MUTANT ANALYSIS OF A POLYOL MONOSACCHARIDE TRANSPORTER IN *ARABIDOPSIS* INVOLVED IN LIGNIFICATION

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ABSTRACT

MUTANT ANALYSIS OF A POLYOL MONOSACCHARIDE TRANSPORTER IN ARABIDOPSIS INVOLVED IN LIGNIFICATION

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Monolignols have important roles in plant development and primarily serve as monomers for lignin polymerization in secondary cell walls. Monolignols are synthesized in the plant cytoplasm prior to entering the apoplast where oxidation occurs. Upon oxidation, monolignols are incorporated into the cell wall. Several mechanisms have been suggested to explain how monolignols cross the plasma membrane, including endocytosis, diffusion, and active transport. However, evidence for those models either do not fully explain translocation for the abundant forms of monolignols, coniferyl alcohol and sinapyl alcohol, relied on theoretical calculations, or used *in vitro* approaches. Only one active transporter protein has been characterized to date. Yet, the transporter was only demonstrably shown to transport *p*-coumaryl alcohol, the least abundant monolignol present in *Arabidopsis*.

Here we show that AtPMT4, a member of the polyol/monosaccharide transporter (PMT) family, which is a subfamily of the major facilitator superfamily (MFS), is a monolignol transporter candidate for coniferyl alcohol and sinapyl alcohol. Gene expression analysis performed on PMT4 in dicots and monocots shows co-expression with lignin biosynthetic genes. Cell-specific expression analysis of the inflorescence stem, a tissue that undergoes intense lignification, shows that AtPMT4 is expressed higher in the lignified cell types, fibers and xylem vessels. We demonstrate that *Arabidopsis* Col-0 plants transformed with a CRISPR-Cas9 construct targeted near the start codon of AtPMT4 displayed lignin phenotypes. We quantified the total lignin, lignin

monomer composition, and digestibility of the inflorescence stem in *pmt4*. Our studies show lowered amounts of lignin, decreased guaiacyl and syringyl units, and increased digestibility. Further, we show that *pmt4* displayed shorter primary root length, a sign of increased sensitivity, when grown in the presence of coniferyl alcohol at millimolar levels. In conclusion, we provide evidence for an understanding of monolignol translocation and lignification by which secondary active transporters are likely involved in a proton-coupled manner.

If the path before you is clear, you're probably on someone else's.

This work is dedicated to two people who took a chance on themselves
to come to America and start all over. To Kich and Qui, thank you for showing me what it means
to be courageous, for it is the force that drives me to find my place
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CHAPTER ONE:

Roles of monolignols in growth, development, and defense

1.1 Abstract

The colonization of land plants that began approximately 540 mya required the development of a vascular system (Delwiche et al., 2015). The vascular system enabled plants to grow upright and compete for sunlight which yielded a competitive advantage to those that could grow rapidly and avoid the canopy of neighbors (de Vries et al., 2018). Accomplishing vertical growth was not trivial. Water needed to be transported to organs not in direct contact with the soil. The specialized tissues of the vascular system feature secondary cell walls (SCWs) that serve two general purposes: 1) to enable efficient transport of water from roots to the rest of the plant body and 2) to provide structural support.

The SCW includes lignin, a polymer that lends rigidity to plants to support secondary growth (Meents et al., 2018; Zhong et al., 2005), along with cellulose, hemicelluloses, and low amounts of structural proteins and enzymes (Kumar et al., 2015). The complexity of the SCW requires coordinated expression of many genes that are mediated by the NAC and MYB transcription factors (Polko et al., 2019). Lignin is present in vascular plants, including seedless vascular plants, gymnosperms, and angiosperms, but not in bryophytes (Zhong et al., 2018). However, lignin is present in rhodophyta, one of the oldest groups of eukaryotic algae. Angiosperm and gymnosperm cell walls contains ~100 times the amount of lignin compared to the red alga, suggesting that land plants require vast amounts of lignin for their anatomical features (Martone et al., 2009). Close homologs of most SCW biosynthetic genes are featured in the genome of *Physcomitrella patens*, a nonvascular plant species that has no lignified SCWs, (Haghighat et al., 2016; Norris et al., 2017). The conservation of lignin biosynthetic genes across

species suggest that vascular plants may have acquired them by gene duplication and functional diversification (Xu et al., 2009; Kulkarni et al., 2012).

1.2 Types of transport tissue in plants: xylem and sclerenchyma

SCW deposition occurs in the xylem and sclerenchyma tissues following increased plant height and width from cell expansion (Ruzicka et al., 2015). In the xylem tissues of plant stem, lignified tracheary elements (TEs) withstand the negative pressure that is generated by tension forces from water during transpiration and photosynthesis (Zimmerman et al., 1994; Shackel, 1996). TEs are comprised of two specialized cell types: tracheids and vessels. Tracheids consist of thin cell walls that form conductive cellular structures through laterally connected hollow cells (Oda and Hasezawa, 2006). Vessels, in contrast, consist of highly thickened cell walls that are perforated and connected end to end (Turner et al., 2007).

Lignification of TEs start at the cell corner of the middle lamella then gradually progress through the SCW layers and will continue after cell death (Terashima and Fukushima, 1993). Less lignification occurs during cellulose and hemicellulose biosynthesis (Somerville, 2006; Scheller and Ulvskov, 2010) while the bulk of lignin deposition takes place after wall synthesis is complete (Pesquet et al., 2013; Smith et al., 2013). The dual supplies of lignin monomers and reactive oxygen species (ROS) are provided by the surrounding precursor cells and xylem parenchyma (Ros Barcelo´ et al., 2005). The post-mortem and non-cell-autonomous nature of lignification likely enable optimal water flow by only replacing dead parenchyma cells that contributed monolignols via cell lysis to the lignifying vascular system (Me´nard and Pesquet, 2015).

Sclerenchyma tissue is simpler than xylem, functioning exclusively for mechanical support (Xie et al., 2021). Sclerenchyma tissue contains sclereids that are highly thickened with lignified

cellular walls that form small bundles in durable layers of most plant tissues (Lev-Yadun, 1997). Both sclerenchyma and xylem tissues contain fiber cells that contribute to the production of wood and strengthen the central axis of plant organs (Phyo et al., 2017). Fibers commonly have thickened SCWs and are classified as either xylary or extraxylary depending on their location (Esau, 1977; Mauseth, 1988). The development of fibers is affected by hormones, where high concentrations of auxin lead to short fibers with thick SCWs (Ranocha et al., 2013). In contrast, high levels of gibberellic acid (GA) lead to long fibers with thin walls (Aleman et al., 2008). In *Arabidopsis*, fibers are in the basal stem section and a lack of interfascicular fibers correlate with a dramatic decrease in stem strength (Zhong et al., 1997).

Compromised lignin formation typically result in a dysfunctional vascular system as observed in *Arabidopsis* mutations associated with lignin reduction and a collapsed xylem where SCWs are unable to resist the forces caused by transpiration (Turner et al., 1997). The overall function of the vascular system that help land plants compete in nature rely on the distribution of lignin across fibers, sclereids, and TEs (Raven, 1977; Weng and Chapple, 2010). In natural habitats, lignin confers lodging resistance, which prevent plant stems from bending or breaking from wind, and therefore important for crop growth and grain yield. Stalk lodging, which is the breakage of plant stems prior to harvest, results in yield loss to many crops under high-density planting (Tanaka et al., 2003, Islam et al., 2007, Zhang et al., 2011). Research into augmenting lignin formation to make plants feasible as a source of raw materials for industrial application is the focus of my dissertation.

1.3 Application of lignin research: bioenergy and agriculture

Advanced biofuels are produced and derived from the conversion of lignocellulosic feedstocks to monosaccharides and their subsequent fermentation to ethanol (Carroll and Somerville, 2009; Youngs and Somerville, 2012; Ponnusamy et al., 2019). Over 50 million tons of lignin are produced annually as a feedstock for industrial processes to create renewable chemicals (Yang et al., 2019; Run-Cang Sun, 2020). Continual improvements to the industrial process are needed to bypass the recalcitrant SCW caused by the strong hydrogen-bonding network between lignin and cellulose crystallinity (Takahashi et al., 2009; Somerville et al., 2010). Pretreatment processes exist to weaken the biomass for enhanced digestibility; however, high enzyme loading is currently required to release glucose from cellulose and its associations with hemicelluloses and lignin (Zhu et al., 2010; Sun et al., 2016).

Comprehensive methods combine physical, chemical, and biological forms of pretreatments to improved enzymatic yields of fermentable sugars by reducing the interactions between lignin and polysaccharides in feedstocks (Balan et al., 2009; Masran et al., 2016; Moniruzzaman and Goto, 2019). Pre-treatment and hydrolysis processes can release fermentation inhibitors (small acids, phenolics, and furans) that reduce bioconversion efficiency at a substantial economic cost (Baral and Shah, 2014; Kumar et al., 2020). Chemical hydrolysis methods using γ-valerolactone (GVL), a small molecule solvent sourced renewably from biomass conversion, offer an enzyme-free route to catalytic delignification that produce a value stream of aromatic products followed by the dissolution of cellulose microfibrils into fermentable sugars (Luterbacher et al., 2014; Tabasso et al., 2016). However, GVL is toxic to fermentative microbes due to adverse chemical effects on membranes and membrane-bound processes (Bottoms et al.,

2018). Engineering a GVL-tolerant, xylose-fermenting yeast will be an important goal to overcoming toxicity, minimizing the costs of reagent recovery, and improving biofuel production (Bottoms et al., 2018).

Depolymerizing heterogeneous lignin is a challenge for downstream processing of manufacturing pure chemicals. However, approaches using low-temperature trifluoroacetic acid have resulted in complete catalytically delignified materials (Yang et al., 2019). These materials were then converted to hydroxymethylfurfural (HMF), a carbon-neutral feedstock for fuels and other chemicals, and levulinic acid (LA), a precursor for pharmaceuticals, plasticizers, and various other additives (Xu et al., 2020; Seretis et al., 2020). Understanding lignin composition, quantity, and distribution combined with pretreatment strategies will help advance agro-industrial applications that currently remain challenging due to the resource intensive separation of lignin from feedstocks (Akin et al., 1986, 1991; Simmons et al., 2010).

1.4 Lignin polymerization and spatial patterning

The assembly of polymers is typically controlled by template polymerization. Cellulose biosynthesis, for example, is conducted by cellulose synthase complexes (CSCs) during which separate homogeneous glucan chains are synthesized and then immediately hydrogen bond with one another to form rigid crystalline arrays, or microfibrils. For lignin, however, polymerization in plants occurs under both chemical and physical controls (Sederoff et al., 1999, Wagner et al., 2011; Sangha et al., 2014; Tobimatsu and Schuetz, 2019). Proteins do not directly influence the chemistry of lignin polymerization beyond the formation of phenoxy radicals. Laccases and peroxidases oxidize the 4-O position of *p*-coumaryl alcohol, sinapyl alcohol, coniferyl alcohol to produce resonance forms of *p*-hydroxyphenyl (H), syringyl (S), and guaiacyl

(G) lignin that undergo radical coupling (Ralph et al., 2004) with the free-phenolic ends of growing lignin polymers. The reaction produces a highly diversified lignin structure with numerous possible linkages that hemicelluloses and proteins participate in (Tobimatsu and Scheutz, 2019). Higher degrees of lignin heterogeneity can be achieved when radicals are produced non-enzymatically by weak substrate specificity of laccases and peroxidases that oxidize monolignol-related compounds (Patel et al., 2019).

Laccase is essential for lignin formation in the major xylem tissues as disruption of *Arabidopsis* laccase genes nearly abolish lignin and causes severe growth arrest (Meents et al., 2018). Laccases are copper-containing glycoproteins that use oxygen while peroxidases use hydrogen peroxide (Herrero et al., 2013; Fernández-Pérez et al, 2015; Barros et al., 2019). Specific laccases and peroxidase are likely secreted to distinct localities of the cell wall to facilitate the spatial and temporal control of lignin deposition (Schuetz et al., 2014). Laccase is associated with root vascular element lignification, and peroxidase is active in the Casparian strip and middle lamella (Steudle, 2000; White and Broadley, 2001; Geldner, 2013). A specific laccase synthesizes G lignin, which is present early in *Arabidopsis* interfascicular fiber lignification (Terashima and Fukushima, 1993), while a peroxidase knockout mutant showed decreased S lignin in the same cells. Laccase-mediated monolignol polymerization is suggested to be essential during early lignification, while peroxidases are required at late stages. Overall, laccases appear more dominant in lignification based on the assessment of mutants studies although genetic redundancy for peroxidases has precluded identification of strong lignin phenotypes.

1.5 Lignin mutant analyses

The effort to map the phenylpropanoid pathway utilized numerous screening techniques on mutagenized populations (Boerjan et al., 2003). Among the first lignin mutants was *sin1*, which was identified by screening for plants unable to accumulate sinapoyl malate (Chapple et al., 1992). Wild-type plants exposed to long wave UV illumimation normally fluoresce a blue-green appearance (Chapple et al., 1992), but mutants displayed a red appearance which resulted from a lack of UV attenuation in the absence of sinapoyl malate. Other approaches included light microscopy screening for altered xylem cell morphology by examining hand-cut stem sections stained with toluidine blue and simpler mutant screens were based on the relative effort required to break a stem by hand (Bharti and Khurana, 1997).

1.6 Lignin composition, distribution, and diversity

Monolignol composition and lignin content vary among plant species, tissues, and stages of development (Bonawitz and Chapple, 2010; Voxeur et al., 2015). Generally, wood of angiosperms and gymnosperms are comprised of 19–33% lignin (Timell, 1967). The monomer ratios are predictive of cell type and function. In the stem, lignin accounts for about 20% of the dry weight and the monomeric subunits H, S, and G are 8%, 5%, and 87%, respectively (Bhargava et al., 2010). Other subunits include caffeyl alcohol and 5-hydroxyconiferyl alcohol, that incorporate into lignin as catechyl units and 5-hydroxyguaiacyl units, respectively (Ralph et al., 2001; Wagner et al., 2011; Chen et al., 2012). The cell wall of gymnosperms is predominantly composed of G-lignin with a small proportion of H-lignin (Yao et al., 2021). Conifers, which produce softwoods, lack syringyl units in their lignins, rendering their lignocellulosic materials difficult to process compared to syringyl-rich hardwood species. The lignin composition in the

xylem cell wall of conifers changes throughout development (Terashima and Fukushima 1988, 1989). The lignin deposited during early differentiation in the middle lamella and the cell corners is enriched in H lignin. G lignin is deposited in the middle lamella and the secondary wall. Small amounts of S lignin may be deposited at later stages. In angiosperm lignin, the composition of fiber cells in the interfascicular bundles has a higher ratio of G-units while vessel elements have a higher ratio of S-units. The lignin in the TEs of vascular bundles is composed primarily of G units. In contrast, the adjacent highly lignified fiber cells that differentiates later during stem development contain mostly S units (Chapple et al., 1992).

Variation in lignin composition found in different plant species affects the degree of crosslinking of the polymer and results in changes in flexibility and hydrophobicity. A reduction in lignin biosynthesis is also observed in *P. trichocarpa* and *M. truncatula* from mutation or down-regulation of *CSE* (Vanholme et al., 2013; Ha et al., 2016; Saleme et al., 2017). *A. thaliana cse* mutants are enriched in H units and deposit less lignin overall than wild-type plants. CSE is a central enzyme in the lignin biosynthetic pathway that works in conjunction with 4CL to bypass the second HCT reaction, and lead to S and G lignin. The role of CSE in lignification, however, may not be universal in all plant species since CSE orthologs were not found in the genomes of *B. distachyon* and corn, and little CSE activity was detected (Ha et al., 2016). The lignin biosynthetic pathway has been worked on extensively since the first committed enzyme of the lignin branch biosynthetic pathway, CCR1, was reported in 1997 (Lacombe et al., 1997), but several recent findings have led to reorganization of the metabolic pathway such as *CSE* published 20 years later (Ha et al., 2016).

1.6.1 Lignin monomer and biosynthesis

Monolignol biosynthesis occurs within the cytosol, or nearby endoplasmic reticulum (ER) (Smith et al., 1994). The cytoplasm was shown as the site of synthesis using fluorescent and radiolabeled techniques to track monolignol localization (Tobimatsu et al., 2013). Enzymes responsible for lignin biosynthesis are in the cytosol. For example, cytochrome P450s (CPRs), which catalyze oxidative steps, are bound to the ER while the catalytic site is accessible to substrates in the cytosol. Tagged monolignols diffuse readily through both PCWs and SCWs (Tobimatsu et al., 2013; Schuetz et al., 2014; Pandey et al., 2016) while intracellular pools of monolignols or intermediates of the pathway were not detected (Chen et al., 2003; Morreel et al., 2004; Laitinen et al., 2017) suggesting rapid incoporation into the lignin polymer. Together, undetectable pools and rapid incorporation of monolignols suggest an efficient export mechanism to the apoplast that passive diffusion alone may not be sufficient to perform.

1.6.2 Monolignol post-modification

The synthesis of conjugated monolignols that lead to glycosylated or other acylated intermediates affects the properties of lignin (Smith et al., 2015; Karlen et al., 2016). Acylation may provide a mechanism to control lignin structure since instances of extensive acylation led to extremely low abundances of β-β linkages (Smith et al., 2015). Monolignols can be acylated by acetate (Ac), *p*-coumarate (*p*CA), ferulate (FA), and *p*-hydroxybenzoate (*p*-BA) in the cytosol and acylated intermediates may be incorporated into the lignin polymer (Karlen et al., 2018). The acylation of monolignols by pCA and FA has been shown to be mediated by cytosol-localized *p*-coumaroyl-CoA monolignol transferases (PMTs) and feruloyl-CoA monolignol transferases (FMTs), respectively (Sibout et al., 2016; Wilkerson et al., 2014).

Monolignol conjugates in grass tissues include ferulate and diferulate cross-links and *p*-coumarate cyclodimers that can account for up to 40% of lignin monomer precursors (Petrik et al., 2014; Eugene et al., 2020). Feruloylated monolignols such as coniferyl alcohol can dimerize (Bunzel et al., 2003) and undergo radical coupling with free monolignols to crosslink and form lignin–hydroxycinnamate–carbohydrate complexes (Grabber et al., 2002; Ralph et al., 1995; Ralph and Helm, 1993) that decrease the digestibility of grass cell walls (Grabber et al., 1998). Cross-coupling products between ferulate, acylating polysaccharides, and a free monolignol support the idea (Ralph et al., 1995) that ferulates on polysaccharides may function as nucleation sites for the lignification process in grasses (Grabber and Lu, 2007). Similarly, tricin, a flavonoid derived from the shikimate and acetate/malonate-derived polyketide pathways, is a monomer in monocot lignification covalently linked with lignin at their starting ends (Lan et al., 2015). It is not clear if tricin is involved in initiation since only chemical analysis has been provided to infer function (Ralph et al., 2020). It would be reasonable to expect that tricin should be in lignifying plants if it is vital to lignification (Lan et al., 2016).

1.6.3 Exotic conjugates and implications for lignin structure and function

Exotic monolignols have been documented across plant species which suggests that lignin is more diverse than previously thought (Karlen et al., 2016). Exotic conjugates were first demonstrated to incorporate into lignin of cell walls using an *in vitro* model system that incorporated cleavable ester bonds into the lignin backbone (Ralph, 2006).

In grasses, monolignol *p*-coumarate conjugates do not polymerize into growing lignin chains due to the reactivity of *p*-coumarate with coniferyl and sinapyl alcohols (Withers et al., 2012). This conjugation results in free-phenolic pendent entities due to an affinity for radical

transfer over radical coupling (Hatfield et al., 2008). Ferulates, however, contain an additional methoxyl group compatible with normal lignification reactions and can incorporate into the polymer. The ester bond from ferulates is a key feature that allows the modified lignin to be depolymerized under mild conditions (Grabber et al., 2008).

Grabber et al. demonstrated the viability of substituting coniferyl alcohol with chemically synthesized coniferyl ferulate in the cell wall of maize. The substitution led to increased susceptibility of the cell wall to alkaline pretreatment. Increased ferulate conjugation *in planta*, however, required the identification of a gene with transferase activity for feruloyl-CoA. *Feruloyl-coenzyme A (CoA) Monolignol Transferase* (*FMT*) was identified from *A. sinensis*, a plant known to produce large amounts of coniferyl ferulate (Xie et al., 2009). The universal 35S promoter and the poplar CesA8 xylem-specific promoter were used to express *FMT* in transgenic poplar and produced coniferyl ferulate and sinapyl ferulate in lignin (Wilkerson et al., 2014). The resulting transgenic plants were susceptible to alkaline pretreatment due to the presence of ester linkages that lead to increase chemical depolymerization. Yet, the CesA8::FMT poplar trees incorporated only an estimated ~7 to 23% of the ferulate conjugates into their lignins. Efforts to increase the levels of FMT expression did not significantly increase incorporation of coniferyl ferulate into poplar. Transporter proteins may be competing against coniferyl ferulate synthesis for coniferyl alcohol (Takeuchi et al., 2018; Väisänen et al., 2020). Increasing the incorporation of coniferyl ferulates by eliminating competition from transporter proteins could lead to lignin engineered with desirable digestibility.

1.7 Monolignol transport: intracellular diffusion, extracellular diffusion, vesicle-mediated, and active transport

Monolignols leave the cell by mechanisms not entirely understood. Many of the proposed mechanisms have been reviewed (Weng et al., 2010; Liu et al., 2011; Liu, 2012; Wang et al., 2013; Barros et al., 2015). If monolignol diffusion was the primary mechanism then diffusion rates would have to satisfy the rate of monolignol consumption during lignification. Diffusion alone is unlikely sufficient for lignification so other mechanisms such as active transport are likely involved.

Low levels of monolignol diffusion across plasma membrane vesicles have been reported (Miao and Liu, 2010). However, limited diffusion could be due to *in vitro* conditions of isolated plasma membrane vesicles that did not include active lignin polymerization. In the absence of lignin polymerization the model could not account for the removal of monomers from the system that is commensurate with laccases and peroxidases *in vivo*. Therefore, there is no unidirectional force driving monolignols. The focus of the dissertation is to provide an update to the current monolignol transport framework and mechanisms for trafficking canonical and exotic monolignols out of the cell exist, including a framework for selective and promiscuous regulatory mechanisms.

Exotic monomers present in various plant lignin lends to the idea that a promiscuous mechanism enables the participation of canonical and exotic monomers in lignification (Karlen et al., 2016). Different cells likely synthesize the three monolignols in varied proportions, and maintain a general mechanism for transporting monolignols indiscriminately. For example, the cell walls of vessels in birchwood are mainly G lignin, whereas the fiber wall are both G and S

lignin (Fergus et al., 1970). In the *Arabidopsis* stem, the lignin of the vascular bundle in vessels is primarily G lignin, whereas the interfascicular fibers are S lignin (Chapple et al., 1994). The differences could be attributed by the level of *Ferulate 5-Hydroxylase* (F5H) that is differentially expressed across tissue, cells, and species (Chapple et al., 1992). We think that a member of the polyol/monosaccharide transporter (PMT) family export canonical and exotic monolignols out of the cytoplasm (Reinders et al., 2005; Klepek et al., 2010; Kong et al., 2020). The focus of our review is to discuss the PMT family and, more specifically, our data on PMT4 and its role in *Arabidopsis* lignification.

Computational models support that monolignols cross the cell membrane by simple diffusion at a rate sufficient for biological activities and cellular metabolism (Vermaas et al., 2019). The model tested the permeability coefficients of monolignols and lignin-related compounds (LRCs) against different membrane models that included *Z. mays*, (1-palmitoyl-2-oleoyl-glycero-3-phosphocholine [POPC]), and *P. putida*. A strong predictor of spontaneous permeation rates was the number of hydroxy or other oxidation sites, which confer increased water-solubility. Glycosylated monolignols, for example, that have more than 10 to 12 oxygens and other larger LRCs such as *p*-coumarate conjugates were unlikely to diffuse through membranes at appreciable rates (Vermaas et al., 2019). These compounds likely require an active transporter in contrast to monolignols (Escamilla-Trevino et al., 2006, Dima et al., 2015).

Monolignol glucosides such as coniferin have been suggested to be the direct precursors of lignin based on measurements of ATP-dependent transport activity with membrane vesicles from differentiating xylem tissues (Tsuyama et al., 2013; Roy et al., 2016). The experiments suggest a common endomembrane-associated proton/coniferin antiport mechanism in the

lignifying tissues of woody plants (Tsuyama et al., 2013). Monolignols are commonly glycosylated immediately to facilitate storage in the vacuole or transported to the apoplast as the glycoconjugate form followed by hydrolytic removal of the carbohydrate (Sulis and Wang, 2020; Speeckaert et al., 2020). Coniferin displayed ATP-dependent transport activity, while coniferyl alcohol showed less than 4% of activity (Miao and Liu, 2010).

Loss-of-function analysis in *Arabidopsis* of beta-glucosidases (BGLU45 and BGLU46) tested with monolignol glucosides inhibited coniferin transport but not coniferyl alcohol transport nor led to a lignin-deficient phenotype (Chapelle et al., 2012). UGTs were up regulated in a laccase triple mutant which suggest demand for monolignol glucosides to the apoplast to account for less monolignols due to decrease oxidation (Lin et al., 2016; Baldacci-Cresp et al., 2020). It is unclear whether monolignols cross the cell membrane in their free, glycosylated form, or both. Monolignols likely passively diffuse across membranes which when lignin synthesis rates are low may be sufficient to avoid observation of a phenotype. Phenotypes could be more pronounced when lignin synthesis rates are high and therefore considerations for stage of lignification should be made for mutant examinations.

In a diffusion model, rates of diffusion depend on the intracellular and extracellular concentration gradients. The intracellular level depends on the rate of monolignol synthesis while the extracellular level depends on the removal of monolignols from the cell during lignin polymerization. Passive diffusion is a likely mechanism but active transport should not be ruled out since transporters for an array of metabolites has been documented in plants (Shoji et al., 2009; Morita et al., 2009; Maron et al., 2010). Should monolignols accumulate intracellularly to toxic levels then perhaps active transporters are involved in detoxification.

Cells that interface with monolignols for lignification are grouped into three communities: autonomous producers, donors to neighboring cells, and acceptors (Smith et al., 2017). Lignification was previously thought to occur only in living cells (Pickett-Heaps, 1968; Takabe et al., 1985; Terashima et al., 1986, 1993; Terashima and Fukushima, 1988; Roberts et al., 2004). Lignification has now been shown to occur before and after programmed cell death.

TEs undergo lignification cell-autonomously by synthesizing monolignols and accepting monolignols from a neighboring cell (Smith et al., 2013). Nonlignifying xylary parenchyma cells donate monolignols in a manner referred to as “good neighbors”. Extraxylary fibers that autonomously lignify were identified by silencing the expression of *Cinnamoyl CoA-Reductase1* (CCR1), an enzyme that reduces a cinnamoyl-CoA, typically either *p*-coumaryl-CoA or feruloyl-CoA, to its corresponding cinnamaldehyde which in this case are, *p*-coumaraldehyde and coniferaldehyde, respectively. A region-specific promoter was used to express microRNAs (miRNAs) to repress translation and accelerate the degradation of CCR1 in TEs and fibers (Smith et al., 2013). Plants transformed with the miRNA construct did not display lignin defects in the xylem tissues. However, supportive fibers outside of the xylem tissue were depleted in lignin. Xylary and extraxylary lignified cells likely employ different lignification mechanisms that have commercial potential. The biosynthesis pathway could be manipulated to produce plants with intact xylem but lower lignin levels so that plants can mature extensively to maximize biomass yet more digestible.

The good neighbor hypothesis defines how and when diverse cell populations contribute monolignols for lignifying *Arabidopsis* inflorescence stem (Smith et al., 2017). In the early stages of stem development, xylary parenchyma cells function as good neighbors to nearby lignifying

TEs. In later stages, xylary fibers act as good neighbors but functionally overlap with parenchyma cells. Fibers, xylary and interfascicular, autonomously produce monolignols and will become the most abundant lignified cells of the stem (Smith et al., 2017). Suppressing monolignol biosynthesis in fibers can reduced recalcitrance and would not penalize TE lignification.

Vesicles act as a transport system in the cell and function in the synthesis of wall materials (Wilkop et al., 2019) but early studies suggest ER-Golgi-vesicle-mediated exocytosis do not play a major role in the transport of the monolignols (Pickett-Heaps, 1968; Wooding, 1968; Takabe et al., 1985). The low resolution autographic techniques did not allow precise localization of radioactivity within the Golgi vesicles and the native proteins in plant cell wall, which are difficult to separate from polysaccharide components, can produce a false positive. To better track intracellular monolignol distribution from the time of synthesis to wall corporation, labeled phenylalanine was used as a precursor and shown to incorporate into xylem vessels at a very early stage in the development of secondary wall (Wooding, 1968). A gradual increase in radiolabel was found in the developing xylem vessel walls, but no label was found in cambium or phloem cell walls. Phenylalanine did not produce a labelled Golgi body and no label was found localized in the cytoplasm of the developing xylem cells after up to 3-4 hours of incubation, suggesting that phenylalanine is consumed rapidly during lignification. However, past this period labelling was found randomly distributed in the cytoplasm of all cells, with no association with any organelle and most likely the result of incorporation into protein.

Techniques to improve tissue preparation in autoradiographic studies include cryofixation of labeled xylem cells of lodgepole pine (Kaneda et al., 2008). Dissected xylem tissue was fed with a (^{3}H)-Phe radiotracer as phenylpropanoid and protein biosynthesis were selectively inhibited.

The Golgi and Golgi-vesicle clusters abundant in the developing xylem cells were not loaded with phenylpropanoids which is not in favor of the role of ER-Golgi in monolignol transport. Further, radiolabel in the ER-Golgi was primarily incorporated into proteins, not monolignols since phenylpropanoid biosynthesis was inhibited. There are still serious drawbacks in the use of (^{3}H)-Phe since metabolic conversions of Phe to monolignols can release tritium to solution and produce false-positive signals.

1.8 Identification of a *p*-coumaryl alcohol transporter

Active monolignol transport is supported by the identification of AtABCG29/PDR1, a transporter protein from the full-size ABCG subfamily transporter family (Alejandro et al., 2012). Plant genomes contain large ABC transporter gene families but only few of the 130 family members that the *Arabidopsis* genome encodes for have been functionally characterized (Theodoulou and Kerr, 2015; Lefèvre and Boutry, 2018). Most members are ATP-driven pumps with two membrane-spanning pores and two nucleotide-binding domains involved in transporting a wide range of molecules (Gräfe and Schmitt, 2021).

AtABC29 was first identified as a potential monolignol transporter based on its co-expression pattern with two 4CL (i.e., 4CL2 and 4CL5) and CCoAOMT and then demonstrated to be involve in *p*-coumaryl alcohol transport (Alejandro et al., 2012). Transformed yeast expressing AtABCG29 exhibited increased tolerance to *p*-coumaryl alcohol likely by exporting the monolignol to the apoplast. This was further supported by isolated membrane vesicles exhibiting *p*-coumaryl alcohol transport activity. Loss-of-function *Arabidopsis* mutants contained less lignin. The data suggested that AtABC29 acts as an monolignol exporter, but specifically for *p*-coumaryl alcohol since the transporter showed no significant activity for coniferyl alcohol or sinapyl alcohol.

Coniferyl alcohol has a high permeability that may not require active transport since diffusive flux can provide roughly 10^9 and 10^{11} molecules per second (Vermaas et al., 2019). An active transporter may be required for *p*-coumaryl alcohol to overcome both the slow rate of synthesis and rate of diffusion.

1.9 Overview of ABC Transporters

Minimal ATP-dependent transporter activity for monolignols was detected in membrane vesicles from differentiating xylem of Poplar, cypress, and pine (Tsuyama et al., 2013). The plasma membrane (PM) H^+-ATPase is an electrogenic pump that exports cellular protons (Morth et al., 2011). In addition to generating a transmembrane chemical gradient of H^+ (acidic on the outside), an electrical gradient is also established (the membrane potential; negative on the inside). Majority of plant PM transport proteins are fueled by combined electrochemical gradient of protons, and therefore the PM H^+-ATPase is thought to be critical for plant growth (Palmgren, 2001, Arango et al., 2003, Sondergaard et al., 2004, Duby and Boutry, 2009). Physiological evidence that PM H^+-ATPase is a driver of growth was mainly based on studies using pump inhibitors, such as vanadate (Parets-Soler et al., 1990; Sondergaard et al., 2004). Studies using inhibitors should be reviewed with attention to define the resulting phenotypes since pump inhibitors can affect a wide variety of transporters.

Comparisons of vacuole and plasma membrane transport activity for monolignols support the premise that ATP was required (Miao et al., 2010). Support for active transport is based on observations that lignification occurs rapidly, and thus, require monolignols immediately upon synthesis to be at the lignin polymerization site (Dean et al., 1987).

Monolignols could accumulate to a high intracellular level prior to lignification in response to stress, which may necessitate a form of active transport to lower toxicity (Bhuiyan et al., 2009). Phenylpropanoids move rapidly through lignifying tissues and are concentrated in secondary cell walls (Kaneda et al., 2008). Glucosides accumulate to millimolar levels in immature xylem before cambial reactivation or when lignification begins (Albersheim et al., 2011). The observations suggest that monolignols and monolignol glucosides do not accumulate inside cells and are redirected quickly upon synthesis. There is no evidence of vacuolar or endomembrane accumulation of monolignols in developing xylem cells before programmed cell death, nor have they been detected in the cytoplasm or vacuoles of TEs or neighbors which indicate that synthesis and transport must be rapid (Smith et al., 2013; Tsuyama et al., 2013).

Evidence for active transport demonstrated the transport of lignin precursors across plasmalemma and their sequestration into vacuoles (Miao and Liu, 2010). The uptake of monolignols and their derivatives were examined using membrane vesicles. A typical Michaelis–Menten kinetic analysis supported a membrane-protein-mediated biochemical process for the uptake of monolignols, or their glucosides, from isolated *Arabidopsis* plasma membrane and vacuolar membrane vesicles tested against different inhibitors and conditions, including without ATP (Miao and Liu, 2010). Plasma and vacuolar membrane vesicles selectively transport different forms of lignin precursors. In the presence of ATP, the inverted plasma membrane vesicles preferentially take up monolignol aglycones, whereas the vacuolar vesicles were selective for glycoconjugates, monolignol 4-O-glucosides. Whether glycosides are the transported form and become deglycosylated prior to crossing the apoplast is unknown. The vacuolar selectivity suggests that different ATP-binding cassette-like transporters recognize different chemical forms.

Poplar and *Arabidopsis* do not normally accumulate *p*-coumaroylated monolignols and presumably do not have the corresponding transporters (Lu and Ralph, 1999; Lapierre et al., 2021; del Río et al., 2008). Yet, the overexpression of grass PMTs in poplar and *Arabidopsis* produced *p*-coumaroylated monolignols that were readily transported to the cell walls for lignin polymerization (Smith et al., 2015; Sibout et al., 2016). Less selectivity from plasma membranes might explain the plasticity of lignin biosynthesis and the ability for promiscuous transport of exotic lignin precursors into the cell wall (Karlen et al., 2016; Zhuo et al., 2019).

1.10 Specific aims

The ABC transporter family have been suggested almost exclusively in the role of monolignol transport. We argue that an agnostic search for monolignol transporters using recent publicly available high resolution RNA-sequencing data would be a more effective strategy. We found that secondary active transporters from the Major Facilitator Superfamily (MFS) should be considered as candidates for monolignol transporters (Remy et al., 2013; Quistgaard et al., 2016; Niño-González et al., 2019). MFS transporters do not directly require ATP and instead rely on electrochemical gradients for their function. Transporters for sugar and nitrate are encoded by ~60% of the reported MFS genes in *A. thaliana* (Eom et al., 2015). Phosphate is also a substrate of MFS, as well as abscisic acid (ABA), GA, and ascorbate (Kanno et al., 2012; Miyaji et al., 2015; Tal et al., 2016). Transporters belonging to the MFS have affinity for a broad range of substrates in several different biological roles that may also include lignification.

The TE induction system using cultured *Arabidopsis* cells helped our understanding of transporters involved in secondary growth (Kubo et al., 2005; Oda et al., 2005). The application of plant hormones to cultured cells can lead to synchronous secondary cell wall thickening that

promote lignification in a short period of time and cause lignin biosynthesis genes to be actively expressed (Church and Galston, 1988; Fukuda and Komamine, 1982). The limited extent of secondary growth of *Arabidopsis*, however, severely limits the spatial resolution at which SCW formation, vascular development, and lignification can be assayed. Instead, the use of woody tree plants, such as *Populus*, helps overcome the limitations of secondary growth in *Arabidopsis* (Farrokhi et al., 2006; Neutelings, 2011; Zhang et al., 2020).

Poplar transcriptomic analyses have enhanced our understanding of the molecular mechanisms underlying cambial growth and wood formation (Sundell et al., 2017; Chao et al., 2019; Kim et al., 2019). Recent studies improved upon earlier transcriptome studies of cambial growth and wood formation in *Populus* that were limited to wood-forming tissues sectioning based on visual anatomical assessment (Sundell et al., 2017). Advancements to the technique using unsupervised approaches (Mutwil et al., 2011; Netotea et al., 2014) by obtaining a continuous sequence of samples extending from differentiated phloem to mature xylem with high spatial resolution that were assigned developmental context using functionally characterized genes relied only on the expression data and not on anatomical annotations or genes with known roles. The experiment represented an unbiased description of the central biological processes underlying cambial growth and wood formation (Sundell et al., 2017).

We utilized several RNA-sequencing datasets to identify monolignol transporter candidates. The development of CRISPR gene editing tools in *Arabidopsis* coupled with high throughput RNA sequencing enabled us to use reverse genetics and establish the gene function of *Polyol Monosaccharide Transporter 4* (PMT4) (Xing et al., 2014; Khurshid et al., 2018; Bewg et al., 2018; Dort et al., 2020). We proposed that PMT4 is an *Arabidopsis* monolignol transporter

based on the co-expression profile of its homolog with lignin biosynthetic genes during poplar cambium development. The function is supported by reduction in lignin and other lignin phenotypes in our *Arabidopsis* mutant. We generated two mutant lines, one which is a heterozygous mutant based on intermediate phenotypes relative to the homozygous mutant. The mutants are described in further detail in Chapter 3.

AtPMT4 belongs to the PMT family which is closely related to the glucose transporter (GLT) family. GLT encodes a plastidic glucose transporter that export starch degradation products from the chloroplast (Cho et al., 2011). Other GLTs have been shown to transport sugar alcohols. It is not clear if monolignols are transported to the apoplast as the aglycone or glycosylated form. Protein sequence alignment of AtPMT4 show 34% similarity to AtGLT1, suggesting that AtPMT4 may have a role in glycosylation. Studies on the other members of the PMT family were informative to understanding AtPMT4 (Scholz-Starke et al., 2003; Büttner, 2007; Klepek et al., 2010; Slewinski, 2011; Geiger, 2020).

Polyol H^+-symporter accumulates linear polyols up to several hundred millimolar into the phloem (Zimmermann and Ziegler, 1975; Lohaus and Fischer, 2002; G. Lohaus, unpublished data). Analyses of reporter genes performed with AtPMT1 or AtPMT2 promoter sequences showed expression in mature (AtPMT2) or germinating (AtPMT1) pollen grains, as well as in growing pollen tubes, hydathodes, and young xylem cells (both genes) and confirmed by an antibody experiment (Klepek et al., 2010). The physiological roles of the proteins could be related to plant cell wall modifications. The data presented suggest that AtPMT1 and AtPMT2 might represent xylitol and fructose transporters in pollen and young xylem. The relative transport rates were similar for both transporters. Xylitol and fructose were among the most permeable and

represented the preferred substrates of AtPMT1 and AtPMT2. pAtPMT2/GFP plants showed intense fluorescence in mature pollen grains. The fluorescence was detected only during the late stages of pollen development and increased further with ongoing anther dehiscence. Anther, germinating pollen grains, and pollen tubes and young are rapidly expanding and typical of lignification.

AtPMT5 is expressed in almost all tissues and organs analyzed, except for pollen and seeds and indicated to transport a wide range of sugars and sugar alcohols (Klepek et al., 2005). AtPMT5 was shown in yeast cells to work by an energy-dependent uptake mechanism where sorbitol accumulated to intracellular concentrations that exceeded the initial outside concentrations. Inward currents demonstrate that a positive charge was symported with each of the tested substrates. AtPMT5 was found to transport sorbitol and glucose with similar rates and comparable K_m-values (Klepek et al., 2005; Reinders et al., 2005). Nevertheless, it seems quite unlikely that sorbitol or glucose represent physiological substrates of AtPMT5 since biologically relevant concentrations of sorbitol have never been reported in *Arabidopsis*, and secondly, glucose is likely to be transported by one of the members of the much more specific, plasma membrane-localized hexose transporters of the AtSTP subfamily. AtSTP are found in most *Arabidopsis* cells and tissues and have 50–100-fold lower K_m-values for glucose than AtPMT5 (Büttner, 2010). Therefore, PMT may be a family of transporter for a range of substrates into sink cells and assist in scavenging sugars from senescing or dying tissue during lignification. ATPMT5 might function in the retrieval of multiple substrates from the apoplast since many of the identified substrates are major components of the cellular metabolism and may leak out of the cells (Klepek et al., 2010). While AtPMT5 accepts a broad spectrum of substrates, it does

discriminate between sorbitol and mannitol which suggest a degree of substrate specificity. In contrast, AtPMT4 is expressed higher in the inflorescence stem and pollen similar to AtPMT1 and AtPMT2. We hypothesize that AtPMT4 functions similarly to AtPMT1, AtPMT2, and AtPMT5 as a plasma membrane broad-spectrum H^+-symporter for a wide range of linear polyols but differ in increased selectivity for monolignols, which is more concentrated in the cytoplasm for both monolignols and protons compared to the apoplastic space where monolignols are consumed for lignification (Kalliampakou et al., 2011; Ramsperger-Gleixner et al., 2004). Our data suggest that AtPMT4 may be involved in lignification as a monolignol transporter based on lignin phenotypes and cell-type specific gene expression profile.

PMT4 is a secondary active plasma membrane transporter powered by a proton motif force created by a proton pump (Cosse and Seidel, 2021). The dominant proton pumps in the plant cell are the plasma membrane ATPase, vacuolar pyrophosphatase (V-PPase), and vacuolar-type ATPase (V-ATPase) that typically work in symport or antiport with a proton and (Morsomme and Boutry, 2000). The pumps act on the cytosolic pH by pumping protons into the lumen of compartments or into the apoplast. To maintain the typical pH and the functionality of the cytosol, the activity of the pumps needs to be coordinated and adjusted on several levels with respect to growth conditions and pathogen-defense, which likely initially involves ROS as a messenger and then as an oxidative defense strategy (Blokhina et al., 2003). Acidification caused by activation of the plasma membrane H^+-ATPase initiates cellular expansion (Morsomme and Boutry, 2000, Falhof et al., 2016). The mechanism known as the “acid growth theory” could be associated with auxin, a hormone assumed to activate the H^+-ATPase. Apoplastic acidification leads to the wall-loosening process (Fry et al., 1992) and promotes osmotic changes allowing

water influx by plasma membrane aquaporins, favoring cell elongation (Maurel, 1997). The strong association between acidification and cell wall development leads us to believe that the transport of monolignol for lignification also relies on secondary active transport and a proton pump.

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CHAPTER TWO:

Gene expression analysis of PMT4 for the involvement in lignification and association with lignified tissue and cells

2.1 Abstract

Phenylpropanoid biosynthesis leading to monolignol products is carried out within the cytoplasm, while the later steps of lignin synthesis involving oxidation and polymerization of the monolignols occur in the apoplastic space. In autonomously lignifying cells, monolignols likely cross the plasma membrane, possibly by an ATP hydrolysis-dependent transport. Searches for a monolignol transporter have been largely not successful. However, the availability of large RNA sequencing datasets obtained by high throughput techniques offer a new opportunity to identify transporter candidates. Analysis of monolignol biosynthetic gene expression patterns in tissues undergoing secondary cell wall development from Poplar, *Brachypodium*, and *Sorghum* displayed several co-expressed plasma membrane transporter candidates. Selection criteria were based on co-expression coefficient with known monolignol biosynthetic genes, number of predicted transmembrane helices, and cell type specific gene expression pattern. In Poplar, At2G20780, which is predicted to belong to the Polyol/Monosaccharide Transporter family was co-expressed with C4H, PAL, and F5H and has a predicted 12 transmembrane helices. Evaluation of the *Arabidopsis* ortholog showed no apparent genetic redundancy and co-expressed with monolignol biosynthetic genes. The homolog is expressed at relatively high levels in the inflorescence stem, root, and pollen, which are lignified tissues. Notably, the candidate is preferentially expressed in fibers and vessel elements of the inflorescence stem which are both highly lignified cell types.

2.2 Introduction

Transcriptome models for secondary growth were initially limited to studies in *Arabidopsis* (Ko and Han, 2004; Minic et al., 2009; Jamet et al., 2009; Hall and Ellis et al., 2013). The small size and narrow secondary growth restricted the spatial resolution necessary to assay SCW formation and vascular development (Ehlting et al., 2005). For example, the xylem fibers that were investigated do not reach full maturity to the extent of trees and instead remain alive for an extended period in laboratory conditions (Bollhöner et al., 2012). The adoption of cell culture systems sought to resolve the small size of the *Arabidopsis* cambial region by promoting artificial secondary growth (Kondo et al., 2015; Saito and Kondo, 2019). Inducible TEs in *Arabidopsis* can undergo extensive SCW thickening in a short period of time that allow for increased resolution of the genes that are active. RNA sequencing performed on *Arabidopsis* cell cultures suggested several transporters belonging to the ABC transporter family are involved in lignin deposition. Genetic evidence for monolignol transport, however, is limited. A *p*-coumaryl alcohol exporter has been characterized but the data did not support transport of the abundant lignin monomers, coniferyl and sinapyl alcohol (Alejandro et al., 2012). The identification of transporters for the abundant monolignols has implications for genetically engineering the composition of H, G, and S lignin deposition in different tissues and cell types.

The developmental transition from non-lignifying tissue to lignifying tissue in poplar cambium offers a high resolution dataset to identify monolignol transporter candidates that may have been missed when using an *Arabidopsis* transcriptome (Sundell et al., 2017). Advancements in sequencing technologies and RNA-seq methodologies provided secondary growth transcriptome data in *Populus* for the identification of all transcribed loci in a sample without

prior knowledge of gene models (Goodwin et al., 2016). Analysis of transcriptional modules and lignification in *Populus* offered an experimental system superior to the *Arabidopsis* cell culture system for studies of major wood cell types. The major wood cell types and their function are as followed: fibers on the xylem side provide structural support, vessel elements transport water and minerals, axial parenchyma cells provide storage, and ray cells perform radial transport and store photosynthates. Both vessel elements and fibers undergo programmed cell death, with death in vessel elements occurring earlier (Courtois-Moreau et al., 2009). Ray cells remain alive for several years (Nakaba et al., 2012).

Monolignol transporter candidates were identified from the poplar dataset using known monolignol biosynthetic genes (Sundell et al., 2017) with the co-expression tool from GxSeq (Genomic expression and Sequence analysis), a bioinformatics web tool made available by the Great Lakes Bioenergy Research Center (https://gxseq.glbrc.org). To maintain a broad search of transporter families, no distinctions were made regarding the class or family of membrane transporter candidates. Phenylpropanoid genes and monolignol pathway specific genes were used to search the poplar dataset for candidates. Candidates were checked for expression patterns during secondary cell wall development in other species such as *Arabidopsis* and *Brachypodium* to determine if the expression pattern is conserved, and presumably functional activity, to obtain a degree of confidence for performing mutant analysis.

2.3 Materials and Methods

2.3.1 Co-expression analysis of lignin genes during SCW development and identification of the gene candidate PMT4

RNA-seq data from *Populus* (Sundell et al., 2017), *Brachypodium* (Jensen and Wilkerson, 2017), and *Sorghum* (McKinley et al., 2016) experiments were analyzed for co-expression and

identification of gene candidates using GxSeq. The genes C4H, F5H, and CAD were used to identified candidates with a compelling r^2 value. The candidates were then aligned against the RNA-seq data to determine if known lignin genes were co-expressed. The TransMembrane prediction using Hidden Markov Models (TMHMM) feature of GxSeq was used to select for candidates with 8 to 12 predicted plasma transmembrane spanning domains since transporters in the ABC and MFS family are reported to have up to 12 transmembrane helices (Yadav et al., 2014; Quistgaard et al., 2016). We set the lower bound for probability of predicted transmembrane helices at 0.8 in case the tool failed to recognize certain nucleotide sequences as representative helices. Co-expressed candidates lacking the predicted transmembrane regions were not considered. Candidate were then selected based on an R^2 value > 0.81 since 0.8107 was the R^2 value of the last lignin gene (CAD) that appeared on the T1 hybrid poplar list when PMT4 was queried. We determined that a 0.81 R^2 value also defined a cutoff suitable to identify genes involved in broad SCW processes based on the following R^2 value of the cellulose synthase involved in secondary cell wall biosynthesis, AT5G17420 (CESA7), AT4G18780 (CESA8), and AT5G44030 (CESA4): 0.8821, 0.8194, and 0.7632, respectively. To find the *Arabidopsis* ortholog of the *P. trichocarpa* PMT4, we performed a nucleotide BLAST search with phytozome (https://phytozome-next.jgi.doe.gov) using BLASTN 2.6.0+. The DNA sequence of Potri.013G135200 (2781 bp) was used to query the TAIR10 genome and the results were as follow: Score = 670 bits (742), Expect = 0.0, Identities = 964/1356 (71%), and Gaps = 6/1356 (0%). We conclude from the BLAST results that AT2G20780 is an ortholog of Potri.013G135200.

2.3.2 GO term enrichment analysis of PMT4

To identify the biological role of PMT4 *in silico*, we analyzed the terms that are over- or underrepresented in the hybrid poplar RNA-seq data co-expression analysis. The *Arabidopsis* Information Resource (TAIR) "GO Term Enrichment for Plants" tool was used to compare the frequency of Gene Ontology (GO) terms from the list of genes co-expressed with PMT4 (sample set) with the frequency of the same set of GO terms in the *Arabidopsis* whole genome set (reference set) (https://www.Arabidopsis.org/tools/go_term_enrichment.jsp). The data was sent to the PANTHER (Protein ANalysis THrough Evolutionary Relationships) Classification System which contains updated GO annotation data for *Arabidopsis*. We selected the annotation data set 'GO biological process complete' and ran the PANTHER Overrepresentation Test (Released 20210224)using the following GO Ontology database: DOI: 10.5281/zenodo.5228828 Release 2021-08-18. The tool applies the Fisher's Exact test to identify over- or underrepresented terms in the sample gene set in comparison to the reference genome set. The default parameters apply a Bonferroni correction, a statistical method that performs multiple statistical tests (one for each pathway, or each ontology term) at the same time. The correction multiplies the single-test P-value by the number of independent tests to obtain an expected error rate. The expected value is the number of genes expected in the sample set list for a particular biological process, based on the reference list. A small p-value indicates that the result is non-random, potentially interesting, and warrants further investigation. In the binomial test we assume that under the null hypothesis, genes in the sample list are sampled from the same general population as genes from the reference set. The probability p(C) of observing a gene for a particular biological process in the uploaded list is the same as in the reference list. We first estimate the probability p(C) from

the reference set assuming that it is large and representative: p(C)=n(C)/N, where n(C) is the number of genes mapped to category C, and N is the total number of genes in the reference set. In our analysis we observed 296 out of 299 uniquely mapped IDS, 109 unmapped IDS, and 4 multiple mapping information.

2.3.3 Tissue and cell type expression analysis of AtPMT4

The Toronto Bio-Analytic Resource (BAR) Expression viewer 'ePlant' was used to generate the visualization for the gene expression intensity of AtPMT4. To generate a visualization for cell type expression within the inflorescence stem, the webtool developed by Shi et al. (https://Arabidopsis-stem.cos.uni-heidelberg.de) was used.

2.3.4 Protein BLAST analysis of PMT4

Protein sequence alignment of AtPMT4 to other members of the PMT family was performed using the TAIR BLAST tool (https://www.Arabidopsis.org/Blast/index.jsp).

2.3.5 Phylogenetic analysis of PMT4

Phylogenetic analysis was performed and organized into a tree using the phylogeny.fr platform (http://www.phylogeny.fr/one_task.cgi?task_type=muscle) (Dereeper et al., 2008). PMTs protein sequences were aligned using the program MUSCLE (v3.8.31), which performs multiple sequence alignment (Edgar, 2004) configured for highest accuracy (MUSCLE with default settings). After alignment, regions containing gaps and/or poorly aligned were removed with Gblocks (v0.91b). The phylogenetic tree was reconstructed using the maximum likelihood method implemented PhyML (v3.1/3.0 aLRT) for phylogeny (Guindon et al., 2005). "Full mode" was selected for MUSCLE run mode which include stages 1 to 3: draft progressive alignment, improved progressive alignment, and alignment refinement, respectively. The MUSCLE program

also used a technique called k-mer extension to find diagonals which are short regions of high similarity between the two sequences to speed up the pair-wise alignment. The maximum number of iterations was 16. Reliability for internal branch was assessed using the aLRT test (SH-Like). Graphical representation and edition of the phylogenetic tree was performed with TreeDyn (v198.3) (Chevenet et al., 2006).

2.3.6 Membrane prediction tool

The membrane prediction tool (https://services.healthtech.dtu.dk/service.php?TMHMM-2.0) was used to predict transmembrane regions and produce a graphical representation of their locations.

2.4 Results

2.4.1 Co-expression analysis of PAL, C4H, CAD, and F5H during hybrid poplar secondary cell wall development revealed a plasma membrane transporter candidate

Several studies have reported searches for monolignol transporter candidates by investigating lignifying tissues often limited to *Arabidopsis*. However, the small size and limited extent of *Arabidopsis* secondary growth restricts the spatial resolution at which SCW formation and vascular development can be assayed. Additionally, the xylem fibers do not fully mature but stay alive even during prolonged growth (Bollhöner et al., 2012). Recently, *Arabidopsis* cell suspensions during TEs differentiation were used to identify monolignol transporter candidates (Takeuchi et al., 2018). However, this model is not representative of lignification in bioenergy productive crops such as poplar. The poplar developmental stage is longer and features more diverse cell types and tissues. During our investigation of potential monolignol transporters, we chose to screen candidates using a *Populus tremula* RNA sequencing dataset under the

assumption that the extensive cell wall development may help reveal transporter candidates not possible when using *Arabidopsis*. We found that CESA4, CESA 7, and CESA 8, which are subunits of the cellulose synthase required for secondary cell wall formation, showed gene expression level changes across the different RNA-seq samples of pooled longitudinal tangential cryosections collected from the trunk of 15 meter high, 45-year-old aspen trees during the middle of the growing season (Figure 2.1). The SCW CESAs are expressed higher in expanding xylem and lignified xylem compared to phloem and cambium tissues. The CESAs were highly expressed in samples 13 through 18 where monolignol biosynthetic genes were also highly expressed. Upon verification that the dataset showed differentially gene expression of the SCW CESAs and lignin biosynthetic genes, we proceeded to use the monolignol biosynthetic genes, PAL, C4H, F5H, and CAD to identify highly co-expressed transporter protein candidates. We identified a predicted membrane transporter that shared expression patterns with PAL, C4H, F5H, and CAD. The candidate, PMT4, was upregulated in the 11^{th} sample, which feature developing vessel elements using assigned developmental context based on functionally characterized genes and estimated tissue composition from anatomical inspection of cross section during sectioning (Figure 2.2). PMT4 is active in lignifying xylem (samples 12 through 19), and less active in both the phloem (samples 1 through 10) and late maturation of xylem cells (20 through 25). The expression levels of PMT4 and known monolignol biosynthetic genes are consistent with CES7 from stages 11 through 21, which feature expanding and lignified xylem. Co-expression analysis of PMT4 in the Populus RNA-seq database followed by GO analysis found that genes co-expressed with PMT4 are involved in lignification. To determine the likelihood that the *Arabidopsis* ortholog would also function in cell wall development similar to poplar, we perform GO analysis of *Arabidopsis*

orthologs from the poplar co-expression data and determined that the set of genes coexpressed with PMT4 were annotated as being involved in the monolignol biosynthetic process (Table 1). F5H was highly co-expressed with PMT4, providing further gene expression support that PMT4 is involved in monolignol biosynthesis and required further exploration.

We used *Arabidopsis* to study the phenotype of PMT4 since we had the appropriate growth chamber space and established techniques to perform CRISPR-Cas9 transformation. We first identified the *Arabidopsis* ortholog of the *P. trichocarpa* PMT4 using a BLAST search against the TAIR 10 genome of *Arabidopsis*. We explored the probability of genetic redundancy underlying the function of AtPMT4 using phylogenetic analysis (Figure 2.4) and found that it belongs to the six-member Polyol/Monosaccharide Transporter (PMT) family (Table 2). Since AtPMT4 sorted into a separate clade away from the other members of PMT there was a probability that AtPMT4 might function uniquely in *Arabidopsis* development and therefore produce a phenotype upon mutation.

We next analyzed the AtPMT4 protein sequence to determine the number of transmembrane domains using a web-based prediction tool running TMHMM 2.0 and determined that AtPMT4 has an estimated 10 transmembrane domain helices which is expected for members from the Major facilitator superfamily (Figure 2.3). The two folding domains predicted for PMT4 are likely encoded by separate exons and undergo homodimerization to form a pore. Together, the co-expression poplar data and metadata of the *Arabidopsis* ortholog suggested that AtPMT4 is a compelling monolignol transporter candidate for functional analysis *in planta*.

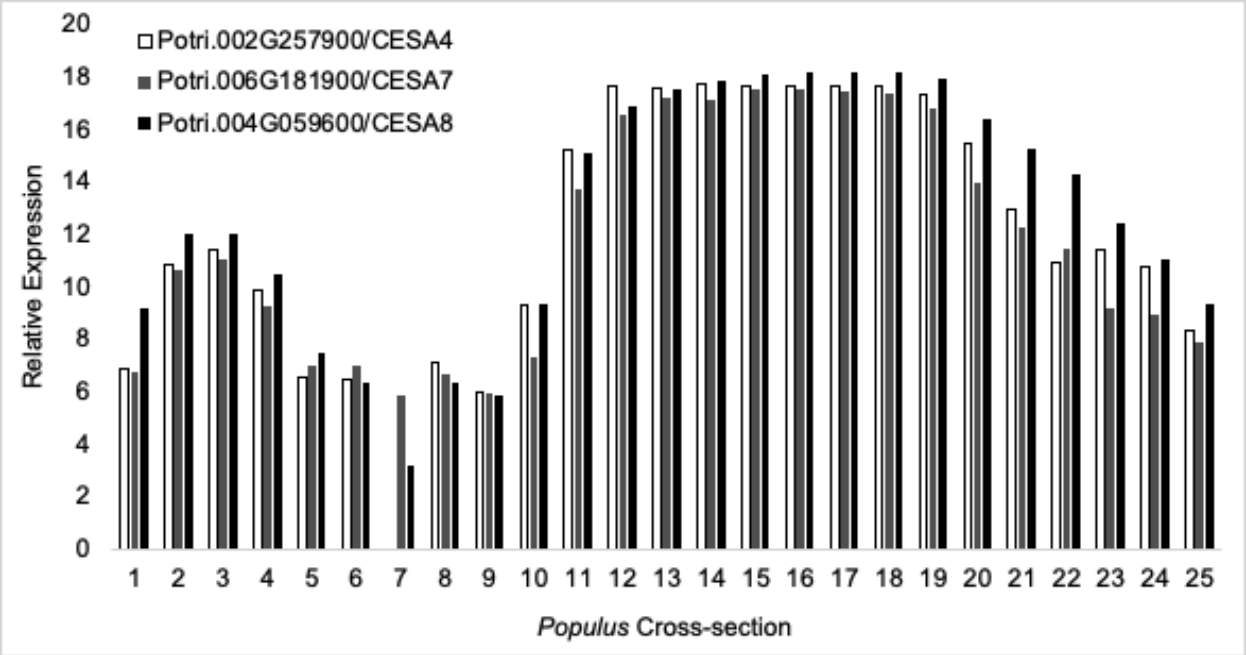

Figure 2.1. SCW CESAs are expressed throughout hybrid poplar cambium development. The data were produced by RNA obtained from pooled longitudinal tangential cryosections obtained from a single (T1), wild growing aspen genotype (*P*. *tremula*). The total number of transcribed mRNA molecules varied along the sample series so the expression values are not absolute transcript amounts but rather estimated relative to the number of reads obtained from each sample. Transverse cross-section from left to right are broadly classified as the following: phloem, cambium, expanding xylem, and lignified xylem from sampled tree T1. CESA 4, CESA7, and CESA8 were used as markers to assess the activity level of SCW genes in the poplar cross-section to determine viability of the RNA-sequencing dataset for investigating monolignol transporter candidates.

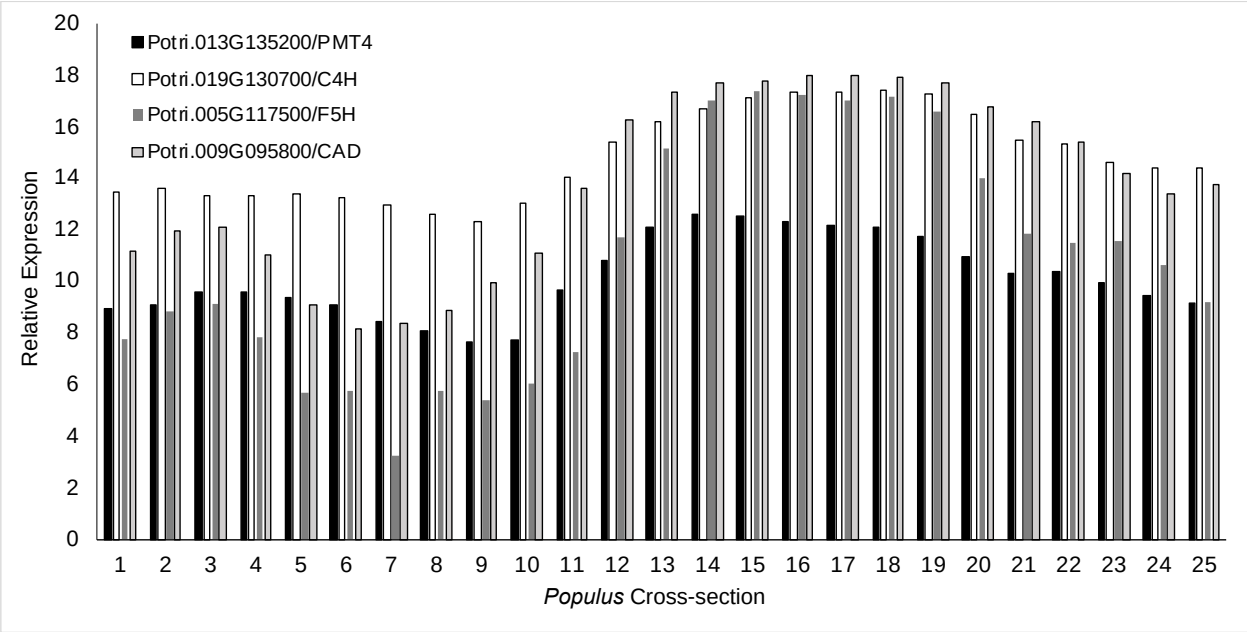

Figure 2.2. AtPMT4 is co-expressed with monolignol biosynthetic genes during hybrid poplar cambium development. The data were produced by RNA obtained from pooled longitudinal tangential cryosections obtained from a single (T1), wild growing aspen genotype (*P. tremula*). The total number of transcribed mRNA molecules varied along the sample series so the expression values are not absolute transcript amounts but rather estimated relative to the number of reads obtained from each sample. Transverse cross-section from left to right are broadly classified as the following: phloem, cambium, expanding xylem, and lignified xylem from sampled tree T1. The genes C4H (lignin specific), CAD (general monolignol biosynthesis), and F5H (sinapyl alcohol specific), were used to identify co-expressed transporters from the poplar transcriptome during cambial growth and wood formation. (All genes shown have an R^2 value >0.88.)

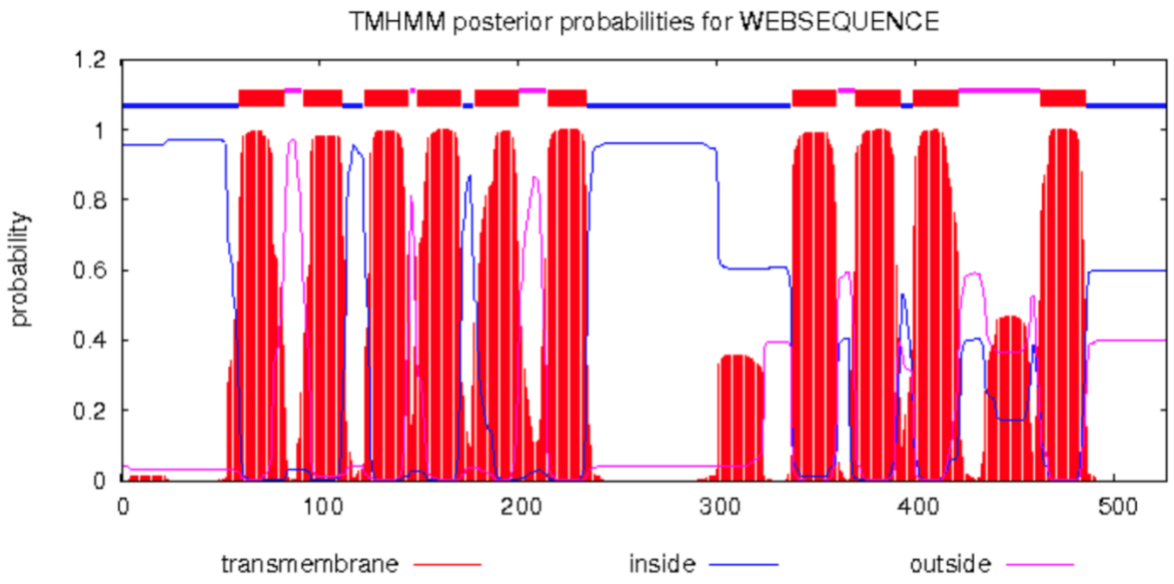

Figure 2.3. AtPMT4 has 10 predicted transmembrane helices.

The membrane prediction tool (https://services.healthtech.dtu.dk/service.php?TMHMM-2.0) predicted transmembrane regions and produced a graphical representation of their locations. The numbers on the y-axis indicate amino acid position while the x-axis is the percent probability (*100) that a transmembrane helices is predicted at that location. The program takes proteins in FASTA format and recognizes the 20 amino acids and B, Z, and X, which are all treated equally as unknown. Any other character is changed to X. For the default format, TMHMM gives some statistics and a list of the location of the predicted transmembrane helices and the predicted location of the intervening loop regions. If the whole sequence is labeled as inside or outside, the prediction is that it contains no membrane helices. The prediction gives the most probable location and orientation of transmembrane helices in the sequence. It is found by an algorithm called N-best (or 1-best in this case) that sums over all paths through the model with the same location and direction of the helices. A number larger than 18 is very likely to be a transmembrane

Figure 2.3 (cont'd)

protein or have a signal peptide. Predicted transmembrane segments in the n-terminal region sometime turn out to be signal peptides.

Table 1. GO Term Enrichment of the first 450 genes (R^2 cutoff value of 0.81) returned when AtPMT4 is queried in the *Populus* Dataset

Gene Ontology Biological Process	Fold Enrichment	P value
Xylan Biosynthetic Process	16.07	1.95E-03
Lignin Biosynthetic Process	15.31	2.61E-03
Plant Secondary Cell Wall Biogenesis	11.66	2.78E-03

	Arabidopsis (Reference List)	Hybrid poplar (Analyzed List)			
GO biological process complete	#	#	expected	Fold Enrichment	P value
lignin biosynthetic process	42	7	0.46	15.31	2.61E-03
→phenylpropanoid biosynthetic process	88	9	0.96	9.39	3.08E-03
⇢⇢phenylpropanoid metabolic process	110	10	1.2	8.35	2.11E-03
→lignin metabolic process	53	10	0.58	17.33	3.99E-06

A gene ontology term enrichment was conducted to interpret the relationship of the candidate and its co-expressed genes in *Arabidopsis*. The fold enrichment of the genes observed in the uploaded (hybrid poplar) list over the expected number (*Arabidopsis* genome). If it is greater than 1, the category is overrepresented in the experiment. If the category is underrepresented, it is less than 1. The first 450 of the 1000 genes returned from the hyrbid poplar co-expression analysis were used for the GO analysis. The "Lignin Metabolic Process" was the second GO biological process that was returned, suggesting that the candidate is involved in lignification. The Fisher's Exact test is applied to determine whether there is a statistical over- or under-representation of genes/proteins in the test list relative to the reference list. The expected value is the number of genes expected in the test list for a particular PANTHER category, based on the reference list.

Table 2. Protein BLAST analysis show that AtPMT4 is similar to other members of the PMT family in *Arabidopsis thaliana*

TAIR ID	Description	Scores (Bits)	E-Value
AT2G20780	PMT4	923	0
AT3G18830	PMT5	397	6.00E-133
AT2G16120	PMT1	369	3.00E-122
AT2G16130	PMT2	364	1.00E-120
AT2G18480	Major facilitator superfamily protein	360	5.00E-119
AT4G36670	PMT6	356	1.00E-117

The protein sequence of AtPMT4 was aligned against the *Arabidopsis* TAIR10 reference. Four members of the PMT family were shown to have protein sequence similarity with AtPMT4. The scores were organized into a phylogenetic tree in Figure 2.4.

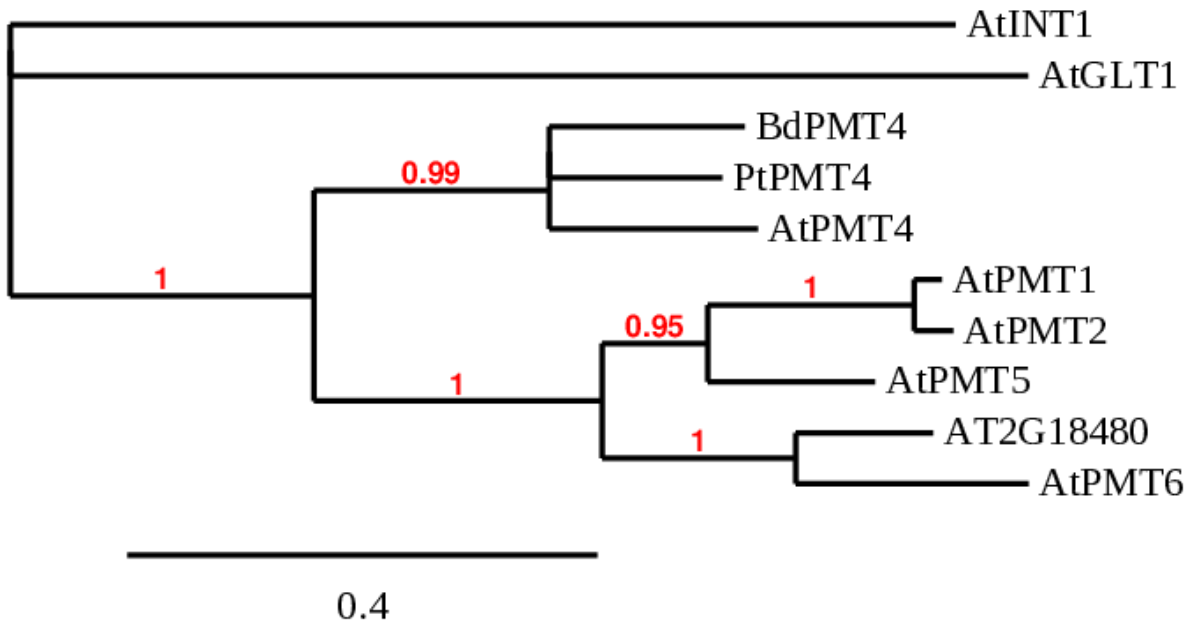

Figure 2.4. Phylogenetic analysis shows AtPMT4 belongs to the Polyol-Monosaccharide Transporter Family. The protein sequence of AtPMT4 was aligned against members of the PMT family and other similar *Arabidopsis* transporters. The numbers shown in red are the distance defined as the fraction of mismatches at aligned amino acid positions, with gaps either ignored or counted as mismatches. The tree also includes the PMT4 ortholog from *P. trichocarpa* and

Figure 2.4 (cont'd)

Brachypodium. Protein sequences were aligned using the program MUSCLE (v3.8.31), which performs multiple sequence alignment (Edgar, 2004) configured for highest accuracy (MUSCLE with default settings). After alignment, regions containing gaps and/or poorly aligned were removed with Gblocks (v0.91b). The phylogenetic tree was reconstructed using the maximum likelihood method implemented PhyML (v3.1/3.0 aLRT) for phylogeny (Guindon et al., 2005).

2.4.2 *Arabidopsis* ortholog is expressed in the inflorescence stem, root, and pollen - all lignified tissues, but higher in fibers and vessel elements of the inflorescence stem

We determine the tissue and cell specificity of AtPMT4 expression using publicly available data. The data for the Toronto BAR Expression Viewer come from Schmid et al., 2005, Nature Genetics 37:501 and Nakabayashi et al., 2005, The Plant Journal, Vol 41:697. Gene expression data generated by the Affymetrix ATH1 array are normalized by the GCOS method, TGT value of 100. Most tissues were sampled in triplicate. AtPMT4 was expressed highly in the pollen and inflorescence stem (Figure 2.4).

Next, we identified which cell types within the inflorescence stem showed higher expression of AtPMT4. We sought out to identify if distinct population of cell types that do or do not lignify are correlated with the expression of AtPMT4. We found that AtPMT4 is differentially expressed among specific cell types from the tissue-specific transcriptome profiling of the *Arabidopsis* inflorescence stem (Shi et al., 2021). AtPMT4 was highly expressed in the fibers and vessel elements (Figure 2.5). The results show association between the expression of AtPMT4 and lignifying tissues and cell types.

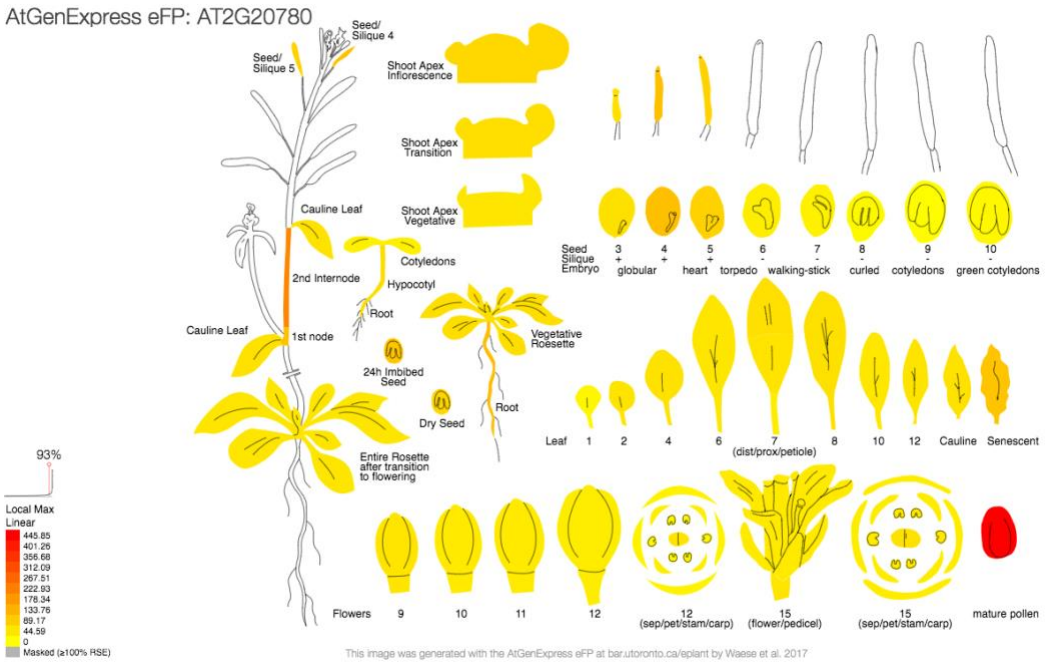

Figure 2.5. AtPMT4 is expressed in the inflorescence stem and pollen tissue. The data come from Schmid et al. (2005) and Nakabayashi et al. (2005). Gene expression data generated by the Affymetrix ATH1 array are normalized by the GCOS method, TGT value of 100. Most tissues were sampled in triplicate. Vectorized image redrawn and programmed by Waese et al. (2017).

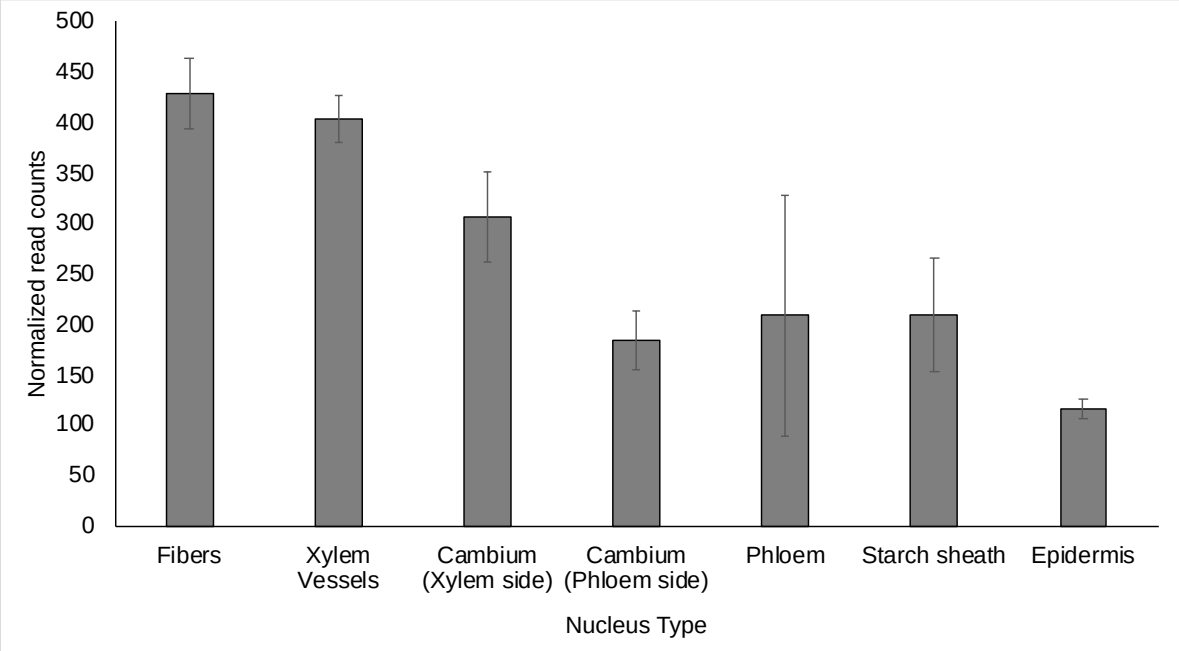

Figure 2.6. AtPMT4 is expressed higher in the fibers and xylem vessels of inflorescence stem. Combined fluorescence-activated nucleus sorting and laser-capture microdissection with next-generation RNA sequencing were used to characterize the transcriptomes of xylem vessels, fibers, the proximal and distal cambium, phloem, phloem cap, pith, starch sheath, and epidermis cells within the mature inflorescence stem. Seven lines expressing Histone 4-GFP under the control of different tissues-specific promoters and collected material from the second internode of the *Arabidopsis* inflorescence stem. GFP-positive nuclei were isolated by fluorescence-activated nucleus sorting (FANS). RNA was extracted from GFP positive nuclei, amplified using the Smart-seq2 method and then RNA-seq analysis was carried out. Comparison of read counts mapped to the GFP sequence to the total number of mappable reads to the *Arabidopsis* genome in GFP-positive and GFP-negative nuclei for each transgenic line. The error bars represent the standard deviation of the normalized read count values by DESeq2 for a minimum of 2 replicates per nucleus type. Clusters containing genes that were very

Figure 2.6 (cont'd)

active in fiber cells were mostly distinct from clusters containing genes whose activity was high in developing vessel elements. Similarly, most genes active in developing phloem cells were distinct from genes active in developing xylem cells. These results suggest that while these cell types partly originate from the same procambial precursors, they quickly establish very distinct profiles.

2.4.3 PMT4 is expressed during SCW development of *Brachypodium*

We next asked the question about whether PMT4 would display SCW-like expression patterns within a monocot plant system to understand if the function may be broadly conserved across plant species. Comparative analysis of PMT4 co-expression with poplar was performed on an RNA-sequencing data set representing *Brachypodium* internode development. For this purpose, we examined *Brachy* CESAs involved in primary and secondary cell wall development (Table 4). Our results show different expression patterns of CESAs across the (early) elongating and fully elongated internode (Figure 2.6). The analysis provided evidence that the RNA-sequencing experiment prepared by Jacob Jensen can be used as a proxy/analog for identifying a monolignol transporter candidate and further gave supportive evidence for PMT4's involved in cell wall function in *Arabidopsis*. Having established a difference between CESA PCW and SCW, we next sought to identify if PMT4 would be expressed similar to CESA SCW in *Brachy*. We found that PMT4 is expressed higher during the elongating stages, not fully elongated, similar to SCW CESAs (Figure 2.7). These results provided gene expression evidence that PMT4 is a good candidate for understanding plant cell wall development and may be a conserved monolignol transporter across dicots and monocots.

Table 3. Primary and Secondary Cell Wall CESAs in *Brachypodium*

	Gene Name	Locus
SCW	BdCESA4	Bradi3g28350
	BdCESA7	Bradi4g30540
	BdCESA8	Bradi2g49912
PCW	BdCESA1	Bradi2g34240
	BdCESA3	Bradi1g54250
	BdCESA6	Bradi1g53207
	BdCESA9	Bradi1g02510
PCW/SCW	BdCESA2	Bradi1g04597
	BdCESA5	Bradi1g29060

The gene name is commonly an abbreviation of the gene function or phenotype. A locus is the specific physical location of a gene or other DNA sequence on a chromosome, like a genetic street address. The plural of locus is "loci".

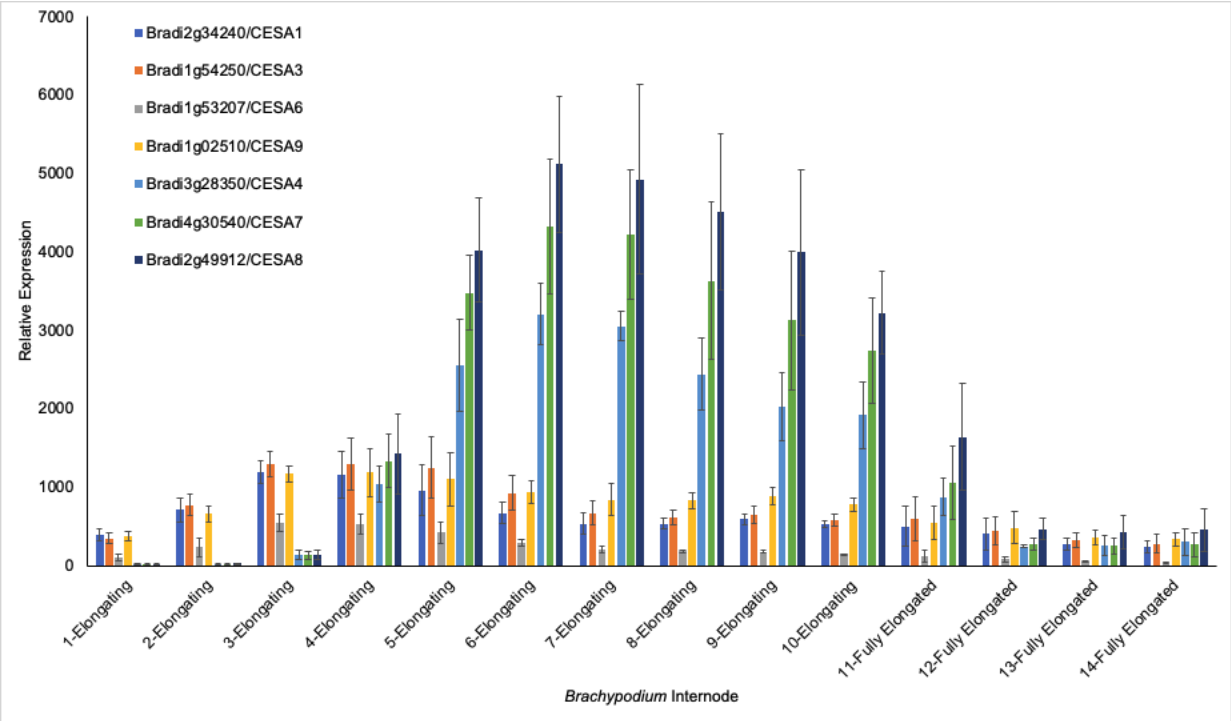

Figure 2.7. Comparison of primary cell wall CESAs and secondary cell wall CESAs expression during *Brachypodium* internode development. The samples consist of elongating internodes (plant age = 9 week) and fully elongated internode (plant age = 12 week). Tissues at the elongating stage were collected in 1mm tissue section at five-hour intervals from 0 to 35 hours. At the 45- and 55-hour timepoint, 2mm tissue sections were collected. For the fully elongated internode, whole tissue was collected at 1-, 3-, 6-, and 10-week intervals. The error bars represent the standard deviations of the three replicates sampled at each stage. The normalization (y-axis) of the expression values were estimated relative to the number of reads obtained from each sample.

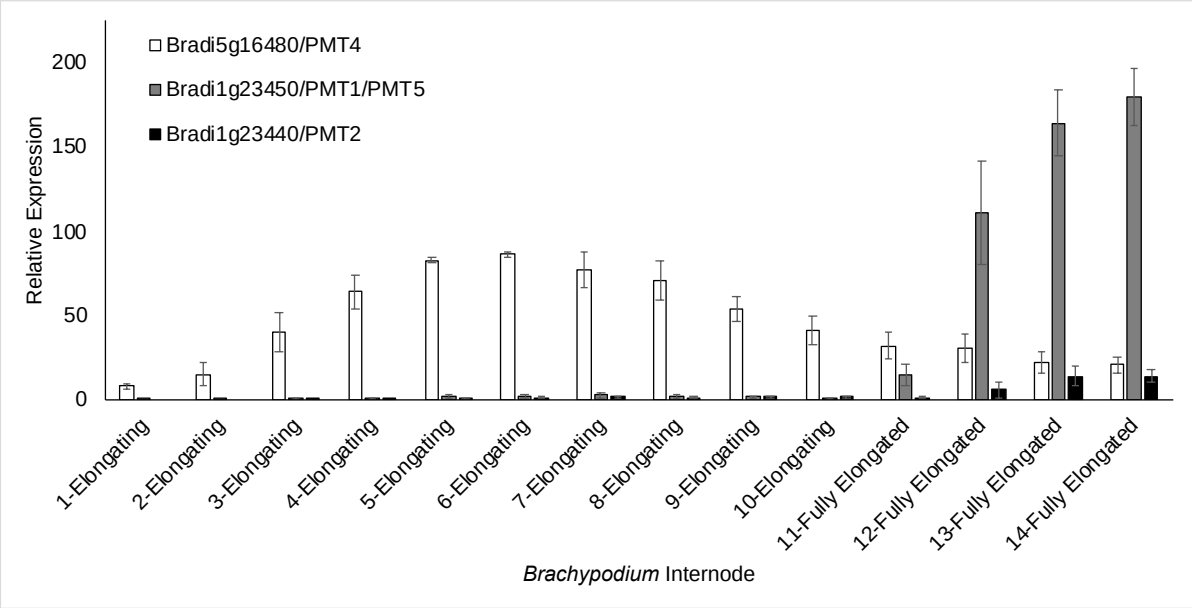

Figure 2.8. PMT4 is expressed throughout the elongating internode. Gene expression of the PMTs were analyzed in *Brachypodium* by RNA-seq on a developmental time series of specific stem internodes.

Table 4. PMTs in *Brachypodium*

Brachy ID	TAIR ID	Gene Name	Expression Level
Bradi5g16480	AT2G20780	PMT4	
Bradi1g23450	AT3G18830, AT2G16120	PMT5, PMT1	
Bradi1g23440	AT2G16130	PMT2	
Bradi1g56700	AT4G36670	PMT6	low expression
Bradi1g56700	AT2G18480	MFS	low expression

2.5 Discussion

Several types of monosaccharide transporter-like (MST) proteins have been characterized in plants; however, PMTs which form a distinct subfamily within the larger family of MST-like have emerged as important regulators of plant metabolites either as a component of growth, as exemplified by sugar transporters, or during subsequent steps of the cell wall formation pathway.

Over the last decade, gene expression information from lignifying tissues in plants have become available which has greatly enhanced our ability to find monolignol transporters.

A critical component of PMTs is the energy-dependent and voltage-dependent symport of their substrate with protons. Several members of the PMT family, including AtPMT5, 1, and 2 have been characterized and shown to be localizing to *Arabidopsis* plasma membranes (Klepek et al., 2005), which are thought to have physiological roles related plant cell wall modifications. While PMTs have been shown to have high-capacity transport of fructose and xylitol it seems unlikely that these or sorbitol and glucose represent physiological substrates of AtPMT5 since noteworthy concentrations of sorbitol have never been reported in *Arabidopsis* and glucose is more likely to be transported by one of the membranes of the much more specific hexose transporters. Together, it seems that the physiology of PMT-type proteins is much more complex in non-polyol translocating species such as *Arabidopsis* than in sorbitol and mannitol translocation plants.

Recent evidence indicates that plants have transporters as key components of monolignol translocation pathways for regulating lignification through the incorporation of different lignin subunits. The ABC transporters show degrees of specificity for plasma and vacuolar membranes and that glycosylation of coniferyl alcohol is necessary for vacuolar storage but not require for direct transport into cell wall in *Arabidopsis*. Important to the focus of this study, a reported ABC transporter did not show a growth response to coniferyl alcohol and is inconsistent to the work done by Miao and Liu. In this work, the selection of AtPMT4, a non-ABC transporter, was made by analyzing gene expression data from several public databases.

In the initial investigation of co-expressed transporters with lignin biosynthetic genes, the reference genes C4H, F5H, PAL, and CAD, which have been functionally characterized as key components of the monolignol biosynthetic pathway, were used for co-expression analysis. C4H converts cinnamic acid into *p*-coumaric acid, F5H converts coniferaldehyde to 5-hydroxy-coniferaldehyde, PAL converts phenylalanine to cinnamic acid, and CAD which is the final enzyme in the monolignol biosynthetic pathway. The analysis was performed on a poplar transcriptome prepared from developing xylem for which CESAs were shown to be differentially expressed (Figure 2.1), indicating that the dataset could be used to identify lignin biosynthetic genes. The queried genes displayed increased transcription in the 11th transverse cross-section which is the segment where the formation of vessel elements in the lignified xylem begins. Of the candidates that were identified, a member of the PMT subfamily, which have been shown to transport a broad range of sugar and alcohols, was co-expressed. The results revealed that AtPMT4, a member of the monosaccharide transporter-like (MST-like) superfamily, exhibited a high co-expression ratio with the four genes of the phenylpropanoid biosynthesis pathway (Figure 2.2). PAL are involved in many biosynthetic pathways. Multiple PAL genes were reported in the co-expression report.

To understand the biological context in *Arabidopsis* of the gene expression analysis performed in hybrid poplar, we performed a Gene Ontology (GO) category enrichment testing of the first 450 genes that were listed in order of co-expression R^2 value to poplar PMT4. The Gene ontology term analysis revealed that a 15.31-fold-enrichment corresponded to lignin metabolic process (Table 1). Further, several CESA genes related to SCW are co-expressed with AtPMT4, albeit with lower co-expression ratios (Figure 2.1). In concordance with our data, the Toronto

BAR Expression viewer shows AtPMT4 expressed in the stem and pollen, both tissues that undergo lignification (Figure 2.3). AtPMT4 was expressed in parenchyma cells, supporting the GNH.

Further the work described here results in the identification of cell-specific expression pattern of AtPMT4 in fibers and xylem vessels cells within the inflorescence stems. These patterns are consistent with the expression of monolignol biosynthetic genes, and cell types that undergo increased lignin deposition (Figure 2.4). This provides support that targeting specific cell specific to mutate secondary active transporters might reduce lignin without gross penalties to the plant.

Along with gene expression analysis of angiosperms, a similarity search using the nucleotide sequence of AtPMT4 was perform with the BLASTP program against other plant species (Table 2). The nucleotide sequence of poplar PMT4 was aligned against AtPMT4, which shared 71% identity (E-value: 0) and BdPMT4 69% (E-value: 8e-162) (Figure 2.5).

In *Brachypodium*, CESA4, involved in secondary cell wall formation, is highly expressed in the mid-stage elongating internode (5-10), while CESA1, involved in primary cell wall formation, is highly expressed in the early stage elongating internodes (1-3). Orthologs of the *Arabidopsis* secondary wall CESA genes have been identified in several vascular plants and their functional roles in cellulose synthesis in xylem vessels and fibers have been confirmed by mutational and/or transgenic analyses in rice, poplar and *Brachypodium* (Tanaka et al., 2003; Aspeborg et al., 2005; Nairn & Haselkorn, 2005; Suzuki et al., 2006; Joshi et al., 2011; Kotake et al., 2011; Handakumbura et al., 2013; Xi et al., 2017), demonstrating the functional conservation of secondary wall CESA genes in secondary wall synthesis in vascular plants and suggesting that the expressions patterns are similar for lignin biosynthetic genes. Once BdPMT4 was determined

to be the likely orthologue to PMT4, we performed gene expression analysis of CESAs in a *Brachypodium* RNA dataset prepared from developing internodes and we showed that CESAs are differentially expressed during development with the secondary cell wall CESAs most active during stages 5 through 10 (Figure 2.6). This confirmed that the dataset could be used to obtain further support of PMT4 involved in secondary cell wall development. BdPMT4 was shown to be most active during stages 5 through 8, similar to SCW CESAs (Figure 2.7). Therefore, these results from our *Brachypodium* study supported PMT4 a good candidate transporter because it may be conserved across plant species for monolignol export. The analysis shows that primary CESAs are expressed predominantly in tissues 1 to 3 while the secondary CESAs are expressed in tissues 5 to 10.

PMT4 was selected for functional analysis *in planta* because of the co-expression analysis from the high-resolution poplar dataset, followed by the tissue and cell specific expression of the candidate in the *Arabidopsis* inflorescence stem, confirmation of the expression level throughout *Brachypodium* stem development, and support from the literature that PMTs are associated with sugar alcohols which suggest that if monolignols are transported by PMT then the transport form could be glycosylated, free, or both. Using these gene expression tools to investigate the function PMT4 proteins *in planta* I hope to further contribute to the understanding of the role of individual PMT proteins in cell wall development.

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CHAPTER THREE:

CRISPR-Cas9 editing and mutant analysis of AtPMT4

3.1 Abstract

It is reasonable to expect that if AtPMT4 is a component of the lignin pathway then it would confer phenotypes similar to other lignin mutants. AT2G20780 is a predicted membrane transporter and cloning the gene for functional analysis such as transport studies in other organisms like yeast would have potentially introduced post-translational events or present a non-native cellular environment that would obfuscate the function of the protein relative to its native conditions. Therefore, phenotypic analysis in Arabidopsis presented a reliable and robust method for characterizing AT2G20780. The capacity for large scale phenotypic analysis was supported by the Michigan State University's Cell Wall Facility where the inflorescence stems from two independent genetic lines generated by CRISPR were submitted for phenotypic analysis upon reaching full senescence.

The homozygous mutant, *pmt4-1*, displays shorter root length than Col-0 when grown on agar media supplemented with 0.5 mM and 1.0 mM coniferyl alcohol while *pmt4-2* was only significantly shorter at the latter concentration. At 1.5 mM and 2.0 mM coniferyl alcohol there is no significant difference between the root length of mutants and Col-0. At 2.5 mM and 3.0 mM coniferyl alcohol, however, the roots of *pmt4-1* are significantly shorter than Col-0. We think that AtPMT4 acts as an exporter for coniferyl alcohol and can nullify some of the toxic effects of coniferyl alcohol at 0.5 mM and 1.0 mM. At 1.5 mM and 2.0 mM coniferyl alcohol, Col-0 and mutants are indistinguishable because transporters are saturated. At 2.5 mM and 3.0 mM, we think the shorter root length phenotype in *pmt4-1* reappears because growth and development penalties are compounding due to knock-on effects from toxicity. In the absence of PMT4, *pmt4-1* cannot export coniferyl alcohol sufficiently, and thus accumulates coniferyl alcohol at

intracellular levels that adversely affect growth.

3.2 Introduction

Phenotypic analysis is a reliable and robust method for characterizing the PMT4 *Arabidopsis* ortholog. A T-DNA line was available from the ABRC but the location of the TDNA insert was not within an exon region and instead upstream of the transcriptional start site. To understand the underlying phenotype associated with AtPMT4, we performed the gene editing technique, CRISPR-Cas9, or clustered, regularly interspersed palindromic repeats-associated endonuclease 9, that allowed us to produce a nonfunctional allele by a frameshift mutation. The CRISPR/Cas9 system has been developed into a powerful genome-editing technology revealing plant gene function and improving crops. The targeted mutagenesis derived from genome editing by CRISPR/Cas9 is valuable for understanding the functions of specific genes. We designed CRISPR-Cas9 constructs that targeted the individual AtPMT4 exons.

Our CRISPR/Cas9 construct featured a single guide RNA (sgRNA) targeted at the first exon of AtPMT4 to generate a DNA double-strand break (DSB) at a position three base pairs upstream of the protospacer adjacent motif (PAM) sequence that we selected using the webtool CRISPR-PLANT (https://www.genome.arizona.edu/crispr/). This typically results in DSBs that are repaired primarily by either nonhomologous end joining (NHEJ) or homology-directed repair (HDR) in vivo. NHEJ occurs most frequently and can introduce insertion/deletion mutations, such as random nucleotide deletions or insertions at the break site that induce a frameshift mutation in the target gene leading to an early stop codon that produces an incomplete protein product and therefore a non-functional allele which is our desired result. Targeting the first exon of PMT4 was of particular interest because the earlier an NHEJ event occurs in the sequence, the higher the

likelihood of generating a phenotype. In the case that a mutation in the first exon was lethal, we decided to also target the second exon, too, to generate a weaker phenotype. However, the likelihood that a lethal event would occur seems unlikely since other similar PMTs exist in *Arabidopsis*.

Coniferyl alcohol has been shown to have toxic effects on *Arabidopsis* (Alejandro et al., 2012). *In vivo*, *Arabidopsis* can transport monolignols and avoid toxic accumulation of the metabolite. However, when high levels of the monolignol are introduced exogenously, *Arabidopsis* develop shorter roots. We added coniferyl alcohol to the growth media to observe how CRISPR-Cas9 plants would respond. We then perform lignin analysis on the inflorescence stem of our CRISPR-Cas9 plants with assistance from the Cell Wall Facility.

The best characterized lignin transporter to date is the ABC transporter, AtABCG29 (Alejandro et al., 2012). The mutant showed no gross lignin phenotypes but did display differences in the composition and quantity of *p*-hydroxyphenyl, guaiacyl, and syringyl groups in lignin. However, transport assays and growth sensitivity assays only showed *p*-coumaryl alcohol as a substrate for AtABCG29. The growth penalty may be due to toxic accumulations of *p*-coumaryl alcohol in the hypocotyl tissue. Similarly in our work, we confirmed that *pmt4* had significant changes in lignin composition and quantity for guaiacyl, and synringyl. We assayed growth sensitivity to coniferyl alcohol and found that *pmt4* displayed shorter root lengths in the presence of the monolignol. While no biochemical evidence was explored, evidence of monolignol transport sensitivity was confirmed via the coniferyl alcohol toxicity assays.

3.3 Materials and Methods

3.3.1 Designing sgRNAs to target *AtPMT4*

The CRISPR-PLANT web tool (https://www.genome.arizona.edu/crispr/) was used to design artificial single-guide RNA (sgRNA) for CRISPR experiments in *A. thaliana* (Tair10). The tool predicted gRNA spacer sequences by genome-wide sequence comparison and scored the best target site based on mismatches and position in their alignments with other spacer sequences (https://www.genome.arizona.edu/crispr/instruction.html). This reduces the probability that the sgRNA will match to a non-specific DNA sequence that is not the target sequence of interest which would lead to an indel event that disrupts a gene that was never meant to be augmented.

The first sgRNA selected was based on proximity to the 5′ coding region of *AtPMT4*. A second sgRNA was also selected to target the second exon of *AtPMT4* in case the first sgRNA resulted in a lethal phenotype. gRNA spacers are limited to protospacer-adjacent motifs (PAMs). Cas9 recognize two types of PAMs: 5′NGG-3′ and 5′NAG-3′. It was critical to select an NGG which is required for the binding of Cas9 to the genome and with no significant sequence identity with other GG-spacers. We selected the following NGG-PAM gRNA spacer that was classified as "class0" for not having significant sequence identity to other NGG-PAM sites and no off-target potential to NAG-PAM sites: 5′-CGCTTGGGGCGGTTGGGAAC-3′. This sequence will be referred to as the: GN_{19} 20 bp sequence.

3.3.2 Annealing and Phosphorylation of sgRNA duplex

The GN_{19} 20 bp sequence was inserted in the sgRNA vector using oligos designed to form a short 20 bp DNA fragment with the necessary overhangs. Complementary oligonucleotide primers were phosphorylated and annealed using T4 PNK from NEB and 10x T4 Ligation Buffer containing

ATP from NEB. The reactions for sgRNA duplexes were ran at 37° C for 30 minutes, 95° C for 5 mins, then moved to 95°C water bath and cooled to 25°C. Final products were ran on 4% agarose gel against oligonucleotide primers to confirm creation of duplex.

3.3.3 Insertion of sgRNA duplex into vector containing Cas9 region

The oligonucleotide duplex formed was inserted in the psgRNA-U6-*At E. coli* plasmid (obtained from Dr. Christoph Benning's lab at Michigan State University). The bacterium containing the psgRNA-U6-*At* plasmid was grown on LB medium with ampicillin (100 µg/mL) at 37 °C overnight. *E. coli* colonies were inoculated in liquid LB containing ampicillin (100 µg/mL) at 37 °C while shaking at 200 rpm overnight. Cultures were purified using Plasmid Miniprep Kit from ThermoFisher Scientific. DNA concentration was checked using **Qubit**. psgRNA-U6-*At* was digested with BbsI from NEB at 37° C. The sgRNA duplex was diluted 1:200, and Quick Ligase (NEB) was used to ligate the sgRNA duplex into the BbsI cut site of psgRNA-U6-*At* at 23° C overnight to create psgRNA-U6-*At* with sgRNA inserted, or psgRNA-U6-*At*-PMT4. Two vectors in total were created, one for each sgRNA duplex. DH5α cells were used to transform psgRNA-U6-*At*-PMT4. The positive transformants were then cultured and purified.

3.3.4 Insertion of U6 promoter + sgRNA duplex + Cas9 fragment into vector (pCambia1300) for agrobacterium-mediated plant transformation

The psgRNA-U6-*At*-sgRNA plasmid was then digested with HindIII-HF (NEB) and EcoRI-HF (NEB) to obtain a 6kb fragment containing the following: U6 promoter, sgRNA oligo duplex, and Cas9 coding sequence. This 6 kb fragment was ligated into the backbone plasmid, pCambia1300. Bacteria containing pCambia1300 plasmid was inoculated on LB and kanamycin (50 µg/mL) medium and grown overnight at 37° C. Liquid cultures were prepared and then purified. The

pCambia1300 plasmid (214.5 ng/µL) was digested with HindIII-HF, EcoRI-HF (NEB), and Cutsmart Buffer for 5 hours. The 6 kb fragment from psgRNA-U6-*At*-PMT4 was ligated into digested pCambia1300 using a 32:1 ratio (6kb fragment:pCambia1300) using Quick Ligase (NEB) at 23° C overnight. The ligation product was transformed using DH5α competent cells as described above and grown overnight at 37° C on LB and kanamycin (50 µg/mL) media. The final plasmid (pCRISPR-*At*-PMT4) was confirmed using colony PCR. The products were run on a 1% agarose gel to confirm correct product. The data were then further confirmed with Sanger sequencing.

3.3.5 Transformation of pCRISPR-*At*-PMT4 into *A. thaliana*

A. thaliana seeds were stratified at 4 °C in the dark for two days before being sown onto 0.6% (w/v) Phyto agar plates containing 4.4 g/L Murashige and Skoog growth medium and 1% (w/v) sucrose (Murashige and Skoog, 1962). Plants were grown on plate, and then at 12 days old, transplanted to soil. Plants were grown under controlled conditions of 100 µE $m^{-2}s^{-1}$ in a 16 h light and 8 h dark cycle at 22 °C.

Agrobacterium competent cells were made by using a GV3101 cell aliquot of 100 uL to grow a 5-10 mL overnight culture in Luria Broth (LB) medium with 25 µg/mL gentamycin and 34 µg/mL rifampicin. A 50 µL aliquot of the culture was then used to grow a 500 mL culture in the same LB medium. The culture was grown at 23° C and shaken at 200 rpm until log phase was reached (OD_{600} of 0.3-0.6) after 15-18 hours. 1 mL aliquots were measured on ice and then centrifuged for 3 minutes at 4° C at 13,000 rpm. The supernatant was removed, and the pellet was suspended in 1 mL cold 10 mM Tris-HCl, pH 7.5 and then centrifuged. The supernatant was removed, and the pellet was suspended in 100 µL of cold LB medium, frozen in liquid nitrogen, and stored at -80°C.

The plasmid pCRISPR-*At*-PMT4 (1-2 µg) and pCambia1300 (empty vector control) plasmid (1-2 µg) were added separately to GV3101 agrobacterium cells using the electroporation method. 1 mL of LB media was then added to the transformation and incubated at 28° C, shaking at 200 rpm for ≥2.5 hours. The cells were then pelleted at 5000 rpm for 5 minutes, and the supernatant was removed. The pellet was suspended in 100 µL LB media. The competent cells were spread on LB media with 1% (w/v) bacto agar, 25 µg/mL gentamycin and 34 µg/mL rifampicin, wrapped in parafilm, and grown at 28° C for two days.

Agrobacterium tumefaciens-mediated floral dip (Clough and Bent, 1998) was used to introduce pCRISPR-*At*-PMT4 into 5 week-old *A. thaliana* Col-0 plants. The *Agrobacterium tumefaciens*-mediated floral dip method was performed again when the plants were 6 weeks old to improve transformation efficiency. Seeds collected from fully grown plants were considered the T_1 generation.

3.3.6 Screening and verifying mutant lines

Upon receiving T1 seeds, the screening process for hygromycin resistant plants began. The T_1 Col-0 seeds for the pCRISPR-*At*-PMT4 and empty-vector control line were plated on Murashige and Skoog medium with 1% (w/v) sucrose and 25 mM/mL hygromycin for antibiotic selection screening.

T1 seeds were cold treated (stratified) for 2 days, exposed to light for 6 hours at 23° C, and then grown in the dark for 5 days at 23° C to stimulate hypocotyl growth.

The plants with an extended hypocotyl and green cotyledons were transplanted to soil and grown under standard growth conditions to maturity.

T2 seeds were collected, and the screening selection process was repeated. T2 seeds displayed a 3:1 hygromycin resistant phenotype. T2 hygromycin resistant plants were transplanted to soil and grown to maturity. T3 seeds were collected and grown without hygromycin on soil in long-day condition. Plants were genotyped to determine if a CRISPR-Cas9 mediated event was successful. Plants that showed an insertion/deletion event that resulted in a frameshift mutation were selected for phenotypic analysis in the T4 generation.

3.3.7 Lignin Content (ABSL assay)

To determine the lignin content, we used a method modified by Foster et al. (2010) that was based on a reported method by Fukushima and Hatfield (2001; 2004).

3.3.8 Lignin Composition (Free-monomer assay)

To determine the lignin composition, or ratio of free lignin monomer, we used a method adopted by Foster et al. (2010) that was based on a reported method published by Robinson and Mansfield. We freeze dried inflorescence stem that was collected from fully matured *Arabidopsis*. The sample was subjected to thioacidolysis. The solubilized phenolics underwent TMS derivatization and the lignin units were then separated and quantified by GC-MS analysis.

3.3.9 Crystalline Cellulose Content (Digestibility assay)

To determine the crystalline cellulose content, we used the method described by Updegraf. The cell wall was isolated from dried inflorescence stem and subjected to pretreatment followed by enzyme digestion. The samples were pretreated with dilute sodium hydroxide (6.25 mM) at 90°C followed by dilute sulfuric acid (2% w/v) at 120°C. After digestion, a separation step removed the soluble digestion fraction from the residual biomass by centrifugation. Enzyme-based assays were performed for glucose and pentose. Enzyme digestion proceeded at a target of one third

or less of the total glucan to maintain the percent of total glucose released below 50%. The threshold provides higher resolution for identifying modified biomass with increased as well as decreased digestibility. Aliquots were removed from the digested solutions, and enzyme-based assays were performed for glucose and xylose and reported as a measure of percentage of dry biomass weight.

3.4 Results

3.4.1 CRISPR-Cas9 transformation results in three independent lines

The first and second exons of AtPMT4 are 211 bp and 1364 bp, respectively. The tool 'CRISPR-PLANT', a platform to help design and construct gRNAs for CRISPR-Cas9 mediated genome editing in plants, identified a total of 4 class0.0 gRNA.

We performed six independent CRISPR transformations. Our transformations resulted in the production of three independent lines: *pmt4-1*, *pmt4-2*, and *pmt4-3*. The genotypes are the following: a 5bp-deletion (*AATGG*) starting after the 28th base pair, a 1bp-insertion (*T*) starting after the 32nd base pair, and a 1bp-instertion (*A*) after the 32nd base pair, respectively. We phenotype the amount of lignin, lignin monomeric composition, amount of sugar released from the stem tissue, and sensitivity of root growth to coniferyl alcohol of *pmt4-1* and *pmt4-2*. *pmt4-3* was identified later and was not included in our phenotypic analysis but may be useful for future work. *pmt4-2* shows a lower peak intensity relative to the rest of the chromatogram at the area of the insertion event, which suggest that *pmt4-2* is not a homozygous line and could be a heterozygous mutant with a weaker phenotype relative to *pmt4-1*. We think that *pmt4-2* is a heterozygous mutant that was still segregating during our phenotype analysis.

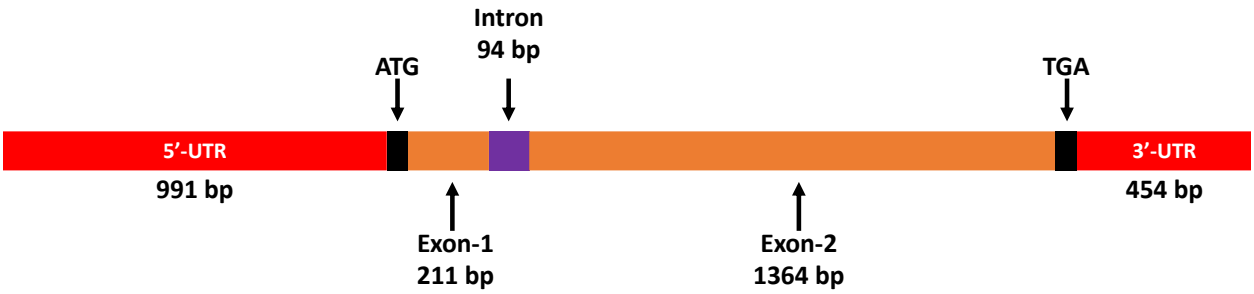
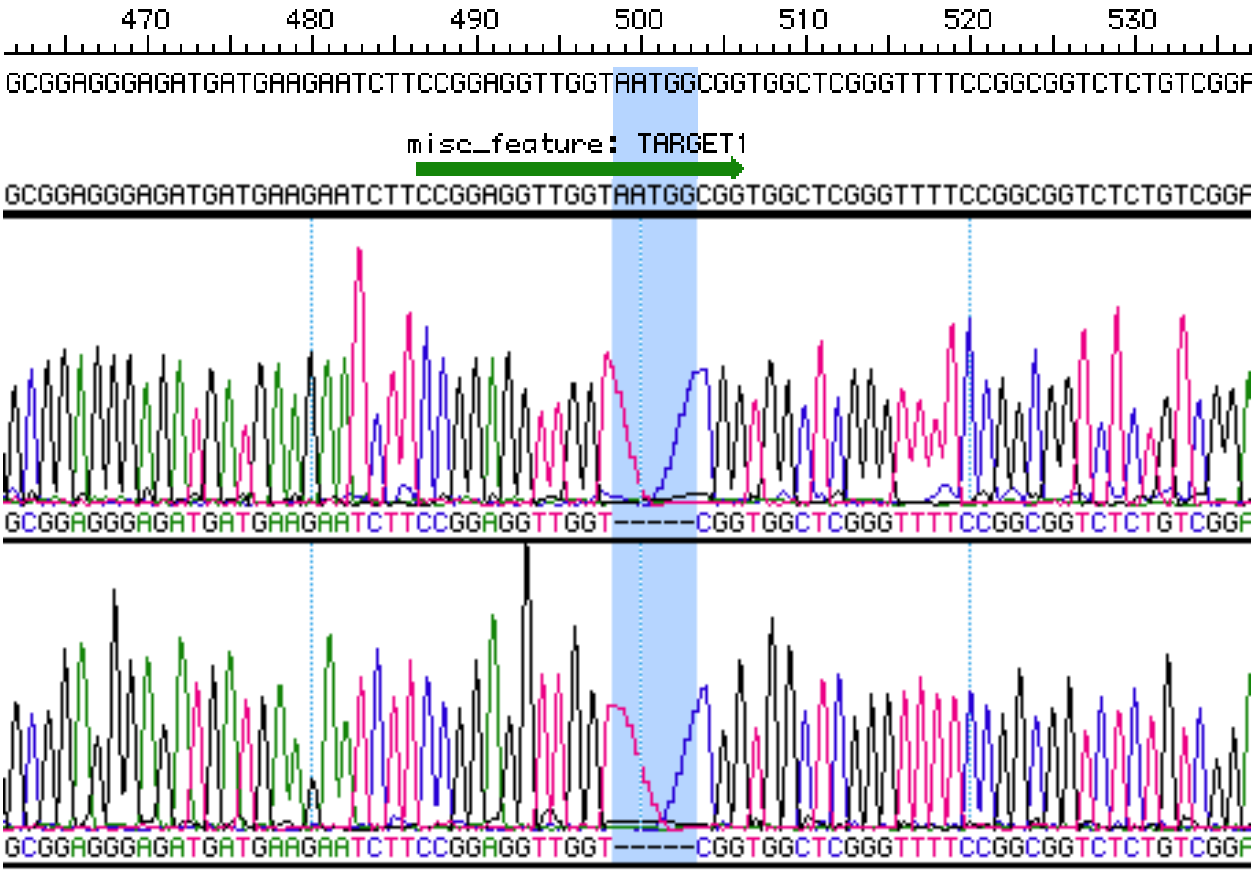
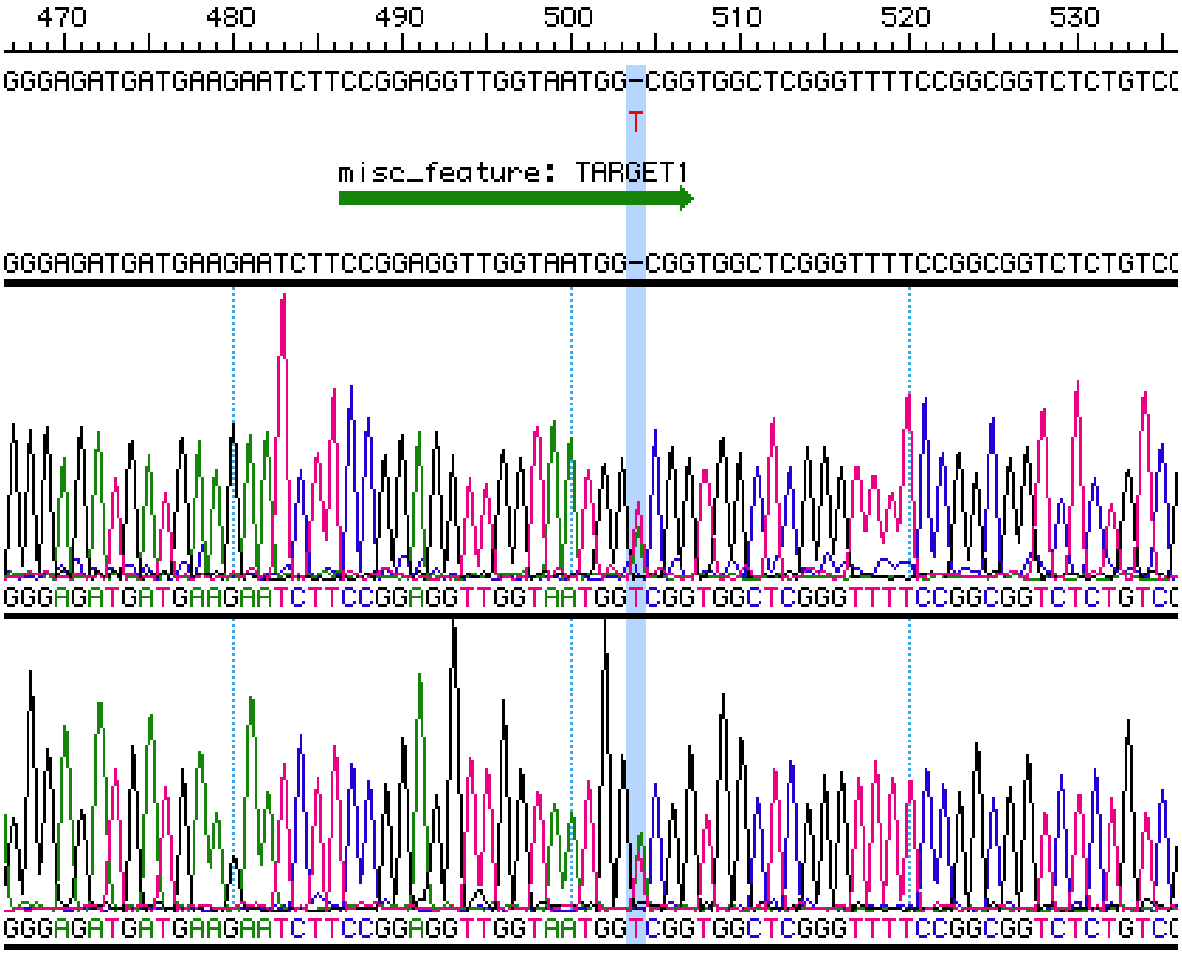


Figure 3.1. Gene model of AtPMT4. The gene contains two exon regions and one intron region. The first exon was selected as the desired target site of mutagenesis since a shift in the reading frame earlier in the coding region has a higher probability of producing a null allele.



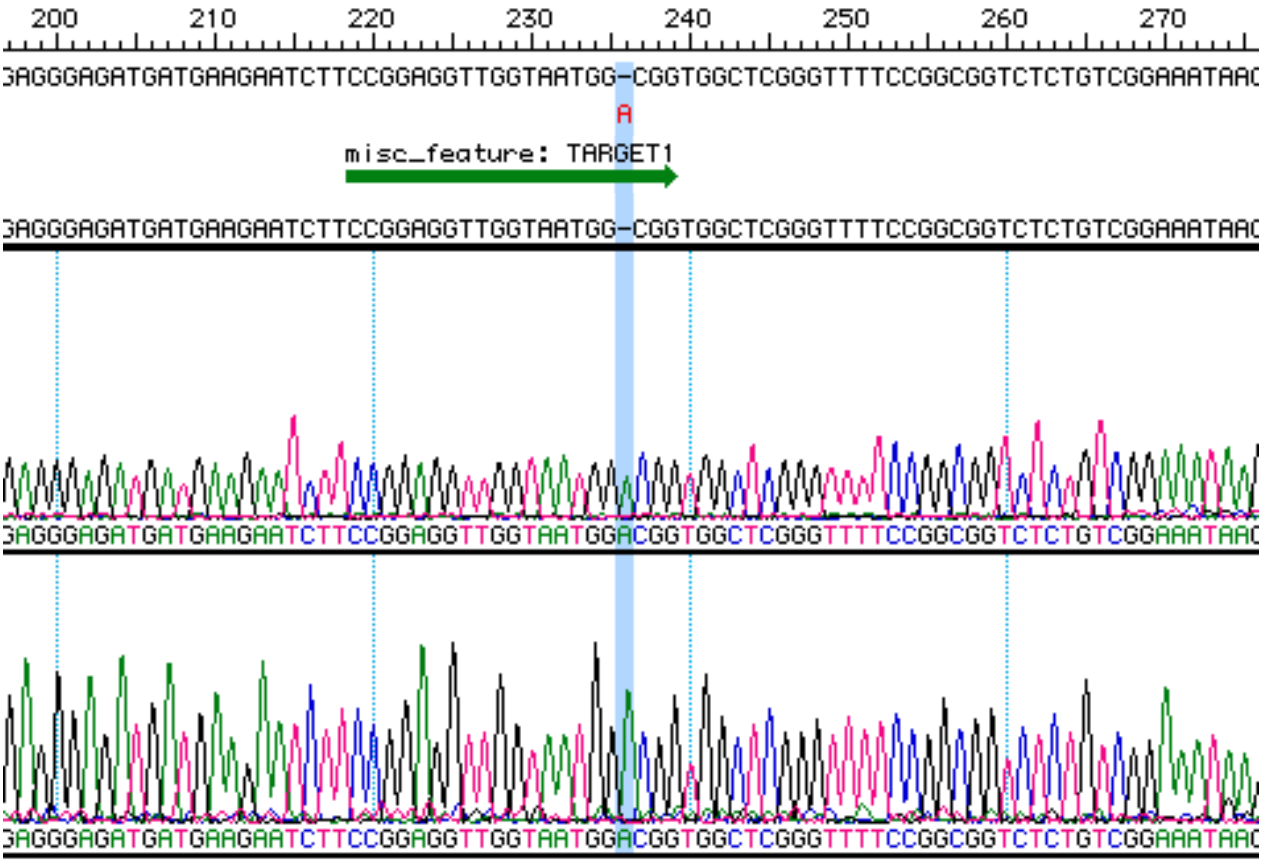
Wild type: ATGATGAAGAATCTTCCGGAGGTTGGTAATGGCGGTGGCTCGGG
pmt4-1: ATGATGAAGAATCTTCCGGAGGTTGGT - - - - - CGGTGGCTCGGG

Figure 3.2. *pmt4-1* is a homozygous mutant. DNA was extracted from leaf tissue and submitted for Sanger sequencing that used fluorescent ddNTPs in the DNA amplification and chain termination reaction. Different length of fragments were produced and separated using capillary electrophoresis. The readout was aligned to a wild type AtPMT4 reference sequence. A 5-bp deletion occurred subsequently following the 24th nucleotide after the start codon. The deletion event was not a multiple of three and likely led to a frame-shift mutation. The following nucleotides were deleted in *pmt4-1*: AATGG.



Wild type: ATGATGAAGAATCTTCCGGAGGTTGGTAATGG-CGGTGGCTCGGG
pmt4-2: ATGATGAAGAATCTTCCGGAGGTTGGTAATGG**T**CGGTGGCTCGGG

Figure 3.3. *pmt4-2* is a heterozygous mutant. DNA was extracted from leaf tissue and submitted for Sanger sequencing that used fluorescent ddNTPs in the DNA amplification and chain termination reaction. Different length of fragments were produced and separated using capillary electrophoresis. The readout was aligned to a wild type AtPMT4 reference sequence. A 1-bp insertion occurred subsequently following the 29th nucleotide after the start codon. The insertion event was not a multiple of three and likely led to a frame-shift mutation. The following nucleotides were inserted in *pmt4-2*: T/A.



Wild type: ATGATGAAGAATCTTCCGGAGGTTGGTAATGG-CGGTGGCTCGGG
pmt4-3: ATGATGAAGAATCTTCCGGAGGTTGGTAATGG**A**CGGTGGCTCGGG

Figure 3.4. *pmt4-3* is a homozygous mutant. DNA was extracted from leaf tissue and submitted for Sanger sequencing that used fluorescent ddNTPs in the DNA amplification and chain termination reaction. Different length of fragments were produced and separated using capillary electrophoresis. The readout was aligned to a wild type AtPMT4 reference sequence. A 1-bp insertion occurred subsequently following the 29th nucleotide after the start codon. The insertion event was not a multiple of three and likely led to a frame-shift mutation. The following nucleotides were inserted in *pmt4-3*: A.

3.4.2 Effect of PMT4 mutation on sensitivity to coniferyl alcohol and primary root length in *Arabidopsis*

In our functional analysis *in planta*, the root lengths of *pmt4-1* and *pmt4-2* were longer compared to Col-0 when grown on Murashige and Skoog agar media. However, the mutants were shorter in the presence of coniferyl alcohol suggesting increased phytotoxicity. At 0.5 mM and 1 mM coniferyl alcohol, the root length of *pmt4-1* and *pmt4-2* were shorter by 23% and 22.4% compared to Col-0 (Figure 4.1). To test if PMT4 obeys normal rate of transport and approaches saturation (Tm), we measured primary root length against increasing concentrations of coniferyl alcohol to determine if no differences in root length for Col-0 and mutants would be observed due to saturated export. We performed a t-test analysis and found that at 1.5 mM and 2.0 mM coniferyl alcohol, the root lengths of Col-0, *pmt4-1*, and *pmt4-2* showed no significant difference. We determined the effect on primary root length with versus without coniferyl alcohol to indicate the amount of coniferyl alcohol that may have diffuse into the plant tissue and cause phytotoxicity (Figure 4.2). Based on this hypothesis, we needed to determine if the composition of lignin monomer as it pertains to coniferyl alcohol were specifically affected.

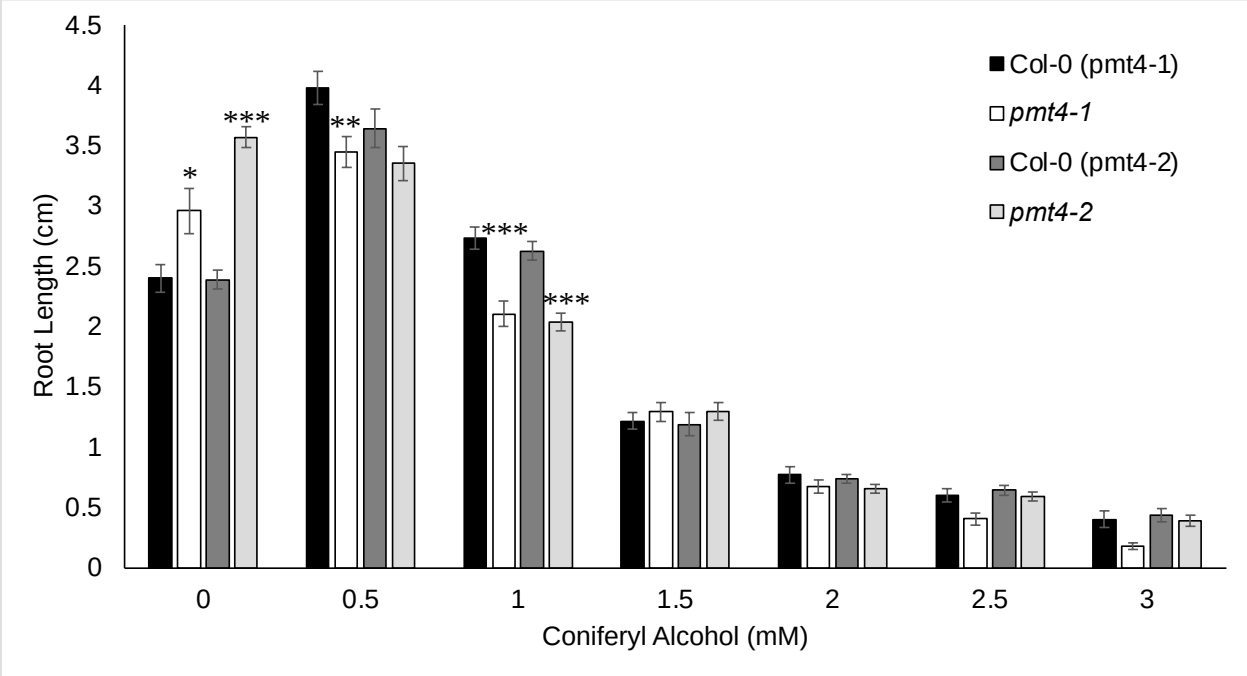


Figure 3.5. Effect of coniferyl alcohol on *Arabidopsis* seedling primary root length. Plants were grown on Murashige and Skoog agar plates at the indicated concentration of coniferyl alcohol. Root length was measured after 2-weeks under long-day conditions (16:8) at 22 ˚C. Biological replicates were characterized by the number of individual plants plated at each concentration which for *pmt4-1* totaled at least 10 individual *Arabidopsis* plants (Control, Col-0 had at least 10). For *pmt4-2*, there were at least 16 biological replicates (Control, Col-0 had at least 17) (shown on Table 5). **Error bars indicate standard error**. Statistical significance was determined by Student's t-test (***P<0.001; **P<0.01; and *P<0.05).

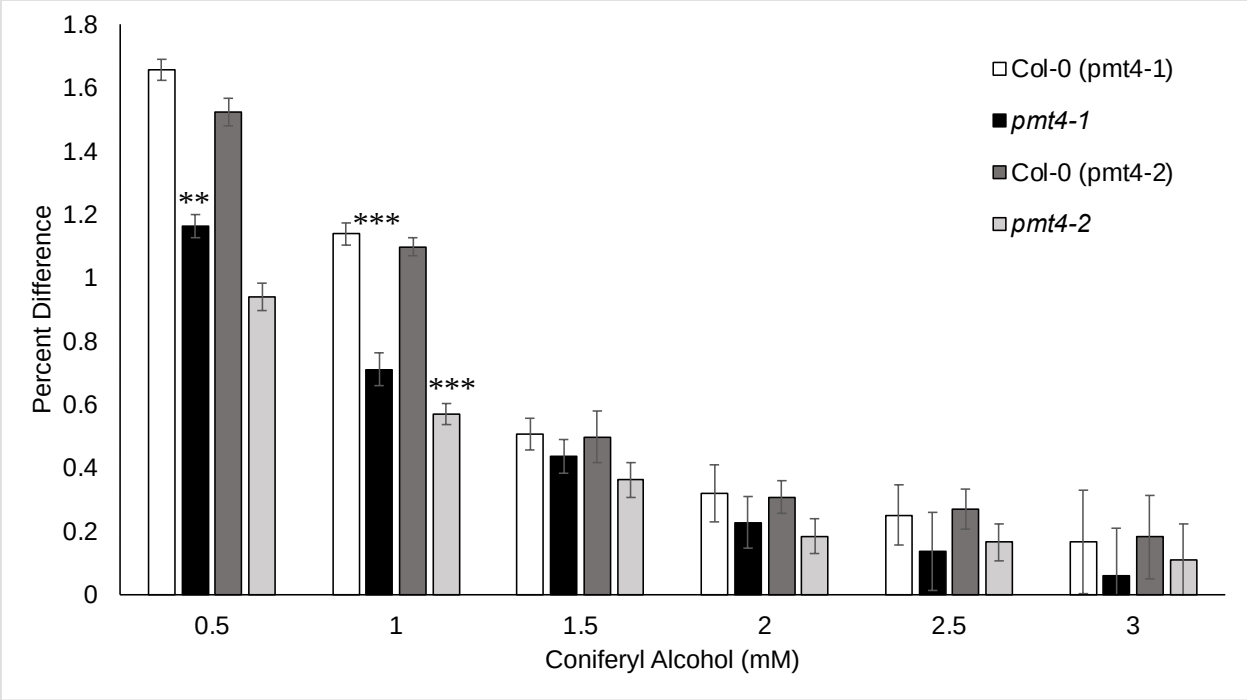


Figure 3.6. Effect of coniferyl alcohol on *Arabidopsis* seedling primary root length expressed as a percentage of root length compared without coniferyl alcohol. Plants were grown on Murashige and Skoog agar plates at the indicated concentration of coniferyl alcohol. Primary root length was measured after 2-weeks under long-day conditions (16:8) at 22 C. The percent difference (y-axis) was calculated by dividing the mean values of the primary root length grown at each coniferyl alcohol concentration by the mean values of primary root length from the identical genotype grown without coniferyl alcohol. Biological replicates were characterized by the number of individual plants plated at each concentration which for *pmt4-1* totaled at least 10 individual *Arabidopsis* plants (Control, Col-0 had at least 10). For *pmt4-2*, there were at least 16 biological replicates (Control, Col-0 had at least 17) (shown on Table 5). **Error bars indicate standard error**. Statistical significance was determined by Student's t-test (***P<0.001; **P<0.01; and *P<0.05).

Table 5. Sample size and replicate information for root analysis

	n = sample size, individual plants			
Coniferyl Alcohol (mM)	*pmt4-1*	*Col-0 vs pmt4-1*	*pmt4-2*	*Col-0 vs pmt4-2*
0	20	19	20	20
0.5	20	20	25	25
1	25	24	17	17
1.5	20	19	24	24
2	10	10	25	25
2.5	15	14	25	24
3	15	15	16	20

3.4.3 Lignin content and composition of inflorescence stems are lowered and altered in *pmt4* affecting overall digestibility

Concentrations at the first two points are inhibitory to *pmt4-1* and *pmt4-2.* After the third point, there is saturation to the response of Col-0 to coniferyl alcohol and suggest there is no longer a transporter functioning quickly enough to protect Col-0 from coniferyl alcohol.

To further investigate the potential relationship of AtPMT4 and monolignols (coniferyl alcohol and sinapyl alcohol), we first determined if *pmt4-1* and *pmt4-2* mutations would lead to a decrease of the overall lignin quantity. A combination of α-amylase and pullulanase were used in the AIR/destarch pretreatment for lignin content assay, lignin monomer composition assay, and matrix polysaccharide composition assay. We quantified acetyl bromide soluble lignin and our results show that *pmt4-1 and pmt4-2* had significantly lowered amounts of soluble lignin by -17.9% and -11.6%, respectively (Figure 4.3). The result further supports that AtPMT4 plays a role in *Arabidopsis* lignification. To further delineate whether AtPMT4 is a broad monolignol

transporter or specific to individual or a subset of monolignols, we next analyzed the lignin monomer composition of the inflorescence stem. Similar although not identical effects were observed where G lignin was lowered in both *pmt4-1* and *pmt4-2* by -16.4% and -10.9%, respectively, while syringyl was only lowered significantly in *pmt4-1* by -18.4% (Figure 4.4). No discernible differences were measured for H lignin in either mutants. The result of the small decrease in lignin content revealed that PMT4 contribute to the incorporation of coniferyl and sinapyl alcohol but it is not essential for lignification. AtPMT4 is likely an element for lignification but diffusion and or other transporters are likely responsible for the rest of the lignin.

Last, we wanted to determine if either change to the amount of lignin or its composition would increase digestibility since lignin imparts rigidity to cell wall tissue by enclosing the rest of the material from chemical hydrolysis. Lignin restricts the access of hydrolytic enzymes to wall polysaccharides and so lowering the amount of lignin should result in higher yields of sugars from such biomass. Cellulose and hemicellulose primarily yield glucose and xylose, respectively, upon efficient hydrolysis. Pretreatment aims to increase the porosity and surface area of the biomass, disrupts the structural integrity of lignin, and reduces cellulose crystallinity (Moodley et al., 2020). Following pretreatment, enzymatic hydrolysis and fermentation will proceed at enhanced rates.

For our other digestibility protocols, we use accellerase, invertase, and α-amylase w/ amyloglucosidase. The free sugars assay uses a zero-pretreatment method, so no enzymes were used. Our results showed that a significant increase in the amount of glucose was released in *pmt4-1* compared to Col-0 (Figure 4.5). Together, the phenotypes observed in our independent alleles, *pmt4-1* and *pmt4-2*, indicate an association between AtPMT4 and coniferyl alcohol and

sinapyl alcohol *in planta* that leads to alteration of lignin and increase digestibility of *Arabidopsis* biomass.

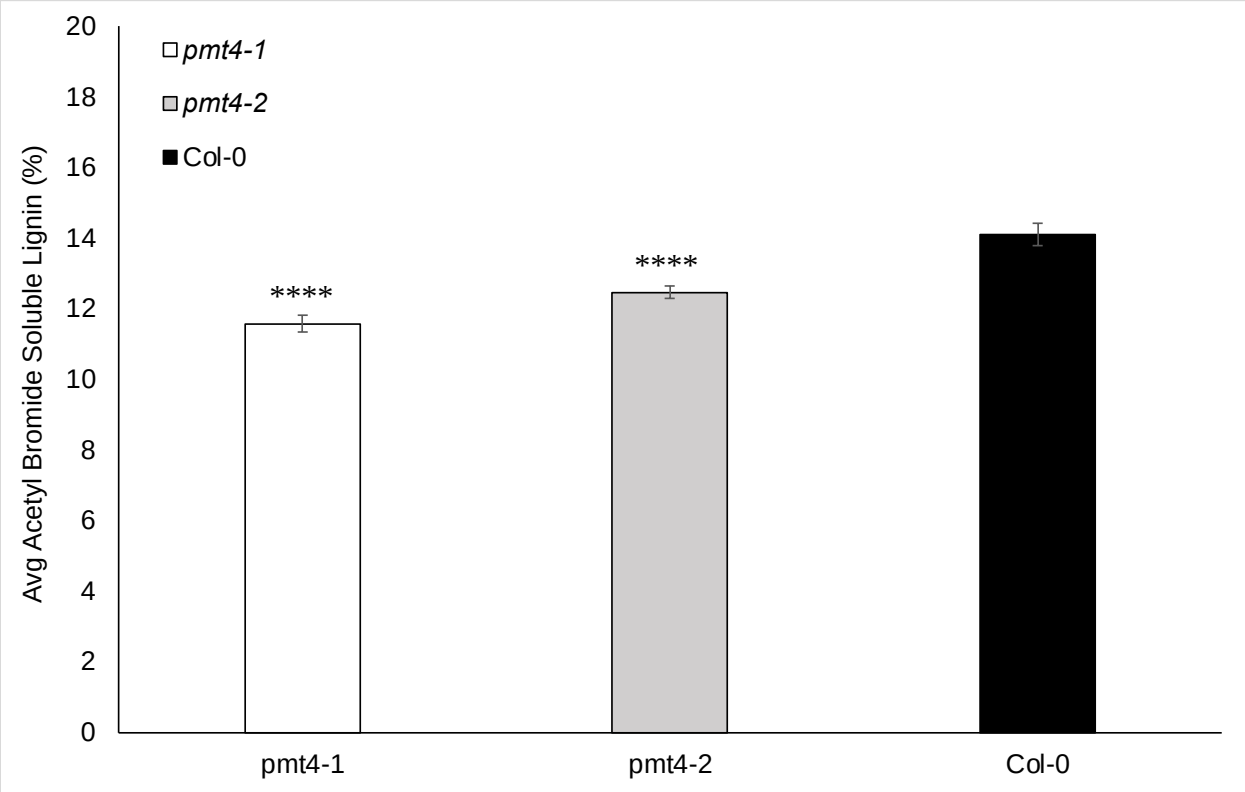


Figure 3.7. Lignin content of inflorescence stems. Plants were grown on soil for 2 months under long day conditions. Inflorescence stems were collected and submitted to the Cell Wall Facility for analysis. Wall material was treated with 25% v/v acetyl bromide in glacial acetic acid. The amount of acetyl bromide soluble lignin (ABSL) was quantified by UV- spectrophotometry and shown as a percentage of the dried material. The biological replicates are represented by the number of individual plants, which for *pmt4-1*, *pmt4-2*, and Col-0 were 14, 17, and 13, respectively (shown in Table 5). **Error bars indicate standard error**. Statistical significance was determined by Student's t-test (***P<0.001; **P<0.01; and *P<0.05).

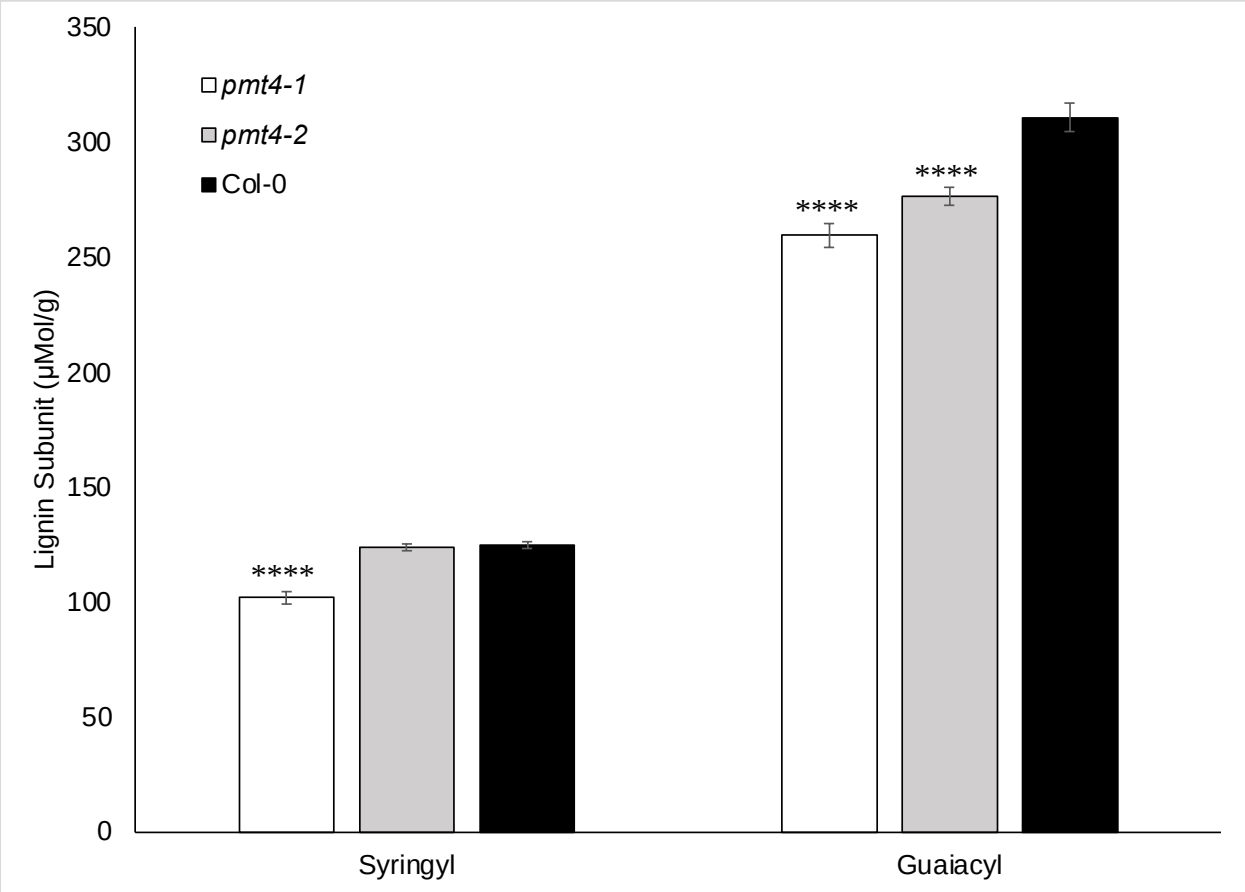


Figure 3.8. Lignin composition of inflorescence stems. Plants were grown on soil for 2 months under long day conditions. Inflorescence stems were collected and submitted to the Cell Wall Facility for analysis. Wall materials were subjected to thioacidolysis and trimethylsilyl derivatization was performed on the solubilized phenolics. The relative abundance of syringyl (S), guaiacyl (G), and *p*-hydroxyphenyl (H) units were quantified by GC-MS analysis. The biological replicates are represented by the number of individual plants, which for *pmt4-1*, *pmt4-2*, and Col-0 were 14, 17, and 14, respectively (shown in Table 6).**Error bars indicate standard error**. Statistical significance was determined by Student's t-test (***P<0.001; **P<0.01; and *P<0.05).

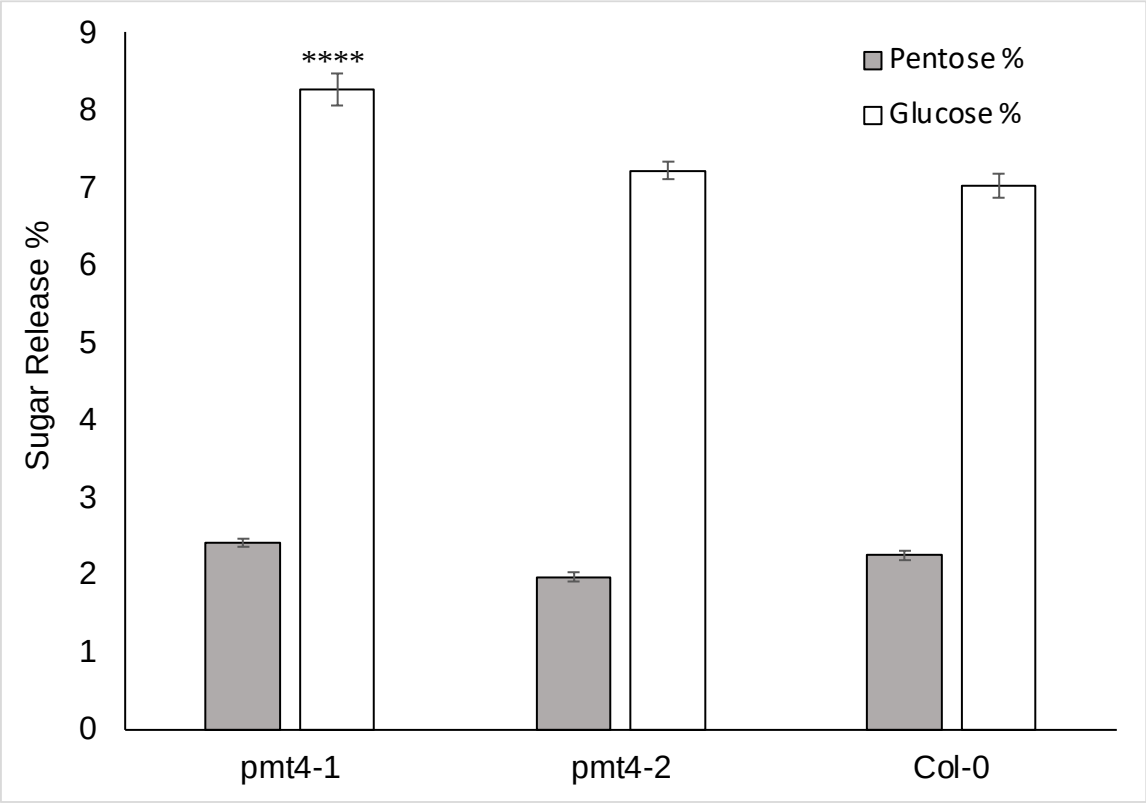


Figure 3.9: Digestibility of the cell wall from inflorescence stems. Plants were grown on soil for 2 months under long day conditions. Inflorescence stems were collected and submitted to the Cell Wall Facility for analysis. The amount of glucose and pentose measured were used to determine the digestibility of the cell wall material. The biological replicates are represented by the number of individual plants, which for *pmt4-1*, *pmt4-2*, and Col-0 were 14, 17, and 13, respectively (shown in Table 6). **Error bars indicate standard error**. Statistical significance was determined by Student's t-test (***P<0.001; **P<0.01; and *P<0.05).

Table 6. Sample size and replicate information for lignin and digestibility analysis

	n = sample size, individual plants		
	pmt4-1	*pmt4-2*	Col-0
ABSL	14	17	13
Lignin Subunit	14	17	14
Digestibility	14	17	14

3.5 Discussion

To perform functional analysis *in planta* we produced a knockout of AtPMT4. We identified a T-DNA insertion line for AtPMT4 in the SALK collection (SALK_142063) where the T-DNA was inserted in the 5' UTR region. To determine the phenotype associated with AtPMT4, however, we needed to produce alleles where the mutation effected the exon region. The CRISPR method has been shown to be an efficient method for targeted disruption of desired gene in plants (Ding et al., 2016) and we used the traditional CRISPR/Cas9 system which is dependent on the RNA polymerase III (Pol III) promoter producing one sgRNA to generate one double stranded break (DSB) to target the first exon in AtPMT4 in the genome. Cas9 most frequently creates a blunt-ended DSB at a position three base pairs upstream of the PAM sequence. One of the challenges to our approach is that mutations introduced by CRISPR/Cas9 using one sgRNA are difficult to identify by PCR analysis, and techniques are limited to homology analysis by DNA sequencing. In contrast, the use of two sgRNAs can be used advantageously to make targeted deletions that can be detected by PCR, making screening for mutants significantly easier than when using one sgRNA. We recommend that future CRISPR-Cas9 experiments in *Arabidopsis* use two or more sgRNAs to achieve targeted loss-of-function deletions.

Our CRISPR experiments led to the production of three independent mutant lines, two homozygous and one heterozygous. We designed sgRNAs that targeted the Cas9 endonuclease to *PMT4* in the first and second exon separately. The sgRNAs were inserted into a plasmid with the Cas9 sequence and the final plasmid was confirmed using colony PCR. PCR was efficient at confirming that the fragment containing the sgRNA was inserted into the destination vector, pCambia1300 because the M13 F primer was designed to amplify a region specific to the

pCambia1300 vector while the sgRNA R primer would begin amplifying at the sgRNA region itself. The transformation of the final CRISPR vector for lines A and B was confirmed to produce transgenic plants. The hygromycin selection technique was used for the transgenic screening (Harrison et al., 2006). The dark treatment caused extended hypocotyls for plants containing a hygromycin resistant gene while the plants that did not harbor the plasmid construct were pale yellow and short in comparison. The screening method made transgenic plants very easy to distinguish from non-transgenic plants by visualization.

Coniferyl alcohol and sinapyl alcohol are important building blocks to lignin polymerization. Recent studies from many laboratories have contributed to several significant advances in the dissection of monolignol translocation mechanism including transporter protein types and the unique spatial and temporal regulation of lignification in individual cell types. Although an ABC transporter mechanism has been linked to monolignol export, direct evidence has been limited to *p*-coumaryl alcohol while an exporter for the major monolignol constituents remain largely enigmatic. We addressed the important issue in our study and show that mutation of PMT4 significantly alters lignin. Our results revealed a dose-dependent response to coniferyl alcohol.

An in-silico diffusion model shows that monolignols could cross a permeable membrane at rates sufficient for biological purposes (Vermaas et al., 2019). Increased solubility would be achieved if sugar molecules were added to monolignols. Secondary active transporters that do not directly hydrolyze ATP such as PMTs have been shown to transport molecules similar to monolignols (Väisänen et al., 2020). The observation was similar to wild-type plants (WT) and an *Arabidopsis pmt5* T-DNA insertion mutant (Klepek et al., 2005). Our results show that the

mutation of a member of the PMT family, a non-ABC transporter, show growth sensitivities to coniferyl alcohol and reduced the recalcitrance of inflorescence stem tissue, which cast doubt on the idea that transporters are not necessary for lignification. It would appear that in Col-0 there is a mechanism to pump out coniferyl alcohol from the cell against the concentration gradient. We found that PMT4 may be required for proper incorporation of coniferyl alcohol and sinapyl alcohol in the secondary cell wall. First, targeted mutagenesis of AtPMT4 in the first exon is sufficient to increase root length by ~19% and ~33% *pmt4-1* and *pmt4-2* when compared to Col-0 in normal growth media. Second, the addition of 0.5 M coniferyl alcohol to the growth medium led to a reduction in root length of *pmt4-1* and *pmt4-2* by ~13% and ~8%, respectively. At 1 M coniferyl alcohol, the root lengths of *pmt4-1* and *pmt4-2* were further reduced by ~23% and ~22%, respectively. At 1.5 mM coniferyl alcohol, there is no significant difference between the mutants and Col-0. Altogether, these results suggest that that the mutants may undergo additional primary growth and cell expansion when components of the secondary cell wall biosynthesis are compromise, and hence display increase root length. However, the mutants may exhibit increased sensitivity to coniferyl alcohol because AtPMT4 is a compromised monolignol exporter that is unable to function in the removal of monolignols that are above normal cellular levels. We therefore propose that coniferyl alcohol is a substrate of AtPMT4 and that the transporter may have a meaningful role in the incorporation of coniferyl alcohol during lignification. This proposal is further supported by cell wall analysis of the mutants by the Michigan State University Cell Wall Facility, which shows multiple cell wall phenotypes including a cell wall that is more susceptible to pre-treatment processes.

A secondary active transporter for sugar alcohols is applicable to other compounds such as monolignols because their diffusion profile is common of many compounds shown experimentally and in silico. Furthermore, while our results are consistent with a dose-dependent transporter response, it is possible that other transporters also influence lignification. Examination of publicly available *Arabidopsis* gene expression databases reveal the expression of other transport genes. For example, AtPMT4 and other PMT members could have specific spatial and temporal expression to regulate lignin polymerization that mask the effects of a null mutation when any one of the transporters are not functional.

Although transgenic mutants of monolignol transporter candidates have been used in the discovery of the transporter function of monolignols, the mechanism by which transporters confers binding to coniferyl alcohol and sinapyl alcohol is still unresolved. Our results now provide an explanation for why non-ABC transporters confer transport and suggest a model in which the ability of the transport is linked to the Good Neighbor Hypothesis. First, we found that *pmt4* lowers the amount of soluble lignin in the inflorescence stem where 11.58 µMol/g and 12.47 µMol/g were measured for *pmt4-1* and *pmt4-2*. This contrasted with 14.11 µMol/g in Col-0. By investigating the amount of soluble lignin, we have determined that mutation of this gene is a key feature that is associated with a lower quantity of lignin. Second, lignin monomer analysis revealed that both G-lignin and S-lignin are lowered in the mutants, whereas H-lignin remains unchanged despite the change to the overall amount soluble lignin. In *pmt4-1*, both the amount of G and S lignin changed with a recorded amount of 260 µMol/g and 102 µMol/g, respectively. In *pmt4-2*, only the G lignin was changed with a recorded amount of 277 µMol/g. In Col-0, 311 µMol/g, 125 µMol/g, and 7.7 µMol/g were recorded for G, S, and H lignin. Third, we identified

changes to the digestibility of the cell wall indirectly by measuring free glucose monomers. Using a weak acid (2M TFA) on the crude dried stem material washed with the Updegraff reagent resulted in insoluble crystalline cellulose that was then solubilized by sulfuric acid. The by-product was quantified by a colorimetric assay which determined that the mutant *pmt4-1* displayed a significant increase in digestibility by 1.24% with an overall 8.3% free glucose compared to Col-0 which had 7.0%. AtPMT4 now exerts a digestibility sensitive phenotype when mutated. The AtPMT4 secondary active transport model not only could explain the lack of ATP, but also the ability to work in conjunction with the Good Neighbor Hypothesis, indicating more than one mechanism for monolignol transport. However, this model could not yet explain the ability of ABC transporter to confer sensitivity to H-lignin and not the more abundant monolignols while AtPMT4 show the opposite effect. It is possible that ATP is required to transport the less abundant monolignol because a sufficient gradient does not exist for H-lignin since it is not synthesized abundantly.

pmt4-2 showed reduced lignification and is likely a result of a heterozygous mutation, reflected by the Sanger sequencing results. For this reason, it is not possible to speculate the function of AtPMT4 with only homozygous line, *pmt4-1*. We need to phenotype the *pmt4-3* homozygous line to strengthen our claim. To our knowledge, secondary active transport of coniferyl alcohol and sinapyl alcohol has not been reported in plants. While monolignol diffusion has been supported, plants likely need an active and passive mechanism in order to respond rapidly to lignification and enable the usage of monolignols immediately after synthesis (active transport in autonomously lignifying cells) and after cell death (passive diffusion to other lignifying cells). Both types may be needed to ensure the deployment of monolignols for

immediate use, minimize unnecessary metabolic waste, and export the accumulation of monolignols that could lead to phytotoxicity.

In the future, it would be important to create a *pmt1/2/3/4/5/6* mutant so the physiological functions of PMTs can be better understood by overcoming the potential redundancy among the PMTs. I expect to see decreased total lignin and a dramatic decreased of coniferyl and sinapyl alcohol in the inflorescence stem compared to *pmt4*. It is reasonable to expect that other PMTs compensate for loss of *AtPMT4* function. The creation of a knockout of the entire family of PMTs will allow me to understand if the redundancy of the PMTs contribute and/or essential to lignification.

While there is no biochemical evidence, we feel that this may be helpful in broadening the search for monolignol transporters by deviating from transporters belonging only to the ABC family. In the future, it would be interesting to explore CRISPR-Cas9 studies of PMT4 in poplar to determine whether the gene target is commercially viable for genetic engineering to improve the digestibility of biofuel crops in addition to other grass systems. We provide further evidence that reverse genetics can lead to interesting findings in plant research, particularly as it pertains to secondary cell wall biosynthesis.

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LITERATURE CITED

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CHAPTER FOUR:

Conclusions and future directions

4.1 Abstract

AtPMT4 comprises of 526 amino acids with the longest N-terminus among the PMT family and is its most distant member. It shares 60% identical amino acids with all other AtPMTs while AtPMT3, AtPMT5, and AtPMT6 share between 70-80% identity. AtPMT1 and AtPMT2 are encoded by adjacent genes on chromosome 2 and show the highest degree of sequence conservation, which may be the result of a duplication event. Our work suggests that AtPMT4 is a monolignol transporter which is supported by studies of the other members of the PMT family.

Members of the PMT family have been shown to transport many classes of substrates. AtPMT5, for example, catalyzes the transport of the cyclic polyol myo-inositol, glucose, and of different hexoses and pentoses, including ribose. AtPMT5 was shown to transport sugars based on inhibition of polyol transport by hexoses. It is possible that the classes of substrates expand to monolignols based on the lignin defect and growth sensitivity to coniferyl alcohol that we observed in our mutant lines. The evidence that members of PMT also catalyze the transport of sugars, however, suggests that monolignols may be transported in their glycosylated form in *Arabidopsis*.

4.2 Conclusions and future directions

Coniferyl alcohol is not very polar and is likely to have some diffusion through membranes when not glycosylated. It is nearly 10 times more soluble in octanol than water, so it should be soluble in phospholipid membranes. However, a transporter might accelerate transport during developmental phases of intense lignification. Therefore, monolignol secondary active transport events that depend on molecular binding between the monolignol and the membrane-embedded channel is likely necessary. Unlike free diffusion, which is linear in relation to

concentration difference, the rate of facilitated diffusion is saturable with respect to the concentration difference between the cytoplasm and apoplast. The temperature dependence of facilitated transport due to the presence of an activated binding event may therefore effect lignification. In some plants, chilling temperatures are shown to promote lignified xylem deposition in cell walls by regulating the transcription of SCW genes (Hao et al., 2021).

Our work suggests that the *Arabidopsis* POLYOL/MONOSACCHARIDE TRANSPORTER (PMT), which was first named POLYOL TRANSPORTER (PLT) gene family back in 2005, are a family of monolignol transporters. Members of the family are shown to transport a broad range of substrates, including sugar alcohols. Work from others suggest that glycosylated monolignols may be the transport form of monolignols. AtPMT5, a polyol/cyclitol/monosaccharide-H+-symporter, is among the best characterized member of the group. A proton-coupled transport mechanism has been shown for AtPMT5. Both in the yeast system and in the Xenopus system, AtPMT5 activity was clearly localized to the plasma membrane, suggesting that other members are also plasma membrane transporter and not on the vacuole. Expression of AtPMT5 was found primarily in sink tissues: in the elongation zone of roots, in the inflorescence stem, and several floral structures, especially in the floral abscission zone. Mechanical wounding and insect feeding induces expression, which is a common characteristic of lignin genes. Wounding, dehiscence, or programmed cell death release metabolites into the cell wall space, and it is expected that surrounding cells have the capability to take up the released compounds as demonstrated by Smith et al. in the case of monolignols. *Arabidopsis*, unlike celery, Plantago, or sour cherry fruit, does not translocate or accumulate sugar alcohol in significant quantities and likely have different physiological roles reserve for secondary cell wall development. AtPMT5 transports a wide range

of monosaccharides and modified sugars indicating a possible role in recovery of a variety of sugars and sugar derivatives. For example, alpha-methylglucose was transported at a high rate and glucuronate was transported at a low rate indicating that alpha-glucosides and sugar acids can serve as substrates. This serves as evidence that if monolignols are transported it would be in the glycosylated form.

We are interested in restoring the WT phenotype in *pmt4-1* and *pmt4-2* lines by using an overexpression construct for PMT4 to determine if the Col-0 lignin phenotype is recovered.

4.3 Mutant analysis of AtPMT4

In summary, *pmt4-1* and *pmt4-2* display increased growth sensitivity to coniferyl alcohol, reduced lignin content, altered lignin composition, and increased susceptibility to cell wall hydrolytic enzymes. For these reasons, we suspect that AtPMT4 is a monolignol transporter, particularly for coniferyl alcohol and sinapyl alcohol. AtPMT4 likely transport coniferyl and sinapyl alcohol with the help of a proton pump, which is in contrast with current models of monolignol translocation that have been characterized as either primary active transport or simple diffusion. Instead, a mechanism that takes advantage of the concentration gradient establish by the synthesis of monolignols and its consumption during the polymerization of lignin synthesis is more likely. We think it is likely that transporters such as AtPMT4 work in conjunction with simple diffusion to supply monolignols to the site of synthesis and during various abiotic stress scenarios where monolignols are required for wounding sites.

Our findings on AtPMT4 combined with the reported literature on PMT1/2 and PMT5 support our hypothesis that transporters likely function to translocate monolignols for lignin synthesis. PMT4 is likely a broad transporter and although it may not transport sugars to any

meaningful extent since the K_m of AtPMT5 is lower than known sugar transporters, it is possible that AtPMT4 transports modified sugars, instead, such as glycosylated monolignols.

AtPMT4 is expressed in the tissues and cell types that undergo lignification similar to PMT1/2, which has been shown to be induced by wounding and is associated with sugar substrates. Perhaps monolignol glucosides are also transported by PMT transporter to areas under stress.

We demonstrated a phenotype with two individual CRISPR lines. One, however, appears to be heterozygous for the frame-shift mutation. This is reflected in the reduce phenotype of *pmt4-2* relative compared to *pmt4-1*. Further, it is unlikely that AtPMT4 is the only transporter with the function of interest in *Arabidopsis*. Other genes with similar functions likely exist, which is why we did not observe a deleterious effect when AtPMT4 was mutated. Instead, the plants were mostly normal, like in other reported cases when PMTs were individually mutated. To further demonstrate and understand the function of AtPMT4 in lignification, additional genetic lines are needed.

4.4 Practical implications of my research

A potentially interesting finding from the research described in this thesis is that single mutation of a member of the PMT family result in a loss of lignin in cell wall analytical experiments, but not total loss of lignin or impaired plant growth, indicating the possibility of gene redundancy or other mechanisms of transport such as diffusion. A AtPMT4 mutant that excludes coniferyl alcohol and sinapyl alcohol but retains an ability to bind to *p*-coumaryl alcohol could conceivably enhance bioenergy crop species that are susceptible to pre-treatment since neither coniferyl alcohol nor sinapyl alcohol are required for *Arabidopsis* growth. In this respect, all currently existing AtPMT4 mutants should be tested for cell wall integrity. To test the possibility that PMT4 may confer

differential lignification in bioenergy crops, the pmt4 mutant reported in this thesis should also be tested for enhanced cell wall pretreatment susceptibility within other plant systems such as *poplar*, *Sorghum*, and *Brachypodium* (Porth et al., 2015; Bryant et al., 2020).

In addition to probing the compounding effects of mutating multiple members of the PMT family, there is also the possibility that zip-lignin in the plant cell wall can be increased. *FMT* poplar lines show limitations to the degree of zip-lignin incorporation. We think this limitation is due to the competition between FMT and transporters such as AtPMT4 for the monolignol substrate. The K_m of FMT for coniferyl alcohol is approximately 100 µM while the km of PMT is either equal or lower. If competition against FMT can be reduced, we think additional amounts of coniferyl ferulate can be synthesized and successfully incorporated into the cell wall. My research implies that PMT is a target that can be mutated, perhaps along with other members of the PMT family, to reduce competition against FMT and generate more superior poplar bioenergy lines. We think this can open a new area of research for combinatorial studies of PMT and FMT for the GLBRC to pursue where mutant studies are not only conducted in poplar, but for *Sorghum* and other bioenergy productive plants of interest. Recently, we have initiated a collaboration with Dr. Shi-You Ding in the Plant Biology department at MSU, to transform our CRISPR lines with an FMT construct to determine if zip-lignin does increase. In a similar approach, we are interested in determining if overexpression of AtPMT4 in a transformed Col-0 background expressing FMT will decrease zip-lignin in the cell wall because of increase competition from the transporter for the coniferyl alcohol substrate. Six genetic lines have been performed and are ready for screening: *pmt4-1* + 35SPMT4, *pmt4-2* + 35SPMT4, *pmt4-1* + FMT, *pmt4-2* + FMT, AtHcFMT1-1 + 35SPMT4, and AtHcFMT1-2 + 35SPMT4. If successful, such combinatorial knockouts of PMT and

introduction of FMT may be further engineered for increased zip-lignin incorporation of the cell wall. Ultimately, we wish to understand if knockout of these genes in poplar would yield similar results/phenotypes. A collaboration with a research group who specializes in performing poplar transformation could aid in the production of a poplar line with PMT4 knockout and another line that is an FMT background with PMT4 knockout to compare the effect. Such a transformation might have biotechnology applications.

4.5 Conclusion

Genetic approaches to understanding the role of monolignol transport in plants have elucidated many important components of monolignol translocation and lignification mechanisms. Since the discovery of monolignols and their important role in plant development and defense many research groups around the world have contributing to the understanding of this transport pathway. During my thesis research, many advances in our understanding of monolignol transport have been made, and it has been exciting to witness, and to be a part of, the progress in this field. Through the identification of a transporter gene outside of the ABC transporter family, my research has contributed to our overall-all understanding of the genetic requirements for transport of coniferyl alcohol and sinapyl alcohol. In the future, I expect that

our knowledge of monolignol transport will be further enhance by studies focusing on mutant analysis of other members of the PMT family and the phenotypic consequences to lignin.

Further, as more components of monolignol synthesis pathways are elucidated, our understanding of the immensely complex lignification pathway and how these polymers integrate growth, developmental, and defense throughout the life of plant will continue to grow alongside our technological advancements to harness their role in bioenergy.

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