ELUCIDATING THE FUNCTION OF ARABINOGALACTAN PROTEINS DURING WOOD FORMATION

by

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Abstract

Arabinogalactan proteins (AGPs) are cell wall proteins with abundant glycosylation, belonging to the large, multi-gene hydroxyproline-rich glycoprotein (HRGP) family. It has been reported that AGPs may contribute to cell expansion, xylem cell differentiation and secondary cell wall deposition. However, the roles of specific AGP in wood developmental processes have never been thoroughly elucidated. Therefore, the objective of this thesis was to investigate the functional role(s) of three AGPs in wood cell wall development. Specifically, the lysine-rich AGP18; a classical AGP, AGP9; and an AGP peptide, AGP14 were studied, because they demonstrated high gene expression levels in the developing xylem of *Populus trichocarpa* during transcriptome re-sequencing initiatives. Based on the phenotypic changes observed when *PtAGP18* was down-regulated in transgenic poplar trees and Arabidopsis *atagp18* T-DNA mutant analyses, I showed roles for *AGP18* in fiber cell shape and fiber secondary cell wall formation (Chapter 2). Moreover, the poplar *PtAGP18* was able to complement the Arabidopsis *atagp18* T-DNA mutants which displayed altered fiber shape and cell wall thickness, indicating that these two genes are functionally equivalent (Chapter 2). Analysis of the growth of Arabidopsis hypocotyls cultivated in darkness revealed that *AGP18* is involved in cell expansion (Chapter 2). In parallel, I showed that the *AGP9* affects xylem vessel differentiation and vessel cell expansion (Chapter 3). A role for *AGP9* in cell expansion was also demonstrated with Arabidopsis *agp9* mutant hypocotyls grown in the dark (Chapter 3). Furthermore, *AGP14* appears to contribute to cell wall formation in poplar (Chapter 4). Taken together, the functional characterization of these AGPs suggests that AGP18 and AGP9 play roles in the development of fibers and vessels, respectively. However, further research is needed to delineate the exact
cellular and molecular mechanisms through which AGPs contribute to secondary xylem development.
Preface

Chapter 2: Li Xi performed the research, analyzed the data and wrote the chapter. Professors Mansfield and Samuels were involved in the identification of the research question, the design of the research, and chapter preparation.

Chapter 3: Li Xi performed the research, analyzed the data and wrote the chapter. Professors Mansfield and Samuels were involved in the identification of the research question, the design of the research, and chapter preparation.

Chapter 4: Li Xi performed the research, analyzed the data and wrote the chapter. Professors Mansfield and Samuels were involved in the identification of the research question, the design of the research, and chapter preparation.
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<th>Description</th>
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<tbody>
<tr>
<td>ABRC</td>
<td>Arabidopsis biological resource center</td>
</tr>
<tr>
<td>AG</td>
<td>arabinogalactan</td>
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<td>GPI</td>
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2×35S duplicated-enhancer cauliflower mosaic virus 35S promoter
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Chapter 1 Introduction

1.1 Overview

Plant cell walls are composed of cellulose, hemicellulose, lignin and pectin, and are located outside the plasma membrane. They function to control cell expansion, offer structural support and act as a barrier to pathogen attack. Based on their morphology and chemistry, plant cell walls can be classified into two broad types: primary cell walls, which are formed by growing cells; and secondary cell walls, which are formed after cell expansion has slowed down and are often lignified. Generally, the role of the primary cell wall is to sustain cell expansion, driven by turgor pressure, by resisting tensile forces. In contrast, secondary cell walls are synthesized adjoining the primary cell walls and create thickened specialized structures in different cell types (i.e. vessels or fibers) for resisting compressive forces (Lee et al., 2011). A significant component of the secondary cell wall is cellulose, a polymer composed of β-1,4-glucose chains, which is a main determinant of cell wall strength and rigidity. Along with cellulose, plant cell walls also contain different proteins, including glycoproteins and proteoglycans. During cell wall remodelling, some proteins are believed to be actively involved in breaking and reforming bonds. Other proteins, such as arabinogalactan proteins (AGPs), are highly glycosylated and may serve as intra- and intermolecular cross-linking moieties.

1.2 Cell wall

1.2.1 Primary cell wall

The characteristic primary cell wall is thin, separated by a middle lamella, and composed of four components, including cellulose, hemicelluloses, pectin and protein. They account for 30, 30, 35 and 1-5% of plant cell wall dry weight, respectively (Cosgrove, 1997). Cell wall polymers have been shown to cross-link after secretion. It has been proposed that hemicelluloses bind to the insoluble surface of cellulose microfibrils by hydrogen bonding (Somerville et al., 2004), while pectin is adjoined to the cell wall matrix by Ca$^{2+}$ bridges, or by forming ester, ionic, glycosidic or H-bonds (Fry, 1986). It is generally
believed that the main function of primary cell walls is to generate and maintain turgor pressure to resist tensile forces and regulate cell expansion.

1.2.2 Secondary cell wall

Following the expansive process of primary cell wall development, the secondary cell wall layers are added, which are generally defined by three distinct layers, namely the S1, S2, and S3. The transition from the S1 layer to S2 layer is marked by a dramatic change in the orientation of the microfibrils and increased lignin deposition. The S2 layer supplies the main mechanical support to the cell, and is the thickest layer in the secondary cell wall, accounting for 75 to 85% of the total thickness. It has been reported that the angles of the cellulose microfibrils in the S2 layer greatly influence the mechanical properties of the cell and wood stem (Barnett and Bonham, 2004). Compared to the primary cell wall, secondary cell walls are thicker, often lignified, and at maturity are very hydrophobic, and can resist compressive forces.

1.2.3 Cell wall composition

1.2.3.1 Cellulose

Cellulose is a linear polymer composed of β-1,4-linked glucose, which is formed by a complex of rosettes embedded within the plasma membrane (Kimura et al., 1999). The rosette complex is composed of cellulose synthase (CesA) proteins that have been shown to be the basic units for cellulose synthesis (Kimura et al., 1999). Each CesA protein synthesizes one β-1,4-glucan chain, and six CesA proteins are thought to comprise one rosette subunit, with six subunits forming one hexameric rosette consisting of a total of thirty-six CesA proteins (Ha et al., 1998; Doblin et al., 2002).

In Arabidopsis thaliana there are 10 CesA genes and 29 cellulose synthase-like (CSL) genes (Richmond and Somerville, 2000), while in poplar there are 18 CesA genes (Djerbi et al., 2005) and 30 CSL genes (Suzuki et al., 2006). In A. thaliana, CesA1, CesA3, and CesA6 have been shown to be responsible for primary cell wall cellulose production, while CesA4, CesA7 and CesA8 contribute to the synthesis of secondary cell wall cellulose (Persson et al., 2005). All of the CesA appear to play important roles in cellulose deposition, but some may be functionally redundant. It has been shown that the primary
wall cellulose synthase complex also contains CesA2, CesA5, and CesA9 which are functionally redundant with CesA6 (Persson et al., 2007a). A similar level of redundancy does not appear to be present in secondary cell wall cellulose synthesis, however. In A. thaliana, mutations in CesA4, CesA7 and CesA8 manifest in a collapsed xylem phenotype, often referred to as an irregular xylem phenotype (irx), and are marked with reduced cellulose contents (Taylor et al., 1999; Taylor et al., 2000; Desprez et al., 2002).

Cellulose confers strength and structure to the cell wall. The individual glucan chains are closely aligned and linked to each other by hydrogen bond to form a highly ordered ribbon, termed the microfibril (Doblin et al., 2002). Relative to primary cell wall cellulose, the secondary cell wall microfibril has a higher degree of polymerization, is more crystalline and forms macrofibrils that strengthen the cell wall (Blaschek et al., 1982; Delmer and Amor, 1995).

1.2.3.2 Hemicelluloses

The hemicelluloses can be classified into five main groups, namely xylan, glucuronoxylan, arabinoxylan, glucomannan and xyloglucan, which have been shown to be cell and species specific in plants (Scheller and Ulvskov, 2010). The main hemicellulosic polysaccharides are xyloglucan which possess a cellulose-like backbone (Shibuya and Misaki, 1978). Xyloglucans have been shown to adhere strongly to cellulose via hydrogen bonds, and therefore reduce the lateral aggregation of microfibrils to large fibrils and prevent cellulose microfibril contact directly (Vincken et al., 1995). In plants, the xyloglucans are likely to aid in withstanding tensile stresses (Cavalier et al., 2008; Baba et al., 2009).

Xylan, an abundant hemicellulose which is different from xyloglucan that is abundant in primary cell walls, plays important roles in secondary cell wall thickening (Northcote et al., 1989). In A. thaliana, it has been shown that mutations in the genes related to xylan biosynthesis, such as irregular xylem 8 (At5g54690), irregular xylem 9 (At2g37090), and irregular xylem 14 (At4g36890) which are glycosyltransferases, resulted in irregular xylem and thinner cell wall phenotype (Persson et al., 2007b). In poplar, suppressed expression of glycosyltransferase PtrlGT8D1 and PtrlGT8D2 also reduced cell wall thickness and those plants displayed brittle stems (Li et al., 2011).
### 1.2.3.3 Pectin

Pectin is the most soluble wall polysaccharide and is rich in D-galacturonic acid. The three main pectic polysaccharides are homogalacturonans (HG), rhamnogalacturonan I (RG-I) and rhamnogalacturonan II (RG-II) (Yapo et al., 2007). HG is the most abundant, and forms a gel within the wall to maintain cell-cell adhesion (Knox, 1992). RG-I is a family of structurally similar molecules, consisting of a linear backbone of rhamnose with multiple side-chains attached, including arabinans and galactans (Obro et al., 2004). RG-II is the most complex polysaccharide, which is composed of an HG backbone decorated by different kinds of sugars (Mohnen, 2008). RG-II may also covalently link to both RG-I and HG via glycosidic bonds. Thus, RG-II likely contributes to a macromolecular cell wall pectic network (Mohnen, 2008).

Pectin is characterized by many large side-chains, and thus can form an amorphous matrix with the cellulose-hemicellulose network and maintain the stability of the cellulose-hemicellulose complex (Cosgrove, 1997; Cosgrove, 2005). Furthermore, the pectin network may control aggregation of the cellulose microfibrils (Jarvis, 1992). Since the components of AGP polysaccharides are similar to pectin, it has been hypothesized that AGPs function closely with pectin to influence cell wall properties. For example, unesterified pectin may contribute to strengthening cell wall cohesion by associating with cellulose, hemicellulose and AGPs (Boughanmi et al., 2010). In *A. thaliana*, Tan et al (2013) demonstrated that APAP1 (At3g45230), a classical AGP, can cross-link with pectin and xylan. In the tension wood of *Liquidambar styraciflua* L. and *Celtis occidentalis* L., AGPs and RG-I were shown to be rich in the G-layer, suggesting that both may function to generate unique properties of the cellulose microfibrils (Bowling and Vaughn, 2008). It has also been speculated that AGPs act as calcium capacitors and potentially bind to calcium via the glucuronic acid in the oligosaccharide side-chains (Lamport and Varnai, 2013; Pickard, 2013).

### 1.2.3.4 Lignin

Lignin is composed of phenolic subunits, and ultimately forms a complex three-dimensional network with different intermolecular linkages (Zhang et al., 2011a). Lignin is composed of three main phenylpropanoids, namely, hydroxyphenyl (H units), guaiacyl (G units) and syringyl (S units). They are...
interconnected by ether and carbon-carbon linkages (Marques et al., 2006; Rencoret et al., 2008). The relative ratios of the monomeric subunits are cell and species specific (Baucher et al., 1998). For example, vessels are rich in G lignin, whereas fibers have S lignin (Saka and Goring, 1985; Patten et al., 2010).

Lignin is hydrophobic and confers substantial mechanical support to the cells, together with other organic compounds (Kohorn, 2000). Cell wall proteins may affect the linkage of cell wall constituents with lignin. For example, hydroxyproline-rich glycoproteins (HRGPs) and glycine-rich proteins (GRPs) were detected and localized with lignin in the secondary cell wall of *Fagus sylvatica* and *Phaseolus vulgaris* L. (Dill et al., 1984; Keller et al., 1989). However, there is no direct evidence to support this hypothesis.

### 1.2.3.5 Cell wall protein

The plant cell wall structural proteins are mainly composed of HRGPs, GRPs, and proline-rich proteins (PRPs) (Cassab, 1998). The functional role of cell wall proteins may be to impart strength, contribute to wall assembly and remodelling, pathogen defence, and wound stress responses (Cassab, 1998).

During plant cell wall biosynthesis, cell wall enzymes also play important roles. In *A. thaliana* and poplar, the genes encoding the carbohydrate-active enzymes (CAZy) are spatially and temporally required for cell wall biosynthesis (Henrissat et al., 2001; Geisler-Lee et al., 2006). Xyloglucan endotransglucosylase/hydrolase (XTH), xyloglucan endotransglucosylase (XET), and pectin methylesterases have been reported to partake in cell wall loosening and rearrangement (Fry et al., 1992; Micheli, 2001; Van Sandt et al., 2007). For example, XET, a member of the xyloglucan XTH family, can mediate the expansion of cells by cleaving and re-integrating xyloglucan chains to the cell wall matrix (Takeda et al., 2002; Cosgrove, 2005).
1.3 Arabinogalactan proteins (AGPs)

1.3.1 Structures of AGPs

AGPs are cell wall proteins belonging to the large HRGP family. The HRGPs contain distinctive hydroxyproline repeats within their polypeptide backbone, and are an integral component of the cell wall in actively growing cells (Lamport and Northcote, 1960). HRGPs are believed to contribute to cell wall rigidity, stability and solubility (Knox, 1997).

AGPs contain 90-99% carbohydrate and 1-10% protein, with arabinose and galactose residues present at approximately a 1:6 (arabinose:galactose) ratio. Arabinogalactan (arabino-3,6-galactan) oligosaccharides, as well as short oligo-arabinan side-chains, decorate the protein backbones that are rich in Pro residues, most of which are hydroxylated (Showalter, 1993; Mau et al., 1995; Gaspar et al., 2001; Ellis et al., 2010). Evidence have been shown that pear PcAGP1 and AtAGP17 protein have 93% and 84% hydroxylated Pro residues, respectively (Oxley and Bacic, 1999; Sun et al., 2005). The “hydroxyproline-contiguity hypothesis” has been proposed based on the observation that repetitive hydroxyproline (Hyp) residues in close contiguity are decorated with oligo-arabinosides, while non-contiguous Hyp residues have arabinogalactan polysaccharides attached to them (Shpak et al., 2001; Sun et al., 2005). According to their core protein compositions, AGPs have been defined as classical and non-classical: classical AGPs are composed of glycosylated Hyp, Ala, Ser, Thr and Gly residues, and possess carbohydrate moieties that are linked to the protein through Ara-O-Hyp and Gal-O-Hyp. In contrast, non-classical AGPs are rich in Asn or Cys residues in addition to Hyp (Showalter, 1993; Mau et al., 1995; Gaspar et al., 2001).

1.3.2 Biosynthesis of AGPs

Many AGPs are glycosylphosphatidylinositol-anchored proteins (GPI), where the GPI anchor is required for the transport of the protein from the endoplasmic reticulum (ER) to the Golgi. The biosynthesis of AGPs is believed to consist of the following steps (Figure 1.1): synthesis of the protein backbone on the rough ER; addition of a GPI anchor to the AGP protein backbone in ER lumen; linkage of monosaccharide units to the glycoproteins (Kieliszewski et al., 1992; Tan et al., 2003); elongation of the
carbohydrate chains; intracellular transport of the AGPs; and, finally the secretion and extracellular transport of the product (Raad et al., 2010). Recently, two of the enzymes involved in AGP post-translational modification have been identified and characterized: prolyl 4-hydroxylase (P4H) catalyzes Pro hydroxylation to create the O-link glycosylation anchor, and hydroxyproline O-galactosyltransferase (HGT) catalyzes the addition of galactose to the oligosaccharide during arabinogalactan formation (Oka et al., 2010).

Figure 1.1: The synthesis and addition of a GPI-anchor to an arabinogalactan protein (AGP). The protein backbone is synthesized on the rough ER. In ER lumen, GPI anchor adds to the AGP protein backbone and monosaccharide units link to the glycoproteins. In Golgi, the carbohydrate chains are elongated and modified. Finally the AGP product is secreted and extracellularly transported. Reproduced with permission from (Ellis et al., 2010); Copyright © 2010 American Society of Plant Biologists.
1.3.3 Types of AGPs

The different types and structural characteristics of AGPs have been studied in *A. thaliana*, where it has been shown that 85 different AGP genes exist in five classes based on protein backbone homology: including, classical AGPs, lysine-rich classical AGPs, AG peptide, fasciclin-like classical AGPs (FLAs), and other chimeric AGPs (*Figure 1.2* and *Figure 1.3*) (Showalter et al., 2010). The classical AGPs contain a C-terminal hydrophobic region and a cleavable N-terminal signal sequence that directs the polypeptide to the endoplasmic reticulum, as well as being rich in Pro residues. Lysine-rich classical AGPs contain lysine-rich regions inserted in the Pro-rich AGP domain, in addition to the N-terminal signal sequences and a hydrophobic C-terminal sequence. Mature AG peptides have short protein backbones consisting of approximately 10 to 13 amino acid residues once the N- and C-terminal signal sequences have been removed (Schultz et al., 2002). In *A. thaliana*, there are 16 AG peptides (Showalter et al., 2010). The FLAs contain fasciclin domains and AGP-like domains characterized by regions rich in Pro residues. Fasciclin-like domains, located between the two AGP-like domains, contain two highly conserved sequence regions of approximately 10 amino acids in length, called H1 and H2. The H1 region is highly conserved in the FLA superfamily of higher plants, while the C-terminus-located H2 domain is rich in hydrophobic amino acids (Liu et al., 2008). Based on genomic sequence analysis, it has been reported that *A. thaliana* has 21 FLAs, while poplar has 15 FLAs (Johnson et al., 2003; Lafarguette et al., 2004).
Figure 1.2: The structure of classical AGPs. Based on the protein backbone homology, there are four types of classical AGPs. The basic form of classical AGPs (A). Lysine-rich AGPs have lysine-rich domain (B). Fasciclin-like AGPs are rich in fasciclin-like domain (C). AG peptide has a short AGP-like domain (D).
Figure 1.3: Phylogenetic tree showing the relationship of classical AGP family members in A. thaliana using Neighbor-Joining method (Saitou and Nei, 1987). Full length of AGP amino acid sequences in A. thaliana are used for alignment. Sequence alignment and polygenetic tree are made in MEGA5 (Tamura et al., 2011). Bootstrap values at branch points were expressed as percentages of 500 replications. Blue arrows indicate the target genes in this research.

1.3.4 Putative roles of AGPs

Previous research has ascribed several putative functional roles to various AGPs (Seifert and Roberts, 2007; Ellis et al., 2010; Tan et al., 2012; Tan et al., 2013), including cell expansion, xylem cell differentiation, and cell wall development. For my research, I am interested in investigating the function of AGPs in cell wall formation in general, with a concentrated focus on three AGPs, namely, classical AGP9, classical AGP14 peptide and lysine-rich AGP18, all which show relatively high gene transcript abundance in the developing xylem of Populus trichocarpa, based on recent transcriptome re-sequencing efforts and gene expression data (Geraldes et al., 2011).

1.3.4.1 A putative role for AGPs in cell wall formation

How AGPs affect cell wall formation is currently not fully understood. During cell wall formation, AGPs appear to be involved in cell expansion, cellulose deposition and cell wall thickening. Given that AGPs are rich in carbohydrate moieties, they may affect the synthesizing cell wall polymer matrix via intra- and inter-molecular cross-linking (Nothnagel, 1997; Schultz et al., 1998; Vaughn et al., 2007; Anand and Tyagi, 2010). A series of studies have been conducted to elucidate the functions of AGPs during plant development; however, precise molecular mechanisms of AGP function are not yet known. A major challenge in elucidating the functional roles of AGPs is their complex chemical structure, and the presence of the GPI-anchor. In addition, the large gene family implies that there is functional redundancy in the superfamily (Tan et al., 2012).
1.3.4.2 A putative role for AGPs in cell expansion

AGPs may contribute to cell expansion, both diffuse cell expansion and tip growth (Seifert and Roberts, 2007; Ellis et al., 2010; Tan et al., 2012; Tan et al., 2013). For example, the A. thaliana Salt Overly Sensitive 5 (SOS5/FLA4) protein contains an AGP-like domain, and the sos5 mutant exhibited abnormal root epidermal, cortical, and endodermal cell expansion after salt stress treatment (Shi et al., 2003).

Furthermore, when A. thaliana seedlings were grown in culture media containing β-glucosyl Yariv reagent (1,3,5-tris (4-β-D-glycopyranosyloxyphenylazo)-2,4,6-trihydroxy-benzene), which can precipitate AGPs and results in a red stain, root epidermal cell elongation was reduced (Willats and Knox, 1996; Ding and Zhu, 1997). Similarly, in carrot suspension culture, cell elongation was reduced after treatment with β-glucosyl Yariv reagent (Willats and Knox, 1996). AGPs have also been shown to be required for apical cell extension in the moss Physcomitrella patens (Lee et al., 2005).

Immunolocalization studies suggest that AGPs may play a role in tip growth. For example, in maize, AGP expression was shown to be highest, using the LM2 antibody, during root hair tip growth and bulge formation. Following the cessation of root hair tip growth, the LM2 epitope label decreased (Samaj et al., 1999). In Physcomitrella patens, LM6 labelling of α-arabinan was detected and shown to be rich in the tips of apical cells (Lee et al., 2005). Furthermore, in in vitro cultivated A. thaliana pollen tubes, AGP epitopes recognized by LM2 were abundant in pollen tips and the pollen tube cell wall (Dardelle et al., 2010). During fiber intrusive tip growth in xylem development, the secretory vesicles move to the growing tips to provide materials for the formation of new cell walls (Franke et al., 1972; Heslop-Harrison et al., 1986). AGPs are highly expressed in developing fibers, as witnessed by cDNA microarrays and antibody labelling, suggesting a role for AGPs in fiber development (Ji et al., 2003; Feng et al., 2004; Knox, 2008; Liu et al., 2008; Roach and Deyholos, 2008; Huang et al., 2013).

A role for AGPs in tip growth has also been postulated, namely pollen tube elongation. Reduced expression, via RNAi-mediated suppression, of AGP6 and AGP11 in A. thaliana partly inhibited pollen tube growth and led to reduced male fertility (Levitin et al., 2008). A. thaliana agp6/11 double mutants showed reduced pollen biogenesis, which included both altered germination and pollen tube elongation.
In addition, kiwifruit and lily pollen tubes exhibited inhibited pollen tube growth and malformations, respectively, after being treated with β-glucosyl Yariv reagent (Roy et al., 1998). This suggests a role for AGPs in maintaining correct pollen tube growth and shape. Based on this indirect evidence, AGPs may play a role in cell expansion; however, the exact mechanism is not yet understood.

### 1.3.4.3 A putative role for AGPs in cellulose deposition

AGPs may act in cell wall biogenesis through an indirect interaction with cortical microtubules, as it has been experimentally demonstrated that cross-linking AGPs may indirectly lead to microtubule disorganization (Driouich and Baskin, 2008). Tobacco BY-2 suspension cells expressing a GFP-microtubule-binding domain were shown to possess disorganized microtubules, and had thicker actin filaments when AGPs were cross-linked by β-glucosyl Yariv reagent (Sardar et al., 2006). Similarly, *A. thaliana* root microtubules were disorganized, and the distance between cortical microtubules and the plasma membrane was greatly increased following treatment with β-glucosyl Yariv reagent (Nguema-Ona et al., 2007).

Given that cellulose microfibril deposition can be influenced by microtubules (Paredez et al., 2006), and AGPs have been shown to influence microtubules, AGPs may also impact cellulose deposition. To this end, previously it was shown that in growing cultivated tobacco cells, there was less cellulose based fluorescence after treatment with β-glucosyl Yariv reagent (Vissenberg et al., 2001).

The question remains, however, how do AGPs affect cellulose microfibril deposition? AGPs may rearrange scaffold proteins and disorganize microtubule organization, thus indirectly influencing cellulose deposition (Driouich and Baskin, 2008). Kohorn et al. (2000) proposed that AGPs could direct cellulose deposition by serving as positional cues and adhesive cell wall components. Schopfer (1991) hypothesized that AGPs promote the separation of microfibrils and decrease the rigidity of cell walls by acting as hydrating molecules to form a gel. Based on the proposed models, this implies that AGPs could act as a lubricant gel for cell wall matrix, or are involved in guiding cellulose deposition by indirectly disrupting the microtubule orientation.
1.3.4.4 Putative role for AGPs in programmed cell death

AGPs may also have a function in programmed cell death. It has been reported that AGPs affected cell death in *A. thaliana* and *Zinnia* embryogenic suspension cultures (Dolan et al., 1995; Groover and Jones, 1999). During maize coleoptile development, AGPs have been shown to be localized to the tracheids by JIM13 antibody which recognizes β-D-GlcA-(1-3)-α-D-GalA-(1-2)-L-Rha (Schindler et al., 1995). In poplar, classical AGPs were found to be enriched during fiber cell death (Moreau et al., 2005). Collectively, these findings point to AGPs playing a role in programmed cell death (Schindler et al., 1995), although their direct or indirect mechanism is not known.

1.3.4.5 AGP expression during xylem development

AGPs may facilitate secondary xylem development and affect xylem fiber intrusive growth; however, the underlying mechanism of AGPs in secondary xylem formation is not apparent. It has been reported that AGPs are spatially and temporally present during xylem development. In maize, based on β-glucosyl Yariv reagent, the location of AGPs was shown to be localized to the outer epidermal wall and xylem elements (Schopfer, 1991). In differentiating loblolly pine, PtX3H6 and PtX14A9, two proteins with amino acid compositions similar to hydroxyproline-rich AGPs, can be detected during xylem development (Loopstra and Sederoff, 1995). PtaAGP6, a lysine-rich AGP in loblolly pine, was localized at early stage of developing xylem via antibody immunolabelling of the lysine-rich domain (Zhang et al., 2003). Motose et al. (2001) suggested that AGPs are required during tracheary element differentiation in *Zinnia* cells, as cells treated with β-glucosyl Yariv reagent were inhibited during differentiation of tracheary elements. These results imply that AGPs may be required for tracheary elements formation in a variety of plants.

1.3.4.6 The expression pattern of FLAs and lysine-rich AGP genes during cell wall formation

1.3.4.6.1 FLAs are highly expressed during cell wall formation

The fasciclin-like (FLA) sub-family of AGPs have been proposed to play a role in cell wall formation based on their expression patterns, which in *A. thaliana* are closely correlated with specific developmental events (Johnson et al., 2003). For example, *AtFLA3* showed elevated expression in pollen grains and
tubes, while AtFLA3 RNAi transgenic plants showed abnormal, aborted pollen. AtFLA3 might, therefore, affect the distribution and deposition of pollen cellulose (Li et al., 2010). The AtFLA11 promoter was shown to be very active during secondary cell wall deposition in A. thaliana inflorescence stems, as demonstrated by a promoter::glucuronidase (Gus) assay, and AtFLA11 was highly expressed in sclerenchyma cells in A. thaliana inflorescence stem and siliques (Ito et al., 2005; MacMillan et al., 2010; Hall and Ellis, 2013). In eucalyptus, FLA2 and FLA3 were specifically expressed in the xylem of stem and branches (MacMillan et al., 2010), while in poplar, FLA1-15 was shown to be expressed abundantly in tension wood and FLA11-15 was specifically present in differentiating xylem compared with mature xylem (Lafarguette et al., 2004). In cotton, all GhFLAs were expressed during fiber development and the transcripts of GhAGP3 and GhAGP4 accumulated during the developmental switch to secondary cell wall biosynthesis (Liu et al., 2008).

1.3.4.6.2 The expression of lysine-rich AGP genes during cell wall formation

The lysine-rich AGP genes have been observed to be expressed during cell wall formation. Specifically, in A. thaliana, three lysine-rich AGP genes, namely AtAGP17, AtAGP18 and AtAGP19, have been shown to display higher expression in stem tissue (Gaspar et al., 2004), and the activity of the AtAGP18 promoter was shown to be specifically associated with vascular bundles (Acosta-Garcia and Vielle-Calzada, 2004). In tomato (Gao and Showalter, 2000) and loblolly pine (Zhang et al., 2003), localization of LeAGP-1 and PtaAGP6 were detected during secondary cell wall thickening.

1.3.4.7 A putative role for FLAs and lysine-rich AGPs in plant growth

The proposed functions of FLAs and lysine-rich AGPs are based on mutant studies in A. thaliana. FLAs have been shown to contribute to stem tensile strength. For example, A thaliana fla11/12 T-DNA knockout double mutants have altered cellulose composition and microfibril angles, as well as compromised stem strength and stiffness (MacMillan et al., 2010). In contrast, the lysine-rich AGP mutant, atagp19, displayed delayed growth, shorter hypocotyls and inflorescence stems, and reduced seed production, suggesting roles for AtAGP19 in cell division, cell expansion and leaf development (Yang et al., 2007b). AtAGP18 may function to initiate female gametogenesis and differentiation of the viable
megaspore, which was demonstrated by the phenotype observed in *A. thaliana* RNAi-*PtAGP18* lines (Acosta-Garcia and Vielle-Calzada, 2004; Demesa-Arevalo and Vielle-Calzada, 2013). When *AtAGP18* was overexpressed in *A. thaliana*, the plants showed smaller rosettes, shorter stems and roots, more branches and fewer viable seeds (Zhang et al., 2011c). The authors suggest that *AtAGP18* may act as a co-receptor for signal transduction during plant development and growth, including binding cytokinins, interacting with receptor kinases or ion channels (Zhang et al., 2011b). How this might actually happen is unsolved, however.

### 1.4 Introduction to secondary xylem (wood)

The secondary xylem of gymnosperms (softwoods) is composed of tracheids and parenchyma cells, while the secondary xylem of woody angiosperms (hardwoods) is more complex, containing several cell types, including vessel elements, tracheids, fibers and parenchyma cells (Figure 1.3). However, the vessel and fiber cells are the main components of secondary xylem of the angiosperms. In poplar, the secondary xylem contains approximately 53-55% fibers, 33% vessel elements and 11-14% rays (Groover et al., 2010).
Figure 1.4: The structure of softwood and hardwood. The main cell type in softwood xylem is tracheid, which has functions in water transport and structural support. Hardwood xylem contains vessels which are water conduits, and fibers which supply supportive strength. Both softwood and hardwood have rays, which are horizontally orientated along the diameter of the tree, and transport water and nutrients radially across the stem. Reproduced with permission from (Tsuchikawa, 2007); Copyright © 2007 Taylor & Francis.
1.4.1 Secondary xylem is produced by the vascular cambium

Secondary xylem development is initiated from the vascular cambium. The vascular cambium itself is made up of dividing cells, only a fewer layers thick with thin-walled, elongated and narrow cells (Lachaud et al., 1999; Groover et al., 2010). The cambial zone contains two distinct cell types: fusiform cambial cells which are radially short but vertically elongated, and ray cambial cells which are small cubical cells (Chaffey et al., 2002a). The fusiform initials differentiate into secondary xylem mother cells and secondary phloem mother cells (Mellerowicz et al., 2001), when periclinal divisions produce secondary xylem toward the inside and secondary phloem (bark) to the outside of the cambium, respectively (Samuels et al., 2006). The ray initials differentiate into ray mother cells that form vascular rays (Mellerowicz et al., 2001). The rays of hardwoods account, on average, for 17% of the volume of the wood, and have a role in the transportation of nutrients between the xylem and phloem (Rahman et al., 2005).

1.4.2 Roles of cytoskeleton in wood formation

The cytoskeleton, such as microtubules, plays an important role in wood formation. In fusiform cambial cells and during early fiber differentiation, microtubules have been shown to be abundant (Chaffey et al., 2002a). The orientation of microtubules during cambial cell expansion and fiber cell expansion was shown to be coordinated with orientation of cellulose microfibrils in primary cell wall to help regulate the cell shape and size (Funada, 2008). During fiber secondary cell wall thickening, the normally randomly organized microtubules appeared to become ordered, and the ordered microtubules displayed parallel and helical patterns that resembled the microfibrillar orientation (Chaffey et al., 1999). Similarly, in the developing xylem of poplar, microtubules were shown to be abundant during fiber secondary cell wall deposition (Kaneda et al., 2010). It has been proposed that the orientation and delivery of cellulose synthase complexes are influenced by microtubules and actin in developing protoxylem (Wightman and Turner, 2008). Given that AGPs may influence the cytoskeletal properties, AGPs may also contribute to differentiation of the cellulosic cell wall.
1.4.3 Fibers

In poplar, fibers act as the primary component of the secondary xylem and confer supportive strength for plant growth. The origin of xylary fibers is the vascular cambium. In contrast, non-xylary fibers such as flax fibers, and fibers in cereals and bamboo, differentiate from the procambium, or from ground meristem tissue such as parenchyma cells that form interfascicular fibers of A. thaliana. Generally, in most species, the fibers are the longest cell type, owing to the intrusive mode of elongation (Lev-Yadun, 2010). Fiber growth in A. thaliana is transcriptionally regulated by the Secondary Wall-Associated NAC Domain Protein1 (SND1) and NAC Secondary Wall Thickening Promoting Factor1 (NST1). SND1 was shown to be specifically expressed in fibers in A. thaliana stem (Zhong et al., 2006). Suppression of SND1 may reduce secondary cell wall thickening, whereas overexpression of SND1 led to ectopically lignified secondary cell wall in A. thaliana stems (Zhong et al., 2006). The roles of NST1 in secondary cell wall thickening in A. thaliana was shown to be similar to SND1 (Mitsuda et al., 2007). In poplar, 16 SND1 (PtrWND) orthologs were recently identified (Zhong et al., 2010a), and appear to play a role in secondary cell wall formation in fibers (Zhong et al., 2010a).

1.4.4 Vessels

Vessels play an important role in conducting water in angiosperms. They, like the fiber cells, initiate from cambial initials. Vessels differentiate and undergo programmed cell death in only a few days with a shorter life span than fibers and tracheids, which have been shown to live for up to a month (Bollhoner et al., 2012). In primary growth of A. thaliana, the xylem vessels originate from procambium and their formation is regulated transcriptionally by the Vascular-Related NAC Domain Protein6 and 7 (VND6, VND7) (Ohashi-Ito et al., 2010; Yamaguchi et al., 2010b; Yamaguchi et al., 2011). VND6 and VND7 regulate the xylem vessel differentiation by controlling the expression of several additional transcription factors and biosynthetic genes (Yamaguchi et al., 2011). Specifically, VND7 and VND6 separately determine protoxylem and metaxylem differentiation (Kubo et al., 2005; Yamaguchi et al., 2010b). Overexpression of AtVND7 can induce protoxylem differentiation in A. thaliana (Yamaguchi et al., 2010b). Similarly, in poplar, PtVNS07/PtrWND6A and PtVNS08/PtrWND6B (orthologous to AtVND7)
were shown to be expressed in the primary xylem vessels and appear to play roles in inducing secondary cell wall formation (Ohtani et al., 2011).

In secondary xylem in woody species, vessel development can be affected by hormones, such as auxin. In stem segments of oak, vessel number was positively correlated with the indole acetic acid (IAA) concentration (Zakrzewski, 1991). Similarly, when the bacterial IAA biosynthetic genes (iaaM or iaaH) were expressed in hybrid aspen to increase IAA concentration, secondary xylem of transgenic plants contained smaller vessels, which occurred in groups (Tuominen et al., 1997). Auxin also influences primary xylem differentiation, as higher concentrations of exogenous IAA applied to greenhouse-grown bean seedlings induced more and smaller vessels in xylem of stem (Aloni and Zimmermann, 1983).

1.5 Research objectives and goals

The primary goal of my research is to ascertain the role(s) of AGPs during xylem development. For this research project, I tested two hypotheses. The first hypothesis is that AGPs facilitate fiber and vessel development by promoting cell expansion. The second hypothesis is that AGPs are required for proper secondary cell wall formation. To address these hypotheses, I have formed the following specific objectives: 1) to elucidate the function of AGP18 in fiber development in poplar and A. thaliana, 2) to test the function of AGP9 in vessel development in poplar and A. thaliana, 3) to assess the function of AGP14 in cell wall development in poplar and A. thaliana.

Chapter 2: AGP18 is required for fiber development in A. thaliana and poplar

The goal of this chapter was to clarify the functions of the lysine-rich AGP18 in fiber development. To achieve this goal, the fiber growth characteristics were investigated in transgenic RNAi poplar lines and A. thaliana mutants. RNAi-PtAGP18 poplar lines and atagp18 mutants were shown to display altered fiber shapes. The poplar PtAGP18 gene was able to complement the A. thaliana mutant, indicating that these two genes are functionally equivalent. In addition, atagp18 mutants showed cell expansion phenotype because the dark-grown hypocotyl did not have normal cell expansion. Furthermore, AGP18 appears to
affect secondary cell wall formation, because down-regulation of AGP18 expression in both poplar and Arabidopsis resulted in thinner secondary cell walls. Since AGP18 is required for cell expansion in hypocotyls, I hypothesize that its role in fiber development is first during cell expansion and morphogenesis, and later in secondary cell wall deposition.

Chapter 3: AGP9 affects xylem vessel development in poplar and A. thaliana

The goal of this chapter was to investigate the role(s) of AGP9 during xylem development. To characterize the function of AGP9 in xylem development of poplar, transgenic RNAi lines and overexpression of AtAGP9 poplar lines were generated. Since the fibers and vessels are the main components in secondary xylem, the traits of these two cell types were studied in transgenic poplar stem cross sections. Based on the observed vessel phenotype, I have shown that AGP9 impacts vessel differentiation in poplar, which appears to occur via an impact on cell expansion and secondary cell wall formation. Again, the role of AGP9 in cell wall expansion was tested in A. thaliana by employing the dark-grown hypocotyl, which showed abnormal hypocotyl expansion, suggesting a role for AGP9 in cell expansion.

In A. thaliana inflorescence stem, AtAGP9 may play roles in primary xylem vessel differentiation. This was demonstrated by the spatial activity of AtAGP9 promoter in the early stage of xylem development using glucuronidase (GUS) assay, and the irregular deposition of multiple protoxylem cells as viewed in stem cross sections of atagp9 mutants and RNAi-AtAGP9 A. thaliana plants.

Chapter 4: AGP14 may affect fiber cell wall formation in poplar

The goal of this project was to examine the role(s) of AGP14 peptide in cell wall development. The expression pattern of AGP14 was characterised in poplar tissues, and AGP14 showed relatively high transcript abundance in developing xylem of poplar. Therefore, the roles of AGP14 in cell wall development were investigated in transgenic poplar RNAi lines and overexpression of AtAGP14 poplar lines, similar to AGP18 and AGP9. There were no changes in wood anatomy in poplar with altered AGP14, but minor changes in cell wall traits in transgenic poplar lines were observed.
Chapter 5: Conclusions and Future Work

In this chapter, I discuss the implications and impact of my research findings, and finally make recommendations for future work to continue to explore a role for AGPs in wood formation.
Chapter 2 *AGP18* is required for fiber development in *Arabidopsis thaliana* and poplar

2.1 Introduction

Arabinogalactan proteins (AGPs) are cell wall proteins that belong to the large hydroxyproline-rich glycoprotein (HRGP) family. Generally, they contain 90-99% carbohydrate and 1-10% protein, with arabinosyl and galactosyl residues commonly found in a 1:6 ratio. Arabinogalactan (Arabino-3,6-galactan), as well as oligo-arabinan side-chains shorter than four residues, decorate the hydroxyproline (Hyp) residues on the protein backbone (Showalter, 1993; Mau et al., 1995; Gaspar et al., 2001; Ellis et al., 2010). According to the core protein composition, AGPs have been categorized as classical and non-classical: classical AGP composition is dominated by glycosylated Hyp, as well as Ala, Ser, Thr and Gly residues, and they possess carbohydrate moieties of branched arabino-3,6-galactan that are linked to the protein through Ara-O-Hyp and Gal-O- Hyp linkages. In contrast, non-classical AGPs are rich in Asn or Cys residues in addition to Hyp residues (Showalter, 1993; Mau et al., 1995; Gaspar et al., 2001). The different types and structural characteristics of AGPs have been studied in *Arabidopsis thaliana*, which possess 85 different AGP genes existing in five broad classes based on the protein backbone homology: classical AGPs, lysine-rich classical AGPs, AG peptides, fasciclin-like classical AGPs, and other chimeric AGPs (Showalter et al., 2010). The complex protein structure and the large gene families have made elucidating the functional roles of AGPs a difficult task. Some suggested functions of AGPs include a contribution to cell expansion, xylem cell differentiation, and cell wall development (Seifert and Roberts, 2007; Ellis et al., 2010; Tan et al., 2012; Tan et al., 2013).

There are several lines of evidence that suggest a role for AGPs in cell expansion. In *A. thaliana* Salt Overly Sensitive 5 (*sos5*) mutants that contain a mutation in an AGP, root elongation has been shown to be significantly reduced when exposed to salt stress (Shi et al., 2003). Reduced root elongation was
also observed in *A. thaliana* seedlings grown in media containing β-glucosyl Yariv reagent, which can cross-link AGPs and disrupt the function of AGPs (Willats and Knox, 1996; Ding and Zhu, 1997). AGPs were detected by cDNA arrays of cotton (Ji et al., 2003) and flax fibers (Roach and Deyholos, 2008). Immunolabelling also demonstrated AGPs in rapidly elongating cotton (1.36 mm per day in average) and flax fibers (2.67 mm per day in average) (Knox, 2008; Liu et al., 2008; Huang et al., 2013). However, the molecular mechanisms of fiber cell elongation have yet to be resolved (Gorshkova et al., 2012).

Transcriptome data collected from developing xylem of *Populus trichocarpa* re-sequencing initiatives detected several AGPs displaying high gene expression (Geraldes et al., 2011) including *PtAGP18*, encoding a lysine-rich AGP with homology to the *A. thaliana AGP18*. In the large multi-gene AGP family, the lysine-rich classical AGPs form a small sub-clade with only three members in *A. thaliana*, namely AtAGP17, AtAGP18 and AtAGP19. The first lysine-rich AGP was reported in tomato (LeAGP-1), and antibody binding localized it to secondary xylem in the root and stem (Gao and Showalter, 2000). In the *atagp19* T-DNA insertion mutants, plants displayed delayed growth, and reduced hypocotyl length by 25% and inflorescence stem length by 40% (Yang et al., 2007b). In loblolly pine, increased expression of a lysine-rich AGP, referred to as PtaAGP6, was detected at the onset of secondary cell wall thickening, suggesting a role in xylem formation (Zhang et al., 2003).

In woody angiosperm xylem, fibers act as important structural components of secondary xylem and confer supportive strength for plant growth. In secondary growth, fiber cells initiate from xylem mother cells in the vascular cambium, follow by cell expansion, secondary cell wall deposition and programmed cell death. Lev-Yadun (2010) suggested that the characteristics of fibers are defined by species, but in most species, fibers are often the longest cell type, owing to the intrusive mode of elongation. However, the exact mechanism of fiber cell elongation has yet to be resolved.

Here, we chose *AGP18* to investigate the function of lysine-rich AGP gene in fiber development in poplar and *A. thaliana*. Several studies have characterized *AGP18* in *A. thaliana*, but a function for *AtAGP18* in fiber development has never been reported. In *A. thaliana*, *AtAGP18* promoter::GUS assays showed activity in vascular bundles and pollen tube, as well as in hypocotyls cultured in the dark (Acosta-
Garcia and Vielle-Calzada, 2004; Yang and Showalter, 2007a). AtAGP18 RNAi-suppressed lines have also previously been reported to display defects in female gametogenesis in A. thaliana (Acosta-Garcia and Vielle-Calzada, 2004). AtAGP18 distribution was correlated with differentiation of the viable megaspore following meiosis (Demesa-Arevalo and Vielle-Calzada, 2013). In contrast, overexpression of AtAGP18 in A. thaliana led to smaller rosettes, more branches and less viable seeds, one fifth shorter stems and one fourth shorter roots compared to wild type (Zhang et al., 2011c). In my study, the putative role of AGP18 in fiber development was investigated by studying interfascicular fiber cell length and shape in atagp18 T-DNA insertion mutants. Transgenic hybrid poplar lines were also generated to test the roles of AGP18 in poplar fiber development by employing RNAi-suppression of PtAGP18 and overexpression of AtAGP18, respectively.

2.2 Results

2.2.1 Lysine-rich PtAGP18

PtAGP18 (Potri.007G051600) was identified as being highly expressed during secondary xylem formation, when 20 actively growing poplar trees were subjected to transcriptome re-sequencing (Geraldes et al., 2011). As such, PtAGP18 and its A. thaliana Columbia-0 (Arabidopsis) ortholog, AtAGP18 (AT4G37450), were separately cloned from P. alba × P. grandidentata (hybrid poplar P39) and Arabidopsis plants, respectively. PtAGP18 shares 64% amino acid sequence similarity with AtAGP18 (Figure 2.1). The structure of PtAGP18, similar to AtAGP18, was characterized as containing an N-terminal signal peptide, potential C-terminal GPI-modification sites, a C-terminal GPI-associated hydrophobic domain, and a specific lysine-rich domain (Figure 2.1 and 2.2). This implies that PtAGP18 belongs to the classical lysine-rich AGP family, similar to AtAGP18. The percentage of Pro residues in the AGP18 mature protein was predicted and shown in Table 2.1A. Most of the Pro residues in the core of other AGP proteins have been shown to be hydroxylated, e.g. pear PcAGP1 and AtAGP17 protein have 93% and 84% hydroxylated Pro residues, respectively (Oxley and Bacic, 1999; Sun et al., 2005).
The Hyp glycosylation characteristics of both the Arabidopsis and poplar AGPs were also predicted by applying the Hyp-contiguity hypothesis to the core protein sequence data (Shpak et al., 2001; Sun et al., 2005) (Table 2.1). Specifically, the “Hyp-contiguity hypothesis” has been proposed based on the observation that more than half of repetitive hydroxyproline residues in close contiguity are decorated with oligo-arabinosides, while non-contiguous hydroxyproline residues usually have arabinogalactan polysaccharides attached to them. PtAGP18 and AtAGP18 have approximately 63% and 70% polysaccharide decorating the Hyp sites and approximately 25% Hyp-arabinosylation sites (Table 2.1B). Similar patterns have been shown for the fasciclin-like AGPs, FLA11 and FLA12, which were highly expressed in fiber cells in Arabidopsis inflorescence stem and siliques (Table 2.1B) (Ito et al., 2005; MacMillan et al., 2010).

Figure 2.1: Alignment of *P. alba* × *P. grandidentata* AGP18 (PtAGP18) amino acid sequences with that of *Arabidopsis thaliana* AGP18 (AtAGP18). Identical conserved amino acids are shaded black, while non-identical amino acids with similar properties are shaded grey. The target sequences show 50% identity and 64% similarity. The conserved lysine-rich domain is highlighted with a red box. Gene identification numbers are: *PtAGP18*, Potri.007G051600; *AtAGP18*, AT4G37450.
**A-PtAGP18**

Signal peptide

MDRNGILGWTLICVLVAGVGGQAAPATPTSTPATPTTPSVPPLAAPAKAKPTTPAPVSSPPAVTPVASSPK

QTVTPVATPLATPPAVTPVSSPPAPVPVSSPEKSSPSVAPTPSSVAAPTAEVPAAPTPSKKKPKKAPA

PGPALLSSPAPPTEAPGSAESVSPGPAALSDNGAETIRCLQKMGGLALGWLALLLIF

Predicted GPI ϖ-site

Predicted C-terminal hydrophobic domain

**B-AtAGP18**

Signal peptide

MDRNFLTTLTICVVAVGQGQSPISSPTKTSPKSPTTAPTSPTKSPVTPSPTTAPAKPTPTASAASSPVEKSPPAP

VSESSPPPTVPESPPVAPMVSSPVPSSPPVAPVADSPAPVADVPAPAPSHKHKTTSKSKKHQA

PAPAPELLGPAPPTEAGPSDNSADFGPSADQSGHASTRVLNRVAVGAVATAWAVLMAF

Predicted GPI ϖ-site

Predicted C-terminal hydrophobic domain

**Figure 2.2:** The predicted protein structures of A) *P. alba × P. grandidentata*, PtAGP18 and B) *Arabidopsis thaliana*, AtAGP18. PtAGP18 and AtAGP18 each contain a signal peptide, GPI-modification site and C-terminal hydrophobic domain, which are indicated by arrows, respectively. The lysine-rich domain is underlined with a solid line. After N-terminal signal peptide processing, the GPI anchor is predicted to be attached to the ϖ site and the C-terminal hydrophobic region is predicted to be cleaved.
Table 2.1: The predicted percentage of Pro residues in the AGP18 mature protein (A). Predicted proportion of hydroxyproline (Hyp) in putative wall-associated AGPs that would be decorated with AG-polysaccharides, or oligo-arabinoside or lack glycosylation based on the Hyp-contiguity hypothesis (B) (Shpak et al., 2001; Sun et al., 2005).

A. The predicted percentage of Pro residues in the AGP18 mature protein

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Predicted the total NO. of Pro residues</th>
<th>Predicted the NO. of non-contiguous Pro residues</th>
<th>Predicted the NO. of Contiguous Pro residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>PtAGP18</td>
<td>46</td>
<td>29</td>
<td>7</td>
</tr>
<tr>
<td>AtAGP18</td>
<td>44</td>
<td>31</td>
<td>5</td>
</tr>
<tr>
<td>PtAGP9</td>
<td>64</td>
<td>14</td>
<td>4</td>
</tr>
<tr>
<td>AtAGP9</td>
<td>63</td>
<td>14</td>
<td>4</td>
</tr>
<tr>
<td>PtAGP14</td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>AtAGP14</td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>AtAGP24</td>
<td>3</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>SOS5</td>
<td>30</td>
<td>14</td>
<td>5</td>
</tr>
<tr>
<td>FLA11</td>
<td>9</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>FLA12</td>
<td>17</td>
<td>13</td>
<td>2</td>
</tr>
<tr>
<td>AtAGP19</td>
<td>78</td>
<td>25</td>
<td>20</td>
</tr>
</tbody>
</table>

B. Predicted proportion of Hyp residues in putative wall-associated AGPs that would be decorated with AG-polysaccharides, or oligo-arabinoside or lack glycosylation

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Hyp-Polysaccharide</th>
<th>Predicted %</th>
<th>None-glycosylated Hyp</th>
</tr>
</thead>
<tbody>
<tr>
<td>PtAGP18</td>
<td>63</td>
<td>25-33</td>
<td>4-13</td>
</tr>
<tr>
<td>AtAGP18</td>
<td>70</td>
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<td>5-10</td>
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<tr>
<td>PtAGP9</td>
<td>22</td>
<td>72-75</td>
<td>3-6</td>
</tr>
<tr>
<td>AtAGP9</td>
<td>22</td>
<td>71-75</td>
<td>2-6</td>
</tr>
<tr>
<td>PtAGP14</td>
<td>100</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>AtAGP14</td>
<td>100</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>AtAGP24</td>
<td>100</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>SOS5</td>
<td>47</td>
<td>40-47</td>
<td>5-13</td>
</tr>
<tr>
<td>FLA11</td>
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<td>NA</td>
</tr>
<tr>
<td>FLA12</td>
<td>76</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>AtAGP19</td>
<td>32</td>
<td>55-62</td>
<td>6-13</td>
</tr>
</tbody>
</table>
2.2.2 Localization of AGPs in developing xylem of poplar

In Arabidopsis, immuno-detection with JIM13 antibody, which recognizes the β-D-GlcA-(1,3)-α-D-GalA-(1,2)-L-Rha-region of diverse AGPs (Yates et al., 1996), suggested that AGPs were strongly localized to the interfascicular fibers (Hall et al., 2013). Similarly, in cotton fibers AGPs were shown to be abundant during fiber elongation (Huang et al., 2013). To determine whether or not AGPs are localized to the developing secondary xylem, the localization of AGPs in poplar was tested with JIM13 antibody. JIM13 antibody labelled the fiber cells of poplar, whereas control sections had no fluorescence signal (Figure 2.3). Furthermore, β-glucosyl Yariv reagent (1,3,5-tris (4-β-D-glycopyranosyloxyphenylazo)-2,4,6-trihydroxy-benzene), an AGP cross-linker, was also used to examine AGP distribution (Yariv et al., 1967). The control α-glucosyl Yariv did not produce a red stain in poplar fibers (Figure 2.4A and B). In contrast, β-glucosyl Yariv stained the poplar fiber cells, but not the vessels (Figure 2.4C and D), similar to the pattern labelled by JIM13 antibody. These results suggest that AGPs are rich in developing xylem, especially fibers, and indirectly implicate AGPs in xylem development in poplar.
Figure 2.3: Immunolocalization of AGPs in fibers using JIM13 antibody on stem cross sections of *P. alba* × *P. grandidentata* hybrid poplar. Freshly harvested stem cross sections were observed using bright field light microscopy (panels A and C) and fluorescence microscopy (B and D). B. No fluorescence was observed in developing fiber cells in control by replacing the JIM13 anti-AGP antibody with PBS buffer. D. Fluorescent label with JIM13 anti-AGP antibody in developing fiber cells and weak label in developing vessels. CA: cambium; V: vessel; F: fiber.
**Figure 2.4:** The location of AGPs in fiber cells detected by β-glucosyl Yariv reagent in developing poplar xylem. A. The control section lacked red Yariv stain when incubated with α-glucosyl Yariv reagent in base of stem cross sections. C. Section showed red stain in fiber cells of poplar xylem after being incubated with β-glucosyl Yariv reagent. The fiber cells in (A) and (C) indicated by the blue arrows are separately shown at higher magnification in (B) and (D). The yellow arrow in A and C indicates the cambium. CA: cambium. F: fiber cell. The trees were approximately three months old. Bars = 0.05 mm.

### 2.2.3 Expression patterns of AGP18 in poplar and Arabidopsis

Quantitative real time PCR (Q-RT-PCR) was used to define the gene expression profile of *PtAGP18* in different tissues of poplar during active xylem deposition. The expression of *PtAGP18* was most abundant in the developing xylem (**Figure 2.5**), which is consistent with data available for poplar on the eFP browser (http://bar.utoronto.ca/efppop/cgi-bin/efpWeb.cgi), showing that *PtAGP18* was highly expressed in xylem (Wilkins et al., 2009). Similarly, the expression of *AtAGP18* in Arabidopsis was evaluated by RT-PCR in different tissues, and shown to have higher expression in flowers and middle stems, with weaker expression at the base of the stems (**Figure 2.6**), which is consistent with previously published work (Yang and Showalter, 2007a).
Figure 2.5: Relative transcript abundance of *PtAGP18* in poplar tissues collected in July, 2007. Transcript levels in each tissue are based on the comparative threshold cycle value relative to the housekeeping gene (actin) transcript level. The expression level of *PtAGP18* is strong in developing xylem, as well as in leaves. Bars show the standard error of the mean (n = 3).

Figure 2.6: The expression pattern of *AtAGP18* in Arabidopsis different tissues tested by semi-quantitative RT-PCR. FL - Flower; L - Leaf; MS - Middle stem; BS - Bottom stem. Transcripts were detected in different tissues. Elongation factor *AtELF5A* (AT5G60390) was used as a control to standardize the sample variations in total RNA amounts of different tissues. Plants were five weeks old.
2.2.4 AGP18 in fiber development in poplar

To test if PtAGP18 plays a role in poplar wood formation, the expression of PtAGP18 was altered by RNAi-mediated suppression of PtAGP18 (RNAi-PtAGP18) driven by the CaMV 35S promoter to achieve post-transcriptional gene silencing. Five transgenic lines were recovered and shown to possess normal height (1.9-2.4 m) (Figure 2.7) and stem thickness (14-16 mm) (Figure 2.8). An assessment of fiber length, as determined by fiber quality analyzer machine (FQA; OpTest Equipment Inc., Hawkesbury, Canada), showed that the RNAi-PtAGP18 poplar lines were not significantly different in length when compared to wild-type trees (Figure 2.9). However, digested RNAi-PtAGP18 fiber preparations stained with toluidine blue O (Obrien et al., 1964) were found to contain broken fibers. Thus, to characterize only intact fibers (possessing two tips) a microscopy-based study was conducted after staining with toluidine blue O. The results showed that the intact fiber length in the RNAi-PtAGP18 poplar lines was significantly shorter (0.07-0.03 mm in length) compared with wild type (P39) in all lines tested (Figure 2.10).

Figure 2.7: Plant height of RNAi-PtAGP18 poplar lines. For each line, there were five biological replicates. Bars show the standard error of the mean. A statistically significant difference at P < 0.05 was evaluated by the Student’s t-test. No significant differences from wild type were detected.
Figure 2.8: Stem thickness of RNAi-PtAGP18 poplar lines. Stem diameter was measured by caliper at the base of stem. For each line, there were five biological replicates. Bars show the standard error of the mean. A statistically significant difference at P < 0.05 was evaluated by the Student’s t-test. No significant differences from wild type were detected.

Figure 2.9: Fiber length of RNAi-PtAGP18 poplar transgenic lines assessed by fiber quality analyzer. Fibers were digested with hydrogen peroxide and acetic acid. For each line, three biological replicates and 10,000 fibers for each replicate were measured per line by fiber quality analyzer. Bars show the standard deviation (n = 3). A statistically significant difference at P < 0.05 was evaluated by the Student’s t-test. No significant differences from wild type were detected.
Figure 2.10: Fiber length of RNAi-PtAGP18 poplar lines assessed by bright-field microscopy. Fibers were digested with hydrogen peroxide and acetic acid, stained with toluidine blue O. For each line, three biological replicates and a total of 900 fibers in all three together were measured by Image J software from light micrograph images captured. Bars show the standard deviation. Asterisks indicate a statistically significant difference compared to wild type at P < 0.05 evaluated by the Student’s t-test.

2.2.5 AGP18 in fiber development in Arabidopsis

Concurrently, I investigated the role of AGP18 in Arabidopsis fiber development using homozygous T-DNA insertion mutant lines (Table 2.2), as well as an RNAi-suppression construct against AtAGP18 (RNAi-AtAGP18) driven by the CaMV 35S promoter. The plant height and stem thickness of the Arabidopsis mutants and transgenic RNAi-AtAGP18 lines were not significantly different compared to wild type. Phenotypic evaluation of the atagp18 mutant lines, atagp18-1 (SALK_117268) and atagp18-2 (CS321385), indicated that the atagp18-2 allele had shorter fibers (0.14 mm shorter in length), as determined by FQA (Figure 2.11). atagp18-1 (SALK_117268) or atagp18-2 (CS321385) both lacked detectable AGP18 transcript (Figure 2.11A). However, fiber lengths in the atagp18-1 allele were not significantly different from wild type. A microscopic evaluation of fiber length showed a statistically
significant decrease in fiber length for both alleles (Figure 2.12). In addition, the RNAi-AtAGP18 Arabidopsis transgenic plants also exhibited slightly shorter fibers assessed by FQA (Figure 2.13) and microscopy (Figure 2.14). This demonstrates that AtAGP18, like the poplar ortholog, PtAGP18, participates in fiber growth. To test if the putative poplar orthologs are functionally conserved, Arabidopsis mutant lines were complemented with the putative poplar orthologs. Specifically, the AtAGP18 promoter was used to drive the expression of both PtAGP18 and AtAGP18 independently in the atagp18-1 and atagp18-2 mutant lines. As assessed by FQA and microscopy, fiber length was restored from 0.55 to 0.7 mm in atagp18-2 mutant complemented lines, and 0.82 to 1.08 mm in the atagp18-1 and atagp18-2 mutant complemented lines, respectively (Figure 2.11 and 2.12).

Using a light microscope, the morphology of the fiber cells was also probed in Arabidopsis using homozygous T-DNA insertion mutant lines and RNAi-AtAGP18 lines using stem sections taken from the base of plants. The interfascicular fibers in the atagp18-1 and atagp18-2 mutant lines were oval-shaped, rather than round as in wild type, and the secondary cell walls appeared thinner (Figure 2.15). When both PtAGP18 and AtAGP18 were expressed under the control of the AtAGP18 promoter in the atagp18-1 and atagp18-2 mutant lines, the shape of interfascicular fibers reverted to the round shape common to wild type (Figure 2.16 and 2.17). Therefore, the cell aspect ratio (i.e. the ratio of width:height of the fiber lumen) was quantified. In each of the mutant lines, the cell aspect ratio was decreased from 0.83 to 0.62, and in the complemented lines, the round interfascicular cell shape was restored to 1.10 (Figure 2.18). Similar interfascicular fiber phenotypes were apparent in stem cross sections of RNAi-AtAGP18 lines (Figure 2.19). This implies that AtAGP18 and PtAGP18 could be involved in establishing fiber cell shape.
Table 2.2: List of mutant alleles and primer sequences used in the present study.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Alleles</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
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<td></td>
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<tr>
<td></td>
<td>Left border primer of the T-DNA insertion</td>
<td>Lb1.3 ATTTTGCAGTTTCGAAC</td>
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A

Genomic DNA

T-DNA

wt 18--1 18--2

B

Fiber length (mm)

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**Figure 2.11:** Fiber length of Arabidopsis *atagp18* mutants assessed by fiber quality analyzer. A. Genotyping of the homozygous *atagp18*-1 and *atagp18*-2 lines. The bands in upper and lower panels were produced with genomic DNA by using *AGP18* specific primers and the combination of T-DNA specific primer and gene primer, respectively. B. Arabidopsis *atagp18* mutants have shorter fibers and complementation lines rescue the shorter fiber phenotype. For each plant line, three biological replicates and 10,000 fibers for each replicate were measured by fiber quality analyzer. Asterisks indicate a statistically significant difference at $P < 0.05$ as evaluated by the Student’s t-test. Bars show the standard error of the mean (n = 3). In 18-1aa/ap, data are shown for complementation lines produced by introducing *AtAGP18* and *PtAGP18* into *atagp18*-1 mutants driven by the *AtAGP18* promoter in each case. In 18-2aa/ap, data are shown for complementation lines produced by introducing *AtAGP18* and *PtAGP18* into *atagp18*-2 mutants driven by the *AtAGP18* promoter in each case.
**Figure 2.12:** Fiber length of Arabidopsis atagp18 mutants assessed by bright-field microscopy. Fibers were digested with hydrogen peroxide and acetic acid, and stained with toluidine blue O. For each line, three biological replicates and average 215 fibers were measured by Image J software on light micrograph images. Bars show the standard error of the mean. A significant difference at P < 0.05 was evaluated by ANOVA with Tukey’s post-hoc test. Different letters on two or more bars indicate significant differences between genotypes. In 18-1aa/ap, data are shown for complementation lines produced by introducing AtAGP18 and PtAGP18 into atagp18-1 mutants driven by AtAGP18 promoter in each case. In 18-2aa/ap, data are shown for complementation lines produced by introducing AtAGP18 and PtAGP18 into atagp18-2 mutants driven by the AtAGP18 promoter in each case.
Figure 2.13: Fiber length of Arabidopsis RNAi-AtAGP18 transgenic plants assessed by fiber quality analyzer. For each plant line, three biological replicates and 10,000 fibers for each replicate were measured by fiber quality analyzer. Asterisks indicate a statistically significant difference compared to wild type at $P < 0.05$ as evaluated by the Student’s t-test. Bars show the standard error of the mean ($n = 3$).

Figure 2.14: Fiber length of Arabidopsis RNAi-AtAGP18 transgenic plants assessed by bright-field microscopy. Fibers were digested with hydrogen peroxide and acetic acid and stained with toluidine blue O. For each line, three biological replicates and 30 fibers were measured by Image J software on light micrograph images. Bars show the standard error of the mean. Asterisks indicate a significant difference compared to wild type at $P < 0.05$ evaluated by the Student’s t-test.
Figure 2.15: Interfascicular fiber cell morphology in Arabidopsis atagp18 mutants. A-C. Cross sections of the lower 1 cm of Arabidopsis stem stained with toluidine blue O. A. Wild type. B. atagp18-1 mutation results in fibers whose shape was altered from round to oval and secondary cell wall appears thinner. C. atagp18-2 mutation results in fibers whose shape was altered from round to oval and secondary cell wall appears thinner. The interfascicular fibers are indicated by arrows. Bar = 0.05 mm.

Figure 2.16: Interfascicular fiber cell morphology in Arabidopsis atagp18-1 mutants and complementation lines. A. atagp18-1 mutation results in fibers (arrow) whose shape is altered and thinner fiber secondary cell wall, as seen in cross sections of Arabidopsis stems stained with toluidine blue O. Fiber roundness (arrow) and secondary cell wall thickness were separately restored by transformation of AtAGP18 into the atagp18-1 mutant, driven by the AtAGP18 promoter (B), and by transformation of PtAGP18 into the atagp18-1 mutant, driven by the AtAGP18 promoter (C). The interfascicular fibers are indicated by arrows. Bar = 0.05 mm.
Figure 2.17: Interfascicular fiber cell morphology in Arabidopsis *atagp18*-2 mutants and complementation lines. A. *atagp18*-2 mutation results in fibers whose shape is altered and thinner fiber secondary cell wall. Fiber roundness (arrow) and secondary cell wall thickness were separately restored by transformation of *AtAGP18* into the *atagp18*-2 mutant, driven by the *AtAGP18* promoter (B), and by transformation of *PtAGP18* into the *atagp18*-2 mutant, driven by the *AtAGP18* promoter (C). The interfascicular fibers are indicated by arrows. Bar = 0.05 mm.
Figure 2.18: Cell shape (cell aspect ratio) of *atagp18* mutants and complementation lines. Fiber cell shape was measured by Image J software from light micrograph images captured at the stem bases. Approximately 125 fiber cells from five independent plants were measured by Image J software on light micrograph images for quantifying the cell aspect ratio (width:height of the fiber cross section). Bars show the standard error of the mean. A significant difference at $P < 0.05$ was evaluated by ANOVA with Tukey's post-hoc test. Different letters on two or more bars indicate significant differences among genotypes. In 18-1aa/ap, data are shown for complementation lines produced by introducing *AtAGP18* and *PtAGP18* into *atagp18*-1 mutants driven by *AtAGP18* promoter in each case. In 18-2aa/ap, data are shown for complementation lines produced by introducing *AtAGP18* and *PtAGP18* into *atagp18*-2 mutants driven by the *AtAGP18* promoter in each case.
Figure 2.19: Cell shape (cell aspect ratio) of Arabidopsis RNAi-AtAGP18 transgenic lines. Cell aspect ratio was measured by Image J software from light micrograph images captured at the stem bases. Bars show the standard error of the mean (n = 50) (from three independent plants). Asterisks indicate a statistically significant difference compared to wild type at P < 0.05 evaluated by the Student’s t-test.

2.2.6 The role of AGP18 in cell expansion in the hypocotyl

Based on the fiber phenotype of atagp18 mutants, AGP18 appears to be required to form longer fibers and is required for the normal cell shape of fibers. We hypothesized that AGP18 may be involved in cell expansion, for both intrusive growth and directional cell expansion contribute to generating the final cell shape. To investigate the function of AGP18 in cell expansion, dark-grown hypocotyls were used which have been previously shown to maintain constant rates of cell expansion, and cell division has no significant effects during growth period (Gendreau et al., 1997). atagp18 T-DNA mutants had shorter hypocotyls compared with wild type after four days growth (Figure 2.20). Again, the complemented lines showed hypocotyl lengths similar to wild-type plants (Figure 2.20). The RNAi-AtAGP18 lines also had shorter hypocotyls after four days growth (Figure 2.21). This evidence supports a general role for AGP18 in a cell expansion process.
Figure 2.20: Hypocotyl length of atagp18 mutants and complementation lines after cultivation for four days in the dark. The growth of hypocotyl was captured by camera, and approximately 25 plants were measured by Image J software. Bars show the standard error of the mean. The quantitative differences are shown to be statistically significant (P < 0.05) evaluated by the ANOVA with Tukey’s post-hoc testing. Different letters on two or more bars indicate significant differences among genotypes. In 18-1aa/ap, data are shown for complementation lines produced by introducing AtAGP18 and PtAGP18 into atagp18-1 mutants driven by AtAGP18 promoter in each case. In 18-2aa/ap, data are shown for complementation lines produced by introducing AtAGP18 and PtAGP18 into atagp18-2 mutants driven by the AtAGP18 promoter in each case.
Figure 2.21: Hypocotyl length of Arabidopsis RNAi-AtAGP18 transgenic plants after cultivation for four days in the dark. The growth of hypocotyl was captured by camera and a total of 30 plants were measured by Image J software. Bars show the standard error of the mean. Asterisks indicate a statistically significant difference compared to wild type at P < 0.05 evaluated by the Student’s t-test.

2.2.7 The role of AGP18 in cell wall development in Arabidopsis and poplar

In Arabidopsis I also quantified the interfascicular fiber secondary cell wall thickness by measuring the fiber double cell wall thickness, i.e. from the edge of the lumen of one cell to the edge of the lumen of the adjacent cell. In interfascicular fibers of atagp18 mutants, double cell walls were thinner than wild type (Figure 2.22). When AtAGP18 and PtAGP18 were introduced into atagp18 mutants, the interfascicular fiber double cell wall thickness phenotype was rescued (Figure 2.22). Furthermore, Arabidopsis RNAi-AtAGP18 lines also had thinner interfascicular fiber cell walls relative to wild type (Figure 2.23). In a parallel study, RNAi-PtAGP18 poplar lines were shown to have reduced double cell wall thickness in secondary xylem fiber cells compared with wild-type trees (Figure 2.24). The overexpression of AtAGP18 driven by duplicated-enhancer cauliflower mosaic virus 35S (2×35S) promoter in poplar
resulted in increased double cell wall thickness in secondary xylem fibers compared with wild type (Figure 2.25). These results suggest that AGP18 also affects cell wall development in poplar.

The effect of AGP18 mis-regulation on cell wall chemistry in poplar and Arabidopsis was assessed according to Cullis et al. (2004). In RNAi-PtAGP18 poplar lines, cell wall carbohydrates were not significantly affected, except for a minor increase in galactose content (Table 2.3). In contrast, overexpression of AtAGP18 in poplar led to significantly increased xylose content (Table 2.4). There were no significant changes in holocellulose or α-cellulose content in either the RNAi-PtAGP18 poplar lines or the AtAGP18 overexpressing poplar lines (Figure 2.26-2.29). However, significantly different lignin contents were apparent in the transgenic poplar trees when compared to wild type. Specifically, five RNAi-PtAGP18 lines were shown to be significantly reduced in acid insoluble lignin content (Table 2.3), while the overexpression of AtAGP18 in poplar increased the acid insoluble lignin content (Table 2.4).

Both in nine-week old Arabidopsis atagp18 T-DNA mutants and the RNAi-AtAGP18 lines, no significant changes in cell wall carbohydrates were apparent compared with wild type, while again the lignin was significantly reduced (Table 2.5 and 2.6). Overexpressing PtAGP18 in Arabidopsis resulted in increased lignin in cell walls (Table 2.7), consistent with the findings in poplar.
Figure 2.22: Fiber cell wall thickness of atagp18 mutant lines and complementation lines. Cross sections of the lower 1 cm of Arabidopsis stem were stained with toluidine blue O, light micrograph images captured at the stem bases from five independent plants, and the double cell wall thickness from average 176 fiber cells was measured using Image J software. Bars show the standard error of the mean. The quantitative differences are shown to be statistically significant (P < 0.05) when evaluated by the ANOVA with Tukey’s post-hoc testing. Different letters on two or more bars indicate significant differences among genotypes. In 18-1aa/ap, data are shown for complementation lines produced by introducing AtAGP18 and PtAGP18 into atagp18-1 mutants driven by AtAGP18 promoter in each case. In 18-2aa/ap, data are shown for complementation lines produced by introducing AtAGP18 and PtAGP18 into atagp18-2 mutants driven by the AtAGP18 promoter in each case.
Figure 2.23: Fiber cell wall thickness of RNAi-AtAGP18 Arabidopsis lines. Cross sections of the lower 1 cm of Arabidopsis stem were stained with toluidine blue O. Approximately 100 cells (from three independent plants) were measured by Image J software from light micrograph images captured at the stem bases. Bars show the standard error of the mean. Asterisks indicate a statistically significant difference compared to wild type at $P < 0.05$ evaluated by the Student’s t-test.
Figure 2.24: Fiber cell wall thickness of RNAi-PtAGP18 poplar lines. Cross sections of the base of poplar stem were stained with toluidine blue O. Approximately 120 cells from three independent plants were chosen and four sides of each cell were measured by Image J software from light micrograph images captured at the stem bases. Bars show the standard error of the mean. Asterisks indicate a statistically significant difference compared to wild type at P < 0.05 evaluated by the Student’s t-test.

Figure 2.25: Fiber cell wall thickness of AtAGP18 overexpressing poplar lines. Cross sections of the base of poplar stem were stained with toluidine blue O. Approximately 150 cells from three independent plants were chosen by measuring the double wall on four sides of each cell by Image J software from light micrograph images captured at the stem bases. Bars show the standard error of the mean. Asterisks indicate a statistically significant difference compared to wild type at P < 0.05 evaluated by the Student’s t-test.
**Table 2.3**: Cell wall chemistry of *RNAi-PtAGP18* poplar lines. Bolded numbers indicate significant differences compared to the wild-type trees. Standard error of the mean is shown in parentheses. Data presented are the average of three biological replicates and each biological replicate contained three technical replicates. Student’s t-test, *P* < 0.05.

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<th>Lines</th>
<th>Arabinose (ug/mg)</th>
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<th>Galactose (ug/mg)</th>
<th>Glucose (ug/mg)</th>
<th>Xylose (ug/mg)</th>
<th>Mannose (ug/mg)</th>
<th>Lignin (mg/100mg)</th>
<th>Insoluble lignin (mg/100mg)</th>
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**Table 2.4**: Cell wall chemistry of *AtAGP18* overexpressing poplar lines. Bolded numbers indicate significant differences compared to the wild-type trees. Standard error of the mean is shown in parentheses. Data presented are the average of three biological replicates. Student’s t-test, *P* < 0.05.

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Figure 2.26: Holocellulose content of RNAi-PtAGP18 poplar lines. Measurements were based on the dry weight of cell wall materials. Bars show the standard error of the mean from three independent plants. Significant differences at P < 0.05 were evaluated by the Student’s t-test. No significant differences from wild type were detected.

Figure 2.27: α-Cellulose content of RNAi-PtAGP18 poplar lines. Measurements were based on the dry weight of cell wall materials. Bars show the standard error of the mean from three independent plants. Significant differences at P < 0.05 were evaluated by the Student’s t-test. No significant differences from wild type were detected.
**Figure 2.28:** Holocellulose content of *AtAGP18* overexpressing poplar lines. Measurements were based on the dry weight of cell wall materials. Bars show the standard error of the mean from three independent plants. Significant differences at P < 0.05 were evaluated by the Student’s t-test. No significant differences from wild type were detected.

**Figure 2.29:** α-Cellulose content of *AtAGP18* overexpressing poplar lines. Measurements were based on the dry weight of cell wall materials. Bars show the standard error of the mean from three independent plants. Significant differences at P < 0.05 were evaluated by the Student’s t-test. No significant differences from wild type were detected.
Table 2.5: Cell wall chemistry of atagp18 mutants and complemented lines in Arabidopsis. Bolded numbers indicate significant differences. Standard error of the mean is shown in parentheses. Each mutant was compared to wild type and each complemented line was compared to its corresponding mutant. Data presented are the three biological replicates. Student’s t-test, P < 0.05. In 18-1aa/ap, data are shown for complementation lines produced by introducing AtAGP18 and PtAGP18 into atagp18-1 mutants driven by AtAGP18 promoter in each case. In 18-2aa/ap, data are shown for complementation lines produced by introducing AtAGP18 and PtAGP18 into atagp18-2 mutants driven by the AtAGP18 promoter in each case.

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Table 2.6: Cell wall chemistry of RNAi-AtAGP18 Arabidopsis lines. Bolded numbers indicate significant differences. Standard error of the mean is shown in parentheses. Data showed as the average of three biological replicates. Student’s t-test, P < 0.05.

<table>
<thead>
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<th>Lines</th>
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<th>Rhamnose (ug/mg)</th>
<th>Galactose (ug/mg)</th>
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</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>9.59 (0.22)</td>
<td>7.29 (0.19)</td>
<td>17.87 (0.4)</td>
<td>290.90 (6.27)</td>
<td>92.62 (0.98)</td>
<td>17.24 (0.2)</td>
<td>22.38 (0.17)</td>
<td>17.92 (0.21)</td>
<td>4.46 (0.05)</td>
</tr>
<tr>
<td>2</td>
<td><strong>8.66</strong> (0.06)</td>
<td>7.4 (0.11)</td>
<td>17.69 (0.14)</td>
<td>295.97 (1.28)</td>
<td><strong>89.46</strong> (0.39)</td>
<td><strong>14.76</strong> (0.65)</td>
<td><strong>21.37</strong> (0.28)</td>
<td><strong>16.94</strong> (0.3)</td>
<td>4.47 (0.03)</td>
</tr>
<tr>
<td>4</td>
<td>10.27 (0.17)</td>
<td><strong>7.92</strong> (0.1)</td>
<td>17.65 (0.08)</td>
<td>291.24 (2.05)</td>
<td>95.57 (2.29)</td>
<td>15.1 (1.43)</td>
<td>21.24 (1.12)</td>
<td>17.35 (1)</td>
<td><strong>3.87</strong> (0.2)</td>
</tr>
</tbody>
</table>

Table 2.7: Cell wall chemistry of Arabidopsis plants expressing PtAGP18. Bolded numbers indicate significant differences. Standard error of the mean is shown in parentheses. Data showed as the average of three biological replicates. Student’s t-test, P < 0.05.

<table>
<thead>
<tr>
<th>Lines</th>
<th>Arabinose (ug/mg)</th>
<th>Rhamnose (ug/mg)</th>
<th>Galactose (ug/mg)</th>
<th>Glucose (ug/mg)</th>
<th>Xylose (ug/mg)</th>
<th>Mannose (ug/mg)</th>
<th>Lignin (mg/100mg)</th>
<th>Insoluble lignin (mg/100mg)</th>
<th>Soluble lignin (mg/100mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>9.63 (0.07)</td>
<td>7.17 (0.13)</td>
<td>16.3 (0.23)</td>
<td>289.74 (1.56)</td>
<td>82.09 (1.08)</td>
<td>6.74 (1.2)</td>
<td>20.83 (0.23)</td>
<td>15.82 (0.27)</td>
<td>5.01 (0.07)</td>
</tr>
<tr>
<td>1</td>
<td>10.34 (0.23)</td>
<td><strong>8.08</strong> (0.19)</td>
<td>17.07 (0.4)</td>
<td>294.75 (2.47)</td>
<td>84.8 (0.76)</td>
<td>8.40 (0.3)</td>
<td><strong>21.61</strong> (0.15)</td>
<td>16.47 (0.15)</td>
<td>5.14 (0.01)</td>
</tr>
<tr>
<td>5</td>
<td><strong>8.83</strong> (0.03)</td>
<td>7.26 (0.04)</td>
<td>16.19 (0.14)</td>
<td><strong>279.67</strong> (1.34)</td>
<td><strong>78.24</strong> (0.74)</td>
<td>6.68 (0.58)</td>
<td><strong>21.91</strong> (0.08)</td>
<td>16.2 (0.14)</td>
<td><strong>5.71</strong> (0.06)</td>
</tr>
<tr>
<td>6</td>
<td><strong>8.13</strong> (0.07)</td>
<td>6.8 (0.14)</td>
<td>16.11 (0.31)</td>
<td><strong>266.66</strong> (1.76)</td>
<td><strong>72.45</strong> (1.31)</td>
<td>5.96 (0.29)</td>
<td>21.76 (0.4)</td>
<td>15.3 (0.34)</td>
<td><strong>6.46</strong> (0.07)</td>
</tr>
<tr>
<td>7</td>
<td>9.44 (0.05)</td>
<td>6.82 (0.07)</td>
<td>16.63 (0.08)</td>
<td>287.65 (1.16)</td>
<td>80.97 (0.73)</td>
<td>7.38 (0.51)</td>
<td><strong>22.59</strong> (0.33)</td>
<td><strong>16.89</strong> (0.14)</td>
<td><strong>5.69</strong> (0.26)</td>
</tr>
</tbody>
</table>

2.2.8 The role of PtAGP18 in wood properties

AGP18 appears to be required for fiber cell wall development in poplar, thus the wood properties of poplar lines with mis-regulated AGP18 were investigated. The results showed that RNAi-PtAGP18 poplar lines were not significantly different in average wood density (Figure 2.30) or microfibril angle, relative to the wild-type trees (Figure 2.31).
Figure 2.30: Wood density of RNAi-PtAGP18 poplar lines. Bars show the standard error of the mean from three independent plants. A significant difference at $P < 0.05$ was evaluated by the Student’s t-test. No significant differences from wild type were detected.

Figure 2.31: Microfibril angles of RNAi-PtAGP18 poplar lines. Asterisk indicates a significant difference compared to wild type at $P < 0.05$ evaluated by the Student’s t-test. Bars show the standard error of the mean ($n = 3$).
2.3 Discussion

2.3.1 AGP18 is expressed in developing xylem of poplar

The expression pattern of AGPs was examined in developing xylem of poplar with JIM13 antibody and β-glucosyl Yariv reagents. Both labelling techniques showed similar findings, confirming that AGPs are abundant in the developing fiber cells. In contrast, developing vessels were only weakly recognized by JIM13 antibody in poplar, while vessels appeared not be labelled by β-glucosyl Yariv reagent. This finding implies that there are distinct AGP patterns. It is known that the β-glucosyl Yariv reagent mainly binds to β-1,3 galactan chains that contain more than five residues (Kitazawa et al., 2013). Given that there is low labelling in the vessels with β-glucosyl Yariv reagent, whereas, AGPs can be detected in developing vessels in Arabidopsis inflorescence stem, such as AGP19 and xylogen (Motose et al., 2004; Yang et al., 2011), the compositions of the chains of functional AGPs in vessels appear to be different from those in fibers, hypothetically containing fewer than five residues in β-1,3-galactan chains.

The expression pattern of PtAGP18, which was assessed by quantitative RT-PCR, showed transcript abundance in the developing xylem, which is consistent with the transcriptome re-sequencing data. A poplar ortholog of AtAGP18 was also detected in developing xylem of hybrid poplar by microarray analysis (Hertzberg et al., 2001). The expression pattern of AGP18 suggests that it may play functional roles in developing xylem of poplar.

2.3.2 AGP18 contributes to fiber development

When AGP18 was down-regulated in both Arabidopsis and poplar, the ensuing fibers were slightly shorter, implicating AGP18 in fiber cell development. Moreover, the Arabidopsis interfascicular fibers were oval, rather than round, in shape. AGP18 may affect the fiber cell shape by influencing cell expansion. Cell expansion is a complex process that involves the balancing of wall hydration, wall extension, mechano-sensing and cross-linking with the wall matrix, and wall deposition (Wolf et al., 2012). In Arabidopsis, the AtAGP18 promoter::GUS assay showed that AtAGP18 promoter activity was highest in the elongation zone of the hypocotyl when plants were grown in the dark for three and five
days (Yang and Showalter, 2007a), a period when the cell expansion process is most active (Gendreau et al., 1997). Commensurately, our results showed that the length of the hypocotyl was shorter in atagp18 mutants compared to wild-type plants when plants were grown in the dark for four days. This supports the idea that AGP18 may promote a cell expansion process, manifesting in the observed altered fiber phenotype in loss-of-function plants. The specific function of AGP18 in cell expansion is, however, not clear.

The AGP18 protein backbone is rich in Hyp-glycosylation sites, with approximately 70% of the hydroxyl-polysaccharide predicted to be decorated (Table 2.1). The hydroxyl-polysaccharide side-chains are hypothesized to be the main locations for glucuronic acid residues, which can potentially bind to calcium ion (Ca\(^{2+}\)) (Lamport and Varnai, 2013; Pickard, 2013). Therefore, according to Lamport and Varnai (2013), AGPs may act as a calcium capacitor, and as such affect calcium signalling to regulate the cell shape. Calcium ion is proposed to play a role in mechano-sensing (Monshausen et al., 2011). In tobacco BY-2 cells, AGPs have been proposed to regulate the calcium ion channels because of the detected elevation of intracellular calcium ion contents after β-glucosyl Yariv reagent binding with AGPs (Pickard and Fujiki, 2005). Zhang et al. (2011) also hypothesized that AtAGP18 may bind to the ion channels via side-chains (Zhang et al., 2011b). One hypothesized mechanism for AGP18 in cell expansion processes is to modulate extracellular calcium ion concentrations and calcium ion channel activities to influence intracellular calcium-mediated signalling.

### 2.3.3 AGP18 has functions in secondary cell wall formation

AGP18 not only has roles in fiber cell expansion, but also in fiber secondary cell wall development. Biochemically, cellulose and hemicellulose contents were not impacted when AGP18 was misregulated, while lignin contents were significantly affected. In poplar, the RNAi-PtAGP18 lines displayed thinner fiber cell walls (Figure 2.24), and the total lignin content was significantly reduced (Table 2.3). When AtAGP18 was overexpressed in poplar, thicker fiber secondary cell wall and increased lignin contents were detected (Figure 2.25 and Table 2.4). The effect of AGP18 in secondary cell wall deposition may
be impacted by abnormal cell expansion. As outlined below, previous research has indicated that there are links between cell expansion, secondary cell wall deposition, and the lignification process.

Previously, AGPs were detected with the MAC207 antibody in the secondary cell wall layer (S1 and S2) of *P. radiata* xylem (Altaner et al., 2010). A possible mechanism by which *AGP18* may directly affect secondary cell wall thickening is by acting as a cross-link between cell wall carbohydrates and the lignin. Sibout et al. (2005) also showed that the expression level of *AtAGP18* was reduced four-fold when the lignin contents were significantly reduced in Arabidopsis mutant stems by down-regulation of cinnamyl alcohol dehydrogenase (CAD), a gene integral to cell wall lignification (Sibout et al., 2005). Alternatively, the side-chain carbohydrates may cross-link with calcium ion to affect the lignification process (Eklund and Eliasson, 1990).

Based on current results, it is still not clear how *AGP18* functions in cell wall deposition. AGPs alter cell wall thickness, which may be a consequence of cross-linking of cell wall components, such as pectin and xylan to fortify the cell wall matrix. The carbohydrate components of AGPs are mainly type II arabinogalactan, which have been reported to cross-link with β-1,4-xylan backbone by O-ether linkages (Kwan and Morvan, 1995). A recent study in Arabidopsis demonstrated that one classical AGP (At3g45230) cross-links with pectin and xylan (Tan et al., 2013): in the apap1 T-DNA insertion mutant, cross-linking carbohydrates such as homogalacturonan (HG), rhamnogalacturonan I (RG-I) and xylan were easily extracted, owing to lack of the attachment sites to the *apap1* carbohydrate chain. There were no morphological phenotypes during plant growth, and the total amount of cell wall materials was not significantly different in mutants compared with wild-type plants (Tan et al., 2013). Based on this evidence it is possible that the expression of *AGP18* in plants impacts the cross-linking with cell wall components. Down-regulation the expression level of *AGP18* in transgenic plants may disrupt the potential ability of AGP18 to cross-link with other cell wall components.

### 2.3.4 *AGP18* in wood properties

My results did not show any correlation between the wood density and fiber double cell wall thickness, but wood density is not only affected by fiber cell wall thickness, but also by fiber cell size. It has been
demonstrated that wood density is positively related to the ratio of fiber cell wall and fiber cell lumen (Makinen et al., 2002). Additionally, the other cell types like vessels, rays or parenchyma cells in xylem may also affect the density of wood.

Microfibril angle in RNAi-PtAGP18 poplar lines also showed no significant differences compared to wild-type trees. Microfibril angle mainly reflects the angle of cellulose microfibrils in the thickest layer of the secondary cell wall (S2) (Barnett and Bonham, 2004). However, the S1 and the S3 layers of secondary cell wall also play important roles in wall thickening and microfibril angels (Bergander and Salmen, 2002). Furthermore, AGPs can be detected in the S1 layer by MAC207 immuno-probing in P. radiata normal wood (Altaner et al., 2010).

In summary, this research primarily focuses on the roles of AGP18 in fiber cell development in poplar and Arabidopsis, and demonstrates that AGP18 is involved in cell morphogenesis and secondary cell wall deposition. Since the main mechanism related to these functions is unknown, future work may need to explore the possible roles of AGP18 in modulating the cytoskeleton array and in the cross-linking of AGP18 carbohydrate side-chains with the cell wall matrix.

2.4 Materials and methods

2.4.1 Plant materials
Arabidopsis thaliana Columbia-0 (Arabidopsis) plants were grown for nine weeks under 17 hours light / 7 hours dark cycle. At maturity, the lower 1 cm of the stem from each homozygous mutant was used for hand sectioning and microscopic evaluation. P. alba × P. grandidentata (hybrid poplar P39) were grown in the greenhouse, equipped with 400 watt high pressure sodium lamps that were used as supplemental lighting from 6:00 am until 10:00 pm (16 hours). The temperature in the greenhouse ranged from 20 ºC to a maximum of 24 ºC, while the humidity ranged from 35-45%. The trees were grown in perennial potting mix composed of 50% peat moss, 25% fine bark, and 25% pumice, and fertilized daily during watering while grown on flood tables. RNAi-PtAGP18 poplar lines were grown between July and November 2011,
while the AtAGP18 overexpressing poplar lines were grown from September to February 2011. Following 4 and 5-months of growth, respectively, the transgenic and paired wild-type trees were harvested. The bottom 10 cm of the stems was used for fiber quality and wood chemical analysis. Fresh wood was used for histological analysis. For each transgenic line and the pair controls, three trees were used to evaluate each phenotype. The poplar tissues used for PtAGP18 transcript expression level analysis were collected by Dr. T. Canam et al. (2008).

2.4.2 DNA and RNA extraction

Genomic DNA was isolated with CTAB (Sigma-Aldrich Co.). Plant tissue was first ground in liquid nitrogen and then combined with 1.0 mL of CTAB buffer (2% (w/v) CTAB (Sigma), 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl, 1% PVP, β-mercaptoethanol, pH 8.0) and mixed with a pestle to fully suspend the tissue in solution. The reactions were incubated for 30-60 minutes at 65 °C. The tubes were centrifuged at 13,000 g for 10 minutes and the supernatant was transferred to a fresh sterile 2.0 mL microtube. One volume of phenol:chloroform:isoamyl alcohol was added to each microtube, mixed thoroughly and finally centrifuged at 13,000 g for 10 minutes. The clear supernatant was transferred to a fresh 1.5 mL tube, and a 1/2 volume of isopropanol was added to each tube. Microtubes were centrifuged at 13,000 g for 10 minutes and washed with 70% ethanol. The pellet was suspended in sterile nanopure water. To screen Arabidopsis homozygous mutants, the quick DNA extraction method was employed (Edwards et al., 1991).

Total RNA was extracted with TRIZOL Reagent (Invitrogen) kit, as per the manufacturer’s instruction, treated with DNase and transcribed into first strand cDNA with SuperScript II reverse transcriptase (Invitrogen).

2.4.3 RT-PCR, semi-quantitative RT-PCR, and quantitative real-time RT-PCR analysis

Reverse transcription polymerase chain reaction (RT-PCR) was performed with cDNA samples isolated from different Arabidopsis and poplar tissues. Semi-quantitative RT-PCR was used to measure the expression levels of target mRNAs from each sample. Elongation factor AtELF5A (AT5G60390) was used as a control to standardize the sample variations in total RNA amounts (Czechowski et al., 2005).
Quantitative real-time RT-PCR (Q-RT-PCR) analysis was used to examine the transcript abundance of *AGP18* in poplar and Arabidopsis tissues using the SYBR Green JumpStart Taq Ready Mix (Sigma-Aldrich), using actin and elongation factor *AtELF5A* as housekeeping genes (Czechowski et al., 2005; Regier and Frey, 2010). Three biological replicates were assayed for each sample. The comparative threshold cycle method was used to quantify mRNA content (Livak and Schmittgen, 2001). The transcript abundance of *PtAGP18* and *AtAGP18* in different tissues was normalized to the actin and *AtELF5A* transcript level, respectively. The primer sequences used are described in Table 2.8.

**Table 2.8**: PCR primer sequences used for *AGP18* gene isolation, cloning, screening, and Q-RT-PCR.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5’ to 3’)</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>AtELF5A</em> FW</td>
<td>TTGCCACACCTCTACACATTTGCAATGCA</td>
<td>Q-RT-PCR</td>
</tr>
<tr>
<td><em>AtELF5A</em> RV</td>
<td>TGTCCTCCTACAGCGAACGGTCTCC</td>
<td>Q-RT-PCR</td>
</tr>
<tr>
<td><em>PiAGP18</em> FW</td>
<td>TTTGTTGGACACTGATTGTC</td>
<td>Q-RT-PCR</td>
</tr>
<tr>
<td><em>PiAGP18</em> RV</td>
<td>TACGGAAAGATCTGTGAGA</td>
<td>Q-RT-PCR</td>
</tr>
<tr>
<td><em>AtAGP18</em> FW</td>
<td>CCAAGCAAGCATAAGAAGATCTAC</td>
<td>Q-RT-PCR</td>
</tr>
<tr>
<td><em>AtAGP18</em> RV</td>
<td>CCGCTACCTCCTCACACC</td>
<td>Q-RT-PCR</td>
</tr>
<tr>
<td>Poplar actin FW</td>
<td>TGCTGAGCGATTTGGGTAC</td>
<td>Q-RT-PCR</td>
</tr>
<tr>
<td>Poplar actin RV</td>
<td>GGGCTAGTGCTAGAGATTTCC</td>
<td>Q-RT-PCR</td>
</tr>
<tr>
<td><em>AtAGP18</em> mutant FW</td>
<td>AGCAACGATGAAAGACTAC</td>
<td>RT-PCR</td>
</tr>
<tr>
<td><em>AtAGP18</em> mutant RV</td>
<td>GCTACATTTCTCACAC</td>
<td>RT-PCR</td>
</tr>
<tr>
<td><em>PiAGP18</em> FW</td>
<td>CACCATGGATCGGAAATGCTCCTGTCTTTG</td>
<td>cloning</td>
</tr>
<tr>
<td><em>PiAGP18</em> RV</td>
<td>CTAGAGATCAAGAAAGAAGCAG</td>
<td>cloning</td>
</tr>
<tr>
<td><em>AtAGP18</em> FW</td>
<td>CACCTTCAGATGGATCGCAATTTGCTCCT</td>
<td>cloning</td>
</tr>
<tr>
<td><em>AtAGP18</em> RV</td>
<td>GTACCATCACCTGACCATAGATAATGATTC</td>
<td>cloning</td>
</tr>
<tr>
<td><em>AtAGP18</em> promoter FW</td>
<td>CTCTGTCTCAACAATGAG</td>
<td>isolation</td>
</tr>
<tr>
<td><em>AtAGP18</em> promoter RV (with ATG)</td>
<td>TTAGGAGGAAATGCGATCC</td>
<td>isolation</td>
</tr>
<tr>
<td><em>AtAGP18</em> promoter FW</td>
<td>AAGCTCTCCTGCTCCTAAGATGAG</td>
<td>cloning</td>
</tr>
<tr>
<td><em>AtAGP18</em> promoter RV</td>
<td>CCAAGCAAGAATTTGCTAAATTTGATCAAA</td>
<td>cloning</td>
</tr>
<tr>
<td>RNAi-<em>AtAGP18</em> FW</td>
<td>GGGGACAAGTTTGTACAAAGGGAGCATGCAATTGTTCCTTCTCC</td>
<td>cloning</td>
</tr>
<tr>
<td><em>RNAl-PiAGP18</em> RV</td>
<td>CACGGGATCGTACATGGAAGGTAAGGGAAGGATGAGAAG</td>
<td>cloning</td>
</tr>
<tr>
<td><em>RNAl-PiAGP18</em> RV</td>
<td>GGCGGATTTCTGTAACAGAAGAGGAGGTAAGG</td>
<td>cloning</td>
</tr>
<tr>
<td><em>RNAl-PiAGP18</em> FW</td>
<td>GGGGACACATTTGTTGTAACAGAGGTTGGTACCTCCAATTCTCCCTTCTCC</td>
<td>cloning</td>
</tr>
<tr>
<td><em>RNAl-PiAGP18</em> FW</td>
<td>GGGGACACATTTGTTGTAACAGAGGTTGGTACCTCCAATTCTCCCTTCTCC</td>
<td>cloning</td>
</tr>
<tr>
<td><em>RNAl-PiAGP18</em> FW</td>
<td>GGGGACACATTTGTTGTAACAGAGGTTGGTACCTCCAATTCTCCCTTCTCC</td>
<td>cloning</td>
</tr>
<tr>
<td><em>RNAl-PiAGP18</em> FW</td>
<td>GGGGACACATTTGTTGTAACAGAGGTTGGTACCTCCAATTCTCCCTTCTCC</td>
<td>cloning</td>
</tr>
<tr>
<td>Kanamycin FW</td>
<td>AGAGGCTTATCGGGCTGAC</td>
<td>screening</td>
</tr>
<tr>
<td>Kanamycin RV</td>
<td>GGAAGTCTCGATAAGAAGG</td>
<td>screening</td>
</tr>
</tbody>
</table>

Footnote: FW: forward primer; RV: reverse primer.
2.4.4 Screening of atagp18 T-DNA insertion mutant homozygous lines

The Arabidopsis atagp18 T-DNA mutant lines were screened for homozygosity using genomic DNA extracted as per Edwards et al (1991). atagp18-1 (SALK_117268) seeds and atagp18-2 (CS321385) seeds were obtained from the Salk Institute Genomic Analysis Laboratory (http://signal.salk.edu/) (Alonso et al., 2003). The primer sequences employed for screening atagp18 T-DNA mutants are listed in Table 2.2, and were designed by T-DNA primer design program (http://signal.salk.edu/tdnaprimers.2.html). Synthesized cDNA was used as a template to test whether or not the expression of AtAGP18 was completely absent in atagp18 homozygous lines. Primer sequences are shown in Table 2.8.

2.4.5 Cloning of the AGP18 gene

Poplar and Arabidopsis tissues were independently used to extract genomic DNA and RNA. Synthesized cDNA from P. alba × P. grandidentata and Arabidopsis thaliana Columbia-0 was used to clone PtAGP18 and AtAGP18 separately by pENTR directional TOPO cloning kit (Invitrogen). The AtAGP18 promoter, which included a segment of DNA 1643 kb upstream of the translational start codon, was amplified from Arabidopsis genomic DNA. The promoter fragment was sequenced after cloning in pDONR201. The upstream ATG was removed in a polymerase chain reaction (PCR) using a reverse primer matching the 5' end of the cDNA. The PCR product was subcloned into pDONR201 and sequenced. The sequences used for cloning the AtAGP18, PtAGP18, and AtAGP18 promoters are listed in Table 2.8.

The poplar and Arabidopsis post-transcriptional gene silencing (RNA-interference) constructs were made using unique 495 and 636 bp coding DNA sequence (CDS) fragments, targeting PtAGP18 and AtAGP18, respectively for each of the poplar and Arabidopsis genes. The representative CDS fragments of PtAGP18 and AtAGP18 were separately cloned as sense and anti-sense fragments into the pHellsigate12 vector containing the CaMV 35S promoter (Helliwell and Waterhouse, 2003). Primer sequences for PtAGP18-RNAi and AtAGP18-RNAi constructs are listed in Table 2.8.
The \textit{PtAGP18} was cloned from synthesized poplar cDNA, while \textit{AtAGP18} was cloned using synthesized Arabidopsis cDNA. The length of each gene was 856 and 978 bp from start codon to 3' UTR, respectively. The \textit{PtAGP18} fragment carrying NcoI and SacI restriction enzyme sites and the \textit{AtAGP18} fragment carrying XbaI and KpnI restriction enzyme sites were separately subcloned into the pSM3 binary vector harbouring the 2×35S promoter (Datla et al., 1993). All primer sequences employed are listed in Table 2.8. For complementation of the \textit{atagp18} mutants, overexpression constructs harbouring \textit{AtAGP18} and \textit{PtAGP18} were used. Target binary vectors were digested by two restriction enzymes HindIII and NcoI to remove the 2×35S promoter. \textit{AtAGP18} promoter carrying HindIII and NcoI sites was inserted into the binary vector.

2.4.6 Bioinformatics analysis of \textit{AtAGP18} and \textit{PtAGP18}

The amino acid identity and similarity of \textit{AtAGP18} (AT4G37450) and \textit{PtAGP18} (Potri.007G051600) were predicted by pairwise alignment of BioEdit sequence alignment editor (http://www.mbio.ncsu.edu/bioedit/bioedit.html). Signal peptides of \textit{PtAGP18} and \textit{AtAGP18} were separately predicted by SignalP 3.0 Server (Petersen et al., 2011). GPI lipid anchor sequences were identified by the big-PI Plant Predictor (Eisenhaber et al., 2003), while the C-terminal hydrophobic domain was predicted by TMPred (http://www.ch.embnet.org/software/TMPRED_form.html).

2.4.7 Plant transformation

Target binary constructs were transformed into \textit{Agrobacterium tumefaciens} GV3101 by the freeze-thaw method (Holsters et al., 1978). Positive clones were incubated in YEP media (5 g/L yeast extract, 10 g/L peptone, 5 g/L NaCl) until an OD$_{600}$ of 0.7 was attained. Arabidopsis plant transformation followed the commonly employed protocol (Bechtold et al., 1993; Clough and Bent, 1998). Positive Arabidopsis transformants were selected by growth on 1/2 Murashige and Skoog medium supplemented with 50 mg/L kanamycin for RNAi lines, and 20 mg/L hygromycin for overexpression lines.

Poplar transformation employed the leaf discs inoculation method (Coleman et al., 2008). Binary constructs carrying the target gene were transformed into \textit{Agrobacterium tumefaciens} EHA105 by the freeze-thaw method (Holsters et al., 1978). Positive clones were selected for incubation in liquid woody
plant media (WPM) overnight (McCown and Lloyd, 1981). Large, fresh leaves from approximately one-month-old poplar were used for transformation. Leaves were cut into discs and cultivated in diluted WPM media containing *Agrobacterium tumefaciens* for 1/2 hour at 28 °C with gentle shaking (100 g). Following incubation, the leaf discs were gently dried with sterilized filter paper to remove the WMP media. The leaves were transferred, abaxial side up, to WPM media supplemented with 0.1 μM of 1-naphthaleneacetic acid (NAA), benzylaminopurine (BA), and thidiazuron (TDZ). After two days, the leaf discs were moved to WPM media with 0.1 μM of NAA, BA, TDZ, 250 mg/L cefotaxine, 500 mg/L carbenicillin, and placed in the dark for three days to kill the *Agrobacterium tumefaciens*. The leaf discs successfully transfected were moved to WPM media with 0.1 μM of NAA, BA, and TDZ, 250 mg/L cefotaxine, 500 mg/L carbenicillin, 50 mg/L kanamycin or 25 mg/L hygromycin, and the plates were placed in subdued light for selecting positive shoots. After the shoots elongated, they were transferred to WPM media with 0.1 μM of NAA, 250 mg/L cefotaxine, 500 mg/L carbenicillin, 50 mg/mL kanamycin or 25 mg/mL hygromycin for rooting. Positive lines were identified by genomic DNA screening, sub-cultured and multiplied. Positive RNAi lines were further confirmed by Q-RT-PCR as per the method described above. Positive Arabidopsis and poplar overexpression lines were separately verified with RT-PCR.

### 2.4.8 Assessment of plant growth

Tree height and caliper at the base of the stem were measured on five trees per construct and the appropriate controls on greenhouse-grown plants.

### 2.4.9 Histology

Nine weeks-old Arabidopsis stems were hand sectioned with a double-sided razor blade. Poplar stems were sectioned with a microtome to a thickness of 64 μm. Fresh sections were stained with either 0.02% aqueous toluidine blue O, or Yariv reagent, and observed via a light microscope (Leica Microsystems Inc., Ontario, Canada). For toluidine blue O staining, 0.02% (w/v) toluidine blue O was used for 5 minutes. For Yariv staining, 2 mg of β-glucosyl Yariv reagent and α-glucosyl Yariv reagent (control) were
separately dissolved in 1 mL of 0.15 M NaCl. The solutions were applied to stem cross sections for 3 hours at room temperature and examined by a light microscope.

2.4.10 Immunofluorescent labeling

Immunofluorescent localization of AGPs with JIM13 antibody (CarboSource, Athens, GA US) was conducted on fresh poplar stem material. 2-3 fresh sections, cut to 64 μm with the microtome, were placed in a microfuge tube and filled with 5% bovine serum albumin (BSA) to block non-specific protein binding. After 20 minutes, diluted primary antibody JIM13 (1:60 dilution in BSA buffer) was added to the sample. A control sample was incubated with 1% BSA. Samples were incubated at 4 °C overnight. Before adding the secondary antibody, the primary antibody solution was removed and samples were washed with 200 μL of tris buffered saline with tween 20 (TBST 1×) solution four times. Diluted secondary antibody anti-rat IgG Alexa Fluor 594 (Invitrogen, USA; 1:200 dilution in PBS buffer) was added to the samples and control. After incubating the samples at 4 °C overnight, antibody tagged sections in TBST 1× solution were viewed with a fluorescence microscope (Leica DMRE, Germany).

2.4.11 Fiber quality analysis

For Arabidopsis fiber length analysis, 5-8 mg stem samples from the base of plants were placed in a 10 ml test tube containing a modified Franklin solution (1:1:1 30% hydrogen peroxide:glacial acetic acid; distilled water) (Franklin, 1945), and incubated at 100 °C for 3 hours. The solution was decanted, the remaining materials were washed and shaken gently by hand until the fibers separated. For poplar fiber length analysis, 200 mg dry wood samples from base of stem were cooked in a modified Franklin solution (1:1 30% hydrogen peroxide: glacial acetic acid) in a 70 °C bath for 48 hours. The materials were washed with distilled water and diluted them into a Falcon Tube. Fibers were separated by blending in a Waring mixer. Diluted fibers were directly analyzed on the fiber quality analyzer (FQA; OpTest Equipment Inc., Hawkesbury, Canada) or observed under microscope after staining with 0.02% w/v toluidine blue O for 5 minutes by loading liquid on a microscopy slide covered by a coverslip.
2.4.12 Cell wall thickness and cell aspect ratio analysis

Poplar stem cross sections (64 µm thickness) and Arabidopsis stem hand sections were used to analyze cell wall phenotype. Stem cross sections were stained with 0.02% w/v toluidine blue O and observed with a light microscope (Leica Microsystems Inc., Ontario, Canada). Double fiber cell wall thickness and interfascicular fiber cell aspect ratio were quantified with Image J software (http://rsb.info.nih.gov/ij/) from at least three replicates for each line. In poplar, gelatinous layers were avoided during cell wall thickness assessment. Measurements were taken across the cell walls of two adjacent fiber cells, which represent one fiber double cell wall thickness; for each cell, four aspects were measured with adjacent cells. Data reported are fiber double cell wall thickness.

2.4.13 Cell wall chemistry

Cell wall carbohydrate compositions were analyzed by Klason as per Cullis et al. (2004). Wood samples were ground to pass a 40-mesh in a Wiley mill and extracted overnight with acetone in a Soxhlet apparatus to remove extractives. 3 mL 72% (w/w) H₂SO₄ was added to 0.2 g samples and allowed to react on the bench for 2 hours with mixing every 10 minutes. The wood hydrolyzate was transferred to septa-sealed serum bottles, to which 112 mL of water was added and autoclaved for 1.5 hours at 121 °C together with the sugar controls. The hydrolyzate was removed and filtered through dry, pre-weighed, medium coarseness sintered-glass crucible to estimate the content of acid-insoluble lignin. Acid-soluble lignin was determined by absorbance at 205 nm on the Bio UV-visible spectrophotometer using quartz cuvettes (Varian, Inc., Australia). Carbohydrates were determined by using a Dx-600 anion exchange high-performance liquid chromatography (Dionex, Sunnyvale, CA, USA).

2.4.14 Holocellulose and alpha-cellulose analysis

Wood samples from the base of poplar stems were ground to pass a 40-mesh in a Wiley mill and extracted overnight with acetone in a Soxhlet apparatus to remove extractives. Extract-free wood samples (195-200 mg) were used for holocellulose quantification. Wood samples were incubated at 50 °C for 14 to 16 hours after adding 3.5 mL buffer (60 mL of glacial acetic acid and 1.3 g NaOH per Liter) and 1.5 mL of 20% sodium chlorite solution. The reaction was quenched by placing it into an ice bath to cool.
After removing the reaction solution by careful pipetting, the procedure was repeated. Finally, the reacted wood meal was transferred to pre-weighed coarse sintered-glass crucible and washed twice with 50 mL of 1% glacial acetic acid and 10 mL acetone. The weight of the crucible was determined after drying, to quantify holocellulose content (Yokoyama et al., 2002).

Approximately 80-100 mg of holocellulose was used for alpha-cellulose isolation. Samples were reacted for 30 minutes after adding 8 mL of 17.5% NaOH. 8 mL of distilled water was added to the samples and stirred for 1 minute. After 29 minutes, the retentate was filtered through a pre-weighed coarse sintered-glass crucible by washing with distilled water. The crucibles were soaked in 1.0 M acetic acid for 5 minutes and washed with distilled water. The dry weight of crucibles was used to quantify the alpha-cellulose contents.

2.4.15 Wood quality

Microfibril angle was assessed by X-ray diffraction using a Bruker D8 Discover (Bruker, Billerica, MA USA). Measurements were taken from three separate samples. Bark to bark samples were first precision cut to 1.68 mm thickness with a twin blade pneumatic circular saw, and extracted overnight in a Soxhlet apparatus with acetone. Sample diffraction patterns were measured with CuKα1 radiation source (λ = 1.54 Å), a 0.5-mm collimator to fit the X-ray source, and GADDS detector. Both the X-ray source and the detector were set to theta = 0°. Microfibril angle was determined by the average T-value of the two 002 diffraction arc peaks according to Megraw et al., (1998).

Wood density was determined by X-ray densitometry (Quintek Measurement System Inc. Knoxville, TN, USA) using both radii (bark to bark) and averaged for each sample.

2.4.16 Statistical analysis

The statistical significance of any difference between experimental results was evaluated either by the Student’s t-test to compare two means or analysis of variance (ANOVA) with Turkey’s post hoc test for multiple comparison among the means (SPSS v11 software, SPSS Inc., Chicago, USA). A value of P < 0.05 was considered statistically significant.
Chapter 3 AGP9 affects xylem development in poplar and Arabidopsis thaliana

3.1 Introduction

In angiosperms, fibers and vessels form the dominant cell types in secondary xylem and play important roles in structure and function. Fibers confer the supportive strength for xylem development, while vessels facilitate water conduction by forming long hollow conduits that are composed of dead lignified cells. Vessel characteristics affect the movement of water, with larger diameter providing higher hydraulic conductance. However, large vessels must balance conductivity with resistance to cavitation which can result in a discontinuous water column (Sperry, 2003). Thinner vessel cell walls are vulnerable to collapse, as witnessed in irregular xylem (irx) mutants of Arabidopsis thaliana with defects in secondary cell wall deposition (Turner and Somerville, 1997; Jones et al., 2001).

Vessels arise from the xylem mother cells in the vascular cambium, and are fully differentiated after cell expansion, cell wall deposition, lignification, and programmed cell death. During vessel cell expansion, the rapid cell enlargement requires vessel cells to generate turgor pressure, and adjust cell wall plasticity (Ray et al., 1972). Vessel cells build the hydraulic system by attracting osmotically active ions such as potassium ions to power cell expansion (Fromm, 2010). After cell expansion, the vessel secondary cell wall is deposited, which is ultimately followed by programmed cell death. Vessels have a shorter life span than fibers or tracheids, which can live for up to one month (Courtois-Moreau et al., 2009; Bollhoner et al., 2012). As a consequence of the numerous cell types in the cambial zone, their distinct cellular activities and short lifespan, the mechanism(s) of vessel differentiation have been difficult to study.

The complexity of xylem vessel development is reflected by the plethora of genes that are up-regulated by the Vascular-Related NAC Domain Protein7 (VND7) and VND6 transcription factors (Kubo et al., 2005; Ohashi-Ito et al., 2010; Yamaguchi et al., 2010b) that are responsible for vessel formation...
during active plant growth. VND7 and VND6 are expressed in the protoxylem and metaxylem vessels, respectively, of A. thaliana (Yamaguchi et al., 2008; Zhong et al., 2008). In addition, genes encoding aquaporins, expansin, pectin methylesterase, and xyloglucan endotransglycosylase can be detected during cambial cell division and expansion (Guglielmino et al., 1997; Schrader et al., 2004). Although some genes responsible for cell wall modification are easily reconciled, there are several arabinogalactan proteins (AGPs) detected during xylem development whose functional roles are yet to be assigned. In maize and cultured Zinnia cells, AGPs have been localized to the xylem elements, using β-glucosyl Yariv reagent (1,3,5-tris (4-β-D-glycopyranosyloxyphenylazo)-2,4,6-trihydroxy-benzene), an AGP cross-linker that stains AGPs red (Yariv et al., 1967; Schopfer, 1991; Motose et al., 2004).

In this study, the functional role of PtAGP9, encoding a poplar classical AGP, and the A. thaliana ortholog AtAGP9 were investigated in xylem development, because of the high transcript abundance of PtAGP9 reported in Populus trichocarpa developing xylem re-sequencing initiatives (Geraldes et al., 2011). These transcriptome data are consistent with the previous results which showed that AGP9 was expressed during cell expansion in developing poplar xylem by microarray analysis (Hertzberg et al., 2001). Furthermore, in developing xylem of loblolly pine, PtX3H6, PtX14A9, and PtaAGP6 were shown to be highly expressed (Loopstra and Sederoff, 1995; Zhang et al., 2003). To investigate the role of AGP9 in xylem development, transgenic poplar trees mis-regulated for AGP9, as well as A. thaliana atagp9 mutant plants, were thoroughly analyzed for the vessel and fiber development.

## 3.2 Results

To characterize the xylem-expressed classical AGPs, PtAGP9 (Potri.009G092300) and its A. thaliana Columbia-0 (Arabidopsis) ortholog AtAGP9 (AT2G14890), each gene was cloned from P. alba × P. grandidentata (hybrid poplar P39) and Arabidopsis, respectively. These genes show 72% amino acid sequence similarity (Figure 3.1). Based on their amino acid composition, PtAGP9 and AtAGP9 are categorized as classical AGPs (Eisenhaber et al., 2003) with an N-terminal signal peptide, potential C-
terminal GPI-modification sites, and a C-terminal GPI-associated hydrophobic domain. The predicted glycosylation pattern of the hydroxyproline (Hyp) residues on PtAGP9 and AtAGP9 predict 22% Hyp-polysaccharide addition sites and approximately 75% Hyp-arabinosylation sites, according to the Hyp-contiguity hypothesis (Shpak et al., 2001; Sun et al., 2005) (Table 2.1).

To elucidate the expression pattern of PtAGP9 in different poplar tissues, quantitative real time PCR (Q-RT-PCR) was employed. PtAGP9 was shown to be highly expressed in the developing xylem, especially in the middle stem (Figure 3.2). These findings are consistent with the expression pattern documented for PtAGP9 on the poplar eFP browser (http://bar.utoronto.ca/efppop/cgi-bin/efpWeb.cgi) (Wilkins et al., 2009).

**Figure 3.1:** Alignment of *P. alba × P. grandidentata* AGP9 (PtAGP9) amino acid sequences with that of *Arabidopsis thaliana* AGP9 (AtAGP9). Identical conserved amino acids are in black shading, non-identical amino acids with similar properties are in grey shading. AtAGP9 has 59% identity and 72% similarity with PtAGP9. Gene identification numbers are: *PtAGP9*, Potri.009G092300; *AtAGP9*, AT2G14890.
Figure 3.2: Relative expression level of \(PtAGP9\) in poplar tissues collected in July 2007. Transcript abundance in each tissue is based on the comparative threshold cycle value relative to the housekeeping gene (actin). Bars show the standard error of the mean \((n = 3)\).

3.2.1 Roles of \(AGP9\) in xylem development in poplar

To explore the functional role(s) of \(AGP9\) in xylem development, transgenic poplar trees with suppressed \(PtAGP9\) were generated by RNAi-mediated suppression of the native gene. Since the introduction of an extra copy of an endogenous gene commonly leads to post-transcriptional gene silencing in plant (Vaucheret et al., 2001), the Arabidopsis ortholog \(AtAGP9\) was overexpressed in poplar, which was driven by the duplicated-enhancer cauliflower mosaic virus 35S promoter \((2\times35S)\). Four \(RNAi-PtAGP9\) transgenic lines were recovered and displayed normal plant growth. However, when the morphology of developing xylem at the base of stem was examined, the \(RNAi-PtAGP9\) poplar lines had smaller, more abundant vessels, and the vessels appeared to group together relative to the vessels in wild-type trees \((P39)\) (Figure 3.3D and E). In Figure 3.3F, the vessels in transgenic line 10 appeared to differentiate earlier compared to wild type, and there appeared to be vessels in the cambial zone (Figure 3.3C). Given the
visual observations, the number and size of vessels were quantified in all lines, with measurements restricted to the first 30 cell files initiating from the vascular cambium. The RNAi-PtAGP9 lines had reduced vessel area from a mean value of 1614 µm² to 1188 µm² and increased vessel numbers from 52 to 66 per area than wild-type trees (Figure 3.4 and 3.5). The five 2×35S::AtAGP9 transgenic poplar lines recovered also had normal plant growth, and the vessels appeared to be larger, fewer, and some showed irregular shape (Figure 3.6). By quantifying the characteristics of the vessels at base of the stem (in cross section), statistically significant differences were observed in the average area occupied by the vessels in three of five transgenic lines overexpressing the Arabidopsis ortholog (Figure 3.7). In those same lines, the number of vessels per area was reduced from a mean value of 52 to a mean value of 42 (Figure 3.8). These findings suggest that AGP9 has an impact on the vessel development in poplar.

Given the vessel phenotype, we also investigated the impact of AGP9 on fiber development in these same transgenic poplar trees. Based on output of the fiber quality analyzer (FQA), both fiber length and fiber width were not significantly different in the RNAi-PtAGP9 (Figure 3.9A and B) or 2×35S::AtAGP9 poplar lines (Figure 3.10A and B) compared to wild-type trees.
Figure 3.3: Vessel morphology of RNAi-PtAGP9 poplar lines. The bases of poplar stems were sectioned, stained with toluidine blue O and observed via a light microscope. Panels A-C. Wild type from three biological replicates. D. RNAi-PtAGP9 line 2. E-F. RNAi-PtAGP9 line 10. Vessels groups are depicted by red circle in (D) and (E). Early initiation of vessel differentiation in cambial zone (F) is indicated by blue arrow compared with wild type in (C). The cambium is indicated by yellow arrows. Wild-type vessel is indicated by black arrow. CA: cambium; V: vessel. Bars = 0.05 mm.
Figure 3.4: Vessel area of RNAi-PtAGP9 poplar lines. The stem base of fresh trees was sectioned and stained with toluidine blue O. For each line, stem cross section images from three biological replicates were captured by microscopy from several aspects of one section (omitting tension wood sections). The size of the vessels was quantified in the first 30 layers of fiber cells from the vascular cambium. Approximately 200 vessels were measured for each line. The significant difference at P < 0.05 was evaluated by the Student’s t-test. Bars show the standard error of the mean. The asterisks indicate a significant difference relative to wild type (wt).
Figure 3.5: Vessel number of RNAi-PtAGP9 poplar lines. The stem base of fresh trees was sectioned and stained with toluidine blue O. For each line, stem cross-section images from three biological replicates were captured by microscope from several aspects of one section (omitting tension wood sections). The vessel numbers per field of view were quantified in the first 30 layers of cells from the vascular cambium in stem cross sections. Around 20 stem cross sections were used for each line. The statistically significant difference at P < 0.05 was evaluated by the Student’s t-test. Bars show the standard error of the mean. The asterisks indicate a significant difference relative to wild type.

![Image of vessel morphology](image)

Figure 3.6: Vessel morphology of AtAGP9 overexpressing poplar lines. The middle stem and stem base of trees were sectioned and stained with toluidine blue O and observed via a light microscope. A. Middle stem cross section of wild type. B-C. Larger, fewer and irregular vessels shown in middle stem cross sections of transgenic poplar line 3 (B) and line 2 (C) compared with wild type (A). D. Base of stem cross section of wild type. E-F. Larger and fewer vessels shown in base of stem cross section of transgenic poplar line 3 (E) and line 2 (F) relative to the wild type (D). Irregular vessel phenotype is indicted by blue arrow in (B) and (C). Cambial zone is indicated by a yellow arrow. Wild-type vessel is indicated by black arrow. V: vessel. Bars = 0.05 mm.
Overexpressed AtAGP9 lines

**Figure 3.7:** Vessel area of AtAGP9 overexpressing poplar lines. The stem base of trees was sectioned and stained with toluidine blue O. Transgenic line 2, 3, 6 and wild type (wt-1) were grown in September 2011. Transgenic line 7, 8 and wild type (wt-2) were grown in February 2012. For each line, stem cross section images from three biological replicates were captured by microscopy from several aspects of one section (excluding tension wood). The size of the vessels was quantified in the first 30 layers of fiber cells from the vascular cambium in stem cross sections. Approximately 300 vessels were measured for each line. The statistically significant difference at $P < 0.05$ was evaluated by the Student’s t-test. Bars show the standard error of the mean. The asterisks indicate a statistically significant difference relative to wild type.
**Figure 3.8:** Vessel number of *AtAGP9* overexpressing poplar lines. The stem base of trees was sectioned and stained with toluidine blue O. Transgenic line 2, 3, 6 and wild type (wt-1) were grown in September 2011. Transgenic line 7, 8 and wild type (wt-2) were grown in February 2012. For each line, stem cross section images from three biological replicates were captured by microscope from several aspects of one section (except tension wood). The vessel number per field of view was quantified in the first 30 layers of fiber cells from the vascular cambium in stem cross sections. Approximately 20 stem cross sections were used for each line. The significant difference at P < 0.05 was evaluated by the Student’s t-test. Bars show the standard error of the mean. The asterisks indicate a statistically significant difference relative to wild type.
Figure 3.9: Fiber length (A) and width (B) of RNAi-PtAGP9 transgenic poplar lines assessed by fiber quality analyzer. For each line, three biological replicates and 10,000 fibers for each replicate were measured per tree by fiber quality analyzer. Bars show the standard error of the mean (n = 3). A significant difference at P < 0.05 was evaluated by the Student’s t-test. No significant differences from wild type were detected.
Figure 3.10: Fiber length (A) and width (B) of AtAGP9 overexpressing poplar lines assessed by fiber quality analyzer. For each line, three biological replicates and 10,000 fibers for each replicate were measured per tree by fiber quality analyzer. Bars show the standard error of the mean (n = 3). A significant difference at P < 0.05 was evaluated by the Student’s t-test. No significant differences from wild type were detected.
3.2.2 AGP9 in cell wall traits in poplar

To examine whether or not the vessel phenotype leads to altered cell wall traits, cell wall carbohydrate and lignin contents were quantified at the base of transgenic stems. In RNAi-PtAGP9 and AtAGP9 overexpressing poplar lines, cell wall lignin content and the total cell wall monomeric carbohydrates were not significantly affected (Table 3.1 and 3.2), this included holocellulose and α-cellulose contents relative to the wild-type trees (Figure 3.11-3.14). These findings imply that the defects in vessel development were not correlated with any obvious changes in cell wall chemistry.

Moreover, wood density was shown not to be significantly changed in the transgenic lines compared to the wild-type trees (Figure 3.15 and 3.16).

Table 3.1: Cell wall chemistry of RNAi-PtAGP9 poplar lines. Data represent the average of three biological replicates. Standard error of the mean is shown in parentheses. Using Student’s t-test, P < 0.05, no significant differences between RNAi-PtAGP9 and wild type were apparent.

<table>
<thead>
<tr>
<th>Lines</th>
<th>Arabinose (µg/mg)</th>
<th>Rhamnose (µg/mg)</th>
<th>Galactose (µg/mg)</th>
<th>Glucose (µg/mg)</th>
<th>Xylose (µg/mg)</th>
<th>Mannose (µg/mg)</th>
<th>Lignin (mg/100mg)</th>
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</thead>
<tbody>
<tr>
<td>wt</td>
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<td>5.24 (0.15)</td>
<td>8.76 (0.91)</td>
<td>513.39 (4.72)</td>
<td>168.29 (2.9)</td>
<td>12.56 (0.89)</td>
<td>20.18 (0.56)</td>
</tr>
<tr>
<td>2</td>
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<td>5.53 (0.18)</td>
<td>8.21 (0.38)</td>
<td>507.61 (14.09)</td>
<td>170.12 (5.17)</td>
<td>13.92 (1.37)</td>
<td>21.24 (0.51)</td>
</tr>
<tr>
<td>3</td>
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<td>5.51 (0.12)</td>
<td>8.45 (0.68)</td>
<td>519.22 (7.95)</td>
<td>166.02 (5.02)</td>
<td>13.45 (1.64)</td>
<td>20.09 (0.5)</td>
</tr>
<tr>
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<td>1.65 (0.02)</td>
<td>5.77 (0.26)</td>
<td>7.75 (0.31)</td>
<td>503.22 (6.15)</td>
<td>173.33 (7.39)</td>
<td>12.39 (0.35)</td>
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<tr>
<td>10</td>
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<td>506.53 (3.32)</td>
<td>173.31 (4.7)</td>
<td>13.42 (0.92)</td>
<td>21.48 (0.13)</td>
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</table>
Table 3.2: Cell wall chemistry of AtAGP9 overexpressing poplar lines. Data represent the average of three biological replicates. Standard error of the mean is shown in parentheses. Using Student’s t-test, P < 0.05, no significant differences between AtAGP9 overexpression and wild type were apparent.

<table>
<thead>
<tr>
<th>Lines</th>
<th>Arabinose (ug/mg)</th>
<th>Rhamnose (ug/mg)</th>
<th>Galactose (ug/mg)</th>
<th>Glucose (ug/mg)</th>
<th>Xylose (ug/mg)</th>
<th>Mannose (ug/mg)</th>
<th>Lignin (mg/100mg)</th>
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<tr>
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<td>156.15(2.69)</td>
<td>26.01(1.09)</td>
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<tr>
<td>6</td>
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<td>wt-2</td>
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<tr>
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<td>10.15(1.34)</td>
<td>458.49(7.92)</td>
<td>150.73(5.89)</td>
<td>25.27(1.8)</td>
<td>21.55(0.73)</td>
</tr>
<tr>
<td>8</td>
<td>3.3(0.16)</td>
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<td>9.51(0.36)</td>
<td>457.96(11.47)</td>
<td>154.71(0.87)</td>
<td>27.75(1.57)</td>
<td>19.52(0.44)</td>
</tr>
</tbody>
</table>

Figure 3.11: Holocellulose content of RNAi-PtAGP9 poplar lines. Measurements were based on the dry weight of cell wall material. Bars show the standard error of the mean from three independent plants. A significant difference at P < 0.05 was evaluated by the Student’s t-test, and no significant differences were observed.
**Figure 3.12:** α-Cellulose content of *RNAi-PtAGP9* poplar lines. Measurements were based on the dry weight of cell wall material. Bars show the standard error of the mean from three independent plants. A significant difference at \( P < 0.05 \) was evaluated by the Student’s t-test, and no significant differences were observed.

**Figure 3.13:** Holocellulose content of *AtAGP9* overexpressing poplar lines. Measurements were based on the dry weight of cell wall material. Bars show the standard error of the mean from three independent plants. A significant difference at \( P < 0.05 \) was evaluated by the Student’s t-test, and no significant differences were observed.
**Figure 3.14:** α-Cellulose content of AtAGP9 overexpressing poplar lines. Measurements were based on the dry weight of cell wall material. Bars show the standard error of the mean from three independent plants. A significant difference at $P < 0.05$ was evaluated by the Student’s t-test. The asterisk indicates a statistically significant difference relative to wild type.

**Figure 3.15:** Wood density of RNAi-PtAGP9 poplar lines. No statistically significant differences were observed, as evaluated by Student’s t-test, ($P < 0.05$). Bars show the standard error of the mean ($n = 3$).
Figure 3.16: Wood density of AtAGP9 overexpressing poplar lines. No statistically significant differences were observed, as evaluated by Student’s t-test, (P < 0.05). Bars show the standard error of the mean (n = 3).

3.2.3 The roles of AGP9 in cell expansion

Since mis-regulation of AGP9 produced changes in vessel size, I hypothesized that AGP9 may impact vessel cell expansion. Dark-grown hypocotyls are a model system for cell expansion because their elongation has been shown to be strictly due to expansion and not cell division (Gendreau et al., 1997). To test if dark-grown hypocotyls were a suitable system to study the role of AGP9 in cell expansion, it was necessary to first demonstrate AGP9 expression in Arabidopsis hypocotyls. As such, the AtAGP9 promoter was fused to the GUS reporter gene, and shown to be present in elongating hypocotyls after three and seven day dark cultivation (Figure 3.17A and B). Similarly, the PtAGP9 promoter showed activity in the elongated hypocotyl when expressed in Arabidopsis (Figure 3.18A and B). As such, atagp9 (SALK_058644c) T-DNA mutants and transgenic Arabidopsis overexpressing PtAGP9 were used to test hypocotyl elongation in dark-grown Arabidopsis. The T-DNA insertion line atagp9 (SALK_058644c) did not contain detectable transcript (Figure 3.19C). In the atagp9 T-DNA mutants, hypocotyl length was significantly shorter after three day (Figure 3.19A) and seven day (Figure 3.19B) growth in the dark. The hypocotyl length was decreased by 7.9% and 5.3%, respectively. Overexpression
of *PtAGP9* in Arabidopsis led to statistically significant increased hypocotyl lengths in some lines compared with wild-type plants after three days growth in the dark. The transgenic plant hypocotyl length was increased by 14% compared to wild type (Figure 3.20A), but no significant difference following seven day (Figure 3.20B) cultivation in the dark. Indirectly this implies that *AGP9* may be associated in hypocotyl elongation.

![Figure 3.17](image)  
**Figure 3.17:** *AtAGP9* promoter activity in Arabidopsis elongated hypocotyl after cultivation in the dark. GUS staining in Arabidopsis hypocotyl after cultivation in the dark for three days (A) and seven days (B). Blue color shows *AtAGP9* promoter activity in elongated hypocotyl.

![Figure 3.18](image)  
**Figure 3.18:** *PtAGP9* promoter activity in Arabidopsis elongated hypocotyls after cultivation in the dark. GUS staining in Arabidopsis hypocotyls after cultivation in the dark for three days (A) and seven days (B). Blue color shows *PtAGP9* promoter activity in elongated hypocotyl.
Hypocotyl length (cm)

A

B

C

Genomic DNA

T-DNA

wt  atagp9 mutants
**Figure 3.19:** Hypocotyl length of *atagp9* mutants after cultivation for three (A) and seven days (B) in the dark. C. Genotyping of the homozygous *atagp9* lines. The bands in upper and lower panels were produced with genomic DNA by using *AGP9* specific primers and the combination of T-DNA specific primer and gene primer, respectively. The hypocotyl length is decreased by 7.9% (A) and 5.3% (B) compared to wild type. The growth of hypocotyls was captured using a camera, and the hypocotyl length of approximately 55 plants was determined using measurements in Image J software. Bars show the standard deviation. Asterisks indicate a statistically significant difference compared to wild type at $P < 0.05$ evaluated by the Student’s t-test.
Figure 3.20: Hypocotyl length in three Arabidopsis lines of *PtAGP9* overexpressing plants after cultivation for three (A) and seven days (B) in the dark. The hypocotyl length is increased by 14% (A), whereas there is no significant difference in hypocotyl length after cultivation for seven days (B) compared to wild type. The growth of hypocotyls was captured using a camera, and then approximately 30 plants were measured by Image J software. Bars show the standard deviation. Asterisks indicate a statistically significant difference compared to wild type at P < 0.05 evaluated by the Student’s t-test.

3.2.4 *AtAGP9* has roles in vascular bundle differentiation in Arabidopsis

The expression pattern of *AtAGP9* was quantified by RT-PCR in Arabidopsis plants, and shown to be most highly expressed in flowers and stems (Figure 3.21). To define the developmental expression pattern of *AtAGP9* in Arabidopsis, the *AtAGP9* promoter was fused to the GUS reporter gene and examined. When the Arabidopsis stem was approximately 15 cm tall, stem segments at different growth stages were harvested as indicated in Figure 3.22A. These representative section largely resemble the developmental stages defined by Hall and Ellis (2013) during kinematic growth profiling. *AtAGP9* promoter displayed activity in developing xylem at a young stage (Figure 3.22B), maximum growth stage (Figure 3.22C), and also in the endodermis and fascicular cambium in mature stems (Figure 3.22E).
Given the AtAGP9 promoter was expressed in developing xylem at young and maximum growth stages, and in cambium at the stem base, the xylem of atagp9 Arabidopsis inflorescence stems was examined for phenotypic changes. The stem cross-sections of atagp9 mutants showed irregular organization of the vascular bundles, especially the irregular deposition of multiple protoxylem areas in vascular bundles (Figure 3.23B and C). Meanwhile, suppression of the expression of AtAGP9 by post-transcriptional gene silencing (RNAi-AtAGP9) resulted in a similar irregular organization of vascular bundles and irregular deposition sites of xylem vessels (Figure 3.23E and F). This suggests that AtAGP9 may be required for proper patterning of differentiating cells of the procambium into xylem in vascular bundles of Arabidopsis.

![AtEIF5A and AtAGP9 expression](image_url)

**Figure 3.21:** The expression pattern of AtAGP9 in Arabidopsis tissues tested by semi-quantitative RT-PCR. FL - Flower; S - Stem; L - Leaf. Elongation factor AtELF5A (AT5G60390) was used as a control to standardize the sample variations in total RNA amounts of different tissues. Plants were five weeks old.
Figure 3.22: Activity of *AtAGP9* promoter in Arabidopsis vascular bundles. Stem cross-sections were stained with GUS solution. A. Arabidopsis inflorescence stem was marked to define developmental stages. Plants were sampled for the activity of *AtAGP9* promoter when the stems were approximately 15 cm tall. Growth stages are indicated in (A). B. At young stage that apical region starts to differentiate (yellow arrow indicates developing xylem). C. At maximum growth-rate stage (yellow arrow indicates developing xylem). D. At cessation stage. E. Old stage at base of stem. Fascicular cambium and endodermis are indicated by black arrow and blue arrow, respectively. FC: fascicular cambium. En: endodermis. Bar = 0.05 mm.
Figure 3.23: The patterning of xylem cells and vascular bundle in *atagp9* T-DNA mutants and RNAi-AtAGP9 Arabidopsis lines. A. Wild type. B-C. Irregular patterning of the xylem vessels and vascular bundles in *atagp9* mutants. D. Wild type. E-F. Irregular patterning of xylem vessels and vascular bundles in RNAi-AtAGP9 lines. The shape and patterning of vascular bundles and xylem cells are indicated by dashed red lines. The arrows indicate the irregular initiation of xylem cells in vascular bundles. Bar = 0.05 mm.

3.2.5 The role of AGP9 in fiber development in Arabidopsis

To examine whether or not fiber development was affected by AGP9 in Arabidopsis, fiber quality was probed. The *atagp9* mutants displayed shorter fibers ranging in length from 0.81 mm to 0.7 mm (Figure 3.24), whereas *CaMV 35S::PtAGP9* Arabidopsis plants had longer fibers, which were increased by 33.3% (Figure 3.25). This implies that in Arabidopsis, *PtAGP9* and *AtAGP9* can promote fiber growth.
**Figure 3.24:** Fiber length of *atagp9* mutants assessed by bright-field microscopy. The *atagp9* mutants displayed shorter fibers ranging in length from 0.81 mm to 0.7 mm. For each line, three biological replicates and a total 260 fibers in the three replicates were measured by Image J software from light micrograph images captured. Bars show the standard error of the mean. Asterisk indicates a significant difference compared to wild type at P < 0.05 evaluated by the Student’s t-test.
Figure 3.25: Fiber length in two Arabidopsis lines of PtAGP9 overexpressing plants assessed by bright-field microscopy. PtAGP9 overexpressing plants had longer fibers, which was increased by 33.3%. For each plant line (1, 2), three biological replicates and a total 150 fibers in the three replicates were measured by Image J software from light micrograph images captured. Bars show the standard error of the mean. Asterisks indicate significant differences compared to wild type at P < 0.05 evaluated by the Student’s t-test.

3.3 Discussion

AGP9 is expressed in both primary xylem in Arabidopsis and secondary xylem development in poplar. The relative transcript abundance of PtAGP9 was highest in developing xylem tissue, which is consistent with earlier work using microarray analysis that demonstrated the expression of AGP9 in the cambium, as well as early and late cell expansion zones in developing xylem (Hertzberg et al., 2001). In Arabidopsis, AGP9 also displays the correct spatial and temporal expression to participate in early primary xylem development, as the AtAGP9 promoter can be detected in developing xylem of inflorescence stem at both young growth and maximum growth stages, which are characterized by active growth of vascular tissues (Schuetz et al., 2013).

3.3.1 AGP9 in vessel differentiation

AGP9 may contribute to vessel development in poplar. The vessels observed in transgenic RNAi-PtAGP9 poplar lines were smaller and more abundant (Figure 3.4 and 3.5), while the 2×35S::AtAGP9 overexpressing lines had larger vessels that were less abundant (Figure 3.7 and 3.8). Moreover, these changes in vessel morphology were specific, in that the fibers were not affected by AGP9 mis-regulation (Figure 3.9 and 3.10). In Arabidopsis, xylem differentiation was also affected, and was best illustrated by an abnormal patterning of the xylem cells in vascular bundles of both the atagp9 mutants and RNAi-AtAGP9 lines.

Currently, there are no published reports addressing the functional mechanism of AGPs in vessel development. However, several hypotheses for the roles for AGP9 in vessel development can be proposed. One hypothesis is that AGP9, located on the cell surface by its GPI anchor, may influence the way in
which xylem mother cells sense developmental signals such as hormones to regulate vessel differentiation. Hormones can affect the vessel-specific transcription factors, VND7 and VND6, whose expression have been shown to be enhanced by auxin and cytokinins (Kubo et al., 2005; Yamaguchi et al., 2011). In the vascular cambium, auxin has been shown to be affiliated with the expansion of xylem initials in trees (Sorce et al., 2013), and in different tree species, auxin concentration can regulate vessel number and size (Aloni and Zimmermann, 1983; Tuominen et al., 1997; Bjorklund et al., 2007; Nilsson et al., 2008). Cytokinin has also been shown to have a role in vessel differentiation in Arabidopsis, where it has been shown to spatially regulate the differentiation and patterning of protoxylem (Mahonen et al., 2006). Furthermore, brassinosteroids (BRs) have a role in vessel differentiation (Yamamoto et al., 1997; Yamaguchi et al., 2010b), cell expansion (Clouse and Sasse, 1998; Bishop and Koncz, 2002), and vascular differentiation (Fukuda, 1997). Alternatively, it has been speculated that AGPs located at the plasma membrane may affect cell development by interacting with transmembrane proteins such as receptor-like kinases (Seifert and Roberts, 2007), which are highly abundant during developing xylem of poplar (Song et al., 2011).

Mis-regulation of AGP9 led to smaller vessels in poplar, suggesting that AGP9 impacts vessel cell expansion. The role of AGP9 in cell expansion was directly tested in dark-grown Arabidopsis hypocotyls, where the hypocotyl length of atagp9 mutants had reduced by 7.9%, while overexpression of the PtAGP9 led to longer (14%) hypocotyls in Arabidopsis. During rapid enlargement of the vessels, wall extensibility is an important factor permitting the cells to reach their full diameter, during which auxin plays important regulatory roles in cell wall loosening events (Rayle and Cleland, 1992; Cosgrove, 2005). One hypothesis related to the role of AGP9 in cell expansion is that at the interface between the cell and the wall, AGP9 may help control expansion by balancing wall loosening and growth in response to hormone signals. Previously, it has been shown that the cross-linking of AGPs with β-glucosyl Yariv reagent led to microtubule disorganization (Nguema-Ona et al., 2007), and microtubules are required for radial expansion. An alternative hypothesis is that AGPs may be acting indirectly through the cytoskeletal network by interacting with transmembrane proteins.
3.3.2 AGP9 in fiber development

Secondary xylem is mainly composed of fibers and vessels. These two cell types in xylem work together, such that the fibers form the bulk of the strength of the xylem and as such restrict the larger vessels from cavitation forces (Jacobsen et al., 2005). Since these two cell types differentiate from the xylem mother cells in vascular cambium, defects in vessel differentiation may directly or indirectly lead to abnormal fiber development. This study demonstrates that AGP9 affects vessel development, but not the fibers in poplar. This is likely due to the fact that these cell types have different developmental programs at the cell enlargement phase initiated from xylem mother cells. In vessels, rapid increases in the cell lumen are formed by a large central vacuole, and this important developmental step is required for vessel radial expansion, while in fibers vacuolization is not as extensive as in vessels, and the fast growth of fibers is characterized as an intrusive mode of elongation (Arend and Fromm, 2003; Lev-Yadun, 2010).

AGP9 mis-regulation had different effects on fiber growth in poplar and Arabidopsis. In poplar, PtAGP9 suppression and AtAGP9 overexpression did not alter fiber growth, whereas in Arabidopsis there were fiber phenotypes in the atagp9 T-DNA mutants and the PtAGP9 overexpressing poplar lines. This inconsistency may be ascribed to the distinct differentiation pattern of fibers in poplar and Arabidopsis. In poplar, fibers are initiated from the vascular cambium as secondary growth, whereas in Arabidopsis, fibers are differentiated from parenchyma cells as primary growth. Previously, in the interfascicular fibreless1 (ifl1) mutant, it was shown that interfascicular fiber differentiation was blocked from parenchyma cells and the shape of the fibers were rectangular rather than containing two sharp tips (Zhong et al., 1997). A similar phenomenon may be manifesting in the atagp9 T-DNA mutants, because phenotypically the fibers were shorter, and the AtAGP9 promoter activity was detected in endodermis and parenchyma cells near endodermis, which indirectly implies that AGP9 may affect fiber differentiation from parenchyma cells. IFL1, a member of the HD–leucine zipper (HD-ZIP) family, was hypothesized to be involved in hormone signalling. Specially, IFL1 was proposed to be involved in auxin flow, which in turn participates in the regulation of interfascicular fiber differentiation (Zhong and Ye, 1999). However, the results described herein cannot delineate how AGP9 regulates fiber differentiation in Arabidopsis.
Based on the hypothesis that AGP9 promotes or, in the very least, is involved in regulating vessel differentiation by transducing hormone signals in poplar, in Arabidopsis inflorescent stem AGP9 may also be part of the response cascade to hormone signalling during the development of fibers.

Herein, I demonstrated a role for AGP9 in vessel and fiber development. AGP9 located at the cell surface, may be a response to the hormone signal events or may interact with transmembrane proteins to affect vessel development. Vessels are important in water transport, and larger vessels can improve the efficiency of this process. However, larger vessels are also prone to embolism, which results in cavitation and inconsistent transport. Thus, vessels with reduced diameter and increased numbers may be beneficial for plant growth and adapting to abiotic stress, such as in environments that are water limited.

3.4 Materials and methods

3.4.1 Plant materials

*Arabidopsis thaliana* Columbia-0 (Arabidopsis) plants were grown for nine weeks under 17 hours light / 7 hours dark cycle. At maturity, the lower 1 cm of the stem from each homozygous mutant was used for hand sectioning and microscopic evaluation. *P. alba × P. grandidentata* (hybrid poplar P39) were grown in a greenhouse in 2011 and 2012, equipped with 400 watt high pressure sodium lamps that were used as supplemental lighting from 6:00 am until 10:00 pm (16 hours). The temperature in the greenhouse ranged from 20 ºC to a maximum of 24 ºC, while the humidity ranged from 35-45%. The trees were grown in perennial potting mix composed of 50% peat moss, 25% fine bark, and 25% pumice, and fertilized daily during watering while grown on flood tables. *RNAi-PtAGP9* lines were grown between September and February 2011, while the *AtAGP9* overexpressing poplar lines were separately grown from September to February 2011 (line 2, 3 and 6 with wild-type trees), and from February to June 2012 for line 7, and 8 with a second set of wild-type trees. Following 5-months of growth, the transgenic and paired wild-type trees were harvested. The bottom 10 cm of the stems was used for fiber quality and wood chemical analyses. Fresh wood was used for histological analysis. For each transgenic line and the pair controls,
three trees were used to evaluate each phenotype. The poplar tissues used for the quantification of PtAGP9 transcript expression level analysis were collected by Dr. T. Canam (Canam et al. 2008).

3.4.2 DNA and RNA extraction
Genomic DNA was isolated with CTAB (Sigma-Aldrich Co.). Plant tissue was first ground in liquid nitrogen and combined with 1.0 mL of CTAB buffer (2% (w/v) CTAB (Sigma), 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl, 1% PVP, β-mercaptoethanol, pH 8.0) and mixed with a pestle to fully suspend the tissue in solution. The reactions were incubated for 30-60 minutes at 65 °C. The tubes were centrifuged at 13,000 g for 10 minutes and the supernatant was transferred to a fresh sterile 2.0 mL microtube. One volume of phenol:chloroform:isoamyl alcohol was added to each tube, mixed thoroughly and finally centrifuged at 13,000 g for 10 minutes. The clear supernatant was transferred to a fresh 1.5 mL tube, and a 1/2 volume of isopropanol was added to each tube. Tubes were centrifuged at 13,000 g for 10 minutes and washed with 70% ethanol. The pellet was suspended in sterile nanopure water. To screen Arabidopsis homozygous mutants, the quick DNA extraction method was employed (Edwards et al., 1991).

Total RNA was extracted with TRIZOL Reagent (Invitrogen) kit, as per the manufacturer’s instruction, treated with DNase and transcribed into first strand cDNA with SuperScript II reverse transcriptase (Invitrogen).

3.4.3 RT-PCR, semi-quantitative RT-PCR, and quantitative real-time RT-PCR analysis
Reverse transcription polymerase chain reaction (RT-PCR) was performed with cDNA samples isolated from different Arabidopsis and poplar tissues. Semi-quantitative RT-PCR was used to examine the expression levels of target mRNAs from each sample. Elongation factor AtELF5A (AT5G60390) was used as a control to standardize the sample variations in total RNA amounts (Czechowski et al., 2005).

Quantitative real-time RT-PCR (Q-RT-PCR) analysis was used to examine the transcript abundance of AGP9 in poplar and Arabidopsis tissues with the SYBR Green JumpStart Taq Ready Mix (Sigma-Aldrich). Three biological replicates were assayed for each sample. The comparative threshold cycle method was used to quantify mRNA content (Livak and Schmittgen, 2001). Poplar actin gene and
Arabidopsis elongation factor *AtELF5A* were chosen as housekeeping genes and used to normalize the transcript abundance of *PtAGP9* and *AtAGP9* in different tissues, respectively (Czechowski et al., 2005; Regier and Frey, 2010). The primer sequences used are described in Table 3.3.

3.4.4 Screening of *atagp9* T-DNA insertion mutant homozygous lines

The Arabidopsis *atagp9* T-DNA mutant (SALK_058644c) lines, obtained from the Salk Institute Genomic Analysis Laboratory (http://signal.salk.edu/) (Alonso et al., 2003), were screened for homozygosity using genomic DNA extracted as per Edwards et al (1991). The primer sequences employed for screening *atagp9* T-DNA mutants were designed by T-DNA primer design program (http://signal.salk.edu/tdnaprimers.2.html). Synthesized cDNA was used as template to examine the extent of *AtAGP9* expression in *atagp9* homozygous lines. Primer sequences are shown in Table 3.3.
Table 3.3: PCR primer sequences used for AGP9 gene isolation, cloning, screening, and Q-RT-PCR.

<table>
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<th>Primer Name</th>
<th>Sequence (5' to 3')</th>
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</tr>
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</tr>
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<td>Q-RT-PCR</td>
</tr>
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</tr>
</tbody>
</table>

Footnote: FW: forward primer; RV: reverse primer; LP: left genomic primer; RP: right genomic primer; LB: left border primer. ov: overexpression.

3.4.5 Cloning of the AGP9 gene

Poplar and Arabidopsis tissues were independently used to extract genomic DNA and RNA. Synthesized cDNA from *P. alba × P. grandidentata* and *Arabidopsis thaliana* Columbia-0 was used to clone *PtAGP9* and *AtAGP9* separately by pENTR directional TOPO cloning kit (Invitrogen). The poplar and Arabidopsis post-transcriptional gene silencing (RNA-interference) constructs were made using unique 455 and 472 bp coding DNA sequence (CDS) fragments, targeting *PtAGP9* and *AtAGP9* respectively for each of the poplar and Arabidopsis genes. The representative CDS fragments of *PtAGP9* and *AtAGP9* were separately cloned as sense and anti-sense fragment into the pHellsgate12 vector containing the
CaMV 35S promoter (Helliwell and Waterhouse, 2003). Primer sequences for the PtAGP9-RNAi and AtAGP9-RNAi constructs are listed in Table 3.3.

PtAGP9 gene was cloned from synthesized poplar cDNA, while AtAGP9 gene was cloned using genomic DNA from Arabidopsis. The length of each gene was 796 and 1381 bp from start codon to 3' UTR, respectively. The PtAGP9 fragment carrying XbaI and SacI restriction enzyme sites and AtAGP9 carrying XbaI and KpnI restriction enzyme sites were separately sub-cloned into the pSM3 binary vector harbouring the 2×35S promoter (Datla et al., 1993). All primer sequences employed are listed in Table 3.3.

The AtAGP9 (1839 kb) and PtAGP9 promoters (1672 bp), which included the translational start codons, were separately amplified from Arabidopsis and poplar genomic DNA. The promoter fragments were sequenced after cloning in pDONR201. The upstream ATG was removed in a polymerase chain reaction (PCR) using a reverse primer matching the 5' end of the cDNA. The PCR products were sub-cloned into pDONR201 and sequenced. Finally, the AtAGP9 and PtAGP9 promoters were separately cloned into the PMDC162 vector, which harboured the β-glucuronidase (GUS) gene (Curtis and Grossniklaus, 2003). The sequences used for cloning the PtAGP9 and AtAGP9 promoters are listed in Table 3.3.

3.4.6 Bioinformatics analysis of AtAGP9 and PtAGP9

The amino acid identity of AtAGP9 (AT2G14890) and PtAGP9 (Potri.009G092300) was predicted by pairwise alignment of BioEdit sequence alignment editor (http://www.mbio.ncsu.edu/bioedit/bioedit.html). Signal peptides of PtAGP9 and AtAGP9 were separately predicted by SignalP 3.0 Server (Petersen et al., 2011). GPI lipid anchor sequences were identified by the big-PI Plant Predictor (Eisenhaber et al., 2003), while the C-terminal hydrophobic domain was predicted by TMPred (http://www.ch.embnet.org/software/TMPRED_form.html).
3.4.7 Plant transformation

Target binary constructs were transformed into Agrobacterium tumefaciens GV3101 by the freeze-thaw method (Holsters et al., 1978). Positive clones were incubated in YEP media (5 g/L yeast extract, 10 g/L peptone, 5 g/L NaCl) until an OD_600 of 0.7 was attained. Arabidopsis plant transformation followed the commonly employed protocol (Bechtold et al., 1993; Clough and Bent, 1998). Positive Arabidopsis transformants were selected by growth on 1/2 Murashige and Skoog medium supplemented with 50 mg/L kanamycin for the RNAi lines, and 20 mg/L hygromycin for the overexpression lines.

Poplar transformation employed the leaf discs inoculation method (Coleman et al., 2008). Binary constructs carrying the target gene were transformed into Agrobacterium tumefaciens EHA105 by the freeze-thaw method (Holsters et al., 1978). Positive clones were selected for incubation in liquid woody plant media (WPM) overnight (McCown and Lloyd, 1981). Large, fresh leaves from approximately one-month-old poplar were used for transformation. Leaves were cut into discs and cultivated in diluted WPM media containing Agrobacterium tumefaciens for 1/2 hour at 28 °C with gentle shaking (100 g). Following incubation, the leaf discs were gently dried with sterilized filter paper to remove the WMP media. The leaves were transferred, abaxial side up, to WPM media supplemented with 0.1 μM of 1-naphthaleneacetic acid (NAA), benzylaminopurine (BA), and thidiazuron (TDZ). After two days, the leaf discs were moved to WPM media with 0.1 μM of NAA, BA, TDZ, 250 mg/L cefotaxime, 500 mg/L carbenicillin, and placed in the dark for three days to kill the Agrobacterium tumefaciens. The leaf discs successfully transfected were moved to WPM media with 0.1 μM of NAA, BA, and TDZ, 250 mg/L cefotaxime, 500 mg/L carbenicillin, 50 mg/L kanamycin or 25 mg/L hygromycin, and the plates were placed in subdued light for selecting positive shoots. After the shoots elongated, they were transferred to WPM media with 0.1 μM of NAA, 250 mg/L cefotaxime, 500 mg/L carbenicillin, 50 mg/mL kanamycin or 25 mg/mL hygromycin for rooting. Positive lines were identified by genomic DNA screening, subcultured and multiplied. Positive RNAi lines were further confirmed by Q-RT-PCR as per the method described above. Positive Arabidopsis and poplar overexpression lines were separately verified with RT-PCR.
3.4.8 Assessment of plant growth

Tree height and caliper at the base of the stem were measured on five trees per construct and the appropriate controls on greenhouse-grown plants.

3.4.9 Histology

Nine weeks-old Arabidopsis stems were hand sectioned with a double-sided razor blade and used for histological assessment. Poplar stems were sectioned with a microtome set to a thickness of 64 µm. Fresh sections were stained with 0.02% aqueous toluidine blue O for 5 minutes, and observed via a light microscope (Leica Microsystems Inc., Ontario, Canada).

3.4.10 GUS staining

AtAGP9 promoter activities were observed in the third generation (T3) transgenic plants. Around 10-15 cm tall Arabidopsis inflorescence stems, at the defined growth stage, were hand sectioned with a double-sided razor blade, and submerged in a GUS staining solution containing 0.2 M NaPO₄, pH 7; 0.5 M EDTA, pH 8; 25 mM potassium ferricyanide; 25 mM potassium ferrocyanide; Triton X-100, and 20 mM X-gluc (5-bromo-4-chloro-3-indoly β-D-glucuronide) dissolved in methanol. The samples were incubated at 37 °C for 2-3 hours. Arabidopsis hypocotyls, grown in the dark for three and seven days respectively, were also stained in the GUS solution for 2-3 hours at 37 °C. Afterwards, the samples were cleared with chloral hydrate (Berleth and Jurgens, 1993), and well-stained samples were mounted on glass slides and observed with a light microscope (Leica Microsystems Inc., Ontario, Canada).

3.4.11 Vessel quality analysis

Poplar stem sections and Arabidopsis stem sections were used to analyze the developing xylem vessel phenotype. Stem cross sections were stained with 0.02% w/v toluidine blue O for 5 minutes and then observed under a light microscope (Leica Microsystems Inc., Ontario, Canada). The total number and area of all vessels per field of view in developing xylem of poplar were quantified in several stem cross sections with Image J software (http://rsb.info.nih.gov/ij/). In poplar, vessels in the gelatinous layers area, associated with tension wood formation, were avoided in the assessment. In Arabidopsis, xylem and
vascular bundle phenotypes were visualized in cross sections at the base of stem under the light microscope.

3.4.12 Fiber quality analysis

For Arabidopsis fiber length analysis, 5-8 mg stem samples from the base of plants were placed in a 10 ml test tube containing a modified Franklin solution (1:1:1 30% hydrogen peroxide:glacial acetic acid; distilled water) (Franklin, 1945), and incubated at 100 °C for 3 hours. The solution was decanted, the remaining materials were washed and shaken gently by hand until the fibers separated. For poplar fiber length analysis, 200 mg dry wood samples from base of stem were cooked in a modified Franklin solution (1:1 30% hydrogen peroxide: glacial acetic acid) in a 70 °C bath for 48 hours. The materials were washed with distilled water and diluted into a Falcon Tube. Fibers were separated by blending in a Waring mixer. Diluted fibers were directly analyzed on the fiber quality analyzer (FQA; OpTest Equipment Inc., Hawkesbury, Canada) or observed under microscope after staining with 0.02% w/v toluidine blue O for 5 minutes by loading liquid on a microscopy slide covered by a coverslip.

3.4.13 Cell wall chemistry

Cell wall carbohydrate compositions were analyzed by Klason as per Cullis et al (2004). Wood samples were ground to pass a 40-mesh in a Wiley mill and extracted overnight with acetone in a Soxhlet apparatus to remove extractives. 3 mL 72% (w/w) H₂SO₄ was added to 0.2 g samples and allowed to react on the bench for 2 hours with mixing every 10 minutes. The wood hydrolyzate was transferred to septa-sealed serum bottles, to which 112 mL of water was added and autoclaved for 1.5 hours at 121 °C together with the sugar controls. The hydrolyzate was removed and filtered through dry, pre-weighed, medium coarseness sintered-glass crucible to estimate the content of acid-insoluble lignin. Acid-soluble lignin was determined by absorbance at 205 nm on the Bio UV-visible spectrophotometer using quartz cuvettes (Varian, Inc., Australia). Carbohydrates were determined by using a Dx-600 anion exchange high-performance liquid chromatography (Dionex, Sunnyvale, CA, USA).
3.4.14 Holocellulose and alpha-cellulose analysis

Wood samples from base of poplar stems were ground to pass a 40-mesh in a Wiley mill and extracted overnight with acetone in a Soxhlet apparatus to remove extractives. Extract-free wood samples (195-200 mg) were used for holocellulose quantification. Wood samples were incubated at 50 °C for 14 to 16 hours after adding 3.5 mL buffer (60 mL of glacial acetic acid and 1.3 g NaOH per Liter) and 1.5 mL of 20% sodium chlorite solution. The reaction was quenched by placing it into an ice bath to cool. After removing the reaction solution by careful pipetting, the procedure was repeated. Finally, the reacted wood meal was transferred to pre-weighed, coarse sintered-glass crucible and washed twice with 50 mL of 1% glacial acetic acid and 10 mL acetone. The weight of the crucible was then determined after drying, to quantify holocellulose content (Yokoyama et al., 2002).

Approximately 80-100 mg of holocellulose was used for alpha-cellulose isolation. Samples were reacted for 30 minutes after adding 8 mL of 17.5% NaOH. Eight mL of distilled water was added to the samples and stirred for 1 minute. After 29 minutes, the retentate was filtered through a pre-weighed coarse sintered-glass crucible by washing with distilled water. The crucibles were soaked in 1.0 M acetic acid for 5 minutes and washed with distilled water. The dry weight of crucibles was used to quantify the alpha-cellulose contents.

3.4.15 Wood density

Measurements were taken from three separate samples. Bark to bark samples were first precision cut to 1.68 mm thickness with a twin blade pneumatic circular saw, and extracted overnight in a Soxhlet apparatus with acetone. Wood density of samples was determined by X-ray densitometry (Quintek Measurement System Inc. Knoxville, TN, USA) using both radii (bark to bark) and averaged for each sample.

3.4.16 Statistical analysis

The statistical significance of any difference between experimental results was evaluated by the Student’s t-test to compare two means (SPSS v11 software, SPSS Inc., Chicago, USA). A value of P < 0.05 was considered statistically significant.
Chapter 4 AGP14, an AG peptide, impacts cell wall development in poplar

4.1 Introduction

There is one group of classical AGPs known as the AG peptides, in which the mature proteins only contain between 10 to 13 amino acid residues following the cleavage of the N- and C-terminal signal sequences (Schultz et al., 2002). *Arabidopsis thaliana* contains 16 AG peptides (Showalter et al., 2010), of which AGP14 is one. To date, little is known about AGP14, however, it has been reported that *AtAGP14* expression is detected in the endodermis and root hairs of *A. thaliana* (Lin et al., 2011). Moreover, *atagp14* T-DNA mutants (SALK_107040) have significantly longer root hairs compared with wild-type plants, suggesting a role in restricting polar cell expansion (Lin et al., 2011). In developing poplar wood, an AG peptide similar to AGP14 was the only AGP gene with reduced transcript abundance in tension wood relative to the normal wood when assessed by microarray analysis (Andersson-Gunneras et al., 2006). However, the functional role of *AGP14* during cell wall development is not clear. Transcriptome data collected from developing xylem of *Populus trichocarpa* re-sequencing initiatives indicated that *AGP14* was among the highly expressed genes, similar to the *AGP9* and *AGP18* (Geraldes et al., 2011). Therefore, in this study, the potential role(s) of *AGP14* in secondary growth were investigated in poplar. In addition, the inflorescence stem of *A. thaliana* was used as a model system for fiber cell and secondary cell wall development to compare and contrast *AGP14*. 
4.2 Results

PtAGP14 (Potri.001G004100) was isolated from *P. alba × P. grandidentata* (hybrid poplar P39), while AtAGP14 (AT5G56540) was isolated from *A. thaliana* Columbia-0 (Arabidopsis) plants. These orthologs share 78% amino acid sequence similarity (Figure 4.1). Following the cleavage of the N-terminal signal peptide and C-terminal GPI-associated hydrophobic domain during protein processing, PtAGP14 shares 75% amino acid sequence similarity with AtAGP14. Bioinformatics analysis suggests that PtAGP14 and AtAGP14 are structurally conserved and highly glycosylated. Both PtAGP14 and AtAGP14 possess 100% Hyp-oligosaccharide addition sites, as predicted by applying the Hyp-contiguity hypothesis to the composition of amino acid sequences (Table 2.1) (Shpak et al., 2001; Sun et al., 2005).

Figure 4.1: Alignment of *P. alba × P. grandidentata* AGP14 (PtAGP14) amino acid sequences with that of *Arabidopsis thaliana* AGP14 (AtAGP14). Identical conserved amino acids are in black shading, non-identical amino acids with similar properties are in grey shading. AtAGP14 has 58% identity and 78% similarity with PtAGP14. Gene identification numbers are: *PtAGP14*, Potri.001G004100; *AtAGP14*, AT5G56540.

4.2.1 Putative roles of the AGP14 in cell wall development in Arabidopsis

Arabidopsis has a very short growth cycle, and as such is a good model plant for investigating cell wall development (Chaffey et al., 2002b; Nieminen et al., 2004). In an attempt to elucidate the specific role(s) of AtAGP14 in cell wall development, the first objective was to establish the expression patterns of AtAGP14 during plant development. In Arabidopsis, AtAGP14 was shown to be expressed in the stem and flowers (Figure 4.2). The reporter gene, β-glucuronidase (GUS), driven by the native AtAGP14 promoter
(AtAGPprom::GUS) showed activity in the endodermis and cortex at the base of the inflorescent stem of Arabidopsis (Figure 4.3).

An Arabidopsis atagp14 T-DNA mutant line (SALK_107040C) was obtained from the Arabidopsis Biological Resource Center (ABRC) and homozygosity was confirmed. However, AtAGP14 expression in the atagp14 homozygous lines was not completely abolished when assessed by RT-PCR. In parallel, AtAGP14 expression was suppressed using an inhibitory RNA (RNAi-AtAGP14) driven by a CaMV 35S promoter, while overexpressing plants were generated by driving PtAGP14 by the duplicated-enhancer cauliflower mosaic virus 35S promoter (2×35S) (Datla et al., 1993). The stem bases of all transgenic Arabidopsis plants from two independent generations were used to evaluate putative effects of AGP14 on cell wall development. In short, there was no morphological change to the stem tissues in the RNAi-AtAGP14 and PtAGP14 overexpressing Arabidopsis lines when assessed by a light microscope. The transgenic Arabidopsis lines were also tested for fiber traits by fiber quality analyzer (FQA). In the RNAi-AtAGP14 lines, the fiber length was decreased by 20.68% and there were no change in fiber width (Figure 4.4A and B), while the overexpression of the PtAGP14 had no effect on fiber length or width (Figure 4.5A and B). The impact of AGP14 on cell wall chemical traits was also assessed, but neither approach showed any significant changes compared to wild-type plants (P39) (Table 4.1 and Table 4.2).

Figure 4.2: The expression pattern of AtAGP14 in five-week-old Arabidopsis tissues tested by semi-quantitative RT-PCR. FL - Flower; L - Leaf; MS - Middle stem; BS - Bottom stem. Elongation factor AtELF5A (AT5G60390) was used as a control to standardize the sample variations in total RNA amounts of different tissues.
Figure 4.3: Activity of AtAGP14 promoter in base of Arabidopsis stems. Stem cross sections are stained in GUS staining solution for 4 hours. Blue color indicates the activity of AtAGP14 promoter in stem cross sections. The yellow arrow indicates endodermis; the black arrow indicates cortex. Bar = 0.05 mm.
Figure 4.4: Fiber length (A) and fiber width (B) of RNAi-AtAGP14 Arabidopsis lines assessed by fiber quality analyzer. Fibers were digested with hydrogen peroxide and acetic acid. For each line, three biological replicates and 10,000 fibers were measured by fiber quality analyzer. Bars show the standard error of the mean (n = 3). Asterisks indicate a statistically significant difference compared to wild type (wt) at P < 0.05 evaluated by the Student’s t-test.
Figure 4.5: Fiber length (A) and fiber width (B) of *PtAGP14* overexpressing Arabidopsis lines assessed by fiber quality analyzer. Fibers were digested with hydrogen peroxide and acetic acid. For each line, three biological replicates and 10,000 fibers were measured by fiber quality analyzer. Bars show the standard error of the mean (n = 3). The significant difference at P < 0.05 was evaluated by the Student’s t-test. No significant differences from wild type were detected.
Table 4.1: Cell wall chemistry in two Arabidopsis lines of RNAi-AtAGP14 plants. Bolded numbers indicate significant differences between the transgenic line (1, 2) and wild type (wt). Standard error of the mean is shown in parentheses. Data presented is the average of three biological replicates. Student’s t-test, P < 0.05.

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Table 4.2: Cell wall chemistry in two Arabidopsis lines of PtAGP14 overexpressing plants. Bolded numbers indicate significant differences between the transgenic line (1, 2) and wild type (wt). Standard error of the mean is shown in parentheses. Data presented is the average of three biological replicates. Student’s t-test, P < 0.05.

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4.2.2 Roles of AGP14 in cell wall development in poplar

The role of PtAGP14 in secondary xylem development in poplar was assessed in parallel. The expression pattern of PtAGP14 was probed using quantitative real-time PCR (Q-RT-PCR), and shown to be expressed in developing xylem of poplar (Figure 4.6), which is consistent with the re-sequencing data (Geraldes et al., 2011). To probe the function of AGP14 in cell wall development, PtAGP14 expression was suppressed by RNA interference (RNAi-PtAGP14), while AtAGP14 was overexpressed in poplar (2x35S::AtAGP14). The RNAi-suppressed lines grew normally (Figure 4.7A and Figure 4.7B), and the ensuing fiber morphology of the RNAi-PtAGP14 poplar lines was not different from wild-type trees.
Similarly, the overexpression of *AtAGP14* did not alter the fiber traits, however, the trees were shorter (Figure 4.8A) and the stems were thinner (Figure 4.8B). The plant height and stem thickness were approximately reduced by 14% compared with wild type. It appears that although the AGP peptide is expressed in the developing xylem, its altered expression does not impact the fiber development in poplar. The morphology of the vessels was also tested at base of the stem, and again there was no change in vessel characteristics.

The secondary cell wall chemical characteristics were also assessed in all transgenic trees and compared to the wild type. The RNAi-*PtAGP14* poplar lines had decreased xylose content and increased mannose content (Table 4.3), while the *AtAGP14* overexpressing lines had lower mannose content compared to wild-type trees (Table 4.4). Cellulose was not affected by *AGP14* mis-expression using either strategy (Figure 4.9A and B; Figure 4.10A and B).

Wood density was also assessed. There was no significant difference in wood density among the RNAi-*PtAGP14* lines (Figure 4.11), whereas overexpression of *AtAGP14* in poplar led to a lower average wood density in three out of seven lines (Figure 4.12). In average, it declined by 19.18% compared to wild type.
Figure 4.6: Relative transcript abundance of *PtAGP14* in poplar tissues collected in July, 2007. Transcript levels in each tissue are based on the comparative threshold cycle value relative to the housekeeping gene (actin) transcript level. *PtAGP14* is expressed in developing xylem, but also in leaf. Bars show the standard error of the mean (n = 3).
Figure 4.7: Plant height (A) and stem thickness (B) of RNAi-PtAGP14 poplar lines. For each line, there were five biological replicates. Asterisks indicate a statistically significant difference compared to wild type at $P < 0.05$ evaluated by the Student’s t-test. Bars show the standard error of the mean.
Figure 4.8: Plant height (A) and stem thickness (B) of AtAGP14 overexpressing poplar lines. For each line, there were five biological replicates. Asterisks indicate a significant difference compared to wild type at P < 0.05 evaluated by the Student’s t-test. Bars show the standard error of the mean.
**Table 4.3:** Cell wall chemistry of RNAi-PtAGP14 poplar lines. Bolded numbers indicate significant differences compared to wild type. Standard error of the mean is shown in parentheses. Data presented is the average of three biological replicates. Student’s t-test, P < 0.05.

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**Table 4.4:** Cell wall chemistry of AtAGP14 overexpressing poplar lines. Bolded numbers indicate significant differences compared to wild type. Standard error of the mean is shown in parentheses. Data presented is the average of three biological replicates. Student’s t-test, P < 0.05.

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Figure 4.9: Holocellulose (A) and α-Cellulose (B) of RNAi-PtAGP14 poplar lines. Measurements are based on dry weight cell wall materials. Asterisks indicate a statistically significant difference compared to wild type at P < 0.05 evaluated by the Student’s t-test. Bars show the standard error of the mean from three independent plants.
Figure 4.10: Holocellulose (A) and α-Cellulose (B) of *AtAGP14* overexpressing poplar lines. Measurements are based on dry weight cell wall materials. Bars show the standard error of the mean from three independent plants. Significant difference at $P < 0.05$ was evaluated by the Student’s t-test. No significant differences from wild type were detected.
Figure 4.11: Wood density of RNAi-PtAGP14 poplar lines. Bars show the standard error of the mean from three independent plants. Significant difference at \( P < 0.05 \) was evaluated by the Student’s t-test. No significant differences from wild type were detected.

Figure 4.12: Wood density of AtAGP14 overexpressing poplar lines. Asterisks indicate a statistically significant difference compared to the wild type trees at \( P < 0.05 \) evaluated by the Student’s t-test. Bars show the standard error of the mean (n = 3).
4.3 Discussion

4.3.1 Roles of AGP14 in cell wall

In Arabidopsis, 16 AG peptides have been identified (Showalter et al., 2010). Except for the longer root hair phenotype that was observed in the atagp14 T-DNA mutants (Lin et al., 2011), no other work has been published in the literature associating a role for AG peptides in plant development.

Our research did not reveal a clear role for AGP14 in cell wall development in Arabidopsis. It is possible that no effect was apparent due to gene redundancy, as AGP13 is a close homolog (95.6%) (Yang and Showalter, 2007a), and has similar expression patterns to AGP14 as indicated on the Arabidopsis eFP Browser. In poplar, cell wall changes were found, however, minor. Specifically, xylose content was decreased by 12%, and mannose content was increased by 24.8% in RNAi-PtAGP14 poplar lines. Moreover, these preliminary results suggest that the fiber cell walls were thinner in RNAi-PtAGP14 poplar lines, which may be ascribed to the reduced xylan content, which has been shown to be important for secondary cell wall thickening (Northcote et al., 1989). Overexpression of AtAGP14 in poplar appeared to affect mannan biosynthesis. There was no change in the cellulose characteristics of the RNAi-PtAGP14 or AtAGP14 overexpressing lines (Figure 4.9 and Figure 4.10).

In conclusion, AGP14 may be involved in cell wall development. The mature peptide has a short backbone decorated with carbohydrates, and as such may act as a cross-linker during cell wall formation. Recently, using the apap1 T-DNA mutant, it was reported that classical apap1 carbohydrate chains supply the attachment sites to cell wall polymers (Tan et al., 2013); the apap1 mutant was shown to possess easily extractable homogalacturonan (HG), rhamnogalacturonan I (RG-I) and xylan (Tan et al., 2013).

AtAGP14 also affected some wood properties, as wood density was slightly lower in AtAGP14 poplar overexpression line 6 and 7, and the corresponding stems were thinner. Wood density is often associated with the overall mechanical properties of trees (Hacke et al., 2001); however, how this manifests from the overexpression of the Arabidopsis gene in poplar is unclear.
4.4 Materials and methods

4.4.1 Plant materials

*Arabidopsis thaliana* Columbia-0 (Arabidopsis) plants were grown for nine weeks under 17 hours light / 7 hours dark cycle. At maturity, the lower 1 cm of the stem from each homozygous mutant was used for hand sectioning and microscopic evaluation. *P. alba × P. grandidentata* (hybrid poplar P39) were grown in a greenhouse, equipped with 400 watt high pressure sodium lamps that were used as supplemental lighting from 6:00 am until 10:00 pm (16 hours). The temperature in the greenhouse ranged from 20 °C to a maximum of 24 °C, while the humidity ranged from 35-45%. The trees were grown in perennial potting mix composed of 50% peat moss, 25% fine bark, and 25% pumice, and fertilized daily during watering while grown on flood tables. *RNAi-PtAGP14* lines and *AtAGP14* overexpressing poplar lines were grown from September to February 2011. Following 5-months of growth, the transgenic and paired wild-type trees were harvested. The bottom 10 cm of the stems was used for fiber quality and wood chemical analyses. Fresh wood was used for histological analysis. For each transgenic line and the pair controls, three trees were used to evaluate each phenotype. The poplar tissues used for *PtAGP14* transcript expression level analysis were collected by Dr. T. Canam (Canam et al. 2008).

4.4.2 DNA and RNA extraction

Genomic DNA was isolated with CTAB (Sigma-Aldrich Co.). Plant tissue was first ground in liquid nitrogen and then combined with 1.0 mL of CTAB buffer (2% (w/v) CTAB (Sigma), 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl, 1% PVP, β-mercaptoethanol, pH 8.0) and mixed with a pestle to fully suspend the tissue in solution. The reactions were incubated for 30-60 minutes at 65 °C. The tubes were centrifuged at 13,000 g for 10 minutes and the supernatant was transferred to a fresh sterile 2.0 mL microtube. One volume of phenol:chloroform:isoamyl alcohol was added to each tube, mixed thoroughly and finally centrifuged at 13,000 g for 10 minutes. The clear supernatant was transferred to a fresh 1.5 mL tube, and a 1/2 volume of isopropanol was added to each tube. Afterwards, tubes were centrifuged at
13,000 g for 10 minutes and washed with 70% ethanol. Finally, the pellet was suspended in sterile nanopure water. To screen Arabidopsis homozygous mutants, the quick DNA extraction method was employed (Edwards et al., 1991).

Total RNA was extracted with TRIZOL Reagent (Invitrogen) kit, as per the manufacturer’s instruction, treated with DNase and transcribed into first strand cDNA with SuperScript II reverse transcriptase (Invitrogen).

4.4.3 RT-PCR, semi-quantitative RT-PCR, and quantitative real-time RT-PCR analysis

Reverse transcription polymerase chain reaction (RT-PCR) was performed with cDNA samples isolated from different Arabidopsis and poplar tissues. Semi-quantitative RT-PCR was used to measure the expression levels of target mRNAs from each sample. Elongation factor *AtELF5A* (AT5G60390) was used as a control to standardize the sample variations in total RNA amounts (Czechowski et al., 2005).

Quantitative real-time RT-PCR (Q-RT-PCR) analysis was used to examine the transcript abundance of *AGP14* in poplar and Arabidopsis tissues using the SYBR Green JumpStart Taq Ready Mix (Sigma-Aldrich), using actin and elongation factor *AtELF5A* as housekeeping genes (Czechowski et al., 2005; Regier and Frey, 2010). Three biological replicates were assayed for each sample. The comparative threshold cycle method was used to quantify mRNA content (Livak and Schmittgen, 2001). The transcript abundance of *PtAGP14* and *AtAGP14* in different tissues was normalized to the actin and *AtELF5A* transcript level, respectively. The primer sequences used are described in Table 4.5.
Table 4.5: PCR primer sequences used for AGP14 gene isolation, cloning, screening, and Q-RT-PCR.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5′ to 3′)</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>AtELF5A FW</td>
<td>TTGCCACACCTCTCACATTGCACTCA</td>
<td>Q-RT-PCR</td>
</tr>
<tr>
<td>AtELF5A RV</td>
<td>TGT CCC TAA CAG CGA AAC GTC CCA A</td>
<td>Q-RT-PCR</td>
</tr>
<tr>
<td>Poplar actin FW</td>
<td>TGCTGAGCGATTTGGAATTC</td>
<td>Q-RT-PCR</td>
</tr>
<tr>
<td>Poplar actin RV</td>
<td>GGGCTATGTGCTGAGATTTCC</td>
<td>Q-RT-PCR</td>
</tr>
<tr>
<td>PiAGP14 FW</td>
<td>GCAACTTCTCTCCTAACAAATCCT</td>
<td>Q-RT-PCR</td>
</tr>
<tr>
<td>PiAGP14 RV</td>
<td>CATCAAGACACCATCAGAC</td>
<td>Q-RT-PCR</td>
</tr>
<tr>
<td>AtAGP14 FW</td>
<td>GGTGCCAGTGTAACCTCTC</td>
<td>Q-RT-PCR</td>
</tr>
<tr>
<td>AtAGP14 RV</td>
<td>AGGAAGACATCGAGATG</td>
<td>Q-RT-PCR</td>
</tr>
<tr>
<td>Piaagp14 mutant FW</td>
<td>ATGGAGGCAATGAGATGAA</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>Piaagp14 mutant RV</td>
<td>TTAAAGAAAAATCAGAGA</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>PiAGP14 FW</td>
<td>CACCAGGAGCAGAATGAA</td>
<td>cloning</td>
</tr>
<tr>
<td>PiAGP14 RV</td>
<td>TCAAGGGAAGGACCCCCAAAGCA</td>
<td>cloning</td>
</tr>
<tr>
<td>AtAGP14 FW</td>
<td>CACCCTTCTGAAATGGGAGAAGGATGAA</td>
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</tr>
<tr>
<td>AtAGP14 RV</td>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
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<td>cloning</td>
</tr>
<tr>
<td>ov-PiAGP14 FW</td>
<td>TCTAAGATGGGACAGAATGAA</td>
<td>cloning</td>
</tr>
<tr>
<td>ov-PiAGP14 RV</td>
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<td>cloning</td>
</tr>
<tr>
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<td>cloning</td>
</tr>
<tr>
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<td>cloning</td>
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</tr>
<tr>
<td>atagp14 mutant RP</td>
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<td>screening</td>
</tr>
<tr>
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<td>screening</td>
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<td>isolation</td>
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<tr>
<td>AtAGP14 Promoter FW</td>
<td>GCCTTTGTTATATGGAAG</td>
<td>isolation</td>
</tr>
<tr>
<td>AtAGP14 Promoter RV</td>
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<td>isolation</td>
</tr>
<tr>
<td>AtAGP14 Promoter FW</td>
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<td>cloning</td>
</tr>
<tr>
<td>AtAGP14 Promoter RV</td>
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<td>cloning</td>
</tr>
<tr>
<td>Kanamycin Forward</td>
<td>AGAGGGCTATCCGGCTATGAC</td>
<td>screening</td>
</tr>
<tr>
<td>Kanamycin Reverse</td>
<td>GAGGAAGACATCGGACAGA</td>
<td>screening</td>
</tr>
</tbody>
</table>

Footnote: FW: forward primer; RV: reverse primer; LP: left genomic primer; RP: right genomic primer; LB: left border primer; ov: overexpression.

4.4.4 Screening of atagp14 T-DNA insertion mutant homozygous lines

The Arabidopsis atagp14 T-DNA mutant lines were screened for homozygosity using genomic DNA extracted as per Edward et al (1991). Arabidopsis atagp14 (SALK_107040C) seeds were obtained from the Salk Institute Genomic Analysis Laboratory ([http://signal.salk.edu/](http://signal.salk.edu/)) (Alonso et al., 2003). The primer sequences employed for screening atagp14 T-DNA insertion homozygous mutants were designed by T-DNA primer design program ([http://signal.salk.edu/tdnaprimers.2.html](http://signal.salk.edu/tdnaprimers.2.html)). Synthesized cDNA was used as template to examine the extent of AtAGP14 expression in atagp14 homozygous lines. Primer sequences are shown in Table 4.5.
4.4.5 Bioinformatics analysis of AtAGP14 and PtAGP14

The amino acid identity of AtAGP14 and PtAGP14 was predicted by pairwise alignment of BioEdit sequence alignment editor (http://www.mbio.ncsu.edu/bioedit/bioedit.html). Signal peptides of PtAGP14 (Potri.001G004100) and AtAGP14 (AT5G56540) were separately predicted by SignalP 3.0 Server (Petersen et al., 2011). GPI lipid anchor sequences were identified by the big-PI Plant Predictor (Eisenhaber et al., 2003), while the C-terminal hydrophobic domain was predicted by TMpred (http://www.ch.embnet.org/software/TMPRED_form.html).

4.4.6 Cloning of the AGP14 gene

Poplar and Arabidopsis tissues were independently used to extract genomic DNA and RNA. Synthesized cDNA from poplar and Arabidopsis tissue was used to clone PtAGP14 and AtAGP14 separately by pENTR directional TOPO cloning kit (Invitrogen). The poplar and Arabidopsis post-transcriptional gene silencing (RNA-interference) constructs were made using unique 174 and 338 bp fragments, targeting PtAGP14 and AtAGP14 respectively for each of the poplar and Arabidopsis gene. The representative fragments of PtAGP14 and AtAGP14 were separately cloned as sense and anti-sense fragments into the pHellsgate12 vector containing the CaMV 35S promoter (Helliwell and Waterhouse, 2003). Primer sequences used are listed in Table 4.5.

PtAGP14 and AtAGP14 were respectively cloned from synthesized P. alba × P. grandidentata and Arabidopsis thaliana Columbia-0 cDNA. The length of each gene was 563 and 341 bp from start codon to 3’ UTR, respectively. The PtAGP14 fragment carrying XbaI and SacI restriction enzyme sites and the AtAGP14 fragment carrying XbaI and KpnI restriction enzyme sites were separately subcloned into the pSM3 binary vector harbouring the duplicated-enhancer cauliflower mosaic virus 35S promoter (2×35S promoter) (Datla et al., 1993). All primer sequences employed are listed in Table 4.5.

The AtAGP14 promoter (1473 kb upstream of the start codon) was amplified from Arabidopsis genomic DNA, cloned into pDONR201, and sequenced. The upstream ATG was removed in a polymerase chain reaction (PCR) using a reverse primer matching the 5’ end of the cDNA. The PCR
product was subcloned into pDONR201 and sequenced. Finally, the \textit{AtAGP14} promoter was cloned into PMDC162 vector which harboured the β-glucuronidase (GUS) gene (Curtis and Grossniklaus, 2003). All primer sequences are shown in Table 4.5.

4.4.7  Plant transformation

Target binary constructs were transformed into \textit{Agrobacterium tumefaciens} GV3101 by the freeze-thaw method (Holsters et al., 1978). Positive clones were incubated in YEP media (5 g/L yeast extract, 10 g/L peptone, 5 g/L NaCl) until an OD\textsubscript{600} of 0.7 was attained. Arabidopsis plant transformation followed the commonly employed protocol (Bechtold et al., 1993; Clough and Bent, 1998). Positive Arabidopsis transformants were selected by growth on 1/2 Murashige and Skoog medium supplemented with 50 mg/L kanamycin for RNAi lines, and 20 mg/L hygromycin for overexpression lines.

Poplar transformation employed the leaf discs inoculation method (Coleman et al., 2008). Binary constructs carrying the target gene were transformed into \textit{Agrobacterium tumefaciens} EHA105 by the freeze-thaw method (Holsters et al., 1978). Positive clones were selected for incubation in liquid woody plant media (WPM) overnight (McCown and Lloyd, 1981). Large, fresh leaves from approximately one-month-old poplar were used for transformation. Leaves were cut into discs and cultivated in diluted WPM media containing \textit{Agrobacterium tumefaciens} for 1/2 hour at 28°C with gentle shaking (100 g). Following incubation, the leaf discs were gently dried with sterilized filter paper to remove the WMP media. The leaves were transferred, abaxial side up, to WPM media supplemented with 0.1 μM of 1-naphthaleneacetic acid (NAA), benzylaminopurine (BA), and thidiazuron (TDZ). After two days, the leaf discs were moved to WPM media with 0.1 μM of NAA, BA, TDZ, 250 mg/L cefotaxime, 500 mg/L carbenicillin, and placed in the dark for 3 days to kill the \textit{Agrobacterium tumefaciens}. The leaf discs successfully transfected were moved to WPM media with 0.1 μM of NAA, BA, and TDZ, 250 mg/L cefotaxime, 500 mg/L carbenicillin, 50 mg/L kanamycin or 25 mg/L hygromycin, and the plates were placed in subdued light for selecting positive shoots. After the shoots elongated, they were transferred to WPM media with 0.1 μM of NAA, 250 mg/L cefotaxime, 500 mg/L carbenicillin, 50 mg/mL kanamycin or 25 mg/mL hygromycin for rooting. Positive lines were identified by genomic DNA screening, sub-
cultured and multiplied. Positive RNAi lines were further confirmed by Q-RT-PCR as per the method described above. Positive Arabidopsis and poplar overexpression lines were separately verified with RT-PCR.

4.4.8 Assessment of plant growth

Tree height and caliper at the base of the stem were measured on five trees per construct and the appropriate controls on greenhouse-grown plants.

4.4.9 Histology

Nine week-old Arabidopsis stems were hand sectioned with a double-sided razor blade and used for histological assessment. Poplar stems were sectioned with a microtome to a thickness of 64 µm. Fresh sections were stained with 0.02% aqueous toluidine blue O for 5 minutes, and observed via a light microscope (Leica Microsystems Inc., Ontario, Canada).

4.4.10 GUS staining

AtAGP14 promoter activities were observed at the base of stem of transgenic plants. Arabidopsis inflorescence stems were hand sectioned with a double-sided razor blade, and submerged in a GUS staining solution containing 0.2 M NaPO₄, pH 7; 0.5 M EDTA, pH 8; 25 mM potassium ferricyanide, 25 mM potassium ferrocyanide, Triton X-100, and 20 mM X-gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronide) dissolved in methanol. The samples were incubated at 37 °C for 3-4 hours. The samples were cleared with chloral hydrate (Berleth and Jurgens, 1993), and well-stained samples were mounted on glass slide and observed with a light microscope (Leica Microsystems Inc., Ontario, Canada).

4.4.11 Fiber quality analysis

For Arabidopsis fiber length analysis, 5-8 mg samples from base of stem were placed in a 10 ml test tube containing a modified Franklin solution (1:1:1 30% hydrogen peroxide:glacial acetic acid; distilled water) (Franklin, 1945), and then incubated at 100 °C for 3 hours. The solution was decanted, the remaining materials were washed and shaken gently by hand until the fibers separated. For poplar fiber length analysis, 200 mg dry wood samples from base of stem were cooked in a modified Franklin solution (1:1 30% hydrogen peroxide: glacial acetic acid) in a 70 °C bath for 48 hours. The materials were washed with
distilled water and diluted into a Falcon tube. Fibers were separated by blending in a Waring mixer. Diluted fibers were directly analyzed on the fiber quality analyzer (FQA; OpTest Equipment Inc., Hawkesbury, Canada).

4.4.12 Cell wall chemistry

Cell wall carbohydrate compositions were analyzed by Klason as per Cullis et al (2004). Wood samples were ground to pass a 40-mesh in a Wiley mill and extracted overnight with acetone in a Soxhlet apparatus to remove extractives. 3 mL 72% (w/w) H₂SO₄ was added to 0.2 g samples and allowed to react on the bench for 2 hours with mixing every 10 minutes. The wood hydrolyzate was transferred to septa-sealed serum bottles, to which 112 mL of water was added and autoclaved for 1.5 hours at 121 °C together with the sugar controls. The hydrolyzate was removed and filtered through dry, pre-weighed, medium coarseness sintered-glass crucible to estimate the content of acid-insoluble lignin. Acid-soluble lignin was determined by absorbance at 205 nm on the Bio UV-visible spectrophotometer using quartz cuvettes (Varian, Inc., Australia). Carbohydrates were determined by using a Dx-600 anion exchange high-performance liquid chromatography (Dionex, Sunnyvale, CA, USA).

4.4.13 Holocellulose and alpha-cellulose analysis

Wood samples from base of poplar stems were ground to pass a 40-mesh in a Wiley mill and extracted overnight with acetone in a Soxhlet apparatus to remove extractives. Extract-free wood samples (195-200 mg) were used for holocellulose quantification. Wood samples were incubated at 50 °C for 14 to 16 hours after adding 3.5 mL buffer (60 mL of glacial acetic acid and 1.3 g NaOH per Liter) and 1.5 mL of 20% sodium chlorite solution. The reaction was quenched by placing it into an ice bath to cool. After removing the reaction solution by careful pipetting, the procedure was repeated. Finally, the reacted wood meal was transferred to a pre-weighed coarse sintered-glass crucible and washed twice with 50 mL of 1% glacial acetic acid and 10 mL acetone. The weight of the crucible was determined after drying, to quantify the holocellulose content.

Approximately 80-100 mg of holocellulose was used for alpha-cellulose isolation. Samples were reacted for 30 minutes after adding 8 mL of 17.5% NaOH. Eight mL of distilled water was added to the
samples and stirred for 1 minute. After 29 minutes, the retentate was filtered through a pre-weighed coarse sintered-glass crucible by washing with distilled water. The crucibles were soaked in 1.0 M acetic acid for 5 minutes and washed with distilled water. The dry weight of crucibles was used to quantify the alpha-cellulose contents.

4.4.14 Wood density

Measurements were taken from three separate samples. Bark to bark samples were first precision cut to 1.68 mm thickness with a twin blade pneumatic circular saw, and then extracted overnight in a Soxhlet apparatus with acetone. Wood density of samples was determined by X-ray densitometry (Quintek Measurement System Inc. Knoxville, TN, USA) using both radii (bark to bark) and averaged for each sample.

4.4.15 Statistical analysis

The statistical significance of any difference between experimental results was evaluated by the Student’s t-test to compare two means (SPSS v11 software, SPSS Inc., Chicago, USA). A value of $P < 0.05$ was considered statistically significant.
Chapter 5 Conclusion

5.1 Thesis summary

In the poplar secondary xylem, fiber and vessel are the primary cells. Prior to this research, it was known that AGPs were present in secondary xylem. For example, PtX3H6, PtX14A9, and PtaAGP6 were shown to be expressed in developing secondary xylem of loblolly pine (Loopstra and Sederoff, 1995; Zhang et al., 2003). However, functional roles for AGPs in xylem development, and more specifically fiber and vessel development were unknown. Therefore, the primary goal of this research was to investigate the contribution of highly expressed AGPs identified through transcriptome re-sequencing efforts in Populus trichocarpa (Geraldes et al., 2011) to xylem development. More specifically, this thesis examined the lysine-rich AGP18; AGP14, which is a peptide AGP; and the AGP9, which is a classical AGP. In this thesis, the phenotypes resulting from the mis-regulation of AGP genes demonstrated putative functional roles for two out of three secondary xylem AGPs: AGP18 is involved in fiber development in wood and AGP9 in vessel development.

The research in chapter 2 showed that AGP18 impacts fiber cell expansion in poplar. In the secondary xylem, fiber cells are initiated from xylem mother cells in the vascular cambium, and are characterized as the longest cell type, because of their mode of intrusive growth (Lev-Yadun, 2010). Here, I demonstrated that in poplar, suppression of PtAGP18 expression through RNA interference (RNAi-PtAGP18) led to shorter fibers. Similarly, atagp18 T-DNA mutants and RNAi-AtAGP18 Arabidopsis lines both had shorter fibers and oval-shaped interfascicular fibers. The PtAGP18 and AtAGP18 appear to be functionally equivalent, because the poplar PtAGP18 gene can complement the altered fiber shape and cell wall thickness phenotype observed in Arabidopsis atagp18 T-DNA mutants. The changes in fiber cell growth may result from the defects in fiber cell expansion in both the axial and radial dimensions. A general role for AGP18 in cell expansion was demonstrated by examining hypocotyl growth patterns after cultivation in the dark, because the hypocotyl growth has previously been shown to maintain constant rates of cell expansion (Gendreau et al., 1997).
This research demonstrated that *AGP18* plays a role in fiber cell expansion, however, the exact mechanism is yet unknown. One hypothesis is that AGPs may be involved in modulating extracellular calcium ion concentrations and calcium ion channel activities to regulate the cell shape (Lamport and Varnai, 2013; Pickard, 2013). This model was developed from experiments that showed when AGPs were precipitated by β-glucosyl Yariv reagent, a higher concentration of intracellular calcium ion was detected in tobacco BY-2 cells (Pickard and Fujiki, 2005). Moreover, the glucuronic acid residues in hydroxyl-polysaccharide side chains, which are decorated on to the hydroxyproline-glycosylation sites of the AGP18 protein backbone, are hypothesized to bind calcium ion in a pH-sensitive manner (Lamport and Varnai, 2013; Pickard, 2013). My bioinformatics query predicted that the AGP18 protein has 70% hydroxyproline-glycosylation sites, which are associated with the correct type of oligosaccharide side chains to contain glucuronic acids. This suggests that AGP18 may be able to bind to calcium ions, but further work is required to demonstrate this experimentally. Additionally, Zhang et al. (2011) also proposed a hypothesis that AtAGP18 may bind to plasma membrane ion channels via AGP side-chains (Zhang et al., 2011b). Finally, an alternative hypothesis is that AGP18 may affect fiber cell shape by indirectly modulating the microtubule array (see summary model, Figure 5.1A). Microtubules are abundant during fiber growth in trees (Chaffey et al., 1999) and during fiber cell expansion, and microtubules impact fiber cell shape by coordinating the orientation of the cellulose microfibrils (Funada, 2008). AGP18 located at the plasma membrane may indirectly affect the microtubule array to influence cell shape, via interactions with transmembrane proteins, as hypothesized by Sardar et al. (2006).
GPI anchor
protein backbone

interact with transmembrane protein
cross-link with cell wall polymers

fiber development

MT?

Part A
fiber cell fate

SND1 and NST1
transcription factors

AGP18

AGP14

Part B

vessel cell fate

VND-INTERACTING2 (VNI2)
hormones

VND7
transcription factor

AGP9
cell expansion

secondary cell wall

Programmed cell death

vessel development

Programmed cell death

AGP9

cell expansion

secondary cell wall

Programmed cell death
**Figure 5.1:** Photomicrograph in a cross section of *P. alba × P. grandidentata*, speculative functional model for AGP18 in fiber development (part A) and AGP9 in vessel development (part B). Fiber and vessel cells differentiate from xylem mother cells in the cambium of *P. alba × P. grandidentata*. Part A illustrates the hypothetical roles for AGP18 and AGP14 in fiber development. The black arrows in part A indicate the hypothesized mechanism on the roles of AGP18 in cell expansion. AGP18 may involve in cell expansion by indirectly affecting the microtubules (MT) array, via interactions with transmembrane proteins. The red arrows in part A show AGP18 may affect fiber secondary cell wall development by cross-linking with cell wall polymers. Part B represents the hypothetical mechanism for AGP9 in vessel development. Yellow arrows in part B indicate the hypothesized mechanism on the roles of AGP9 in vessel cell fate and vessel cell expansion. Bold words in part A and part B signify the roles of AGP18 and AGP9 in fiber and vessel development, respectively. Blue circles indicate arabinogalactan carbohydrate side-chains. CA: cambium. MT: microtubules.
The phenotypes for poplar with mis-regulated AGP18 not only showed changes in fiber morphogenic growth, but also in fiber secondary cell walls. Once fiber cell expansion has ceased, the secondary cell wall is thickened and this is followed by programmed cell death. It has been shown that the development of fiber secondary cell walls are transcriptionally regulated by the Secondary Wall-Associated NAC Domain Protein1 (SND1) and NAC Secondary Wall Thickening Promoting Factor1 (NST1) in Arabidopsis, poplar, and the legume Medicago truncatula (Zhong et al., 2006; Mitsuda et al., 2007; Zhong et al., 2007; Zhao et al., 2010; Zhong et al., 2010). Here, AGP18 appeared to affect the fiber cell wall thickening, because down-regulation of AGP18 in Arabidopsis and poplar led to thinner cell walls, respectively. Biochemically, the lignin content in the transgenic poplar and Arabidopsis lines was decreased, but without an observable change in cell wall carbohydrate components. Lysine-rich AGP18, located at the plasma membrane, is proposed to cross-link with cell wall components to regulate fiber cell wall development (Figure 5.1A). Cell wall polymers have been shown to cross-link after secretion, such as hemicelluloses binding to the cellulose via hydrogen bonding (Somerville et al., 2004), while pectin is cross-linked to itself by Ca$^{2+}$ bridges (Fry, 1986), and pectin and xyloglucan can be covalently linked together in primary cell walls (Popper and Fry, 2008). Secondary walls deposit inside the primary walls, ultimately forming three layers and lignify into the cell wall matrix through various intermolecular linkages (Zhang et al., 2011). The cross-linked polymers can stabilize and stiffen cell walls. For example, defects in cellulose and xylan biosynthesis can lead to collapsed cell phenotypes and thinner cell walls (Taylor et al., 1999; Taylor et al., 2000; Desprez et al., 2002; Persson et al., 2007a). AGPs have been reported to cross-link with cell wall polymers. Type II arabinogalactan, as the main carbohydrates of AGPs, can cross-link with β-1,4-xylan backbone and contributes to secondary cell wall thickening (Northcote et al., 1989; Kwan and Morvan, 1995). Moreover, APAP1 was recently shown to cross-link with xylan and pectin (Tan et al., 2013). Specifically, the cross-linking cell wall components, xylan, rhamnogalacturonan I (RG-I), and homogalacturonan (HG), were easily extracted in the apap1 T-DNA insertion mutant, owing to their defective attachment with the APAPI carbohydrate chains. However, plant growth and the total cell wall carbohydrate compositions were not significantly altered (Tan et al.,
This research and previous evidence together permits the formulation of a hypothesis that suggests that AGP18 affects fiber cell wall development by cross-linking with cell wall polymers.

In parallel, AGP14, which is an AGP peptide, appears to act as a cross-linker to contribute to cell wall formation in poplar (Chapter 4). Biochemically, decreases in xylose content and increases in mannose content were detected in RNAi-PtAGP14 poplar lines, while overexpression of AtAGP14 in poplar resulted in lower mannose content. AGP14 is rich in carbohydrate side chains decorating a short protein backbone, and AGP14 is predicted as a GPI anchor protein which locates at the cell surface. Possibly, AGP14 acts within the carbohydrate matrix to supply a platform to cross-link with cell wall polymers for facilitating cell wall formation.

This research also revealed a role for PtAGP9 and AtAGP9 in xylem vessel development (Chapter 3). The classical PtAGP9 which is rich in Populus trichocarpa developing xylem, and an ortholog of AtAGP9 was previously reported to be expressed during cell expansion in developing xylem of poplar by microarray analysis (Hertzberg et al., 2001). To elucidate the role(s) of AGP9 in xylem development, the expression of PtAGP9 was suppressed in poplar by post-transcriptional gene silencing (RNAi-PtAGP9). Phenotypically, the RNAi-PtAGP9 poplar lines had smaller and more vessels than wild type, while overexpression of AtAGP9 in poplar led to larger and fewer vessels. The vessel phenotypes are not due to changes in cell wall traits, as neither the RNAi-PtAGP9 poplar lines nor AtAGP9 overexpressing poplar lines displayed any other detectable traits inconsistent with wild type. Normal cell wall traits may be ascribed to the dominance of fibers in this tissue, and the normal development of fibers in the transgenic AGP9 poplar lines.

Altered vessel number suggests that AGP9 plays a role in vessel differentiation. Vessel cell fate, like the fiber cells, is specified in xylem mother cells, which are produced by periclinal divisions of fusiform initials in the cambium (Mellerowicz et al., 2001; Samuels et al., 2006). Vessel differentiation is positively regulated by the transcription factors Vascular-Related NAC Domain Protein6 and 7 (VND6, VND7) (Ohashi-Ito et al., 2010; Yamaguchi et al., 2010; Yamaguchi et al., 2011; Ohtani et al., 2011), and hormones, such as auxin (Aloni and Zimmermann, 1983; Tuominen et al., 1997; Nilsson et al., 2008),
cytokinin (Mahonen et al., 2006), and brassinosteroids (Yamamoto et al., 1997; Yamaguchi et al., 2010). Furthermore, hormones have been shown to induce the expression of VND6 and VND7 to affect vessel differentiation (Ohashi-Ito et al., 2010; Yamaguchi et al., 2010; Yamaguchi et al., 2011). Consequently, based on the changed vessel number phenotype observed in transgenic poplar lines, it is hypothesized that the GPI-anchored AGP9 on the cell surface may somehow contribute to the cell’s ability to respond to hormone signals that regulate vessel cell differentiation (Figure 5.1B). Another possibility is that AGP9 may interact with transmembrane proteins, such as receptor kinases, to affect vessel development (Figure 5.1B). Transmembrane receptor kinases play important roles in cell differentiation and are proposed to be sensors of cell wall integrity (De Smet et al., 2009; Steinwand and Kieber, 2010). Abundant receptor-like kinases can be detected in developing xylem, when isolated poplar plasma membrane proteins from developing xylem were analyzed by mass spectrometry-based proteomics (Song et al., 2011).

Once vessel cell fate is determined in the cambium, the size of vessels rapidly increases, during this process, vessels need to adjust their cell wall plasticity to expand (Ray et al., 1972) and AGP9 could influence this process. Vessel expansion occurs by building turgor pressure through osmotically active ions (Fromm, 2010), and by loosening the cell wall (Cosgrove, 2005). Additionally, microtubules are present in the developing vessels of angiosperm trees, and microtubules appear to correlate with the orientation of cellulose microfibrils (Abe et al., 1995; Chaffey et al., 1999). Therefore, microtubules can also affect vessel cell expansion through coordinating with orientation of cellulose microfibrils in primary cell walls (Funada, 2008). I hypothesize that AGPs may indirectly affect the cytoskeleton to regulate cell expansion (Figure 5.1B). Supportive evidence for an important association between AGPs and the cytoskeleton is that disorganized microtubule organization was apparent when AGPs were cross-linked by β-glucosyl Yariv reagent in tobacco BY-2 suspension cells and Arabidopsis roots (Sardar et al., 2006; Nguema-Ona et al., 2007). The connection between these extracellular AGPs and cytoskeleton is not known, but another hypothesis is that unknown transmembrane proteins with kinase activity could link both cellular components.
In conclusion, this research demonstrated different functional roles for three AGPs in wood development. AGP18 has a role in fiber cell expansion and fiber secondary cell wall thickening. In parallel, AGP14 may also play roles in cell wall development. Moreover, AGP9 affects vessel differentiation and vessel expansion. The AGP9 findings might be considered surprising since the JIM13 antibody label was weak in vessels, but JIM13 binds galactan side chains found on Hyp-polysaccharides, which are relatively low in AGP9 (22% Hyp-polysaccharides compared to AGP18 with 70%) (Table 2.1). Therefore, JIM13 immunolabelling might only have demonstrated a subset of the AGPs expressed in developing xylem, i.e. those with low galactan content. The expression levels of the various AGPs and distinct AGP patterns detected by JIM13 antibody and β-glucosyl Yariv reagents in developing poplar xylem suggests that the function of AGPs could be cell-type specific.

5.2 Future works

This research demonstrated unique roles for the different AGPs in wood development. However, the functional mechanisms explaining how these AGPs act in woody cell development are not known. Future work may consider addressing the questions listed below:

5.2.1 The specific location of AGP18 in xylem

My research clearly shows the expression of PtAGP18 in developing poplar xylem, but the antibodies and Yariv reagent do not identify the specificity of the AGP bound. To resolve the location of PtAGP18 in a specific cell type such as in fibers, an antibody that labels the lysine-rich domain of PtAGP18 could be made. Similarly, in Arabidopsis, the location of AtAGP18 in fibers could be tested with a similar specific antibody, which has been produced by Yang and Showalter (2007a), and kindly provided by Professor Showalter. Moreover, the specific antibody could also be used to detect the spatial localization pattern of AtAGP18 during fiber elongation in Arabidopsis florescence stem. Stems harvested following the defined growth stages of Arabidopsis identified by Hall and Ellis (2013), and then sectioned and immunolabelled with the antibody should be observed under confocal microscopy to elucidate the location of the epitope.
5.2.2 AGP18 in cell expansion

AGP18 may be involved in modulating extracellular calcium ion to regulate cell expansion. During my thesis, I found preliminary data to show that pollen tubes were significantly shorter in atagp18 mutants relative to wild type, when in vitro pollen tube germination was induced. However, after supplying 1 mM calcium ion to the media, it significantly induced pollen tube growth in the atagp18 mutants compared with wild type, whereas the growth of pollen tubes in the wild-type plants was retarded. In the future, the interaction(s) of AGP18 and calcium ion in cell expansion should be addressed in detail. Pollen tubes may be a suitable model system to conduct this research. It has been shown that the growth of a pollen tube can be affected by pectin and calcium ion (Rojas et al., 2011). Calcium ion binds to the de-esterified pectin residues, and higher concentrations of calcium ion in the extracellular media results in less uncross-linked pectin, which in-turn leads to the cessation of the cell expansion process (Rojas et al., 2011). Therefore, immunolabelling with specific antibodies for pectin could be employed to detect whether or not there are potential phenotypic changes in pollen tube cell wall after treatment with calcium ion in atagp18 mutants. It is possible that AGP18 may modulate the calcium ion to intervene with cell wall polymers such as pectin to regulate cell expansion.

Another hypothesis is that AGP18 may affect the fiber cell shape by influencing the cytoskeletal array, such as microtubules that have determinant roles in cell shape and are rich in developing fibers in poplar (Monshausen and Gilroy, 2009; Kaneda et al., 2010; Hamant et al., 2010). During secondary vascular cambium initial expansion and morphogenesis, microtubules show distinct array patterns (Samuels et al., 2006). AGP18 acts in cell shape formation in Arabidopsis interfascicular fibers, thus I hypothesize that AGP18 influences early development of fiber differentiation from parenchyma cells in Arabidopsis by affecting the cortical microtubule array. A direct link between the extracellular AGP and cortical microtubules inside the plasma membrane has never been reported. However, treatment of tobacco BY-2 suspension cells and Arabidopsis roots with the AGP cross-linker β-glucosyl Yariv reagent led to disorganized microtubule organization (Sardar et al., 2006; Nguema-Ona et al., 2007). Future work may consider testing the effects of AGP18 in the microtubule array in Arabidopsis. The stems of atagp18
T-DNA mutant carrying yellow fluorescent protein (YFP)::α-tubulin fusions driven native promoter could be generated and followed during the defined developmental stages suggested by Hall and Ellis (2013). In parallel, AGP9 is hypothesized to affect vessel cell expansion via the cytoskeleton network. Therefore, the same technique could be employed to learn whether or not loss-of-function of AGP9 leads to the abnormal array of microtubules in atagp9 T-DNA mutants.

5.2.3 AGP18 in fiber cell wall

This research demonstrated that AGP18 affects the fiber cell wall thickness. The thickened secondary cell wall is rich in cellulose and hemicellulose, while xylan and mannan are the main forms of hemicellulose in the secondary cell wall. Moreover, xylan, mannan, and glucomannans were abundant in fiber secondary cell wall detected by immunofluorescence localization (Kaneda et al., 2010; Kim and Daniel, 2012; Kim et al., 2012). However, this research did not show significant changes in the cell wall traits in transgenic poplar trees and Arabidopsis plants. Since the overall composition did not change, I hypothesize that AGP18 can strengthen the cell wall by cross-linking with cell wall polymers, similar to the role of APAP1. apap1 mutants did not have altered cell wall traits, however, cell wall polymers were easily extractable in apap1 T-DNA mutants (Tan et al., 2013). Therefore, the cross-linking of AGP18 with cell wall components in transgenic poplar and Arabidopsis lines could be tested following the approach employed by Tan et al. (2013). Specifically, the fresh developing xylem from transgenic RNAi-PtAGP18 lines and fresh Arabidopsis samples from atagp18 T-DNA mutants together with the wild-type poplar trees and wild-type Arabidopsis could be separately collected to prepare the alcohol-insoluble cell wall residues. The total sugars, which are separately extracted by increasingly harsh reagents (Tan et al. 2013), could then be identified using an anion exchange high-performance liquid chromatography. Finally, based on the monosaccharide composition in various cell wall extractions, the extractable cell wall polymers could be determined. This research has shown that there is no significant change in total cell wall carbohydrate compositions. Therefore, if there are significant differences in monosaccharide compositions in Arabidopsis atagp18 mutant plants and RNAi-PtAGP18 poplar lines compared to the controls after different solvent treatments, it would demonstrate how the corresponding cell wall polymers
are cross-linked with AGP18. Similarly, a potential role for AGP14 in cell wall development may be detected using this approach.

5.2.4 AGP9 in hormone signalling

Based on the observed vessel phenotype in the transgenic poplar lines, AGP9 is proposed to affect vessel differentiation, perhaps by influencing the hormone signal perception systems. Vessel differentiation can be affected by auxin and cytokinins (Aloni and Zimmermann, 1983), but it is not known how AGP9 could interact with the hormone perception systems. Since poplar RNAi-PtAGP9 lines have more vessels (Figure 3.5), it would imply more auxin is present or the xylem cells are more sensitive to auxin. In contrast, overexpression lines had fewer vessels (Figure 3.8), so I hypothesize less auxin or reduced auxin sensitivity. Future work could explore the regulatory roles of hormones in the function of AGP9 during vessel differentiation by exogenously supplying hormones to transgenic trees following the approach of Bjorklund et al. (2007) for influencing the secondary growth resulting from the vascular cambium. Specifically, exogenous hormones, such as auxin and the polar auxin efflux inhibitor 1-N-naphthylphthalamic acid (NPA), could be carried by lanolin paste and separately applied to decapitated poplar RNAi-PtAGP9 and AtAGP9 overexpressing lines at the site of vascular cambium initiation, which is above the youngest fully expanded leaf at the top of each stem. After three to four weeks, samples could be harvested approximately 0.5-1 cm below the decapitation position and vessel morphological changes in developing xylem observed. If AGP9 responds to hormones to regulate vessel development, after supplying exogenous auxin, it may lead to more vessels in RNAi-PtAGP9 lines compared to the control, or to fewer vessels in AtAGP9 overexpressing lines relative to the corresponding control. However, by applying NPA, it may have a reverse effect in the transgenic poplar trees. The other possibility is that no changes in vessel development appear after applying the exogenous hormones, it indirectly suggests that the role of AGP9 may be affected by transcription factors, such as VND7.

5.2.5 AGP9 in vessel cell wall traits

AGP9 may affect vessel cell wall development. However, biochemically AGP9 has no effect on cell wall traits. It may be due to the fact that the total cell wall constituents from the transgenic poplar lines were
used for cell wall chemistry analysis and fibers are dominant in the secondary xylem, and the
development of fibers are normal in transgenic poplar trees. Thus, these results may not reflect the vessel
cell wall characteristics. Since xylan and glucomannans can be detected (by immunolabelling) in the
secondary cell wall of vessels, especially in the S2 layer (Kim and Daniel, 2012; Kim et al., 2012), future
work should consider using immunolabelling techniques to characterize vessel cell wall in transgenic
poplar RNAi-PtAGP9 and AtAGP9 overexpressing lines with greater resolution. LM10 or LM11
monoclonal antibody for xylan, and LM21 or LM22 antibody for mannan could be employed in concert.

5.2.6 AGP9 in interfascicular fiber differentiation in Arabidopsis

In chapter 3, I also found that AGP9 affects fiber differentiation in Arabidopsis. In Arabidopsis, fibers are
differentiated from parenchyma cells and normal differentiation of fibers from parenchyma cells includes
intrusive fiber growth. In the interfascicular fiber mutant (ifl1), when the interfascicular fiber
differentiation was blocked from parenchyma cells, the shapes of cells were rectangular rather than
containing two sharp tips (Zhong et al., 1997). Given that the activity of AtAGP9 promoter can be
detected in endodermis and parenchyma cells near the endodermis, it indicates that AtAGP9 may impact
fiber differentiation at an early stage. Therefore, stem longitudinal sections could be employed to further
test the possible differentiation defects, over a developmental gradient, on the fibers of the atagp9
mutants and RNAi-AtAGP9 Arabidopsis lines via light microscopy.

In summary, the main goal of this thesis was to investigate the functional role(s) of three AGPs in
secondary xylem development. Based on the phenotypic changes observed when the target genes were
down-regulated in transgenic poplar trees and Arabidopsis T-DNA mutant analyses, I am now able to
show a role for AGP18 in fiber development, both cell expansion and secondary cell wall formation
(Chapter 2). Moreover, the poplar PtAGP18 gene and AtAGP18 gene appear to be functionally
equivalent, because the alter fiber shape and cell wall thickness phenotypes in Arabidopsis atagp18 T-
DNA mutants can be complemented by PtAGP18 (Chapter 2). I also showed that the AGP9 affects xylem
vessel differentiation, and vessel cell expansion (Chapter 3). Furthermore, AGP14 may affect cell wall
formation in poplar (Chapter 4). Collectively, AGP18 and AGP9 affect the development of fibers and vessels in poplar, respectively.


Altaner, C.M., Tokareva, E.N., Jarvis, M.C., and Harris, P.J. (2010). Distribution of (1 -> 4)-beta-galactans, arabinogalactan proteins, xylans and (1 -> 3)-beta-glucans in tracheid cell walls of softwoods. Tree Physiology 30, 782-793.


Appendices

Appendix A  Supplemental data for chapter 2

A.1 *PtAGP18* transcript abundance in transgenic RNAi-*PtAGP18* poplar plants as determined by quantitative real-time RT-PCR. Leaf transcript levels are based on the comparative threshold cycle value relative to the housekeeping gene (actin). *PtAGP18* was down-regulated in transgenic lines (6, 7, 12, 14, and 15) relative to wild-type (WT) levels. Bars represent the standard error of the mean (n = 3).

A.2 Leaf transcript abundance of transgenic poplar plants overexpressing the Arabidopsis *AGP18* (2×35S::AGP18) as determined by RT + PCR. *AtAGP18* transcripts can be detected in transgenic poplar lines (1, 2, 4, and 10), but not in wild type (WT).
A.3 *AtAGP18* transcript abundance in transgenic *RNAi-AtAGP18* Arabidopsis plants as determined by quantitative real-time RT-PCR. Leaf transcript levels are based on the comparative threshold cycle value relative to the housekeeping gene elongation factor *AtELF5A* (AT5G60390). *AtAGP18* was down-regulated in transgenic lines (2 and 4) relative to wild-type (WT) levels. Bars represent the standard error of the mean (n = 3).

![Graph showing transcript abundance](image)

A.4 Leaf transcript abundance of transgenic Arabidopsis plants overexpressing the poplar *AGP18* (2×35S::AGP18) as determined by RT + PCR. *PtAGP18* transcripts can be detected in transgenic Arabidopsis lines (1, 5, 6, and 7), but not in wild type (WT).

![Image of gel electrophoresis](image)
Appendix B Supplemental data for chapter 3

B.1 Real-time RT-PCR analysis of PtAGP9 transcript abundance in leaf tissue of RNAi-PtAGP9 transgenic poplar plants. Transcript levels of PtAGP9 are based on the comparative threshold cycle value relative to actin gene. PtAGP9 was down-regulated in transgenic lines (2, 3, 9, and 10) compared to wild-type (WT) levels. Bars represent the standard error of the mean (n = 3).

B.2 Leaf transcript abundance of transgenic poplar plants overexpressing the Arabidopsis AGP9 (2×35S::AGP9) as determined by quantitative real-time RT-PCR. AtAGP9 transcript levels are based on the comparative threshold cycle value relative to actin gene. AtAGP9 transcripts are apparent in transgenic lines (2, 3, 6, 7, and 8). Bars represent the standard error of the mean (n = 3).
B.3 *AtAGP9* transcript abundance in *RNAi-AtAGP9* transgenic Arabidopsis leaves as determined by quantitative real-time RT-PCR. *AtAGP9* transcript levels are relative to the housekeeping gene elongation factor *AtELF5A* (AT5G60390). Compared to the expression level of *AtAGP9* in wild-type plants (WT), *AtAGP9* was down-regulated in *RNAi-AtAGP9* transgenic plants. Bars represent the standard error of the mean (n = 3).

B.4 RT + PCR results of *PtAGP9* expression in leaf tissue of *PtAGP9* overexpressing Arabidopsis plants (3, 5, and 9) and wild type (WT). The expression level of *PtAGP9* can be detected in transgenic lines, but not in wild type.
Appendix C  Supplemental data for chapter 4

C.1 PtAGP14 transcript abundance in leaves of RNAi-PtAGP14 transgenic poplar plants as determined by quantitative real-time RT-PCR. PtAGP14 transcript levels are based on the comparative threshold cycle value relative to actin gene. Transgenic polar lines 5, 6, 21, and 22, show PtAGP14 down-regulation compared to wild-type (WT) level. Bars represent the standard error of the mean (n = 3).

C2 RT + PCR results showing the expression levels of AtAGP14 in leaf tissue of AtAGP14 overexpressing poplar plants. AtAGP14 was expressed in transgenic poplar lines 2, 6, 7, 11, 12, 13, and 17, which is absent in the wild-type (WT) plants.
C3 *AtAGP14* transcript abundance in the leaves of RNAi-AtAGP14 transgenic Arabidopsis plants as determined by quantitative real-time RT-PCR. *AtAGP14* transcript levels are based on the comparative threshold cycle value relative to elongation factor *AtELF5A*. *AtAGP14* was down-regulated in Arabidopsis transgenic lines 1 and 2, relative to wild type (WT). Bars represent the standard error of the mean (n = 3).

C4 *PtAGP14* expression level in leaf tissue of Arabidopsis plants overexpressing *PtAGP14* as assessed by RT + PCR. The expression level of *PtAGP14* is shown in transgenic lines 1, 2, and 4, but absent in wild-type (WT) plants.