Investigating the Transcriptional Regulation of Secondary Cell Wall Synthesis and Thigmomorphogenesis in the Model Grass *Brachypodium distachyon*

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Investigating the transcriptional regulation of secondary cell wall synthesis and thigmomorphogenesis in the model grass *Brachypodium distachyon*

A Dissertation Presented
by
JOSHUA H. COOMEY

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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Plant Biology
Investigating the transcriptional regulation of secondary cell wall synthesis and thigmomorphogenesis in the model grass *Brachypodium distachyon*

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I would like to first give thanks to my family for all their love and support. To my father, the first scientist in my life, who instilled me with a ‘can do’ attitude and who taught me that any task can be accomplished with the right tools. To my mother, whose enthusiasm for education and clear communication shaped so much of my approach to academics and mentoring. To my sister, who’s drive, determination, and commiseration has always been a source of inspiration and support.

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A key aspect of plant growth is the synthesis and deposition of cell walls. In specific tissues and cell types including xylem and fiber, a thick secondary wall composed of cellulose, hemicellulose, and lignin is deposited. Secondary cell walls provide a physical barrier that protects plants from pathogens, promotes tolerance to abiotic stresses, and fortifies cells to withstand the forces associated with water transport and the physical weight of plant structures. Grasses have numerous cell wall features that are distinct from eudicots and other plants. Study of the model species *Brachypodium distachyon* has helped us begin to understand the internal and external cues that regulate the synthesis of grass secondary cell walls. In this dissertation, I investigate the function of two transcription factors in regulating cell wall biosynthesis, *SWIZ* and *KNOB7*. *SWIZ* controls wall synthesis and plant growth in response to external mechanical force. In response to touch, SWIZ protein moves into the nucleus, a translocation that is modulated by the level of bioactive gibberellic acid in the cell. Positive and negative perturbation of *SWIZ* results in shorter plants with thicker fiber cell walls, phenotypes that are enhanced
in plants treated with regular mechanical stimulus during growth. KNOB7 is orthologous to the characterized cell wall regulator AtKNAT7 in Arabidopsis thaliana. KNOB7 negatively regulates fiber wall thickness and lignification, as is observed in AtKNAT7, but KNOB7 shows unique control of lignin composition, hydroxycinnamic acid content, and cell wall polysaccharide content. These observations may reflect control of grass specific cell wall characteristics not present in eudicots, such as high levels of wall bound hydroxycinnamic acids and the prevalence of heteroxylan polysaccharides. Together, these insights from SWIZ and KNOB7 function further our understanding of how grasses regulate their growth and secondary cell wall synthesis.
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CHAPTER 1

TANSLEY REVIEWS: GRASS SECONDARY CELL WALLS, BRACHYPODIUM DISTACHYON AS A MODEL FOR DISCOVERY.

1.1 Introduction to the secondary cell wall

The secondary plant cell wall provides mechanical strength that allows plants to stand upright, resist pest and pathogen invasion, and transport water over long distances. Both plants and humans have found this abundant matrix of crosslinked polymers useful as durable building material, with timber featuring in human construction around the world for generations. The secondary wall is distinct from other cell wall types in composition as well as the developmental timing and tissue types where it is deposited. Secondary walls form in thick layers, rich in cellulose, hemicelluloses, and lignin. Cellulose microfibrils have a tensile strength rivaling steel, and form crystalline structures. Hemicelluloses include a variety of polysaccharides, but in grass secondary walls these are mostly mixed linkage glucans and heteroxylans, a defining aspect of this plant lineage. Finally, lignin is a recalcitrant and heterogeneous mixture of randomly polymerized phenolic monolignols that is interspersed and cross linked with wall polysaccharide polymers. Lignification is a hallmark of secondary walls, and unique chemistry and synthesis of this polymer continues to be uncovered (Fig. 1.1).

Following cell expansion of cells surrounded by a primary wall, secondary walls are deposited in a highly specific spatio-temporal manner in certain cell types over development. Unlike eudicots, grass stem growth is a result of iterative division and
elongation events from stacked intercalary meristems called nodes (Esau, 1977; Langer, 1979). New cells generated from the node elongate, pushing up the nodes above with the final node transitioning to the flowering meristem. Thus, the internode regions are most mature at the bottom of the stem, while cells within an internode are most mature at the top of that region, just before the next node (Langer, 1979). Secondary wall deposition occurs between cell elongation and senescence, with cellulose, lignin, and hemicellulose content increasing with maturity (Rancour et al., 2012; Matos et al., 2013; Kapp et al., 2015). Grass stems account for the majority of secondary wall forming sclerenchyma tissues. The interfascicular fibers develop thick secondary walls and provide mechanical strength for the upright stem. Grasses form discrete vasculature with the xylem and phloem contained by bundle sheath cells, unlike eudicots where a cambium separates collateral xylem and phloem (Fig. 2). Depending on the species, stem vascular bundles can be arranged in peripheral rings or dispersed throughout the stem (Esau, 1977). Xylem develops strong secondary walls that can tolerate the high pressures caused by evapotranspiration. Phloem cells do not have secondary walls. Besides phloem, grass stem parenchyma tissue can be found in the pith and in cortex pockets, which have been shown to function as carbon storage tissues during development (Jensen & Wilkerson, 2017). While this review focuses on secondary cell walls in B. distachyon and other grasses, wall synthesis has also been investigated using B. distachyon as a model system for callus tissue, young vegetative growth, and endosperm development (Christensen et al., 2010; Guillon et al., 2011a; Liu et al., 2016; Betekhtin et al., 2018; Francin-Allami et al., 2019).
Figure 1.1. General schematic of grass secondary cell wall matrix. The grass secondary cell wall matrix is made up of cellulose microfibrils, mixed-linkage glucans, heteroxylans, and lignins. (a) A generalized cartoon of grass secondary wall polymer interactions. (b) Schematic fine structure of the pink circled region in (a). Cellulose microfibrils consist of multiple, organized, β(1,4)-linked glucose chains. Mixed-linkage glucans are also glucose chains, but include β(1,3) linkages. Heteroxylan has a xylose backbone that is decorated with sugar and phenolic side chains of xylose, arabinose, glucuronic acid, and hydroxycinnamates (FA and pCA). These polysaccharides can be interwoven with lignins, branched phenolic polymers made of three main lignin units, syringyl (S), guaiacyl (G), and p-hydroxyphenyl (H). Lignins can also contain ferulic and p-coumaric acids.

1.2 *Brachypodium distachyon*, a model grass system.

*B. distachyon* is a model for cereal crops and temperate grasses because of its small stature, simple growth requirements, short life cycle, relatively small and sequenced genome, and close phylogenetic relation to those species (Scholthof *et al.*, 2018). *B. distachyon* has a ‘finished’ reference genome with the only ambiguity being the placement of some centromeric repeats.
(https://phytozome-next.jgi.doe.gov/info/Bdistachyon_v3_1). In addition, there is a growing atlas of gene expression profiles (Trabucco et al., 2013b; Sibout et al., 2017; MacKinnon et al., 2020). It is also remarkable in terms of the resources available for experimental molecular genetics. Genetic transformation protocols are well-developed; current efficiency makes B. distachyon a grass highly amenable to transformation (Bragg et al., 2012). Mutant resources consist of 23,000 T-DNA mutants and 1,200 sequenced chemical mutants (Bragg et al., 2012; Granier et al., 2015). Given that these mutations are more-or-less randomly distributed across the genome and chemical mutagenesis typically induces multiple mutations per mutant line, this large collection likely includes loss-of-function mutations in the majority of B. distachyon genes and multiple nonsynonymous mutations in virtually every gene (Dalmais et al., 2013). This latter category of mutations may be particularly interesting because it can help elucidate the function of cell wall genes, as well as the importance of specific amino acids and protein domains, information that cannot be inferred from knock-out mutants. A large natural variation population exists for B. distachyon, with sequenced genomes for many accessions. These resources have been applied in several studies on growth and biomass related traits (Lee et al., 2012; Tyler et al., 2014; Kapp et al., 2015; Gordon et al., 2017). Thus, B. distachyon is well positioned for gaining fundamental insights into cell wall biosynthesis (Coomey & Hazen, 2015). This knowledge can then be leveraged for agronomic gains in more experimentally recalcitrant grass species.
Figure 1.2. Transverse section of *B. distachyon* and *A. thaliana* stems. Transverse stem cross sections of *A. thaliana* (a) and *B. distachyon* (b) stained with phloroglucinol-HCl, a general stain for lignified tissues. Most eudicots, such as *A. thaliana*, have vascular bundles of xylem separated from phloem by cambium layers, and flanked by interfascicular fibers. In *B. distachyon*, the stem vascular bundles also contain xylem and phloem, but there is no cambial layer, and the vasculature is encased by a lignified bundle sheath layer of mestome cells and surrounded by interfascicular fiber cells. In both species, a cortex region of less lignified cells separates the interfascicular region from the epidermis. Abbreviations: vascular bundles (VB), epidermis (Ep), cortex (Co), cambium area (Ca), interfascicular fibers (IF), phloem (Ph), mestome (Mes), metaxylem (MX), protoxylem (PX), parenchyma (P). Scale bar = 100 µm.

1.3 Cellulose

Cellulose is perhaps the most abundant polymer in the world, found in the walls of every plant cell. It is made of β-1-4 linked glucose monomers, and these glucan chains are synthesized at the plasma membrane by the cellulose synthase complex (Fig. 1). Extruded cellulose chains form organized microfibrils with crystalline structure; the degree of this organization impacts wall mechanics, with greater crystallinity resulting in stiffer walls.
The cellulose synthase complex consists of multiple Cellulose Synthase A (CesA) subunits and associated proteins (Pear et al., 1996; Polko & Kieber, 2019). CesA genes are a subclade of the cellulose synthase superfamily, along with the Cellulose Synthase-like (Csl) clades. Across plant species, seven major lineages have been identified in the CesA genes, which separate into the CesAs associated with primary or secondary wall synthesis (Little et al., 2018). This distinction between primary and secondary wall synthesis is conserved across most vascular plants. In B. distachyon, BdCesA4, 7, and 8 have been shown to function in secondary wall synthesis, and these proteins are highly similar to those characterized in other species for secondary wall function, such as Arabidopsis thaliana and rice (Oryza sativa) (Handakumbura et al., 2013). In B. distachyon, loss-of-function in the secondary CesAs results in reduced crystalline cellulose content, compromised wall integrity, and reduced plant growth (Handakumbura et al., 2013; Petrik et al., 2016). Interestingly, the secondary CesA lineage contains a Poacea-specific clade, which in B. distachyon is represented by BdCesA10. This CesA10 group does not contain the canonical UDP-glucose binding motif (D,D,D,QxxRW) found in glucosyltransferases (Handakumbura et al., 2013). While phylogenetic analysis clearly places these proteins in the CesA clade, it is not clear what role they play, if any, in cell wall synthesis.

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

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

1.4 Mixed-linkage glucans

One of the salient differences that defines grass secondary cell walls is the composition
and utilization of non-cellulosic polysaccharides. These can generally be thought of as pectins and hemicellulose, but discussion of these polymers is often better suited to classification by backbone structure (Scheller & Ulvskov, 2010; Atmodjo et al., 2013).

In eudicots, the predominant polysaccharide polymer after cellulose is xyloglucans, β-1-4-linked glucose chains that contain numerous 1-6 xylose substitutions. The xylose side chains can be further decorated with other sugars such as galactose or fucose (Bauer et al., 1973; Fry, 1989; Scheller & Ulvskov, 2010). In grasses, the role of xyloglucans is largely replaced by mixed-linkage glucans (MLGs) and heteroxylans.

Mixed-linkage glucans are, as their name suggests, β-1-4 linked glucose chains that are interrupted with β-1-3 linkages (Fig. 1 & 3). (1,3)-β-glucans are typically separated either by two or three (1,4)-β-glucans, forming oligosaccharide units of β-cellotriosyl or β-cellotetraosyl (Fig. 3), although longer chains of (1,4)-β-glucans are also observed (Bulone et al., 2019). Almost no evidence of adjacent (1,3)-β-glucan bonds has been found (Buliga et al., 1986). These altered linkages result in the polymer having kinks and bends, unlike the linear glucan chains that form cellulose. As a result, MLG does not form crystalline structures. The relative amounts of β-cellotriosyl and β-cellotetraosyl units strongly relate to the solubility of the overall polymer and are expressed as ratios of degrees of polymerization of tri- and tetrasaccharides (DP3:DP4). Solubility of the polymer decreases at either end of the ratio spectrum. Longer stretches of either β-cellotriosyl or β-cellotetraosyl units increases the overall order of the polymer with more undisturbed regions of (1,4)-β-glucan linkages, and thus decreases solubility. Greater solubility occurs with DP3:DP4 ratios that range from 1:1 to 2.5:1 (Lazaridou &
MLGs were once thought to be unique to grass cell walls, but several examples have now been observed outside of the commelinid monocots, and indeed outside of green plants. Polysaccharides containing (1,3;1,4)-β-glucans have been observed in green, red, and brown algae, lichens, fungi, bryophytes, and the monophyletic genus *Equisetum* (*Bulone et al.*, 2019). Genomic data further supports the idea that MLGs are not specific to the Poaceae, with enzymes capable of synthesizing (1,3;1,4)-β-glucan linkages identified across monocots and in isolated cases in other species. MLG has been shown to be synthesized by members of the *CslF*, *CslH*, and *CslJ* families (*Bulone et al.*, 2019). All three of these groups have co-evolved independently in monocots from sister Csl clades (*Little et al.*, 2018). Members of *CslF/H/J* clades have been shown to be capable of synthesizing (1,3;1,4)-β-glucan when heterologously expressed, but it is not clear whether all of these groups are responsible for native MLG synthesis. By far the best characterized enzyme in MLG synthesis is CslF6, which has been studied in barley, wheat (*Triticum aestivum*), rice, maize, and *B. distachyon* (*Nemeth et al.*, 2010; Vega-Sanchez *et al.*, 2012; *Kim et al.*, 2015, 2018). BdCslF6 protein is localized to the Golgi membrane, with an external catalytic domain (*Kim et al.*, 2015, 2018). Antibody detection of (1,3;1,4)-β-glucan in maize also supports a Golgi localized synthesis of MLG (*Carpita & McCann*, 2010). However, evidence in other grasses suggests that MLG synthesis occurs at the plasma membrane. In barley and wheat, antibody detection of MLG showed localization at the plasma membrane and cell wall, as did antibody detection of HvCslF6 and TaCslF6 (*Trethewey & Harris*, 2002; *Trethewey et al.*, 2005; *Biliaderis*, 2007; *Burton et al.*, 2010a).
Wilson et al., 2006, 2015). The N-terminus region of the CslF6 protein in barley, maize, and sorghum (Sorghum bicolor) influence total MLG synthesis activity and the C-terminal region appears to influence the ratio of DP3:DP4 linkages (Jobling, 2015; Dimitroff et al., 2016)

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

The utility of increased MLG as a bioenergy source and the effect of increased MLG on wall content and plant health has been explored in studies overexpressing MLG synthesis in barley and A. thaliana. Excess MLG synthesis under constitutive promoters was detrimental to plant health, but tissue or developmentally specific promoters driving MLG synthesis resulted in plants with higher MLG content in grain or stem without such deleterious effects (Burton et al., 2011; Vega-Sánchez et al., 2015) In barley, MLG and starch levels have been shown to be inversely related in the developing coleoptile (Roulin et al., 2002), and MLG levels have been shown to dynamically rise and fall over the course of development in vegetative tissue (Gibeaut et al., 2005). The grain cell walls of
B. distachyon differ from cultivated cereals with exceptionally high levels of MLG and relatively lower starch levels (Guillon et al., 2011b; Opanowicz et al., 2011; Trafford et al., 2013; Burton & Fincher, 2014). This shift in carbon storage suggests that B. distachyon may rely on MLG to a greater extent than starch for endosperm carbon storage (Trafford et al., 2013; Burton & Fincher, 2014). It has been suggested that MLG metabolism is enzymatically simpler than starch metabolism, requiring fewer enzymes in more available cellular spaces than the multi-step, amyloplast specific process of starch metabolism (Roulin et al., 2002; Burton & Fincher, 2012; Trafford et al., 2013; Bulone et al., 2019). While this has yet to be explored experimentally, it has been noted that a fast, alternative glucose storage pathway from (1,3;1,4)-β-glucan metabolism may confer an advantage to the grasses, as evidenced by the development of this mechanism in a group with such widespread success.
F6, a Golgi membrane bound protein with cytoplasmically active catalytic sites. (b) Miniature of Fig 1a cell wall schematic highlighting the mixed linkage glucan component.

1.5 Grass heteroxylans

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

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

The presence of glucuronic acid (GlcA) side chains differentiates heteroxylans into glucuronoarabinoxylans and arabinoxylans. In *A. thaliana*, GlucUronic acid substitution of Xylan (AtGUX)-1 adds GlcA at evenly spaced intervals of 8-10 xylose residues, although greater spacing has been observed. AtGUX2 appears to preferentially add GlcA more frequently, at 5-7 residue intervals without regard for even spacing (Bromley *et al.*, 2013). The evenly spaced xylan regions form the major xylan domain, and the less organized GlcA spacing populates the minor domain. The major domain has been shown to interact with cellulose microfibrils, an interaction that is also mediated by xylan acetylation. Similar GUX function has yet to be observed in grasses, but presumably a mechanism for adding GlcA to heteroxylan exists. Additionally, 4-O-methylation of GlcA by AtGXMT (glucuronoxylan methyltransferase), a DUF579 protein, has been characterized in *A. thaliana*, but not in any grasses to date (Urbanowicz *et al.*, 2012). The addition of GlcA and its methylation have been implicated in eudicots in mediating xylan interaction with other wall polymers, and this phenomenon is ripe for investigation in grasses.
1.6 Xylan acetylation

Xylan acetylation has long been observed, but only recently has the role of these modifications been revealed. In *A. thaliana*, recent work has shown that acetylation pattern influences xylans-cellulose interaction. Regularly spaced acetylation on every other xylose monomer in regions of the xylan backbone results in the polymer forming a two-fold helix that closely bonds with the hydrophilic side of cellulose microfibrils (Busse-Wicher *et al.*, 2014). The modification of xylan with acetate has strong implications for the solubility of the polymer, as well as the strength of xylan-cellulose interactions. Xylan-O-acetyltransferases (XOATs) are DUF231 family proteins, and carry out 2-O- and 3-O-monoacetylation and 2,3-di-O-acetylation (Fig. 1.4). In *A. thaliana*, 9 XOATs have been identified and genetically characterized, including the Trichome Birefringence protein, TBR-like proteins, and ESKIMO1 (Zhong *et al.*, 2017). In grasses, there has been an expansion of the DUF231 XOATs, with rice containing 14 members. *OsXOAT1* and *OsXOAT7* complement the *A. thaliana* *esk1* xylan acetylation mutant, and all 14 rice XOATs can acetylate xylohexose *in vitro* (Zhong *et al.*, 2018a).

While the degree of xylan acetylation has been shown to play a critical role in wall integrity, evidence of deacetylation activity has not yet been shown in eudicots. However, *rice brittle sheath 1* (*OsBS1*) encodes a GDSL lipase/esterase that functions as an acetyesterase in the Golgi, removing acetyl groups from xylans (Zhang *et al.*, 2017). Mutation in *OsBS1* results in greater 2-O and 3-O-acetylation, which compromises secondary wall patterning and integrity.
Figure 1.4. Grass heteroxylan structure. (a) Fine structure of heteroxylan and biosynthetic enzymes. The major non-cellulosic polysaccharides in grasses are xylans. A xylose (blue) backbone is decorated with side chains of xylose, arabinose (green), glucuronic acid (teal), and ferulic acid (purple). The enzymes responsible for forming certain linkages on the heteroxylan polymer are depicted in either yellow, pink, or both, having been characterized respectively in grass systems, eudicots, or showing conserved function. (b) Miniature of Fig 1a cell wall schematic highlighting the heteroxylan component.

1.7 Lignins

Lignins are large phenolic polymers mainly deposited in the primary and the secondary cell wall of xylem and sclerenchyma cells. These polymers provide the hydrophobicity and mechanical properties necessary for the development of land plant vasculature. Lignins embed polysaccharides in the cell wall and are a major barrier for biomass usages such as saccharification for biofuel production (Marriott et al., 2014). Unlike other wall polymers, lignins contain many types of inter-unit bonds (aryl beta-aryl ether, phenyl coumaran, resinol, biphenyl) randomly formed during polymerization, some being more (C-C) or less (C-O-C) resistant to degradation (Mnich et al., 2020). Consequently, lignin structure is not predictable, although the abundance of each monomer seems to influence the occurrence of certain linkages (Stewart et al., 2009).
Lignins are synthesized from three monolignols, *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol, that differ by their degree of methoxylation. Once incorporated into lignin polymers, these phenolics give rise to *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units, respectively. In *B. distachyon* stems, lignin content accounts for 18-25% of the dry cell wall residue, and in the wild-type Bd21-3 accession, stem lignin is comprised of about 62% S, 34 % G, and 4 % H units (Bouvier d’Yvoire *et al.*, 2013; Trabucco *et al.*, 2013b). Within the grasses, *B. distachyon* has one of the highest proportions of S units reported (Clarke *et al.*, 1933; Méchin *et al.*, 2014; Herbaut *et al.*, 2018).

Lignin biosynthesis results from a branch of the phenylpropanoid pathway and has long been thought to rely on the aromatic amino acid L-phenylalanine (L-Phe) as a starting substrate (Fig. 5). The standard convention in most studied plant systems has been that L-Phe is first deaminated by phenylalanine ammonia lyase (PAL), yielding cinnamate, which is then C4-hydroxylated by coumarate-4-hydroxylase (C4H) to make coumarate. Coumarate is a common branch point for all three main monolignols. However, this conventional pathway has recently been challenged by work in *B. distachyon* demonstrating that L-tyrosine (L-Tyr) can also serve as an initial substrate for lignin synthesis as it already contains a C4 hydroxylation. Indeed, tyrosine ammonia lyase (TAL) activity in grasses (Higuchi *et al.*, 1967) suggests that C4H activity can be bypassed to produce coumarate (Fig. 1.5). In grasses, PAL and TAL activities are controlled by the same protein, but clear evidence for a genuine PTAL activity in the phenolic pathway was poorly documented until recently. In plants expressing a *BdPAL* RNAi
hairpin construct to knock down expression of multiple BdPAL genes, both PAL and TAL activities were affected and plants contained 43% less lignin (Cass et al., 2015; Barros et al., 2016). Only one predicted PTAL (PTAL1) was identified in this family and nearly half of the total lignin deposited in B. distachyon occurs via TAL activity (Barros et al. 2016). Interestingly, BdPTAL1 is mainly involved in the biosynthesis of S units and cell wall linked coumarates, with less effect on G units as revealed by plants fed with C13-labelled L-Phe or L-Tyr. A biological role for PTAL has only been shown in B. distachyon to date, but putative orthologs to BdPTAL1 have been identified in several other grasses (Barros et al. 2016). Further characterization is needed to confirm whether this alternate initiation of lignin synthesis is shared broadly amongst grasses, or indeed present in other groups.

Other recent discoveries are further changing our understanding of lignin biosynthesis in grasses. Very recently, (Barros et al., 2019) proposed that a cytosolic ascorbate peroxidase with genuine 4-coumarate 3-hydroxylase (C3H) activity oxidizes coumarate into caffeate in the phenylpropanoid pathway. Decreased expression of this novel C3H in B. distachyon results in significantly reduced lignin content and structure. This “acid” route to caffeic acid and thus to caffeoyl CoA through the activity of 4-hydroxycinnamatate:CoA ligase (4CL) would be complementary to the C3’H pathway where 4CL, 4-hydroxycinnamoyl CoA:shikimate / quinate hydroxycinnamoyltransferase (HCT), 4-coumaroyl shikimate/quinate 3’-hydroxylase (C3’H), function sequentially to convert coumarate to caffeoyl CoA (Fig. 5). Feruloyl-CoA produced by the methoxylation of caffeoyl CoA by Caffeoyl CoA O-methyl transferase (CCoAOMT) is a
substrate of cinnamoyl-CoA reductase (CCR). The proposition of an alternate “acid” route to monolignol synthesis is not new, and but the discovery of a cytosolic ascorbate peroxidase with 4-coumarate 3-hydroxylase activity in planta was lacking until now. Interestingly, Barros et al. 2019 showed this is not unique to grasses, as the null allele of the Arabidopsis C3H ortholog is lethal. The lignin pathway that involves the membrane bounded C3’H also plays a critical role in grass lignin synthesis. Indeed, C3’H knockout rice mutants were severely affected in their development and displayed typical C3’H phenotypes with lignins largely enriched in H units at the expense of G and S units (Takeda et al., 2018). Interestingly, caffeoyl shikimate esterase (CSE) activity was not detected in B. distachyon stem crude extract and this result is supported by the absence of close orthologs of AtCSE in B. distachyon (Ha et al., 2016). CCR is a cornerstone step to monolignol biosynthesis. CCR activity converts CoA-conjugated intermediaries into the aldehyde precursors of monolignols. While ccr mutants with decreased lignin and increased monolignol conjugates were studied in maize there are no reports on cell wall properties of CCR deficient lines in B. distachyon (Tamasloukht et al., 2011; Cass et al., 2015).

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

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

Once exported into the apoplast by an unknown mechanism, monolignols are oxidized by peroxidases and/or laccases (Vanholme et al., 2012; Wang et al., 2013; Perkins et al., 2019; Vermaas et al., 2019). Laccases are multi-copper oxidases that use oxygen as an electron acceptor, while peroxidases use H₂O₂. Once oxidized, the monolignols radically
polymerize into the branched lignin polymer with multiple bond types resulting from the various positions of the oxygen radical on the monolignol subunit. There are 17 laccases in *A. thaliana* and 29 in *B. distachyon* (Berthet et al., 2011; Le Bris et al., 2019). *B. distachyon* LACCASE 5 and 8 were identified as orthologs of *AtLAC17* and were shown to be responsible for lignification in interfascicular fibers (Wang et al., 2015; Le Bris et al., 2019). A laccase gene from sugarcane (*SofLAC*) also genetically complemented an *A. thaliana lac17* mutant (Cesarino et al., 2013). Lignin content decreased by 30% in the double *lac5lac8* mutant, and saccharification increased by 140% compared to the wild type. Lignin deposition was less affected in vascular bundles compared with fibers suggesting that different laccases or peroxidases are recruited for lignification of these tissues.

### 1.8 Hydroxycinnamic acids

The presence of hydroxycinnamic acids, namely ferulic acid (FA) and *p*-coumaric acid (*pCA*), in the cell wall is a defining feature of grass secondary cell walls (Ralph et al., 1994; Hatfield et al., 2009). FA is predominantly linked to heteroxylan through an ester bond. The oxidation of FA in the cell wall, probably by peroxidases, generates esterified dehydrodiferulates which serve as linkages between two arabinoxylan polymers. In lignified tissues, xylan-esterified ferulates can be etherified to G units of lignin and thus serve as a covalent linkage between hemicelluloses and lignins (Hatfield et al., 2016; Lapierre et al., 2019). An esterified ferulate on arabinoxylan is considered as a nucleation site of lignification in grasses and thus an important mechanism for cell wall reinforcement (Ralph et al., 1995, 1998). *pCA* is esterified on arabinoxylan to a lesser
extent than FA and tends to be found esterified to S units in B. distachyon lignins. Plant-specific acyl-CoA dependent acyltransferases of the BAHD (BEAT, AHCT, HCBT, and DAT) family are the enzymes responsible for the acylation of the arabinose side chains of heteroxylans and monolignols with hydroxycinnamates (D’Auria, 2006; Mitchell et al., 2007). An expanded grass-specific BAHD clade (also called the “Mitchell clade”) was identified by bioinformatic analysis in rice as candidates for hydroxycinnamate transfer (D’Auria, 2006; Mitchell et al., 2007; Bartley et al., 2013). Consequently, BAHD enzymes with feruloyl transferase activity were first explored in rice and have also been investigated in B. distachyon (Piston et al., 2010; Bartley et al., 2013). BAHD01 in B. distachyon and Setaria viridis appear to be involved in feruloylation of arabinoxylans (de Souza et al., 2018). Downregulation of SvBAHD01 significantly reduced FA on arabinoxylan, with an increase in pCA-Araf acylation and no substantial change in lignin content while in B. distachyon only a moderate decrease in FA-arabinoxylan was observed (de Souza et al., 2018). Interestingly, BdBAHD01 downregulation lines showed increased saccharification efficiency, despite unchanged lignin content, highlighting the role of FA in maintaining cell wall integrity. Overexpression of BdBAHD05 (also called BdAT1) caused a moderate increase in FA content and downregulation showed a moderate decrease (Buanafina et al., 2016; de Souza et al., 2018). Analysis in sugarcane revealed six BAHD genes, one of which is homologous to SvBAHD01, and downregulation of SacBAHD01 similarly reduced stem FA content and increased biomass digestibility (de Souza et al., 2019).

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

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

1.9 Tricin

As evidenced by their pCA and FA content, grasses are remarkable in their capacity to incorporate phenolic compounds other than the typical coumaryl, coniferyl, and sinapyl alcohols into lignin. Tricin, an O-methylated flavone, was first characterized in wheat straw lignin (del Río et al., 2012). Tricin is incorporated into grass lignin in varying amounts across grass species, with oat (Avena sativa), wheat, and B. distachyon straw being particularly enriched in this compound (Lan et al., 2016). Tricin is incorporated in lignin polymers via 4’-O-β coupling (Lan et al., 2018). Biomimetic radical coupling reactions give evidence that tricin may serve as a possible nucleation site for lignification, as has been suggested for ferulate (Ralph et al., 1995, 1998; Lan et al., 2015). Tricin and monolignols come from two different branches of the phenylpropanoid pathway, and consequently their synthesis shares some common enzymes. This is particularly true for enzymes involved in the metabolic flux upstream of p-coumaric acid synthesis.

CHALCONE SYNTHASE, a pivotal enzyme for flavonoids production, uses malonyl-CoA and p-coumaryl-CoA as substrates. Silencing this enzyme in maize resulted in strongly reduced levels of apigenin- and tricin-related flavonoids, and also strongly reduced incorporation of tricin into the lignin polymer (Eloy et al., 2017). The impact of the flavonoid pathway on the production of cell wall tricin content was also demonstrated in rice (Lam et al., 2017, 2019). It is also possible that some of the cell wall changes
observed in BdPMT overexpression lines may stem from the depletion of \( p \)-coumaroyl-CoA pool, as both chalcone synthase and PMT act on this substrate. O-methyltransferases involved in the O-methylation of 5-hydroxy-coniferaldehyde to produce sinapyl alcohol were also shown to be involved in the methylation of tricin in rice, maize, and sorghum (Fornalé et al., 2017; Eudes et al., 2017; Lam et al., 2019). The bi-functionality of COMT in the lignin and flavonoid pathways is not unexpected since an COMT involved in lignification of *A. thaliana* stems also O-methylates isorhamnetin, a flavonoid structurally similar to tricin (Do et al., 2007). There is now abundant evidence that other molecules, called “nontraditional monomers” like tricin or hydroxycinnamic acids, can be incorporated into the lignin polymer (del Río et al., 2018; Vanholme et al., 2019). The biological role of these novel lignin components remains to be determined.
Figure 1.5. Alternative routes to lignin biosynthesis. (a) In some grasses, phenylalanine tyrosine ammonia lyase (PTAL) can bypass cinnamic acid 4-hydroxylase (C4H) activity and thus directly produce p-coumarate from tyrosine.
An ascorbate peroxidase-like gene with coumarate 3-hydroxylase (C3H) activity can directly synthesize caffeate from coumarate. Red arrows illustrate a grass specific lignin pathway unique from other lignin pathways identified in most studied eudicots. Green arrows show routes potentially bypassed or absent in B. distachyon, such as caffeoyl shikimate esterase (CSE). Blue arrows are paths believed to be common to eudicots and grasses. Asterisks highlight enzymes characterized in B. distachyon. The mechanism for monolignol export across the plasma membrane remains unclear. Phenylalanine ammonia lyase (PAL), 4-hydroxycinnamoyl CoA ligase (4CL), hydroxycinnamoyl CoA:shikimate hydroxycinnamoyl transferase (HCT), p-coumaroyl shikimate 3'-hydroxylase (C3'H), caffeoyl CoA O-methyltransferase (CCoAOMT), hydroxycinnamoyl CoA reductase (CCR), ferulic acid 5-hydroxylase (F5H), caffeic acid/5-hydroxyferulic acid O-methyltransferase (COMT), cinnamyl alcohol dehydrogenase (CAD), laccase (LAC), peroxidase (PRX). R- in the monolignol hydroxycinnamnics could be substituted by a hydrogen or methyl group. (b) Miniature of Fig 1a cell wall schematic highlighting the lignin component.

1.10 Silicon

Poaceae accumulate high quantities of silicon in the cell wall of their shoots. This phenomenon is particularly remarkable in rice (Ma & Yamaji, 2006). The main role of silicon is to provide plant resistance to many biotic and abiotic stresses (Hattori et al., 2005; Deshmukh et al., 2017). However, silicon may interact with polysaccharides, which consequently impact plant biomass processing in biorefineries (Perry & Lu, 1992; Kido et al., 2015). For biofuel production, there is a tradeoff between soil amendment with silicon that can increase polysaccharide yield with a negative effect on the conversion of biomass into biofuels (Głazowska et al., 2018b). Silicon content in rice and maize can be modulated by changing the expression of silicon transporters (Ma et al., 2007; Mitani-Ueno et al., 2016; Bokor et al., 2017). The analysis of different silicon transporter mutants showed that silicon availability may impact the morphology and patterning of stem and leaf macrohairs (Głazowska et al., 2018b). The Bd low silicon 1 (Bdslsi1) mutant is impaired in silicon transporter function and has reduced silicon uptake, with 93% less silicon present in the shoot. Mixed-linkage glucan content is drastically
modified in Bdlsi1 ((Kido et al., 2015; Głazowska et al., 2018a). This result is in agreement with previous studies suggesting that (1;3,1;4)-β-glucan is involved in silicon-dependent strengthening of the rice cell wall (Kido et al., 2015). The Bdlsi1 mutant also displayed an altered degree and pattern of homogalacturonan methyl esterification. Despite the relatively low amount of pectins found in grasses, this change in homogalacturonan represents a significant alteration to the wall matrix. Lastly, Bdlsi1 mutant FA extrability was lower with only minor changes in lignin content (Kido et al., 2015; Głazowska et al., 2018a). These data highlight the important role silicon plays in cell wall integrity in B. distachyon and grasses in general, and presents interesting avenues for further study.

1.11 Transcriptional regulation of secondary cell wall thickening

Canonical transcription factors that directly bind DNA play a prominent role in the regulation of plant secondary cell wall thickening. The cis-regulatory regions of genes associated with cellulose, hemicellulose, and lignin biosynthesis interact directly with numerous MYB and NAC family transcription factors (Fig. 6)(Nakano et al., 2015). Many of the R2R3-MYB protein family subgroups appear to bind a similar sequence motif, the AC element, also known as the M46RE (MYB46 responsive cis-regulatory element) and the SMRE (secondary wall MYB-responsive element) (Kim et al., 2012; Zhong & Ye, 2012; Zhao & Bartley, 2014; Handakumbura et al., 2018). In A. thaliana, AtMYB46 and the close paralog AtMYB86 activate the expression of cellulose, hemicellulose, and lignin biosynthetic genes, as well as other MYBs capable of activating secondary cell wall related genes (Zhong et al., 2007b; Zhong & Ye, 2007). Some of the
downstream MYB activators, among them *AtMYB58/63* and *AtAtMYB42/85*, activate only lignin genes (Rao & Dixon, 2018; Zhang *et al.*, 2018a). However, in sorghum, rice, and switchgrass (*Panicum virgatum*), ectopic expression of *OsMYB58/63, PvMYB58/63*, and the sorghum ortholog *SbMYB60* results in the activation of cellulose and hemicellulose genes as well as lignin (Noda *et al.*, 2015; Scully *et al.*, 2016; Rao *et al.*, 2019). A potential ortholog to *OsMYB42/85, ZmMYB167*, was overexpressed in maize and heterologously in *B. distachyon* to similar effect (Bhatia *et al.*, 2019). Similar functions have been resolved for the *A. thaliana* and rice orthologs *AtMYB61* and *OsMYB61* as well as *AtMYB103* and *OsMYB103* (Hirano *et al.*, 2013; Huang *et al.*, 2015; O’Malley *et al.*, 2016; Zhao *et al.*, 2019). These downstream MYBs bind the AC element and activate both lignin and wall polysaccharide biosynthesis genes. Overall, there are few distinctions in the transcription factor targets for these genes between grasses and *A. thaliana*. Those that have been observed may be the outcome of low-resolution experimental designs that sample one tissue type at one time point for a limited number of outputs.

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

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

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

While the regulatory network is dominated in number by MYB and NAC transcription activators, several repressors have also been described; namely TALE (Three Amino acid Loop Extension), zinc-fingers, HD-ZIP III, WRKY, LATERAL ORGAN BOUNDARY (LBDs) and some MYBs. Among the many types of cells that do not have secondary cell walls are pith, which reside inside the stem and among the cells in the plant with the thickest walls. A WRKY transcription factor, *WRKY12*, is a repressor of wall thickening in pith and other cells. It can directly bind the promoters of *AtNST2* and poplar *C4H* and broadly repress wall thickening in *A. thaliana*, poplar, and switchgrass (Wang et al., 2010; Yang et al., 2016; Rao et al., 2019). The five class III HD-ZIPs in *A. thaliana* (*REVOLUTA, PHABULOSA, PHAVOLUTA, CORONA*, and *HB8*) and some orthologs in
poplar have been shown to play a role in cambium cell initiation and vascular bundle organization (Floyd & Bowman, 2006). However, to our knowledge, there are no reports describing a function for this group of genes in grasses. Several LBD family transcription factors, *AtLBD15/18/30*, can activate the expression of *AtVND7* and induce wall thickening and differentiation into tracheary cells (Soyano *et al.*, 2008; Ohashi-Ito *et al.*, 2018). *AtLBD29*, on the other hand, is a repressor of stem secondary wall thickening and is activated by the phytohormone auxin (Lee *et al.*, 2019a). Repression is also supplied by the MYB G4 clade and are the best characterized in grasses. These include ZmMYB11/31/42, PvMYB4/32, and OsMYB108, which are orthologous to AtMYB4/32 (Zhao & Bartley, 2014; Rao & Dixon, 2018; Miyamoto *et al.*, 2019). These were first described in a grass as direct repressors of lignin gene expression (Fornalé *et al.*, 2006; Sonbol *et al.*, 2009). In switchgrass, PvMYB4 is a direct repressor of lignin associated genes (Shen *et al.*, 2012; Rao *et al.*, 2019). The MYB31/42 MYBs in sorghum, rice, and maize directly bind to the cis-regulatory regions of various lignin biosynthetic gene, but there appears to be variation in phenylpropanoid gene expression and protein-DNA interactions across species (Agarwal *et al.*, 2016). Wounding induced lignification occurs in maize by the degradation of ZmMYB11/31/42 protein and a protein interacting partner ZML2 (Vélez-Bermúdez *et al.*, 2015). Thus, wounding and the subsequent activation of MeJA signaling will remove MYB G4 clade repression in maize and induce lignin gene expression. The repressing MYB G4 clade interacts with AC-like sequence motifs, similar to the wall activating MYBs (Fornalé *et al.*, 2010; Shen *et al.*, 2012; Agarwal *et al.*, 2016). The exact targets of the phenylpropanoid pathway vary across system and study, which suggests there may be some transcription factor sub-functionalization.
Members of two different classes of the TALE super family, KNOX and BEL, have been shown to regulate secondary wall synthesis. The class II KNOX gene *KNOTTED OF ARABIDOPSIS THALIANA 7 (AtKNAT7)* was initially identified as an irregular xylem mutant (*irx11*) (Brown et al., 2005a). KNAT7 orthologs are generally described in the literature as repressors, and while there is substantial evidence for this, there are also some outstanding issues raised by data indicating a role as an activator of wall deposition. *Atknat7* mutants have thicker interfascicular fiber walls, as expected for a repressor mutant, but this mutant also shows collapsed xylem (Brown et al., 2005a; Li et al., 2012). *Atknat7* mutants have greater lignin content, but reduced xylan, suggesting that *AtKNAT7* may differentially regulate aspects of wall polymer synthesis. In conflicting reports, one group has shown xylan biosynthetic genes upregulated in *Atknat7* lines, while another shows downregulation (Li et al., 2012; He et al., 2018). AtKNAT7 protein can bind to the *AtIRX9* promoter, a gene responsible for xylan backbone synthesis.

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

Among the genes co-expressed with *B. distachyon CSLF6*, a predominant MLG synthase, was a trihelix family transcription factor (*BdTHX1*) (Kim et al., 2018; Fan et al., 2018). This is the first THX protein associated with cell wall biosynthesis and the first shown to bind directly to a CSLF gene. *In planta* and *in vitro* assays showed that BdTHX1 protein binds to the GT element in the second intron of *BdCSLF6* and the 3’ region of glycoside hydrolase family 61 endotransglucosylase/hydrolase 8 (*BdXTH8*), a grass-specific enzyme that uses MLG as a substrate (Fan et al., 2018). Attempts to recover viable transgenic plants were unsuccessful and suggest a strong selection against the perturbation of *BdTHX1*; thus, it is uncertain if it is a transcriptional activator or repressor.

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

There is nearly complete overlap between the regulatory network components between eudicots and grasses. The distinctions between grass and eudicot walls are difficult to assign to differences in varying functions or members of the regulatory network. While no LBD, BLH, or HD-ZIP III have been described as regulators of cell wall biosynthesis in grasses, it is possible that they have simply not been studied or reported. Additionally, THX1 is likely unique to grasses since it regulates a hemicellulose gene not present in eudicots. Meta-analysis of microarray gene expression data, to make a combined mutual ranked network for rice and A. thaliana, has revealed differences in the relative importance or each regulator (Zhao et al., 2019). The degree of connectivity among genes, which is the number of edges for each network node, can suggest the importance of each transcription factor. Some highly connected genes in A. thaliana, including VND1/2/6/7 and AtMYB46/83 have a two to five-fold decrease in connectivity in rice. On the other hand, transcription factors with considerably more connections in rice than A. thaliana are OsSND2/3, the rice ortholog of KNAT7, KNOR1 as well as OsSWN1, the ortholog of AtNST1.
Figure 1.6. Transcriptional regulation of secondary cell wall deposition. Secondary cell wall transcription regulatory network in A. thaliana (a) and grasses (b). Bean shape indicates DNA binding transcription factors. Ovals indicate protein interactors. Circles are hormones. Orthology between A. thaliana and grasses is denoted by color. Blue, red, and green arrows indicate regulation of cellulose, lignin, and hemicellulose, respectively. Arrows indicate activation and bars repression. Grey shaded items have not been described in both systems.
1.12 Conclusions

Much progress has been made in recent years to better understand grass cell wall composition and regulation, in large part thanks to the numerous genetic and genomic resources that have been developed. A case in point, *B. distachyon* as a model grass system has been central to these efforts, and provides fertile ground for future studies. The unique features of grass cell walls, such as MLG synthesis and the integration of hydroxycinnamates into lignin and xylan are beginning to be uncovered in detail. Elements that were thought to be more common between grasses and eudicots, such as lignin synthesis, continue to show evidence that there is yet unexplored diversity in plant cell wall chemistry, with alternate lignin biosynthetic pathways and atypical monomers components. Regulation remains an area of much overlap, but rather than playing catch-up with eudicots, grass networks now offer new insights of their own that expand the cell wall network. Uncovering grass specific functions, such as *BdTHX1* regulation of MLG, highlight the opportunities to advance this important area of plant biology.
CHAPTER 2

SECONDARY WALL INTERACTING BZIP (SWIZ) IS A TOUCH-SENSITIVE REGULATOR OF PLANT HEIGHT AND CELL WALL THICKENING

2.1 Introduction

2.1.1 Mechanosensing and thigmomorphogenesis

Forces both internal and external to a cell influence growth. Turgor pressure in conjunction with anisotropic cell wall dynamics maintain a cell’s physical shape and direction of growth. Force perception between neighboring cells plays a critical role in the development and maintenance of tissue form and function, such as in the lobed, interlocking pavement cells on the leaf epidermis, or the developmental hotpots in the apical meristem (Hamant et al., 2008; Uyttewaal et al., 2012; Bidhendi et al., 2019). Specific inter-cell forces result in dynamic remodelling of the cortical cytoskeleton, with subsequent changes in cellulose microfibril alignment and alterations to other cell wall components such as pectin methyl esterification (Hamant et al., 2008; Uyttewaal et al., 2012; Bidhendi & Geitmann, 2018; Bidhendi et al., 2019; Altartouri et al., 2019).

Beyond cell to cell response to force, whole plants perceive and respond to mechanical force through thigmomorphogenesis (Jaffe, 1973; Jaffe et al., 1980, 2002). The classic hallmarks of touch responsive growth include reduced plant height, increased radial growth in plants with a cambial meristem, increased branching, and delayed flowering time (Jaffe, 1973; Braam, 2004). These attributes have been leveraged by farmers for hundreds of years, with the mugifumi tradition documented in Japan in 1680. Farmers tread on young wheat and barley seedlings to elicit increased branching and seedset, along with stronger roots, a practice that still continues today with mechanized rollers.
The molecular and genetic mechanisms in plants that perceive and translate force into remain somewhat poorly understood. Touch induced gene expression has been studied for some time, identifying numerous transcripts upregulated by mechanical stimulation. The first touch-induced (TCH) genes identified in *A. thaliana* were found to encode calmodulin (*AtTCH1 / AtCaM2*), calmodulin -like proteins (*AtTCH2 / AtCML24, AtTCH3 / CML12*), and a xyloglucan endotransglycosylase/hydrolase (*AtTCH4 / AtXTH*) (Braam & Davis, 1990). Subsequent studies identified numerous other genes with touch responsive expression, often showing overlap with induction from other stimuli such as dark, cold, and hormones (Polisensky & Braam, 1996; Lee *et al.*, 2005). Together, around 600 genes were found to be upregulated by touch, primarily coding for proteins involved in calcium binding and signaling, cell wall modification, and a variety of transcription factors and kinases. The nucleus plays a key physical role in mechanoperception (Fal *et al.*, 2017). In animal cells, the nucleus participates in force perception through interactions between the cytoskeleton and nucleoskeleton. Microtubules and actin filaments interact with nuclear membrane Linker of Nucleoskeleton and Cytoskeleton (LINC) complexes, composed of Klarsicht/ANC-1/Syne-1 homology (KASH), and Sad1p and UNc-84 (SUN) domain proteins that internalize force applied to the cytoskeleton to the lamin protein nucleoskeleton (Chambliss *et al.*, 2013; Fal *et al.*, 2017). In this way, forces applied to the exterior of the cell are translated to the nucleus, where modulation of nuclear shape and chromatin structure are related to gene expression events. In plants, similar molecular players have been implicated, but fewer have been experimentally validated. KASH and SUN domain complexes exist in plants, similarly connecting the cytoskeleton and nucleoskeleton (Graumann *et al.*, 2014; Fal *et al.*, 2017).
Mechano-sensitive ion channels are capable of cellular mechanoperception (Monshausen & Haswell, 2013; Leblanc-Fournier et al., 2014; Basu & Haswell, 2017). These membrane embedded complexes dynamically open and close in response to lateral membrane tension, allowing ions to flow across the membrane. When force is applied to a cell, such as through tissue bending or cell expansion, the membrane tension increases and opens the ion channel. These types of mechanically gated signalling events have been best described in animal and bacterial systems, but plants also contain genes encoding similar channels, AtMCA1&2 and AtMsl1-10, that have been implicated in sensing mechanical signals such as gravity, osmotic pressure, pathogen invasion, and touch (Haswell et al., 2008; Peyronnet et al., 2008; Leblanc-Fournier et al., 2014; Hamilton et al., 2015b). AtMsl family proteins are localized not only to the plasma membrane, but to mitochondrial and chloroplast membranes as well, and these organelles have been shown to function in mechanoperception signaling and osmotic shock protection (Hamilton et al., 2015a; Lee et al., 2016, 2019b; Basu et al., 2019). The function of these membrane channels strongly implies that ions play a role as mechano-signalling molecules, of which calcium is at the forefront. Cytosolic Ca2+ fluxes have been observed in roots following mechanical bending (Monshausen et al., 2009), is required for lateral root formation (Richter et al., 2009), and a maize calcium dependent protein kinase, ZmCPK11, has been shown to be activated rapidly following mechanical stimulation (Szczegielniak et al., 2012). Additionally, AtTCH1, 2, and 3 are all calmodulin and calmodulin like proteins, rapidly upregulated in expression following touch (Braam & Davis, 1990; Braam, 2004; Lee et al., 2005). Despite these many observations, it is not yet known where Ca2+ fluxes originate from or by what mechanism they cross the membrane. The
dynamic control of bioactive cellular gibberellic acid (GA) has been linked to mechanoperception as well as cytosolic calcium levels. GA has also recently been shown to control the cytosolic accumulation of Ca2+ following mechanical stress, and perhaps more interestingly, that this Ca2+ accumulation occurred even in a della quintuple mutant line (Okada et al., 2017). This suggests that GA levels mediate cytosolic calcium flux in response to mechanical stress independent of DELLA mediated pathways. Furthermore, it has been demonstrated in A. thaliana that mechanical force application inactivates cellular GA (Lange & Lange, 2015). The addition of exogenous bioactive GA to mechanically stressed plants remediates their height phenotype, and mutants in Atga20ox, an enzyme that inactivates cellular GA, do not show thigmomorphogenic phenotypes under mechanical stress treatment (Lange & Lange, 2015). Recent work studying the translocation dynamics of several bZIP family transcription factors has provided new links for calcium and GA signalling in mechanoperception signalling.

2.1.2 bZIPS

In plants, the basic leucine zipper (bZIP) proteins family is a relatively large group of transcription factors. In B. distachyon there are 96 bZIPS, divided into 9 groups, compared to A. thaliana with 78 bZIPS placed into 13 groups (Jakoby et al., 2002; Liu & Chu, 2015; Dröge-Laser et al., 2018). Group I bZIPS in A. thaliana function pleiotropically in aspects of biotic and abiotic responses, as well as plant development. The best characterized Group I bZIP is A. thaliana VirE2-interacting protein 1 (AtVIP1), which was identified for its role in agrobacterium mediated T-DNA transfer (Djamei et al., 2007). AtVIP1 also regulates leaf growth, as does bZIP29 and 30 (Van Leene et al., 2016; Lozano-Sotomayor et al., 2016). Other Group I bZIPS have been shown to play
roles in anthocyanin synthesis in response to abiotic stress, pollen fertility, and vascular development (Torres-Schumann et al., 1996; Yin et al., 1997; Ringli & Keller, 1998; Dai et al., 2004, 2008; Van Oosten et al., 2013; Gibalová et al., 2017). All of these bZIPs are also implicated in mechanosensing, in the form of osmosensing. AtVIP1 and other Group I bZIPs have been shown to translocate from the cytoplasm to the nucleus in response to a variety of biotic and abiotic stimuli, including hypoosmotic conditions (Tsugama et al., 2012, 2014, 2016). This translocation appears to be dependent on protein phosphorylation, either from mitogen activated protein kinase 3 (MPK3) during pathogen invasion, or via calcium dependent protein kinase (CDPK). AtVIP1 has recently been shown to interact with calmodulins, the calcium binding proteins involved in many calcium signalling events. Calcium dynamics have long been implicated in touch response signalling, but as mentioned above, the connection between stimulus, calcium fluctuation, and biological response has remained elusive. AtVIP1 translocation was found to depend on cytosolic calcium levels. Treatment with ion scavengers such as EDTA and EGTA strongly limited AtVIP1 nuclear translocation (Tsugama et al., 2018). Looking further upstream in the touch signalling cascade, calcium ion flux across the membrane may be controlled by membrane pore complexes, a number of which have been shown to be mechanically responsive. AtMCA1 and AtMCA2 mutants were tested for impact on AtVIP1 translocation, but no effect was shown, indicating that other ion channels may be responsible for touch mediated Ca2+ import (Tsugama et al., 2018). The Group I bZIP Nt REPRESSOR OF SHOOT GROWTH (NtRSG) in tobacco has been implicated in playing a GA homeostasis maintenance role and has been shown to translocate from the cytoplasm to the nucleus in response to cellular GA levels. When
GA levels are nominal, NtRSG is largely in the cytoplasm, and to some extent in the nucleus. At nominal GA levels when NtRSG was partially localized to the nucleus, it directly activates expression of *NtEnt-kaurene oxidase* (*NtKO*), an enzyme at an early step in the GA biosynthesis pathway. After cellular GA levels were reduced, NtRSG translocated to the nucleus and also activated *NtGA20oxidase1* (*NtGA20ox1*), an enzyme further down the GA biosynthesis pathway that is in part responsible for converting GA species to their bioactive form (Fukazawa et al., 2010, 2011). This suggests that NtRSG acts to promote bioactive GA synthesis in response to low GA conditions. As with AtVIP1, NtRSG translocation relies on CDPK mediated phosphorylation (Ishida et al., 2008; Ito et al., 2017). Both AtVIP1 and NtRSG associate with 14-3-3 proteins in the cytoplasm while phosphorylated (Ishida et al., 2004; Ito et al., 2014, 2017; Tsugama et al., 2018). Phosphatase activity causes NtRSG to dissociate from the 14-3-3 protein and enters the nucleus, with PP2A complexes implicated in this process for AtbZIP29 in (Van Leene et al., 2016). *NtRSG* is named due to the dwarf phenotype observed when a dominant negative form of the protein is over expressed. In the dwarf plants, there are reduced levels of bioactive GA and reduced elongation of stem internode cells (Fukazawa et al., 2000). These phenotypes were later explained by the work described above on *NtRSG* positive regulation of GA synthesis. Transverse sections of the *NtRSG* dominant negative mutant do not show obvious cell wall phenotypes (Fukazawa et al., 2000). Two rice bZIP genes, *OsRF2a* and *OsRF2b*, have also been shown to regulate GA levels and impact elements of cell wall synthesis and vascular development. The role of these genes in vascular development has been described in the context of rice tungro bacilliform virus. This double stranded DNA badnavirus contains a promoter region with vascular
tissue-specific cis-elements, particularly phloem-specific elements, that OsRF2a/b were found to bind and active (Yin et al., 1997; Dai et al., 2003, 2004, 2008). Similar to NtRSG, dominant negative forms of OsRF2a/b cause stunted growth. A OsRF2a dominant negative transgene in tobacco is reported to have altered phloem development and reduced xylem lignification (Dai et al., 2003). In rice leaf vasculature, overexpression and knockdown of OsRF2a resulted in more sclerenchyma development around the vascular bundle, and the presence of large air pockets flanking the vascular bundle. The vasculature itself was somewhat smaller in both cases (Yin et al., 1997).

2.1.3 Summary
Thigmomorphogenesis is a widely observed phenomenon that results in reduced height, increased radial growth, and increased branching. The mechanisms behind this form of growth are not yet fully understood, but involve aspects of hormone regulation, Ca2+ signalling, Group I bZIP intracellular translocation, and changes in gene expression. Here I describe the function of a B. distachyon bZIP transcription factor, SECONDARY WALL ASSOCIATED bZIP and its role in touch response and cell wall biosynthesis.

2.2 Methods
2.2.1. Plasmid construction
Overexpression constructs were built using the Invitrogen Gateway cloning system. PCR amplified coding sequences were cloned into the pENR-D-TOPO or appropriate pDONR vector for multisite recombination, and further subcloned into a modified pOl001 destination vector (Vogel et al., 2006). Artificial microRNA constructs were built by sequential PCR amplification from the pNW55 plasmid, replacing the native rice microRNA osaMIR528 with the target sequence of interest, and later subcloned into a
modified pOL001 destination vector (Vogel et al., 2006; Warthmann et al., 2008). Target sequences were derived from the Web MicroRNA Designer platform (http://wmd3.weigelworld.org).

Sequence confirmed clones for all destination vectors were electroporated into *Agrbacterium tumefaciens* strain AGL-1.

### 2.2.2. Plant transformation

Transformation was performed according to Vogel & Hill 2008 (Vogel & Hill, 2008). Immature seeds were collected from ~6 week old plants, dehulled, and surface sterilized with a solution of 1.3% NaClO and 0.01% Triton-X100 for four min. Sterilized seeds were rinsed three times in sterile water. Embryos were dissected from the seeds and placed on callus initiation media (CIM) for four weeks, then subcultured to fresh CIM for two more weeks, the subcultured a final time onto fresh CIM for one week. Seven week old calli were co-cultivated in a suspension of *A. tumefaciens* for ~5 min, then thoroughly dried on sterile filter paper for 3-5 days at 22°C in the dark. Calli were moved onto CIM media containing 50 mg/L hygromycin B and 150 mg/L timentin, where they were grown for 3-5 weeks with selective subculture of healthy callus at week 4. After selection, healthy calli were moved to Linsmaier and Skoog media supplemented with 50 mg/L hygromycin B, 150 mg/L timentin, and kinetin to promote shoot growth. Calli that produced green tissue within 3-5 weeks were moved to Murashige and Skoog media supplemented with 50 mg/L hygromycin B and 150 mg/L timentin to allow root growth. After 1-3 weeks, calli that established roots were transplanted to soil and grown as described below.
2.2.3. **Plant growth**

*Brachypodium distachyon* line Bd21-3 was used for all experiments. Seeds were stratified on wet paper towel wrapped in foil to exclude light for 10 days at 4°C before being planted in Promix BX potting mix in SC10 Ray Leach Cone-tainers (Stuewe & Sons Inc, https://www.stuewe.com/products/rayleach.php). Plants were grown in a Percival PGC-15 growth chamber with day/night conditions of 20h light at 24°C and 4h dark at 18°C respectively.

2.2.4. **Transverse stem sections, histology**

The main stem of senesced plants was taken and the internode of interest removed and embedded in 8% agarose. Samples were sectioned using a Leica VT1000 Vibratome, making 55um thick sections. Multiple sections of each internode were collected and stored in water at 4°C. Histochemical staining was carried out using toluidine blue, phloroglucinol-HCl, and Maule reagent as described in Mitra & Loque (2014)(Pradhan Mitra & Loqué, 2014). Images were obtained at 4, 10, and 20x using a Nikon Eclipse E200MV R light microscope and PixeLINK 3 MP camera.

2.2.5 **Measuring cell wall thickness**

Transverse sections imaged at 20x were used for cell wall thickness measurements. Interfascicular fiber cells separated by one cell layer from the mestome cells on the phloem side of major vascular bundles were targeted for measurement. Using ImageJ, lines were drawn across two walls of adjoining cells. The resulting line length was divided by two to give one cell wall width. ~15 measurements were made for each plant.
2.2.6. Translocation assay

2.2.6.1. Plant growth
Seeds were deglumed and surface sterilized in an aqueous solution of 1.3% NaClO and 0.1% Triton-X100 for 4 min with agitation. The sterilization solution was removed and seeds were washed three times in aseptic conditions with sterile water. Using sterile forceps, the seeds were placed on agar containing 1X MS salts, pH 5.7, without sucrose. The seeds were placed in a row across the upper third of the plate, convex side up, with the embryo oriented down when the plates were held vertically. 8-12 seeds were placed per plate. The plates were wrapped in foil to exclude all light and incubated vertically at 28°C for 6 d.

2.2.6.2. Pharmacology treatments
After the 6 day growth period, seedlings were moved to segmented petri plates with four quadrants. Each quadrant contained 1x MS media, pH 5.7, without sucrose, plus a different concentration of the pharmacological agent being tested, including a no-treatment control quadrant. GA4 and paclobutrazol treatment concentrations of 10 mM, 50 mM, and 100 mM were used. After transfer to the treatment media, roots were gently pressed into the media so that they were completely covered, and positioned close to the bottom of the plate in an even plane for better imaging. Plants were left in the treatment media for 6h to acclimate from the touch stimulus of movement, and to allow for chemical update of the treatment compound.

2.2.6.3. Confocal microscopy
All observations were made on a Nikon A1R scanning confocal microscope using a 10x objective. Plates were fixed to the microscope stage with tape and their lid removed. Root
areas to be observed were located by eye and then confirmed under confocal conditions. The coordinate stage locations of each region were programmed into the Nikon NIS Elements Advanced Research V6 software package for automated imaging. After all target regions were programmed, 30 min of imaging began pre-treatment, with images captured every 2 min.

2.2.6.4. Touch treatment
To elicit the touch response, the time lapse capture program was paused and the microscope switched back to the eyeport. Each observed root region was gently probed for 5 sec with a blunt metal probe while observing through the eyepiece, as one would with a dissecting scope. After all regions were probed, each field of view was quickly refocused under confocal to assure the root had not moved in the x, y, z planes, repositioning the stage and reencoding the location if necessary, and then resuming time lapse capture every 2 min. From start to finish, the touch treatment takes no more than 5 min. Images are captured for 60-90 min post treatment. For experiments with multiple stimulus events, the timelapse sequence was paused and roots were probed as described for the relevant stimulus events.

2.2.6.5. Analysis
Analysis of GFP signal was done using the Nikon NIS Elements Advanced Research V5 software package. For each time series of images for one root, the frame showing maximal nuclear signal was selected and used as a reference point. Using the General Analysis tool, a channel for GPF signal was established and thresholded for intensity and particle size to identify the nuclear regions. These regions were added to the timelapse image series as a binary layer and then converted to static regions of interest (ROIs) for
quantification. The GFP intensity under each nuclear ROI was calculated for the course of the timelapse and the average signal from each nucleus was plotted for intensity over time.

2.2.7. Cell wall material insoluble in alcohol (MIA)

The main stem of mature, senesced plants was collected and cut into small pieces (~2cm) into a 2ml tube. Two metal beads were added and the stem was ground to a fine powder using a Retsch 440 bead beater. Ground material was transferred to a glass screw cap tube. Cell wall material was washed with 5ml of water in an 80°C water bath for 10 min with agitation. The cell wall material was pelleted by centrifugation at 3700 rpm for 10 min and the supernatant aspirated by vacuum. This was repeated for a second water wash. The cell wall material was then washed three times with 100% ethanol at 80°C for 15 min per wash, with collection by centrifugation and aspiration of the supernatant between washes as described above. The cell wall material was then washed twice with acetone for 15 min per wash at room temperature, then left to dry under a fume hood overnight. Modified from INARE protocols.

2.2.8. ABSL quantification

Beginning with dry cell wall MIA samples, 4.5-5.5mg of each sample was weighed into a 2mL glass vial using a precision balance. 1ml of acetyl bromide solution (25% acetyl bromide, 75% acetic acid) was carefully added to each vial under a fume hood. The vials were capped and inverted several times to mix. Samples were incubated in a drying oven at 55°C for 2h 30 min, with mixing by gentle inversion every 30 min to ensure full sample solubilization. The samples were cooled to room temperature before proceeding. 0.1 ml of sample was diluted into 1.2ml of acidified 2M NaOH, then mixed with 0.3ml of
0.5M hydroxylamine chlorhydrate and 1.4ml of acetic acid. Using a glass pipette, air was bubbled through the sample to ensure full homogenization of the reaction mixture. Absorbance at 280nm was measured on a SpectraMaX M6 plate reader, and the lignin content was calculated using the following equation: \( \% \text{lignin} = 100 \times \frac{(A_{280} \times \text{Vol reaction} \times \text{Vol dilution})}{(20 \times \text{Vol sample solution} \times \text{Mass sample (mg)})} \). Modified from INARE protocols.

2.2.9. Thigmomatic construction and operation

The Thigmomatic is a very basic robotic device that sweeps a flat of plants with a metal bar at regular intervals to elicit a touch response (Fig 2.1). The device was constructed from aluminum V-Slot linear rail (https://openbuildspartstore.com/linear-rail/) and bracket joints for the upright supports (20x20 mm), cross bars (20x20 mm), and tracks (20x40mm).


The stepper motor provides fine spatial control over the gantry cart position with bi-directional motion. Motor function is controlled by a Raspberry Pi 3B microcomputer equipped with a stepper motor HAT (https://www.adafruit.com/product/2348). The Thigmomatic was programmed to cover a specified distance in one direction once every 90 min.
Figure 2.1 Thigmomatic. (A) Overview of Thigmomatic setup inside a Percival PGC-15 growth chamber, showing linear rail based frame (1), gantry carts (2), NEMA17 stepper motor (3), 12V 18W AC/DC power supply (4), and Raspberry Pi 3b microcomputer (5). (B) Thigmomatic in action, making contact with *B. distachyon*.

2.2.10. DAP-seq

DNA affinity purification was carried out by collaborators at the JGI according to the protocol established by O’Malley et al 2016 (O’Malley *et al.*, 2016). In brief, transcription factor coding sequences were HALO tagged and mixed with Bd21 genomic DNA for in vitro binding. Protein-DNA was crosslinked, fragmented, immunoprecipitated using the HALO tag, barcoded, and sequenced. Reads were mapped
back to the Bd21 genome to identify binding target loci. The nearest annotated gene to a bound peak was used for GO analysis.

2.2.11. HOMER identification of motifs

HOMER v4.10 (Heinz et al., 2010) was used to compute enrichment scores for transcription factor binding motifs previously identified using DNA-affinity-purified sequencing (DAP-seq) (O’Malley et al., 2016) among each group of cycling transcripts. Motif enrichment was calculated against the hypergeometric distribution; the significance threshold was set to $p < 0.05$. Similar motifs were determined using the compareMotifs.pl function of HOMER against the global list of known motifs with the default threshold cutoff of 0.6. Modified from MacKinnon et al 2020 (MacKinnon et al., 2020).

2.2.12. GO analysis

NCBI BLAST and Phytozome (Altschul et al., 1990; Goodstein et al., 2011) were used to find orthologs for all *B. distachyon* v3.1 genes as the reciprocal best match to *A. thaliana* TAIRv10 protein sequences. Genes that did not significantly match a corresponding gene in *A. thaliana* were discarded from this analysis. *Arabidopsis thaliana* biological process gene ontology (GO) annotations were obtained from http://ge-lab.org/gskb/. For Kuiper’s test, the distribution of GO terms was compared with an empirically-determined background distribution. Gene identifiers were submitted to g:Profiler (Raudvere et al., 2019) for KEGG and Wiki pathway enrichment analysis. Modified from MacKinnon et al 2020 (MacKinnon et al., 2020).

2.2.13. Yeast one-hybrid assay for protein DNA interaction

In a yeast one-hybrid assay, the interaction between a transcription factor (TF) of interest
and a DNA sequence of interest are assayed by measuring the activation of a reporter gene in a heterologous yeast system.

The DNA sequence of interest is usually the promoter region of a gene representing approximately 1kb of sequence upstream from the gene’s start codon. This sequence is cloned and tested in three overlapping fragments for approximately 400bp each. The promoter sequence fragment is cloned upstream of a reporter gene, in this case encoding a B-galactosidase reporter. The finished vector is linearized by restriction digest, and then stably transformed into YM4271 yeast to generate reporter lines. Homologous recombination of the linearized vector targets the insertion to a specific locus in the yeast genome, controlling for transcriptional effects based on insertion point. A number of these lines are grown on selective media and then tested for reporter activity. Lines that do not show reporter activity without an effector added are selected for use in testing protein-DNA interaction.

The coding sequence for the TF of interest is cloned into the pDEST22 expression vector in frame with the Gal4 activation domain (Gal4AD), which induces transcriptional activity when bound to DNA. This vector is then transformed into the yeast reporter lines. If the Gal4AD:TF fusion protein binds to the promoter fragment being tested, the Gal4AD will activate transcription of the luciferase gene. B-galactosidase activity is measured by adding ONPG, a substrate that the B-galactosidase enzyme cleaves, causing a colorimetric change from clear to yellow. Protein-DNA interactions were tested in triplicate, with a positive/negative determination made for colorimetric change after a 3 h incubation period.
2.2.14. Genomic DNA Extraction

Tissue samples were collected in 1.5ml tubes with two metal beads and flash frozen in liquid nitrogen. They were ground to a fine powder in frozen blocks in Retsch 440 bead beater. 600ul of DNA extraction buffer (100mM NaCl, 50mM Tris, 25mM EDTA pH8, 1% SDS, a 10mM 2-mercaptoethanol) was added to each sample while still frozen, then vortexed vigorously to mix. Samples were incubated at 65°C for 10 min. 250ul of potassium acetate was added, the samples were mixed by inversion and then incubated on ice for 20 min. Tubes were centrifuged at 12,000 rpm for 10 min and the supernatant was carefully removed and placed in a new tube containing 600ul of isopropanol. The samples were incubated on ice for 20 min to precipitate the nucleic acids, then centrifuged at 10,000rpm. The supernatant was removed and the pellet was washed once with 300ul of 70% ethanol followed by a centrifugation at 15,000rpm for 1 min. The supernatant was removed and the pellet was dried under a fume hood for 1h before resuspending in 30ul of DNase free water.

2.2.15 RNA extraction and RT-qPCR

RNA was extracted from the main stem of plants one day after flowering using the Qiagen RNeasy Plant Mini Kit with on-column DNA digestion with RNase-free DNase I (Qiagen). First strand cDNA synthesis was performed using 500ng of total RNA with the Invitrogen SuperScript™ III First-Strand Synthesis SuperMix for qRT-PCR. cDNA samples were diluted by a factor of 10 with Rnase-free water. Quantitative PCR was done in 10ul reactions with 1ul of diluted cDNA using the Qiagen QuantiFast SYBR Green PCR Kit. Reactions were run in triplicate on an Eppendorf RealPlex2 Mastercycler.
2.3 Results

2.3.1 SWIZ is a Group I bZIP transcription factor and candidate cell wall regulator.

To identify genes potentially involved in cell wall regulation, microarray analysis of transcript abundance was conducted on RNA extracted from *B. distachyon* leaf, root, and stem tissue (Trabucco et al., 2013a). A gene annotated as a bZIP transcription factor, *Bradi1g17700* was highly expressed in root and stem relative to leaf (Fig 2.2).

Additionally, expression was highly correlated with other genes associated with wall biosynthesis, including secondary wall cellulose synthases and members of the lignin biosynthesis pathway. Phylogenetic analysis of the *Bradi1g17700*, henceforth referred to as *SECONDARY WALL INTERACTING bZIP* (SWIZ), amino acid sequence shows it to be an ortholog of the *A. thaliana* Group I bZIPS (Jakoby et al., 2002; Dröge-Laser et al., 2018), and most closely related to *AtbZIP18/52* (Figure 2.3).

![Bar graph showing expression levels of SWIZ in leaf, root, and stem tissue](image)

**Figure 2.2.** SWIZ is highly expressed in maturing stem and root. SWIZ transcript measured by microarray from *Brachypodium distachyon* leaf, root, and stem tissue. Mean +/- standard deviation of three biological replicates.
Figure 2.3 SWIZ phylogeny. Comparison of amino acids sequences from *B. distachyon*, *O. sativa*, *A. thaliana*, *N. tabacum*, and *L. solanum* shows SWIZ (blue) is similar to *A. thaliana* Group I bZIPs.

To further support the candidacy of SWIZ as a potential secondary cell wall regulator, it was screened for interaction with cell wall gene promoters by a yeast one-hybrid assay. Regulatory regions ~1000 bp upstream of the start codon of genes involved with cell wall biosynthesis were divided into three ~400 bp overlapping fragments. These regions were cloned upstream of the *B-galactosidase* reporter gene and stably integrated into yeast. The SWIZ coding sequence was cloned in frame with a Gal4 activation domain and transformed into the reporter lines. Interaction of SWIZ protein with the DNA region
being tested would allow the Gal4 activation domain to promote transcription of the reporter gene. B-galactosidase activity was determined by colorimetric assay as either a positive or negative interaction (Fig 2.4A). SWIZ interacted positively with one fragment of **CAFFEIC ACID 3-O-METHYLTRANSFERASE 6 (COMT6)** promoter, two fragments of **CINNAMYL ALCOHOL DEHYDROGENASE 1 (CAD1)** promoter, and one fragment of **CELLULOSE SYNTHASE A4 (CESA4)** promoter (Fig 2.4B, full sequences listed in Appendix). **CAD1** and **COMT6** are members of the lignin biosynthetic pathway, while **CESA4** is a secondary cell wall cellulose synthase.

**Figure 2.4.** SWIZ protein interacts with cell wall gene regulatory regions. Yeast one-hybrid assay of protein-DNA interaction using a B-galactosidase colorimetric reporter. SWIZ coding sequence fused with the Gal4 activation domain was transformed into yeast lines containing promoter:reporter constructs of cell wall promoter fragments driving B-galactosidase expression. A) Schematic of the yeast one-hybrid assay. Reporter lines were tested for self activity prior to screening, with two non-self active lines of each construct chosen for assay. Activation of the reporter was measured in triplicate, with positive/negative determination for colorimetric change. B) Schematic showing SWIZ positive interactions with promoter regions from **CAD1**, **COMT6**, and **CESA4**.
2.3.2 SWIZ binds a conserved motif

In determining the direct and indirect effects of transcription factor activity, assaying the targets directly bound by the protein of interest is of immense value. DNA affinity purification sequencing (DAP-seq) is an in vitro approach to identifying genome wide binding sites of a protein of interest. Collaborators at the Joint Genome Institute performed DAP-seq for SWIZ protein with Bd21 genomic DNA. Based on statistically significant enrichment of sequences purified with SWIZ protein, we identified 3,302 loci across the genome. The genes downstream of the binding sites were identified for further analysis (Supplemental File 1). From the bound DNA sequences, the HOMER program was used to identify binding motif sequences enriched in these fragments compared to the genomic background. From de novo analysis, nine sequence motifs were identified as significantly enriched above the background. The two most significant motifs were found in 24% and 17% of the enriched regions, and strongly resemble an A. thaliana Group I bZIP binding motifs (O’Malley et al., 2016) (Table 1). Gene ontology (GO) analysis was performed on both the full set of binding targets identified genome wide (Table A1), as well as the subset of 812 genes containing the top two binding motifs (Supplemental File 2), essentially CAGNCTG and CAGCTG (Table A2).
Table 1. SWIZ binding motifs identified by DNA affinity purified sequencing. Motif analysis using the HOMER program identified conserved sequences between regions interacting with SWIZ. Motif column displays the binding motif logo with nucleotide size depicting conservation at that position. p-value represents significance of enrichment in bound sites compared to the genomic background.

<table>
<thead>
<tr>
<th>Motif</th>
<th>p-value</th>
<th>% of bound targets</th>
<th>% of background</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAG-cTG</td>
<td>1e-1357</td>
<td>23.93</td>
<td>0.21</td>
</tr>
<tr>
<td>CAGctG</td>
<td>1e-737</td>
<td>16.98</td>
<td>0.36</td>
</tr>
</tbody>
</table>

2.3.3 SWIZ genetic reagents

To investigate the role of SWIZ in plant growth and secondary cell wall development, transgenic lines with enhanced or perturbed SWIZ function were generated. Overexpression lines were created using the maize ubiquitin promoter to drive expression of the SWIZ coding sequence as well as the SWIZ coding sequence fused with engineered green fluorescent protein (GPF), hereafter referred to as SWIZ-OE or SWIZ:GFP-OE (Figure 2.5A). Two independent events of SWIZ-OE and three independent events of SWIZ:GFP-OE were isolated and analyzed. Knockdown of SWIZ transcript abundance was done by expressing an artificial microRNA construct with homology to the first exon of the SWIZ mRNA, hereafter referred to as swiz-amiRNA (Fig 2.5B). Two independent events were isolated and analyzed. Transcript abundance analysis by RT-qPCR showed that SWIZ was significantly over expressed in the SWIZ-OE lines compared to wildtype (p < 0.05, Fig 2.5C). SWIZ expression was reduced in the swiz-amiRNA line, but not significantly at the p < 0.05 threshold (p=0.065, Fig 2.5D).
Figure 2.5. Diagram of SWIZ transgenic reagents. A) Constructs for SWIZ overexpression. The maize ubiquitin promoter was used to drive expression of the SWIZ coding sequence, either alone or fused in frame with eGFP. B) Artificial microRNA construct for SWIZ knockdown. The SWIZ mRNA schematic (exons - yellow blocks, introns - grey lines, UTR - gray blocks) shows the target site for amiRNA interference, indicated by the arrow in the first exon. Below is a diagram of the amiRNA construct used for SWIZ knockdown. The maize ubiquitin promoter was used to drive expression of an amiRNA that has homology with SWIZ exon 1, and forms a hairpin after transcription. The 21-mer nucleotide sequence used for target specificity is listed, with red postions showing hairpin mismatches that are predicted to interact with the DICER complex. C) and D) Relative level of SWIZ gene expression measured by RT-qPCR in SWIZ-OE (C) and swiz-amiRNA (D) lines. Whole stem tissue was collected 1 day after inflorescence emergence. LB, left border; ZmUbi prom, maize ubiquitin promoter; Hyg, hygromycin phosphotransferase gene; NOS, nopaline synthase terminator; RB, right border. ns: p>0.05, : *: p<=0.05.
2.3.4 SWIZ effects cell wall thickness, lignification, and plant morphology

Wildtype, SWIZ-OE, and swiz-amiRNA plants were grown and phenotyped for plant morphology, stem biology, and cell wall deposition. Transverse sections were taken from the second elongated internode of the main stem and stained for lignin using phloroglucinol-HCl. SWIZ-OE and swiz-amiRNA plants had dramatic interfascicular fiber phenotypes (Fig 2.6A-C). Compared to wildtype, (Fig 2.6A), SWIZ-OE plants (Fig 2.6B) had comparable intensity of phloroglucinol staining, but with an uneven distribution of color between neighboring fiber cells. The walls also appeared to be thicker than those of wildtype, again with an uneven pattern of thickening among cells. Some epidermal cells in the SWIZ-OE lines were also aberrantly thickened. The swiz-amiRNA plants (Fig 2.6C) also showed thicker interfascicular fiber walls, but in a more even distribution. These fiber cells also stained far more lightly for lignin compared to wildtype (Fig 2.6C).

Interfascicular fiber wall thickness was quantified in fiber cells, two layers distal from the major vascular bundle sheath layer, and showed significantly thicker walls in both SWIZ-OE and swiz-amiRNA compared to wildtype (Fig 2.6D). Lignin content was measured by the acetyl bromide soluble lignin (ABSL) method, which showed no change in lignin between wildtype and SWIZ-OE, but a significant reduction in swiz-amiRNA plants (Fig 2.6E). This quantification is in agreement with the visual measure of lignification observed by phloroglucinol-HCl staining, which showed lighter staining and thus less lignin in swiz-amiRNA plants. Additionally, both SWIZ-OE and swiz-amiRNA lines were significantly shorter in height than wildtype plants (Fig 2.6F).
Figure 2.6. SWIZ reagent phenotypes. Plants were grown in standard long day conditions without consideration for mechanical stimuli. Wildtype Bd21-3 (A), SWIZ-OE (B), and swiz-amiRNA (C) stems were sectioned in the second elongated internode after senescence and stained with phloroglucinol-HCl to show lignin deposition in pink-red hues. (D) Cell wall thickness was quantified for interfascicular fiber cells. (E) Acetyl bromide soluble lignin measured in extractive free cell wall material prepared from the main stem after senescence. (F) Plant height measured at senescence. Scale bar = 100µm. ns: p>0.05, *: p<=0.05. n=4-6 plants per genotype.
2.3.5 SWIZ translocates into the nucleus in response to mechanical stimulus and cellular GA levels

Some Group I bZIP proteins have been described as mechano- and osmosensing (Tsugama et al., 2014, 2016). I hypothesized that SWIZ protein may translocate within the cell in response to mechanical force. To test this, SWIZ:GFP-OE and GFP-OE plants were grown on tilted MS plates for 6 d in the dark and GFP localization was observed in their roots following a mechanical stimulus. The nuclear localized GFP signal was tracked and quantified over a 75 min period following the application of mechanical force with a metal probe (Fig 2.7A). GFP-OE signal was present in both the cytosol and nucleus, and nuclear accumulation remained static over the imaging period. The GFP protein was mostly observed in the cytosol in SWIZ:GFP-OE plants, but following mechanical force treatment, nuclear GFP signal increased substantially, reaching a peak around 30 min post stimulus (Fig 2.7B). The nuclear signal then decreased, returning to near starting levels by the end of imaging period (Fig 2.7A).
Figure 2.7. SWIZ translocates to the nucleus in response to mechanical stress. (A) Roots of SWIZ:GFP-OE and GFP-OE were observed after mechanical stress application. Images were taken every 2 min. Nuclear GFP signal was quantified in selected nuclei at each time point. The average nuclear GFP signal is represented by the trend line, with error bars indicating standard deviation of the mean. (B) Still image of SWIZ:GFP-OE and GFP-OE roots at 0 min (pre-stimulus) and 30 min (post stimulus). n=14-20 nuclei.

The dynamics and repeatability of SWIZ nuclear translocation were further investigated by sequential stimulus events. Touch response to stimulus can saturate at a certain number of treatments (Martin et al., 2010; Leblanc-Fournier et al., 2014; Moulia et al., 2015). To see if SWIZ translocation dynamics varied after repeated treatments, I applied mechanical force to SWIZ:GFP-OE roots as described above. A second stimulus was given 80 min after the first stimulus was applied, and again at 160 min. Following each mechanical stimulation, SWIZ translocated from cytoplasm to nucleus with maximum nuclear signal achieved in about 30 min post stimulus (Fig 2.8). The maximum intensity of each of these three translocation events did not differ significantly from each other, nor did the time between reaching maximum intensity or returning to pre-stimulus levels.
This suggests that SWIZ translocation dynamics are not impacted by repeated stimulus events, at least not over ~3 h.

![Graph showing SWIZ translocation dynamics](image)

**Figure 2.8.** SWIZ translocation dynamics are similar in amplitude and timing following repeated stimuli. SWIZ:GFP-OE roots were imaged by confocal microscopy with stimulus applied to the field of view at 0, 90, and 180 min. Images were taken every 2 min. Nuclear GFP signal was quantified in selected nuclei at each time point. The average nuclear GFP signal is represented by the trend line, with error bars indicating standard error of the mean. n=124 nuclei.

When conducting these translocation assays, root in the field of view was treated with mechanical force and all nuclei in that region were tracked and quantified for fluorescence. To determine if the mechanically stimulated SWIZ translocation signal is transferable to regions outside of the specific tissue that receives the force, two regions of the same SWIZ:GFP-OE root were imaged over the same timespan. The two regions were separated by 3 cm of undisturbed tissue. After a 45 min acclimation period, where nuclear GFP signal was static, the upper root region was given a mechanical stimulus (Fig 2.9A). The upper stimulated region showed typical SWIZ:GFP nuclear signal accumulation. At the same time, the lower root region was also imaged, and no translocation was observed (Fig 2.9B). The nuclear signal in the lower region remained...
constant at the acclimation period levels. At 120 min, the treatments were reversed, with
the lower root region receiving a stimulus while the upper region was unperturbed. The
lower region showed the expected SWIZ::GFP nuclear translocation while the upper
region did not. These results suggest that the signal that triggers SWIZ nuclear
translocation following a mechanical force event is localized to the cells that directly
receive that stimulus.
Figure 2.9 SWIZ translocation is localized to the region of tissue that experiences mechanical stimulus. SWIZ:GFP -OE roots were imaged by confocal microscopy with stimulus applied to the field of view at 30 and 120 min. At 30 min, stimulus was applied to the upper section of the root, while at 120 min it was applied to the lower section. SWIZ:GFP translocation was monitored in the upper section in (A) and the lower section in (B). Upper and lower regions were separated by ~3 cm of root tissue. Images were taken every 2 min. Nuclear GFP signal was quantified in selected nuclei at each time point. The average nuclear GFP signal is represented by the trend line, with error bars indicating standard error of the mean. n=109, 184 nuclei respectively for upper and lower regions.
SWIZ translocation dynamics were further investigated in response to cellular gibberellic acid (GA) levels. Mechanical forces have been shown to inactive cellular GA, and that cellular GA levels dictate cytosolic versus nuclear localizations of *A. thaliana* Group I bZIPs similar to SWIZ in amino acid sequence (Fukazawa *et al.*, 2010; Lange & Lange, 2015). To test if SWIZ translocation following mechanical force application was dependent on bioactive GA levels, *SWIZ:GFP-OE* roots were grown on MS media for 6 d before being transferred to GA treatment plates. Seedlings were transferred to media plates containing MS + 0 mM (control), 10 mM, 50 mM, or 100 mM bioactive GA (GA4) and were incubated on this media for 6 h prior to imaging. After a 10 min acclimation period, in which no nuclear translocation was observed, a mechanical force was applied and nuclear signal tracked. SWIZ:GFP signal amplitude was reduced in roots treated with GA, particularly at the 50 and 100 mM level. After 60 min the nuclear signal had returned to near pre-stimulus levels, and a second stimulus was applied (Fig 2.10). This time all the GA treated roots showed a similar reduction in nuclear signal, nearly half of the untreated control. These results suggest that the addition of exogenous GA4 reduces SWIZ translocation to the nucleus following mechanical force application.
Figure 2.10. SWIZ translocation to the nucleus is dampened by the addition of exogenous GA4. SWIZ:GFP-OE roots were grown on MS media for 6 d then moved to media supplemented with 10, 50, or 100 mM of GA4. Plants were left on supplemented media for 6 h then imaged following stimulus at 10 and 60 min. Images were taken every 2 min. Nuclear GFP signal was quantified in selected nuclei at each time point. The average nuclear GFP signal is represented by the trend line, with error bars indicating standard deviation of the mean. n= 85-125 nuclei per treatment.

The effect of reduced GA levels on SWIZ translocation was also investigated.

Paclobutrazol is an inhibitor of GA synthesis, and is known to reduce the level of bioactive GA in treated cells. SWIZ:GFP-OE roots were grown on MS media for 6 d before being transferred to GA treatment plates. Seedlings were transferred to media containing MS + 0 mM (control), 10, 50, or 100 mM paclobutrazol and were incubated on this media for 6 h prior to imaging. After a 10 min acclimation period, in which no nuclear translocation was observed, a mechanical force was applied and nuclear signal tracked. Roots treated with 10 mM of paclobutrazol did not show altered translocation dynamics compared to the untreated control, but roots treated with 50 or 100 mM...
paclobutrazol showed greater SWIZ:GFP nuclear localization to begin with, even prior to mechanical stimulus (Fig 2.11). Following stimulus, the nuclear GFP signal in the 100 mM paclobutrazol treated roots did increase to a similar maximum as the control, 10, and 50 mM treated roots, but the net degree of translocation was far less. SWIZ:GFP signal in the 100 mM paclobutrazol treated roots was high prior to treatment and remained high even after the other nuclear signals diminished. This suggests that pharmacological reduction of bioactive GA levels triggers SWIZ nuclear translocation even without the application of mechanical force.

Figure 2.11. SWIZ translocation to the nucleus is enhanced by the addition of paclobutrazol (PBZ), a GA synthesis inhibitor. SWIZ:GFP-OE roots were grown on MS media for 6 d, then moved to media supplemented with 10, 50, or 100 mM of PBZ. Plants were left on supplemented media for 6 h, then imaged by confocal microscopy with stimulus at 10 and 60 min. Images were taken every 2 min. Nuclear GFP signal was quantified in selected nuclei at each time point, and then normalized to their value at t=0 min. The average normalized nuclear GFP signal is represented by the trend line, with error bars indicating standard deviation of the mean. n= 64-142 nuclei per treatment
2.3.6 Thigmomorphogenesis in *B. distachyon*

The thigmomorphogenic response in *B. distachyon* was assessed by treating plants with regular mechanical force for two or three weeks during development. Wildtype Bd21-3 plants were grown for 7 d and then either grown without mechanical perturbation as controls or brushed with a metal bar once every 90 min for two or three weeks. After two weeks, a subset of the treated plants were removed from stress and allowed to recover. Another set of plants remained under the treatment for an additional week, totalling three weeks of mechanical force application (Fig 2.12A). After the stress period, all plants were allowed to recover and grow without intentional mechanical perturbation until senescence, after which they were phenotyped. Two week stressed plants were significantly shorter than controls, and three week stressed plants were shorter still (Fig 2.12B). Despite this difference in height, there was not a significant difference between the groups in terms of aboveground biomass (Fig 2.12C). Three week stressed plants had significantly more branches, with an increase also observed in two week stressed plants that was not statistically significant (Fig 2.12D).
Figure 2.12. *B. distachyon* displays classic thigmomorphogenic phenotypes. Wildtype Bd21-3 was grown for one week prior to treatment and then brushed once every 90 min with a metal bar for two or three weeks, then allowed to grow without mechanical stimulus until senescence. (A) Left to right, no stress, two weeks stress, three weeks stress. Height (B), (C) aboveground non-grain biomass, and (D) branch number were measured at senescence. ns: p>0.05, *: p<=0.05. n=5 plants per treatment. n=5 plants per treatment.

The effect of repeated mechanical stress on stem biology and interfascicular fiber cell walls was assessed by making transverse sections of the peduncle (Fig. 2.3.13A-C) and the third internode (Fig. 2.3.13D-F) of control plants (Fig. 2.3.13A,D) or plants treated with two (Fig. 2.3.13B,E) or three weeks (Fig. 2.3.13C,F) of mechanical perturbation. Sections were stained with phloroglucinol HCl to detect secondary cell walls, with reddish-pink coloration identifying lignification. The degree of staining and stem morphology in the third internode did not appear to be impacted by mechanical treatments, but the peduncle showed distinct morphological changes in the three week
stressed plants (Fig 2.13C). Interfascicular fiber cell walls stained lighter for lignin, and large parenchyma pockets were evident around the stem periphery, between the minor bundles. These parenchyma pockets contain starch granules, as evidenced by staining with Lugol’s iodine (Fig 2.14). There was no significant difference in fiber wall thickness between the internode regions or from differing touch treatments (Fig 2.13G).

Figure 2.13. *B. distachyon* stem biology under mechanical stress conditions. Wildtype Bd21-3 was grown for one week prior to treatment and then brushed once every 90 min with a metal bar for two or three weeks. Transverse sections of the peduncle (A-C) or 3rd internode (D-F) were taken from control (no stress) (A, D), 2 week stressed (B, E), and 3 week stressed (C, F) plants and stained with phloroglucinol-HCL to identify lignin. (G) Quantification of interfascicular fiber wall thickness. Scale bar = 100µm. ns: p>0.05, *: p<=0.05. n=3 plants per treatment.
2.3.7 Thigmomorphogenesis in SWIZ genetic reagents

Given the nature of SWIZ translocation in response to mechanical stimulus, and the aberrant wall phenotypes observed in SWIZ-OE and swiz-amiRNA lines, I wanted to test the hypothesis that SWIZ translocation in response to mechanical stimulus was responsible for the observed cell wall and plant growth phenotypes. Wildtype Bd21-3, SWIZ-OE, and swiz-amiRNA were established for one week then placed under mechanical stress as described above for two weeks, or grown carefully without mechanical stimuli as a control group. After senescence, plant height (Fig 2.15A), branching (Fig 2.15B), and aboveground biomass (Fig 2.15C) were measured. In control conditions, there was no difference in these traits. In mechanically stressed conditions, both SWIZ-OE and swiz-amiRNA were significantly shorter than wildtype, and swiz-amiRNA also showed a significant increase in branching.
Figure 2.15. Thigmomorphogenic whole plant phenotypes are enhanced in SWIZ reagents under mechanical stress. Wildtype Bd21-3, SWIZ -OE, and swiz-amiRNA plants were grown for one week and then placed under control conditions or two weeks of mechanical stress with metal bar contact once every 90 min. After senescence, plants were phenotyped for (A) height (cm), (B) branch number, and (C) aboveground biomass (g). ns: p>0.05, *: p<=0.05. n=5 plants per treatment.
Transverse sections of the stem were made in the third elongated internode and the peduncle for wildtype, SWIZ-OE, and swiz-amiRNA plants in control and touch conditions, and stained with phloroglucinol-HCl (Fig 2.16). The largest effect was seen in the peduncle (Fig 2.16). Control SWIZ-OE and swiz-amiRNA plants showed lighter lignin staining compared to wildtype. In mechanically stressed conditions, both showed an increase in phloroglucinol-HCl staining, particularly in swiz-amiRNA. SWIZ-OE interfascicular fiber walls also appear thicker in the stressed peduncle. In the third internode, SWIZ-OE and swiz-amiRNA both showed less lignin staining than wildtype in control and stressed conditions (Fig 2.16). Interfascicular fiber wall thickness was quantified, showing that thickness increased with mechanical stress treatment in the peduncle of SWIZ-OE compared to wildtype. In the third internode there was no difference in SWIZ-OE compared to wildtype, although SWIZ-OE walls were thicker in the touched plants compared to the control. swiz-amiRNA fiber cells did not show a significant difference in touched versus control conditions, or compared to wildtype.
Figure 2.16. Stem biology of SWIZ transgenic reagents under touched conditions. Wildtype Bd21-3, SWIZ-OE, and swiz-amiRNA plants were grown for one week and then placed under control conditions or two weeks of mechanical stress with metal bar contact once every 90 min. After senescence, the main stem was collected and transverse sections made of the peduncle and third elongated internode. Sections were stained with phloroglucinol-HCl. Scale bar = 100µm.
Figure 2.17 Quantification of interfascicular fiber wall thickness under touch and control conditions in SWIZ genetic reagents. n=4-6 plants per genotype, per treatment.

The changes observed in cell wall thickening and staining in SWIZ-OE and swiz-amiRNA lines under touched conditions, coupled with the protein binding of cell wall associated promoters in the yeast one hybrid prompted me to investigate cell wall gene expression in SWIZ transgenic plants in response to touch stimulus. The entire main stem from wildtype, SWIZ-OE, and swiz-amiRNA plants in control and mechanically stimulated conditions was collected one day after flowering, and approximately 20 min following mechanical stimulus. Relative expression of cell wall biosynthesis genes was measured for CAD1, COMT6, and CESA4 (Fig 2.18). While not statistically significant, COMT6 expression decreased in both SWIZ-OE and swiz-amiRNA with a larger decrease in swiz-amiRNA when touched.
Figure 2.18. Cell wall gene expression in SWIZ transgenic plants following mechanical stimulus. Wildtype Bd21-3, SWIZ-OE, and swiz-amiRNA plants were grown for one week and then placed under control conditions or two weeks of mechanical stress with metal bar contact once every 90 min. One day after flowering, the entire main stem was collected approximately 20 min after a touch stimulus event. Transcript abundance measured by RT-qPCR relative to ubiquitin conjugating enzyme 18 expression. ns: p>0.05, *: p<=0.05. n=3 plants per genotype.
2.4 Discussion

2.4.1 SWIZ is a likely cell wall regulator

SWIZ was first identified as a candidate regulator of secondary wall synthesis based on gene expression pattern and subsequently by protein-DNA interactions with cell wall associated cis-regulatory regions. SWIZ is clearly a Group I bZIP transcription factor, and is the closest B. distachyon ortholog to A. thaliana bZIP18/52 and O. sativa bZIP61, closely related to OsRF2b (Jakoby et al., 2002; Liu & Chu, 2015; Dröge-Laser et al., 2018). Several families of transcription factors are known to directly regulate secondary wall thickening, chief among them are NAC and MYB proteins (Coomey et al., 2020). As with SWIZ, many of these genes were first identified based on their expression patterns being correlated with secondary wall biosynthetic enzymes. Recent studies in A. thaliana, B. distachyon, and O. sativa have shown protein-DNA interactions with the promoters of secondary wall associated genes, as I have demonstrated for SWIZ and CAD1, COMT6, and CESA4 (Zhong & Ye, 2007; Taylor-Teeples et al., 2015; Handakumbura et al., 2018).

Surprisingly, I observed a similar phenotypic response in both gain and loss of function lines with reduced height and thicker interfascicular fiber walls. SWIZ gain-of-function mutants were generated constitutively expressing SWIZ transcript and loss-of-function was achieved by knockdown of SWIZ expression using an artificial microRNA construct targeting SWIZ transcript. After confirming the presence and expression of the transgenes, SWIZ-OE and swiz-amiRNA were assayed for growth and cell wall defects. The swiz-amiRNA lines had lower lignin content in fiber cells compared to wildtype, while SWIZ-OE was unchanged. These data suggest a role for SWIZ in activating lignin
deposition. Furthermore, the decrease of lignin in swiz-amiRNA supports a role as a direct regulator of CAD1 and COMT6. Given the unusual pattern of wall thickening seen in SWIZ-OE sections and the reports of Group I bZIPS translocating in response to hypo-osmotic stress (Tsugama et al., 2014, 2016), I hypothesized that SWIZ may respond to mechanical stimuli to regulate aspects of growth and secondary wall deposition.

2.4.2 SWIZ translocation dynamics are consistent with reported bZIP dynamics in response to mechanical stimulus and GA

I began to test this hypothesis by investigating the cellular localization of SWIZ protein in response to mechanical stimulus. Consistent with reports of Group I bZIPs in root tissue (Tsugama et al., 2014, 2016), SWIZ translocated from the cytosol to the nucleus in response to mechanical stimulus. In A. thaliana, translocation was stimulated by treatment with a hypo-osmotic solution, which proved technically challenging on our confocal microscope platform. Since hypo-osmotic pressure simulates external force on the cell, I opted to apply force directly to the root by light contact with a metal probe. Prior to touch, SWIZ was mostly localized to the cytosol, with some nuclear localization. Following touch, SWIZ translocated to the nucleus quite rapidly, with peak nuclear signal intensity around 30 min after stimulus. By 75 min post-stimulus, the nuclear signal had returned to pre-stimulus levels. This timing is consistent with the observations made for A. thaliana Group I bZIPs (Tsugama et al., 2014, 2016), although those reports only provide information at 0, 30, and 120 min post-treatment, rather than the measurements taken every 2 min for SWIZ translocation. I also investigated the repeatability of SWIZ translocation following sequential stimuli. The timing of touch response has been investigated in various systems on the order of min to days, and have shown acclimation of the touch response to successive stimuli (Coutand et al., 2009; Martin et al., 2010;
Leblanc-Fournier et al., 2014). For SWIZ, the translocation dynamics are consistent at least over three successive cycles of stimulus and return to baseline. The dynamics observed in SWIZ-OE suggest that SWIZ translocation is not dampened by subsequent stimulus. While the kinetics of touch response acclimation have not been studied in B. distachyon, we may consider at least that input from SWIZ remains consistent for multiple stimulus events.

2.4.3 Thigmomorphogenesis in B. distachyon.

Before testing the idea that SWIZ functioned in touch responsive growth, I first wanted to examine thigmomorphogenesis in B. distachyon to establish a mechanical stimulus assay and provide a baseline of phenotypic response in wildtype plants. In light of the timing and repeatability of SWIZ translocation dynamics, regular stimulus at 90 min intervals was chosen for the treatment. Touch stimulus is generally an inhibitor of plant elongation growth, but promotes branching and radial growth (Jaffe, 1973; Braam, 2004; Chehab et al., 2009). In eudicots, this radial growth occurs in the cambium meristem, a cell type that is not present in grasses. Therefore, they do not undergo such changes in radial growth. Our study of wildtype B. distachyon plants with two or three weeks of regular touch revealed a decrease in plant height similar to that previously reported (Gladala-Kostarz et al., 2020). We also observed an increase in branching with increasing touch treatment. However, we did not observe a change in total biomass at senescence, perhaps due to compensation by increased branching.

2.4.4 SWIZ touch responsive phenotypes

SWIZ gain- and loss-of-function transgenic lines were subjected to two weeks of touch stimulus alongside control groups that were treated carefully to minimize possible
sources of mechanostimulation from watering and handling. In touch conditions, both SWIZ-OE and swiz-amiRNA plants were shorter than wildtype, while in control conditions they were not different. Total biomass was not affected by genotype or touch, but swiz-amiRNA did show increased branching under touched conditions. While the touch treatment did affect wildtype plants similar to control conditions, the influence of touch was greater in the SWIZ transgenics.

Under touched conditions, the peduncle of SWIZ-OE had thicker interfascicular fiber walls compared to wildtype, while in the control there was no difference. In the third internode there was no difference in fiber wall thickness between lines. The peduncle also showed a difference in lignin staining in touched conditions compared to control, with SWIZ-OE and swiz-amiRNA showing an increase in staining intensity. The prevalence of phenotypic changes in the peduncle but not the third internode is attributed to the nature of the touch treatment. The metal bar passed over the plants at a set height that was above the position of the third internode, but came into contact with the peduncle. Thus, the entire plant did not experience the mechanical stimulus evenly, and the touch induced response was limited to the contacted tissue. This is consistent with how SWIZ translocation is restricted to the stimulated region. In A. thaliana, AtVIP1 translocation was limited to just a few cells perturbed by contact with a pin (Tsugama et al., 2014). SWIZ translocation was similarly specific to the cells that experienced direct mechanical stimulus. Regions of the root 3 cm away from a touched section did not show translocation. Given this specificity, I assume that the peduncle cells of touched plants experienced SWIZ translocation while lower internodes did not.
Bioactive GA status may act in SWIZ mechanosignaling and explain touch responsive height phenotypes.

Several lines of evidence implicate bioactive GA status as part of the mechanoperception pathway that modulates SWIZ translocation, and may help explain the SWIZ touch responsive phenotypes. Dominant negative NtRSG transgenic tobacco has a dwarf phenotype due to misregulation of GA synthesis that inhibits cell elongation (Fukazawa et al., 2000). In tobacco protoplasts, NtRSG translocates into the nucleus in response to low bioactive GA, where it then promotes the synthesis of bioactive GA (Ishida et al., 2004; Fukazawa et al., 2010, 2011). Lange & Lange (2015) demonstrated that touch inactivates cellular GA (Lange & Lange, 2015). Together, these observations can provide a model relating touch, GA, and cell elongation. Touch inactivates GA, a hormone that promotes elongation, and in response bZIP proteins translocate into the nucleus and act to re-establish bioactive GA and elongation. SWIZ translocation is consistent with these observations. When SWIZ-OE roots were treated with exogenous bioactive GA, the nuclear translocation following touch was dampened. When SWIZ-OE roots were treated with paclobutrazol to chemically inhibit bioactive GA synthesis, SWIZ protein localized to the nucleus even in the absence of touch.

Applying this model to SWIZ-OE and swiz-amiRNA provides possible mechanisms for the reduction in height observed in both types of transgenic plant under touch treatment. If SWIZ is indeed an activator of wall synthesis, the large influx of protein to the nucleus following touch may trigger activation of cell wall thickening, resulting in reduced cellular elongation. If SWIZ regulates GA synthesis genes similarly to NtRSG, then the reduced SWIZ population in amiRNA lines may not be able to restore proper GA levels following touch, resulting in an inability to continue elongation. This sort of activity in
the swiz-amiRNA lines would be consistent with the dwarf phenotype observed in NtRSG dominant negative mutants (Fukazawa et al., 2000). Similar dwarfing is also seen from expression of an OsRF2a dominant negative mutant (Dai et al., 2003).

2.4.6 SWIZ touch responsive cell wall phenotypes may depend on interacting partners

In touched peduncles, SWIZ-OE and swiz-amiRNA showed increased lignin staining in interfascicular fiber cells compared to untouched controls, while the third internode of both mutants stained more lightly in both touched and control conditions. Gene expression analysis did not show a significant difference in COMT6, CAD1, or CESA4 expression in stems of either SWIZ-OE or swiz-amiRNA in touched or control conditions. COMT6 expression did appear to be somewhat reduced in the SWIZ transgenics, which would be consistent with the lighter staining seen in the third internode. However, this experiment was conducted on whole stems prior to my understanding of the specific nature of SWIZ translocation in directly contacted regions. It is possible that the gene expression signal in the touched cells was diluted by homogenization with the entire stems, and thus we did not observe a change in touched conditions.

SWIZ may regulate wall thickening by directly binding upstream of genes associated with secondary wall biosynthesis. We identified a sequence motif commonly reported for bZIP-DNA interactions (O’Malley et al., 2016). Yeast one-hybrid data shows SWIZ protein interacting with regulatory regions of CAD1, COMT6, and CESA4, yet none of these appear in the in vitro DAP-seq binding results. Interestingly, one of the top two binding motifs from DAP-seq is present in the overlapping region of two CAD1 promoter fragments that SWIZ interacted in yeast. One explanation for why this and perhaps other
cell wall relevant genes do not appear in the DAP-seq results comes from the homo and heterodimerization of bZIP proteins. The leucine zipper domain of bZIPS has been shown to dictate the interactions between dimerizing partners (Schütze et al., 2008), and in A. thaliana the combinatorial interactions of different bZIP groups has been fairly well defined (Deppmann et al., 2004; Grigoryan & Keating, 2006; Ehlert et al., 2006; Weltmeier et al., 2006; Schütze et al., 2008). Group I bZIPS are known to interact with one another as well as with Group E members (Schütze et al., 2008; Van Leene et al., 2016). These interactions can have a synergistic effect on transcriptional activity, and can result in unique binding interactions (Schütze et al., 2008; Van Leene et al., 2016). A study of AtbZIP29 showed heterodimerization specifically within a subset of seven Group I bZIPS that are orthologous to bZIPS implicated in cell wall synthesis and remodelling in other systems, including NtRSG, OsRF2a, OsRF2b, and SlVSF-1 (Yin et al., 1997; Ringli & Keller, 1998; Fukazawa et al., 2000; Dai et al., 2003, 2004; Van Leene et al., 2016). SWIZ is part of the same clade of protein, and SWIZ’s role in cell wall synthesis may also rely in part on interaction with other bZIP partners, a role that would not be identified through the in vitro DAP-seq assay.

2.4.7 SWIZ in vitro binding targets relate to hormone cross talk and mechanoperception pathways

Despite the caveats of biological relevance for bZIPS in in vitro binding assays, it is still tempting to speculate on the implications of the SWIZ binding targets identified from DAP-seq. Perhaps the most interesting avenue suggested from this data involves the crosstalk of auxin, gibberellin, and ethylene signaling. A number of these sites are associated with genes annotated as auxin response factors (ARFs) and small auxin upregulated RNA (SAUR) genes. Gibberellin metabolism and signalling genes in this
data set include GRAS family transcription factors and an ortholog of AtGA2OX2.

Ethylene responsive binding proteins are implicated in touch response, and an annotated APETALA2/Ethylene responsive binding protein is present in the DAP-seq results. Cross talk between these hormone signalling pathways is implicated in secondary wall development. In maize stems ethylene signalling controls auxin and GA, resulting in altered cell wall synthesis (Zhang et al., 2020).

Aspects of force perception and signalling such as wall associated kinases, receptor like kinases, mitogen activated protein kinases, calcium dependent protein kinases, calmodulin, and calmodulin-like genes. Calcium fluxes have been described in response to touch (Lee et al., 2005; Monshausen et al., 2009), and both calcium dependent protein kinases and mitogen activated protein kinases are known to phosphorylate bZIPs (Djamei et al., 2007; Ishida et al., 2008; Pitzschke et al., 2009; Ito et al., 2014). Wall integrity sensors such as wall associated kinases and receptor like kinases have been suggested to play a role in force perception as well (Monshausen & Haswell, 2013; Leblanc-Fournier et al., 2014; Kohorn, 2016). These putative SWIZ regulatory targets suggest a scenario where SWIZ translocation and transcriptional activation may prime the cell for future sensing events by upregulating possible sensing and signalling components.

2.5 Conclusions

SWIZ is a bZIP family transcription factor orthologous to proteins in other species that have been implicated in cell wall development and remodelling. SWIZ appears to control elongation and cell wall thickening in response to mechanical stimuli, which regulates SWIZ translocation into the nucleus by acting on the cellular bioactive GA pool. Touch decreases cellular bioactive GA levels, which causes SWIZ to translocate from the
cytoplasm into the nucleus. This translocation can be reduced by supplementing with exogenous bioactive GA, or induced by chemically inhibiting GA synthesis.
3.1 Introduction

The *three-amino-acid-loop-extension (TALE)* homeodomain family of transcription factors includes several well characterized regulators of growth and development in eudicots and grasses. The *KNOTTED1 HOMEBOX (KNOX)* and *BEL* class *TALEs* have been shown to regulate secondary wall synthesis. The founding member of the *KNOX* gene family is *ZmKNOTTED1 (KN1)*, which was first identified in maize as the causative gene in a gain of function mutant whose phenotype was of bumpy, or knotted looking leaves (Vollbrecht *et al.*, 1991). *KNOX* genes include Class I and Class II types. Class I *KNOX* genes including *KN1*, *SHOOT MERISTEMLESS*, and *GNARLY*, regulate aspects of organ development and meristem maintenance (Hake *et al.*, 2004; Hay & Tsiantis, 2010). Class II *KNOX* genes are not as well characterized, with most work focusing on the function of *KNOTTED OF ARABIDOPSIS THALIANA 7 (Zhong *et al.*, 2008a; Li *et al.*, 2012; Liu *et al.*, 2014; He *et al.*, 2018). *AtKNAT7* was first identified as *IRREGULAR XYLEM 11 (IRX11)* in a screen for transcripts co-expressed with secondary cell wall associated genes (Ehlting *et al.*, 2005; Persson *et al.*, 2005; Brown *et al.*, 2005b). The irregular xylem (irx) phenotype, as the name implies, consists of collapsed xylem vessel cells. This phenotype can vary in severity, and has been associated primarily with cellulose defects, but also with altered hemicellulose and lignin biosynthesis (Turner & Somerville, 1997; Brown *et al.*, 2005b). *AtKNAT7* has been shown repeatedly to be a
target of the so-called master regulators of cell wall biosynthesis; SND1, NST1, NST2, VND6, and VND7 all positively regulate AtKNAT7 expression (Zhong et al., 2006b, 2007a, 2008a). MYB46, another target of SND1, has also been shown to be a positive regulator of KNAT7 expression (Zhong et al., 2007a; Zhong & Ye, 2007). All of these master regulators are known to be capable of activating the entire suite of cell wall biosynthetic enzymes, either directly or through other downstream regulators, such as AtKNAT7. AtKNAT7 and orthologs in other species, such as poplar (PtKNAT7) and cotton (GhKNL1), are generally described in the literature as repressors of cell wall synthesis in fiber cells (Li et al., 2012; Ma et al., 2019). AtKNAT7 contains a canonical LxLxL EAR motif, known for its role in repressing gene expression. EAR containing proteins recruit histone modifying complexes to their DNA binding sites, resulting in condensation of the chromatin and subsequent transcriptional repression (Kagale & Rozwadowski, 2011).

While there is substantial evidence for AtKNAT7 repressor function, there is also data indicating a role in positive wall regulation. Atknat7 mutants have thicker interfascicular fiber walls, as expected for knocking out a repressor, but this mutant also shows collapsed xylem with thinner walls. Atknat7 mutants have greater lignin content, but reduced xylan, suggesting that AtKNAT7 may differentially regulate aspects of hemicelluloses compared to lignins. AtKNAT7 protein can bind to the promoter of AtIRX9, a gene responsible for xylan backbone synthesis as well as control the expression of several enzymes in the lignin biosynthesis pathways (He et al., 2018).

AtKNAT7 is known to interact with several other protein partners, including another class II KNOX protein, AtKNAT3. The double mutant, knat7/knat3 has more severe cell wall phenotypes than Atknat7 alone, while the Atknat3 knockout has no reported
phenotype. AtKNAT7 is also involved in seed coat development through interaction with AtMYB75 (Bhargava et al., 2010, 2013). In other systems such as alfalfa and peach, other class II KNOX genes not orthologous to AtKNAT7 play roles in leaf and seed coat development (Testone et al., 2009; Chai et al., 2016).

Another TALE transcription factor, BEL1-LIKE HOMEODOMAIN6 (BLH6), can repress secondary wall formation in interfascicular fiber cells. Similar to Atknat7, Atblh6 mutants have collapsed xylems and thicker fiber cell walls. Indeed, AtBLH6 and AtKNAT7 protein physically associate, and as a complex repress fiber wall thickening. Genetic analysis has shown that this complex regulates fiber wall development by directly binding the AtREV promoter (Liu et al. 2014). The blh6/rev and knat7/rev double mutants exhibit the collapsed xylem phenotype similar to the single blh6 or knat7 mutants, but the rev double mutants do not have thicker interfascicular fiber walls. This suggests that xylem wall regulation relies on distinct mechanism from interfascicular fibers, and that the direction of transcriptional action by known wall regulators may vary between these tissues.

In grasses, rice OsKNOR1 (also known as OsKNAT7) is the closest ortholog of AtKNAT7 and has also been shown to negatively regulate cell wall synthesis in interfascicular fiber cells (Zhao et al., 2019; Wang et al., 2019). As in Atknat7, Osknor1 loss-of-function mutants have thicker interfascicular fiber walls, but no reported xylem phenotype. These plants also had an increase in grain size that was attributed to variation in cell size (Wang et al., 2019). Gene similar to BLH6 have not been described as regulators of wall thickening in grasses. However, OsKNOR1 analysis revealed functions unique from AtKNAT7. It was shown to physically interact with OsNAC31 (also known as OsVND7/
OsSWN3) and Os GROWTH REGULATING FACTOR 4 (OsGRF4). Transient gene expression analysis in protoplasts showed that OsKNOR1-OsNAC31 jointly regulated OsMYB61/103 expression, with OsKNOR1 mitigating the positive regulation by OsNAC31. Similarly, OsGRF4 is known to activate expression of cell expansions genes, and OsKNOR1 interaction reduces this expression (Wang et al., 2019).

3.2 Methods

3.2.1. Plasmid construction

Overexpression constructs were built using the Invitrogen Gateway cloning system. PCR amplified coding sequences were cloned into the pENR-D-TOPO or appropriate pDONR vector for multi site recombination, and further subcloned into a modified pOl001 destination vector (Vogel et al 2006). CRISPR-Cas9 guide RNA sequences were designed using the CRISPR-PLANT web resource (http://www.genome.arizona.edu/crispr/CRISPRsearch.html). The knob7-1 allele was targeted by the 5’-CCTGCAGCTGAAGCAAATCAAGA-’3 guide RNA. Guide RNA oligos were annealed by heating to 95C for 2 min followed by slowly cooling to 25C at 2 degrees per minute. Annealed guide RNAs were cloned into the pENTR_OsU3B_sgRN vector by BsaI digestion and ligation. Sequence confirmed clones were recombined using the Invitrogen Gateway cloning system into the pOs-cas9_RC_of_L destination vector. Sequence confirmed clones for all destination vectors were electroporated into Agrbacterium tumefaciens strain AGL-1.

3.2.2 Identification of mutants

Sodium azide mutant population was generated by the Sibout lab at INRAE Versailles-
Grignon (Dalmais et al 2013). Briefly, wildtype Bd21-3 seeds were treated with sodium azide to induce point mutations. Genomic DNA from M2 plants from each family was pooled and sequenced to identify mutation sites. To confirm specific mutations in subsequent generations, PCR primers were designed to flank the mapped mutation locus, and sequencing of the PCR product determined the presence of the mutation. The knob7-3 allele was identified in line NaN451.

3.2.2. Plant transformation
Transformation was performed according to Vogel & Hill 2008. Immature seeds were collected from ~6 week old plants, deglumed, and surface sterilized with a solution of 1.3% NaClO and 0.01% Triton-X100 for four min. Sterilized seeds were rinsed three times in sterile water. Embryos were dissected from the seeds and placed on callus initiation media (CIM) for four weeks, then subcultured to fresh CIM for two more weeks, the subcultured a final time onto fresh CIM for one week. Seven week old calli were co-cultivated in a suspension of A. tumefaciens for ~5 min, then thoroughly dried on sterile filter paper for 3-5 days at 22C in the dark. Calli were moved ont CIM media containing 50 mg/L hygromycin B and 150 mg/L timentin, where they were grown for 3-5 weeks with selective subculture of healthy callus at week 4. After selection, healthy calli were moved to Linsmaier and Skoog media supplemented with 50 mg/L hygromycin B, 150 mg/L timentin, and kinetin to promote shoot growth. Calli that produced green tissue within 3-5 weeks were moved to Murashige and Skoog media supplemented with 50 mg/L hygromycin B and 150 mg/L timentin to allow root growth. After 1-3 weeks, calli that established roots were transplanted to soil and grown as described below.
3.2.3. Plant growth

*Brachypodium distachyon* line Bd21-3 was used for all experiments. Seeds were stratified on wet paper towel wrapped in foil to exclude light for 10 days at 4C before being planted in Promix BX potting mix in SC10 Ray Leach Cone-tainers (Stuewe & Sons Inc, https://www.stuewe.com/products/rayleach.php). Plants were grown in a Percival PGC-15 growth chamber with day/night conditions of 20h light at 22C and 4h dark at 18C respectively.

3.2.4. Transverse stem sections, histology

The main stem of senesced plants was taken and the internode of interest removed and embedded in 8% agarose. Samples were sectioned using a Leica VT1000 Vibratome, making 55um thick sections. Multiple sections of each internode were collected and stored in water at 4C. Histochemical staining was carried out using toluidine blue, phloroglucinol-HCl, and Maule reagent as described in Mitra & Loque (2014). Images were obtained at 4, 10, and 20x using a Nikon Eclipse E200MV R light microscope and PixeLINK 3 MP camera.

3.2.5 Measuring cell wall thickness

Transverse sections imaged at 20x were used for cell wall thickness measurements. Interfascicular fiber cells separated by one cell layer from the mestome cells on the phloem side of major vascular bundles were targeted for measurement. Using ImageJ, lines were drawn across two walls of adjoining cells. The resulting line length was divided by two to give one cell wall width. ~15 measurements were made for each plant.

3.2.6. Cell wall material insoluble in alcohol (MIA)

The main stem of mature, senesced plants was collected and cut into small pieces (~2 cm)
into a 2 ml tube. Two metal beads were added and the stem was ground to a fine powder using a Retsch 440 bead beater. Ground material was transferred to a glass screw cap tube. Cell wall material was washed with 5ml of water in an 80°C water bath for 10 min with agitation. The cell wall material was pelleted by centrifugation at 3700 rpm for 10 min and the supernatant aspirated by vacuum. This was repeated for a second water wash. The cell wall material was then washed three times with 100% ethanol at 80°C for 15 min per wash, with collection by centrifugation and aspiration of the supernatant between washes as described above. The cell wall material was then washed twice with acetone for 15 min per wash at room temperature, then left to dry under a fume hood overnight. Modified from INRAE protocols.

3.2.7. ABSL quantification

Beginning with dry cell wall MIA samples, 4.5-5.5 mg of each sample was weighed into a 2 mL glass vial using a precision balance. 1ml of acetyl bromide solution (25% acetyl bromide, 75% acetic acid) was carefully added to each vial under a fume hood. The vials were capped and inverted several times to mix. Samples were incubated in a drying oven at 55°C for 2 h 30 min, with mixing by gentle inversion every 30 min to ensure full sample solubilization. The samples were cooled to room temperature before proceeding. 0.1 ml of sample was diluted into 1.2 ml of acidified 2MNaOH, then mixed with 0.3 ml of 0.5 M hydroxylamine chloride and 1.4 ml of acetic acid. Using a glass pipette, air was bubbled through the sample to ensure full homogenization of the reaction mixture. Absorbance at 280 nm was measured on a SpectraMaX M6 plate reader, and the lignin content was calculated using the following equation: %lignin= 100 x (A280 x Vol reaction x Vol dilution) / (20 x Vol sample solution x Mass sample (mg)). Modified from
INRAE protocols.

3.2.8. Mild alkaline hydrolysis

Beginning with solvent free cell wall samples, 10mg per sample was weighed out into tubes. 100 ul of o-coumaric acid (1mg/ml) was added to each sample as an internal control. 1ml of 1N NaOH was added to each sample and mixed well by inversion. The samples should turn a bright, fluorescent yellow. The tubes were wrapped in foil to protect them from light and placed on a rotating agitator overnight. The next day, samples were removed from the agitator and acidified with 250 ul of 6M HCl. Samples were centrifuged at 2000g for 5 min to remove any remaining cell wall debris, and the supernatant transferred to a clean tube. Hydroxycinnamate extraction was done on silica columns bound with C21 hydrocarbons. Columns were washed with methanol, then primed with 2 ml of acidic water (H2O + 0.1% formic acid). 0.5 ml of sample was run through the column. Excess salts were flushed out with 2 ml of acidic water, then the column was eluted into glass vials with 1ml of methanol. Eluted samples were chilled at -20°C for 15 min to precipitate any insoluble components. Samples were analyzed by HPLC-MS with a diode array detector using a MachereyNagel - EC 50/2 (mm) nucloshell - RP (rev phase) C18, 2.7 mm granularity column. Modified from INARE protocols.

3.2.9 Thioacidolysis

Thioacidolysis reagent was carefully prepared in a chemical fume hood as previously described (Méchin et al., 2014), resulting in a 9:1 dioxane:ethanethiol mixture with 0.1M tetrafluoroboric acid dimethylether. For each sample, 10 mg of cell wall material insoluble in alcohol was weighed into screw cap tubes. 7 ml of thioacidolysis reagent was
added, along with 2.5 mg/ml of heneicosane C21 as an internal standard. The tubes were
incubated in a 100°C oil bath for 4 h and then cooled in ice water. 0.2M NaHCO3 was
added to each tube, followed by 0.1 ml of 6M HCl. 7 ml of dichloromethane was added
and tubes were gently mixed. The lower organic fraction was taken and dried over
anhydrous sodium sulfate, then concentrated by rotoevaporation to approximately 0.5 ml.
5 ul of the concentrated sample was taken for trimethylsilylation with 100ul N,O-
bis(trimethylsilyl)trifluoroacetamide and 10ul pyridine for 1h. Samples were analyzed by
GC-MS. Modified from INARE protocols.

3.2.10. Neutral sugar analysis

Beginning with cell wall material insoluble in alcohol, 10 mg of each sample was
hydrolized in 2.5M trifluoroacetic acid for 2h at 100°C, as described in (Harholt et al.,
2006). To determine the cellulose content, the residual pellet obtained after the
monosaccharide analysis was rinsed twice with ten volumes of ethanol and once with 10
volumes of acetone and hydrolysed with H2SO4 as described (Updegraff, 1969). The
released monosaccharides of hemicellulose were diluted 500 times and the released
glucose of cellulose was diluted 1000 times. Then the monosaccharides were quantified
using an HPAEC-PAD chromatography as described in (Harholt et al., 2006). Modified
from INARE protocols.

3.2.11. Yeast one-hybrid assay for protein DNA interaction

In a yeast one-hybrid assay, the interaction between a transcription factor (TF) of interest
and a DNA sequence of interest are assayed by measuring the activation of a reporter
gene in a heterologous yeast system.
The DNA sequence of interest is usually the promoter region of a gene representing approximately 1kb of sequence upstream from the gene’s start codon. This sequence is cloned and tested in three overlapping fragments for approximately 400bp each. The promoter sequence fragment is cloned into the pLUC vector, just upstream from the luciferase reporter gene. The finished vector is linearized by restriction digest, and then stably transformed into YM4271 yeast to generate reporter lines. Homologous recombination of the linearized vector targets the insertion to a specific locus in the yeast genome, controlling for transcriptional effects based on insertion point. A number of these lines are grown on selective media and then tested for luciferase activity. Lines that are not “self active” are selected for use in testing protein-DNA interaction.

The coding sequence for the TF of interest is cloned into the pDEST22 expression vector in frame with the Gal4 activation domain (Gal4AD), which induces transcriptional activity when bound to DNA. This vector is then transformed into the yeast reporter lines. If the Gal4AD:TF fusion protein binds to the promoter fragment being tested, the Gal4AD with activate transcription of the luciferase gene. Luciferase activity is measured by adding coelenterazine, a substrate that the luciferase enzyme cleaves. This cleavage releases light, which is quantified on a plate reader. By normalizing the luminescent reading by the density of cells being tested, we generate a value for relative luciferase activity that indicates the degree of protein-DNA interaction. Protein-DNA interactions are tested in triplicate.

3.2.12. Genomic DNA Extraction
Tissue samples were collected in 1.5 ml tubes with two metal beads and flash frozen in liquid nitrogen. They were ground to a fine powder in frozen blocks in Retsch 440 bead
beater. 600 ul of DNA extraction buffer (100 mM NaCl, 50 mM Tris, 25 mM EDTA pH8, 1% SDS, a 10mM 2-mercaptoethanol) was added to each sample while still frozen, then vortexed vigorously to mix. Samples were incubated at 65°C for 10 min. 250ul of potassium acetate was added, the samples were mixed by inversion and then incubated on ice for 20 min. Tubes were centrifuged at 12,000 rpm for 10 min and the supernatant was carefully removed and placed in a new tube containing 600 ul of isopropanol. The samples were incubated on ice for 20 min to precipitate the nucleic acids, then centrifuged at 10,000 rpm. The supernatant was removed and the pellet was washed once with 300ul of 70% ethanol followed by a centrifugation at 15,000 rpm for 1 min. The supernatant was removed and the pellet was dried under a fume hood for 1h before resuspending in 30 ul of DNase free water.

3.2.13. RNA extraction and RT-qPCR

RNA was extracted from the main stem of plants one day after flowering using the Qiagen RNeasy Plant Mini Kit with on-column DNA digestion with RNase-free DNase I (Qiagen). First strand cDNA synthesis was performed using 500ng of total RNA with the Invitrogen SuperScript™ III First-Strand Synthesis SuperMix for qRT-PCR. cDNA samples were diluted by a factor of 10 with Rnase-free water. Quantitative PCR was done in 10ul reactions with 1ul of diluted cDNA using the Qiagen QuantiFast SYBR Green PCR Kit. Reactions were run in triplicate on an Eppendorf RealPlex2 Mastercycler.
3.3 Results

3.3.1 *KNOB7* is a candidate wall regulator and a Class II KNOX gene

To identify genes involved in cell wall regulation, microarray analysis of transcript abundance was measured in *B. distachyon* leaf, root, and stem tissue. The *Bradi1g76970* transcript was highly abundant in root and stem compared to leaf. Additionally, *Bradi1g76970* expression was highly correlated with other genes known to function in cell wall biosynthesis, including secondary cellulose synthases and members of the lignin biosynthesis pathway, as well as other known and candidate cell wall regulators such as *SWAM1* and *SWAM4*. Phylogenetic analysis of *Bradi1g76970*, henceforth referred to as *KNOTTED OF BRACHYPODIUM DISTACHYON 7 (KNOB7)* showed it to be an ortholog of *AtKNAT7*, a Class II KNOX gene.
Figure 3.1 *Bradi1g76970* is highly expressed in maturing stem and root. *Bradi1g76970* transcript measured by microarray from *Brachypodium distachyon* leaf, root, and stem tissue. Mean +/- standard deviation of three biological replicates.
Figure 3.2 KNOX protein phylogeny. Class I and II KNOX genes from B. distachyon, Z. mays, O. sativa, A. thaliana, and H. neglectus. KNOB7 (orange) falls in the Class II clade as an ortholog of AtKNAT7. Phylogeny is based on amino acid sequence, bootstrap values listed on branches.
DNA binding data from a yeast one-hybrid assay further supported KNOB7 as a candidate cell wall regulator. From a panel of cell wall gene regulatory regions, KNOB7 protein was found to interact with elements of the COMT6 and SWAM1 promoters. COMT6, as described in Chapter 1, is a biosynthetic enzyme involved in monolignol biosynthesis. SWAM1, also described in Chapter 1, is an R2R3 MYB transcription factor that activates secondary cell wall biosynthesis in B. distachyon interfascicular fiber cells (Handakumbura et al., 2018). Interestingly, when the KNOB7 promoter was screened against our transcription factor library, SWAM1 protein interacted with KNOB7 regulatory sequences. This suggests that SWAM1 and KNOB7 may regulate each other through protein-DNA interactions.
Figure 3.3 KNOB7 protein interacts with cell wall gene regulatory regions. Yeast one-hybrid assay of protein-DNA interaction using a luciferase reporter. KNOB7 coding sequence fused with the Gal4 activation domain was transformed into yeast lines containing promoter:reporter constructs of cell wall promoter fragments driving luciferase expression. A) Schematic of three yeast one-hybrid assay. Reporter lines were tested for self activity prior to screening, with two non-self active lines of each construct chosen for assay. Activation of the reporter was measured in triplicate, with quantitative determination luciferase activity. B) Schematic showing KNOB7 positive interactions with promoter regions from COMT6 and SWAM1. SWAM1 protein was also found to interact with the KNOB7 promoter.
3.3.2 *KNOB7* genetic reagents

To investigate the role of *KNOB7* in plant growth and secondary cell wall development, transgenic lines with enhanced or perturbed *KNOB7* function were generated (Figure 3.4). Overexpression lines employed the maize ubiquitin promoter to drive expression of the *KNOB7* coding sequence fused with engineered green fluorescent protein (*GFP*), hereafter referred to as *KNOB7:GFP-OE*. Three independent events were selected for analysis. Two mutant lines were isolated, *knob7-1* and *knob7-3*. In *knob7-1*, CRISPR-Cas9 was used to create a mutation in the DNA binding homeobox domain. This editing resulted in a single base pair insertion, causing a frameshift in the homeobox domain. *knob7-3* was identified from a sodium azide (NaN) mutagenized population (Dalmais et al., 2013). Whole genome sequencing of mutants identified families with multiple mutations. PCR amplification of the mutation site in multiple individuals of those families followed by sequencing identified plants homozygous for the *knob7-3* allele, which results in a change from a glycine to a serine.
Figure 3.4 Diagram of KNOB7 genetic reagents. A) Schematic of the KNOB7 protein showing domain annotation and position of mutations. Numbers represent amino acid position. knob7-1 contains a frameshift in the DNA binding homeobox domain (HB) caused by targeted mutagenesis with CRISPR/Cas9. knob7-3 is a sodium azide (NaN) induced point mutation causing a change from a wildtype glycine to a serine near the ELK domain. B) KNOB7 overexpression transgene. The maize ubiquitin promoter was used to drive expression of the KNOB7 coding sequence fused in frame with eGFP. LB, left border; ZmUbi prom, maize ubiquitin promoter; Hyg, hygromycin phosphotransferase gene; NOS, nopaline synthase terminator; RB, right border.
In both knob7-1 and knob7-3 mutant lines, expression of KNOB7 transcript was not affected by the mutations. In KNOB7:GFP OE lines, the KNOB7 transcript was overexpressed compared to the wildtype (Fig 3.5.)

Figure 3.5 Expression of KNOB7 in mutant and overexpression lines. Relative level of KNOB7 gene expression measured by RT-qPCR in KNOB7 mutant (A) and KNOB- OE (B) lines. Whole stem tissue was collected 1 day after inflorescence emergence.
3.3.3 KNOB7 localizes to the nucleus

KNOB7 localization was first examined by transient expression in *Physcomitrella patens* (Figure 3.6A). Transformation with *KNOB7:GFP-OE* resulted in a localized GFP signal that resembles a nuclear localized GFP control (*Ubi::NLS::GFP*). Constitutive GFP lacking a nuclear localization signal (*Ubi::GFP*) was present throughout the cell, and overlapped with the constitutive mCherry control (*Ubi::mCherry*). KNOB7 localization was further confirmed in planta when analyzing transgenic plants regenerated from tissue culture. *KNOB7:GFP-OE* plants showed nuclear localized GFP signal in leaf tissue, compared with wildtype non-transgenic controls (Figure 3.6B).

Figure 3.6 KNOB7 localizes to the nucleus. A) Transient expression of *KNOB7:GFP* in *P. patens* shows nuclear localization. *Ubi:mCherry* and *Ubi::GFP* are used as constitutively localized controls. Nuclear localized GFP (*Ubi::NLS::GFP*) showed the same localization pattern as KNOB7:GFP. B) *KNOB7:GFP-OE* transgenics show nuclear localization in leaf epidermal cells. GFP nuclear signal is seen in *KNOB7:GFP-OE* leaf and not in wildtype. Red signal is chlorophyll autofluorescence.
3.3.4 KNOB7 is a negative regulator of interfascicular fiber wall thickening and lignification

Cell wall deposition and stem biology was measured in the KNOB7 reagent panel.

Transverse sections of the senesced second stem internode and stained with phloroglucinol-HCl and Toluidine blue (Figure 3.7). Phloroglucinol-HCl acts as a general stain for lignin, causing a reddish-pink pigmentation when reacting with lignified cinnamyl aldehydes. Toluidine blue is a polychromatic stain that interacts with both lignin and polysaccharides. Lignin stains a blueish-green hue, while polysaccharide components such as cellulose and hemicelluloses stain a darker blue-purple. Compared to wildtype, knob7-1 and knob7-3 appeared somewhat similar when stained with phloroglucinol, but both showed greater blue-green coloration when dyed with toluidine blue, indicating a higher lignin content in the interfascicular fiber walls. KNOB7:GFP-OE plants showed almost no red coloration when stained with phloroglucinol, and toluidine blue staining resulted in a dark blue/purple coloration. Both of these observations indicate a relative lack of lignin in the interfascicular fiber walls. Lignin content was further measured by the acetyl bromide soluble lignin method (ABSL).

Results quantitatively reflected what was observed by histology; that there was greater lignin content in knob7-1 and knob7-3 stems, with significantly less lignin in KNOB7:GFP OE (Figure 3.8).
Figure 3.7 KNOB7 negatively regulates lignification in interfascicular fibers. (A-D) Second internode sections of senesced stems stained with phloroglucinol-HCL, with red coloration as a proxy for lignin. (E-H) Second internode sections of senesced stems stained with Toluidine blue, with blue-purple indicating polysaccharides and teal-green indicating lignin. Compared to wildtype Bd21-3 (A,E), knob7-1 (B,F) and knob7-3 (C,G) mutants have more lignin staining, particularly evident in the toluidine blue stained sections. KNOB7:GFP OE plants (D,F) have drastically less lignin, evident in both phloroglucinol and toluidine blue staining. Scale bar = 100um.
Figure 3.8 KNOB7 is a negative regulator of lignin. Acetyl bromide soluble lignin measured in extractive free cell wall material prepared from the main stem after senescence. ns: p>0.05, *: p<=0.05. n=8-12 plants per genotype.

Interfascicular fiber wall thickness was also quantified in the KNOB7 reagent panel. 

knob7-1 plants had thicker walls compared to wildtype, while knob7-3 and KNOB7:GFP-OE walls were not significantly different (Figure 3.9).
Figure 3.9 KNOB7 is a negative regulator of interfascicular fiber wall thickness. Cell wall thickness was quantified for interfascicular fiber cells in transverse sections of the second elongated internode. ns: $p>0.05$, *: $p<=0.05$. n=5-8 plants per genotype.

3.3.5 KNOB7 alters lignin composition and levels of wall bound hydroxycinnamic acids

Other aspects of KNOB7 reagent secondary cell wall content was assessed. For knob7-1 and knob7-3, measurements were made of hydroxycinnamic acid content by mild alkaline hydrolysis followed by HPLC-MS. Ferulic acid (FA) and $p$-coumaric acid ($p$CA) content was measured, and both mutants showed significantly higher levels of FA compared to their respective wildtype controls. knob7-3 also showed significantly higher $p$CA levels, while knob7-1 showed a non-significant increase (Figure 3.10).
Figure 3.10 Wall-bound hydroxycinnamic acids are increased in knob7-1 and knob7-3 mutant lines. Mild alkaline hydrolysis on extractive free cell wall material prepared from the main stem of knob7-1 plants one day after flowering (green tissue) or knob7-3 plants after senescence (dry) released ferulic acid (FA) p-coumaric acid (pCA) from secondary cell wall polymers and quantified by HPLC-MS. Mean comparison by pairwise t-test after ANOVA testing. ns: p>0.05, *: p<=0.05. n=4-7 plants per genotype.

Lignin composition was measured in wildtype and knob7-3 plants by thioacidolysis and GC-MS. This method allows quantification of the three main monolignol subunits, S, G, and H. Compared to wildtype, knob7-3 plants had more S lignin and less G lignin (Figure 3.11A). This was further reflected in the entire KNOB7 genetic reagent panel stained with Maule reagent. This stain identifies S lignin units with a cherry red coloration (Figure 3.11B). Compared to wildtype, knob7-1 and knob7-3 stems show brighter red color in the interfascicular fibers, while KNOB7:GFP OE still stains red, but a duller hue than the mutants or wildtype.
Figure 3.11 KNOB7 mutant alleles have increased S lignin content. Thioacidolysis of extractive free cell wall material prepared from the main stem of knob7-3 plants after senescence released lignin monomer components (A-C), which were quantified by GC-MS. D) Transverse sections of the second elongated internode of the main stem after senescence were stained with Maule reagent to identify S lignin. Cherry-red coloration represents greater quantities of S lignin. ns: p>0.05, *: p<=0.05. n=3 plants per genotype.

3.3.6 KNOB7 alters cell wall polysaccharide content

In knob7-1 plants, neutral sugar analysis was conducted to look at the secondary cell wall polysaccharide content. Compared to wildtype, knob7-1 plants had significantly more xylose (Figure 3.12). knob7-1 also showed a significant decrease in arabinose and rhamnose levels, with no change in glucose, non-cellulosic glucose, or galactose.
Figure 3.12 Neutral sugar analysis of knob7-1. Hydrolysis of lyophilized, extractive free cell wall material prepared from the main stem of knob7-1 plants one day after flowering. Trifluoroacetic acid hydrolysis released cellulosic glucose, while sulfuric acid hydrolysis was employed to release arabinose, galactose, glucose, rhamnose, and xylose. Compounds were identified and quantified by HPLC. ns: p>0.05, *: p<=0.05. n=4-6 plants per genotype.
3.4 Discussion

*KNOB7* was identified as a candidate wall regulator based on homology with the well-characterized ortholog in *A. thaliana*, *AtKNAT7*. As with *AtKNAT7*, *KNOB7* is highly enriched for secondary cell walls. The *KNOB7* protein interacted with the promoter of genes that encode the lignin pathway enzyme *COMT6* in a yeast one-hybrid assay. *AtKNAT7* is a known regulator of secondary cell wall synthesis, including regulation of lignin synthesis (Zhong *et al.*, 2008a; Li *et al.*, 2012; Liu *et al.*, 2014; He *et al.*, 2018). This evidence prompted us to further characterize *KNOB7* function as a regulator of wall thickening.

*KNOB7* overexpression and two mutant alleles did not display any overt growth or stature phenotypes, but stem cross section histology revealed distinct changes in cell wall composition and morphology. Histo-chemical staining of *KNOB7-OE* plants showed a drastic decrease in interfascicular fiber lignin content, while *knob7-1* and *knob7-3* showed an increase. These observations were further substantiated by measuring lignin content in mature stems. *KNOB7-OE* showed significantly less lignin content, while *knob7-1* showed significantly more, supporting the histochemical staining. *knob7-3* did not show a significant difference, possibly due to large variance among the replicates. These data support the role of *KNOB7* as a negative regulator of lignin deposition in interfascicular fiber cells. Similarly, *AtKNAT7* and *PoptrKNAT7* mutants exhibited an increase in lignin accumulation (Li *et al.*, 2012; Wang *et al.*, 2020). A lignin phenotype was not observed in mutants of *KNOB7* the rice ortholog *OsKNOR1* (Wang *et al.*, 2019). Yeast data also substantiates *KNOB7*’s role in regulating lignin synthesis. *KNOB7* bound
the \textit{COMT6} promoter, a key enzyme in the final steps of lignin synthesis. Repression of \textit{COMT6} would be consistent with the observed changes in lignin quantity in the \textit{KNOB7} reagents, with less accumulation in the \textit{KNOB7-OE} and more in \textit{knob7-1}. \textit{COMT6} regulation is also consistent with the observed increase in S lignin in \textit{knob7-1} and \textit{knob7-3} by Maule staining and thioacidolysis. \textit{COMT6} mediates the conversion of coniferaldehyde to sinapaldehyde, and de-repression of this activity in \textit{knob7-1} and \textit{knob7-3} could result in more S lignin derived from sinapaldehyde.

Cell wall thickness is another hallmark trait impacted by \textit{AtKNAT7} and orthologs in other species (Zhong \textit{et al.}, 2008a; Li \textit{et al.}, 2012; Wang \textit{et al.}, 2020). Interestingly, \textit{AtKNAT7} appears to have an opposite effect on interfascicular fibers and xylem. Xylem cells have thin walls and some will collapse, while the interfascicular fibers are significantly thicker. We did not observe a collapsed xylem phenotype in either \textit{knob7-1} or \textit{knob7-3} mutant lines. However, while collapsed or irregular xylem is a common feature of wall mutants in \textit{A. thaliana} and a trait used to identify cell wall mutants, it has only been reported once in a grass to my knowledge, in the \textit{B. distachyon spaghetti 1 (spa1)} mutant. (Turner \& Somerville, 1997; Ehlting \textit{et al.}, 2005; Persson \textit{et al.}, 2005; Brown \textit{et al.}, 2005b; Timpano \textit{et al.}, 2015). The causative gene behind the \textit{spa1} phenotype has yet to be determined (Timpano \textit{et al.}, 2015). \textit{OsKNOR1} mutants do not have irregular xylem (Wang \textit{et al.}, 2019). There may be differences in grass vasculature such as a surrounding layer of mestome and bundle sheath cells or differences in xylem physical properties (Coomey \textit{et al.}, 2020). Fiber wall thickness is impacted in \textit{knob7-1} plants, with thicker walls than wild type. This is consistent with the observation in both \textit{AtKNAT7} and \textit{OsKNOR1} mutants (Zhong \textit{et al.}, 2008a; Li \textit{et al.}, 2012; Wang \textit{et al.}, 2020).
Overexpression of AtKNAT7 or OsKNOR1 resulted in thinner fiber cell walls. In my study, KNOB7-OE fiber walls are not significantly thinner.

Other aspects of cell wall chemistry were also assayed in KNOB7 mutants. The hydroxycinnamates pCA and FA are derivatives of the lignin biosynthetic pathway and in grass secondary cell walls they can be linked to either heteroxylans and lignins (Bartley et al., 2013; Petrik et al., 2014; Smith et al., 2017). Both knob7-1 and knob7-3 had a significant increase in cell wall bound FA, and knob7-3 also had a significant increase in pCA. These measurements were not made in OsKNOR1 or AtKNAT7. There was also a shift in lignin chemistry observed in KNOB7 reagents. The proportions of each main monolignol, S, G, and H, was measured in knob7-3, to reveal an increase in S lignin with a commensurate decrease in G lignin. Maule staining detects S lignin, and both knob7-1 and knob7-3 showed increased Maule staining intensity while KNOB7:GFP-OE showed less. This is the opposite phenotype that was described in AtKNAT7 mutants, where an increase in G lignin was observed (Wang et al., 2020). Together with the increase in FA and pCA, the shift towards S lignin in KNOB7 mutants suggests a somewhat different mechanism for lignin regulation in grasses. Several aspects of grass lignin biosynthesis are distinct from eudicots and the difference in KNOB7 mutant phenotypes may reflect such differences (Lan et al., 2015; Barros et al., 2016, 2019; Coomey et al., 2020).

Polysaccharide content was also measured by trifluoroacetic acid and sulfuric acid hydrolysis to release neutral sugars. knob7-1 showed a significant increase in xylose content, and a significant decrease in arabinose and rhamnose. As with S and G lignin content, these measures of xylose and arabinose show the opposite trend reported for AtKNAT7 mutants. In A. thaliana, AtKNAT7 activates xylan biosynthesis, and mutants
had less xylose and more arabinose (He et al., 2018). Again, this may be a result of the fundamental difference in grass secondary cell walls compared to eudicots such as a xylose backbone decorated with side chains of xylose, arabinose, and glucuronic acid in grasses and xyloglucan in eudicots (Coomey et al., 2020). The decrease in arabinose and increase in pCA is noteworthy, as the addition of pCA to heteroxylans is through arabinose linkage, which may suggest that the increased pCA in knob7-3 may be associated with lignin (Petrik et al., 2014).

Reciprocal binding between KNOB7 and SWAM1 in yeast one-hybrid assays raises the interesting question of negative feedback regulation. SWAM1 is a characterized activator of interfascicular fiber secondary cell walls (Handakumbura et al., 2018), and my data suggests that KNOB7 is a repressor in this tissue. In SWAM1-OE plants KNOB7 is upregulated, and similarly down regulated in the SWAM1 dominant repressor lines (Hazen lab unpublished data), further suggesting KNOB7 is a direct target of SWAM1 regulation. I would expect to find an increase in SWAM1 expression in KNOB7 mutants, and a decrease in KNOB7-OE, although these measurements have not yet been made. It would be interesting to investigate the SWAM1-KNOB7 relationship over time to better understand how these two transcription factors with similar expression patterns, reciprocal binding, but opposite transcriptional polarities may act in a feedback loop to fine tune fiber wall synthesis.
CHAPTER 4

CONCLUSIONS

The focus of my research was to better understand the factors that regulate growth in grasses. While we have uncovered many aspects of growth dynamics in eudicot systems, grasses represent an understudied region of the plant kingdom that is of great ecological, agricultural, and economic importance. I chose to study the genetic regulation of secondary cell wall synthesis. Grasses have distinct secondary cell wall properties from eudicots, and are critical to proper growth and development, and as such drew my interest to better understand this aspect of growth. To this end, I chose two candidate cell wall regulators, SWIZ and KNOB7, to characterize for their roles in the regulation of secondary cell wall synthesis.

SWIZ is a Group I bZIP that is highly expressed in root and stem tissue. Gain of function and loss of function lines both had reduced height and thick fiber cell walls. Like other characterid Group I bZIPS, SWIZ translocates within the cell in response to mechanical stimuli and cellular bioactive GA levels. This mode of action prompted me to look at the connection between SWIZ function and touch responsive growth. B. distachyon shows classic thigmomorphogenic traits such as reduced height and increased branching in response to mechanical stimulus. SWIZ appears to play a role in regulating cell wall thickening in response to touch, specifically the touch induced inactivation of cellular GA. Under touched conditions, SWIZ-OE lines had thicker cell walls in the touched portion of their stems. The direct genetic targets of SWIZ regulation that are responsible for these phenotypic outputs have yet to be identified, but components of lignin and
cellulose biosynthesis identified in yeast as binding targets are likely candidates. I found SWIZ to bind a motif similar to close orthologs in A. thaliana, and in vitro protein-DNA binding has identified a number of regulatory candidates with suggestive roles in other aspects thigmomorphogenesis.

*KNOB7* is the ortholog of *AtKNAT7*, a known regulator of cell wall thickening and lignification in A. thaliana. The role of similar genes in cell wall synthesis have been shown in other species such as rice and poplar, and *KNOB7* phenotypes are largely consistent with these reports. I generated gain of function lines overexpressing *KNOB7* and loss of function lines with a frameshift in the DNA binding domain and a non-synonymous point mutation. I showed distinct and reciprocal changes in interfascicular fiber cell wall thickness and lignification in these lines, as well as changes in lignin and polysaccharide chemistry that are all consistent with the role of *KNOB7* as a negative regulator of secondary cell wall synthesis in interfascicular fiber cells.

In summary, I characterized SWIZ, a novel component of thigmomorphogenic signalling that impacts stem elongation and fiber wall thickening, as well as *KNOB7*, an ortholog of an established negative regulator of cell wall synthesis and lignification. These findings contribute to our growing understanding of secondary cell wall synthesis and growth dynamics in grasses.
Table A1. GO terms from BdSWIZ DAP-seq, all peaks. BP: biological process, CC: cellular compartment, MF: molecular function

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### Table A3. SWIZ protein-DNA interactions from yeast one hybrid assay

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