACTTGGAAGCGCCGGCGGGT
TCCCGATGTCGGCCAGCGTGGGGAGGATGCGATTTCGTAGTAATGATGGGTTC
ACTACGTCGGTCAGCCGCCCCGCGGTCTGTTGATCGCTGAAACGGAGCCCGG
GCGCGGCGTCGCAGCCGGGAAAAAACCGATCTGGGAGGAACGCGACGCGGA
GCGGCTGATCTGATCGTTTCATGAGTGCACGCACCGACGCACGCATCCGATC
CGACACGATGCGGTGCCCATTTTCGGGGCCGGATCGGCGATGGGCGGTTCTTT
GCCGCTTCACCTGAAGAGTCAAGATTTCGCTAAAAAAAAGCATGAAGAGTCA
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CGCGGCAGATCACCCTCGGTTCCATGCTTTGGTTTACGCCATTTTGTTCAGC
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CGCGCTTATGCTTACACCAAGCCACCGACGGTAACGTGCTGCCCATATTTTT
GCCCTGACGCGTGCAAATTAAGGCCGATCAGTCGTCCAGTACAACAGTGGC
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TCCTCCTCCTAATCGACCGGCCAGCTTAATTAATTTTTTTCATAATAAAATAAA
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GCATGCATTTTTTCAAATGTTCTTTGCAAGGATACTCGTACGTACGTACAACC
CACCGATCACCATTAGTTTTCTCAAAAAATCGAAAAAAGGAGATGGCGGGAG
ACCCAAGCTTGGGTTGGTCTGGAAGGGGGTTGGTCAGGACTCAGGAGAGACG
CGCGCACCTTGCTTGAGCCTGACAACGGCAGCAGAATCCAGCGCAGCGCACC

GGCAAAACCAAACCTTTCGCTTCACTTCGCGGCAACGGGGACTCCACACCCA
ACCCGCCAAGCTCCCCCTACCGTCCGGCCACGTCGATCCACAGACCAAATC
ACAACTCCTTTCTTCGAACTTCGAACAGCCACCCCTCACCTCTCCCCACCCG
CCAAAACACCTAACCTCTCATATAGCCCCCAGCCCCTGCCACAAGCGAAC
CAACTAAGCACCCACACTTAGCCCCCGTCTCCTCTCGCCCTGCGCTGCGCTGC
ACCGGCGCTGCGGCGGAGATCGACCGGCCGGCG

Supplemental Figure S2. Sequence of 2,385 bp cloned *BdCESA7* promoter fragment. Begins at (-) 2,385 and extends to (-) 1 of Bradi4g30540.1. Annotated 5' UTR sequence was unavailable in either Phytozome or Gramene.

[illegible]

Supplemental Figure S3. Sequence of 1,890 bp cloned *BdCESA8* promoter fragment. Begins at (-) 1,896 and extends to (-) 1 of Bradi2g49912.1. Included 5' UTR sequence is marked in boldface.

Supplemental Figure S5. BdPMT and BdCESA8 phenotypic rescue constructs plasmid maps. The BdPMT and BdCESA8 cDNA sequences were Gateway recombination based LR cloned into the BdPMT and BdCESA8 promoter empty vector constructs shown in Supplemental Figure S2. The entire expression cassette was then removed from each construct using SfiI and cloned into the B258 p9io ACT vector from DNA cloning services. This vector allowed for paromomycin to be used for transgenic plant selection, as the mutant genetic background into which these T-DNAs were to be inserted already had hygromycin resistance from the incorporation of the T-DNA insertion in the native BdCESA8 gene. Further, the use of the rice Actin promoter rather than the maize Ubiquitin promoter to drive the plant selectable marker reduced the chance of silencing due insertion of repetitive sequence elements.

Supplemental Figure S6. BdPMT, BdCESA7, and BdCESA8 promoter empty vector constructs. These plasmids have the cloned promoter sequences described, followed by the ZmUbi Intron 1. This is followed by the ccdB gene flanked by Gateway recombinations sites to allow LR cloning to be used to insert a desired gene of interest downstream of the promoter plus intron. The backbone vector is pIKb001, which uses hygromycin as the plant selectable marker.

Supplemental Figure S7. X-Gluc stained juvenile stage stem cross sections taken from the longitudinal median of the first basal internode 18 days after transfer from Hygromycin selection plates to soil. Note that at 18 DAP, there was only one internode present, and it was covered by a leaf sheath. To make sectioning easier and less damaging to the stem, the leaf sheath was often removed prior to cross-sectioning of the young stem. Left panels' scale bars represent 170 um, center panels' scale bars represent 110 um, right panels' scale bars represent 29.5 um.

Supplemental Figure S8. Comparison of expression of *BdPMT* promoter driving *GUSPLUS* expression across transgenic lines in juvenile and adult leaves. Differences are likely due to positional effects of the transgenic cassette within the genome, including enhancer and repressor sequences located distal from the insertion sites. (A, C, E, G) Apical leaf snips of *BdPMT* promoter::*GUSPLUS* Lines 2, 3, 6A, and 11A, respectively, taken 18 days after planting. (B, D, F, H) Leaf snips taken from the flag leaf of the same transgenic lines sampled 32 days after planting. Scale bar represents 1.0 mm.

Supplemental Figure S9. Range of *GUSPLUS* expression in 3 days after germination seedling roots across multiple transgenic lines of *BdCESA7* promoter (A-D), *BdCESA8* promoter (E-H), and *BdPMT* promoter (I-L). (M) *ZmUBQ* promoter, (N) WT. Differences in expression are likely due to positional effects of the transgene in the genome. Scale bar represents 1.0 mm.

Supplemental Figure S10. Comparison of expression across *BdCESA7* promoter driving *GUSPLUS* transgenic lines in adult leaves. Differences are likely due to positional effects of the transgenic cassette within the genome, including enhancer and repressor sequences located distal from the insertion sites. (A,B) *BdCESA7* promoter::*GUSPLUS*. Line 149A. (C,D) *BdCESA7* promoter::*GUSPLUS*. Line 153A. (E,F) *BdCESA7* promoter::*GUSPLUS*. Line 156A. (G,H) *BdCESA7* promoter::*GUSPLUS*. Line 122A. (I,J) *BdCESA7* promoter::*GUSPLUS*. Line 38A. (K,L) *BdCESA7* promoter::*GUSPLUS*. Line 91A. All leaves were viewed at 11.2X. Scale bar represents 1.0 mm. Addition of potassium ferri- and ferro-cyanide to the X-Gluc staining solution was performed when staining *BdCESA7* promoter::*GUSPLUS* Lines 149A and 122A to limit blue product diffusion into neighboring cells and outward into the staining solution.

Supplemental Figure S11. Comparison of expression across *BdCESA8* promoter driving *GUSPLUS* transgenic lines in adult leaves. Differences in expression are likely due to positional effects of the transgene in the genome, including the presence of distally located cis-acting enhancers or repressors. (A,B) *BdCESA8* promoter::*GUSPLUS*. Line 113A. (C,D) *BdCESA8* promoter::*GUSPLUS*. Line 111A. (E,F) *BdCESA8* promoter::*GUSPLUS*. Line 41A. (G,H) *BdCESA8* promoter::*GUSPLUS*. Line 3A. (I,J) *BdCESA8* promoter::*GUSPLUS*. Line 5B. (K,L) *BdCESA8* promoter::*GUSPLUS*. Line 46A. (M,N) *BdCESA8* promoter::*GUSPLUS*. Line 55A. (O,P) *BdCESA8* promoter::*GUSPLUS*. Line 89A. Scale bar represents 1.0 mm.

Supplemental Figure S12. Range of expression across multiple transgenic lines of *BdCESA7* (Top panel), *BdCESA8* (Center panel), *BdPMT* (Bottom panel), promoters driving *GUSPLUS* expression in adult stems. Stem sections were taken from the apical internode, 2 cm above the apical node of adult *Brachypodium distachyon*, 32 days after planting. Samples viewed at 100X magnification; scale bar represents 170 μ m.

Homozygous	Heterozygous	
<i>Bdcesa8</i>	<i>Bdcesa8</i>	WT

Supplemental Figure S13. Height comparison of homozygous *Bdcesa8* mutant, heterozygous *Bdcesa8* mutant, and wild-type control *Brachypodium distachyon*. Homozygous *Bdcesa8* mutants are stunted, whereas the *Bdcesa8* heterozygous mutant appears similar to wildtype height.

Supplemental Figure S14. Crystalline cellulose quantitations as performed by Updegraff assay on homozygous *Bdcesa8* mutants, heterozygous *Bdcesa8* mutants, and wild-type controls. Homozygous *Bdcesa8* demonstrate a decreased amount of cellulose, whereas *Bdcesa8* heterozygotes contain a similar amount of cellulose as wild-type controls.

Supplemental Figure S15. Quantitation of ug pCA/ mg extracted sample of *PMT* phenotypic rescue transgenic lines versus wild-type and *Bdpmt-1* knockout controls.

Supplemental Figure S16. Genomic DNA PCR results of *BdCESA8* phenotypic rescue transgenic lines compared to B258p9ioACT empty vector and wild-type plant controls. The PCR product for the presence of the *Bdcesa8* T-DNA insertional mutation using the T3 TDNA LB and GSP R Mandy primer pair is approximately 800 bp due to partial truncation of one of the two tandem insertions. The PCR product for the presence of the endogenous, unaltered *BdCESA8* gene (WT allele) is 628 bp using the JJ18282 F1 and R1 primer pair. The PCR product which indicates the integration of the *BdCESA8* phenotypic rescue T-DNA, obtained using the NOST primer up and *BdCESA8* EX13 F1 primer pair, is 408 bp. Loading order is as follows: 1) *BdCESA8* phenotypic rescue line 58A PCR'd for the *Bdcesa8* T-DNA insertional mutation. 2) *BdCESA8* phenotypic rescue line 58A PCR'd for the endogenous *BdCESA8* WT allele. 3) *BdCESA8* phenotypic rescue line 58A PCR'd for the presence of the *BdCESA8* Phenotypic Rescue T-DNA. 4) *BdCESA8* phenotypic rescue line 57A PCR'd for *Bdcesa8* T-DNA insertional mutation. 5) *BdCESA8* phenotypic rescue line 57A PCR'd for the endogenous *BdCESA8* WT allele. 6) *BdCESA8* phenotypic rescue line 57A PCR'd for the presence of the *BdCESA8* Phenotypic Rescue T-DNA. 7) 100 bp DNA ladder. 8) B258p9io empty vector transgenic line 29A PCR'd for the *Bdcesa8* T-DNA insertional mutation. 9) B258p9io empty vector transgenic line 29A PCR'd for the endogenous *BdCESA8* WT allele. 10) B258p9io empty vector transgenic line 29A PCR'd for the presence of the *BdCESA8* Phenotypic Rescue T-DNA. 11) Wild-type (WT) PCR'd for the *Bdcesa8* T-DNA insertional mutation. 12) Wild-type (WT) PCR'd for the endogenous *BdCESA8* WT allele. 13) Wild-type (WT) PCR'd for the *BdCESA8* Phenotypic Rescue T-DNA. For simplicity, this figure shows only 2 phenotypic rescue transgenic lines plus controls; in total, 10 such transgenic lines (independent regenerants) were obtained which were homozygous for inheritance of the *Bdcesa8* mutation and had integrated at least one copy of the *BdCESA8* phenotypic rescue T-DNA in a heterozygous fashion.