TRANSGENIC MANIPULATION OF TUBULINS IN *POPULUS* ALTERS CELL WALL PROPERTIES AND DROUGHT RESPONSE CHARACTERISTICS

by

PRASHANT SHIDRAM SWAMY

(Under the Direction of Chung-Jui Tsai)

ABSTRACT

Microtubules (MTs) are dynamic cytoskeletal polymers of alpha-(TUA) and beta-(TUB) tubulins. Various processes including the deposition of cellulose microfibrils in the developing cell wall are thought to depend on MT function. In turn, post-translational modifications (PTMs) at the C-terminal end of the tubulin monomers contribute to the regulation of MT growth and stability. In order to investigate the importance of MTs during cell wall formation, a small subset of xylem-abundant tubulins (*TUA1*, *TUA5*, *TUB9* and *TUB15*) including PTM mimics of *TUA1* (dY and dEY) were expressed ectopically in transgenic *Populus*. Out of several *TUA* - *TUB* gene pairs used for transformation, only combinations containing PTM mimics of *TUA1* led to transgenic plants. Plants expressing the TUA1dY+TUB9 or TUA1dEY+TUB15 combinations were used for further characterization. Transgenic plants exhibited changes in cell wall properties, although cellulose, hemicellulose and lignin contents were unaffected. Based on cell wall glycome profiling, the pectin-xylan polysaccharide matrix was altered in stem wood of both transgenic groups. Lignin composition was altered in the transgenics which exhibited decreased S/G monomer ratios.

Guard cell dynamics are highly dependent upon MT dynamics, and therefore, leaf gas exchange characteristics were determined in plants exposed to short- or long-term water deficits. During a short-

term, acute drought stress, source leaf transpiration and net photosynthesis rates were higher in transgenic than wild type plants. Leaf gas exchange characteristics did not differ between transgenic and wild type plants maintained at chronically reduced soil water potential. Mature leaves also exhibited greater width-to-length ratios in TUA1dY+TUB9 compared to the other plant lines. The results suggested that tubulin manipulations in *Populus* had pleiotropic effects on cell wall deposition, guard cell dynamics and cell expansion.

INDEX WORDS: *Populus*, tubulin, microtubules, posttranslational modification, cell wall property, microfibril angle, wood density, acute drought, chronic drought, photosynthesis, stomatal conductance, transpiration rate, plant cell expansion

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Introduction

The secondary cell wall is critical for strength and load bearing capacity of large plants like trees. Cell wall biogenesis depends in large part on a coordination of cytoskeletal dynamics with biosynthesis of structural macromolecules. While numerous advances have contributed to our understanding of lignocelluose biosynthesis (Gilbert, 2010) and its master regulators (transcription factors, Zhong et al., 2008), the function of the cytoskeleton in cell wall formation remains comparatively understudied (Chan, 2012; Shaw, 2012). A role for cortical microtubules (MTs) in cellulose microfibril (MF) deposition was first implied nearly 50 years ago, based on the physical parallelism between MTs and MFs in root cells (Green, 1962; Ledbetter and Porter, 1963). Higher MT densities and co-alignment of MTs and MFs were also observed at sites of secondary cell wall thickening in differentiating tracheids or wood fibers (Abe et al., 1995; Chaffey et al., 1997; Chaffey, 1999). The angle between MFs and the longitudinal axis of the cell is referred to as the microfibril angle (MFA). The MFA underlies an important wood quality trait, tensile strength (Reiterer et al., 1999), and MF with a low MFA therefore provides greater strength to the cell wall (Barnett and Bonham, 2004). The secondary cell wall of wood fibers is very thick, and consists of three distinct layers (S1-S3). The MFA is lowest in the thickest layer (S2) of the secondary wall, and is generally random in the primary cell wall (Abe and Funada, 2005).

In addition to a role for MTs in cellulose deposition, there may be some interaction with pectin polysaccharides as well. For example, dense aggregates of MTs in developing seed coats of *Arabidopsis* coincide with localized areas of pectin secretion (McFarlane et al., 2008). It was demonstrated that normal distribution of methylated pectin is necessary for normal MT-MF co-alignment and anisotropic growth in

Arabidopsis hypocotyls (Yoneda et al., 2010). According to these findings, MTs appear to physically interact with pectins or other cell wall polymers apart from their well-known connection in cellulose microfibril orientation during the cell wall assembly.

Microtubules are composed of heterodimeric alpha-(TUA) and beta-(TUB) tubulin subunits (Nogales et al., 1998; Nogales, 2001). Both TUA and TUB are encoded by multi-gene families in eukaryotes. The TUA and TUB gene families are mostly similarly sized in lower eukaryotes, human and mouse (Sullivan, 1988; Gray et al., 2013). In plants, however, the gene families generally differ from each other in size. For example, *Populus* has, 20 *TUB* versus 8 *TUA* genes. Two *TUA* and two *TUB* genes comprise a small subset of these that are strongly expressed in developing wood tissues (Oakley et al., 2007). A unique feature among the *Populus* tubulin genes is their sequence hyper-variability at the C-terminus (Oakley et al., 2007). C-terminal regions of animal, lower eukaryote and parasite TUAs and TUBs contain sites for various post-translational modifications (PTMs) which affect MT stability and association with MT-interacting proteins (Xia et al., 2000; Westermann and Weber, 2003; Belmadani et al., 2004). However, less is known about PTMs in plant tubulins. Transgenic approaches that target PTM function can be applied to *Populus* to learn whether PTM function is conserved from animal models, and whether tubulin PTMs play a role in a process such as wood formation.

Elucidation of MT function that can be linked to MF deposition has largely been based on pharmacological approaches (DeBolt et al., 2007) and mutant analysis (Ishida et al., 2007). Transgenic manipulation of tubulins has also been utilized, but that approach has been fraught with difficulties. For instance, acute cytotoxic effects were observed when *TUB*, but not *TUA*, was overexpressed in yeast (Weinstein and Solomon, 1990). In work with transgenic maize or tobacco, no transformants were recovered by manipulating individual *TUA* or *TUB* gene members, but transformants were obtained when a balanced *TUA* and *TUB* expression was achieved (Anthony and Hussey, 1998; Anthony et al., 1999). By contrast, transgenic *Arabidopsis* with mis-regulated *TUA* or *TUB* were viable, although plant morphology was affected (Bao et al., 2001; Burk et al., 2006). Such outcomes may highlight the risks of tubulin manipulation by transgenic means in general. The risks are probably inherent due to the role of

tubulins in so many fundamental cellular processes (Johnson and Porter, 1968; Thissen et al., 1997). The possibilities highlight a need to recognize in advance that several transgenic strategies may have to be employed to successfully manipulate tubulin expression.

The present research aims to address whether *Populus* tubulins can be transgenically manipulated, and whether such manipulation can impact MT properties, wood formation and plant development. *Populus* was chosen as the experimental system because of the unusual characteristics of its tubulin families, its rich genomics resources and amenability to genetic transformation. The specific objectives are to:

1. Produce transgenic *Populus* plants that over-express xylem-preferential *TUA* and *TUB* isoforms and their PTM mimics in combination.
2. Analyze the transgenic effects on wood development and chemical composition.
3. Investigate the effects of tubulin perturbation on MT-mediated cell expansion and guard cell behavior under different drought regimes.

Literature review

Microtubule structure and dynamic instability

Microtubules (MTs) are proteinaceous cytoskeletal structures present in all eukaryotes. MTs consist of 13 protofilaments, each made up of polymers of TUA-TUB heterodimers arranged in a head-to-tail manner (Nogales, 2000). The head-to-tail assembly of the heterodimers underlies MT polarity: the plus (growing) end corresponds to the TUB end of the dimer; while TUA is exposed at the minus end of MTs. Both TUA and TUB are GTP-binding proteins, but only the TUB-bound GTP is hydrolysable to GDP (Nogales et al., 1998; Nogales, 2000). MTs constantly undergo polymerization and de-polymerization in a process called dynamic instability, which is modulated by the hydrolysis state of GTP (Nogales, 2001). Polymerization occurs at the plus end with the addition of a GTP-bound TUB, this end is kinetically more dynamic than the minus end containing the TUA subunit (Wilson and Jordan, 1995). Dynamically unstable MTs rapidly undergo alternating phases of growth and shrinkage. This happens

when the GTP cap is lost or hydrolyzed at the plus end of the MT (Panda et al., 2002; Caplow and Fee, 2003). It has been shown that the *Arabidopsis* MTs also exhibit some degree of growth-biased "treadmilling", owing to MT bundling and repositioning at the cell cortex (Shaw et al., 2003; Ehrhardt and Shaw, 2006). Hybrid treadmilling in MTs is a combination of constant addition and removal of heterodimers at the plus and minus end, respectively.

Microtubule organization is closely aligned with cellulose microfibril orientation

Various natural and synthetic anti-mitotic drugs are either MT-stabilizing or MT-de-stabilizing agents. MT-stabilizing agents, including taxol, promote MT polymerization while de-stabilizing agents, such as colchicine and dinitroaniline-related compounds, prevent heterodimer assembly into MTs. The observation that treatment of algal cells with colchicine disrupted not only MT organization, but also the orientation of MFs is what led Green (1962) to the MF-MT co-alignment hypothesis.

Various anti-MT agents have been shown to alter MT dynamics and cause morphological anomalies. Some of these compounds specifically target plant MTs and have been exploited as potent herbicides. One example is dinitroanilines, such as oryzalin which binds to tubulin dimers to form a tubulin-oryzalin complex for co-polymerization with tubulins, and inhibit subsequent polymerization (Hugdahl and Morejohn, 1993). Structural modeling (Ishida et al., 2007) as well as analysis of naturally occurring dinitroaniline-resistant goosegrass (Anthony and Hussey, 1999) pointed to TUA as the potential binding target of dinitroanilines. The benzamide herbicides, propyzamide and RH-4032, have been found to cause a swollen root phenotype by binding to TUB and inhibiting MT assembly (Young and Lewandowski, 2000). Screening of small-molecule chemical libraries for inhibitors of cell wall synthesis in *Arabidopsis* identified morlin as another potent anti-MT drug (DeBolt et al., 2007). Morlin appears to act directly on MTs, with pleiotropic effects on cellulose synthase (CesA) function and cell morphogenesis (DeBolt et al., 2007). The well-known anti-cancer drug taxol functions by stabilizing cortical MT arrays (Schiff and Horwitz, 1981; Bajer et al., 1982). Taxol causes a swollen root phenotype in both the meristematic and elongation zones of *Arabidopsis* roots, whereas the polymerization inhibitor

oryzalin affects mainly dividing meristematic cells (Baskin et al., 1994). Together, these results support roles for MTs and MT dynamics in MF organization and plant morphogenesis.

Mutants and transgenic plants defective in MT organization

Taxol, oryzalin and other anti-MT pharmacological agents have been used to facilitate screening of MT-relevant genes or gene modifications in mutants or transgenic plants with MT defects (Burk and Ye, 2002; DeBolt et al., 2007; Ishida et al., 2007). For example, a single missense mutation at the Drp (dinitroaniline response phenotypes) locus in goosegrass provided natural resistance against dinitroaniline herbicides and this locus was found to harbor a *TUA* gene (goosegrass TUA1). This mutation affected herbicide binding to TUA, thereby rendering the herbicide ineffective (Yamamoto et al., 1998). A suite of 32 tubulin mutants with altered anisotropic growth phenotypes or sensitivity to propyzamide treatment were identified by screening EMS (ethane methyl-sulfonate) mutagenized M2 or T-DNA insertional mutant populations, followed by genetic mapping (Ishida et al., 2007). Of those 32 mutants, 17 were due to the mutations in *TUA* while the others had *TUB* mutations. A majority of the strong helical mutants that were isolated carried mutations in either *TUA4* or *TUB4* genes (Ishida et al., 2007). These mutations mapped to amino acids at the intra or inter-dimer contacts of tubulin, the lateral interacting regions of adjacent protofilaments, or the GTPase activating region, regardless of the subunit affected (Ishida et al., 2007). The severity of the twisting phenotype depended on location of the point mutations. The same mutant screen approach has also led to the identification of other proteins involved in MT organization, including a PHS1 phosphatase, which regulates phosphorylation of mitogen-activated protein kinase (MAPK-Pase, Naoi and Hashimoto, 2004) and two MT-associated proteins, SPIRAL1 (SPR-1, Nakajima et al., 2004) and SPIRAL2 (SPR-2, Shoji et al., 2004).

These findings highlight the importance of tubulin protein structure and PTM sites for normal cytoskeletal functioning and plant morphology (reviewed in Thitamadee et al., 2002; Buschmann and Lloyd, 2008). *MOR1* (Microtubule Organization 1) was discovered as an important microtubule-associated protein (MAP) in *Arabidopsis*, essential for organizing cortical MTs at various developmental

stages and maintaining cell anisotropy (Whittington et al., 2001). A point mutation in this gene caused several temperature-dependent developmental defects due to MT shortening and loss of ordered alignments at the cell cortex. Although the mutant did not have any apparent defects in cell division, it exhibited isotropic cell expansion, organ twisting, impairment in root hair polarity and floral defects which caused sterility. A mutation in the gene encoding katanin severing protein, *fra2*, revealed that ordered MT orientation is necessary for normal cellulose microfibril deposition in the primary and all layers of the secondary cell wall (Burk et al., 2001). In the *fra2* mutant, cellulose and hemicellulose contents were reduced because of altered MT dynamics (Burk and Ye, 2002). *Arabidopsis* EMS mutant screening with oryzalin identified two loci linked to orientation of MT and apparent cell swelling in root hypocotyl cells. These two mutations were mapped to *PROCUSTE1*, which encodes CESA6, a cellulose synthase, and KORRIGAN, which encodes endo-1,4-b-D-glucanase, both involved in cellulose biosynthesis (Bhandari et al., 2006; MacKinnon et al., 2006). These cell wall mutants exhibited randomized MT organization upon treatment with the cellulose biosynthesis inhibitor, isoxaben, but MT organization was not affected by a limited cellulase treatment (Paredez et al., 2008). All these data provided genetic evidence that cross talk between MT and MF is essential for normal orientation of MTs and ordered deposition of cellulose MFs in the developing cells.

In addition to the MT-MF correlation, recent studies demonstrate that MTs have prominent roles in the deposition of non-cellulosic cell wall constituents, especially pectins. Pectins are derived from galacturonic acids and are important components of the primary cell wall (Ridley et al., 2001). Pectins function in various stages of plant growth and development, including plant defense (Mohnen, 2008). The development of *Arabidopsis* seed contain a short but intense period of pectin deposition at a small domain of plasma membrane. Although the normal distribution of actin network was observed, cortical MTs concentrate at those small areas of the plasma membrane while their densities were quite low elsewhere (McFarlane et al., 2008). The normal distribution of methylated pectin polysaccharides in the cell wall also influences the anisotropy of cell expansion, possibly by physical interaction with MT-MF alignment (Yoneda et al., 2010). In addition to the pectins, disruption of arabinogalactan proteins was achieved by

binding with active Yariv that disorganized cortical MTs and disturbed MT-MF co-alignment in *Arabidopsis* (Nguema-Ona et al., 2007). Associations of cortical MTs with pectins or proteoglycans in the cell wall indicate that this physical interaction may be necessary in and/or precede the cellulose deposition. Whether MTs regulate the pectin assembly into cell wall or pectins/proteoglycans regulate the MT-MF co-alignment remains to be investigated.

Transgenic manipulations of tubulins

Transgenic manipulation of individual tubulin genes did not produce viable transgenic plants in maize or tobacco but double transformations (*TUA* and *TUB*) did (Anthony and Hussey, 1998; Anthony et al., 1999). In transgenic yeast, artificially high ratios of *TUB* to *TUA* led to arrested cell division and to cytotoxicity due to aberrant MT structures, whereas overexpression of *TUA* resulted in slower loss of cell viability and suppressed cytotoxicity due to excess TUB (Weinstein and Solomon, 1990). Transgenic *Arabidopsis* expressing a modified *TUA* with an N-terminal GFP fusion or a hemaglutinin epitope tag were viable, but exhibited twisted growth (Abe and Hashimoto, 2005). Similar findings were reported for *Arabidopsis* that over-expressed a *GFP-TUA* (Burk et al., 2006) or that exhibited reduced *TUA* expression (Bao et al., 2001). In *Arabidopsis* plants expressing TUA with N-terminal tags, the dynamics of cortical MT arrays were compromised due to inhibition of the GTPase activating domain (Abe and Hashimoto, 2005). Overall, disruption of cortical MT arrays by mutation, transgenesis or pharmacological interventions resulted in various developmental defects in cell morphogenesis, cell anisotropy and growth (Bao et al., 2001; Abe and Hashimoto, 2005; Burk et al., 2006; Ehrhardt and Shaw, 2006; DeBolt et al., 2007).

Differential expression of tubulin genes: Expression in vegetative tissues

Due to the fact that tubulins are found in all tissues, they have been used routinely in expression studies as housekeeping controls. However, many TUA and TUB isoforms are spatiotemporally regulated in response to various developmental and environmental cues (Wasteneys, 2004; Radchuk, 2008). In

Arabidopsis, five of six TUAs are expressed in vegetative tissues at different levels (Kopczak et al., 1992; Abe et al., 2004), while *AtTUA1* is expressed exclusively in pollen (Carpenter et al., 1992). In barley, most TUA isoforms are differentially expressed during the development of leaf mesophyll cells (Schroder et al., 2001). Of the eight *Populus* TUAs, six are expressed at various levels in different vegetative tissues, including developing xylem, while *PtTUA6* and *PtTUA8* are predominantly expressed in pollen (Oakley et al., 2007). In the case of *Populus TUBs*, different sets of genes are preferentially expressed in pollen and developing xylem that outnumber the genes observed at these tissues in other species. For example, *Arabidopsis AtTUB1* and *AtTUB5* are expressed in seedlings, leaves, stems, roots, *AtTUB8* is expressed in vascular tissues, while *AtTUB9* is pollen-specific (Chu et al., 1998). Differential expression of *TUB* in different tissues has also been reported in maize (Villemur et al., 1994) and rice (Yoshikawa et al., 2003). Taken together, the data underscores the importance of spatiotemporal regulation of tubulins during various developmental stages.

Expression during secondary cell wall synthesis

Considering the importance of MTs during cellulose MF deposition, it is not surprising that several tubulin genes exhibit strong expression in cells undergoing secondary cell wall thickening. Developing cotton fiber has been a model system for cellulose biosynthesis research. The fibers originate from epidermal cells of ovules via a diffuse-growth elongation mechanism (Tiwari and Wilkins, 1995). The secondary cell wall of cotton fibers is composed entirely of cellulose, and MT organization patterns correlate with cellulose MFs (Seagull, 1992). Seven *TUAs* are expressed during cotton fiber elongation (Whittaker and Triplett, 1999). Three of them, *GhTUA1*, *GhTUA5* and *GhTUA9* are abundant in the initial fiber elongation stage, which coincides with primary cell wall synthesis (Li et al., 2007). Of these genes, *GhTUA9* was shown to promote cell elongation in yeast when expressed ectopically (Li et al., 2007). The expression of *GhTUA2*, *GhTUA3* and *GhTUA4* remained strong at the onset of secondary cell wall synthesis and continued through the later stages of fiber development (Whittaker and Triplett, 1999). This is consistent both with the continued importance of tubulin synthesis during cellulose accrual, and with

the idea of functional differences between tubulin isoforms. Similar fiber-preferential expression was also observed for a suite of nine *GhTUB* genes (He et al., 2008). The *Zinnia* cell culture system has also served as a model for vascular development, because cultured mesophyll cells can be induced to trans-differentiate into tracheary elements (Yoshimura et al., 1996). Consistent with a role for MTs in vascular development, transcripts of two *TUB* (*ZeTUB1* and *ZeTUB3*) genes were elevated in cultured mesophyll cells during trans-differentiation (Yoshimura et al., 1996). In *Populus*, *TUB* genes are expressed at much lower levels compared to *TUAs* (Oakley et al., 2007). Distinct patterns of *TUB* expression in developing xylem (*PtTUB9, PtTUB15, PtTUB16* and *PtTUB13*), pollen (*PtTUB19, PtTUB20, PtTUB7, PtTUB8* and *PtTUB15*) and roots (*PtTUB15*) were observed, while remaining *TUB* genes were expressed at very low levels in vegetative tissues (Oakley et al., 2007). Furthermore, the expression of xylem-abundant genes was elevated in the tension wood which contains high amounts of crystalline cellulose, supporting their association with cellulose MF arrays (Oakley et al., 2007). The *PtTUB9* ortholog in *Euculyptus grandis*, *EgTUB1*, also exhibited wood fiber specific expression (Spokevicius et al., 2007).

Expression during reproductive stages

Phylogenetically conserved, pollen-specific TUA and TUB isoforms were reported in *Arabidopsis* (Carpenter et al., 1992; Snustad et al., 1992; Cheng et al., 2001), rice (Yoshikawa et al., 2003), maize (Villemur et al., 1994) and *Populus* (Oakley et al., 2007). *Arabidopsis AtTUA1* is exclusively expressed in pollen grains and accumulates to higher levels during pollen tube growth (Carpenter et al., 1992). Expression of *TUA1* in pollen of Chinese cabbage has been associated with cell division processes (Zhang et al., 2009). Its expression was significantly lower in cytoplasmic male sterile (CMS) line, indicating that this gene is involved in regulation of male fertility (Zhang et al., 2009). The phylogenetically related *PopulusPtTUA6* and *PtTUA8* were abundantly expressed in pollen but absent elsewhere (Oakley et al., 2007). Pollen expression was also observed for class I members, such as *PtTUA1*, *PtTUA5* and *PtTUA7* that were also abundant in other tissues (Oakley et al., 2007). Pollen-specific expression has also been reported for classes III and IV TUBs. Localization of class IV *AtTUB9*

to pollen grains was confirmed using both reporter gene analysis and *in situ* hybridization (Chen et al., 2011). In rice, class III *OsTUB8* was found exclusively in pollen (Yoshikawa et al., 2003), similar to its maize ortholog *ZmTUB4* (Villemur et al., 1994). In *Populus*, the most abundant *TUB* transcripts in pollen, i.e., *TUB19* and *TUB7* also belong to classes III and IV, respectively (Oakley et al., 2007).

Microtubules and stress response

Plants face various environmental stresses, and the stress responses likely involve MTs. In angiosperms, for example, tension wood is produced in response to gravitropic stimuli which can be altered by tree-bending or leaning (Pilate et al., 2004). Tension wood is enriched with highly crystalline cellulose (Timell, 1969), and exhibits increased MT density (Prodhan et al., 1995). Consistent with this, the mRNA transcripts of a suite of xylem-abundant tubulin genes in *Populus* are induced upon tension wood stress (Oakley et al., 2007). It was proposed that MTs act as gravity mechano-sensors, able to change their orientation and, in turn, the orientation of cellulose MFs in response to gravity (Wymer et al., 1996).

Salt adaptive shoots of *Eucalyptus microcorys* exhibited several defects in the chloroplast ultrastructure and respiration capabilities that included loss of cell turgor pressure (Keiper et al., 1998). Later study determined that one of the *TUA* gene was strongly down-regulated under salt stress and adaptation (Chen and De Filippis, 2001), suggesting the possible effects on MTs upon salt exposure. In spring wheat, expression of several *TUA* genes was responsive to temperature changes in varying patterns (Farajalla and Gulick, 2007). Two *TUA* paralogs identified in freeze-tolerant variety were found to be differentially regulated during cold acclimatization of winter wheat (Christov et al., 2008). Low temperature, freezing and dehydration have been reported to cause MT destabilization and lead to cell death in different plant species (Bartolo and Carter, 1991).

In plants, guard cells regulate stomatal aperture size for gas exchange and transpiration. Aperture control is mediated by various signal transduction cascades and involves MTs and other MT-interacting proteins (Kim et al., 1995; Zhang et al., 2008). Stomatal aperture size is primarily controlled by the turgor

pressure of the flanking guard cells, in conjunction with dynamic cellulose MF (Palevitz, 1976) and MT rearrangements (Cyr, 1994). For example, the diurnal regulation of stomatal opening and closure is correlated with the respective rearrangements of MTs (Fukuda et al., 1998). While the cross-talk between pectins or related polysaccharides and MTs is known (Andeme-Onzighi et al., 2002; Nguema-Ona et al., 2007; Yoneda et al., 2010), pectin components also contribute to the flexibility of guard cells (Jones et al., 2003). Biosynthesis of ABA is sensitive to changes in MT dynamics and osmotic stress in the root cells of *Zea mays* (Lu et al., 2007). Overexpression of Translationally Controlled Tumor Protein (TCTP) that binds to MTs and affect the MT stability enhances drought tolerance by ABA and calcium mediated stomatal closure (Kim et al., 2012). These data provided support for the involvement of MTs in preserving cell physiology during stress conditions.

Tubulin gene families: TUA and TUB isoforms

Tubulins are highly conserved, both within and between the TUA and TUB families, and across kingdoms (Dutcher, 2001). Co-polymerization of TUAs or TUBs chimeras from different species into functional MTs further supports their evolutionary conservation (Anthony and Hussey, 1999; Anthony et al., 1999). In higher plants, tubulin proteins are encoded by multiple gene families with unequal numbers. TUA and TUB isoform numbers range from six TUA and nine TUB in *Arabidopsis* (Kopczak et al., 1992; Snustad et al., 1992) to eight TUA and twenty TUB in *Populus* (Oakley et al., 2007). In the monocot maize, six TUA and eight TUB genes were identified (Villemur et al., 1992; Villemur et al., 1994), while four TUA (Yuan et al., 2005) and eight TUB (Yoshikawa et al., 2003) genes were reported in rice. As many as 15 TUA genes are present in the hexaploid wheat genome, corresponding to five homologous groups of three genes each descending from the three ancestral genomes (Farajalla and Gulick, 2007). Of the eight *Populus* TUAs, four originated from gene duplication events (Oakley et al., 2007). The predicted TUA proteins range from 449 to 451 amino acids in length and share 88-98% sequence similarity. Significant expansion is also observed in the TUB family of *Populus*, involving both whole genome duplication and tandem gene duplication events. These have given rise to 10 highly

homologous pairs of TUB genes in *Populus*. The predicted TUB proteins have 444-451 amino acids and share 89-98% overall sequence similarity. Although the cause of this complexity is unknown, the differential expansion between TUA and TUB families may contribute to a variety of specialized functions e.g. secondary growth during evolution of higher plants.

Phylogenetic analysis

Previous phylogenetic analyses showed that plant TUAs fall into two distinct classes, suggesting their origin from two ancestral genes (Villemur et al., 1992). Within each class, monocot and dicot sequences typically form separate clusters. The eight *Populus* TUAs are evenly distributed in both classes, with four members each. PtTUA1, PtTUA3, PtTUA5 and PtTUA7 belong to class-I, while PtTUA2, PtTUA4, PtTUA6 and PtTUA8 belong to class-II (Figure 1, Oakley et al., 2007). Class-I includes several members that were found to be highly expressed in cells undergoing secondary wall thickening, including the xylem-preferential PtTUA1 from aspen and the cotton fiber-abundant GhTUA2/3 and GhTUA4 (Whittaker and Triplett, 1999). Several class-II TUAs were found to express specifically in reproductive tissues, such as the pollen-specific AtTUA1 (Carpenter et al., 1992) and the PtTUA6/8 paralogs (Oakley et al., 2007). Phylogenetic analysis revealed that plant TUBs clustered into four classes (Figure 2, Oakley et al., 2007). The *Populus* TUB family is unevenly distributed, with half of its members present in the class-I and class-I like group. This group includes the xylem-preferential aspen PtTUB9 PtTUB15, PtTUB16 and PtTUB13 (Oakley et al., 2007).

The class-II cluster is dominated by dicot members with a lower representation of monocots, while the reverse is true for Class IV. Class-III members contain distinguishable sequence variations (insertions or substitutions) at or near position 39. Some of the class III and class IV members were found to be pollen-specific, such as AtTUB9 (Carpenter et al., 1992), OsTUB8 (Yoshikawa et al., 2003), ZmTUB2 and ZmTUB4 (Villemur et al., 1994) and poplar PtTUB19/20 (Oakley et al., 2007).

Gene structure

Analysis of the *Populus* genomic sequence indicated that class-I and class-II TUA members have three and four introns, respectively. The intron positions are conserved within each family, but only the second intron position is conserved in all TUAs. In contrast, all TUBs have two introns located at conserved positions, except class-I members (PtTUB13-PtTUB18) that contain an additional intron in the 5' UTR region (Oakley et al., 2007). This intron was also found in several other Class-I members, and in rice it was shown to positively regulate the transcription of rice *TUB16* (Morello et al., 2002).

Tubulin post-translational modifications

Although highly conserved, distinct isoforms within both TUA and TUB gene families contribute to functional heterogeneity. This heterogeneity of tubulins is thought to underlie the diverse functions of MTs during cell development. However, tubulin functional heterogeneity can also arise from various post-translational modifications (PTMs). Documented tubulin PTMs include those more commonly found in other proteins, such as phosphorylation and acetylation, as well as those restricted to or first identified on tubulins, such as polyglutamylation (Edde et al., 1990), polyglycylation (Redeker et al., 1994), and detyrosination (Thompson, 1982). All these PTMs, except acetylation which occurs near the N-terminus, affect the C-terminal domain of TUAs and TUBs. The C-terminus of these proteins is exposed on the outside of the microtubules where they are well positioned to influence interactions with other proteins. All of these PTMs occur on tubulins after their assembly into MTs, except serine phosphorylation (S172) of TUBs, which inhibits TUB incorporation into MTs (Fourest-Lieuvin et al., 2006).

Acetylation occurs on the lumen side of MTs (Nogales et al., 1998) at the ε-amino group of lysine at position 40 (K40) (Lhernault and Rosenbaum, 1985). Although acetylation was thought to be evolutionarily conserved (Lhernault and Rosenbaum, 1985), some TUAs in vertebrates do not contain K40 where acetylation might occur (Ledizet and Piperno, 1987). In plants, class-I TUA isoforms contain K40, but not class-II TUAs. This suggests that the function of TUA acetylation may depend on other developmental or environmental cues. For example, lack of acetylated TUA did not have any observable

phenotypes in protozoa and nematodes (Kozminski et al., 1993; Gaertig et al., 1995; Fukushige et al., 1999). However, the acetylation and deacetylation modes of PTM have been linked to flagellar assembly and disassembly, respectively, in *Chlymadomonas* (Lhernault and Rosenbaum, 1985; Shea et al., 1990). In vertebrates, the movement of brain-derived neurotropic factor (BDNF)-associated vesicles (Dompierre et al., 2007) and the migration and branching of cortical neurons were both affected by TUA acetylation (Creppe et al., 2009). More recently, *Caenorhabditis elegans* mutants defective in acetylation of TUA were found to exhibit impaired vesicle movement and TUA protein turnover (Solinger et al., 2010). Furthermore, disruption of MEC-17, an acetyl transferase important in K-40 acetylation in *C. elegans*, resulted in defective touch neurons (Akella et al., 2010).

PTMs, such as polyglycylation (up to 34 glycine residues) and polyglutamylation (1-20 glutamyl units), occur at the C-terminal ends of TUAs and TUBs via formation of an isopeptide bond with the γ-carboxyl group of glutamic acid (E) (Wloga et al., 2009). Since both of these poly-residue side-chain modifications occurred at similar sites on TUA and TUB, their presence is speculated to be complementary (Xia et al., 2000; Rogowski et al., 2009; Wloga et al., 2009). Polyglycylation in *Tetrahymena termophila* TUA was found to be dependent on sexual parasitic cell type (Fennell et al., 2008). In similar organism, mutations at TUB glycylation sites had various effects ranging from defects in cell motility, cytokinesis to cell death where axonemes lacked the central tubulin pair (Thazhath et al., 2002). By contrast, overexpression of TTLLs (Tubulin Tyrosine Ligase-like) with tubulin elongase activity in *Tetrahymnea thermophila* increased accumulation of polyglutamylated stable MTs and provided nocodazole resistance (Wloga and Gaertig, 2010). Apart from its interactions with ciliary dyneins (motor proteins) and its role in flagellar motility (Gagnon et al., 1996), polyglutamylation of tubulins has been found to orchestrate protein function in maintaining mitotic spindles during cell division in HeLa cells (Regnard et al., 1999).

The tyrosination and detyrosination cycle is unique to TUAs, and has not been reported for other proteins thus far. In animals and other species including *Chlamydomonas*, the cycle involves removal and reattachment of the terminal tyrosine residue of TUA, catalyzed by tubulin-specific carboxypeptidase and

tubulin tyrosine ligase (TTL), respectively (Idriss, 2000). In some cases, detyrosinated (referred to as dY or Glu-tub, Figure 3) TUA is susceptible to further removal of the penultimate glutamine, which leads to non-tyrosinable TUA (Δ2 isoform, or dEY). As such, the dEY-TUA can no longer participate in the tyrosination-detyrosination cycle due to the lack of TTL recognition motif (GEE) (Figure 3, Ruediger et al., 1994). Several developmental programs are regulated by the tyrosination-detyrosination cycle in animals. Detyrosinated TUAs have been associated with stable MTs in neuroblastoma cells (Wehland and Weber, 1987); although stable MTs may not necessarily contain detyrosinated TUAs as seen in cultured fibroblast cells (Khawaja et al., 1988; Webster et al., 1990). The presence of excess Δ2-TUAs in mammalian brain has also been linked to higher MT stability (Paturle-Lafanechere et al., 1991; Alonso et al., 1993; Paturle-Lafanechere et al., 1994; Janke and Kneussel, 2010).

In plants, relatively little is known about tubulin PTMs. For example, tyrosine phosphorylation of tobacco TUAs and TUBs has been detected based on DEAE chromatography and immuno-precipitation, but no function has been speculated upon (Blume et al., 2008). In maize, the use of 2D PAGE followed by immuno-blotting with PTM-specific antibodies (of animal TUA origin) showed evidence of tubulin tyrosination, acetylation, and polyglutamylation in various tissues, but polyglycylation was not detected (Wang et al., 2004). The occurrence of tubulin PTMs in tobacco cell suspension cultures was also reported using PTM-specific antibodies (against tyrosinated, detyrosinated, Δ2, acetylated, polyglutamylated or acetylated animal TUAs), and is thought to contribute to the high degree of tubulin heterogeneity (Smertenko et al., 1997). Acetylated TUA signals were detected in mitotic spindles, phragmoplasts and cortical microtubules in pine root cells (Gilmer et al., 1999), supporting the importance of tubulin PTMs during cell division and other cell differentiation processes.

Populus harbors TUA and TUB genes with unusually high degrees of amino acid sequence variability at their C-terminal ends (Oakley et al., 2007). The C-termini are sites for all but two (e.g., acetylation and phosphorylation) tubulin PTMs described above. *Populus TUA* genes encoding unusual C-terminal ends (M-, E- or Q-types) outnumber (5 vs. 3) the more typical and evolutionarily conserved Y-type (tyrosinated) isoforms. One implication is that these isoforms may not participate in a tyrosination-

detyrosination cycle that is still hypothetical for plants, but which regulates tubulin dynamics in other systems. The M-type isoforms predominate in class I (3 out of 4), and include the xylem-abundant *PtTUA5*. The fact that both M-type and Y-type *TUA* genes are abundantly expressed in xylem and are up-regulated in tension wood (Oakley et al., 2007), raises questions about the complexity of tubulin regulation in wood forming tissues. Whether the genetically encoded variability in *Populus* substitutes or supplements PTM-derived tubulin diversity as reported in other species remains to be investigated. Development of an extensive secondary cell wall is characteristic of woody perennials where placement and integration of complex wall polysaccharides is regulated by many genetic components including tubulins. Interest in how tubulins (modified or non-modified isoforms) contribute to the assembly of complex polysaccharides and/or their integration into the cell wall matrix comprises the background rationale for the present investigation. The transgenic approach was employed in *Populus* to understand the possible roles of tubulins along with their PTM mimics on cell wall characteristics and overall plant development.

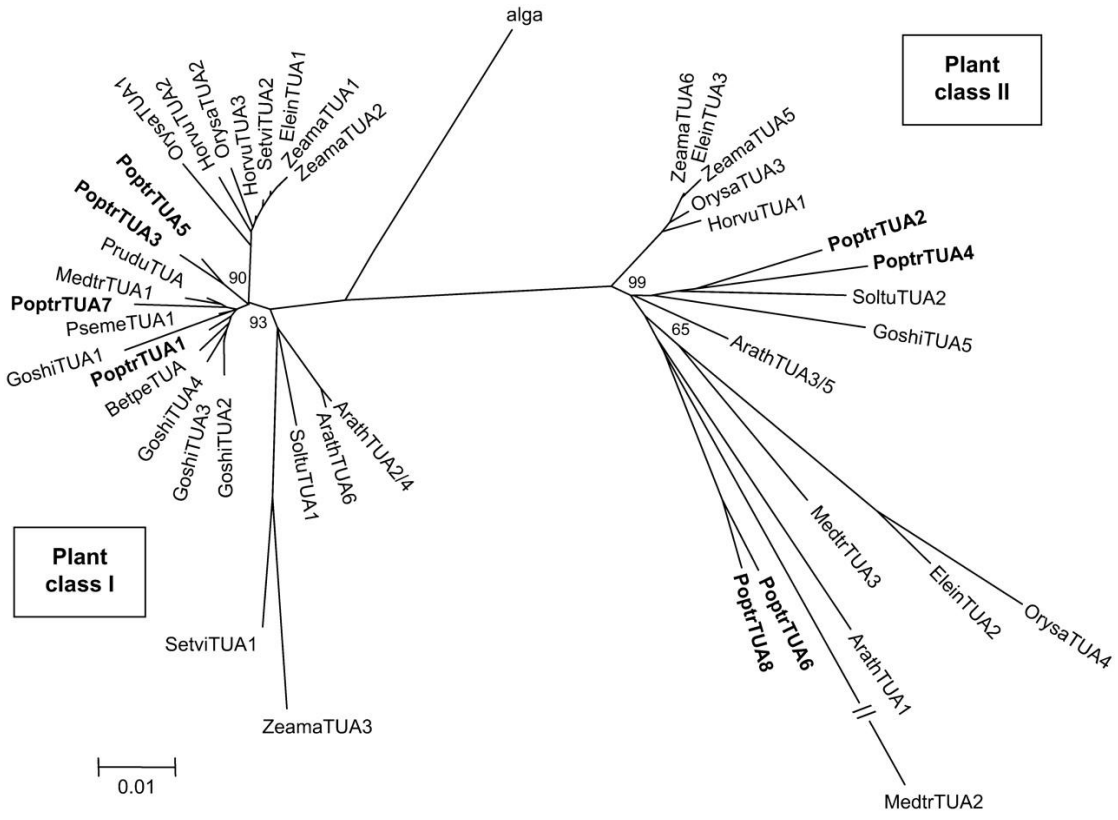

Figure 1.1. Minimum-evolution tree of representative full-length plant TUA proteins. Figure used from Oakley et al., (2007).

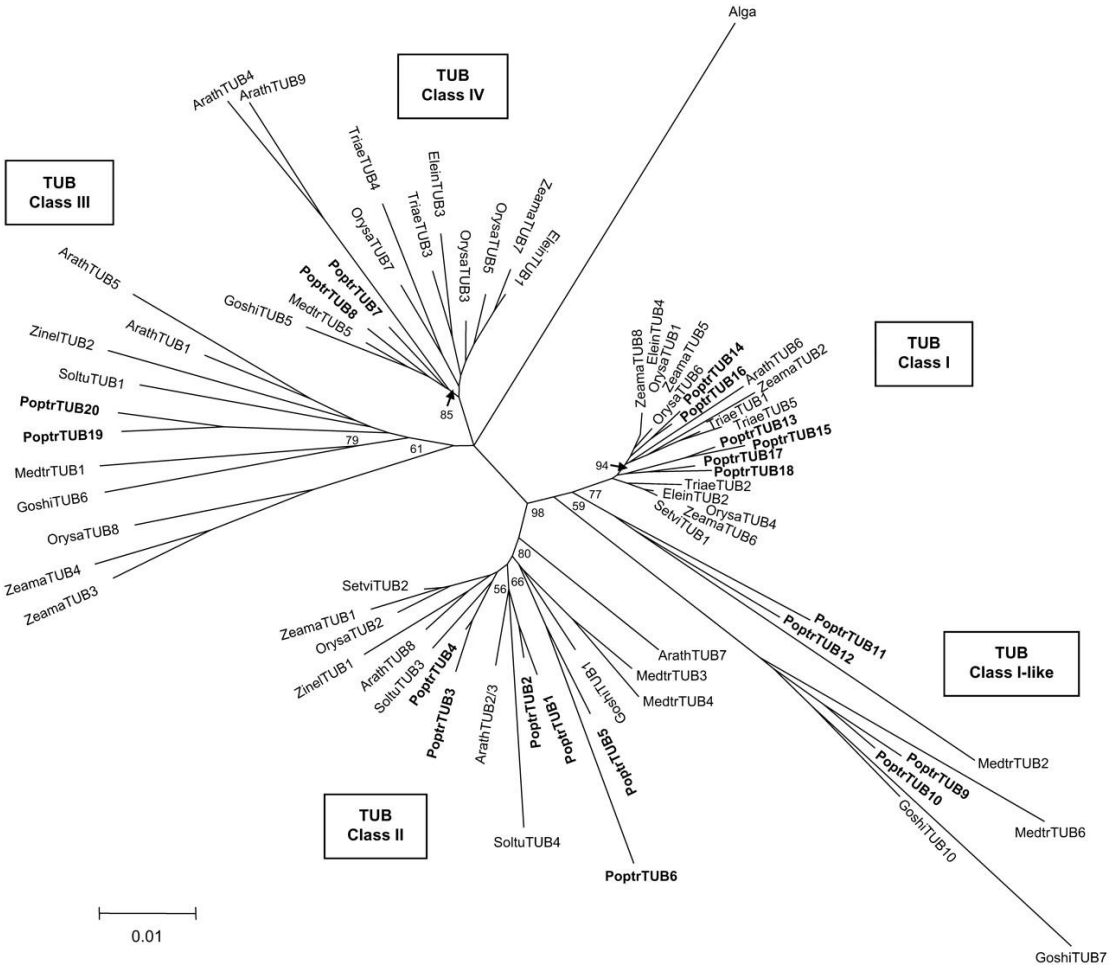

Figure 1.2. Minimum-evolution tree of representative full-length plant TUB proteins. Figure used from Oakley et al., (2007).

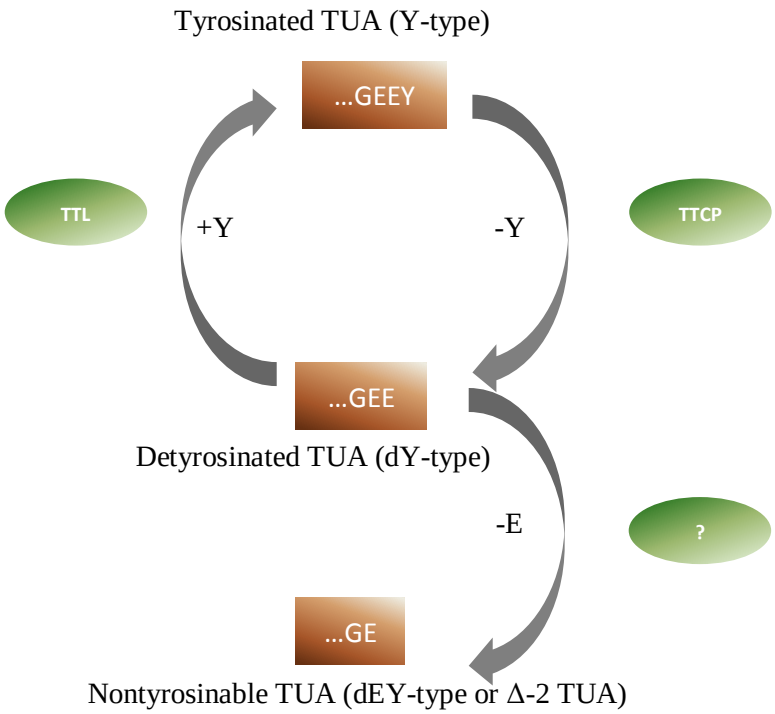

Figure 1.3. Tyrosination- detyrosination cycle and generation of Δ-2 (dEY) TUA. Figure modified from Westermann and Weber (2003).

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CHAPTER 2

TRANSGENIC MANIPULATIONS OF *POPULUS* TUBULINS AFFECT CELL WALL PROPERTIES[1]

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Abstract

Microtubules (MTs) are dynamic cytoskeletal polymers of alpha-(TUA) and beta-(TUB) tubulin heterodimers. Various processes including the deposition of cellulose microfibrils in the developing cell wall are thought to depend on MT function. MT growth, stability and function depend in part on post-translational modifications (PTMs) at the C-terminal end of the TUA and TUB monomers. In order to investigate MT function during wood formation, a small subset of xylem-abundant tubulins (*TUA1*, *TUA5*, *TUB9* and *TUB15*), including PTM mimics of detyrosinated (dY) or nontyrosinatable (dEY) *TUA1* were co-transformed in combinatorial *TUA*-*TUB* pairs into *Populus*. The gene combinations that gave rise to viable transformants contained PTM mimics (*TUA1dY* + *TUB9* and *TUA1dEY* + *TUB15*) while all other combinations, including a synthetic gene pair, failed to produce rooted shoots. Gene expression analysis in leaf and developing xylem tissues revealed that the transcript levels of *TUA* transgenes were present at higher levels than *TUB* transgenes, despite both being controlled by the same constitutive promoter. The patterns were similar to those of their endogenous counterparts, suggesting that *Populus* tubulins are under translational or co-translational autoregulation. Cellulose, hemicellulose and lignin abundance were not quantitatively altered in the transgenic stem wood. However, there were pleiotropic transgenic effects on the pectin-xylan matrix, and the changes were most striking in the TUA1dEY+TUB15 lines. Lignin S/G ratio and wood density were comparatively low in the TUA1dY+TUB9 lines. The phenotypic variations observed in the two transgenic groups may be attributed to the distinct TUA1dY or TUA1dEY PTM variants that they possess. The results support an active tubulin tyrosination-detyrosination pathway in *Populus*, and suggest that tubulin PTM manipulation may be a useful strategy to perturb MT dynamics and function. This study highlights the potential of manipulating tubulins to alter woody biomass characteristics without compromising plant growth.

Introduction

Microtubules (MTs) are polymers composed of heterodimers of alpha- (TUA) and beta-tubulin (TUB) subunits (Stephens, 1970). Apart from their involvement in critical cellular processes, such as cytokinesis and subcellular trafficking (Johnson and Porter, 1968; Thissen et al., 1997), cortically-arranged MTs are thought to guide the deposition of nascent cellulose microfibrils (MF) during cell wall formation (Baskin, 2001).

Ledbetter and Porter (1963) first coined the term "microtubules" to describe the cytoplasmic elements that reside on the inner side of the cell wall and parallel the MF, as reported by Green (1962). This led to the MT-MF co-alignment model, which describes a pattern consistently observed during different stages of cell wall development. The orientation of both MTs and MFs in the primary cell wall is generally random, but the two achieve orderly arrangement during cell wall thickening (Abe and Funada, 2005). MT density also increases as secondary cell wall deposition progresses (Chaffey et al., 1999). Highly dense, parallel arrays of MTs have been observed in the specialized gelatinous (G)-layer, formed in tension wood (TW) fibers of angiosperms in response to gravitropic stimuli (Prodhan et al., 1995; Chaffey et al., 2002), consistent with the near-exclusive presence of crystalline cellulose in the G-layer (Norberg and Meier, 1966). The disruption of MT-MF co-alignment by anti-MT drugs or mutation of MT-associated proteins causes severe growth anomalies, including impaired cell wall development, in *Arabidopsis* (Yamamoto et al., 1998; Burk and Ye, 2002; Burk et al., 2006; DeBolt et al., 2007; Ishida et al., 2007). *In vivo* visualization of GFP-tagged tubulins in *Arabidopsis* hypocotyls has recently provided experimental evidence for the proposed role of cortical MTs in directing the deposition of MFs across the plasma membrane (Paredez et al., 2006; Crowell et al., 2009). MTs aggregate near the site of pectin secretion during primary seed coat maturation, suggesting that MTs may contribute to pectin deposition (McFarlane et al., 2008). Disruption of MT-MF co-alignment by the chemical inhibitor cobtorin was accompanied by abnormal pectin distribution, further supporting the role of MTs in pectin deposition (Yoneda et al., 2010).

In higher plants, tubulin proteins are encoded by multi-gene families with uneven numbers, ranging from six *TUAs* and nine *TUBs* in *Arabidopsis* (Kopczak et al., 1992; Snustad et al., 1992) to eight *TUAs* and twenty *TUBs* in *Populus* (Oakley et al., 2007). Significant expansion of the *Populus TUB* family can be traced to both whole-genome and tandem duplication events, giving rise to 10 paralogous *TUB* pairs, while only four of the eight *TUAs* are so derived (Oakley et al., 2007). Plant TUAs fall under two distinct phylogenetic classes having different exon/intron structures, suggesting an origin from two ancestral genes (Oakley et al., 2007). Most species, including *Populus*, show an even distribution of TUA family members between the two classes (Oakley et al., 2007). Plant TUBs, on the other hand, are classified into four phylogenetic classes with largely conserved gene structure, likely descending from one ancestral gene (Oakley et al., 2007). Unlike the TUA family, taxon-biased representation is evident among the four TUB phylogenetic groups. For instance, four of the nine *Arabidopsis* TUBs are found in Class II, while half of the *Populus* TUB members belong to the Class I and Class I-like groups (Oakley et al., 2007). For both TUA and TUB families, distinct phylogenetic grouping has been linked to differential expression and functional association. Class I TUAs have generally been associated with secondary cell wall formation, whereas Class II TUAs contain several pollen-specific isoforms. Likewise, several Class I and Class II *TUBs* are highly expressed in cells undergoing secondary cell wall thickening, while a few of Classes III and IV members exhibit pollen-biased expression (Whittaker and Triplett, 1999; Oakley et al., 2007).

In addition to spatiotemporal regulation of tubulin gene expression, tubulin protein synthesis is subject to autoregulation and post-translational modifications (PTMs) (Pachter et al., 1987; MacRae, 1997). In animals, autoregulation involves co-translational degradation of tubulin mRNAs in response to increased tubulin monomer concentration (Pachter et al., 1987), thereby ensuring a precise control of tubulin levels. This autoregulation is modulated by specific N-terminal sequences in TUBs (Gay et al., 1987). While autoregulation of TUA mRNAs has not been consistently supported (Bachurski et al., 1994), a translational feedback regulation route has been proposed (Gonzalez-Garay and Cabral, 1996). Mammalian tubulins are known to undergo extensive PTMs, especially at the acidic carboxy (C)-termini

(Westermann and Weber, 2003). Tubulin PTMs impact MT dynamics and exhibit spatiotemporal specificity, thus serving important roles during development of animals and lower eukaryotes (Wloga and Gaertig, 2010). One of the most common PTMs involves the enzymatic removal and reattachment of the C-terminal Tyr, known as the tyrosination-detyrosination cycle (Idriss, 2000). The C-terminal Tyr is evolutionarily conserved among TUAs across kingdoms and species (Little and Seehaus, 1988). However, only three of eight predicted *Populus* TUA proteins contain the conserved C-terminal Tyr that may participate in the tyrosination-detyrosination cycle (Oakley et al., 2007). Given the high degree of C-terminal sequence heterogeneity of the *Populus* TUA and TUB families (Oakley et al., 2007), the importance of tubulin diversity during secondary growth within the context of PTMs remains to be investigated.

The multiple, complex modes of tubulin regulation likely contribute to the reported difficulty in genetic manipulation of tubulins, which is exacerbated by their essential roles in cellular and developmental processes. Attempts to overexpress the *TUB* gene in *Saccharomyces cerevisiae* resulted in loss of cell viability, while overexpression of *TUA* reversed the cytotoxicity effects (Weinstein and Solomon, 1990). Although it has been possible to obtain tubulin mutants or transgenics in *Arabidopsis* via mutagenesis or epitope/GFP tagging, many of these plants have displayed developmental anomalies, such as left- or right-handed helical growth (Abe and Hashimoto, 2005; Burk et al., 2006; Ishida et al., 2007). In support of the MT-MF co-alignment hypothesis, transgenic *Arabidopsis* over-expressing a GFP-tagged *AtTUA6* exhibited alterations not only in growth but also in cell wall properties (Burk et al., 2006). In GFP-TUA6 overexpressors, the cell wall thickness of pith and interfascicular fiber cells was substantially reduced and the composition of structural sugars was altered, indicating a role for tubulins in cell wall synthesis (Burk et al., 2006). Attempts to overexpress eucalyptus *EgTUB1* in somatically-derived wood sectors led to gene silencing, perhaps due to homology-dependent co-suppresion (Spokevicius et al., 2007). The resultant downregulation of the *TUB1* gene was responsible for increased microfibril angle (MFA), opposite to what was expected through overexpression of *TUB1* (Spokevicius et al., 2007). Although single tubulin gene manipulation in *Arabidopsis* has been reported in many instances, a

TUA+TUB co-transformation strategy appears to be critical in other species, such as maize and tobacco (Anthony and Hussey, 1998; Anthony et al., 1999). By introducing a combination of *TUA* and *TUB* genes in tobacco, the tubulin overexpressing plants accumulated higher levels of functional tubulins without observable effects on plant development (Anthony et al., 1999). The present study sought to investigate the effects of tubulin perturbation on wood formation using stably transformed *Populus* plants. The co-transformation strategy (Anthony and Hussey, 1998) was adopted here to target xylem-abundant *TUA* and *TUB* genes for ectopic expression under control of a constitutive promoter. The results revealed that inclusion of PTM mimics was beneficial in obtaining transgenic plants from co-transformation experiments. Transgenic plants exhibited altered wood properties and cell wall matrix involving pectin but not cellulose.

Materials and methods

Plant materials and growth condition

Populus tremula X *Populus alba* clone 717-1B4 genotype was used unless otherwise indicated. Clonal propagation and greenhouse maintenance were followed as described previously (Frost et al., 2012). The Leaf Plastochron Index (LPI) numbers were followed as per Larson and Isebrands (1971) where first fully unfurled leaf was considered LPI-0. At harvesting, mature leaf (LPI 15) and developing xylem scraped from mature stem were snap-frozen in liquid nitrogen and stored at -80 ^{o}C until use. The remaining stem wood portion was air-dried and stored at room temperature for further analysis.

PCR, cloning and construction of transformation vectors

Full-length TUA and TUB sequences were amplified from *Populus tremula* X *Populus alba* xylem cDNA using the primers listed in Appendix 2A. PCR-amplified *TUA1, TUA5, TUB9* and *TUB15* along with amplification of PCR-introduced PTM mimics of *TUA1- TUA1dY* and *TUA1dEY* cDNAs were cloned in pCR2.0-TOPO (Invitrogen Inc, Carlsbad, CA, USA). The sequence-confirmed *TUA* and *TUB* clones were later inserted into pCAMBIA 1302 or modified pCAMBIA 1302 (contains *nptII* gene for

bacterial selection, cloned from pCAMBIA 2301), called PCM hereafter. All *TUA* or PTM mimics of *TUA1* were directionally cloned into pCAMBIA 1302 while the *TUB* genes were cloned into PCM vectors. The PCR- or restriction digestion-confirmed binary clones containing *TUA* and *TUB* genes were transformed into *Agrobacterium* strain C58-pMP90 using the method described by Holsters et al. (1978). The correct orientations of cloned *TUA* or *TUB* gene cassettes were confirmed by colony PCR using primers described in Appendix 2A.

Plant transformations

The leaves from one month-old micro-propagated plants of *Populus tremula* X *Populus alba* clone 717-1B4 were used as explants for plant transformations as described previously (Ma et al., 2004). The *Agrobacterium* cultures at the active growth stage carrying the *TUA* or *TUB* gene construct in different tubes were mixed in 1:1 ratio and used for leaf disc transformation. The putative transformants were selected on tissue culture media containing kanamycin and hygromycin antibiotics. The regenerated transgenic shoots derived from calli were transferred onto root induction media in magenta boxes (Caisson Labs, USA). The rooted plantlets were then hardened off in the growth room before they were transferred to the greenhouse in one gallon pots.

Tension wood induction and wood processing

Wild type and transgenic plants were potted in large three gallon pots and fertilized regularly. At one meter height, plants were inclined against metal rails at approximately 30^0 angles and tied to avoid accidental movements during routine maintenance. At the end of three week experimental period, the stem containing the gelatinous layer (tension wood, Figure 12) was de-barked and developing xylem was collected and preserved immediately in liquid nitrogen and stored at -80^0C. The stems were then air-dried, split lengthwise into tension wood-rich stems. The straight wood trees were used as controls and harvested and processed exactly as tension wood samples. The harvested stem wood materials were air dried and milled through a 40-mesh sieve using a Thomas Wiley Mini-Mill (Thomas Scientific,

Swedesboro, NJ, USA). The wood meal was then extracted with 100% ethanol for 99 cycles using a Büchi extraction unit E816 (BÜCHI Labortechnik AG, Flawil, Switzerland). The extractive-free samples were then air dried and used for subsequent analysis.

Transgene confirmation and gene expression analysis

A. Transgene confirmation by PCR

Small pieces (1 cm^2) of putative transgenic calli were used for genomic DNA extractions as described previously (Dellaporta et al., 1983). The genomic DNAs were then subjected to PCR using gene-specific primers (Appendix 2A) to confirm the presence of transgenes. The wild type calli and plasmid bearing the *TUA* or *TUB* were used as controls in the experiment. The confirmed calli were then advanced to the shoot induction media.

B. Total RNA extraction

Total RNA extraction from mature leaves (LPI 15) or developing xylem was carried out as described by Chang et al. (1993). Briefly, frozen tissues were ground in liquid nitrogen to a fine powder via mortar and pestle. The powder volume of 100 µl was then added to 1.3 ml of pre-warmed CTAB buffer containing 2M NaCl, 25 mM EDTA, 0.1 M Tris base (pH 9.0), 2% w/v PVP (K-30), 2% w/v CTAB with 2% β-mercaptoethanol added just before the extraction and mixed by vortexing. Samples were then incubated for 10 minutes at 65^0C with intermittent mixing and allowed to cool to room temperature. To this tube, chloroform: isoamyl alcohol (24:1) were added to a filling volume just below the lid, vortexed and centrifuged for five minutes at 16,000 g to separate the phases. The aqueous phase was then mixed with one third volume of 8M $LiCl_2$ and incubated on ice for 3-5 hours to precipitate the RNA. The total RNA was harvested by centrifugation at 4^0C for 20 min at 16,000 g. The RNA pellet was resuspended in 500 µl of RNAse free water and the LiCl2 precipitation was repeated by incubating the tube on ice for two hours. The tubes were then subjected to centrifugation at 4^0C for 10 min at 15,000 g to pellet RNA. The resultant pellet was then dissolved in 300 µl of RNAse-free water and re-precipitated

using 0.1 volume of 3M sodium acetate and 2.5 volumes of 100% ethanol by incubation at -80^{0}C for 30 min and centrifuging at 4^{0}C for 10 min. The RNA pellet was then washed with 1 ml of 70% ethanol and centrifuged for one min and the pellet was air-dried and suspended in 50 µl RNAse-free water. The RNA quantity was estimated using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and quality was assessed by equal RNA loading onto 1% agarose.

C. First strand cDNA synthesis and real-time RT-PCR

The cDNA synthesis from one µg total RNA was carried out with anchored oligo (dT) 20 primers, superscript II reverse transcriptase (Invotrogen, Carlsbad, CA, USA) according to manufacturer's recommendations. For real-time quantitative PCR assay, one ng of cDNA was used along with gene specific primers (Appendix 2A), ABsolute SYBR Green mix (Abgene, Rochester, NY, USA) and ROX as an internal reference marker. Amplification was carried out in Stratagene MX 3005P (Stratagene Inc, La Jolla, CA, USA) as follows: 95^{0}C for 15 min followed by 40 cycles of 95^{0}C for 15 sec, 58^{0}C for one min and 72^{0}C for one min. Two technical replications were included for each sample. The relative transcript levels were estimated by $2^{-\Delta Ct}$ method by normalizing the expression levels of the gene of interest to the average expression level of three housekeeping genes (elongation factor 1β, actin and actin-related protein) as described in Tsai et al. (2006). The amplicon specificity was assessed by dissociation curve analysis (MxPro-Mx3005P, Stratagene Inc., La Jolla, CA, USA).

Microfibril angle and wood density analysis

Intact 2-3 cm stem pieces were used to measure the microfibril angle (MFA) and wood density as described previously (Coleman et al., 2009) in the laboratory of Shawn Mansfield at the University of British Columbia, Canada.

Metabolic profiling and analysis

The xylem samples were ground to fine powder via liquid nitrogen in mortar and pestle. A small aliquot of the powder was freeze-dried. For metabolite profiling, 10 mg freeze dried powder was used to extract plant metabolites. The metabolite extraction, sample derivatization and GC-MS loading was performed as described in Frost et al. (2012). The resulting peak data was matched to NIST08 (Babushok et al., 2007), Fiehnlib (Agilent Technologies, Kind et al., 2009) and Tsai lab in-house libraries. The abundance of the compounds was normalized to the internal standard and the sample dry weight. Compounds with at least 70% mass spectral peak identities to the above databases were retained and analyzed in the Metalab platform (Tsai Lab, AspenDB web portal).

Wood chemistry analysis

A. Total lignin determination

Total lignin from extractive-free samples was determined by the Klason method (laboratory analytical procedure, NREL, USA) with modifications. Briefly, the extractive-free wood meal was oven dried for two hours at 105^0C and 200 mg of wood meal was transferred to 10 ml glass tube and 2 ml of 72% (w/w) H_2SO_4 was added. The tubes were incubated at room temperature (1 h) for digestion. Later, 1 ml of myo-inositol (46.4 mg/ml) was added to the tubes, mixed and diluted to 58 ml with deionized water to achieve 4% H_2SO_4 final concentration in an Erlenmeyer flask. The slurry was autoclaved for one hour and allowed to cool at room temperature. The acid-soluble lignin was calculated by measuring the absorbance of diluted (10X) hydrolysates at A205.

Acid soluble lignin = A205 ÷ a x df x vol (unit = g)

A205 = absorbance at UV 205 nm

A, (extinction coefficient) = 110 L/g-cm

df, dilution factor (10X)

vol = hydrolysate volume (58 ml)

The remaining hydrolysates containing insoluble lignin were then filtered through pre-dried glass-microfiber filters (VWR International LLC, Radnor, PA, USA) and washed until acid-free. The insoluble debris (Klason lignin) was then dried with the filter paper at 105^0C until constant weight was obtained. The total lignin was determined as follows:

Total lignin % = (Klason lignin + acid soluble lignin) ÷ sample weight x 100%

B. Syringyl-to-guaiacyl ratio of lignin monomer analysis

Extractive-free wood samples were subjected to pyrolysis GC-MS to determine syringyl-to-guaiacyl lignin ratio in the laboratory of Jeng-Der Chung, Taiwan Forestry Research Institute, Taiwan.

C. Structural carbohydrate analysis

Klason hydrolysates from the lignin analysis were used to estimate the structural carbohydrates using sugar standards in the laboratory of Shawn Mansfield, University of British Columbia, Canada.

D. Hemicellulose composition and glycosyl linkage analysis

Non-cellulosic glycosyl (hemicellulose) composition and glycosyl linkage analysis were determined by GC-MS using method as reported (Merkle and Poppe, 1994) and (York et al., 1986) by Tina Thomas in the laboratory of Parastoo Azadi, Complex Carbohydrate Research Center (CCRC) at the University of Georgia.

E. Glycome profiling and data analysis

Extractive-free wood meal was used for glycome profiling and analysis was carried out as described in Pattathil et al. (2010) in the laboratory of Michael Hahn at the CCRC, University of Georgia. The data from this analysis was subjected to quality control filtering prior to analysis. If the values of two

samples from three replicates were larger than 0.1 and present in at least one of six extraction fractions, the corresponding antibody signal was considered detected in that genotype. Using this criterion, 127 of 154 antibodies were carried forward for one-way ANOVA analysis to identify antibodies that were significantly changed their recognition patterns in at least one extraction. Of the 127 antibody recognition patterns, 109 antibody signals were clustered according to their distinct epitope binding pattern in self-organizing maps (SOM). The analysis was performed with the assistance of Liangjiao Xue.

Results

Populus transformations

Two xylem-abundant genes each from the *TUA* (*TUA1* and *TUA5*) and *TUB* (*TUB9* and *TUB15*) families were selected for co-transformation in four different combinations, hereafter abbreviated as A1B9, A1B15, A5B9 and A5B15. To test the feasibility of perturbing PTM homeostasis *in planta*, two PTM mimics encoding de-tyrosinated (dY) and non-tyrosinatable (dEY) isoforms of TUA1 were included, resulting in four more combinations, denoted as A1dYB9, A1dYB15, A1dEYB9, A1dEYB15. To address possible homology-dependent gene silencing effects due to over-expression of endogenous genes (Meyer and Saedler, 1996), synthetic *TUA1* and *TUB9* with altered codon usage (<67.9% nucleotide sequence identity with the native genes, Appendix 2B) were included as a single additional combination (synA1B9). In all cases, *TUA* and *TUB* constructs were cloned into two different vector backbones having either a hygromycin- or kanamycin selectable marker to facilitate selection of co-transformants (see Methods). The nine *TUA*+*TUB* construct combinations along with the vector control were transformed into *Populus tremula* × *alba* clone 717-1B4 using an established *Agrobacterium*-mediated transformation protocol (Ma et al., 2004). Putative transgenic calli were obtained from all nine construct combinations grown on kanamycin-hygromycin double selection media. However, the overall transformation efficiency was very low in multiple transformation trials relative to what was routinely obtained for this *Populus* genotype using the vector control (Table 1). PCR confirmation of transgenes in the genomic DNA of representative lines is shown in Figure 1. While most calli from the vector control successfully

regenerated into whole plants, this was not the case for calli derived from the tubulin constructs. In the latter case, the majority of the callus lines failed to advance to shoot regeneration, elongation or rooting stages (Table 1). Some regenerated shoots exhibited various developmental anomalies (Figure 2). Interestingly, the four construct combinations containing PTM mimics of *TUA1* (dY or dEY) had the highest transformation efficiency at the callus stage regardless of whether *TUB9* or *TUB15* was co-transformed, and three of these combinations were the only ones that produced viable transgenic plants (Table 1). Neither native nor synthetic gene combinations, the latter of which was predicted to produce native proteins if transcribed and translated, led to viable transformants. These results concur with previous reports that manipulation of tubulin expression may be detrimental to growth and development, although co-transformation of *TUA* and *TUB* has been successfully applied in other systems (Anthony and Hussey, 1998; Anthony et al., 1999). In the case of *Populus*, the PTM mimic-containing gene pairs appeared more permissive to transgenic manipulation and whole plant regeneration.

Relative transcript level analysis

Three independent events each from the A1dYB9 and A1dEYB15 transgenic groups and a wild-type control line were micropropagated, transplanted to soil and maintained in a greenhouse for subsequent characterization. The relative abundance of endogenous and transgenic tubulin transcripts in mature leaves and developing xylem was assessed using quantitative reverse transcription PCR (qRT-PCR). In mature leaves, endogenous levels of *TUA1, TUB9* and *TUB15* transcripts were low, as expected from previous work (Figure 3; Oakley et al., 2007). The transcript levels of *TUA1dY* or *TUA1dEY* transgenes were increased by 5- to 30- fold relative to endogenous *TUA1* in both transgenic groups (Figure 3a, c). Expression was more variable for *TUB* transgenes. Although both *TUA* and *TUB* transgenes were driven by the same CaMV 35S promoter, transcripts of *TUB* transgenes were detected at much lower levels than for *TUA* transgenes. Except for the A1dYB9-4 line where the *TUB9* transgene was up-regulated by 5-fold, transcript levels of the *TUB* transgenes were either similar to (for A1dYB9 lines, Figure 3b) or less than the endogenous levels (for A1dEYB15 lines, Figure 3d).

In developing xylem, endogenous *TUA1, TUB9* and *TUB15* transcripts were present at higher levels than seen in mature leaves, as reported by Oakley et al. (2007). In contrast, transgene transcripts were detected at much lower levels in all cases (Figure 4). As was the case in mature leaves, relative abundance of *TUB* transgene transcripts was lower than that of *TUA* transgenes in xylem. Homology-dependent co-suppression was a concern, due to the high level of endogenous tubulin gene expression. However, possible evidence of co-suppression was only observed for A1dYB9 lines 2 and 9, where the endogenous *TUB9* levels were 30-48 % lower than the wild type (Figure 4b). Endogenous *TUA1* transcripts in A1dEYB15 line 12 were 2-fold higher relative to wild type levels, and *TUA1dEY* transgene levels were 2.5-fold higher compared to the other two A1dEYB15 lines. Although endogenous *TUB15* transcript levels in this line remained unchanged relative to wild type, *TUB15* transgene transcripts in this line were 10- to 18- fold higher than in the two other A1dEYB15 lines. Together, the results suggest that these *TUA* and *TUB* transgenes were, much like their endogenous counterparts, differentially regulated in a gene family- and tissue-dependent manner.

Stem and petiole anatomy

Histological analysis was carried out to examine the vascular structure of young stem (at LPI 5) and mature leaf petiole (LPI 15) of wild-type and transgenic plants. No obvious anatomical differences were observed in the cross sections of stems or leaf petioles between plant lines (Figure 5).

Wood density and microfibril angle analysis

Wood density and microfibril angle (MFA) are important physical properties of wood. MFA is of particular interest for the present study, as it reflects the arrangements of cellulose microfibrils within the secondary cell wall. Both analyses were carried out in the laboratory of Shawn Mansfield, University of British Columbia, Canada. Wood density was significantly reduced in A1dYB9 transgenic lines, but was largely unaffected in A1dEYB15 trees (Figure 6). MFA in most transgenic lines did not change significantly relative to the wild type, with the exception of A1dEYB15 line #5, which had a lower MFA

compared to all other plants (Figure 6d). The possibility of a sampling artifact associated with #5 (such as development of tension wood during early growth) cannot be ruled out because the low MFA was not consistent in this transgenic group. Additional analyses using an independent cohort of plants are needed to confirm the MFA data.

Wood chemistry analysis

Total lignin content was estimated by the Klason method using extractive-free wood meal (40-mesh). The lignin content was not appreciably changed in the transgenic lines relative to wild type, with small increases in the A1dYB9 lines that were not statistically significant (Figure 7). The syringyl-to-guaiacyl (S/G) monolignol ratio as determined by pyrolysis GC-MS in the laboratory of Jeng-Der Chung (Taiwan Forestry Research Institute, Taiwan) was significantly lower, by 16-18%, in A1dYB9 lines compared to the wild type (Figure 7b). The differences, however, were much smaller and not consistently significant in A1dEYB15 lines (Figure 7d).

Acid-soluble hydrolysates from the Klason lignin analysis were utilized for cell wall carbohydrate analysis by HPLC in the laboratory of Shawn Mansfield (Figure 8). The most abundant sugars in the poplar wood samples are glucose (Glc, ~42%) and xylose (Xyl, ~18%), the major components of cellulose and hemicelluloses, respectively. Their levels did not differ between wild type and transgenic trees (Figure 8a). The other, less abundant sugars, galactose (Gal, ~1%), mannose (Man, ~1.5%), arabinose (Ara, ~0.3%) and rhamnose (Rha, ~0.4%) are components of hemicelluloses and pectins. Their levels did not differ significantly between wild type and transgenic lines. The only exception was Man, which showed small, statistically significant changes in some transgenic lines that were inconsistent within the two transgenic groups. Overall, the results suggested no notable changes in the main structural carbohydrates of stem wood due to tubulin perturbation in the transgenic trees.

Glycome profiling

Glycome profiling was employed to provide a high resolution analysis of cell wall carbohydrate composition (performed by Sivakumar Pattathil in the laboratory of Michael Hahn, Complex Carbohydrate Research Center at the University of Georgia). Sequential chemical extractions in increasingly harsh solvents were performed on extractive-free wood meal to separate different cell wall polysaccharide fractions based on solvent accessibility. These fractions were then subjected to automated ELISA procedures using a comprehensive plant cell wall glycan-directed monoclonal antibody (mAb) toolkit (Pattathil et al., 2010). The epitope signal intensities and carbohydrate recovery from each fraction are shown in Figure 9. Data from the entire 154 mAb were further filtered by an arbitrary detection threshold of signal intensity >0.1 in two of three biological replicates; and the resulting 127 mAb were subjected to clustering analysis by self-organizing map (SOM) with the help of Lianjiao Xue from the Tsai laboratory. The analysis identified six major clusters of epitope distribution profiles across cell wall fractions and genotypes (Figure 10). Clusters 1 and 2 consist primarily of xyloglucans and xylans, respectively, while the other clusters were dominated by rhamnogalacturonan I (RG-I) and arabinogalactan (AG) epitopes derived from pectins. In Cluster 1, both the relative abundance and distribution pattern of xyloglucans, either fucosylated or non-fucosylated, were similar between transgenic and wild type samples, although there was a noticeable increase in the post-chlorite (PC) fractions of the transgenics (Figure 10). In Cluster 2, a consistent increase in xylan-6 and xylan-7 epitopes was observed in all fractions of the A1dEYB15 group compared to the wild type. Interestingly, A1dYB9 line 4 behaved more like the A1dEYB15 group, while the other two A1dYB9 lines were similar to wild type. The four pectin-associated clusters (Clusters 3 to 6) showed a general trend of increased epitope abundance in the transgenics relative to the wild type. Cluster 3 included all xylan-5 epitopes in the mAb panel and seven RG-I/AG epitopes, suggesting the correlation of xylan-5 glycans with the pectin network. In general, the observed increases were greater in the A1dEYB15 lines than in the A1dYB9 lines, except for the A1dYB9 line 4, which was similar to the A1dEYB15 group. Increased pectin and xylan epitopes were found in the more easily extracted cell wall fractions (particularly the relatively mild sodium

carbonate fraction, Clusters 2, 3, 4 and 6), as well as in the more tightly bound fractions extracted by 4M KOH, chlorite or post-chlorite 4M KOH (Clusters 2-6). After removal of lignin and its associated cell wall polymers by chlorite treatment, additional pectin epitopes were more abundant in all transgenics compared to wild type in the post-chlorite fraction. Thus, although cellulose and hemicellulose components were not affected by tubulin manipulation, glycome profiling results revealed that the xylan and pectin polysaccharide networks, and possibly their interactions with lignin polymers, were altered in the secondary cell wall of the transgenics.

Metabolic profiling of the developing xylem

The overall status of soluble metabolites in developing xylem was assessed by GC-MS profiling. The analysis revealed changes in some soluble sugars. For example, sucrose levels were significantly higher in most A1dYB9 and A1dEYB15 transgenic lines than in the wild type (Figure 11a, d). Glucose levels were reduced in two of three A1dYB9 lines while showing a slight but not always significant increase in A1dEYB15 lines. A similar pattern was seen for fructose. A less abundant sugar, xylose, was significantly increased in all A1dYB9 lines relative to the wild type but remained unchanged in A1dEYB15 lines (Figure 11b, e). The levels of galacturonic acid (GalA), a component of pectins, were significantly increased in several of the transgenic lines. The metabolites shikimic acid and catechin increased in lines #9 and #2 of A1dYB9 and in line #11 of A1dEYB15, but the response was weaker in A1dYB9 line 4 and in the remaining A1dEYB15 lines. No other metabolites from GC-MS profiling evidenced any genotype-specific trends in transgenics compared to the wild type (Appendix 2C).

Tension wood analysis

Tension wood (TW) was induced in transgenic and wild type trees by growing the trees at an angle for three weeks (Figure 12). Quantitative RT-PCR was used to assess transcript levels of endogenous and transgenic tubulins in a subset of straight wood (SW) and TW samples (Figure 13). Levels of endogenous *TUA1, TUB9* and *TUB15* transcripts were very high in both SW and TW samples,

as previously reported (Oakley et al., 2007). However, only *TUB15* transcript levels were clearly elevated by TW treatment, in contrast to a previous report where *TUA1*, *TUB9* and *TUB15* were all up-regulated in TW (Figure 13d & h; Oakley et al., 2007). This discrepancy may be attributed to the difference in TW induction length (three months in the previous study vs. three weeks in the present study) or genotypic differences (*Populus tremuloides* in the previous study vs. *Populus tremula* × *alba* in this study) in tubulin transcript response. By comparison to the endogenous transcripts, *TUA1dY, TUA1dEY, TUB9* and *TUB15* transgene transcript levels were very low and did not exhibit any treatment effect (Figure 13).

Wood chemistry analysis of SW and TW

Total lignin content of SW and TW was determined by the Klason method using extractive-free wood meal. As shown for the original transformants, the total lignin content was similar in the straight wood of wild type, A1dYB9 and A1dEYB15 lines (Figure 14). On average, lignin content was reduced by 25% in TW of all three groups. When compared with the wild-type TW, a significant but slight increase in the lignin content of A1dEYB15-11 TW was noted (Figure 14). The results indicate that tubulin manipulation did not affect lignin response to TW treatment.

The major structural sugars, Glc and Xyl, exhibited the predicted TW responses (Norberg and Meier, 1966), with Glc increased and Xyl reduced relative to SW, without any transgenic effects (Figure 15a & b). The less abundant structural sugars, Ara, Rha and Gal, were increased by approximately 25%, 60% and 300%, respectively, in TW (Figure 15c-e). On the other hand, levels of Man were reduced by approximately 50% in TW (Figure 15f). Consistent with results from the original transformants, none of the structural carbohydrates were altered in the A1dYB9 or A1dEYB15 groups relative to wild type.

Non-cellulosic glycosyl composition and linkage analyses

The results from structural carbohydrate and glycome profiling analyses suggested that tubulin manipulation plays an important role in the composition of cell wall polysaccharides other than cellulose. To more deeply understand the effects of tubulin manipulation on non-cellulosic polysaccharide structure,

SW and TW samples from wild type and one representative line each of A1dYB9 and A1dEYB15 were used for glycosyl residue composition analysis. The extractive-free wood samples were analyzed using GC-MS by Tina Thomas in the laboratory of Parastoo Azadi at the CCRC, the University of Georgia. The hemicelluloses comprised primarily of Xyl (ca. 75%) and Glc (ca. 12%; Figure 16a & b). Neither of these sugars differed among the SW samples, except for a slight decrease of Xyl in A1dEYB15-11 (Figure 16a). Increases in Gal and Rha (Figure 16c & e), and decreases in Xyl and Man in TW (Figure 16a & f) relative to SW were consistent with the total structural carbohydrate analysis of Klason lysates. Levels of GalA increased slightly in SW of both transgenic groups compared to wild types (Figure 16d), consistent with glycome profiling results described above. However, this difference between transgenic and wild type lines was not observed in TW.

The same suite of samples was subjected to glycosyl linkage analysis as previously described (York et al., 1986). The most abundant linkages in SW samples were 4-Glc, 4-Man, 2-Xyl and 2, 3, 4-Xyl (Figure 17a, c, d, and e). None of these linkages differed significantly between SW of transgenics and wild type. The proportion of 4-Gal linkages was absent from the wild type, but low levels were consistently detected in both transgenic lines (Figure 17b). In TW samples, levels of 4-Man and 2,3,4-Xyl generally decreased (Figure 17c & d) while 4-Gal generally increased relative to SW (Figure 17b). In all cases, the differences between SW and TW were statistically significant in a majority of glycosyl linkages detected.

Discussion

Tubulin transformations

Previous studies have shown that manipulation of tubulins can be lethal or cause developmental defects in transgenic yeast, mammalian cells, *Arabidopsis*, maize, and tobacco (Weinstein and Solomon, 1990; Gonzalez-Garay and Cabral, 1996; Anthony and Hussey, 1998; Bao et al., 2001). In the cases of maize and tobacco, maintaining a balanced *TUA*:*TUB* expression by co-transformation was essential in order to obtain viable transformants (Anthony and Hussey, 1998; Anthony et al., 1999). In the present study,

however, transformation efficiency of *Populus* was very low even with co-transformation of *TUA* and *TUB*. The vast majority of the transgenic callus lines showed abnormal development during organogenesis, consistent with reported developmental difficulties associated with tubulin perturbations. Unexpectedly, the only co-transformation combinations that produced viable transgenic plants contained a PTM mimic of *TUA1* (dY or dEY), with either *TUB9* or *TUB15*. In fact, in terms of transformation efficiency, the four PTM mimic-containing construct combinations consistently outperformed the other native or synthetic gene pairs throughout various tissue culture stages (Table 1). These results suggested that while the co-transformation strategy did not work as efficiently for *Populus* as reported for maize and tobacco (Anthony and Hussey, 1998; Anthony et al., 1999), inclusion of PTM mimics appeared to be beneficial in improving co-transformation efficiency for tubulin manipulation in *Populus*.

Tubulin regulation

Despite the fact that both *TUA* and *TUB* transgenes were driven by the same constitutive CaMV 35S promoter, their transcript abundance differed by 10- to 37-fold in the two tissues examined (Figure 3, 4). In all transgenic lines examined, the overall transcript levels of *TUA* transgenes (*TUA1dY* or *TUA1dEY*) were higher than those of *TUB* transgenes (*TUB9* or *TUB15*). This is reminiscent of the observation that endogenous levels of *TUA* transcripts are always higher than *TUB* transcripts in different *Populus* tissues (Oakley et al., 2007). These data suggested that both endogenous and introduced *TUA* and *TUB* genes are subject to post-transcriptional or co-translational regulation in *Populus*, perhaps via autoregulation or autofeedback regulation as reported in animal systems, to maintain a proper balance of the two subunits (Pachter et al., 1987; Gonzalez-Garay and Cabral, 1996). The precise 'balance' of *TUA:TUB* transcript levels in *Populus* is unknown, but our data suggested that high levels of *TUB* transgene expression were not permissible.

Although most of the construct combinations produced multiple callus lines, shoot regeneration from calli was severely impaired and many regenerated shoots did not survive. A plausible explanation is that mis-regulation of tubulin subunits disturbing the functional MT arrays led to developmentally lethal

phenotypes. The cytotoxic effects of tubulin mis-regulation were observed in yeast (Weinstein and Solomon, 1990) and mammalian cell lines (Gonzalez-Garay and Cabral, 1996), especially due to higher accumulation of TUB subunits. Although *Arabidopsis* appears more permissive to tubulin manipulations, helical growth and various developmental defects have been frequently observed in the resulting mutant or transgenic plants (Bao et al., 2001; Burk et al., 2006; Ishida et al., 2007). Given the observed difficulty during organogenesis but not callus induction, and based on the overall low levels of tubulin transgene expression in developing xylem from six viable transgenic lines, recovery of developmentally normal *Populus* transformants appeared to depend on permissible levels of tubulin perturbation. Excessive levels of tubulin transgene expression may have impeded regeneration, as no such lines were obtained.

Tubulins and wood properties

Our results showed that the tubulin perturbation in transgenic poplars altered lignin composition but not cellulose content in the stem wood. Glycome profiles of cell wall fractions derived from sequential chemical extractions showed highly distinguishable changes in the transgenic plants (Figure 10). Specifically, enhanced extractability of pectin-related epitopes rhamnogalacturonan-I (RG-I) and arabinogalactan (AG) was observed in the transgenic samples, particularly from the A1dEYB15 lines. Profiles of xyloglucan (XG), the predominant primary cell wall hemicellulose in eudicots and nongraminaceous monocots (O'Neill and York, 2003), were not altered, but several xylan epitopes (xylan-3, 4, 5, 6 and 7) also exhibited improved extractability in the transgenics. The results pointed to a role of MTs in deposition of pectin and xylan polysaccharides.

During cell wall biogenesis, primary cell wall consisting of proteins, cellulose, hemicelluloses and pectins, is deposited immediately after cell division. Synthesis of lignin then follows, filling in between primary cell wall polysaccharides. The primary cell wall is pushed outwards at the onset of secondary cell wall deposition, with a pectin-rich layer known as middle lamella interconnecting two adjoining cells (Jarvis et al., 2003). The middle lamella later becomes highly lignified, especially in the cell corners (Donaldson, 2001). The synthesis of cellulose microfibrils takes place at the plasma

membrane, while pectin and hemicelluloses are synthesized in Golgi and transported via vesicles to the plasma membrane for deposition into the cell wall (Picketth, 1968; Kimura et al., 1999; Nebenfuhr et al., 1999; Donaldson, 2001; Nebenfuhr and Staehelin, 2001; Mohnen, 2008). Thus, in addition to their proposed role in directing cellulose microfibril deposition (Paredez et al., 2006), cortical MTs may also regulate assembly of other cell wall matrix polysaccharides via their function in vesicle trafficking (Crowell et al., 2009), or by virtue of their close association with plasma membrane onto which new cell wall layers are synthesized. Indeed, a link between cortical MTs and pectin mucilage deposition during *Arabidopsis* seed coat maturation was reported (McFarlane et al., 2008). Pectins are involved in complex polysaccharide networks, including both alkali-labile and alkali-stable pectin-xyloglucan linkages in primary cell wall, as well as pectin-xylan-xlyloglucan complexes in lignified secondary cell wall (Femenia et al., 1999; Thompson and Fry, 2000; Popper and Fry, 2005). This is consistent with the glycome profiling results where pectin extractability was altered both in easily-extracted cell wall fractions as well as those that required harsh chemical treatments. Thus, while cellulose content was not affected, compelling evidence suggested that perturbation of tubulins imposed widespread alterations in pectic polysaccharides, xylans and lignin composition in *Populus* wood.

Differential phenotypic characteristics of the two transgenic groups

The two transgenic groups, A1dYB9 and A1dEYB15, exhibit distinct wood properties. While most of the transgenic lines showed altered cell wall pectin and xylan distribution relative to the wild type, the degree of change was greater in A1dEYB15 than in A1dYB9 lines. The total lignin content in all genotypes was quantitatively similar, while syringyl-to-guaiacyl monolignol ratios were consistently lower in A1dYB9 compared to wild-type and A1dEYB15 plants. The differences in the wood properties of the two transgenic groups may be explained by the different *TUA* (*dY* versus *dEY*) rather than *TUB* (*TUB9* versus *TUB15*) transgenes that they harbor, as transcript levels of the latter were barely detected. In animals, PTMs of TUAs via the tyrosination and detyrosination cycle have been associated with MT dynamics, as stable and long-lived MTs are enriched with dY and/or dEY TUA isoforms (Thompson,

1982; Kreis, 1987; Paturle-Lafanechere et al., 1991; Paturle-Lafanechere et al., 1994; Peris et al., 2009; Janke and Kneussel, 2010). Long-lived MTs rich in detyrosinated tubulins have also been co-localized with Golgi complex, implicating a role of tubulin PTMs in the organization and transport of Golgi vesicles (Thyberg and Moskalewski, 1999). Although mass spectrometry-based proteomic evidence is still lacking, antibody-based detection of Y and dY tubulin isoforms has been reported in plants (Wiesler et al., 2002; Wang et al., 2004). The differential phenotypic responses observed between the A1dYB9 and A1dEYB15 transgenics are consistent with a functioning tubulin tyrosination-detyrosination pathway in *Populus*. Under this scenario, the ectopic TUA1dY isoform in A1dYB9 plants may participate in the reversible tyrosination-detyrosination cycle to produce both Y-type and dY-type isoforms of TUA1, or be irreversibly converted to the dEY isoform (Chapter 1, Figure 3). However, the ectopic dEY isoform in A1dEYB15 lines cannot be further modified. Thus, while both dY and dEY variants of TUA1 can impart MT dynamics, the effects could vary due to their differential capacity to participate in the PTM. These factors might explain the wood property differences of the two transgenic groups.

Future directions

Several unanswered questions limit a thorough understanding of tubulin regulation during *Populus* cell wall deposition. In this study, the transgenes were driven by a constitutive CaMV35S promoter that might be responsible for the low transformation rates due to pleiotropic effects on other MT-mediated cellular processes. To study developmental-specific effects, tissue-specific promoters (e.g. xylem-specific promoter) should be employed to scrutinize the role of MTs during cell wall development. Whether or not dY and dEY modifications of TUA occur in *Populus* remains unknown. Since it is not possible to distinguish between tubulins synthesized from native genes and transgenes at the protein level, it would be difficult to distinguish their spatiotemporal localization and developmental regulation. Some gaps in our current knowledge can be filled by protein localization studies in the cell wall including estimates of alpha-cellulose, hemicellulose and pectin content. Transgenic trees should be thoroughly

characterized in field conditions to assess their potential usefulness. Further in-depth analyses are needed to understand MTs regulation during cell wall deposition of woody perennials.

Acknowledgements

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Table 2.1. Summary of *Populus* co-transformation from multiple independent experiments.

Construct pairs	# explants	callus	shoot regeneration	Rooting	viable events
TUA1+TUB9	106	8	1	0	0
TUA1+TUB15	100	6	1	0	0
TUA5+TUB9	98	3	0	0	0
TUA5+TUB15	100	6	1	0	0
TUA1dY+TUB9	114	13	5	3	3
TUA1dY+TUB15	90	10	2	0	0
TUA1dEY+TUB9	118	15	2	2	2
TUA1dEY+TUB15	103	17	11	4	4
synTUA1+synTUB9	89	3	0	0	0
Vector control	42	>15	>5	5	5

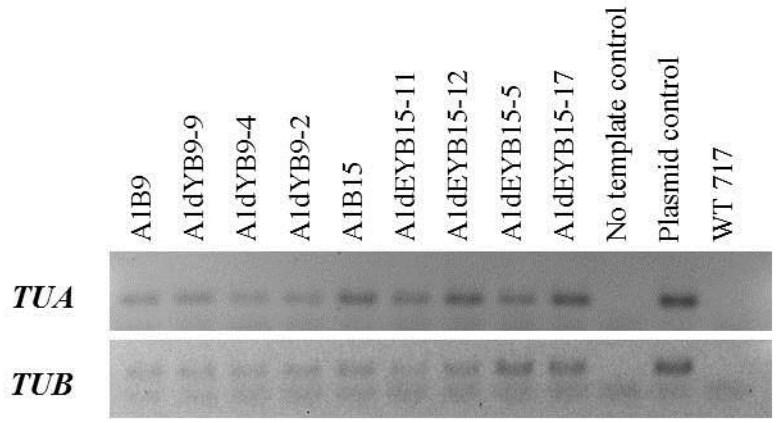

Figure 2.1. PCR confirmation of putative transgenic calli. Putative transformants were confirmed by PCR amplification of callus DNA using *TUA* (top panel) and *TUB* (bottom panel) transgene-specific primers. Non-template sample, vector plasmid and wild type callus were included as controls.

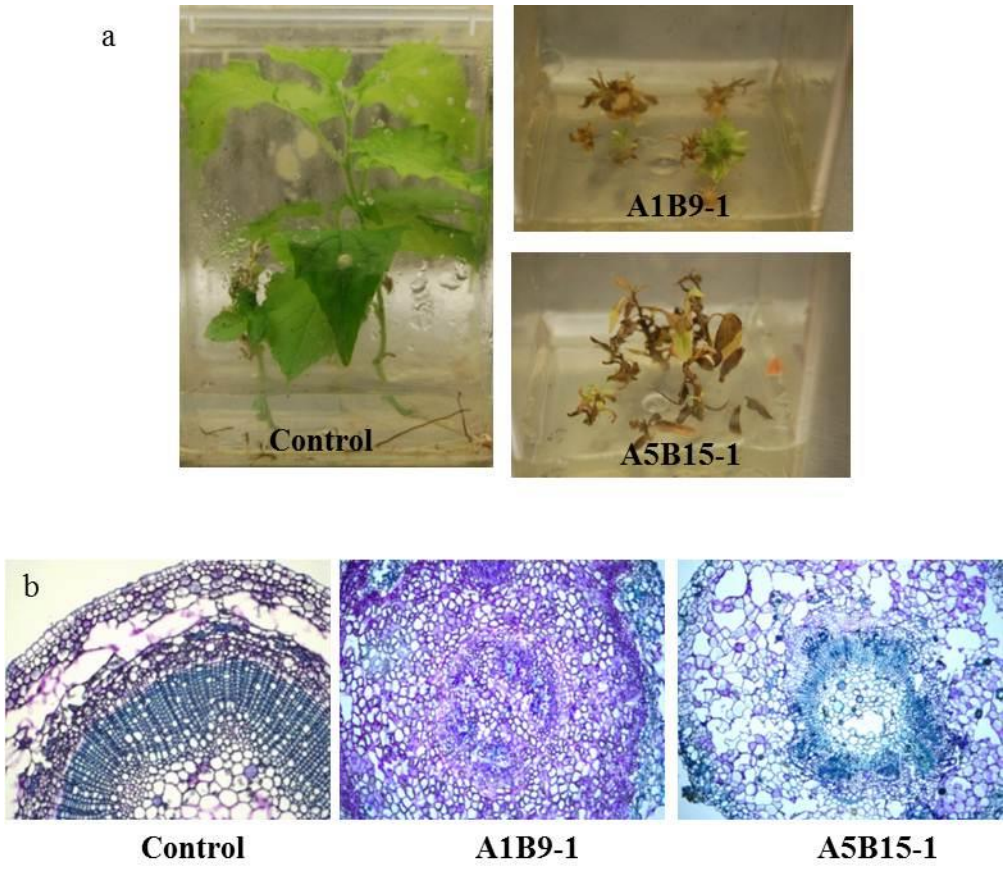

Figure 2.2. Developmental anomalies in some difficult-to-regenerate transformants. Developmental problems observed during regeneration (A1B9-1) and elongation (A5B15-1) stages of two transgenic lines are shown relative to a normally elongating wild type line (a). Anatomical abnormalities were observed in stem cross-sections from plants shown in panel (b).

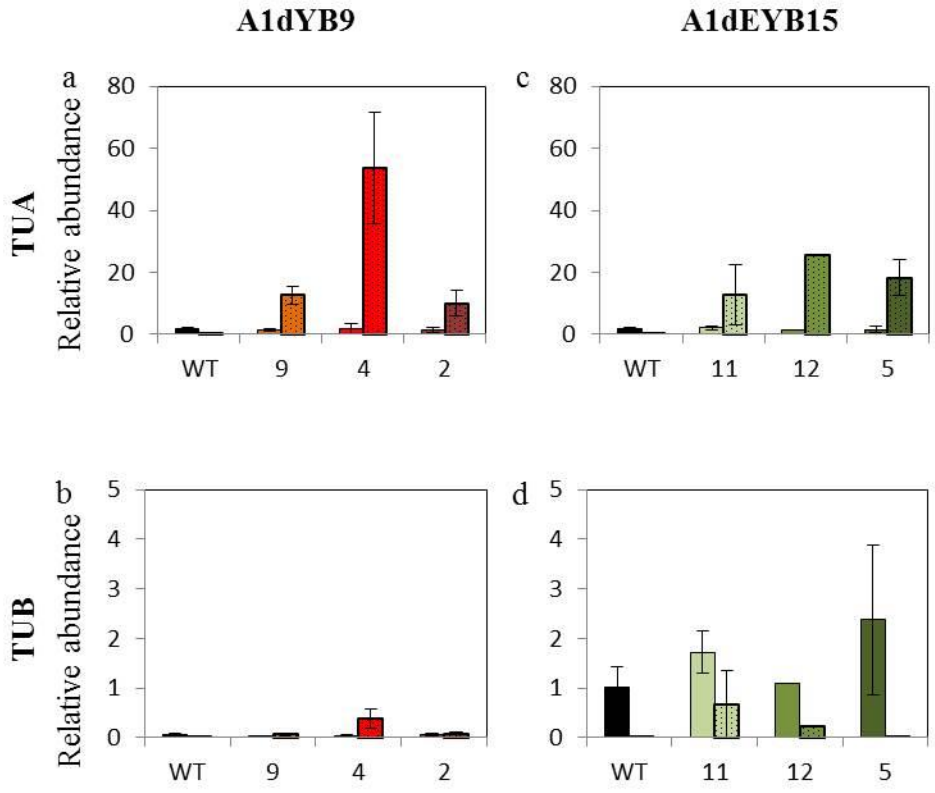

Figure 2.3. QPCR analysis of tubulin gene expression in mature leaves. Expression analysis of endogenous (solid bars) and transgene (dotted bars) tubulins in mature leaves (LPI 15) of the A1dYB9 (a, b) and A1dEYB15 (c, d) transgenic groups. Bars represent mean ± SD expression of at least three biological replicates, except lines 12 and 5, which included one and two biological replicates, respectively.

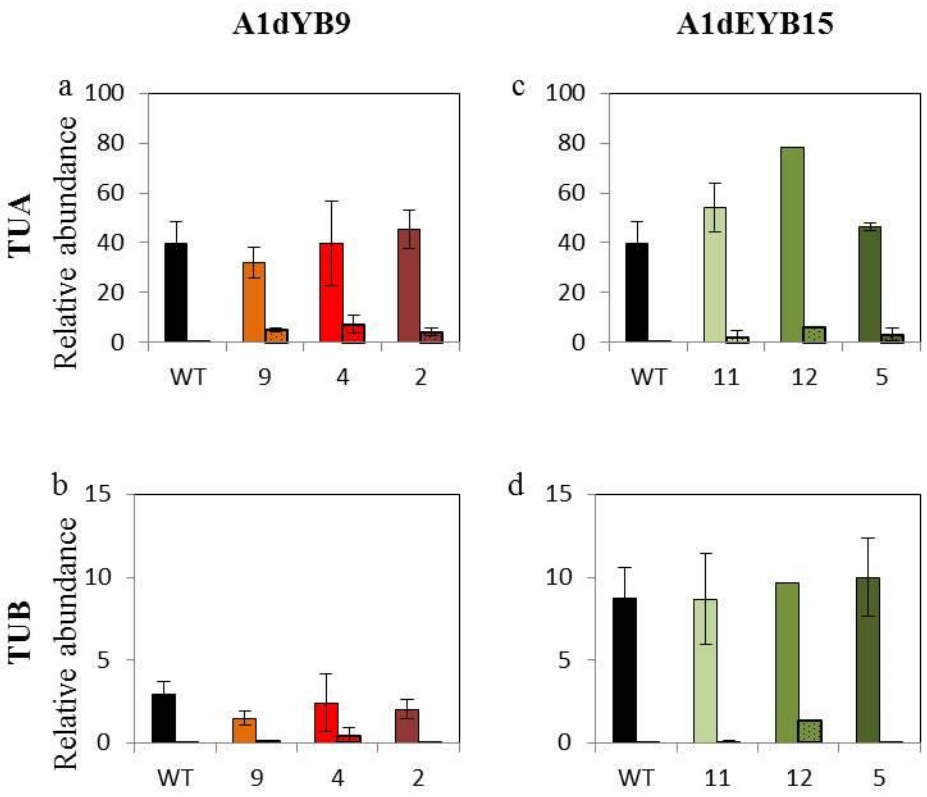

Figure 2.4. QPCR analysis of tubulin gene expression in developing xylem. Expression analysis of endogenous (solid bars) and transgene (dotted bars) tubulins in developing xylem of the A1dYB9 (a, b) and A1dEYB15 (c, d) transgenic groups. Bars represent mean ±SD expression of at least three biological replicates, except lines 12 and 5, which included one and two biological replicates, respectively.

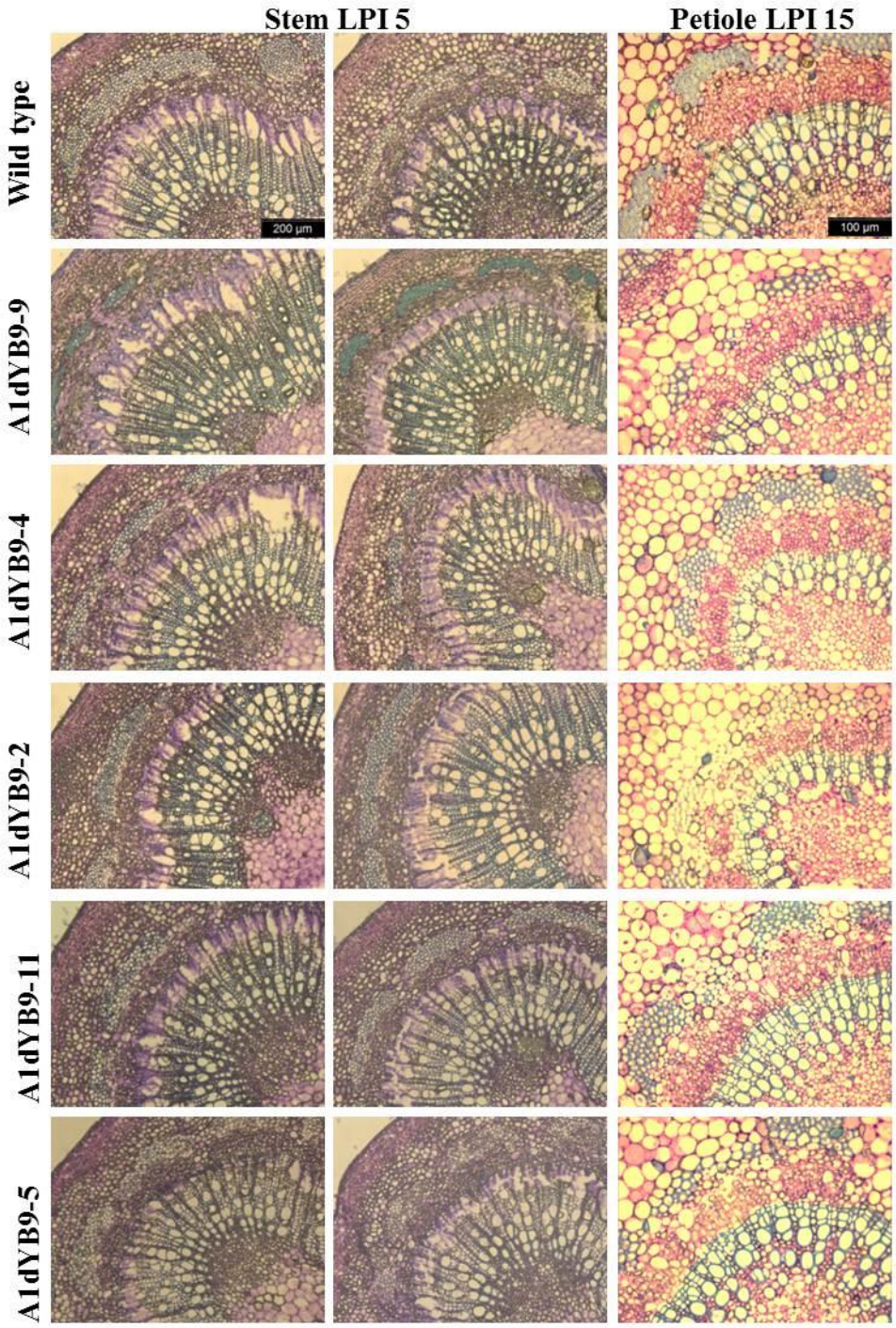

Figure 2.5. Histological analysis of stem (at LPI 5, two biological replicates) and petiole (LPI 15) cross sections of wild type and transgenic *Populus*. Tissues were stained with toluidine blue and visualized under microscope.

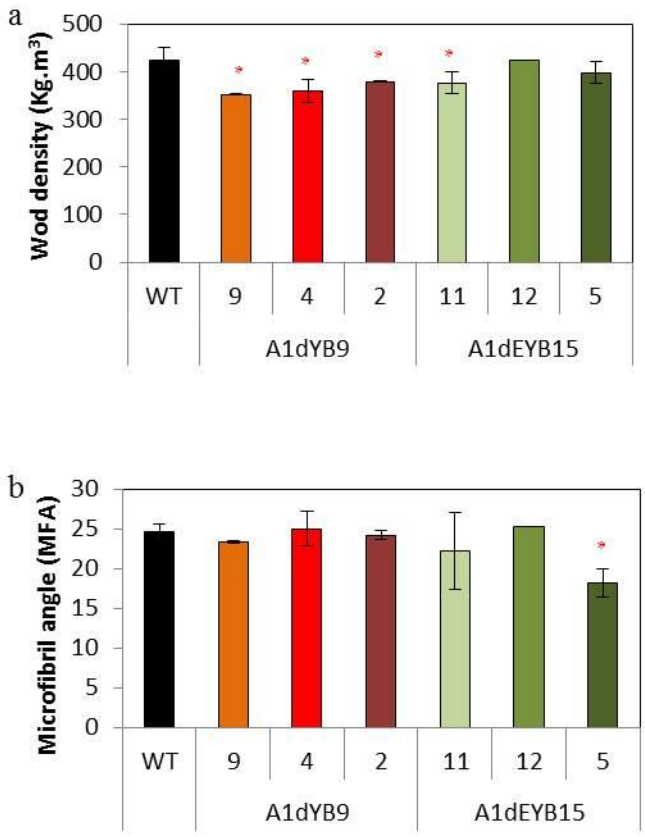

Figure 2.6. Physical properties of wood samples from wild type and transgenic *Populus* lines. Wood density was significantly lower in all A1dYB9 lines, while it was not consistently affected in A1dEYB15 (a). Microfibril angle did not exhibit any consistent trends in the transgenic lines (b). Bars represent the mean ±SD of at least three biological replicates, except lines 12 (n=1) and 5 (n=2). Significant differences as determined by student's-*t* test (*$p \leq 0.05$).

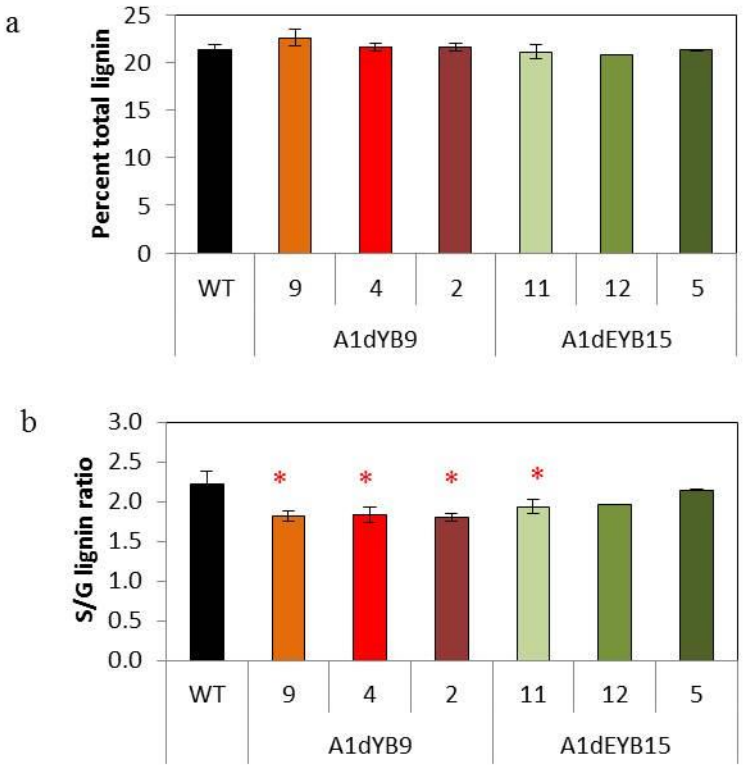

Figure 2.7. Wood chemistry analyses from wild type and transgenic *Populus* lines. Estimated total percent lignin as determined by Klason method (a). Ratio of syringyl-to-guaiacyl lignin monomer content (b). Bars represent mean ±SD of at least three biological replicates except lines 12 (n=1) and 5 (n=2). Significant differences as determined by student's-*t* test (*$p \leq 0.05$).

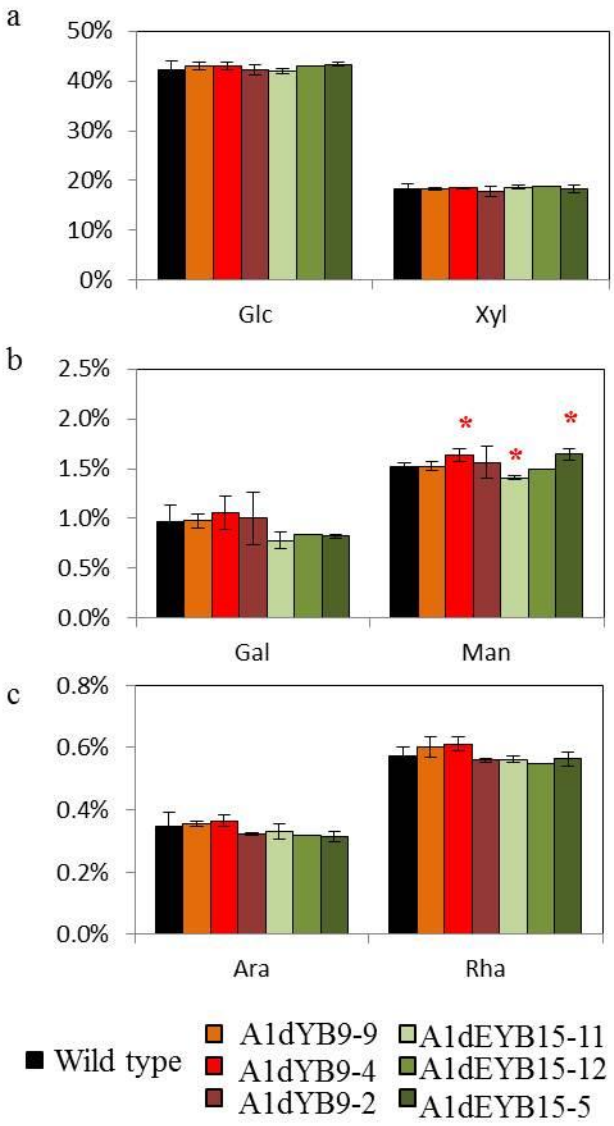

Figure 2.8. Structural carbohydrate analysis of secondary cell wall samples from wild type and transgenic *Populus* lines. Glucose and xylose (a); galactose and mannose (b); arabinose and rhamnose (c). Bars represent mean ±SD of at least three biological replicates except lines 12 (n=1) and 5 (n=2). Significant differences as determined by student's-*t* test are indicated by red asterisks (*p≤0.05).

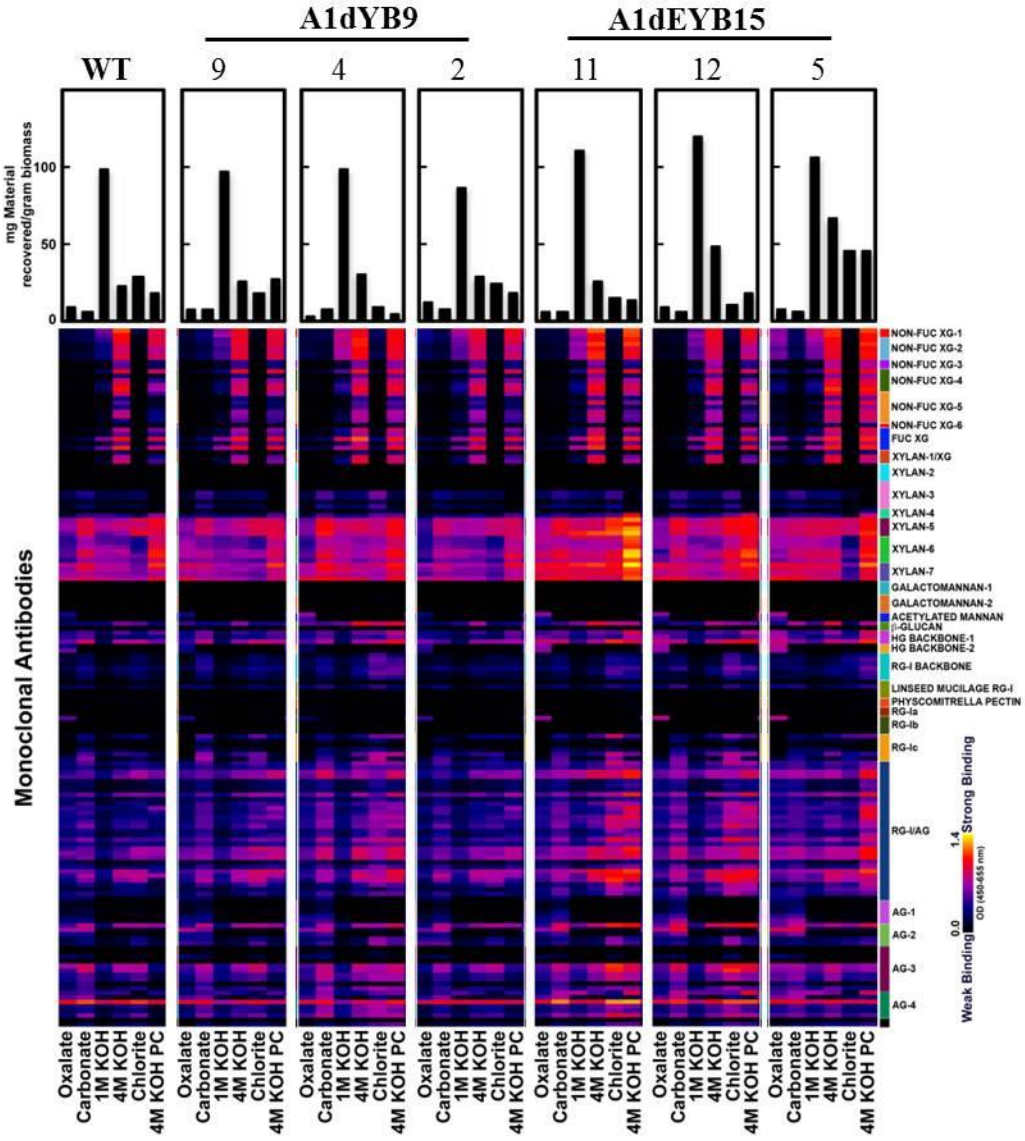

Figure 2.9. Glycome profiling of extractive-free wood biomass in wild-type and transgenic *Populus* lines. Representative profiles of three independent transgenic lines from A1dYB9 and A1dEYB15 group are shown along with wild type control. The six sequential extractions are arranged in columns, while monoclonal antibodies recognizing specific epitopes are arranged in rows. The carbohydrate recovery from each fraction is indicated by bars over the respective profiles. Figure is generated by Sivakumar Pattathil.

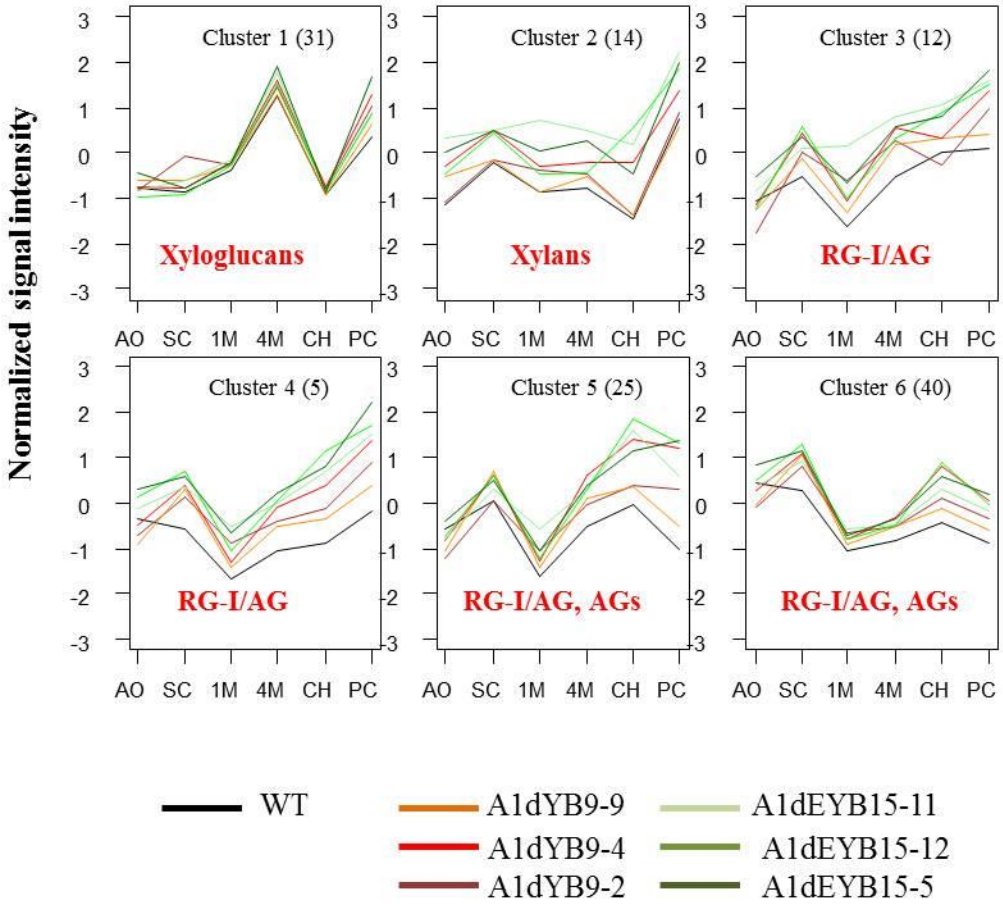

Figure 2.10. Self-organizing map (SOM) clustering of polysaccharide antibody binding patterns in wild type and transgenic *Populus* lines. Six extractions are arranged on the x-axis and normalized signal intensities are on the y-axis. Representative antibody groups are shown within each cluster. RG-I, Rhamnogalacturonan-I,/AG, arabinogalactans. Extraction fractions are AO, ammonium oxalate; SC, sodium carbonate; 1M, 1M potassium hydroxide; 4M, 4M potassium hydroxide; CH, chlorite and PC, post-chlorite 4M potassium hydroxide. SOM clustering analysis was performed with the assistance from Lianjiao Xue.

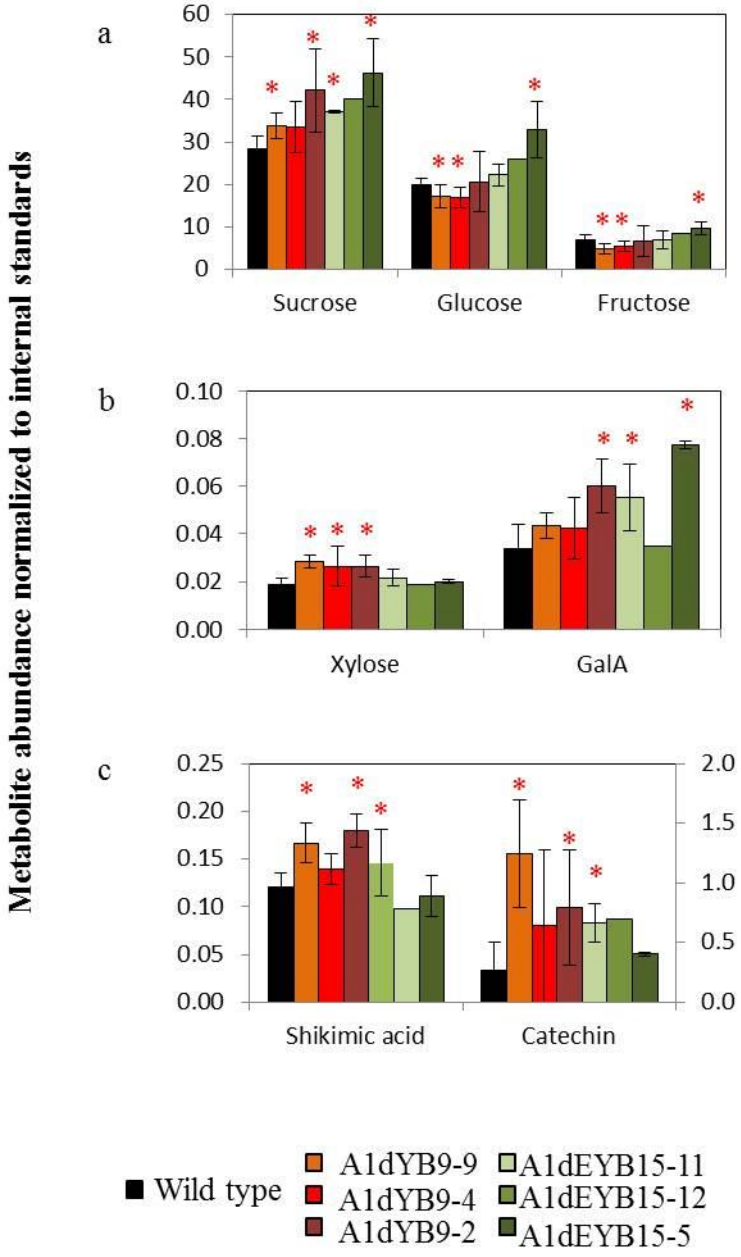

Figure 2.11. Abundance of soluble metabolites in wild-type and trangenic *Populus* identified by GC-MS analysis. Bars represent mean ±SD of at least three biological replicates. Significant changes are indicated by red asterisks (*$p<0.05$). GalA, galacturonic acid.

a

b

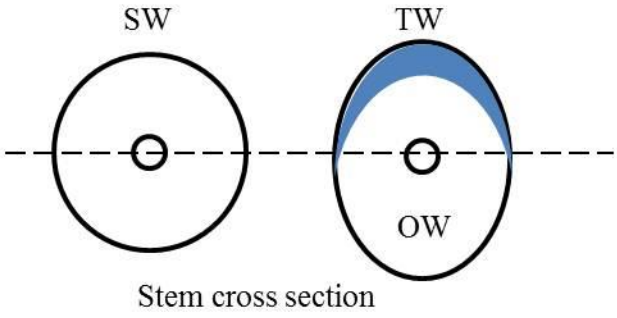

Figure 2.12. Artificial tension wood (TW) induction in *Populus*. Trees were inclined at an angle for three weeks to induce TW formation (a), schematic illustration of stem cross sections of SW and TW (b). Note the distinct G-layer in TW induced stems. SW, Straight wood from non-inclined trees, OW, opposite wood.

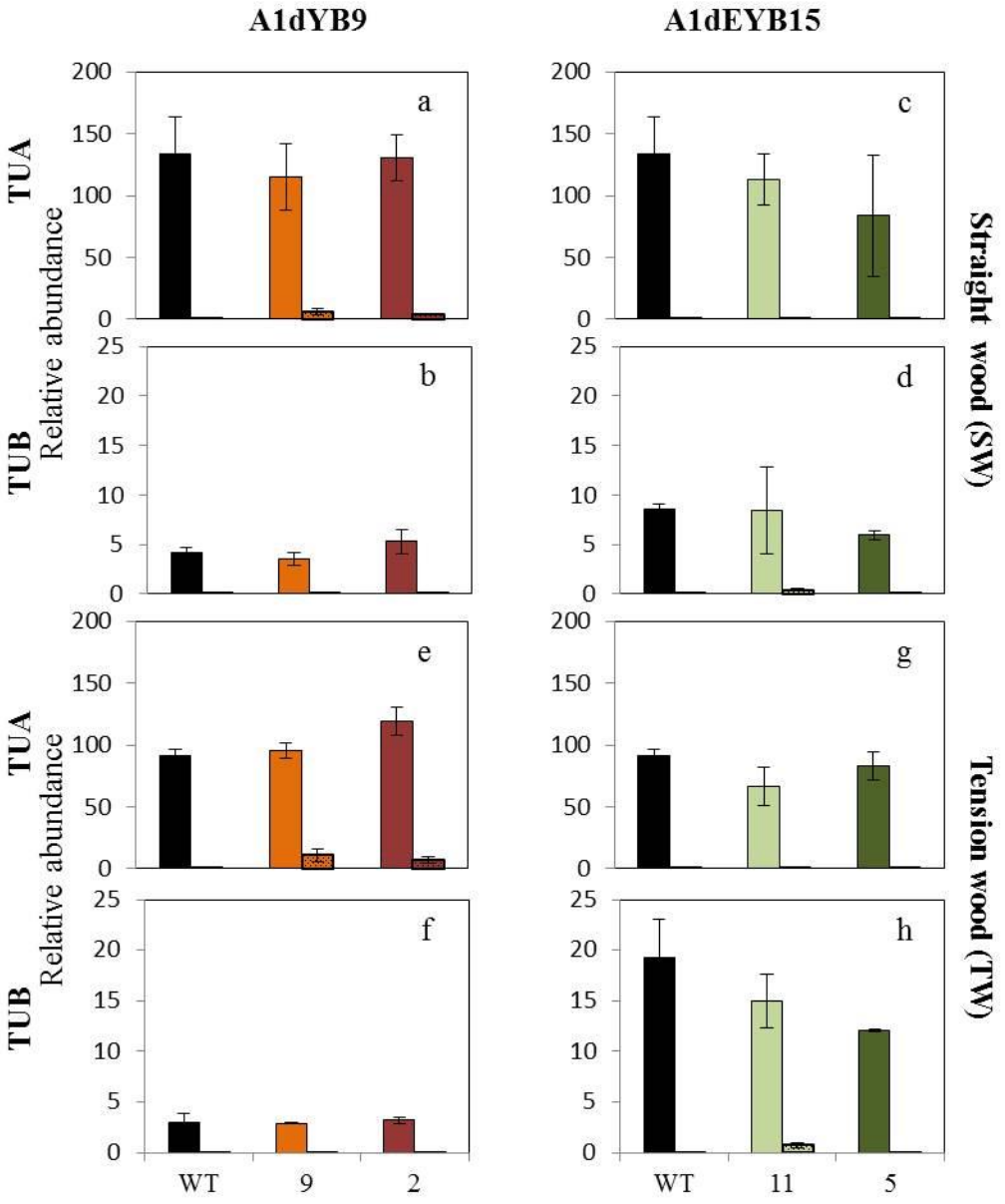

Figure 2.13. Expression of tubulins in straight and tension wood from wild-type and transgenic *Populus* lines. Expression of endogenous (solidd bar) and transgenic (dotted bar) *TUA1* in straight wood (a, c). Expression of endogenous and transgenic *TUB9* and *TUB15*, respectively, in straight wood (b, d). Expression of endogenous and transgenic *TUA1* in tension wood (e, g). Expression of endogenous and transgenic *TUB9* and *TUB15*, respectively, in tension wood (f, h). Bars represent mean ±SE of at least three biological replicates.

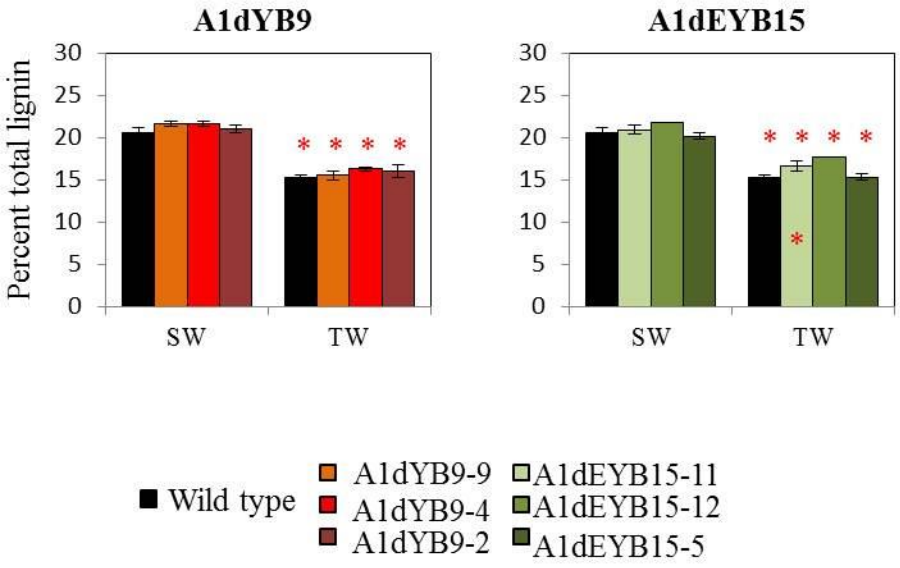

Figure 2.14. Total lignin analysis by Klason method in SW and TW samples. Bars represent mean ±SE of at least three biological replicates. SW, Straight wood; TW, tension wood. Significant differences are indicated by red asterisks above (treatment effect) or within the bar (genotypic effect) as determined by student's-*t* test (*p≤0.05)

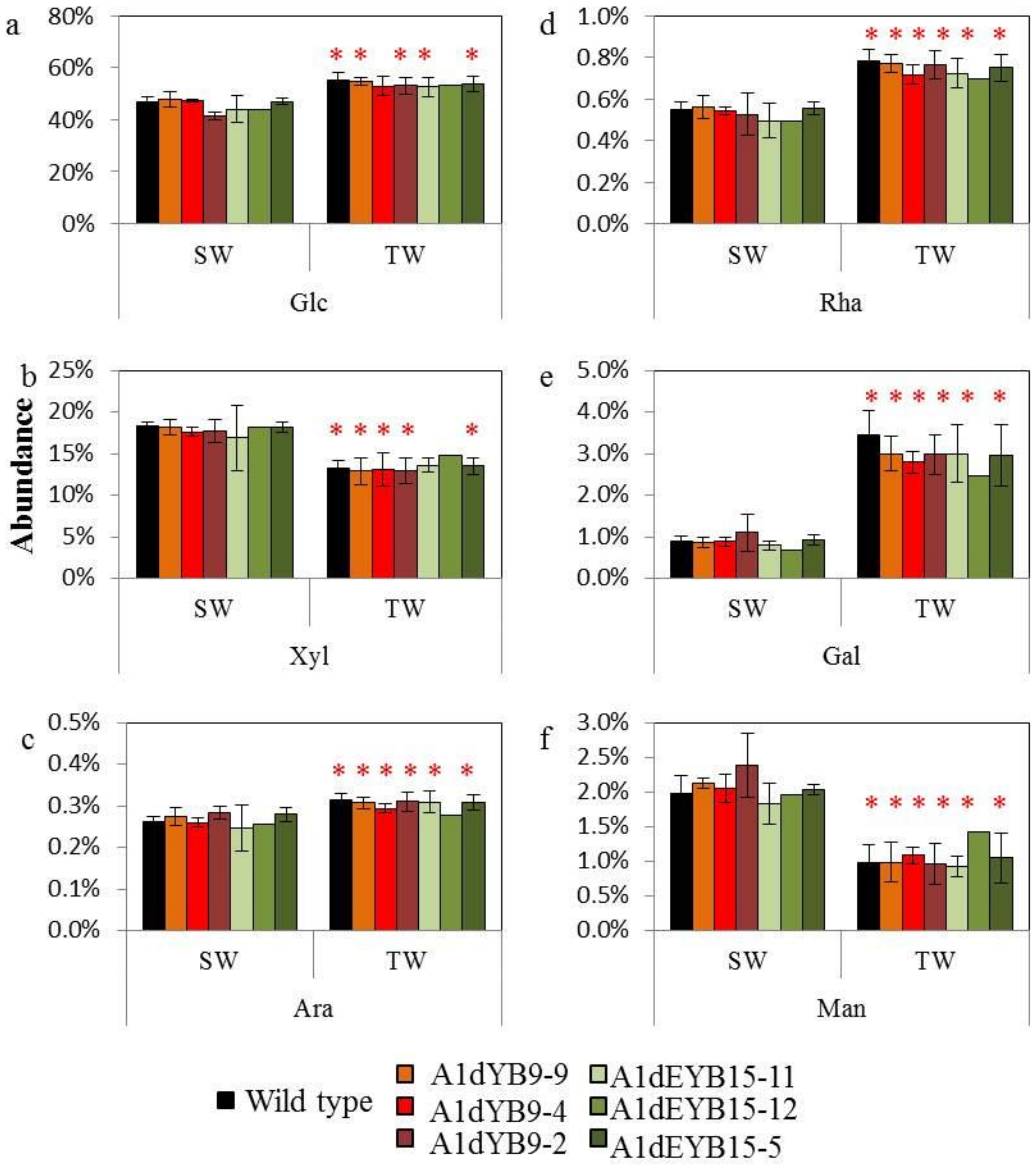

Figure 2.15. Structural carbohydrate analysis of straight and tension wood in wild type and transgenic wood samples. Bars represent mean ±SE of at least three biological replicates, except for line 12 (n = 1). Significant treatment effects are indicated by red asterisks (*$p<0.01$). Abbreviations of sugars are the same as in Figure 8.

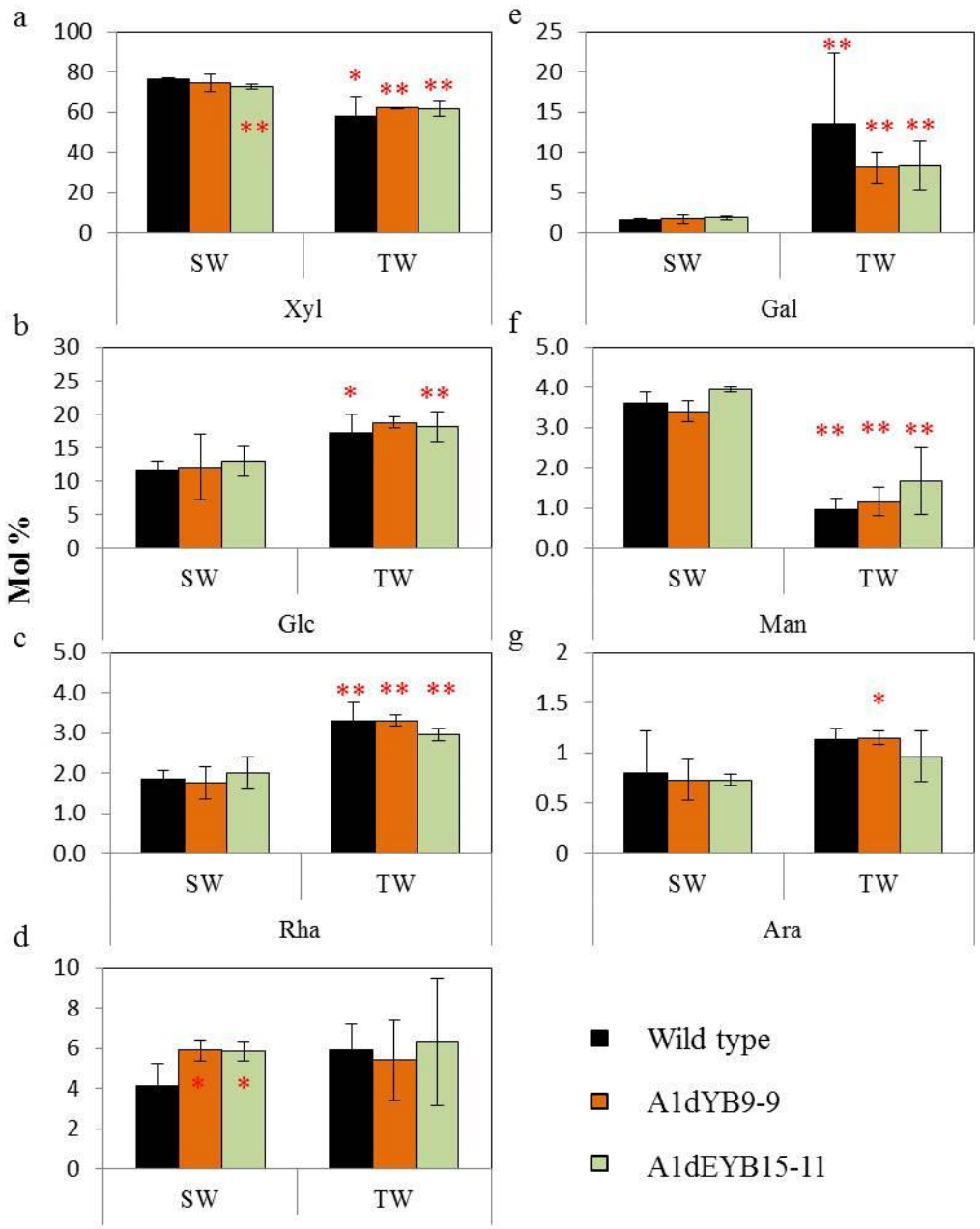

Figure 2.16. Hemicellulose composition analysis of SW and TW from wild type and transgenic *Populus* lines. One representative line each of A1dYB9 and A1dEYB15, in addition to wild type, were analyzed. Bars represent mean ±SD of at least two biological replicates. Significant differences are indicated by red asterisks over the bars (treatment effect) or inside the bars (genotype effects) as determined by student's-*t* test (*$p0.05<p\leq0.1$, **$p\leq0.05$). Abbreviations are the same as in Figure 8, plus GalA, galacturonic acid.

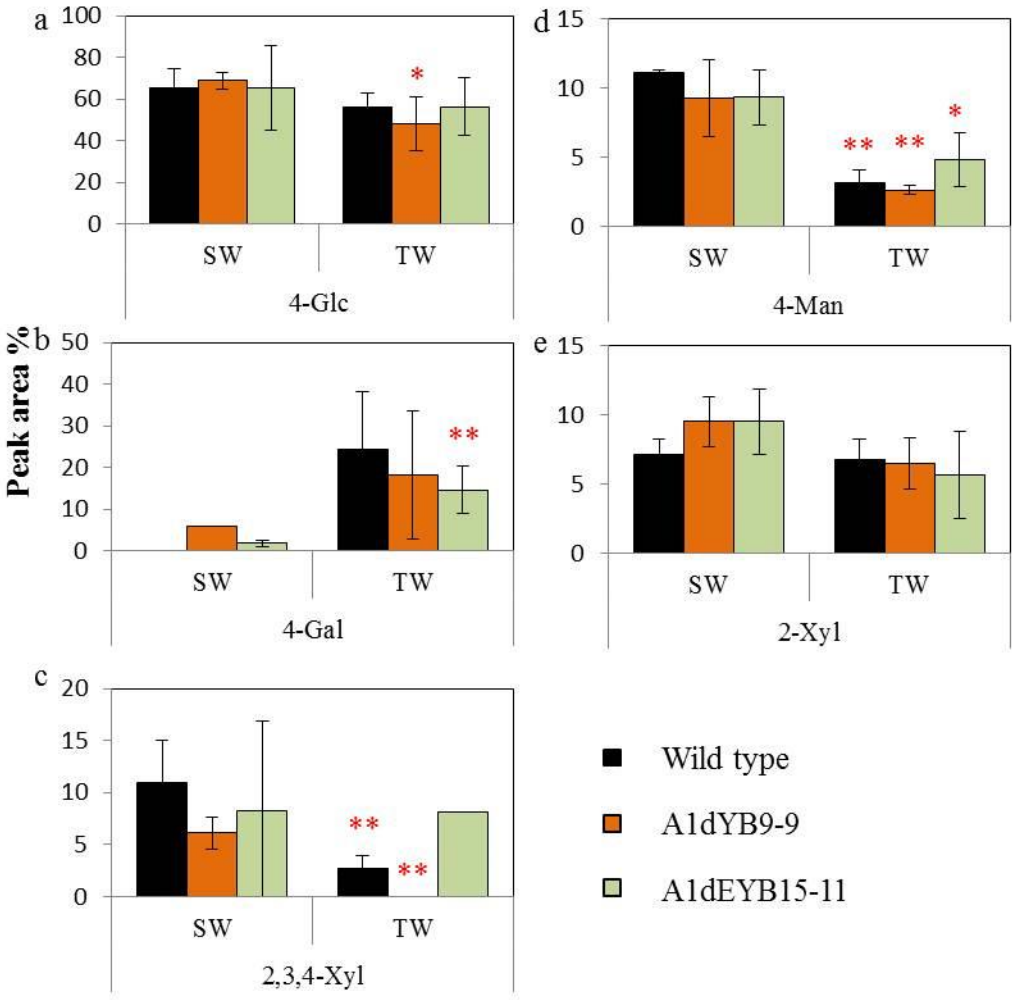

Figure 2.17. Glycosyl linkage analysis of SW and TW samples. One representative line each of A1dYB9 and A1dEYB15, in addition to wild type, were analyzed. Bars represent mean ±SD of at least two biological replicates. Significant linkage differences between SW andTW treatment are shown by red asterisks as determined by student's-*t* test (*$p0.05<p\leq0.1$, **$p\leq0.05$).

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Appendix 2A. List of primers used for cloning and real-time RT-PCR analysis. Underlined nucleotides correspond to the restriction sites for directional cloning in the binary vector.

Gene	Forward (F) and reverse (R) primers (5' to 3')		Amplicon (bp)
Cloning			
TUA1	(F)	GCTCTAGATGAGAGAGTGCATTTCGATTCA	1356
	(R)	TCCCCCGGGTCAGTACTCATCTCCTTCAT	
TUA5	(F)	GCTCTAGATGAGAGAGTGCATTTCG	1356
	(R)	GGCGTCGACTCACATGTACTCGTCACCA	
TUA1dY	(F)	TUA1-F	1353
	(R)	ACGCGTCGACTCACTCATCTCCTTCATCACCAT	
TUA1dEY	(F)	TUA1-F	1350
	(R)	ACGCGTCGACTCAATCTCCTTCATCACCATCCT	
TUB9	(F)	GCTCTAGATGAGAGAAATCCTTCATG	1335
	(R)	TCCCCCGGGTTAGTTCTCCATAGGCTCTT	
TUB15	(F)	GCTCTAGATGAGAGAAATCCTTCACATTC	1338
	(R)	TCCCCCGGGCTAGGCAGCCTCTTCCTCCT	
RT-PCR			
Endo_TUA1	(F)	TGGAGAGGATGGTGATGAAGGAGATG	109
	(R)	CACGTACCAACAGACATGGTCTAAGC	
Endo_TUB9	(F)	CAGTCATGTTTAGGAGAAARGCGT	230
	(R)	CACCAGAAACAACTCATCCTT	
Endo_TUB15	(F)	ACAGCTATGTTCAGGAGGAAGGCT	371
	(R)	TCACACCAGCCACATGGCTTAMTA	
Transgenic TUA1dY/ dEY	(F)	Endo-TUA1 (F)	129 *(dY)*, 126 *(dEY)*
	(R)	TAATCATCGCAAGACCGGCAACAG	
Transgenic TUB9/ TUB15	(F)	Endo-TUB9 (F)/ Endo-TUB15 (F)	280 *(TUB9)*, 284 *(TUB15)*
	(R)	TAATCATCGCAAGACCGGCAACAG	
Housekeeping genes			
Actin	(F)	GCGGTGATGGTGAGTTCTTTCT	213
	(R)	ATCGAGAGGGAGGACCATTACAGT	
Elongation factor-1B	(F)	GACCTKGTATCAGTGGATTCCCTC	213
	(R)	GAACAGAGGCACAAGATTACCAGG	
Actin related Protein	(F)	ACTGTGAGGAGATGCAGAAACGCA	194
	(R)	GCTGTGTCACGGGCATTCAATGYT	

Appendix 2B. Nucleotide sequence alignment of reference and synthetic *TUA* and *TUB* genes.

1. Nucleotide sequence alignment of *TUA1* and synthetic *TUA1* (codon altered), 67.9% identity.

```
               10        20        30        40        50        60
TUA1   ATGAGAGAGTGCATTTCGATTCACATTGGTCAAGCCGGTATTCAGGTCGGCAATGCTTGC
       ::::: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: ::
SynTUA ATGAGGGAATGTATCTCTATCCATATCGGACAGGCTGGAATCCAAGTTGGAAACGCATGT
               10        20        30        40        50        60

               70        80        90       100       110       120
TUA1   TGGGAGCTTTACTGCCTCGAACACGGCATTCAGCCTGATGGCCAGATGCCAAGTGACAAA
       :::::  : :: ::  : :: :: :: :: :: :: :: :: :: :: :::::   ::: ::
SynTUA TGGGAATTGTATTGTTTGGAGCATGGAATCCAACCAGACGGACAAATGCCTTCTGATAAG
               70        80        90       100       110       120

              130       140       150       160       170       180
TUA1   ACTGTTGGCGGAGGTGATGATGCTTTCAACACCTTTTTCAGTGAAACTGGTGCGGGAAAG
       :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: ::
SynTUA ACAGTGGGAGGTGGAGACGACGCATTTAATACTTTCTTTTCTGAGACAGGAGCTGGTAAA
              130       140       150       160       170       180

              190       200       210       220       230       240
TUA1   CACGTCCCTCGTGCTATCTTTGTTGATCTTGAGCCCACTGTTATTGATGAAGTCAGGACT
       :: :: ::  : :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: ::
SynTUA CATGTTCCAAGAGCAATTTTCGTGGACCTCGATCCAACAGTGATCGACGAGGTTAGAACC
              190       200       210       220       230       240

              250       260       270       280       290       300
TUA1   GGAGCATACCGCCAGCTTTTCCACCCCGAGCAACTCATCAGTGGAAAGGAAGATGCTGCC
       :: :: ::  : :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: ::
SynTUA GGTGCTTATAGACAACTCTTTCATCCTGAACAGCTTATTTCTGGTAAAGAGGACGCAGCT
              250       260       270       280       290       300

              310       320       330       340       350       360
TUA1   AACAACTTTGCCCGTGGCCACTATACCATTGGGAAAGAGATTGTTGATCTCTGCTTGGAT
       :: :: :: ::  : :: :: :: :: :: :: :: :: :: :: :: :: :: ::  : ::
SynTUA AATAATTTCGCTAGAGGACATTACACTATCGGAAAGGAAATCGTGGACCTTTGTCTTGAC
              310       320       330       340       350       360

              370       380       390       400       410       420
TUA1   CGTATCCGAAAGTTAGCTGATAACTGCACTGGCCTTCAAGGGTTTTTGGTGTTCAATGCT
        : :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: ::
SynTUA AGAATTCGTAAATTGGCAGACAATTGTACAGGATTGCAGGGATTCTTGGTTTTTAACGCA
              370       380       390       400       410       420

              430       440       450       460       470       480
TUA1   GTTGGAGGTGGTACTGGTTCTGGTCTTGGGTCACTTCTCCTGGAGAGGCTCTCTGTTGAC
       :: :: :: :: :: :: :: :: ::  : :: :: ::::: :: :: :: :: :: :: ::
SynTUA GTGGGTGGAGGAACAGGATCAGGATTGGGATCTCTTCTTCTTGAAAGACTTTCAGTGGAT
              430       440       450       460       470       480

              490       500       510       520       530       540
TUA1   TATGGCAAAAAATCAAAGCTTGGTTTCACTGTATATCCATCCCCGCAAGTTTCCACATCA
       :: :: :: :: :: ::  : :: :: :: :: :: :: :: :: :: :: ::::: :: ::
SynTUA TACGGAAAGAAGTCTAAATTGGGATTTACAGTTTACCCTTCTCCACAGGTTTCTACTTCT
              490       500       510       520       530       540
```

```
             550       560       570       580       590       600
TUA1   GTTGTAGAGCCCTACAACAGTGTCCTTTCAACTCACTCTCTCCTTGAGCATACTGATGTT
       :: :: :: :: :: ::   ::: :: :: :: :: :: :: ::::: :: :: :: :::
SynTUA GTGGTTGAACCATATAATTCTGTTCTCTCTACACATTCACTTCTTGAACACACAGACGTT
             550       560       570       580       590       600

TUA1   GCTGTGCTCCTTGACAATGAGGCCATCTATGACATTTGCAGGCGCTCTCTTGACATTGAG
       ::::: :: ::::: :: :: :: :: :: :: :: :: ::  : ::  : :: :: ::
SynTUA GCTGTTCTTCTTGATAACGAAGCTATTTACGATATCTGTAGAAGATCATTGGATATCGAA
             610       620       630       640       650       660

TUA1   CGTCCCACTTACACCAATCTTAACCGCCTTGTTTCTCAGGTGATCTCATCTTTGACTGCC
        : :: :: :: :: ::  : ::  :  : :: ::::: :: ::::: ::: : :: ::
SynTUA AGACCTACATATACTAACTTGAATAGATTGGTGTCTCAAGTTATCTCTTCTCTTACAGCT
             670       680       690       700       710       720

TUA1   TCATTAAGGTTTGATGGAGCTCTTAATGTGGATGTTACTGAGTTCCAAACCAACTTGGTT
       :: :: :: :: :: :: :: ::  : :: :: :: ::::: :: :: :: :: ::: : :::
SynTUA TCTTTGAGATTCGACGGTGCATTGAACGTTGACGTTACAGAATTTCAGACTAACCTTGTT
             730       740       750       760       770       780

TUA1   CCATACCCCAGGATCCATTTCATGCTTTCCTCTTATGCCCCTGTCATCTCCGCAGAGAAG
       :: ::::: :: :: :: :: :::::::: ::::: :: :: :: :: :: :: :: :::
SynTUA CCTTACCCTAGAATTCACTTTATGCTTTCTTCTTACGCTCCAGTTATTTCTGCTGAAAAG
             790       800       810       820       830       840

TUA1   GCATACCATGAGCAGCTCTCTGTGGCTGAGATAACCAACAGTGCTTTTGAGCCATCATCC
       :: :: :: :: :: :: :: :: ::::: :: :: ::   :::::: :: :: :: ::
SynTUA GCTTATCACGAACAACTTTCAGTTGCTGAAATTACTAATTCTGCTTTCGAACCTTCTTCT
             850       860       870       880       890       900

TUA1   ATGATGGCCAAGTGTGACCCACGTCATGGCAAGTACATGGCTTGCTGCCTGATGTATAGA
       :::::::: ::::: :: ::  : :: :: :: :: :::::::: :: :: ::::: ::
SynTUA ATGATGGCTAAGTGCGATCCTAGACACGGAAAATATATGGCTTGTTGTCTTATGTACAGG
             910       920       930       940       950       960

TUA1   GGTGATGTTGTGCCCAAGGATGTGAATGCAGCTGTGGCTACCATCAAGACCAAGCGCACA
       :: :: :: :: :: :: :: :: :: :: :: ::::: :: :: :: :: :: ::  : ::
SynTUA GGAGACGTGGTTCCTAAAGACGTTAACGCTGCTGTTGCAACTATTAAAACTAAAAGAACT
             970       980       990      1000      1010      1020

TUA1   ATCCAGTTTGTTGATTGGTGCCCAACTGGGTTCAAGTGTGGCATCAACTACCAGCCACCA
       :: :: :: :: :: ::::: :: :: :: :: :: :: :: :: :: :: :: :: :: :::
SynTUA ATTCAATTCGTGGACTGGTGTCCTACCGGATTTAAATGCGGAATTAATTATCAACCTCCA
            1030      1040      1050      1060      1070      1080

TUA1   ACTGTTGTTCCAGGAGGCGACCTTGCTAAGGTTCAGAGGGCTGTTTGCATGATTTCCAAT
       :: :::::::: :: :: :: :: :: :: :: ::::: ::::: :: ::::: :: ::
SynTUA ACAGTTGTTCCTGGTGGAGATCTCGCAAAAGTGCAGAGAGCTGTGTGTATGATCTCTAAC
            1090      1100      1110      1120      1130      1140

TUA1   TCCACAAGTGTTGCAGAAGTCTTCTCTCGCATTGACCACAAGTTTGATCTCATGTATGCC
       :: ::   ::::::::: :: :: ::  : :: :: :: :: :: :: :: ::::: ::
SynTUA TCTACTTCTGTTGCAGAGGTTTTTTCAAGAATCGATCATAAATTCGACCTTATGTACGCT
            1150      1160      1170      1180      1190      1200
```

```
TUA1   AAGCGTGCGTTTGTGCACTGGTATGTTGGCGAGGGTATGGAGGAAGGAGAGTTCTCAGAG
       ::  : :: :: :: :: ::::: :: :: :: :: ::::: :: :: :: ::::: ::
SynTUA AAAAGAGCTTTCGTTCATTGGTACGTGGGTGAAGGAATGGAAGAGGGTGAATTCTCTGAA
            1210      1220      1230      1240      1250      1260

TUA1   GCTCGTGAGGATCTTGCTGCCCTGGAGAAGGATTATGAGGAGGTTGGGGCTGAATCTCCC
       ::: : :: ::  : ::::: :: :: :: :: :: :: ::::: :: ::::: :: ::
SynTUA GCTAGAGAAGACTTGGCTGCTCTTGAAAAAGACTACGAAGAGGTGGGAGCTGAGTCACCA
            1270      1280      1290      1300      1310      1320

TUA1   GATGGAGAGGATGGTGATGAAGGAGATGAGTAC
       :: :: :: :: :: :: :: :: :: :: :::
SynTUA GACGGTGAAGACGGAGACGAGGGTGACGAATAC
            1330      1340      1350
```

2. Nucleotide sequence alignment of *TUB9* and synthetic *TUB9* (codon altered), 66.8% identity.

```
TUB9   ATGAGAGAAATCCTTCATGTCCAAGCTGGTCAGTGTGGTAACCAAATTGGTGGCAAGTTT
       ::::: :: ::  : :: :: :: :: :: :: :: :: :: :: :: :: :: :: ::
SynTUB ATGAGGGAGATTTTGCACGTTCAGGCAGGACAATGCGGAAATCAGATCGGAGGAAAATTC
              10        20        30        40        50        60

TUB9   TGGGAGGTTGTGTGTGATGAACACGGGATTGATCCCACAGGGAATTATGCTGGCAACTCT
       ::::: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: ::
SynTUB TGGGAAGTGGTTTGCGACGAGCATGGAATCGACCCTACTGGAAACTACGCAGGAAATTCA
              70        80        90       100       110       120

TUB9   AATGTTCAACTTGAGAGGGTTAATGTTTACTACAATGAGGCTAGTGGTGGTCGCTATGTG
       :: :: ::  : :: :: :: :: :: :: :: :: :: ::   ::: ::  : :: ::
SynTUB AACGTGCAGTTGGAAAGAGTGAACGTGTATTATAACGAAGCATCTGGAGGAAGATACGTT
              130       140       150       160       170       180

TUB9   CCTAGAGCTGTGCTAATGGACCTTGAGCCAGGGACCATGGACAGCTTGAGGACTGGTCCC
       :: :: :: :: :: :::::  : :: :: :: :: :::::     : :: :: :: ::
SynTUB CCAAGGGCAGTTCTTATGGATTTGGAACCTGGAACTATGGATTCTCTTAGAACAGGACCT
              190       200       210       220       230       240

TUB9   TACGGGCAAATCTTTAGGCCTGACAATTTTGTTTTCGGCCAAAATGGAGCTGGAAATAAC
       :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: ::::: :: ::
SynTUB TATGGACAGATTTTCAGACCAGATAACTTCGTCTTTGGACAGAACGGTGCTGGTAACAAT
              250       260       270       280       290       300

TUB9   TGGGCTAAGGGACATTACACTGAAGGAGCTGAACTGATCGATTCTGTTCTTGATGTTGTT
       ::::: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: ::
SynTUB TGGGCAAAAGGTCACTATACAGAGGGTGCAGAGCTTATTGACTCAGTGCTCGACGTGGTG
              310       320       330       340       350       360

TUB9   CGAAAAGAAGCTGAGAATTGTGATTGCTTACAAGGCTTCCAAATCTGTCATTCTCTGGGA
        :::: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: ::
SynTUB AGAAAGGAGGCAGAAAACTGCGACTGTTTGCAGGGATTTCAGATTTGCCACTCACTTGGT
```

```
             370       380       390       400       410       420

TUB9   GGTGGAACTGGATCAGGAATGGGGACTCTGCTCATATCAAAGATCAGGGAAGAATACCCT
       :: :: :: :: :: :: ::::: :: :: :: :: :: :: :: :: :: :: :: ::
SynTUB GGAGGTACAGGTTCTGGTATGGGAACACTTCTTATCTCTAAAATTAGAGAGGAGTATCCA
             430       440       450       460       470       480

TUB9   GATAGGATGATGTTAACTTTCTCAGTATTTCCATCTCCCAAGGTTTCTGATACTGTGGTT
       :: :: :::::::: :: :: :: :: :: :: :: :: :: :: ::::: :: :: ::
SynTUB GACAGAATGATGTTGACATTTTCTGTTTTCCCTTCACCAAAAGTGTCTGACACAGTTGTG
             490       500       510       520       530       540

TUB9   GAGCCCTACAATGCAACCCTCTCTGTACACCAACTAGTTGAAAATGCTGATGAGTGTATG
       :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :::
SynTUB GAACCATATAACGCTACTCTTTCAGTTCATCAGCTTGTGGAGAACGCAGACGAATGCATG
             550       560       570       580       590       600

TUB9   GTCCTTGACAATGAAGCTCTCTATGATATCTGCTTTCGAACTCTCAAGCTCACCAATCCA
       ::  : :: :: :: :: :: :: :: :: :: :: ::  :::: :: :: :: :: :: ::
SynTUB GTTTTGGATAACGAGGCACTTTACGACATTTGTTTCAGAACACTTAAACTTACTAACCCT
             610       620       630       640       650       660

TUB9   AGCTTTGGTGATCTTAACCACTTGATCTCAGCAACCATGAGTGGAGTAACATGTTGCCTT
          :: :: ::  : :: ::  : :: :: :: :: :::  ::: :: :: :: :: ::  :
SynTUB TCTTTCGGAGACTTGAATCATCTTATTTCTGCTACTATGTCTGGTGTTACTTGCTGTTTG
             670       680       690       700       710       720

TUB9   CGGTTCCCGGGCCAATTGAACTCTGATCTTCGGAAACTAGCCGTGAACTTAATCCCCTTC
        : :: :: :: ::  : :: :: ::  :  : :: :: :: :: :: :: :: :: :: ::
SynTUB AGATTTCCTGGACAGCTTAATTCAGACTTGAGAAAGCTTGCTGTTAATTTGATTCCATTT
             730       740       750       760       770       780

TUB9   CCGCGTCTCCATTTCTTCATGGTTGGTTTTGCACCATTAACCTCCCAAGTCTCACAACAG
       ::  :  : :: :: :: ::::: :: :: :: :: :: :: :: :: :: :: :: :: ::
SynTUB CCAAGATTGCACTTTTTTATGGTGGGATTCGCTCCTTTGACTTCTCAGGTTTCTCAGCAA
             790       800       810       820       830       840

TUB9   TACCGTGCCTTAACCATCCCGGAGCTGACACAACAAATGTGGGATGCTAAAAACATGATG
       ::  : :: :: :: :: :: :: ::  :::: :: :: :::::::: :: :: :: ::::::
SynTUB TATAGAGCTTTGACTATTCCAGAATTGACTCAGCAGATGTGGGACGCAAAGAATATGATG
             850       860       870       880       890       900

TUB9   TGTGCAGCTGACCCTCGGCACGGTAGGTACTTGACAGCCTCAGCTATGTTCCGAGGCAAA
       :: :: :: :: ::  : :: :: :: ::  : :: :: :: :: :::::  :::: ::
SynTUB TGCGCTGCAGATCCAAGACATGGAAGATATCTTACTGCTTCTGCAATGTTTAGAGGAAAG
             910       920       930       940       950       960

TUB9   ATGAGCACTAAGGAAGTTGATGAACAAATGATAAATGTGCAAAACAAGAACTCATCATAT
       :::   :: :: :: :: :: :: :: :: ::::: :: :: :: :: :: :: :: :: ::
SynTUB ATGTCTACAAAAGAGGTGGACGAGCAGATGATCAACGTTCAGAATAAAAATTCTTCTTAC
             970       980       990      1000      1010      1020

TUB9   TTTGTTGAGTGGATTCCAAATAATGTTAAATCAAGTGTTTGTGACATTCCACCAACTGGG
       :: :: :: ::::: :: :: :: :: :: ::   ::: :: :: :: :: :: :: ::
SynTUB TTCGTGGAATGGATCCCTAACAACGTGAAGTCTTCTGTGTGCGATATCCCTCCTACAGGA
            1030      1040      1050      1060      1070      1080
```

```
TUB9   TTAGCAATGTCATCAACATTTATGGGAAATTCTACGTCTATTCAAGAAATGTTTAAGCGT
        : :: ::::: :: :: :: ::::: :: :: :: ::::: :: :: ::::: ::  :
SynTUB CTTGCTATGTCTTCTACTTTCATGGGTAACTCAACTTCTATCCAGGAGATGTTCAAAAGA
            1090      1100      1110      1120      1130      1140

TUB9   GTTTCGGAACAATTTACAGTCATGTTTAGGAGAAAGGCGTTTTTGCACTGGTACACTGGG
       :: :: :: :: :: :: :: ::::: :: :: :: :: ::  : :: ::::: :: ::
SynTUB GTGTCTGAGCAGTTCACTGTTATGTTCAGAAGGAAAGCTTTCCTTCATTGGTATACAGGA
            1150      1160      1170      1180      1190      1200

TUB9   GAAGGAATGGATGAAATGGAGTTTACTGAGGCTGAAAGTAACATGAACGATTTGGTTTCT
       :: :: ::::: :: ::::: :: :: :: :::::   ::: ::::: ::  : :: :::
SynTUB GAGGGTATGGACGAGATGGAATTCACAGAAGCTGAGTCTAATATGAATGACCTTGTGTCT
            1210      1220      1230      1240      1250      1260

TUB9   GAATATCAACAATATCAAGATGCCGCAGCCGATAATGAGGGGGAGTATGACGAAGAAGAG
       :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: :: ::
SynTUB GAGTACCAGCAGTACCAGGACGCTGCTGCTGACAACGAAGGAGAATACGATGAGGAGGAA
            1270      1280      1290      1300      1310      1320

TUB9   CCTATGGAGAACTAA
       :: ::::: :: :::
SynTUB CCAATGGAAAATTAA
            1330
```

Appendix 2C. List of metabolites detected by GC-MS in wild type and transgenic developing xylem.

Metabolites	RI	RT	WT			A1dYB9-9			A1dYB9-4			A1dYB9-2			A1dEYB15-11			A1dEYB15-12-5		
Sucrose	2621.19	18.68	28.42	±	2.94	33.77	±	2.92	33.48	±	6.13	42.14	±	9.78	37.11	±	0.15	44.27	±	6.64
Glucose	1876.05	12.55	19.95	±	1.41	17.05	±	2.71	16.90	±	2.35	20.61	±	7.11	22.18	±	2.58	30.49	±	6.19
Fructose	1846.65	12.3	10.43	±	1.05	7.06	±	1.28	7.87	±	1.32	9.67	±	3.62	10.05	±	2.12	13.69	±	1.49
Tagatose	1855.85	12.38	7.00	±	0.67	4.85	±	0.94	5.37	±	0.94	6.49	±	2.25	6.84	±	1.43	9.21	±	1.05
Threose	1884.53	12.62	0.42	±	0.14	0.47	±	0.06	0.55	±	0.28	0.65	±	0.10	0.76	±	0.16	1.02	±	0.33
Erythrose	1431.4	8.88	0.02	±	0.02	0.09	±	0.06	0.05	±	0.04	0.01	±	0.01	0.02	±	0.01	0.02	±	0.01
Ribose	1623.54	10.37	0.02	±	0.00	0.01	±	0.00	0.02	±	0.00	0.02	±	0.01	0.02	±	0.00	0.02	±	0.00
Xylose	2358.86	16.6	0.02	±	0.00	0.03	±	0.00	0.03	±	0.01	0.03	±	0.00	0.02	±	0.00	0.02	±	0.00
GalA	1918.24	12.91	0.03	±	0.01	0.04	±	0.01	0.04	±	0.01	0.06	±	0.01	0.06	±	0.01	0.06	±	0.02
Galactose	2271.28	15.87	0.16	±	0.02	0.16	±	0.01	0.15	±	0.01	0.17	±	0.03	0.16	±	0.02	0.18	±	0.04
Xylose	1630.28	10.43	0.01	±	0.00	0.01	±	0.00	0.01	±	0.00	0.01	±	0.00	0.01	±	0.01	0.01	±	0.00
Arabinose	1739.81	11.37	0.76	±	0.30	0.90	±	0.29	0.74	±	0.27	0.71	±	0.27	0.74	±	0.26	0.53	±	0.18
Gulose	2374.27	16.72	1.13	±	0.31	0.90	±	0.35	1.25	±	0.43	0.93	±	0.25	0.95	±	0.47	0.69	±	0.35
l-Alanine	1327.41	8.24	0.08	±	0.01	0.06	±	0.01	0.06	±	0.01	0.09	±	0.04	0.07	±	0.00	0.07	±	0.02
Asparagine	1647.65	10.58	0.21	±	0.15	0.06	±	0.02	0.23	±	0.33	0.03	±	0.00	0.06	±	0.07	0.10	±	0.04
L-Aspartic acid	1503.22	9.33	0.24	±	0.09	0.17	±	0.04	0.15	±	0.11	0.12	±	0.06	0.12	±	0.06	0.16	±	0.11
L-Serine	1312.16	8.14	0.05	±	0.02	0.05	±	0.01	0.05	±	0.03	0.06	±	0.02	0.06	±	0.01	0.07	±	0.03
L-Threonic acid	1532.25	9.58	0.08	±	0.03	0.14	±	0.03	0.10	±	0.01	0.10	±	0.01	0.11	±	0.01	0.09	±	0.01
L-Tyrosine	1928.5	13	0.01	±	0.01	0.01	±	0.00	0.01	±	0.00	0.01	±	0.00	0.01	±	0.00	0.01	±	0.00
L-Valine	1158.54	7.19	0.02	±	0.01	0.02	±	0.01	0.03	±	0.03	0.03	±	0.02	0.03	±	0.01	0.04	±	0.01
L-glutamine	1753.43	11.49	0.35	±	0.20	0.14	±	0.10	0.41	±	0.48	0.13	±	0.08	0.19	±	0.08	0.17	±	0.13
Hexahomoserine	1596.42	10.14	0.03	±	0.01	0.01	±	0.01	0.02	±	0.02	0.02	±	0.01	0.02	±	0.00	0.03	±	0.02
Threonine	1342.02	8.33	0.05	±	0.03	0.09	±	0.04	0.06	±	0.04	0.04	±	0.02	0.05	±	0.01	0.07	±	0.01
Citric acid	1793.3	11.84	0.48	±	0.14	0.44	±	0.06	0.47	±	0.20	0.36	±	0.03	0.36	±	0.07	0.39	±	0.10
Glc-6-phosphate	2310.29	16.2	0.10	±	0.04	0.07	±	0.01	0.08	±	0.03	0.09	±	0.03	0.08	±	0.00	0.08	±	0.02
Fumaric acid	1304.25	8.09	0.03	±	0.03	0.14	±	0.07	0.02	±	0.02	0.02	±	0.00	0.02	±	0.00	0.03	±	0.01
Oxalic acid	1333.47	8.27	0.05	±	0.01	0.05	±	0.03	0.04	±	0.04	0.05	±	0.02	0.03	±	0.01	0.04	±	0.02
Succinic acid	1265.06	7.85	0.15	±	0.04	0.11	±	0.03	0.11	±	0.04	0.14	±	0.04	0.12	±	0.02	0.12	±	0.04
Phosphoric acid	1215.86	7.54	2.56	±	1.03	4.12	±	1.89	2.40	±	1.58	2.38	±	0.28	3.27	±	2.16	5.82	±	0.78
Oxaloacetic acid	1702.45	11.05	0.03	±	0.02	0.03	±	0.01	0.03	±	0.01	0.03	±	0.01	0.03	±	0.01	0.04	±	0.01

Metabolites	RI	RT	WT			A1dYB9-9			A1dYB9-4			A1dYB9-2			A1dEYB15-11			A1dEYB15-12-5		
Malic acid	1462.11	9.07	5.66	±	4.27	7.33	±	2.58	9.27	±	1.71	8.62	±	3.56	9.11	±	2.38	8.30	±	1.51
Arabinitol	1669.15	10.76	0.06	±	0.01	0.08	±	0.02	0.06	±	0.02	0.06	±	0.01	0.05	±	0.01	0.05	±	0.02
Galactitol	1898.59	12.74	4.02	±	0.41	1.29	±	1.74	3.06	±	0.24	3.95	±	1.30	4.05	±	0.55	5.62	±	1.18
palatinitol	2526.7	17.97	0.19	±	0.02	0.22	±	0.03	0.19	±	0.07	0.22	±	0.02	0.18	±	0.02	0.22	±	0.06
Allo-inositol	2086.82	14.34	11.57	±	2.42	15.02	±	1.69	11.95	±	2.33	14.22	±	3.17	10.01	±	1.77	10.92	±	3.01
Phthalic acid	1963.2	13.3	0.03	±	0.00	0.02	±	0.00	0.02	±	0.00	0.02	±	0.01	0.03	±	0.01	0.02	±	0.00
Palmitic acid	2042.4	13.97	2.24	±	0.94	1.35	±	0.60	2.15	±	1.19	2.13	±	0.89	1.66	±	0.15	2.38	±	0.51
Linoleic acid	2217.72	15.42	0.19	±	0.17	0.09	±	0.03	0.21	±	0.22	0.13	±	0.02	0.12	±	0.07	0.15	±	0.08
stearic acid	2247.21	15.67	5.11	±	2.16	2.71	±	1.14	4.87	±	3.16	4.85	±	2.13	3.33	±	0.20	5.27	±	1.03
Myristic acid	1835.69	12.2	0.19	±	0.06	0.23	±	0.05	0.25	±	0.10	0.18	±	0.07	0.18	±	0.06	0.18	±	0.05
Quinic acid	1835.92	12.2	8.28	±	2.12	12.02	±	0.17	9.82	±	3.83	11.24	±	1.88	10.71	±	3.93	9.92	±	2.35
Shikimic acid	1777.4	11.7	0.12	±	0.02	0.17	±	0.02	0.14	±	0.02	0.18	±	0.02	0.15	±	0.03	0.11	±	0.02
Catechin	2851.09	20.4	0.27	±	0.24	1.24	±	0.45	0.64	±	0.63	0.79	±	0.48	0.66	±	0.16	0.40	±	0.25
Coniferyl alcohol	1931.71	13.02	0.38	±	0.20	0.25	±	0.06	0.63	±	0.34	0.42	±	0.17	0.43	±	0.12	0.45	±	0.09
Sinapyl alcohol	2080.9	14.29	5.62	±	1.97	2.74	±	1.72	6.05	±	1.48	4.94	±	2.30	6.03	±	2.01	7.94	±	2.80
Salicin	2494.06	17.72	3.91	±	2.64	4.04	±	1.38	3.71	±	1.44	2.72	±	0.56	2.47	±	0.79	1.75	±	0.59
Mucic acid	1800.35	11.9	0.08	±	0.01	0.11	±	0.01	0.09	±	0.02	0.10	±	0.00	0.08	±	0.00	0.08	±	0.01
Glyceric acid	1276.43	7.92	0.02	±	0.01	0.04	±	0.01	0.02	±	0.00	0.02	±	0.00	0.02	±	0.00	0.02	±	0.00
Tranexamic acid	1410.55	8.75	0.01	±	0.00	0.01	±	0.00	0.01	±	0.00	0.01	±	0.00	0.01	±	0.00	0.01	±	0.00
Tartaric acid	1574.75	9.95	0.05	±	0.01	0.04	±	0.01	0.05	±	0.02	0.05	±	0.02	0.05	±	0.01	0.04	±	0.01
Ascorbic acid	1932.4	13.04	2.13	±	1.48	1.27	±	0.92	2.27	±	0.73	3.22	±	0.90	2.45	±	0.67	2.19	±	0.58
Lactic acid	2562.21	18.24	0.02	±	0.01	0.02	±	0.00	0.02	±	0.00	0.02	±	0.00	0.02	±	0.00	0.02	±	0.01
Saccharic acid	2049.11	14.03	0.03	±	0.01	0.04	±	0.01	0.03	±	0.01	0.03	±	0.00	0.02	±	0.00	0.03	±	0.01
Guanidinobutyric A	1521.57	9.49	0.19	±	0.15	0.23	±	0.04	0.13	±	0.10	0.12	±	0.08	0.23	±	0.12	0.26	±	0.21
Mannonic acid	1863.83	12.44	0.28	±	0.07	0.15	±	0.04	0.17	±	0.10	0.13	±	0.03	0.22	±	0.04	0.17	±	0.01
Cellobiose	2388.19	16.85	0.13	±	0.03	0.13	±	0.00	0.10	±	0.01	0.12	±	0.01	0.11	±	0.04	0.14	±	0.03
Dibutyl phthalate	1962.84	13.29	0.23	±	0.10	0.18	±	0.03	0.23	±	0.09	0.18	±	0.04	0.23	±	0.04	0.22	±	0.06
Glycerol	1211.85	7.52	0.56	±	0.17	0.38	±	0.09	0.62	±	0.16	0.51	±	0.14	0.51	±	0.14	0.44	±	0.29

CHAPTER 3

PERTURBATION OF TUBULINS ALTERS LEAF DEVELOPMENT AND SHORT-TERM DROUGHT RESPONSE IN *POPULUS*[1]

[1] Swamy, P.S., Harding, S.A., Frost C.J., Nyamdari, B and Tsai, C-J. *To be submitted.*

Abstract

The dynamic organization of cortical microtubules (MTs) is essential in regulation of cell expansion. In plants, cortical MT arrangement also influences stomatal guard cell responses to environmental stimuli. Thus, altered MT dynamics brought about by transgenic tubulin manipulations are likely to produce pleiotropic effects. Previously, transgenic *Populus* plants with perturbed tubulin expression and post-translational modifications were found to exhibit altered cell wall properties during wood formation. These transgenic plants afforded an opportunity to investigate the effects of tubulin manipulations on other MT-dependent traits during leaf development. Prolonged leaf expansion was observed in transgenic A1dYB9 (harboring detyrosinated *TUA1dY* in conjunction with *TUB9*) but not A1dEYB15 (nontyrosinatable *TUA1dEY* and *TUB15*) plants. Epidermal impression analysis suggested that mature leaves of A1dYB9 plants exhibited a greater expansion of pavement cells in the leaf-width direction compared to the other plant lines. To investigate the effects of tubulin manipulations on guard cell dynamics, transgenic plants were subjected to drought treatments, a stress known to induce MT-mediated stomatal closure. Under acute drought conditions, transgenic plants exhibited a delayed stomatal closure and, as a result, maintained higher rates of gas exchange and transpiration than the wild type at the time of measurement. However, under prolonged drought treatments, transgenic and wild type plants had similar stress adaptation responses. The results suggested that perturbation of tubulin homeostasis affected cell expansion during leaf development and caused short-term changes in guard cell dynamics under water-limited conditions.

Introduction

As vital cytoskeletal components, microtubules (MTs) are essential to cell division, cell expansion, organelle trafficking, cell wall formation and stomatal behavior. MTs are dynamic structures that depend on the regulated polymerization and depolymerization of alpha-(TUA) and beta-(TUB) tubulin heterodimers for their function (Shaw et al., 2003). As dynamic structures, cortical MTs control the directional growth of cellulose microfibrils (MFs), thereby constraining radial expansion and conferring growing cells with their shape (reviewed in Lloyd and Chan, 2004). MT function also depends on various MT-interacting proteins, as mutations in many of those proteins result in severe defects in cell expansion (Bichet et al., 2001; Burk et al., 2001; Whittington et al., 2001; Sedbrook et al., 2004) and leaf shape (reviewed in Tsukaya, 2006). Similarly, mutations that directly affect MT structure result in a plethora of morphological phenotypes (Baskin, 2001; Whittington et al., 2001; Ishida et al., 2007). In plants, tubulin mutations and ectopic expression of tubulins with C- or N-terminal tags can lead to helical growth and various organ twisting phenotypes (Bao et al., 2001; Abe et al., 2004; Burk et al., 2006; Ishida et al., 2007).

The opening and closing of leaf stomata is mediated by guard cells as they respond to changes in light conditions, water availability and hormonal flux (Schroeder et al., 2001). Such environmental changes trigger various signal transduction cascades, some of which appear to involve MTs and MT-associated proteins (Kim et al., 1995; Marcus et al., 2001; Zhang et al., 2008). The orientation of guard cell MTs exhibits a diurnal cycle which suggests that MT function links gas and water exchange with diurnal regulation of stomatal aperture (Fukuda et al., 1998). During stomatal opening, the MTs of the guard cells organize into arrays that guide MF reorientation (Palevitz, 1976; Lahav et al., 2004; Eisinger et al., 2012). In fact, GFP tracking of MT-plus ends revealed rapid changes in MT instability and/or orientation during natural stomatal movements, while manipulation of guard cell behavior by absicic acid or light treatments also led to changes in MT organization (Eisinger et al., 2012).

Both TUA and TUB subunits undergo extensive post-translational modifications (PTMs) after their assembly into MTs in response to various developmental and/or external cues (Duckett and Lloyd,

1994; MacRae, 1997; Westermann and Weber, 2003). One of the most extensively studied PTMs of animal tubulin is reversible removal and reattachment of C-terminal tyrosine (tyrosination and detyrosination cycle) of TUA subunits (Thompson, 1982). The detyrosinated TUA subunit may be irreversibly converted to a non-tyrosinatable TUA isoform by removing the penultimate glutamate, referred to as dEY or Δ2-modification (Paturle-Lafanechere et al., 1991). The presence of dY or dEY modifications has been associated with long-lived or stable MTs in mammalian brain cells (Kreis, 1987; Wehland and Weber, 1987; Alonso et al., 1993; Paturle-Lafanechere et al., 1994; Peris et al., 2009; Janke and Kneussel, 2010). Detyrosinated TUAs have been putatively detected in a few plants using animal PTM-specific antibodies, but their effects on MT function have not been described (Wiesler et al., 2002; Wang et al., 2004). In the current study, transgenic plants described in Chapter 2 that overexpress PTM mimics of *TUA1* (*dY* or *dEY*) in combination with *TUB9* or *TUB15* were chosen for further investigation. Transgenic *TUA* transcripts were comparatively abundant in leaves, and this presented an opportunity to investigate the sensitivity of epidermal cell expansion and guard cell behavior to MT perturbation. In the present study, leaf expansion and aspect were monitored in wild type and transgenic *Populus* trees. To explore the possibility of changed MT dynamics due to tubulin manipulations on guard cell behavior, plants were subjected to short- and long- term drought conditions. The transgenic effects due to water stress were analyzed by leaf photosynthetic measurements and metabolic profiling analysis and compared to wild type plants.

Materials and methods

Plant materials and growth conditions

The greenhouse grown wild type and transgenic *Populus* plants were used from the previous experiments (Chapter 2). Three individual lines each of A1dYB9 and A1dEYB15 transgenic group along with wild type plants were used in all the experiments. Plants were watered normally unless otherwise indicated for acute or chronic drought treatments. Fertilizer application and other routine maintenance such as spraying were carried out as needed on a weekly basis.

Leaf epidermal and stomata impression analysis

The epidermal impressions were obtained by applying a thin layer of nail polish over the reinforcement label attached to the leaves. After a few minutes of drying, the labels were carefully removed, adhered to a microscope slide and stored at room temperature until use. To obtain the stomata counts, the leaf was briefly immersed in liquid nitrogen and immediately brushed off the densely packed trichomes from the abaxial surface. The stomatal impressions were taken from the abaxial side. The impressions were viewed under a 40X objective and images were acquired using camera mounted on Zeiss Axioskop 50 microscope (Zeiss, USA). Further analyses to estimate the number of epidermal and/stomatal cells were carried out using the 'ImageJ' image processing program.

Drought treatments

Three transgenic events each of A1dYB9 and A1dEYB15 groups along with the wild-type controls were grown in standard greenhouse conditions. For the acute drought experiment, trees were watered and fertilized normally until they grew to 1 m height. The leaf gas exchange measurements of LPI 15 were taken at noon from all genotypes with at least four biological replications. Following the measurements, water was withheld until the visible drought symptoms (typically 16-18 hours) occurred on the younger leaves. Immediately, the trees were subjected to gas exchange measurements on the same leaf (LPI 15) from each tree and leaf was harvested for metabolic profiling. Gas exchange measurements from the Licor 6400*XT* instrument were imported into Microsoft Excel and the data was analyzed. For the chronic drought experiment, one set of trees comprising of all transgenic lines, transgenic controls and wild-type were watered normally to maintain a soil relative water content (RWC) of >0.3, while the another set was watered minimally (~0.1 RWC). The chronic drought experiment was conducted for two weeks and the gas exchange measurements were taken just before harvesting.

Harvesting and biomass analysis

The mature leaf (LPI 15) from acute and chronic drought experiments was harvested immediately after the photosynthetic measurements and snap-frozen in liquid nitrogen until use. For biomass analysis, plant tissues from apex to LPI 20 were harvested after two weeks of chronic drought treatment. Fresh weight was measured immediately after harvest and the plant tissues were air-dried for two weeks to estimate dry biomass. Well-watered plants from all genotypes were included as control in the experiment.

Metabolite profiling and analysis

Mature leaves (LPI 15) were ground to fine powder in liquid nitrogen and freeze dried. Metabolite extractions and profiling were performed as described by Frost et al. (2012).

Results

Leaf development in A1dYB9 and A1dEYB15 trees

Transgenic plants with perturbed tubulin expression and/or post-translational modification described in Chapter 1 were used for analyzing leaf expansion and guard cell dynamics, two traits that are known to be regulated by microtubule (MT) arrays. As presented in Chapter 2, transcript levels of the transgenes in mature leaves were 5- to 30- fold higher than their respective endogenes, except for the *TUB15* transgene which was detected at lower levels compared to its endogenous counterpart (Chapter 2, Figure 3). Leaf development of greenhouse-grown transgenic and wild type plants was monitored for morphological differences. After transplanting to soil, the original cohort of transgenic plants (from tissue culture) developed various leaf curling phenotypes under growth room conditions. The phenotypes were observed for mature but not newly emerged or rapidly-expanding leaves. In A1dYB9 leaves, moderate downward curling or shallow angular twisting occurred in all independent lines (Figure 1a). A more severe curling with twisting of the distal part of leaf occurred in all independent A1dEYB15 lines (Figure 1a). The leaf curling phenotype continued and progressed upwards, although at a much reduced pace, after the plants

were transferred to a greenhouse. However, the phenotype was not observed in any of the vegetatively propagated plants that were maintained in greenhouse conditions over a ~3-year span. Occasionally, the leaves of A1dYB9 plants exhibited a severe necrosis on the distal part of the leaves, followed by deformation of the distal half and cupping of the remaining leaf lamina (Figure 1b). This phenotype was observed only in summer of 2011 and 2012 when temperatures were extremely high, suggesting environmental conditions were a causative factor. Curling affected primarily young and rapidly expanding leaves. After cut-back, new sprouts typically resumed normal growth and morphology.

Cell expansion requires intact arrays of cortical MTs, and perturbations in the tubulin subunits (TUA or TUB) are known to affect the cell anisotropy (Bao et al., 2001; Burk et al., 2006; Ishida et al., 2007). Over the course of multiple propagation trials by rooted cuttings, it was observed that mature leaves of some transgenic plants appeared to have larger leaf areas than the wild types. For this reason, leaf expansion was monitored for wild-type as well as developmentally-normal transgenic plants (without any of the leaf twisting, curling or necrotic phenotypes). Leaf length and width were measured for the fifteen youngest leaves (leaf plastochron index LPI-1 to LPI-15) of each plant. The growth of young leaves (LPI-1 to approximately LPI-6) showed a similar developmental trajectory in all plant groups. However, the mature leaves (LPI-7 and older) of A1dYB9 lines exhibited a greater width-to-length ratio, while the A1dEYB15 and wild-type patterns were indistinguishable (Figure 2a). This pattern was consistently observed in multiple experiments (Figures 3-4).

To investigate whether the greater width-to-length ratio of mature A1dYB9 leaves was caused by greater widths and/or shorter lengths, length and width measurements from a subset of young and mature leaves were analyzed separately (Figures 3-4). The data from two independent cohorts of plants indicated that the altered aspect ratio of A1dYB9 leaves most likely resulted from a significant increase in leaf width relative to the wild type, as shown for LPI-10 and LPI-15 (Figures 3b and 4b). To further dissect whether the increased leaf expansion was due to altered cell expansion or cell division, nail polish impressions of adaxial LPI-10 leaves were used to estimate epidermal cell sizes. The calculated LPI-10

epidermal cell areas of two A1dYB9 lines were significantly larger than those of wild type and two A1dEYB15 lines (Figure 2b). The results suggested that the wider leaves of A1dYB9 plants were likely caused by prolonged cell expansion in mature leaves that had otherwise ceased to expand in the wild-type plants. However, the possibility that the wider leaves could also be caused by a higher rate of cell division cannot be completely ruled out.

Biomass analysis

A portion of the above-ground biomass of leaves and stem internodes from apex to LPI-15 of all genotypes was measured (Figure 5). Although the dry weight of young leaves from all genotypes was similar, the biomass of mature leaves (LPI-11-14) from A1dYB9 trees was significantly higher than the other plant lines, consistent with the greater width of these leaves (Figure 5). The stem biomass did not differ significantly among genotypes, except line #11 of A1dEYB15 which had a greater stem biomass due to the overall larger tree sizes in that experiment.

Drought responses of transgenic trees

Acute drought stress

The dynamic rearrangement of MTs during opening and closure of stomata suggests that MTs are integral to regulating the physiological behavior of guard cells (Fukuda et al., 1998). Putative changes in MT stability in the guard cells, due to tubulin perturbations, would be expected to alter stomatal conductance, which may in turn affect transpiration and gas exchange capacity of photosynthetically active leaves. To test this possibility, wild-type and transgenic plants were subjected to water deficit (drought) stress. In the acute drought experiments, water was withheld until LPI-5 to 6 (rapidly expanding leaves) showed wilting symptoms, which ranged from 18-20 h under the experimental conditions. At this stage, maximum photosynthetic rate (A_{max}), stomatal conductance and transpiration were measured in a mature

leaf (LPI-15) that remained turgid. These measurements were compared with those taken from LPI-15 of well-watered plants prior to the drought stress.

The A_{max}, stomatal conductance and transpiration rate in wild-type plants decreased significantly under acute drought stress, as compared to the pre-stress condition (Figure 6). The transgenic lines, however, did not show significant photosynthetic responses to drought in most cases. The three A1dYB9 transgenic lines exhibited a weak trend towards decreased photosynthesis, stomatal conductance and transpiration rates under drought stress, but the differences were significant only in line #4 for stomatal conductance and transpiration (Figure 6). The trend of reduction was less evident in A1dEYB15 lines except for transpiration rates that were significantly decreased in line #12 (Figure 6c). These results suggested that transgenic plants exhibited differential responses to acute drought, relative to the wild type, that may be attributed to altered microtubule dynamics in the guard cells.

Chronic drought stress

Chronic drought stress experiments were carried out to further investigate the effects of tubulin perturbation on leaf photosynthetic properties under a longer, but less severe, water- deficit regime. For logistic reason, vegetatively propagated plants were divided into three cohorts by plant size, with a staggered experimental schedule each starting one week apart. Plants in each cohort were further divided into well-watered and chronic drought treatment groups. Plants subjected to the chronic drought treatment were maintained at 0.05-0.15 RWC, while the well-watered plants were maintained at > 0.3 RWC during a two-week experimental period. A_{max}, stomatal conductance and transpiration rate were measured on LPI-15 of each plant at the end of the experimental period. The first and second cohorts consisted mostly of wild type and A1dEYB15 transgenic plants, while the third cohort consisted of wild type, A1dYB9 and A1dEYB15 plants.

The photosynthetic responses to drought were variable among the three cohorts of plants, likely due to the changing weather patterns during the staggered experimental periods. A_{max} did not differ

significantly between well-watered and chronic drought-stressed plants in any of the cohorts, regardless of plant type (Figure 7a). Stomatal conductance generally decreased, though not always significantly, in drought-stressed plants (Figure 7b). Similarly, the transpiration rates did not differ between well-watered and drought-stressed plants in most cases (Fig 7c). Despite the variations among cohorts, no clear differences between WT and transgenic plants were observed in their photosynthetic responses to chronic water deficit. Compared to the acute drought experiment, which lasted about 20 hours, plants under chronic drought stress can adapt to reduced water availability (Kozlowski and Pallardy, 2002). Thus, the results were consistent with the interpretation that transgenic plants exhibited a delayed stomatal closure relative to the wild types under acute drought treatment, but responded similarly to wild types during chronic drought stress. Additional experiments are necessary to confirm these findings by repeating the drought trials under more uniform environmental conditions.

A portion of the above-ground biomass from drought-stressed plants was analyzed and compared to the well-watered plants. The results showed that the combined leaf and stem biomass of all genotypes were reduced under chronic drought, as expected (Figure 5b). However, there was no genotypic difference in leaf or stem biomass between drought-stressed wild-type and transgenic plants.

Metabolic profiling of leaves subjected to acute drought stress

Preliminary GC-MS metabolic profiling of mature leaves (LPI-15) from wild-type and transgenic plants under acute drought stress was performed to assess the metabolite changes. Metabolites with a mass spectral matching confidence of at least 70% against the NIST library were selected for further analyses. The metabolites with significantly different abundance between wild-type and at least one of the transgenic lines ($*p \leq 0.1$, analyzed by two-sample *t*-test) were retained for comparative analysis. The list of these 31 metabolites along with their retention time (RT) and retention index (RI) is provided in Appendix 3A.

The metabolic changes in A1dYB9 and A1dEYB15 transgenic lines relative to the wild type were displayed as abundance ratios in a heatmap (Figure 8). Most of the metabolites did not exhibit strong genotypic differences, suggesting that the overall metabolic adjustments to acute drought stress were similar among genotypes. The A1dYB9 plants exhibited a slightly stronger response than the A1dEYB15 plants. Within the A1dYB9 group, the response patterns of line #4 were quite different from those of the other two lines. These included higher levels of chlorogenic acid, catechin and kaempferol and reduced levels of fatty acids such as steric and palmitic acids. The different metabolic response of line #4 coincided with its different photosynthetic response under acute drought (Fig 6). Interestingly, line #4 also differed from the other two A1dYB9 lines in its *TUA* and *TUB* transgene expression (Chapter 2, Figure 3). Whether the metabolite changes under drought reflected a constitutive differences of line #4 relative to wild-type or the other plant lines is not known, since well-watered samples were not collected. In order to more fully investigate the effects, new experiments are needed to compare the metabolite status of plants under well-watered conditions with that of drought-stressed plants.

Discussion

Transgenic tubulin perturbation affected cell expansion

Tubulin manipulation led to altered aspect ratios in mature leaves of A1dYB9 transgenics. This transgenic phenotype appeared to be caused by prolonged widthwise (mediolateral) expansion relative to the wild type counterparts that have already ceased their lengthwise (proximodistal) and widthwise expansion during leaf development. The epidermal cell cast analysis revealed an overall increase in pavement cell size of mature A1dYB9 leaves, pointing to increased cell expansion as the cause of increased leaf width. Aberrant MT arrays that resulted from mutations (Ishida et al., 2007) or transgenic manipulations (Bao et al., 2001; Abe and Hashimoto, 2005; Burk et al., 2006) of the tubulin subunits have been shown to alter cell expansion and lead to constitutive twisting of various organs. In *Arabidopsis*, leaf lamina growth is regulated by two independent processes of polarized cell expansion, as illustrated by the narrow-leaf *angustifolia* (*an*) and round-leaf *rotundifolia3* (*rot3*) mutants (Tsuge et al., 1996; Tsukaya, 2006). The *an*

mutant exhibits restricted epidermal and spongy mesophyll cell expansion width-wise, resulted in thick and narrow leaves, while the *rot3* mutation restricts cell polarity length-wise during early development, leading to short and round leaf phenotypes (Tsuge et al., 1996). Interestingly, only the *an* phenotype is linked to cell wall formation, as *ANGUSTIFOLIA* encodes a homolog of animal carboxy-terminal binding protein (CtBP) implicated in regulation of MT arrangement (Kim et al., 2002). On the other hand, *ROT3* encodes a plant-specific cytochrome P450, CYP90C1 that is involved in brassinosteroid biosynthesis (Kim et al., 1998; Kim et al., 2005). Given the altered width-wise leaf expansion observed in the mature leaves of A1dYB9 plants, it will be of interest to examine whether expression of the *Populus ANGUSTIFOLIA* homolog is altered in response to tubulin perturbation in the transgenics.

Unlike the narrow-leaf phenotype of the *Arabidopsis an* mutant that persists throughout development, the altered epidermal cell expansion of the transgenic *Populus* was observed only in mature leaves, and only in the A1dYB9 lines. The difference between *Arabidopsis* and *Populus* may be related to their distinct leaf phyllotaxy, or may reflect a more complex developmental regulation of leaf blade expansion in *Populus*. The phenotypic differences between the A1dYB9 and A1dEYB15 transgenic *Populus* may be attributed to differential effects of the two TUA1 PTM isoforms (dY versus dEY) on MT dynamics, as expression levels of transgenes were similar between the two plant groups. Further investigation of MT dynamics in leaves should shed light on distinct mechanistic effects of dY or dEY on cell expansion.

Tubulin manipulation altered guard cell behavior in transgenic *Populus*

Plants use a myriad of strategies, including drought avoidance and tolerance mechanisms, in response to water deficits for survival and/or adaptation (Chaves et al., 2003). One of the most common and earliest leaf responses to short-term water limitation is stomatal closure to reduce water loss, accompanied by reduced photosynthetic gas exchange (A_{max}) and transpiration rates. Accordingly, wild-type *Populus* showed the expected decrease in leaf A_{max} and water loss under acute drought conditions relative to well-watered plants. However, a majority of the transgenic lines exhibited delayed photosynthetic responses to

short-term water deficits, able to maintain higher rates of A_{max}, stomatal conductance and transpiration than the drought-stressed wild type at the time of measurement. The results are consistent with an effect of altered MT homeostasis on stomatal dynamics, due to tubulin perturbation.

The opening and closure of the stomatal aperture is regulated by changes in the turgor pressure of the surrounding guard cells (Brodribb and Holbrook, 2003). The swelling and shrinking of guard cells are intimately linked to dynamic MT rearrangements, and are controlled by several factors that include environmental conditions such as light and moisture (Lahav et al., 2004), activation of K+ channels (Zhou et al., 1999) and action of several signaling molecules (Zhang et al., 2008). Based on live cell imaging of MT dynamics, it was shown that perturbation of MT arrays by pharmacological agents disrupts guard cell functions: oryzalin treatments impeded guard cell opening, whereas taxol treatments delayed stomatal closure (Eisinger et al., 2012). The delayed photosynthetic responses of transgenic poplars to acute drought can be explained by altered MT stability due to ectopic expression of tubulin PTM mimics that interfered with the tyrosination and detyrosination cycle. Plants respond to long-term drought stress by various mechanisms that include adjustments in osmotic potential, metabolites, cell wall rigidity, plant growth rate, and shoot-to-root ratio via hormonal signaling (reviewed in Chaves et al., 2003). Indeed, transgenic and wild type *Populus* trees grown under chronic low water availability exhibited reduced leaf and stem biomass (Figure 5). Unlike under the acute drought conditions, wild-type and transgenic plants responded similarly to chronic drought conditions in their photosynthetic and stomatal conductance rates. The results suggested that tubulin manipulation altered the stomatal dynamics of transgenic leaves in response to short-term water deficits, but did not affect the adaptation responses of transgenic plants to chronic drought stress.

Future directions

The A1dYB9 and A1dEYB15 transgenic *Populus* exhibited overlapping but not identical leaf phenotypes. Although the underlying mechanisms remain elusive, the results appear to implicate differential responses of transgenically altered MT homeostasis to developmental and environmental cues. Because stomatal

guard cells are enriched in pectin polymers, and because pectin networks were altered in the stem wood of both transgenic groups (Chapter 2), it will be of interest to investigate pectin polysaccharide composition and distribution in the guard cell wall of wild-type and transgenic plants by immunolocalization studies. Similarly, high-resolution glycome profiling analysis of leaf cell walls is necessary to understand the compositional differences, if any, between the two transgenic groups that may affect guard cell dynamics and epidermal expansion. Additionally, leaf histological studies are needed in order to investigate whether epidermal, palisade and spongy mesophyll cell shapes, or other aspects of leaf development, such as leaf blade thickness, are altered. Since all transgenes are driven by a constitutive CaMV 35S promoter, the pleiotropic effects on leaf development can be separately investigated by targeting specific tissues (e.g. transgene expression driven by epidermal or guard-cell specific promoters). More detailed time scale monitoring and other strategies as outlined in Buschmann et al. (2010) are needed to understand the transgenic response in long-term water stress. This study highlights the importance of MTs in regulating leaf cell expansion and guard cell behavior. A better understanding of the mechanisms underlying the observed phenotypes may lead to new strategies for agronomic trait improvements.

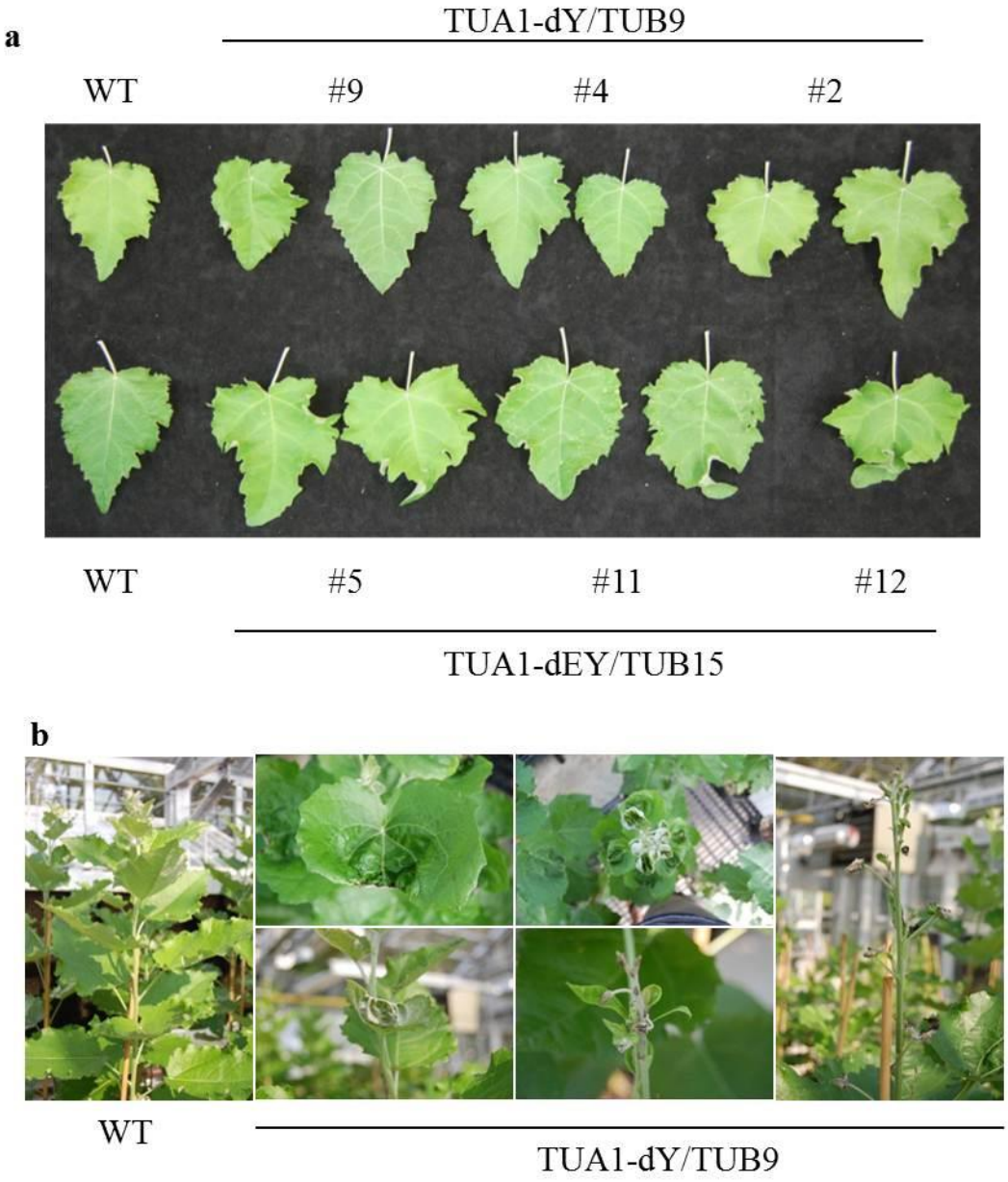

Figure 3.1. Leaf developmental phenotypes observed in transgenic plants. Leaf twisting and curling was more severe in original (tissue culture regenerated) A1dEYB15 lines (**a**); in two consecutive summer trials, A1dYB9 leaves exhibited severe phenotypes with burning of distal part of the leaf, cupping, and emergence of secondary shoots (**b**).

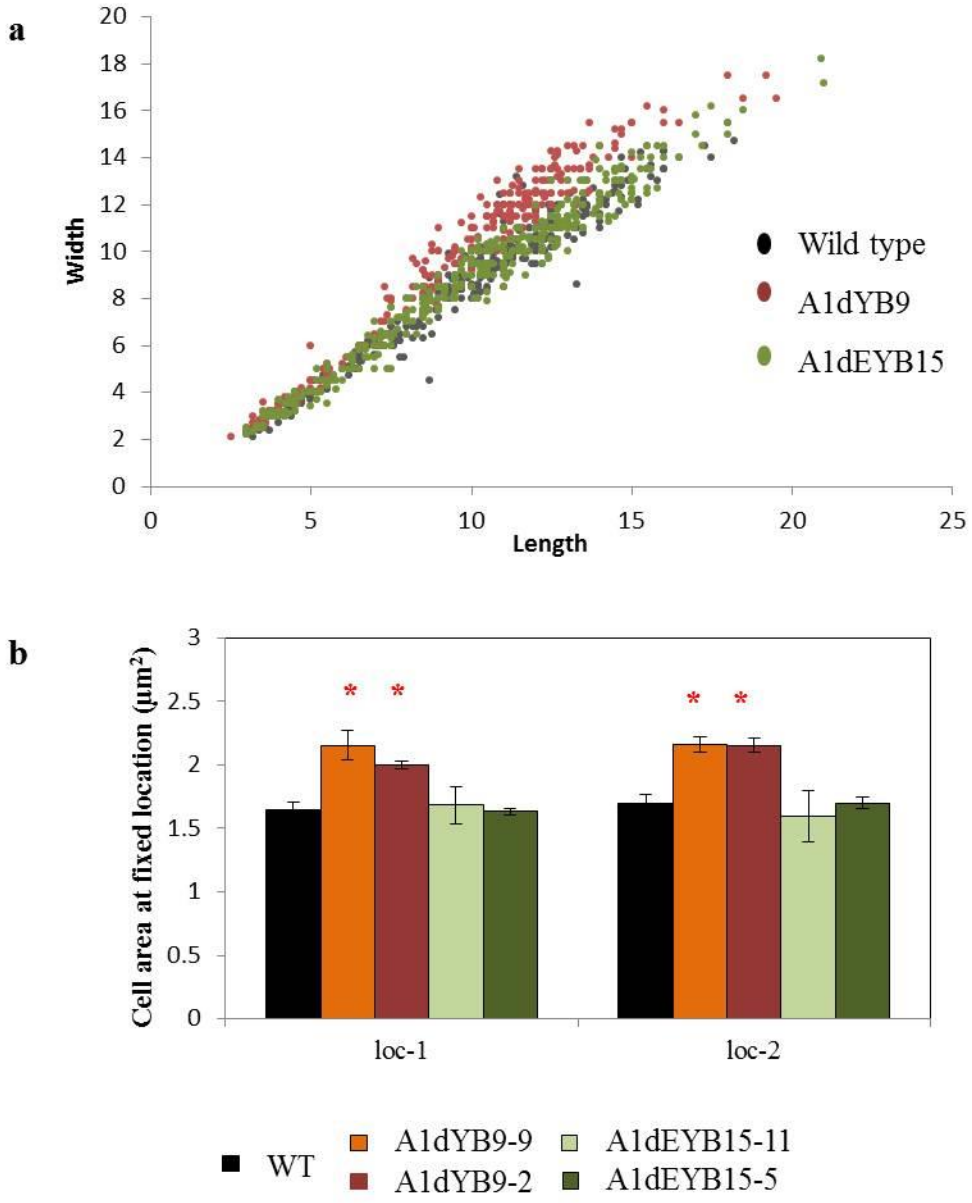

Figure 3.2. Phenotypic analysis of mature leaves in cohort 1. Scatterplot (a) of length and width measurements of LPI 1-LPI 15 leaves from transgenic and wild type trees. Epidermal impressions were used to measure average leaf cell area (b) at two different locations on leaves (loc-1 and 2). Bars represents mean ± standard deviations from n=3. *$p \leq 0.05$ as determined by Student's *t*-test.

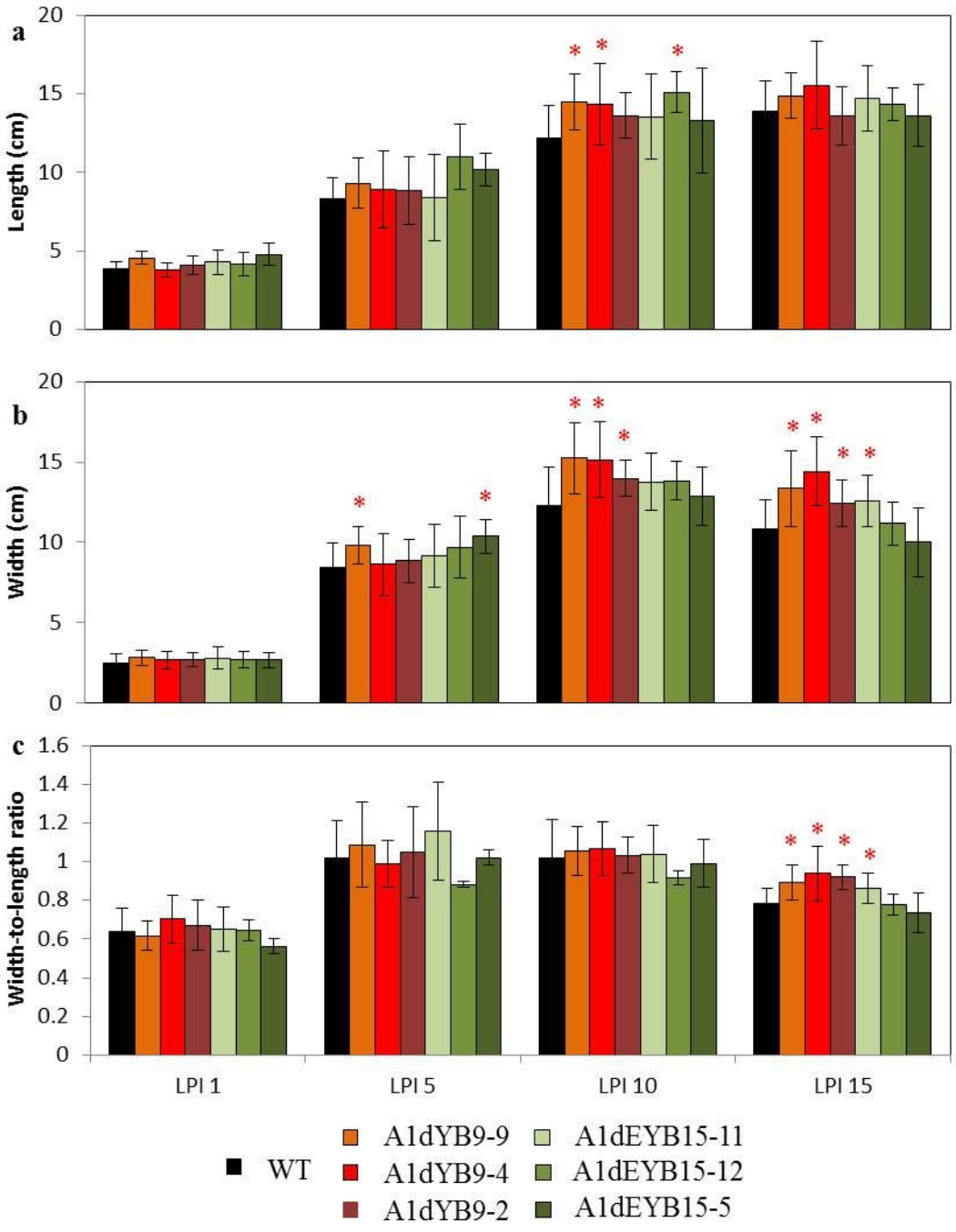

Figure 3.3. Phenotype analysis in cohort 1. Length (a), width (b) and width-to-length (c) measurements of representative leaves in cohort 1. Bars represent mean ± standard deviations from n=3. *$p \leq 0.05$ as determined by Student's *t*-test.

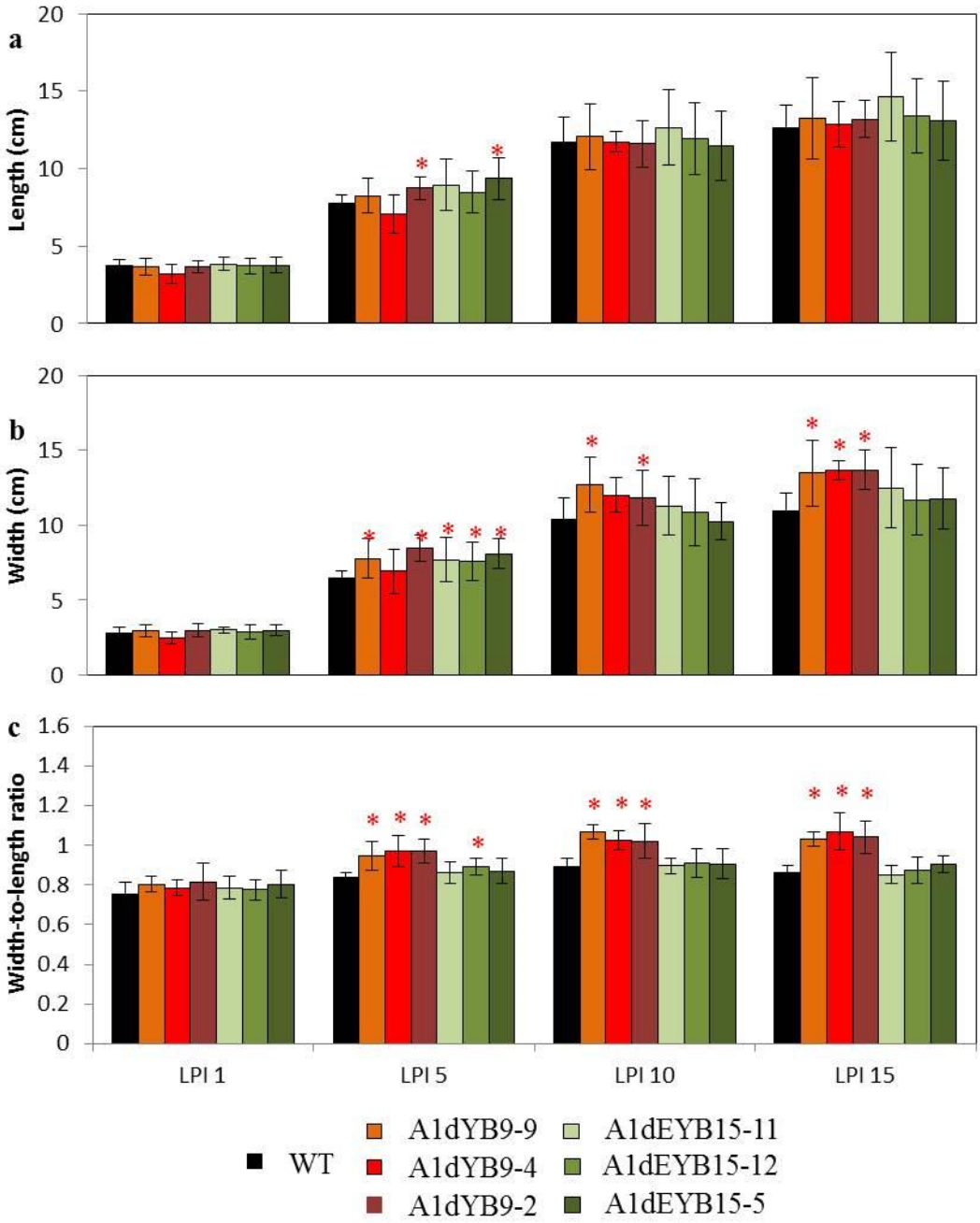

Figure 3.4. Phenotype analysis in cohort 2. Length (a), width (b) and width-to-length (c) measurements of representative leaves in cohort 2. Bars represent mean ± standard deviations from n=3. *$p \leq 0.05$ as determined by Student's *t*-test.

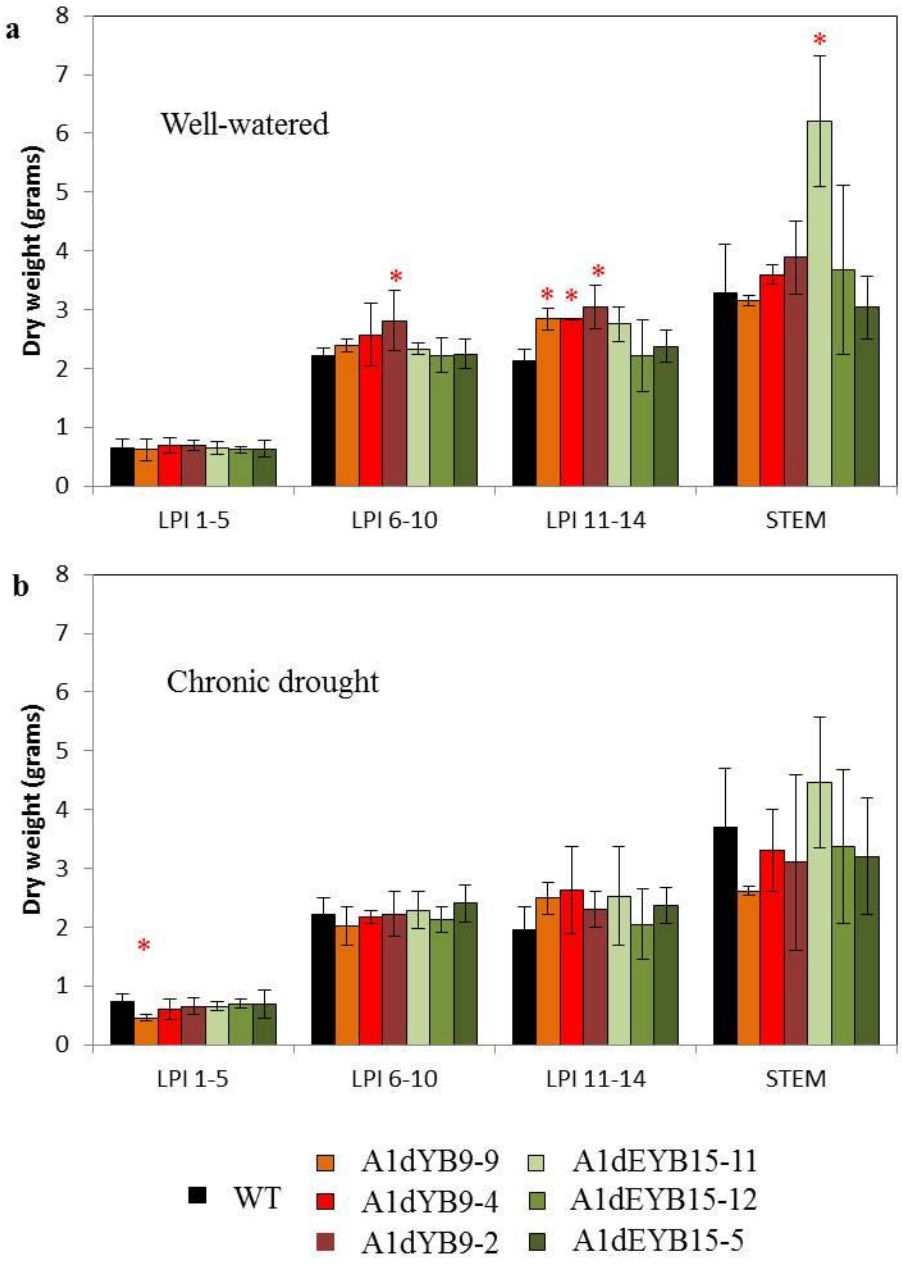

Figure 3.5. Representative biomass analysis in wild type and transgenic trees. Bars are mean ± SD from n≥3. *$p \leq 0.05$ as determined by Student's *t*-test.

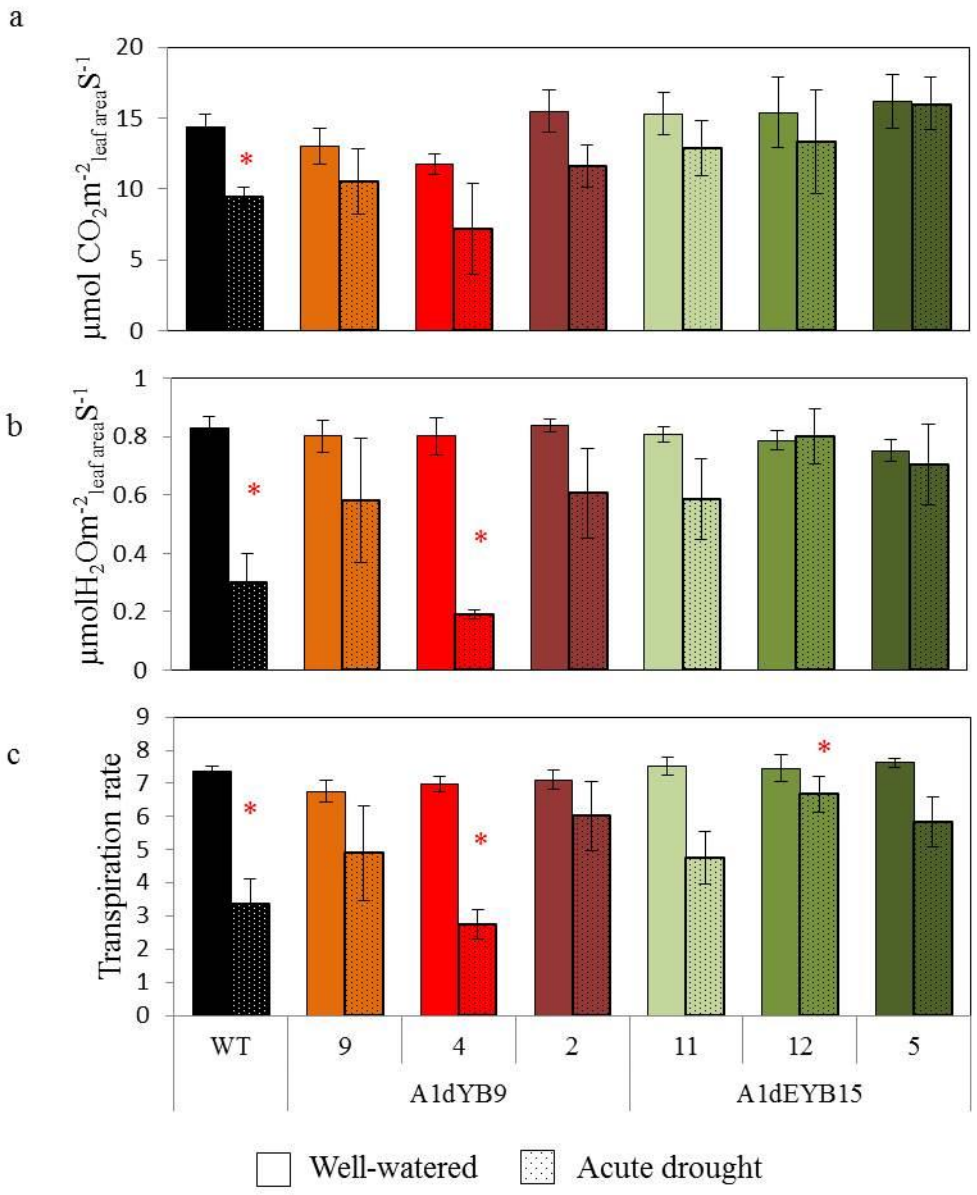

Figure 3.6. Analysis of plants under acute drought stress. Photosynthetic parameters were measured in wild type and transgenic trees**.** Bars represent mean ± standard deviation values measured under well-watered (solid) or acute drought (dotted). Significant changes between well watered and acute drought are indicated by red asterisks as determined by Student's *t*-test *p≤0.05. n=>3.

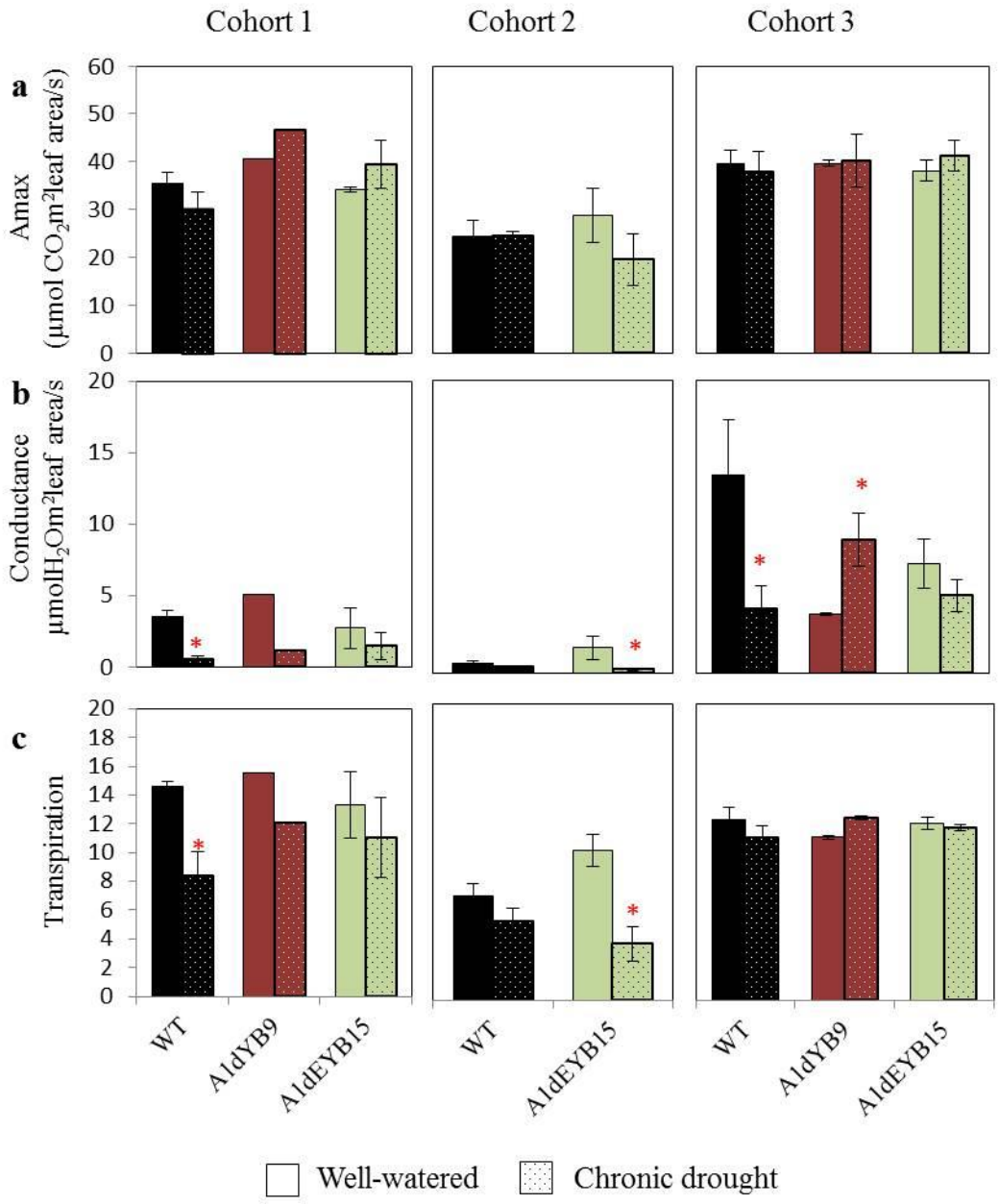

Figure 3.7. Analysis of plants under chronic drought stress. Photosynthetic parameters were measured in wild type and transgenic trees**.** Bars represent mean ± standard deviations values measured under well-watered (solid) or chronic drought (dotted). Significant changes between well watered and acute drought are indicated by red asterisks as determined by Student's *t*-test *p≤0.05. n=>3 (except in A1dYB9 of cohort 1).

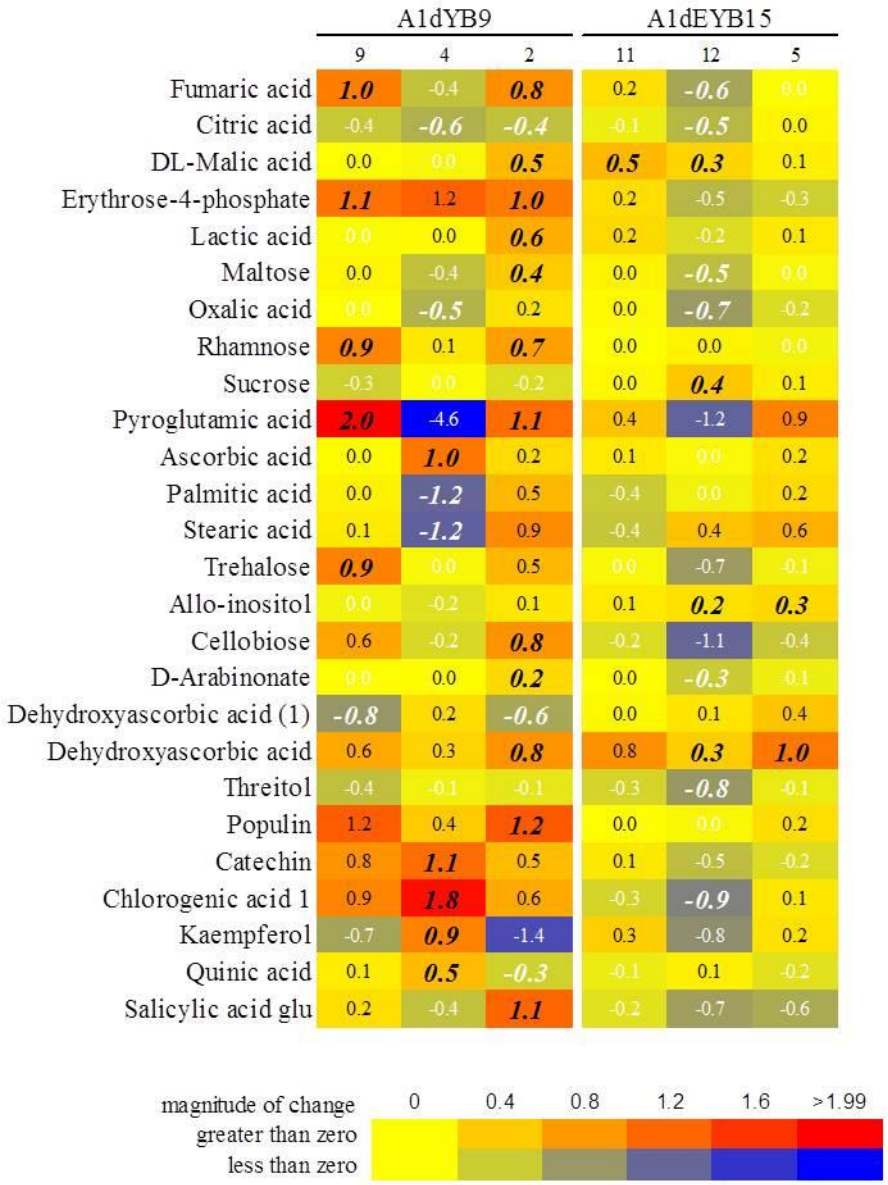

Figure 3.8. Metabolite profiles of mature leaves subjected to acute drought stress. Heatmap is generated by $\log_2$ transformed abundance ratios of transgenics relative to the wild type. Bold and italicized numbers represent significant changes as analyzed by pairwise students' *t*-test. $p \leq 0.05$, n=>3.

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Appendix 3A. List of metabolites in mature leaves detected by GC-MS under acute drought stress conditions.

Compounds	**RI**	**RT**	**WT**			**A1dYB9-9**			**A1dYB9-4**			**A1dYB9-2**			**A1dEYB15-11**			**A1dEYB15-12**			**A1dEYB15-5**		
Shikimic acid	1781	10.0	0.1	±	0.0	0.2	±	0.0	0.1	±	0.0	0.2	±	0.0	0.2	±	0.1	0.1	±	0.0	0.2	±	0.1
Quinic acid	1839	10.4	9.1	±	1.3	10.6	±	3.3	13.7	±	1.6	7.1	±	1.8	8.2	±	1.4	9.9	±	1.4	7.7	±	1.4
Salicin	2503	16.0	1.6	±	0.4	0.9	±	0.4	1.3	±	0.2	2.1	±	0.6	1.3	±	0.5	1.3	±	0.0	1.6	±	0.6
Catechin	2805	18.6	25.7	±	11.5	73.3	±	55.1	63.7	±	25.6	27.3	±	6.2	31.2	±	20.0	20.2	±	7.8	27.8	±	8.8
Chlorogenic acid	3003	20.4	20.5	±	5.2	21.4	±	10.8	29.5	±	3.2	17.5	±	6.3	16.8	±	4.9	14.8	±	4.1	24.5	±	8.1
Populin	2947	19.9	6.5	±	1.9	13.8	±	15.4	8.2	±	1.6	15.2	±	4.5	6.3	±	2.9	6.7	±	1.7	6.3	±	2.3
Kaempferol	2965	20.0	1.8	±	0.5	0.8	±	0.6	2.7	±	0.3	0.5	±	0.2	1.8	±	0.4	0.9	±	0.4	2.1	±	1.0
SAGs	2693	17.4	0.0	±	0.0	0.1	±	0.0	0.0	±	0.0	0.1	±	0.0	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0
Methoxysalicylic	2959	20.0	2.0	±	1.0	2.6	±	1.0	2.2	±	0.5	1.9	±	0.5	1.6	±	0.5	1.4	±	0.5	1.8	±	0.4
Salicortin	3066	21.5	353.1	±	77.7	332.2	±	55.6	268.7	±	10.5	333.3	±	21.3	305.4	±	69.8	254.5	±	57.9	285.5	±	60.1
Chalcone	1576	8.3	0.3	±	0.0	0.3	±	0.0	0.3	±	0.0	0.4	±	0.0	0.3	±	0.0	0.3	±	0.0	0.3	±	0.1
Fumaric acid	1296	6.6	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0
Succinic acid	1253	6.4	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0
Citric acid	1796	10.1	2.2	±	0.5	1.8	±	1.1	1.4	±	0.2	1.6	±	0.5	2.0	±	0.3	1.5	±	0.5	2.4	±	0.8
Oxalic acid	1029	5.2	9.3	±	1.7	9.5	±	1.6	6.2	±	0.1	10.9	±	2.8	9.3	±	3.3	5.2	±	0.6	7.4	±	1.6
Malic acid	1468	7.5	5.0	±	0.8	4.5	±	1.3	4.1	±	1.4	7.2	±	1.4	7.2	±	1.3	6.3	±	0.8	5.0	±	0.7
Lactic acid	1573	8.3	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	0.1	±	0.0	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0
Saccharic acid	2015	11.9	0.1	±	0.1	0.2	±	0.1	0.0	±	0.0	0.2	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0
Erythrose-4-P	1706	9.3	0.1	±	0.0	0.3	±	0.1	0.5	±	0.2	0.3	±	0.2	0.1	±	0.1	0.1	±	0.1	0.1	±	0.0
Phosphoric acid	1205	6.1	2.2	±	1.5	2.2	±	1.1	3.1	±	0.6	1.8	±	1.0	2.8	±	1.1	2.3	±	1.6	2.2	±	0.6
Sucrose	2635	17.0	35.7	±	6.9	27.9	±	0.6	35.0	±	1.0	29.9	±	1.9	38.3	±	11.7	48.3	±	5.0	36.3	±	9.1
Glucose	1882	10.8	1.9	±	1.0	2.6	±	1.3	2.0	±	1.0	2.0	±	1.0	2.6	±	0.8	1.8	±	0.9	2.3	±	1.4
Fructose	1852	10.6	3.3	±	1.2	3.9	±	1.8	3.4	±	1.4	3.6	±	1.2	4.5	±	1.0	3.6	±	1.5	4.0	±	1.8
Xylose	1685	9.2	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0
Galactose	2114	12.7	0.1	±	0.0	0.1	±	0.1	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.1
Lactose	2435	15.4	0.7	±	0.2	0.9	±	0.2	0.6	±	0.0	0.7	±	0.3	0.6	±	0.3	0.4	±	0.1	0.7	±	0.3
Maltose	2542	16.3	0.1	±	0.0	0.2	±	0.0	0.1	±	0.0	0.2	±	0.0	0.1	±	0.1	0.1	±	0.0	0.1	±	0.0

Compounds	RI	RT	WT			A1dYB9-9			A1dYB9-4			A1dYB9-2			A1dEYB15-11			A1dEYB15-12			A1dEYB15-5		
Rhamnose	3035	20.9	2.9	±	0.7	5.0	±	2.4	3.1	±	0.1	4.6	±	1.3	2.6	±	0.7	2.7	±	0.9	2.3	±	0.7
Gulose	2231	13.7	3.2	±	2.7	6.8	±	7.7	3.9	±	0.9	5.9	±	4.0	2.2	±	2.3	1.8	±	1.4	0.7	±	0.1
Lyxose	1685	9.2	0.2	±	0.1	0.2	±	0.0	0.3	±	0.0	0.3	±	0.1	0.3	±	0.1	0.3	±	0.1	0.3	±	0.1
Cellobiose	2398	15.1	0.0	±	0.0	0.1	±	0.0	0.0	±	0.0	0.1	±	0.0	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0
Arabiofuranose	1745	9.7	0.8	±	0.5	0.6	±	0.2	1.2	±	0.2	0.6	±	0.1	0.9	±	0.3	1.1	±	0.6	1.3	±	0.8
Trehalose	2746	17.8	0.0	±	0.0	0.1	±	0.0	0.0	±	0.0	0.1	±	0.0	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0
Threonic acid	1545	8.0	0.7	±	0.2	0.7	±	0.4	0.5	±	0.1	0.5	±	0.1	0.7	±	0.2	0.7	±	0.2	0.8	±	0.2
Glutamic acid	1519	7.8	0.0	±	0.0	0.2	±	0.1	0.0	±	0.0	0.1	±	0.0	0.1	±	0.1	0.0	±	0.0	0.1	±	0.1
allo-inositol	2095	12.6	0.2	±	0.1	0.1	±	0.0	0.2	±	0.1	0.2	±	0.0	0.2	±	0.1	0.2	±	0.1	0.2	±	0.1
Glycine	1146	5.8	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0
Butyric acid der.	1242	6.3	0.0	±	0.0	0.1	±	0.0	0.0	±	0.0	0.1	±	0.0	0.1	±	0.0	0.0	±	0.0	0.1	±	0.0
Ascorbic acid	1939	11.3	0.2	±	0.1	0.2	±	0.2	0.5	±	0.1	0.3	±	0.1	0.3	±	0.0	0.3	±	0.1	0.2	±	0.1
Gluconic acid lac.	1923	11.1	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0	0.1	±	0.0
Nonanoic acid	1315	6.7	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0
Phenyl-Glucoside	3040	21.0	11.4	±	3.3	15.3	±	7.2	12.2	±	1.7	14.4	±	1.9	11.5	±	2.5	9.5	±	2.2	10.4	±	2.2
Galactopyranoside	3027	20.8	11.3	±	3.8	10.9	±	1.8	7.3	±	1.6	15.2	±	1.7	11.4	±	2.0	8.2	±	0.4	11.9	±	2.2
Dehydroascorbic	1832	10.4	0.6	±	0.2	0.4	±	0.1	0.7	±	0.2	0.4	±	0.1	0.7	±	0.2	0.7	±	0.2	0.8	±	0.2

CHAPTER 4

CONCLUSIONS AND FUTURE WORK

Conclusions

Previous research in our laboratory identified a small subset of *TUA* (*TUA1* and *TUA5*) and *TUB* (*TUB9* and *TUB15*) genes that are abundantly expressed in xylem (Oakley et al., 2007). In the present study, these genes along with two PTM mimics of *TUA1*: detyrosinated TUA1 (dY) and nontyrosinatable TUA1 (dEY) were co-transformed into *Populus* and characterizations of viable transgenic trees are presented in **Chapter 2** and **Chapter 3**. The conclusions of the present investigation are summarized below:

- Although the transformation efficiency in *Populus* was quite low even with the co-transformation strategy, inclusion of PTM mimics of *TUA1* was beneficial in obtaining viable transgenic plants. Many of the putative transformants (calli), as well as those derived from the native or synthetic gene combinations failed to regenerate into plants, indicative of intolerable levels of tubulin perturbations during organogenesis. The regulation of *TUA* and *TUB* transgenes in *Populus* appeared to be more complex, since the expression levels of *TUA* were always higher than *TUB* genes, either under the native (endogenous genes) or the constitutive CaMV 35S promoter (transgenes). This may explain the low rates of transformation and regeneration of viable transgenic plants, due to drastically altered *TUA:TUB* expression.
- Comprehensive cell wall analysis of stem wood from transgenic plants revealed that cellulose and hemicelluloses were not affected by tubulin manipulations. Furthermore, wood MFA did not change consistently in two transgenic groups. However, the extractability of pectins and, to a lesser extent, xylan, from stem wood of transgenic plants was changed, both by mild as well as by harsh chemical treatments in the glycome profiling analysis. Thus, compelling evidence

suggested that tubulin manipulations affected non-cellulosic cell wall deposition in stem wood of transgenic *Populus*.

- Mature leaves from A1dYB9 plants exhibited greater pavement cell expansion resulting in greater width-to-length ratio than the other plant lines. The effects on leaf development are consistent with a role of MTs during cell expansion. However, this phenotype was not observed in A1dEYB15 lines, suggesting distinct *in vivo* functions between dY and dEY-type tubulins.
- Mature leaves from transgenic plants showed delayed stomatal closure in response to acute drought stress, thereby maintaining higher rates of transpiration and photosynthesis relative to the wild types at the time of measurements. However, under chronic drought conditions, transgenic plants exhibited similar leaf photosynthetic capacity and stomatal conductance as those observed in wild types. This suggested that altered tubulin homeostasis had a greater effect on acute drought response that may be attributed to altered MT dynamics, than on longer-term, adaptation response to chronic water deficits.
- The two transgenic groups showed a range of overlapping but non-identical phenotypes. This may be due to the effects of *TUA* rather than *TUB* transgenes, since the expression levels of the *TUA* PTM mimics were higher than the *TUB9* or *TUB15* transgenes. Specifically, only the A1dY-tubulin may participate in tyrosination and detyrosination cycle for conversion to either Y-type or dEY-type, while dEY-tubulin may not be further modified. Therefore, different phenotypes displayed by the two transgenic groups may be attributed to differential *in vivo* functions of the PTM mimics that they contain.

Future work

Several unanswered questions limit a mechanistic understanding of tubulin manipulations on wood and leaf properties *in planta*. The following analyses are proposed for further characterization of the transgenic *Populus* lines:

- Protein characterization of wild type and transgenic plants using plant-specific anti-PTM antibodies or by *de novo* C-terminal sequencing of tubulins using mass-spectrometry approaches.
- Immunolocalization of tubulins to investigate transgenic effects on MT dynamics in developing xylem, pavement cells and guard cells.
- *In-depth* analysis of leaf structural carbohydrates to characterize transgenic effects on pectin composition.
- Timescale analysis of guard cell behavior under inductive conditions, such as ABA, drug and light treatments.
- Refined tubulin perturbations using tissue-specific promoters, such as xylem-, epidermal-, mesophyll- and guard cell-specific promoters, to minimize widespread effects during plant development.