INVESTIGATING THE ROLE OF CELLULOSE SYNTHASES IN THE BIOSYNTHESIS AND PROPERTIES OF CELLULOSE IN SECONDARY CELL WALLS

by

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Abstract

Cellulose synthases are the enzymes responsible for the production of cellulose in plant cell walls. Mutations in any one of the Arabidopsis cellulose synthase (CesA) AtCesA4, AtCesA7, and AtCesA8 genes cause plants to develop collapsed xylem as a result of reduced cellulose content, demonstrating their critical role in secondary cell wall biosynthesis. A thorough characterization of the growth, cell wall properties, and cellulose ultrastructure of the AtCesA4irx5-1, AtCesA7irx3-1, and AtCesA8irx1-1 mutants, presented herein, is the first report of the changes to cellulose microfibril angle, cell wall crystallinity, and cellulose degree of polymerization (DP) in these mutants. This study suggests that the non-redundant functions of individual CesAs may be related to CesA-specific thresholds required for the formation of a cellulose synthesizing complex (CSC), and CesA-specific roles in regulating crystallinity and DP. Additionally, the results illustrate the importance of a fully formed CSC in regulating cellulose microfibril angle.

By identifying and characterizing three new CesA genes from spruce (Picea glauca), PgCesA1, PgCesA2, and PgCesA3, which are homologous to the Arabidopsis AtCesA8, A4, and A7 and the Populus trichocarpa PtiCesA8-A, A4, and A7-A genes, respectively, the degree of functional conservation among AtCesA homologs was explored. Expression of PgCesA1 or the PtiCesAs in AtCesAirx plants rescued the collapsed xylem phenotype, thus demonstrating for the first time that orthologs of AtCesA4, A7, and A8 have conserved functions.

Lastly, in planta techniques were used to measure interactions between AtCesAs to investigate if specific and consistent interactions exist. The results suggest that CesA8 and A4 can form homodimers in planta, and that there might be weak or transient interactions between AtCesA7-A4 and AtCesA7-A8.

Collectively, the results presented suggest, indirectly, an unequal ratio of CesA subunits (AtCesA4:A7:A8) is required for proper cellulose biosynthesis, and that each CesA likely has a unique function which ultimately affects cellulose properties such as cell wall crystallinity and DP. Our conclusions shed new light on the role of CesAs in cellulose biosynthesis in secondary cell walls and elicit questions about the current model of CSC form and function.
Preface

This thesis contains three chapters written with the intent of publication in peer-reviewed journals.

Chapter 2: Lisa McDonnell designed the research project, performed the research, conducted data analysis, and prepared the manuscript. Laura Haley and Ilga Porth assisted in performing research. Shawn Mansfield was involved with design of the research program, providing research opportunity, and editing the manuscript.

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Chapter 4: Lisa McDonnell designed the research project, performed the research, conducted data analysis, and prepared the manuscript. Shawn Mansfield was involved with design of the research program, providing research opportunity, and editing the manuscript.
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AI</td>
<td>acid insoluble lignin</td>
</tr>
<tr>
<td>AS</td>
<td>acid soluble lignin</td>
</tr>
<tr>
<td>BiFC</td>
<td>bimolecular fluorescence complementation</td>
</tr>
<tr>
<td>BRET</td>
<td>bioluminescent resonance energy transfer</td>
</tr>
<tr>
<td>CesA</td>
<td>cellulose synthase</td>
</tr>
<tr>
<td>CSC</td>
<td>cellulose synthesizing complex</td>
</tr>
<tr>
<td>CSR</td>
<td>class-specific region</td>
</tr>
<tr>
<td>DCB</td>
<td>2, 6-dichlorobenzonitrile</td>
</tr>
<tr>
<td>DP</td>
<td>degree of polymerization</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GPI</td>
<td>glycosylphosphatidylinositol</td>
</tr>
<tr>
<td>GX</td>
<td>Glucuronoxylan</td>
</tr>
<tr>
<td>HVR</td>
<td>hyper-variable region</td>
</tr>
<tr>
<td>IRX</td>
<td>irregular xylem</td>
</tr>
<tr>
<td>KOR</td>
<td>Korrigan</td>
</tr>
<tr>
<td>LUC</td>
<td>Luciferase</td>
</tr>
<tr>
<td>MAP</td>
<td>microtubule associated protein</td>
</tr>
<tr>
<td>MASC</td>
<td>microtuble-associated cellulose synthase compartment</td>
</tr>
<tr>
<td>MF</td>
<td>Microfibril</td>
</tr>
<tr>
<td>MFA</td>
<td>microfibril angle</td>
</tr>
<tr>
<td>MT</td>
<td>Microtubule</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>real-time PCR</td>
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<tr>
<td>SmaCC</td>
<td>small CesA compartment</td>
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<tr>
<td>SUSY</td>
<td>sucrose synthase</td>
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<td>UDP</td>
<td>uridine diphosphate</td>
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<tr>
<td>WT</td>
<td>wild-type</td>
</tr>
<tr>
<td>YFP</td>
<td>yellow fluorescent protein</td>
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Dedication

I dedicate this thesis to my family.
CHAPTER 1 Introduction

1.1 Overview

The inherent properties of the secondary cell walls of woody tissue ultimately affect the final properties of wood. Thus, understanding the molecular and mechanical mechanisms governing cellulose biosynthesis is an important and intriguing area of research. The research described herein focuses on the process of cellulose production in the secondary cell wall, with an emphasis on the cellulose synthase enzymes responsible for its biosynthesis. Elucidating the roles of cellulose synthase enzymes in regulating cellulose properties not only offers a means to understand the fundamentals of plant and wood cell wall development, but also may offer a unique means to improve one of the most abundant industrially used natural polymers.

1.2 The cell wall

The natural structure and chemistry of plant cell walls allow cells to expand, become rigid, provide immense structural support, allow water to be transported great distances, and protect plants from pathogens. The diversity of cell wall structures and functions immediately signifies the complex nature of cell wall biosynthesis. Of interest in the research presented in this thesis is the secondary cell wall formation of xylem and fibres. However, this does not undermine the importance of the work done on primary wall synthesis, which provides the scaffold for the process of secondary cell wall formation. Primary cell walls differ from secondary walls in both composition and function. Both primary and secondary walls contain a great deal of cellulose and polysaccharides. However, the hemicellulose found in primary walls of dicots is mostly xyloglucans, whereas xylans are the predominant hemicellulose in secondary walls (Mellerowicz and Sundberg, 2008). Additionally, primary walls contain significantly more pectins and proteins compared to secondary walls (Cosgrove, 2005). In general, primary walls are thinner and more flexible, allowing for cell expansion. In contrast, secondary walls are thick, rigid, and lignified.

Hemicelluloses are heteropolymeric polysaccharides, and most commonly include xyloglucans, xylans, mannans, and glucomannans in vascular plants.
Xyloglucan is the dominant hemicellulose in primary walls of dicots (Scheller and Ulvskov, 2010), composed of 1,4-β-linked glucan chains with frequent integrations of xylosyl residues and side chains composed of xylosyl, galactosyl, and fucose (Liepman et al., 2010). Glucuronoxylan (GX), a 1, 4-β-linked xylan is an example of a hemicellulose found in Arabidopsis and woody plants (Liepman et al., 2010), and is the most common hemicellulose in secondary walls of angiosperms (Scheller and Ulvskov, 2010). Mannans (e.g. glucomannan) are also found in dicot secondary cell walls (Mellerowicz et al., 2001) including those of Arabidopsis xylem and fibres (Handford et al., 2003) and several functional mannann-synthases have been identified in Arabidopsis (Liepman et al., 2005).

Hemicelluloses form a cross-linking network between cellulose microfibrils (Cosgrove, 2005), which can be modified during cell development (Cosgrove, 2005; Scheller and Ulvskov, 2010). In secondary cell walls, the most likely function of hemicellulose appears to be associated with load-bearing capacity and structural support. Some Arabidopsis irregular xylem mutants, with reduced xylan content and alterations to xylan backbone structure, exhibit thinner secondary cell walls and collapsed xylem, illustrating the role of xylans in providing structural integrity to the walls of fibres and vessels (Zhong et al., 2005; Brown et al., 2005; Brown et al., 2007). For example, the putative xylan glycosyltransferases FRA8, IRX8, and IRX9 were shown to be co-expressed with Arabidopsis secondary cell wall biosynthetic genes (Brown et al., 2005). Changes to GX properties in these mutants suggested that IRX9 might be involved in GX chain elongation, whereas FRA8 and IRX8 might be involved in the addition of glucuronic acid residues to the GX reducing ends (Zhong et al., 2005; Brown et al., 2007). Cross sections of stems of irx8 plants revealed an early onset of collapsed xylem, compared to the AtCesA6^{irx1-1} mutant, and a double irx8- AtCesA8^{irx1-1} mutant exhibited a more severe irx-like phenotype, emphasizing the importance of xylans in maintaining cell wall structural integrity especially in cellulose deficient mutants (Persson et al., 2007a). Recently, Wu et al. (2010) reported redundant and partially redundant functions of some glycosyltransferases involved in xylan chain elongation. With such a diversity of hemicelluloses found in plant cell walls, it is not surprising that the hemicellulose glycosyltransferase gene families are extremely large, and that the
functions of the coded enzymes are diverse, including (but likely not limited to) chain elongation, side-chain addition, and reducing end synthesis (reviewed by Scheller and Ulvskov, 2010).

Lignins are another heteropolymer found in secondary cell walls, which are composed of phenylpropanoid subunits assembled in a random amorphous macromolecule. Cross-linkages between lignin and hemicelluloses, or lignin-carbohydrate complexes (Jeffries, 1990) likely add additional stiffness to plant cell walls. A correlation between lignin content, wood density, and stiffness has been reported along with reductions in stiffness in transgenic trees with reduced lignin content (Bjurhager et al., 2010). The hydrophobic nature of lignins likely enhances the capacity for vessels and tracheids to transport water. Lignin polymers are composed of alcohol monomers: p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol, which produce “H”, “G”, and “S” lignins, respectively (Boerjan et al., 2003). Analysis of mutants with defects in lignin biosynthesis has played an important role in elucidating some of the key steps in the lignin synthesis pathway (for a review see Boerjan et al., 2003). Similar to plants with defects in polysaccharide biosynthesis, lignin-deficient mutants often exhibit collapsed xylem phenotypes, highlighting the importance of lignin in cell wall integrity. For example, the irx4 mutant of Arabidopsis (a cinnamoyl-CoA reductase mutant), which contains up to 50% less lignin than wild-type plants, exhibits stunted growth, and collapsed xylem (Jones et al., 2001). The ref8 mutant of Arabidopsis (a p-coumarate 3-hydroxylase, C3’H, mutant) also exhibits reduced lignin content, and consequently collapsed xylem, but additionally it was found that the lignin produced in the mutant varied in composition compared to wild-type plants (Franke et al., 2002) illustrating how changes to lignin are very dependent on the step within the biosynthetic pathway that is altered. Cell wall defects as a result of reduced lignin are not limited to Arabidopsis. For example, lignin reductions in hybrid poplar, the result of RNAi-suppression of p-coumaryl-3’-hydroxylase caused a collapsed xylem phenotype in tree stems and an increase in susceptibility to vessel cavitation (Coleman et al., 2008). Some lignin-deficient mutants also exhibit a concomitant increase in cellulose (Coleman et al., 2008a; Hu et al., 1999), suggesting potential compensatory feedback between the regulation of lignin and cellulose biosynthesis.
Cellulose is the most abundant polymer in secondary cell walls, as well as a large component of primary cell walls next to pectins (Mellerowicz et al., 2001). Cellulose is a homopolymer composed of 1, 4-β-linked glucose molecules. Cellulose is polymerized from the UDP (uridine diphosphate)-glucose precursor, that can originate from the phosphorylation of free glucose, or by the action of sucrose synthase to produce UDP-glucose and fructose from UDP and sucrose. The long chains of cellulose can lie parallel to one another and form hydrogen bonds between chains, forming strong and often crystalline microfibrils (Delmer and Amor, 1995; Saxena and Brown, 2005). The rigidity and hydrophobicity of crystalline cellulose provides structural support to cell walls and aids in the ability of xylem vessels to transport water.

Two crystalline forms of cellulose exist in nature, designated as allomorphs I and II. Cellulose chains are aligned in parallel or anti-parallel in cellulose I and II, respectively. Allomorph II has additional hydrogen bonding, resulting in a more thermostable form of cellulose (Brown, 2004). Cellulose II is found in some algae and bacteria, but allomorph I is the most common form and found in vascular plants. The parallel alignment allows for the microfibril structure to form (Brown 2004). Cellulose I has two additional sub-forms: Iα and Iβ. Iα and Iβ cellulose have different states of crystal packing, such that the crystalline unit cell of Iα is triclinic, whereas Iβ is monoclinic. Triclinic refers to a unit cell with three axes (in the x, y, and z plane of a three dimensional unit cell) that are of different lengths, whereas monoclinic unit cells have at least two axes that are the same length but differ from the third axis. The differences in crystal packing result in varied hydrogen bonding between chains in the Iα and Iβ forms (Delmer, 1999). Iβ is the most common form of cellulose found in plants, whereas Iα can be found in bacteria and algae (Delmer, 1999; Saxena and Brown, 2005).

Groups of chains come together, perhaps spontaneously (Guerriero et al., 2010), to form microfibrils. The number of individual chains in a microfibril varies between plants and cell wall types. Typical microfibrils that have been measured in a variety of plant types have a diameter between 2 nm and 4 nm (Donaldson, 2007; Frey-Wyssling, 1968). Aggregates of cellulose have been known to form large sheets and ribbons in bacteria and algae (Brown et al., 1976; Herth, 1983). Additionally, microfibrils can
aggregate even further to form larger macrofibrils, between 50 and 250 nm in diameter in primary cell walls of maize parenchyma (Ding and Himmel, 2006), and between 14 nm and 27 nm in secondary cell walls of poplar and pine (Donaldson, 2007).

The number of glucose units in a cellulose chain is described by the degree of polymerization (DP). Cellulose chain DP may affect the strength of a microfibril, and therefore how a cell wall grows and expands (Wasteneys and Fujita, 2006), however this has not yet been determined experimentally. It is feasible to speculate that the strength of a microfibril would be affected if the constituting glucose chains were shorter (lower DP): such a microfibril would likely be more susceptible to breakage, bending, or separation during expansion (Wasteneys and Fujita, 2006). Expanding to the cell wall, lower inherent DP could negatively affect cell wall rigidity, or increase cell wall flexibility, and therefore change the way a cell expands or withstands physical stresses.

The range of DP of cellulose chains is quite large, from near 8000 in some primary walls to greater than 15000 in secondary cell walls (Brown, 2004). The factors, physical, chemical or enzymatic that dictate DP are not completely clear. Wasteneys and Fujita (2006) postulated that the life span of cellulose synthesizing complexes in the membrane, which could be influenced by the organization of microtubules, could affect DP. The differences in DP between cell wall types within a single plant, or perhaps even within a single cell, suggest that there are indeed specific mechanisms that affect and perhaps regulate cellulose DP.

The primary cell wall is composed of many layers of cellulose microfibrils (MF), of which the organization can be irregular, but generally transverse with respect to the axis of elongation. The highly cellulosic secondary cell wall is made up of three layers (S1 to S3), which are deposited successively inside of the primary cell wall. The longitudinal angle of cellulose MFs in the cell wall, with respect to the axis of elongation, is described as microfibril angle (MFA). Large MFAs provide flexibility in the cell wall required for growth and bending early in development, whereas small angles confer the strength and rigidity needed to withstand turgor pressure (Barnett and Bonham, 2004). The S2 layer is the thickest of the secondary cell wall layers and has a small MFA compared to the thinner S1 and S3 layers, which generally have a higher MFA (Barnett and Bonham, 2004).
1.3 Cellulose synthase genes

1.3.1 Bacteria and non-vascular plants

An operon containing four genes linked to cellulose biosynthesis was the first genetic identification of what came to be known as the cellulose synthases. The operon, identified in *Acetobacter xylinium*, was determined to be essential for cellulose biosynthesis (Wong *et al.*, 1990), and further mutant studies revealed changes in cellulose crystallinity upon mutation in the fourth gene, *acsD* (Saxena *et al.*, 1994).

More recently, Roberts and Bushoven (2007) genetically characterized the CesA and CesA-like (CSL) gene family of the moss, *Physcomitrella patens*. The full-length CesA genes identified contain typical motifs common amongst all other known CesAs: a zinc-binding domain, a plant-conserved region, a class specific region, and eight transmembrane domains. They also show that many of the introns of the moss CesAs are conserved with *Arabidopsis* CesAs. Phylogenetic analysis places the *P. Patens* CesAs in separate clades from those of the higher plants. It is interesting that there are many CesAs in this moss, even though it does not form vasculature with thick secondary cell walls. When comparing the *P. patens* CesAs to those of Arabidopsis, Roberts and Bushoven (2007) highlighted the fact that there were no *P. patens* orthologs of the *Arabidopsis* primary and secondary wall CesAs, suggesting that the specialized functions of AtCesAs in primary versus secondary cell wall biosynthesis is not present in *P. patens*, at least not at the genetic level. For this reason, the current model of CesA specialization of function (primary versus secondary cell wall) may have evolved with vascular plants.

1.3.2 Vascular plants

Evolutionarily, CesA gene families appear to have emerged prior to the divergence of gymnosperms from angiosperms. The cotton (*Gossypium hirsutum*) cellulose synthase 1 (*GhCesA1*) and *GhCesA2* were the first CesA genes to be identified in higher plants (Pear *et al.*, 1996). Compared to the CesAs identified in bacteria, higher plant CesAs contain additional sequences termed the plant-conserved region, a hypervariable region, and a larger N-terminal sequence (Doblin *et al.*, 2002;
Pear et al., 1996; Delmer et al., 1999). These additional motifs could be responsible for putative diversified functions of plant CesAs (Delmer et al., 1999).

1.3.2.1 Arabidopsis thaliana

The CesA gene family of Arabidopsis is by far the most extensively studied, greatly advanced by the sequencing of the Arabidopsis genome in 2000. From genomic data available, 10 individual CesA genes and 31 cellulose-synthase-like genes were identified (Richmond and Somerville, 2000). The function of many of the AtCesAs has been under investigation. Studies often utilize mutant phenotypes and large scale gene expression analysis to further characterize the putative functions of each of the 10 CesAs.

Tissue specific expression of the known CesA genes resulted in the classification of CesAs as either primary or secondary cell wall-specific (Hamann et al, 2004). In Arabidopsis, AtCesA4, A7, and A8 are often described as displaying reduced expression in leaves, roots, flowers, and seedlings but elevated expression in stem tissue (Hamann et al, 2004). Analysis of AtCesA4, A7, and A8 mutants revealed that these three CesA subunits are required for proper formation of the cellulose network in secondary cell walls. Screening of mutagenized plants for alterations in xylem structure identified the first two secondary cell wall-cellulose deficient CesA-mutants: irregular xylem 1 (irx1) and irx3 (Turner and Somerville, 1997), later identified as mutations in the AtCesA8 and AtCesA7 genes, respectively (Taylor et al, 2000). A decrease in cellulose contributed to the collapsed xylem phenotype. It has been suggested that some CesA mutants might not produce cellulose at all in the secondary cell wall (Taylor, 2008); however, there is not yet any evidence to prove this, and thus it remains only speculative. Instead, it is likely that mutants produce cellulose in the secondary cell wall, but at dramatically reduced levels and quality.

Of the 10 CesA genes identified in Arabidopsis, only three have thus far been conclusively linked to secondary cell wall formation, whereas the remaining seven appear to be primary cell wall-specific. AtCesA1 was first identified as a primary cell wall-specific CesA in the rsw1-1 mutant, which showed defects in cell elongation and reduced levels of cellulose synthesized at a restrictive temperature (Arioli et al, 1998).
In addition to CesA1, CesA3 (Sheible et al, 2001) and CesA6 (Fagard et al, 2000) have also been implicated in cellulose production in the primary cell wall. Furthermore, it has been shown that expression of CesA6 was not sufficient to rescue the rsw1-1 mutant phenotype, suggesting non-redundant functions between these two CesA proteins (Robert et al, 2004). Examination of double- and triple-mutant phenotypes revealed partial redundancy between AtCesA2, A5, A9 and A6 depending on stage of growth (Desprez et al., 2007; Persson et al., 2007b). Phylogenetic analysis also suggests that their functions could be slightly redundant. Interestingly, Stork et al. (2010) recently suggested that AtCesA9 could have a unique role in the formation of secondary cell walls in seed coats. Also, it has been postulated that AtCesA7 might have some role in primary cell wall formation (Bosca et al., 2006; Zhong et al., 2007). These latter two observations advocate some overlap in function of a CesA in both primary and secondary cell walls, and these results also imply that CesA function can vary depending on cell type, or developmental stage.

1.3.2.2 Monocots

As in Arabidopsis, there are ten identified CesAs in rice, Oryza sativa. An investigation of the brittle culm mutants lead to the characterization of three rice CesAs proposed to be involved in secondary cell wall cellulose biosynthesis (Tanaka et al., 2003): OsCesA4, OsCesA7, and OsCesA9. Phylogenetic analysis confirmed the similarity to Arabidopsis secondary cell wall-specific CesAs; however, the cell-wall deficient phenotype differed slightly from that of the Arabidopsis mutants. The rice brittle culm mutants exhibited thinner walls in fibre cells, but no collapsed xylem. This has been attributed to inherent differences in cell wall composition between the monocot rice and dicot Arabidopsis (Tanaka et al., 2003). Another OsCesA4 mutant, bc11, exhibited both primary (increased callose, pectin) and secondary cell wall alterations suggesting a link between biosynthesis of the two cell walls (Zhang et al., 2009), as was also suggested in the case of the Arabidopsis AtCesA7mnr10 mutant (Bosca et al., 2006).

At least eight CesA genes have been shown to exist in barley, Hordeum vulgare (Burton et al., 2004). Expression profiling of the HvCesA gene family suggested two co-
expressed groups: *HvCesA1*, *A2*, and *A6* (group 1), and *HvCesA4*, *A7*, and *A8* (group 2). These groups are most similar to the *Arabidopsis* primary- and secondary cell wall-specific CesAs, respectively. Two *HvCesA4* mutants have reduced cellulose crystallinity, but also exhibited a slight up-regulation of *HvCesA7* and *HvCesA8* in the mutant internodes (Burton et al., 2010), further suggesting non-redundant functions between members of a putative CesA group, as the *A7* and *A8* up-regulation could not rescue the mutant phenotype.

Twelve CesA gene family members have been identified in *Zea mays* (Appenzeller et al., 2004). Gene expression analysis suggests that *ZmCesA10, 11, 12* are co-ordinately expressed in tissues undergoing secondary cell wall formation, similar to the CesA4, *A7*, and *A8* group in Arabidopsis (Appenzeller et al., 2004). The available expression data, however, shows a great deal of overlap between putative CesA-group members (e.g.: primary versus secondary cell wall-specific) in both the elongating and maturing regions. The overlap of expression, and likely protein activity, is surely not limited to corn. Further characterization of CesAs in monocot plants may reveal functional orthology to the known CesAs in *Arabidopsis*, for example, but could also reveal varied cell wall-specificity, perhaps as a result of the divergence between monocot and dicot species.

### 1.3.2.3 Trees

#### 1.3.2.3.1 Poplar

An evaluation of the *Populus trichocarpa* genome suggests there are 17 CesA genes (Kumar et al., 2009). The CesA nomenclature proposed by Kumar et al. (2009) will be used throughout this thesis when referring to poplar CesAs, whereby the *PtiCesA* genes are numbered based on sequence homology to the *Arabidopsis CesAs*, such that *PtiCesA8-A* and *A8-B* are homologs of *AtCesA8*. The added complexity of a tree genome due to genome duplication is highlighted by putative multiple copies of individual *PtiCesAs*. For example, there are currently six copies of *PtiCesA6* (Kumar et al., 1999), and multiple copies of all other *PtiCesAs* except for *PtiCesA4*. Interestingly, some duplicates of a CesA are both transcribed, such as *PtiCesA8-A* and -*B* (Djerbi et
Separation of some of the CesA duplicates will likely occur with thorough functional characterization of the gene products, or classification of some as non-functional.

In poplar, most of the information regarding the role of CesAs is limited to expression profiles. For example, analysis of transcript abundance in both normal and tension wood of *P. trichocarpa* display an up-regulation of *PtiCesA4*, *PtiCesA6*, and *PtiA8-A and A8–B* in developing xylem and tension wood (Djerbi et al., 2004). Further, Suzuki *et al.* (2006) suggested that *PtiCesA8-B* is the most highly expressed in young developing xylem. However, the other highly expressed CesAs were *PtiCesA3-C* and *PtiCesA1-A*, which are orthologous to *AtCesAs* normally ascribed as primary cell-wall specific (Suzuki *et al.*, 2006). In aspen, *P. tremuloides*, *PtdCesA7-B* and *PtdCesA8-A* were found to be are highly up-regulated in developing xylem (Samuga and Joshi, 2002; Joshi, 2003). Expression of secondary cell wall CesAs is altered in tension wood, however, the extent of change observed varies between studies. In hybrid aspen (*Populus tremula x tremuloides*), *PttxCesA8-A* is the only gene confirmed to have increased expression using both PCR and microarray analyses (Djerbi et al, 2004 and Anderson-Gunneras *et al.*, 2006, respectively). The tension wood gelatinous cell wall layer (G-layer) is highly cellulotic, with larger macrofibrils, more crystalline cellulose, and a very small MFA (Mellerowicz *et al.*, 2001; Mellerowicz and Sundberg, 2008). The differences in cellulose ultrastructure between the S2 and G-layers suggest there are mechanisms in place that control these properties. Whether or not individual CesAs contribute to these properties is not known. Also, the degree to which various CesAs are tension wood-specific is not completely clear, nor is it apparent if particular CesAs have a distinct role in altering the cell wall properties to deposit a G-layer. Perhaps small changes in gene expression represent substantial alterations to the ratio of CesA subunits in a given cell, which could contribute to altered cellulose of tension wood.

From just a few reports it is clear that a variety of CesAs are putatively involved in cellulose biosynthesis in xylem tissue of poplars. Further characterization of CesA specificity and functionality will be required to clearly elucidate any specificity among poplar CesAs.
1.3.2.3.2 Eucalyptus

*Eucalyptus*, another economically important tree, has also been investigated to identify and classify the *CesA* genes (Lu et al., 2008; Ranik and Myburg, 2006). Six full-length *EgCesA* genes have been identified in *Eucalyptus grandis* (Ranik and Myburg, 2006), and were shown to be expressed at varying levels depending on tissue type. Gene expression profiling resulted in clustering of the identified *CesAs* into two groups: one that was most highly expressed in developing xylem (*EgCesA1, EgCesA2*, and *EgCesA3*) and the other group more highly expressed in primary-wall forming tissues of *Eucalyptus* (*EgCesA4, EgCesA5, EgCesA6*). It was further shown that the *EgCesAs* were less similar to each other than they were to the *AtCesAs* (at the nucleotide level) suggesting the six *EgCesAs* are distinct from one another. Additionally, GUS-*EgCesA*-promoter fusions expressed in *Arabidopsis* showed that *EgCesA1* and *A3* expression is limited to secondary cell wall-forming cells in the stem (Creux et al., 2008). Creux et al. (2008) also identified many cis-elements within the *EgCesA* promoters that are conserved with *AtCesA* promoters. In another independent study, (Lu et al., 2008), the results of Ranik and Myburg were supported by expression profiling of three putative secondary cell wall-specific *EgCesA* genes, homologous to *AtCesA4, AtCesA8*, or *AtCesA3*. It was found that only the *EgCesAs* homologous to *AtCesA4* and *AtCesA8* were up-regulated during xylem maturation. As with the characterization of CesAs in poplar, a great deal of functional characterization is required for the EgCesA proteins to identify if wall-specific functions exist.

1.3.2.3.3 Pine

There are fewer reports of coniferous CesAs, despite the economic importance of many coniferous species worldwide. A query of *Arabidopsis* CesA gene sequences against publically available sequence data, returned several hits to pine and spruce sequences. However, most of the matches proved to be mRNA fragments and putative CesA sequences. To date only a handful of full-length CesAs have been identified in conifers; and those characterized are from *Pinus taeda* (Nairn and Haselkorn, 2005; Nairn et al., 2008) and *Pinus radiata* (Krauskopf et al., 2005). As in the other tree species, there has been an emphasis on isolating secondary cell wall-specific CesAs.
Gene expression analysis has revealed at least three CesAs in *Pinus taeda* that are up-regulated in wood-forming tissues (Nairn and Haselkorn, 2005; Nairn *et al.*, 2008), and one gene (*PrCesA10*) in *Pinus radiata* that is homologous to *AtCesA7*, which is expressed in tracheids undergoing secondary cell wall formation (Krauskopf *et al.*, 2005).

### 1.4 CesA protein structure

CesA proteins have a conserved structure containing a zinc-binding domain, eight transmembrane domains (two located at the amino terminus and six at the carboxyl terminus), a plant-conserved region, a class-specific region (CSR), and two highly conserved domains, A and B (Richmond, 2000; Krauskopf *et al.*, 2005). Within domains A and B there are four amino acid motifs that are characteristic of processive glycosyltransferases: three aspartic acid residues (D) and a QXXRW motif (Joshi and Mansfield, 2009). All components of domains A and B are necessary for the binding of UDP-glucose (Pear *et al.*, 1996), suggesting that this region is part of the CesA catalytic domain (Pear *et al.*, 1996; Doblin *et al.*, 2002). *In vitro* experiments also indicated that the zinc-binding domains of cotton CesA1 and A2 are required for proper interactions (Kurek *et al.*, 2002).

Predicted CesA amino acid sequences from genes within a given plant are less similar than comparative CesA sequences from other plants (orthologs) (Ranik and Myburg, 2006; Joshi *et al.*, 2004; Nairn and Haselkorn, 2004; Samuga and Joshi, 2002). Although the conserved regions are highly similar (90%) within and among plants, CSRs tend to have extremely low similarity within a plant (11 - 40%) and slightly higher similarity (70%) among orthologs (Ranik and Myburg, 2006). The CSR may dictate differences in function among CesAs within a plant, and has been used to aid in classification.

### 1.5 Cellulose synthase complex

Hexameric complexes associated with cellulose production, described as rosettes or terminal complexes, were first visualized in bacteria (Brown *et al.*, 1976) and have been described in some plants as scattered throughout the plasma membrane.
Some of the first images of rosettes were in the plasma membrane of algae (Brown and Montezinos, 1976). Rosettes and terminal complexes have been observed in a variety of arrangements within plasma membranes of diverse species (Tsekos, 1999). For example, in Oocystis, the terminal complexes have been shown to be arranged in linear arrays, and these organisms tend to produce large microfibrils (Tsekos 1999) and sheets of cellulose (Brown and Montezinos, 1976). Comparatively, large arrays consisting of dozens of six-lobed rosettes have been visualized in the plasma membrane of the algae Micraterias denticulata (Giddings et al., 1980) and Spirogyra (Herth, 1983), and according to Gidding et al. (1999) the size of cellulose microfibril bundles was proportional to the size of rosette arrays observed in M. denticulata. Furthermore, the arrangement of the rosettes in the plasma membrane can differ between primary and secondary cell walls in the same organism (Tsekos, 1999). It has been postulated (Tsekos, 1999) that differences in terminal complex arrangement may be involved in the production of Iα cellulose versus Iβ cellulose. The diversity of rosette arrangements in various species and during different stages of cell wall biosynthesis might explain the inherent variability in cellulose microfibril characteristics, such as size and shape (Tsekos, 1999), and perhaps crystallinity, thus affecting cell wall properties.

The active unit responsible for the production of cellulose is called the cellulose synthesizing complex (CSC). The CSC contains cellulose synthases (Kimura et al., 1999a), and approximately six CSCs associate to form a hexameric complex described as a rosette (Saxena and Brown, 2005). It is postulated that at least three unique CesAs are required for secondary cell wall CSCs to form in Arabidopsis: AtCesA4, A7, and A8 (Taylor et al., 2003).

1.6 The link between CesAs, rosettes, and the CSC

The first link between CesA genes and the rosette structure was provided by analysis of the AtCesA1 mutant, rsw1-1 (Arioli et al., 1998). When grown at the restrictive temperature, the mutants had reduced cellulose content and subsequently cell wall defects that affected growth such as stunted growth in seedlings and oddly-
shaped epidermal cells. In addition, the plants were shown to have fewer rosettes in the plasma membrane. In particular, after a long exposure to the restrictive temperature there were no rosettes visible, just a dense scattering of single particles. This implies that in the absence of a functional CesA, the CSCs and rosette structures are altered.

Mutant forms of *AtCesA4* (*irx5*) have been used to examine the interactions among CesA proteins and confirm that three CesA subunits are required for CesA-CesA interactions, detected *in vitro* using immunoprecipitation (Taylor *et al.* 2003). Null mutations of *AtCesA4* caused a reduction in AtCesA8 and A7 protein levels, and eliminated *in vitro* detection of interactions between AtCesA8 and AtCesA7. However, interactions were apparent in a non-null mutant of *AtCesA8*. These results suggest that the three CesAs interact to form the CSC, and all three are required for interactions to occur.

In the CesA mutants, reduced cellulose production is believed to be the result of improper assembly and function of the CSC, which is supported by the results of Arioli *et al.* (1998). However, it is interesting to note that cellulose is still produced in the absence of one CesA. It has been postulated that in the absence of a functioning CesA, aberrant CSCs may form and produce β-1,4-glucan chains that do not crystallize properly (Arioli et al, 1998). Labelling of AtCesA7 with GFP allowed Gardiner *et al.* (2003) to visualize the location of CesA-containing complexes or CesA-containing organelles within developing roots. They showed that the GFP-CesA signal was localized along the spiral cell wall thickenings of root xylem vessels, which was reduced, but not completely eliminated, in a null-*AtCesA4* or null-*AtCesA7* mutant background. This suggests that the remaining CesAs in a mutant background are likely present, and functioning, during cell wall biosynthesis.

**1.7 Composition of the CSC**

Elucidating the components of the CSC and how they affect cellulose production and properties remains an intriguing area under investigation. Some *in vitro*, and a few *in vivo* studies, have shown that the CesA proteins form homodimers and heterodimers (Taylor et al, 2003; Atanassov et al, 2009; Timmers *et al.*, 2009; Desprez *et al.*, 2007), and putative CSCs have been isolated and characterized (Atanassov *et al.*, 2009; Song
et al., 2010). In Arabidopsis, the use of immunoprecipitation methods lead to the conclusion that all secondary cell wall-specific CesAs interact (Taylor et al., 2000; Taylor et al., 2003) within a large complex (Atanassov et al., 2009), but yeast-two hybrid assays by Timmers et al. (2009) suggested that AtCesA7 does not form homodimers. Furthermore, in vivo experiments (using bimolecular fluorescence complementation, BiFC) suggested all CesA-CesA interaction combinations are possible except CesA7 homodimers (Timmers et al., 2009).

Very recently, Gu et al. (2010) reported that a novel protein, cellulose synthase interactive-1 (CSI1) interacts with AtCesA1, A3, and A6 (interactions detected using yeast-two hybrid assays). The function of CSI1 is not yet determined, although mutant CSI1 plants have a reduced cellulose phenotype. In the developing xylem of poplar (Populus deltoides x trichocarpa) Song et al. (2010) reported on the use of immunoprecipitation to isolate protein complexes containing CesAs, and showed that two types of putative CSCs might exist during secondary cell wall formation: a type I CSC that contains PdxtCesA4, A7(A/B), A8(A/B), and a type II CSC that contains PdxtCesA1 (A/B), A3(C/D), and A6 (E/F). Additionally, they reported that sucrose synthase (SUSY), korrigan (KOR), and the GPI-anchored protein COBRA interacted with the CesA complexes during immunoprecipitation. Contrary to this, the interaction of KOR with Arabidopsis CesAs has not been found using immunoprecipitation (Desprez et al., 2009) and yeast-two hybrid assays (Maloney, 2010). Additionally, using the same technique (immunoprecipitation of large complexes), Atanassov et al. (2009) reported only CesA-CesA interactions and no detection of non-CesA proteins. However, it must be noted that detection of CesA-interacting proteins may differ between plants and cell types. Additionally, the techniques and conditions used to isolate interacting proteins or complexes could affect the ability to detect interactions. As evidenced by the recent reports of CSI1 (Gu et al., 2010), there may be additional proteins interacting with CesAs that have not previously been reported. The particularly large, globular, cytoplasmic domain of rosettes observed by Bowling and Brown (2008) also lends support to the idea that additional proteins could be components of the CSC. The size of the putative CSC isolated by Song et al. (2010), however, was not reported, thus it is difficult to interpret whether the size of isolated CSCs supports the idea of a
large, diverse, multi-protein complex. Clearly, further investigation is required to fully elucidate the composition of CSCs.

1.8 Transport of CSCs

The abundance and organization of CSCs, and rosettes, within the plasma membrane during cellulose biosynthesis is believed to greatly affect cellulose properties, leading to an important question: how do CSCs ultimately end up in the plasma membrane at sites of cell wall thickenings? Haigler and Brown (1986) visualized rosette structures within Golgi vesicles (and in the plasma membrane) of Zinnia cells undergoing secondary cell wall thickening and it has been suggested that complete CSCs are formed, but inactive, within the Golgi. Control of CSC localization from the Golgi has been attributed to microtubules, pre-existing cellulose networks, and a variety of proteins. The mechanisms of delivery, and consequent distribution of CSCs in the plasma membrane may affect the final abundance and properties of cellulose produced, and is thus an intriguing area of research.

The use of fluorescence confocal microscopy has facilitated the visualization of active CSC movement in live cells, and the trafficking of CSCs via Golgi vesicles and other transport vesicles (Paredez et al., 2006; Wightman and Turner, 2008; Crowell et al., 2009; Gutierrez et al., 2009; Wightman and Turner, 2010). Changes in the pattern of Golgi-CSCs after treatment with an actin-disrupting drug have been shown to alter the distribution of CSCs in the plasma membrane, suggesting that CSC organization is dependent on the actin cytoskeleton, perhaps by affecting the delivery of Golgi-CesA complexes to particular sites or regions of the plasma membrane (Wightman and Turner, 2008; Gutierrez et al., 2009; Crowell et al., 2009). Crowell et al. (2009) also observed that CesA-Golgi complexes move and pause at various sites along MTs, and that pause events are often associated with presumable CSC delivery into the plasma membrane, and that the distribution of CSCs was dependent on a functional MT array. Wightman and Turner (2008) have also reported on CesA-Golgi compartments which the authors postulated also pause to deliver CSCs to sites of secondary cell wall thickening. Furthermore, reports of smaller CesA-containing particles have been observed below the plasma membrane plane in the cytosol, and are described as
MASCs (microtubule-associated cellulose synthase compartments, Crowell et al., 2009) and SmaCCs (small CesA compartments, Gutierrez et al., 2009). Both MASCs and SmaCCs have been suggested to be another, mostly non-Golgi vesicular compartment involved in regulating the distribution of CSCs and therefore cellulose biosynthesis during primary cell wall formation (Crowell et al., 2009; Gutierrez et al., 2009). MASC abundance and distribution were increased in response to drug-induced disruptions to cellulose biosynthesis and osmotic stress, further suggesting that they are involved in regulating cellulose biosynthesis by perhaps internalizing CSCs from the plasma membrane (Crowell et al., 2009). The same microtubule-associated localization and drug-induced accumulation was observed for SmaCCs (Gutierrez et al., 2009), and a comparison of images presented by Crowell et al. (2009) and Gutierrez et al. (2009) suggest that MASCs and SmaCCs are similar in size: between 300 and 500 nm in diameter. Together, these results strongly suggest that SmaCCs and MASCs could be the same population of CSC-containing cortical compartments (Whightman and Turner, 2010). Occasional association of SmaCCs (and possibly MASCs) with the trans-Golgi network has also been observed (Gutierrez et al., 2009; Whightman and Turner, 2010). Recent increases in the use of confocal fluorescence microscopy to observe CSCs in live cells has greatly enhanced our understanding of the dynamic nature of CSC delivery (and perhaps removal) to the plasma membrane, and that a variety of CSC-containing compartments exist in plant cells. However, it is clear that further characterization of the composition and function of MASCs/SmaCCs is required to completely understand their role in CSC regulation, as well as determine if they are the same population of CSC-compartments.

1.9 The cytoskeleton and cellulose microfibril orientation

The organization of cellulose microfibrils is crucial for proper cell formation, growth, anisotropy, and ultimately plant development. The mechanism, by which CSCs are localized within the plasma membrane, and the regulation of cellulose microfibril (MF) orientation within the cell wall, are not yet known. In addition, whether or not the localization of CSCs within the plasma membrane solely dictates cellulose MF orientation or which, if any, post-deposition remodeling occurs is still under question.
The role of microtubules (MTs) in cellulose MF organization has been the topic of much debate (Baskin, 2001; Wasteneys, 2004; Paredez et al., 2008; Crowell et al., 2010). It has been many years since microtubules were observed to lay parallel to cell wall components, such as cellulose microfibrils (Ledbetter and Porter, 1963), and since then various models have been proposed to explain the role of MTs in cellulose MF organization, such as the alignment hypothesis and template-incorporation hypothesis (reviewed by Baskin, 2001) and the microfibril-length-regulation hypothesis (Wasteneys, 2004; Wasteneys and Fujita, 2006). In combination with previous reports, the more recent use of fluorescently-tagged CesAs (often in combination with fluorescently labeled microtubules) provides more information about the relationship between MT and MF organization.

Colocalization of CSCs and MTs has been observed in xylem vessels (Gardiner et al, 2003; Wightman and Turner, 2008) and during primary cell wall formation (Paredez et al, 2006). Disruption of the MT array (using MT-depolymerizing drugs) causes CSC trajectories and localization to become disorganized (Gardiner et al, 2003; Paredez et al, 2006; Crowell et al., 2009), suggesting that proper localization of the CSC is dependent on MTs. However, Himmelspach et al. (2003) demonstrated that in the mor1-1 mutant grown under restrictive temperature conditions, causing microtubule disorganization, and after the application of DCB (2, 6-dichlorobenzonitrile), resulting in disorganization of the cellulose microfibril array, newly formed cellulose microfibrils aligned in an organized, parallel fashion, indicating that neither the MT network nor a pre-existing cellulose network was required for organized microfibrils to form. Additionally, Paredez et al. (2006) showed that after several hours of MT disruption by oryzalin treatment, CSC patterns and trajectories become highly organized, despite the lack of an organized MT array, further supporting the idea that MT organization is not required for CSC organization (Paredez et al., 2006) and hence cellulose MF organization (Himmelspach et al., 2003).

These results suggested that the organization of cellulose MFs was not dependent on organized MTs or pre-existing MFs, and that other factors such as a self-assembly mechanism, CSC density, or the rate of cellulose production may regulate cellulose MF organization (Himmelspach et al., 2003). However, it is hard to argue that
MTs are not, at least indirectly, involved in the regulation of cellulose deposition or cellulose MF properties at particular times in cell wall development. As detailed by Fujita (2008) MT disorganization in the mor1-1 mutant affects cellulose crystallinity, CSC density, and CSC velocity in primary cell walls. This clearly indicates a role for MTs in regulating aspects of cellulose microfibril properties, potentially by affecting CSC distribution and movement, but perhaps not microfibril orientation. It has been proposed that MTs could regulate the length of cellulose MFs, (Wasteneys 2004; Wasteneys and Fujita, 2006), which could affect cell expansion dynamics. Additionally, the alteration of the patterning and movement of non-Golgi CesA compartments (MASCs and SmaCCs, described above) in response to changes in the MT array further illustrates that some aspect of CSC organization is affected by the MT network (Crowell et al., 2009; Gutierrez et al., 2009), which could ultimately determine properties of the cellulose microfibrils produced. It should also be considered that MT-CesA associations (whether direct or indirect) could differ in primary and secondary cell walls, or between developmental stages.

The existence of secondary mechanisms involved in MT-aided CSC organization is supported by the phenotype of the Arabidopsis fra1 mutant (Zhong et al., 2002). A mutation of a kinesin-like protein resulted in slight disorganization of the cellulose microfibrils in the secondary cell walls of fibers, resulting in reduced fiber strength. No change in cellulose content or MT organization was observed, and therefore it was suggested that the kinesin-like protein may guide CSCs along MTs, or may regulate the positioning of other barrier proteins along MTs that would restrict the localization of CSCs and therefore cellulose MFs (Zhong et al., 2002). An increase in the expression of a kinesin-like protein in tension wood of Eucalyptus (Paux et al., 2005) also suggests a role for such a protein in cellulose biosynthesis in secondary cell walls.

Microtubule associated proteins (MAP) may act as an intermediate between MTs and CSCs. For example, it has been shown that treatment of plants with DCB inhibits cellulose production causing abnormal cell wall formation (Taylor et al., 1992), and more recently a MAP20 protein found in developing xylem of hybrid poplar was shown to bind DCB (Rajangam et al., 2008). Additionally, mis-regulation of an Arabidopsis MAP (AtMAP70-5) caused alterations to microtubules and secondary cell wall thickening in
xylem cells (Pesquet et al., 2010). Wightman et al. (2009) observed that the movement of YFP-CSCs in secondary cell wall forming cells was slower following treatment with DCB. Taken together, it is feasible to speculate that there may be a population of proteins that facilitate linkages between MTs and CSCs, potentially affecting CSC movement and therefore cellulose deposition and potentially MF organization.

Disruption of KORRIGAN or CesA6 (AtCesA6<sup>prc</sup> mutant) in Arabidopsis was found to increase the sensitivity of plants to a microtubule destabilizing drug, oryzalin, and the mutant plants had disorganized MT arrays (Paredez et al., 2008). Previously, it had been shown that disruption of the cellulose MF network using DCB (Himmelspach et al., 2003) or isoxaben (Fisher and Cyr, 1998) causes MT-disorganization. Combined, these results strongly suggest there is feedback in response to perturbations of the cellulose network that ultimately affects the MT array.

1.10 Goals and hypothesis

Although a great deal of research has been conducted around the fact that a group of conserved genes are required for cellulose biosynthesis in the secondary cell wall of Arabidopsis (CesA4, A7, and A8), there is little to no information available to identify if each CesA has a unique role in cellulose biosynthesis, and if their precise roles confer specific cellulosic properties. The main goal of my research was to contribute to our understanding of the role of secondary cell wall-specific cellulose synthases during cellulose production. These questions were investigated in three separate studies, detailed in Chapters 2, 3, and 4. The scope and aim of each study is briefly described here.

1.10.1 Chapter 2: Characterization of Arabidopsis CesA secondary cell wall-specific mutants

The goal of this research was to characterize the growth and cell wall properties of AtCesA secondary cell wall-specific cellulose synthase mutants. The emphasis was on understanding how the individual CesAs contribute to cellulose biosynthesis and cellulose properties in plant secondary cell walls. The hypothesis was that each AtCesA has a unique role in cellulose production, such that a mutation in any one
protein will result in altered cellulose properties. To examine the effect of each mutation, I measured growth rates and cell wall properties of plants grown under both long and short-day lighting conditions. Additionally, I examined the distribution of AtCesAs \textit{in planta} using YFP-CesA fusions.

1.10.2 Chapter 3: Identification of secondary cell wall-specific cellulose synthase genes from \textit{Picea glauca} and conservation of function among CesA orthologs

The goal of this research was to identify secondary cell-wall specific CesA genes in a gymnosperm, spruce (\textit{Picea glauca}), that are orthologous to the \textit{Arabidopsis} secondary cell wall-specific CesAs. It was hypothesized that the isolated genes would share similar conserved domains and would exhibit varied transcript abundance such that the highest expression would correspond to tissues undergoing significant secondary cell wall formation. Additionally, I tested the hypothesis that true orthologous CesAs would be functionally conserved. This was tested by transforming \textit{Arabidopsis AtCesA}^{irx} mutants with candidate CesA genes from spruce and poplar, to determine if presence of the spruce or poplar CesA could recover the mutant phenotype, thus demonstrating conservation of function among CesA orthologs.

1.10.3 Chapter 4: Measuring CesA-CesA interactions \textit{in planta}

The goal of this work was to examine if specific and consistent interactions exist among Arabidopsis CesA proteins \textit{in vivo} during secondary cell wall formation. The hypothesis was that CesA subunits form homodimers and heterodimers within the CSC, and that these interactions are specific and consistent during a defined developmental stage. I utilized two methods, bioluminescence energy transfer (BRET) and bimolecular fluorescence complementation (BiFC) to study CesA-CesA interactions \textit{in vivo}. 

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CHAPTER 2 Characterization of Arabidopsis CesA secondary cell wall-specific mutants

2.1 Introduction

Although a variety of cellulose synthase (CesA) mutants have been studied and characterized, the precise mechanism by which the cellulose synthase proteins and the cellulose synthesizing complex (CSC) affect cellulose properties such as abundance, crystallinity, microfibril angle, and degree of polymerization are still relatively unknown. It has been shown that at least three CesAs are required for proper secondary cell wall formation in Arabidopsis: AtCesA4, AtCesA7, and AtCesA8 (Taylor et al., 2000; Taylor et al., 2003; Gardiner et al., 2003; Taylor et al., 2004). Mutations in any one of these genes results in reduced cellulose synthesis and altered secondary cell wall structure (for review see Taylor, 2008). However, a comparison of all three mutants has not been provided in a single, unified study. Instead, several, independent reports provide a general summary of the effects of each mutant, all of which generally lack a complete evaluation of the effects of each mutation on cellulose properties. In an effort to better understand the role of AtCesA4, A7, and A8 in determining cellulose properties, a complete and thorough characterization of three Arabidopsis secondary cell wall CesA-mutants was performed: AtCesA4\textsuperscript{irx5-1}, AtCesA7\textsuperscript{irx3-1}, and AtCesA8\textsuperscript{irx1-1}. Growth phenotype, secondary cell wall composition, and cellulose ultrastructure were determined for each mutant, and a critical review of the information collected provides some new insight into the complex process of cellulose biosynthesis.

Cellulose is a linear, unbranched, homopolymer composed of $\beta$-1,4-linked glucan units, polymerized from the UDP (uridine-diphosphate)-glucose precursor (Delmer and Amor, 1995). Cellulose chains are aligned parallel to one another and form hydrogen bonds between chains, and among fibrils, ultimately generating a polymer with both crystalline and amorphous regions (Delmer and Amor, 1995; Saxena and Brown, 2005). Bundles of glucan chains, putatively 16 to 36 glucan chains in vascular plants (Saxena and Brown, 2005), form cellulose microfibrils. Microfibrils with diameters of 3 to 5 nm were observed in maize cell walls, a size which supports the idea that microfibrils could contain a range of glucan chains (Ding and Hemmel, 2006).
The orientation, and thus microfibril angle (MFA) of cellulose microfibrils in a cell wall, or layers of a cell wall, is thought to greatly affect cell wall structure and function. Cellulose MFA is quite diverse between plant species (Lichtenegger et al., 1999), tissue, cell type, and developmental stage (Donaldson, 2007; Barnett and Bonham, 2004; Mansfield et al., 2009), and cell wall layer (Chan et al., 2010; Barnett and Bonham, 2004; Donaldson, 2007). Such diversity in MFA illustrates the importance of cellulose MFA in cell wall structure and function. Variations in MFA are thought to affect cell wall stiffness and extensibility (Reiterer et al., 1999). For example, in secondary cell walls, the cellulose microfibrils of the dominating S2 layer have a small MFA to provide the structural integrity required for water transport (Barnett and Bonham, 2004; Mellerowicz and Sundberg, 2008; Fang et al., 2004). Comparatively, the S1 and S3 layers of secondary cell walls, as well as microfibrils in primary cell walls, have a larger MFA (Barnett and Bonham, 2004; Seagull, 1992) and alterations to cellulose microfibril orientation in primary walls affects the degree and direction of cell expansion (Himmelspach et al., 2003; Wasteneys and Fujita, 2006; MacKinnon et al., 2006). The present study aims to determine the MFA of secondary cell wall cellulose in whole Arabidopsis stems, providing novel information regarding the ultrastructure of cellulose in a very important model plant.

Cellulose ultrastructure has a prominent effect on cell wall structure and function. For example, the degree of polymerization and extent of crystallization dictate the strength of the individual microfibril. Consider, for example, the differences in cellulose ultrastructure between primary and secondary cell walls. The primary cell wall cellulose of some plants is less crystalline than secondary cell walled tissues (Harris and DeBolt, 2008; M. Fujita, personal communication). Harris and DeBolt (2008) also found that cellulose crystallinity varied greatly among diverse species, and the percentage of crystalline cellulose in stems decreased when plants were exposed to wind stress. These results infer a relationship between cellulose crystallinity and the function of the cell wall (for example decreased stiffness under wind conditions). Cellulose microfibrils of primary walls may be composed of glucan chains with a lower degree of polymerization (DP) compared to those of secondary walls, although experimental evidence comparing cellulose DP in various tissues and cell walls is lacking.
Mechanically, it is thought that microfibrils composed of short glucan chains (low DP) would be more susceptible to breakage or separation, therefore affecting cell wall strength and expansion (Wasteneys and Fujita, 2006). In synthetic and bacterial produced cellulose films, a decrease in DP is correlated with reduced cellulose strength (Henriksson and Berglund, 2007). Variation in the properties of cellulose microfibrils, such as MFA and crystallinity, between cell wall layers evokes many questions pertaining to how, what, and when these qualities are determined, and to what extent the CesA complexes affect these properties.

In CesA mutants, reduced cellulose production is believed to be the result of improper assembly and function of the CSC, or impaired function of a CesA subunit. In fact, the loss of hexagonal rosettes at the plasma face of plants with a mutant form of a primary cell wall CesA, AtCesA1, has been observed (Arioli et al., 1998). However, cellulose is still produced in the absence of one CesA. It has been postulated that in the absence of a functioning CesA, aberrant CSCs may form and produce β-1,4-glucan chains that do not crystallize properly (Arioli et al., 1998).

A number of secondary cell wall-specific CesA mutants have been described in the literature, and Figure 2.1 summarizes an overview of the types of mutants reported. Table 2.1 provides details of the mutant phenotype characterization for some of the mutants for which cell wall composition has been reported. Of the information currently available, the most critical observations revealed thus far are that all three CesAs (AtCes4, A7, and A8) are required at the same time for proper cellulose biosynthesis, such that a mutation in any one CesA results in a dramatic reduction in cellulose content. Due to the diversity of published information available, only a limited comparison of the mutants can be made, mostly limited to broad changes in phenotype. Also, discrepancies in reports on changes to cellulose content further limit the comparisons that can be made about each mutant. For example, the same AtCesA7irx3-1 mutant has been reported to have both a 70% decrease (Taylor et al., 1999) and a 92% decrease (Taylor et al., 2003) in cellulose compared to wild-type plants. Such differences could be due to many factors, including the age of plants harvested for analysis, the region of stem analyzed, and the growth conditions. In an effort to gain more insight into the role of each CesA in cellulose biosynthesis, it is
prudent to thoroughly investigate the CesA4, A7, and A8 mutants simultaneously. Additionally, cellulose properties such as MFA, crystallinity, and DP have not been measured in any of the mutants. Our study, therefore, provides the means for direct comparison of each mutant and novel information about cellulose structure in these mutants, and such a thorough comparison may shed new light on the individual role of each protein in cellulose biosynthesis.
Figure 2.1. A schematic diagram illustrating the location of various mutations in Arabidopsis AtCesA4 (A4), AtCesA7 (A7), and AtCesA8 (A8) genes (modified from Fujita, 2008; Zhong et al., 2003). Only mutants for which cell wall properties have been published are shown. Mutants have been given new labels (e.g.: A7fra5 instead of fra5), as shown in Table 2.1. Further details about the amino acid substitutions can be found in Table 2.1.

HVR – hypervariable region
Zn – zinc-binding domain
PM – plasma membrane
TMD – transmembrane domain
CSR – class-specific region
D1, D2, D3, QXXRW – conserved residues within the catalytic domain.
Table 2.1. A summary of \( \text{AtCesA}^{ir\times} \) phenotypes of \( \text{AtCesA}4, \ A7, \) and \( \text{A8} \) mutants reported in the literature. The summary is restricted to reports of cell wall composition. Values are a percentage of the levels reported for wild-type plants from within the same literature source. The new labels presented are a nomenclature suggestion (Taylor, 2008). Ds - dissociation insertion mutation; arrow – substitution event; OE – 35S promoter-directed over-expression; *conserved residue in catalytic site.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Genotype</th>
<th>Mutation</th>
<th>New Label</th>
<th>% of WT values</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{AtCesA}4 )</td>
<td>( \text{irx}5-1 )</td>
<td>Ds insertion after L(^{653} )</td>
<td>( \text{A4}^{\text{irx5-1}} )</td>
<td>Cellulose 8%</td>
<td>Taylor et al., 2003</td>
</tr>
<tr>
<td>( \text{AtCesA}4 )</td>
<td>( \text{irx}5-2 )</td>
<td>W(^{995} \rightarrow \text{STOP} )</td>
<td>( \text{A4}^{\text{irx5-2}} )</td>
<td>Lignin 121%</td>
<td>Taylor et al., 2003</td>
</tr>
<tr>
<td>( \text{AtCesA}7 )</td>
<td>( \text{fra}5 )</td>
<td>P(^{557} \rightarrow \text{T} )</td>
<td>( \text{A7}^{\text{fra5}} )</td>
<td>Cellulose 46%</td>
<td>Zhong et al., 2003</td>
</tr>
<tr>
<td>( \text{AtCesA}7 )</td>
<td>( \text{irx}3 )</td>
<td>no description</td>
<td>( \text{A7}^{\text{irx3}} )</td>
<td>Cellulose 20% stem</td>
<td>Turner &amp; Somerville, 1997</td>
</tr>
<tr>
<td>( \text{AtCesA}7 )</td>
<td>( \text{irx}3 )</td>
<td>no description</td>
<td>( \text{A7}^{\text{irx3}} )</td>
<td>Lignin 121%</td>
<td>Ha et al., 2002</td>
</tr>
<tr>
<td>( \text{AtCesA}7 )</td>
<td>( \text{irx}3 )</td>
<td>W(^{659} \rightarrow \text{STOP} )</td>
<td>( \text{A7}^{\text{irx3-1}} )</td>
<td>Lignin 152%</td>
<td>Taylor et al., 1999</td>
</tr>
<tr>
<td>( \text{AtCesA}7 )</td>
<td>( \text{irx}3 )</td>
<td>W(^{659} \rightarrow \text{STOP} )</td>
<td>( \text{A7}^{\text{irx3-1}} )</td>
<td>Xylose 107%</td>
<td>Taylor et al., 2003</td>
</tr>
<tr>
<td>( \text{AtCesA}7 )</td>
<td>( \text{irx}3-4 )</td>
<td>TDNA insertion</td>
<td>( \text{A7}^{\text{irx3-4}} )</td>
<td>Xylose 19%</td>
<td>Brown et al., 2005</td>
</tr>
<tr>
<td>( \text{AtCesA}7 )</td>
<td>( \text{OE-fra5} )</td>
<td>OE of fra5 in WT background</td>
<td>( \text{A7}^{\text{OE-fra5}} )</td>
<td>Cellulose 24%</td>
<td>Zhong et al., 2003</td>
</tr>
<tr>
<td>( \text{AtCesA}8 )</td>
<td>( \text{fra}6 )</td>
<td>R(^{352} \rightarrow \text{K} )</td>
<td>( \text{A8}^{\text{fra6}} )</td>
<td>Cellulose 40% stem</td>
<td>Zhong et al., 2003</td>
</tr>
<tr>
<td>( \text{AtCesA}8 )</td>
<td>( \text{irx}1 )</td>
<td>no description</td>
<td>( \text{A8}^{\text{irx1}} )</td>
<td>Cellulose 40% stem</td>
<td>Turner &amp; Somerville, 1997</td>
</tr>
<tr>
<td>( \text{AtCesA}8 )</td>
<td>( \text{irx}1-1 )</td>
<td>*D(^{683} \rightarrow \text{N} )</td>
<td>( \text{A8}^{\text{irx1-1}} )</td>
<td>Cellulose 15%</td>
<td>Taylor et al., 2003</td>
</tr>
<tr>
<td>( \text{AtCesA}8 )</td>
<td>( \text{irx}1-2 )</td>
<td>S(^{679} \rightarrow \text{L} )</td>
<td>( \text{A8}^{\text{irx1-2}} )</td>
<td>Cellulose 10%</td>
<td>Taylor et al., 2003</td>
</tr>
<tr>
<td>( \text{AtCesA}8 )</td>
<td>( \text{irx}1-5 )</td>
<td>TDNA insertion</td>
<td>( \text{A8}^{\text{irx1-5}} )</td>
<td>Cellulose 21%</td>
<td>Brown et al., 2005</td>
</tr>
<tr>
<td>( \text{AtCesA}8 )</td>
<td>( \text{OE-fra6} )</td>
<td>OE of fra6 in WT background</td>
<td>( \text{A8}^{\text{xOE-fra6}} )</td>
<td>Cellulose 100%</td>
<td>Zhong et al., 2003</td>
</tr>
</tbody>
</table>
2.2 Materials and methods

2.2.1 AtCesA mutant nomenclature

In order to integrate the gene identity with previously assigned *irx* labels a modified nomenclature will be used throughout this chapter and thesis, as proposed by Taylor (2008). The revised nomenclature is reviewed in Figure 2.1 and Table 2.1. The following nomenclature will be used to describe the mutants that were used in the subsequent studies, *irx*1-1: *AtCesA*\(^{8\text{irx1-1}}\) (*A*\(^8\text{irx1-1}\)); *irx*3-1: *AtCesA*\(^{7\text{irx3-1}}\) (*A*\(^7\text{irx3-1}\)); and *irx*5-1: *AtCesA*\(^{4\text{irx5-1}}\) (*A*\(^4\text{irx5-1}\)).

2.2.2 Plant growth

Wild-type *Arabidopsis* (Landsberg ecotype, WT), *AtCesA*\(^{8\text{irx1-1}}\) (Turner and Somerville, 1997), *AtCesA*\(^{7\text{irx3-1}}\) (Turner and Somerville, 1997), and *AtCesA*\(^{4\text{irx5-1}}\) (Taylor *et al.*, 2003) were grown and used for these studies. Seeds were first surface sterilized by washing for 2 minutes in 70% (v/v) ethanol followed by an 8 minute wash in a 30% (v/v) bleach and 0.2% (v/v) Triton X-100 solution. Washed seeds were rinsed ten times with sterilized water, after which, seeds were stratified by storing in water, in the dark, at 4°C for at least 2 days. Stratified seeds were germinated on half-concentration MS medium (Mirashige and Skoog, 1962) with no sucrose under continuous light. Seedlings were transferred to soil approximately 7 days post germination, and grown in a growth chamber at 21°C under either a long-day light cycle (16-h light/8-h dark) or a short-day light cycle (8-h light/16-h dark). Plants under both long and short-day conditions were monitored and measured regularly for growth analysis. For growth, rosette diameter and stem height were measured weekly after the first rosette leaf exceeded 0.5 cm. The appearance of flower buds, open flowers, siliques, and mature siliques was also recorded. When plants were fully mature (yellowed siliques), they were harvested for cell wall analysis. For live-cell imaging, inflorescence stems of mature (15-day to 30-day old) plants were used.

2.2.3 Xylem morphology

Toluidine blue staining was employed to assess xylem morphology. Stem bases (approximately 1cm from the soil level) from plants (21-30 days old) were hand-
sectioned using a double-edged razor blade. Sections were stained for 5 minutes in a 0.25% (w/v) Toluidine blue solution, and rinsed for 5 minutes in water. Hand-sections were viewed through a Leica Light Microscope, and pictures taken with a Q-imaging camera.

2.2.4 Structural carbohydrate and starch analysis

Two and three-month old *Arabidopsis* stems were harvested from fully mature plants that had grown under long-day or short-day conditions. Dried stems were ground in a Wiley mill to pass a 0.4-mm mesh screen (40 mesh). Extractives were removed by subjecting the ground tissue to a hot acetone extraction for 16 hours using a Soxhlet apparatus. Lignin and carbohydrate content was determined using a modified Klason technique. Approximately 200 mg of dried, acetone-extracted tissue was macerated at 10 minute intervals in 3 mL of 72% sulphuric acid for a total of 2 hours. Samples were then diluted to 3% acid by the addition of 112 mL deionized water and autoclaved for 1 hour at 121°C. The acid-insoluble lignin fraction was isolated by filtration of the filtrate through a pre-weighed medium coarseness sintered-glass crucible, the retentate thoroughly rinsed with distilled water, dried at 105°C for 24 hours, and weighed to determine the amount of acid insoluble lignin. The acid soluble lignin was determined by measuring the absorbance at 205 nm according to TAPPI Useful Method UM-250. The filtrate was then used to determine the amount of structural monosaccharides using HPLC analysis. Specifically, samples were analysed using a DX-600 anion-exchange HPLC (Dionex) equipped with an ion exchange PA1 column (Dionex). Concentrations of glucose, xylose, mannose, galactose, arabinose, rhamnose, and fucose were determined using regression equations from calibration curves that were derived from external standards.

Starch content of mature stems was also determined. To extract starch, approximately 20 to 50 mg of the acetone-extracted tissue described above was hydrolyzed in 5 mL of 4% sulphuric acid at 121°C for 3.5 minutes. Glucose content was determined by HPLC analysis and represents starch content.
2.2.5 Microfibril angle and crystallinity by X-ray diffraction

Microfibril angle (MFA) was measured using an X-ray diffraction technique (Megraw et al., 1998). Measurements were taken from at least five individual mature stems (as described in 2.2.3) for each genotype. The first 3 cm of the stem base were used for X-ray diffraction. The 002 diffraction spectra from at least five individual mature (see section 2.2.3) stems for each genotype were screened for T value distribution and symmetry on a Bruker D8 discover X-ray diffraction unit. Wide-angle diffraction was used in the transmission mode, and measurements were made with CuKα1 radiation (λ represents 1.54 Å). The X-ray source was fit with a 0.5 mm collimator and the scattered photon was collected by a GADDS detector. The X-ray source and the detector were set to a theta angle of 0°. The diffraction data were integrated using GADDS software and further analyzed to estimate MFA values.

Cellulose crystallinity was determined from the same stems used for MFA analysis. Crystallinity estimates were generated by analysis of X-ray diffraction. The X-ray parameters used were the same as those described for MFA determination with the exception of the source theta being set at 20°. Diffraction intensities were counted at 0.1° increments between 4 and 40° in the 2 theta angle range. The diffraction data were integrated using GADDS software and the output data further analyzed using a crystallinity calculation program based on the Vonk method (Vonk, 1973) to estimate the degree of crystallinity.

2.2.6 Preparation of α-cellulose

Ground, acetone-extracted stem tissue samples (as described in 2.2.3.2) were employed for quantitative assessment of α-cellulose content. First, to remove lignin, approximately 50-200 mg of ground tissue was mixed with 3.5 mL of buffer solution (per 1L: 60 mL glacial acetic acid, 1.3 g NaOH) and 1.5 mL of 20% (w/v) sodium chlorite solution in a capped and parafilm-sealed glass tube and incubated with gentle shaking for 16 hours in a 50°C water bath. The overnight reaction was then quenched by placing the tubes in an ice bath for 8 hours. The reaction solution was then carefully removed, leaving the wood meal in the tube. A second identical reaction was set-up and left to react overnight. The wood meal was then washed twice with 50 mL of 1% (v/v) glacial acetic acid followed by washing with 10 mL of acetone and filtered through
a pre-weighed, coarse, sintered glass crucible. Samples were dried overnight in a 50 °C oven and weighed to determine the mass of hollocellulose.

Approximately 25-100 mg of hollocellulose was used to isolate α-cellulose. The hollocellulose was placed in a small beaker with 8 mL of 17.5% (v/v) NaOH. After a 30 minute incubation, 8 mL of deionized water was added and the sample stirred and left to react for an additional 29 minutes. The solution was then filtered through a pre-weighed, coarse, sintered glass crucible and washed three times (3 x 50 mL) with deionized water. The samples, in the crucible, were soaked in 1.0 M acetic acid for 5 minutes and then washed again (3 x 50 mL) with deionized water. The α-cellulose was dried overnight in a 50°C oven and weighed.

2.2.7 Degree of polymerization of cellulose fibres

The molecular weight distributions of α-cellulose isolated from wild-type and the AtCesA10x mutant plant lines were measured using gel permeation chromatography (GPC) coupled to a multi angle light scattering detector (MALS; Dawn Helos-II, Wyatt Technologies). Between 8.5 mg to 40 mg of α-cellulose was individually weighed into glass vials. Prior to dissolution, solvent exchange from water (Nanopure), to anhydrous ethanol to N,N-Dimethylacetamide (DMAC) was performed in series. In brief, the cellulose was stirred for three days in water, followed by two days in ethanol and, finally two days in DMAC. At each solvent change, the solvent was removed by centrifugation for 20 minutes at 6000 rpm. The final pellet of cellulose was resuspended in a 9% (w/v) lithium chloride in DMAC solution (LiCl/DMAC) at a ratio of 6 mg of cellulose per 1 mL of solvent. This suspension was then stirred for three days at room temperature, and stored at 4°C prior to GPC analysis. The cellulose in LiCl/DMAC was diluted 1:20 with HPLC grade DMAC. Samples were filtered through a 0.45 μm nylon filter and run on an Agilent 1100 series GPC instrument equipped with two Styragel columns in series (Waters; 5E, 4E) maintained at room temperature with an isocratic flow rate of 1mL/min of 0.9% LiCl/DMAC. Following separation, cellulose polymer size was estimated by multi-angle light scattering and processed using the ASTRA program (Wyatt Technologies) to determine the average molecular weights.
2.2.8 RNA extraction and cDNA synthesis for gene expression analysis

Plant tissues (namely 21-30 day-old stems) were harvested and immediately frozen in liquid nitrogen. Total RNA was extracted using TRIzol (Invitrogen) according to the manufacturer’s instructions. RNA was treated with TURBO DNase (Ambion) to remove DNA. First-strand cDNA was synthesized from 1 μg of DNase-treated RNA using Superscript II Reverse Transcriptase (Invitrogen) with dT\textsubscript{18} oligonucleotides.

2.2.9 Real-time PCR analysis

Gene expression was measured using quantitative real-time PCR (RT-PCR). RT-PCR reactions were set-up in triplicate for each sample with Platinum SYBR Green qPCR Master Mix (Invitrogen) and run on an Mx3000p real-time PCR system (Stratagene). The primers used to detect \textit{AtCesA} and the housekeeping control gene \textit{AtUBQ5} are listed in Table 2.2.
### Table 2.2. PCR primers used for gene isolation, cloning, screening, and real-time (RT) PCR.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5' to 3')</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>A4HVRIIFW</td>
<td>TGACATGTGATTGGTTGCGTGCTG</td>
<td>RT</td>
</tr>
<tr>
<td>A4HVRIIRV</td>
<td>AATCGCCTCCGCTGATGATCGTT</td>
<td>RT</td>
</tr>
<tr>
<td>A7HVRIIFW</td>
<td>ACATGAATTGATGACGTAGACCGCCCTT</td>
<td>RT</td>
</tr>
<tr>
<td>A7HVRIIRV</td>
<td>ACCGCAGCTTATGACATGGATTGCTCT</td>
<td>RT</td>
</tr>
<tr>
<td>A8HVRIIFW</td>
<td>GCAAAGCAGAGAAGACTTGATGCTGCTG</td>
<td>RT</td>
</tr>
<tr>
<td>A8HVRIIRV</td>
<td>TTAACTAGTGCTCAGAGACCGGCAATT</td>
<td>RT</td>
</tr>
<tr>
<td>UBQ5FW</td>
<td>ACACAAAGCAGAGAAGATCAAGAC</td>
<td>RT</td>
</tr>
<tr>
<td>UBQ5RV</td>
<td>AATGACCTCAGCATGAAAAGTCCCAGC</td>
<td>RT</td>
</tr>
<tr>
<td>LUC3'FW</td>
<td>AGAGTGCTGAAGAAGACAGACGAGAGA</td>
<td>screening/RT</td>
</tr>
<tr>
<td>YFP3'FW</td>
<td>ATCACAATGCTGGACTGAGATGCTGCT</td>
<td>screening/RT</td>
</tr>
<tr>
<td>A45'RV</td>
<td>TACAAAGGACATGAGACGAGACGAGA</td>
<td>screening/RT</td>
</tr>
<tr>
<td>A75'RV</td>
<td>TGACAGCTTTGCTCCTATGCTGTTG</td>
<td>screening/RT</td>
</tr>
<tr>
<td>A85'RV</td>
<td>TCACACAGTGTTTCAGATGGGA</td>
<td>screening/RT</td>
</tr>
<tr>
<td>A4GWFW</td>
<td>CACGGAACCAACACACACATGCCGCCAC</td>
<td>isolation/pENTR cloning</td>
</tr>
<tr>
<td>A4GWRV</td>
<td>TTAACAGTGCGCCACATTTGCTTCCCA</td>
<td>isolation/pENTR cloning</td>
</tr>
<tr>
<td>A7GWFW</td>
<td>CACGGAAGCTAGCGCCGGCTTGTGTC</td>
<td>isolation/pENTR cloning</td>
</tr>
<tr>
<td>A7GWRV</td>
<td>TGACAGCTTTGCTCCTAGCAGCCTGGA</td>
<td>isolation/pENTR cloning</td>
</tr>
<tr>
<td>A8GWFW</td>
<td>CACCGAGTCTAGGTTCTCCCA</td>
<td>isolation/pENTR cloning</td>
</tr>
<tr>
<td>A8GWWR</td>
<td>TTGAAGACTCAAGTACAAAGACA</td>
<td>isolation/pENTR cloning</td>
</tr>
<tr>
<td>PA7FWSDAI</td>
<td>CCTGCAGGGGCTAGAGATTTGGGAGGTGATGG</td>
<td>promoter cloning</td>
</tr>
<tr>
<td>PA4FWCLAI</td>
<td>AGTCATCGATGGTGACAGATAGTTGGAGGTGATGG</td>
<td>promoter cloning</td>
</tr>
<tr>
<td>PA4RVAVRII</td>
<td>AGTCCTAGTGCTGGAGAGCAGAGAGAGGTGAG</td>
<td>promoter cloning</td>
</tr>
<tr>
<td>PA7FWSDAI</td>
<td>CCTGCAGGGCGACAACACAGCAGAGAGGTACG</td>
<td>promoter cloning</td>
</tr>
<tr>
<td>PA7FWCLAI</td>
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<td>promoter cloning</td>
</tr>
<tr>
<td>PA7RVAVRII</td>
<td>AGTCCCTAGGAGGGACGCCGAGAGATTAGCAG</td>
<td>promoter cloning</td>
</tr>
<tr>
<td>PA8FWSHINDIII</td>
<td>AAGCTTACAGATCTACATCTCTCTCGTGCGC</td>
<td>promoter cloning</td>
</tr>
<tr>
<td>PA8FWCLAI</td>
<td>ATGCTAGCTCAACACATCTCTCCCTGGTCG</td>
<td>promoter cloning</td>
</tr>
<tr>
<td>PA8RVAVRII</td>
<td>CCTAGGCCCTGTGGAGAAACAGAGAAATGAACCC</td>
<td>promoter cloning</td>
</tr>
<tr>
<td>LUCFWAVRII</td>
<td>AGTCCCTAGGACAACAGAATCTCAGAGCAATCAAGC</td>
<td>LUC-YFP cloning</td>
</tr>
<tr>
<td>Term-YFPRVKPN</td>
<td>AGTCGGTACCTCCGCGCTATGTGCTGGAAT</td>
<td>LUC-YFP cloning</td>
</tr>
</tbody>
</table>
Conditions for the RT-PCR reactions were 95°C for 10 minutes followed by 35 cycles of 95°C for 30 seconds, 59°C for 1 minute, and 72°C for 30 seconds. Relative gene expression was calculated using the equation described by Pfaffl (2001), where \( \Delta ct \) represents \( 2^{-\Delta Ct} \), in which CesA gene expression is relative to that of the control gene, \( AtUBQ5 \). Melting curve analysis, agarose gel electrophoresis, and DNA sequencing of RT-PCR products was used to confirm that the RT-PCR amplicons represented the expected RT-PCR products.

2.2.10 Plant transformation vectors

Arabidopsis thaliana cellulose synthases (AtCesA) 4, 7, and 8 cDNAs were isolated from cDNA generated from wild-type Arabidopsis (Colombia ecotype) total RNA. PCR was used to isolate the cDNAs lacking a start codon for N-tagging in the plant transformation vectors, and adding a CACC sequence to the N-terminus for ligation into the pENTR cloning vector. The primers used for AtCesA isolation by PCR are listed in Table 2.2. PCR products were cloned into the pENTR-D/TOPO cloning vector (Invitrogen) and sequenced using universal M13 FW and RV primers, as well as gene-specific internal primers (Table 2.2) to verify identity and confirm that there were no PCR-induced mutations. Confirmed pENTR-CesA plasmids were subsequently used for Gateway-Clonase insertion of the CesA cDNA into plant transformation vectors.

Expression vectors containing Luciferase under the 35S promoter, pPZPhRLuc-attR (P35S::Luc), or enhanced Yellow Fluorescent Protein under the 35S promoter, pBin19YFP-attR (P35S::YFP) are described by Subramanian et al. (2006). LR Clonase II (Invitrogen) reactions between pENTR-CesA plasmids and the destination expression vectors were performed according to manufacturer’s instructions to create P35S::Luc-CesA and/or P35S::YFP-CesA expression vectors. Vectors were sequenced to ensure placement of the AtCesA cDNA in frame, downstream from the Luc or YFP tag.

To create expression vectors containing AtCesAs under the control of native Arabidopsis cellulose synthase promoters, the 35S promoter from the above Luc or YFP vectors were removed and replaced with AtCesA promoters. For PAtCesA::LUC destination vectors the Arabidopsis CesA promoters were amplified from DNA (between 1 and 2 kb upstream of gene start codons) using PCR (primers in Table 2.1), adding
SdaI (PAuCesA4, PAuCesA7) or HindIII (PAuCesA8) to the 5’ terminus and AvrII (PAuCesA4, 7, 8) to the 3’ terminus of the promoter fragment. The promoter fragments were digested from cloning vectors with SdaI/AvrII or HindIII/AvrII and ligated into the P35S::Luc-CesA backbone which had the 35S promoter removed by digestion with SdaI/AvrII or HindII/AvrII. LR Clonase II reactions were performed between the promoter-modified destination vectors and pENTR-CesA vectors to create PAuCesA4::Luc-CesA4 and PAuCesA8::Luc-CesA8. PAuCesA::YFP-CesA vectors were created in a similar fashion, except that XmaJI and AvrII were used to replace the 35S promoter and replace with the CesA promoter, followed by an LR Clonase II reaction with pENTR-CesA vectors to create PAuCesA7::YFP-CesA7 and PAuCesA8::YFP-CesA8. The promoter-tag-gene fragments of all binary vectors were confirmed with sequencing.

A binary construct containing a Luciferase-YFP fragment under the control of the AtCesA4 promoter was also created. The Luc-YFP fragment was amplified by PCR adding an AvrII site to the 5’ terminus and a KpnI site to the 3’ terminus and cloned into the pBLUNT cloning vector (Invitrogen). The YFP fragment was removed from the PAuCesA4::YFP binary vector using endogenous AvrII/KpnI sites. The Luc-YFP fragment was then cut and isolated from the cloning vector by a AvrII/KpnI double digest, and subsequently ligated into the previously linearized PAuCesA4::YFP vector, to create PAuCesA4::Luc-YFP.

2.2.11 Plant transformation

Plants were transformed using a method modified from Clough and Bent (1998). Agrobacterium tumefaciens GV3101-pMP90 (Hellens et al., 2000) were transformed with a binary vector using a freeze-thaw method. Cultures were grown overnight at 28°C in Luria-Bertani medium containing 50 μg L⁻¹ kanamycin or 50 μg L⁻¹ spectinomycin, 25 μg L⁻¹ rifampicin, and 25 μg L⁻¹ gentamycin. The overnight culture was centrifuged to pellet the cells and resuspended in a 5% (w/v) sucrose solution to an OD₆₀₀ of at least 0.400. Silwet L-77 (LEHLE Seeds) was added to each resuspended culture at a final concentration of 0.02% (v/v). Newly flowering plants (approximately 4 weeks old) were sprayed with the Agrobacterium solution using a fine mist spray nozzle. Sprayed plants were placed in a dark, humid environment for 16 to 24 hours and then returned to the light. A second spraying was often conducted 5 days after the first spray
to increase the transformation rate. After all spray treatments, plants were maintained as usual and seeds were harvested from mature, dried plants. Seeds were screened for putative transformants by germinating on half-concentration MS medium (no sucrose) with the addition of either 75 $\mu$g L$^{-1}$ kanamycin or 50 $\mu$g L$^{-1}$ glufosinate ammonium sulfate. Seedlings that successfully grew on antibiotics were grown in soil for further analysis. Transformed plants were confirmed by PCR on genomic DNA using gene specific oligonucleotides (Table 2.1)

2.2.12 Live-cell image acquisition of YFP-CesAs

Both seedlings and stem sections were used for live cell imaging. For seedlings, whole, dark- or light-grown, five to ten-day-old seedlings were mounted in water between a 44 x 22 mm (#1.5) cover slip and a glass slide. For stem sections, a segment of stem from plants 15 to 21-days old was excised and longitudinal hand sections were made and immediately mounted in water as above.

YFP fluorescence was detected via a Leica DMI6000 inverted microscope with a Quorum Wave FX system which had a modified Yokogawa CSU-10 spinning disk scan head (Yokogawa Electric Corporation). YFP was excited with a 491 nm laser and emissions passed through a 528/38 band filter (Chroma Technology). Images were acquired using a Hamamatsu 9100-13 EMCCD camera (Hamamatsu) controlled by Volocity software (Improvision).

2.3 Results
2.3.1 AtCesA$^{irx}$ phenotype

The AtCesA8$^{irx1-1}$, AtCesA7$^{irx3-1}$, and AtCesA4$^{irx5-1}$ mutants were used in this study (Figure 2.1 and Table 2.1). These mutants are of the Arabidopsis Landsberg ecotype. The AtCesA8$^{irx1-1}$ mutant contains an amino acid substitution at a conserved residue in the catalytic domain, likely rendering the mutant CesA8 protein non-functional but still produced (Taylor et al., 2003). The AtCesA7$^{irx3-1}$ and AtCesA4$^{irx5-1}$ mutants are believed to be null-mutations (Taylor et al., 1999; Taylor et al., 2003) as a result of truncation of the protein just after the fourth transmembrane domain. These mutants were used for three reasons: 1) previous studies had reported some changes to cell wall content in these mutants, providing some background and context for the work
presented in this thesis; 2) homozygous mutant seed stocks were readily available; and 3) These mutants grew more easily than other AtCesA\textsuperscript{irx} mutants which exhibited very weak and minimal growth and reproduction, which would have hindered some of the experiments conducted (namely genetic transformations and the potential for crossing).

Homozygous \textit{A8}\textsuperscript{irx1-1}, \textit{A7}\textsuperscript{irx3-1}, and \textit{A4}\textsubscript{irx5-1} mutants have a distinctive stunted growth and rounded-leaf phenotype compared to wild-type plants grown under the same conditions (Figure 2.2). Compared to wild-type, the AtCesA\textsuperscript{irx} plants are 20 to 42\% shorter (Table 2.3). AtCesA\textsuperscript{irx} plants grown under short-day conditions were more stunted than those grown under long-day conditions. Xylem vessels of all mutants were often collapsed and irregularly shaped (Figure 2.2, arrowheads).
Figure 2.2. Stature and xylem phenotype of wild-type and AtCesA\textsuperscript{rx} plants. Mature, dried plants harvested after growth under short-day conditions (\textminus S labels) or long-day conditions (\textminus L labels). Leaf inset pictures are from 15-day-old plants grown under long-day conditions. Wild-type (WT) plants (A) looked nearly indistinguishable under long and short-day conditions, so only short-day plants are imaged here. B, E, I: \textit{AtCesA8}\textsuperscript{rx1-1}. C, F, J: \textit{AtCesA7}\textsuperscript{rx3-1}. D, G, K: \textit{AtCesA4}\textsuperscript{rx5-1}. Cross sections of the lower portion of inflorescence stems (within the bottom 4 cm) show normal xylem in wild-type (H) and collapsed xylem in the mutants (I-K). Arrowheads indicate xylem vessels. Scale bar represents 50 \( \upmu \text{m} \).
Table 2.3. Maximum stem height and rosette-leaf diameter of wild-type and $AtCesA^{irx}$ plants grown under long-day and short-day conditions. Averages were calculated from measurements taken from 35-50 individual plants. Growth was measured for a total of 42 days (long-day, all lines), 98 days (short-day, wild-type), or 63 days (short-day, $AtCesA^{irx}$ lines) after which time growth ceased. Bold values represent averages that are statistically different from wild-type averages (t-test $p<0.05$).

<table>
<thead>
<tr>
<th>Line</th>
<th>Rosette Diameter</th>
<th>Stem height</th>
<th>Rosette Diameter</th>
<th>Stem height</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Max. Days</td>
<td>Avg. (cm)</td>
<td>S.D.</td>
<td>Max. Days</td>
</tr>
<tr>
<td>Wild-type</td>
<td>28</td>
<td>7.3</td>
<td>1.1</td>
<td>42</td>
</tr>
<tr>
<td>$A8^{irx1-1}$</td>
<td>28</td>
<td>4.0</td>
<td>0.8</td>
<td>42</td>
</tr>
<tr>
<td>$A7^{irx3-1}$</td>
<td>28</td>
<td>3.8</td>
<td>0.8</td>
<td>42</td>
</tr>
<tr>
<td>$A4^{irx5-1}$</td>
<td>28</td>
<td>4.0</td>
<td>0.8</td>
<td>42</td>
</tr>
</tbody>
</table>
Under long-days, stem heights increased steadily for 30 days, after which there was a brief growth plateau. Under short-day conditions $AtCesA^{irx}$ stems reached a maximum height by day 55, whereas the wild-type plants showed a steady increase in stem height after 60 days of growth and reached a maximum height after day 80. As seen in Figure 2.3, the stem growth rate of wild-type and $AtCesA^{irx}$ plants differ depending on day length growth conditions. Long-day $AtCesA^{irx}$ plants exhibit a reduced growth rate compared to wild-type plants. Contrastingly, short-day $AtCesA^{irx}$ plants exhibited an increased growth rate during the first 8 weeks of growth compared to wild-type plants. In particular, $A7^{irx3-1}$ and $A4^{irx5-1}$ plants experienced more rapid growth than wild-type and $A8^{irx1-1}$ plants. Day length conditions also affected reproductive stages of growth (Figure 2.4). Under long day conditions all lines formed buds and siliques at approximately the same times post-planting. However, under short-day conditions all the mutant lines formed buds and mature siliques significantly earlier than wild-type plants, and had fully matured nearly a month prior to wild-type plants.

2.3.2 Carbohydrate and lignin content

Total lignin (soluble plus insoluble) and structural carbohydrate content of mature $AtCesA^{irx}$ stems were determined and compared to wild-type plants to identify and quantify any differences in cell wall composition (Figure 2.5 and 2.7). Additionally, stems from plants grown under both long and short-day light conditions were compared.

2.3.2.1 Cellulose

Whole, mature, dried stems were evaluated for cellulose content. As seen in Figure 2.5, the $AtCesA^{irx}$ mutant lines had significantly lower levels of total glucose when compared to wild-type stems. Under both day lengths, $A8^{irx1-1}$ plants had more glucose than $A7^{irx3-1}$ and $A4^{irx5-1}$ plants, as compared to wild-type. $A8^{irx1-1}$ stems contained between 58% and 66% the amount of glucose found in wild-type plants, whereas $A7^{irx3-1}$ and $A4^{irx5-1}$ contained on average 65% less glucose than wild-type.
Figure 2.3. Stem growth rate of wild-type and AtCesA\textsuperscript{irx} plants grown under varied day-length conditions. (A) Plants grown under long-day conditions (16 hours light). (B) Plants grown under short-day conditions (8 hours light). Plant height was measured weekly once the primary stem was at least 0.5 cm tall, until plants ceased growing. The average stem height calculated each week is from a population of approximately 50 plants. Error bars represent standard deviations.
Figure 2.4. Chronological progression of flower and siliques formation of wild-type and AtCesA\textsuperscript{irx} Arabidopsis plants. Growth stages were monitored weekly for plants grown under long-day (A) and short-day (B) light conditions. Bars represent the time period during which 50% of the plants monitored were exhibiting that stage of growth. The red line indicates when siliques maturation began (based on complete yellowing of siliques on 50% of the plants monitored). The legend in box A also applies to box B.
**Figure 2.5.** Structural carbohydrate content of wild-type and AtCesA<sup>irx</sup> Arabidopsis stems. Plants were grown under long-day conditions (A) and short-day conditions (B). Error bars represent standard deviation, n represents 3 (3 pools of at least 25 plants in each pool). *values are statistically significant compared to wild-type, **values are statistically significant compared to AtCesA<sup>8<sup>irx1-1</sup></sup>, (p<0.05, ANOVA followed by Tukey’s post-hoc test).
Interestingly, $A8^{irx1-1}$ plants had increased starch levels under both long and short-day growth conditions, whereas $A7^{irx3-1}$ and $A4^{irx5-1}$ plants had reduced total glucose and starch under long-day conditions (Figure 2.7).

To determine what proportion of the altered glucose content can be ascribed to the cellulose in $AtCesA^{irx}$ stems, the amount of $\alpha$-cellulose was determined from extractive-free stem samples (Figure 2.6). All the $AtCesA^{irx}$ lines, under both short and long-day conditions, showed significantly reduced levels of $\alpha$-cellulose compared to wild-type stems. The reductions were more significant in short-day plants, such that $AtCesA^{irx}$ stems contained on average 62% less $\alpha$-cellulose than wild-type stems. Under both day-length conditions $A7^{irx3-1}$ showed the greatest reduction in $\alpha$-cellulose. Interestingly, the glucose levels in $A8^{irx1-1}$ plants seemed least affected (compared to the other $AtCesA^{irx}$ lines), however, the $\alpha$-cellulose levels for all the $AtCesA^{irx}$ lines were similar suggesting an equivalent cellulose reduction in all mutant lines.

### 2.3.2.2 Lignin

Acid-soluble (AS) and acid-insoluble (AI) lignin were determined for $AtCesA^{irx}$ and wild-type stems, and summed to represent total lignin content (Figure 2.5). Lignin content was elevated in all the $AtCesA^{irx}$ lines under both short and long day conditions. The greatest changes in total lignin, above wild-type levels, were observed in $A7^{irx3-1}$ and $A4^{irx5-1}$ stems grown under long-day conditions (a 32% and 24% increase over wild-type, respectively). The $A8^{irx1-1}$ stems also contained elevated lignin, but only significantly under short-day conditions, with a 15% increase over wild-type. Under long-day conditions the increases in lignin in $AtCesA^{irx}$ stems was due to an overall increase in both AS and AI lignin (Figure 2.7). A trend observed in all long-day mutant lines was a higher proportion of AI to AS lignin such that the ratio of AS:AI decreased from 8.1 in wild-type to between 5.5 and 7.3 in $AtCesA^{irx}$ lines. The observed changes in AI and AS proportions were only statistically significant in $A4^{irx5-1}$ stems, and can be attributed more specifically to an excessive increase in AI lignin compared to all other lines (Figure 2.7).
Figure 2.6. α-cellulose content of wild-type and AtCesA<sup>irx</sup> stems. Stems were harvested from mature, dried plants grown under long-day (white bars) and short-day (grey bars) conditions. Error bars represent standard deviation, n represents 3 (3 pools of at least 25 plants each). *values are statistically significant compared to wild-type (p<0.05, ANOVA followed by Tukey's post-hoc test).
Figure 2.7. A summary of growth and cell wall changes measured in \( \text{AtCesA8}^{\text{irx1-1}} \), \( \text{AtCesA7}^{\text{irx3-1}} \), and \( \text{AtCesA4}^{\text{irx5-1}} \) plants grown under long and short day conditions. Values represent the fraction percentage compared to wild-type plants (e.g.: 0.66 glucose corresponds to 66% of the wild-type level), such that values above and below 1.0 represent an increase and decrease compared to wild-type, respectively. Red colours indicate a large decrease, green colours a large increase. Orange and yellow colours represent a range of moderate decreases, no change, to moderate increases. MFA – microfibril angle, DP – degree of polymerization, AS – acid soluble, AI – acid insoluble.
2.3.2.3 Hemicellulose and pectin

Components of secondary cell wall hemicelluloses were estimated by HPLC determination by quantifying the amount of fucose, arabinose, galactose, xylose, and mannose in stem extractions (Table 2.4). All the AtCesA\textsuperscript{irx} lines exhibited an overall increase in hemicellulose content compared to wild-type, under both long and short-day growth conditions (Figure 2.7). The greatest change was in stems of A7\textsuperscript{irx3-1} and A4\textsuperscript{irx5-1} plants which had a 34% and 30% increase above wild-type, respectively. A8\textsuperscript{irx1-1} stems had a slight, but statistically significant, increase (8% above wild-type) in hemicelluloses, but only under long-day conditions. A more careful inspection of the monosaccharide composition (Table 2.4) indicates that the increase in hemicellulose content of AtCesA\textsuperscript{irx} stems is due to broad increases in most of the monosaccharide components analyzed, with the greatest increase being in fucose, arabinose, and xylan, of which the latter two may represent an increase in arabinoxylan. The only exception was mannose, which generally decreased in all mutant lines, particularly under short-day conditions. Rhamnose values, which form part of the pectic components, remained similar to wild-type levels except in A4\textsuperscript{irx5-1} stems, which contained 17% to 22% greater levels of rhamnose under long and short-day conditions, respectively.

2.3.3 Cellulose characterization

To broaden our understanding of how the AtCesA mutations affect cellulose ultrastructure, cellulose crystallinity, microfibril angle (MFA), and degree of polymerization (DP) were measured and compared to wild-type stems (Table 2.5). Cellulose MFAs in the A7\textsuperscript{irx3-1} and A4\textsuperscript{irx5-1} lines were significantly larger than the MFAs of wild-type and A8\textsuperscript{irx1-1}, when grown under long-day conditions. Wild-type and A8\textsuperscript{irx1-1} MFA was approximately 14\textdegree, whereas A7\textsuperscript{irx3-1} and A4\textsuperscript{irx5-1} MFA was between 17\textdegree and 20\textdegree. Interestingly, there were no significant differences in MFA among wild-type and the AtCesA\textsuperscript{irx} lines when grown under short-day conditions. In general, the MFAs were larger in all plants when grown under short-day conditions compared to long-day.
Table 2.4. Cell wall carbohydrate content of wild-type (WT) and AtCesA
Arabidopsis inflorescence stems from plants grown under long-day and short-day conditions. Standard deviation is in parenthesis. Values in bold are statistically significant compared to wild-type values, *indicates a significant difference compared to A8
Arabidopsis, ♦indicates a difference compared to A4
Arabidopsis (p<0.05, ANOVA followed by a Tukey’s post-hoc test), n represents 3.

<table>
<thead>
<tr>
<th>Line</th>
<th>Fucose (µg/mg dry weight)</th>
<th>Arabinose (µg/mg dry weight)</th>
<th>Rhamnose (µg/mg dry weight)</th>
<th>Galactose (µg/mg dry weight)</th>
<th>Glucose (µg/mg dry weight)</th>
<th>Xylose (µg/mg dry weight)</th>
<th>Mannose (µg/mg dry weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Long-day</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>3.02 (0.15)</td>
<td>10.71 (0.40)</td>
<td>8.04 (0.53)</td>
<td>12.36 (0.58)</td>
<td>433.06 (14.44)</td>
<td>144.28 (3.22)</td>
<td>17.41 (0.89)</td>
</tr>
</tbody>
</table>
| A8
Arabidopsis | 3.25 (0.07)               | **13.75 (0.12)**              | 7.60 (0.38)                 | 13.12 (0.27)                 | **287.80 (5.76)**         | 156.24 (3.76)             | 16.70 (0.45)              |
| A7
Arabidopsis | **3.51 (0.11)**           | **13.21 (0.77)**              | 7.94 (0.70)                 | 13.47 (0.63)                 | **163.78 (5.72)**         | **206.94 (7.88)**         | **13.42 (0.37)**          |
| A4
Arabidopsis | **4.45 (0.50)**           | **15.19 (1.31)**              | 9.44 (1.07)                 | **13.67 (0.31)**             | **138.35 (13.95)**        | **195.28 (6.42)**         | 15.29 (1.19)              |
| **Short-day**|                           |                               |                             |                              |                           |                           |                           |
| WT           | 3.58 (0.12)               | 15.41 (0.64)                  | 8.48 (0.64)                 | 15.69 (0.67)                 | 439.79 (24.72)            | 141.61 (9.51)             | 20.66 (1.57)              |
| A8
Arabidopsis | **4.15 (0.16)**           | **20.91 (0.71)**              | 8.34 (0.35)                 | 15.45 (0.63)                 | 255.99 (11.39)            | 146.79 (5.11)             | **15.93 (1.18)**          |
| A7
Arabidopsis | **4.41 (0.19)**           | 15.78 (1.29)                  | 8.64 (1.18)                 | 14.42 (0.56)                 | **152.66 (1.77)**         | **183.73 (3.02)**         | **12.43 (1.31)**          |
| A4
Arabidopsis | **4.61 (0.13)**           | **17.99 (0.25)**              | **10.31 (0.43)**            | 15.00 (0.35)                 | **153.54 (1.97)**         | **186.68 (3.25)**         | **14.55 (0.52)**          |
Table 2.5. Microfibril angle, cell wall crystallinity, and relative degree of polymerization (DP) of cellulose from stems of wild-type (WT) and AtCesA⁴ plants. The DP values presented are relative to WT (WT set to 100). Plants were grown under long-day and short-day light conditions. Standard deviation is in parenthesis. Values in bold are statistically significant compared to wild-type values, *indicates a significant difference compared to A⁸irx⁴⁻¹, *indicates a difference compared to A⁴irx⁵⁻¹ (p<0.05, ANOVA followed by a Tukey’s post-hoc test), n represents 6 for MFA and crystallinity, n=2 or 3 for relative DP.  a represents no day-length effect and b represents a day-length effect (p<0.05, two-way ANOVA followed by a Bonferroni post-hoc test).

<table>
<thead>
<tr>
<th>Line</th>
<th>MFA</th>
<th>% Crystalline</th>
<th>Relative DP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Long-Day</td>
</tr>
<tr>
<td>WT</td>
<td>13.5 (0.8)ᵇ</td>
<td>42.6 (2.3)ᵃ</td>
<td>100.0 (17.5)</td>
</tr>
<tr>
<td>A⁸irx⁴⁻¹</td>
<td>13.9 (2.1)ᵇ</td>
<td>32.6 (4.3)ᵃ</td>
<td>117.4 (10.2)</td>
</tr>
<tr>
<td>A⁷irx³⁻¹</td>
<td>17.2 (1.6)*ᵇ</td>
<td>25.5 (5.5)ᵃ</td>
<td>72.7 (12.6)*</td>
</tr>
<tr>
<td>A⁴irx⁵⁻¹</td>
<td>19.9 (1.0)*ᵃ</td>
<td>34.3 (8.5)ᵃ</td>
<td>38.6 (11.6)*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Short-Day</td>
</tr>
<tr>
<td>WT</td>
<td>20.8 (2.1)</td>
<td>46.1 (7.3)</td>
<td>100.0 (17.5)</td>
</tr>
<tr>
<td>A⁸irx⁴⁻¹</td>
<td>19.7 (1.4)</td>
<td>37.6 (9.3)</td>
<td>45.9 (16.6)</td>
</tr>
<tr>
<td>A⁷irx³⁻¹</td>
<td>20.8 (3.0)</td>
<td>35.8 (6.7)</td>
<td>44.5 (3.7)</td>
</tr>
<tr>
<td>A⁴irx⁵⁻¹</td>
<td>21.2 (2.1)</td>
<td>44.1 (8.6)</td>
<td>43.2 (16.8)</td>
</tr>
</tbody>
</table>
From long-day to short-day conditions, wild-type and \( A8^{irx1-1} \) plants exhibited an average increase in MFA of 6.5\(^{\circ}\), whereas \( A7^{irx3-1} \) and \( A4^{irx5-1} \) only had an average 2\(^{\circ}\) increase in MFA when grown under short-day conditions. Two-way ANOVA analysis revealed a significant day-length effect on MFA in wild-type, \( A8^{irx1-1} \), and \( A7^{irx3-1} \) plants. Interestingly, no day-length effect was found for MFA of \( A4^{irx5-1} \) plants, suggesting that the \( A4^{irx5-1} \) mutation may mute the effect of day-length on MFA.

Under long-day conditions, cell wall crystallinity was lower in the \( AtCesA^{irx} \) mutants compared to wild-type plants, although only the decreases in \( A8^{irx1-1} \) and \( A7^{irx3-1} \) were statistically significant. Under short-day conditions, a similar trend was observed but none of the values were significantly different, and the decreases (compared to wild-type) were less than those observed for long-day grown plants. Although the general trend in cell wall crystallinity values was increased for all lines when grown under short-days, compared to long-days, there was no statistically significant effect of day-length effect on cell wall crystallinity.

The degree of polymerization (DP) of the isolated \( \alpha \)-cellulose from the mutant lines was calculated relative to the wild-type samples. As seen in Table 2.5, under long-day conditions the \( \alpha \)-cellulose from the \( A7^{irx3-1} \) and \( A4^{irx5-1} \) mutants had a significantly lower DP compared to wild-type and the \( A8^{irx1-1} \) mutant plants. In particular, the relative DP of \( A4^{irx5-1} \) plants was approximately 60\% lower than that of wild-type. Under short-day conditions, a more uniform reduction in DP was observed in the mutant lines, as all the mutants had relative DP values that were approximately 55\% lower than that of the wild-type plants. Reduced \( \alpha \)-cellulose quantities limited the replications that could be performed for DP, and for this reason a two-way ANOVA was not performed to statistically determine the presence of a day-length effect on DP. However, comparison of the raw DP values (data not shown) suggests there may only be a negative day-length effect on DP of \( A8^{irx1-1} \) mutants grown under short-days.

A summary of the changes to growth and cell wall properties in the \( AtCesA^{irx} \) is presented in Figure 2.7. In general, the \( A7^{irx3-1} \) mutant plants exhibit a more severely altered cell wall phenotype compared to the \( A8^{irx1-1} \) and \( A4^{irx5-1} \) mutants.
2.3.4 Expression of AtCesAs in AtCesA\textsuperscript{irx} and wild-type plants

Various constructs were created to assess the expression of the wild-type AtCesA genes in both wild-type and AtCesA\textsuperscript{irx} mutant backgrounds. Gene expression was controlled by either the constitutive cauliflower mosaic 35S promoter (P\textsubscript{35S} or the native AtCesA promoters (P\textsubscript{AtCesA}). For all constructs, the AtCesA cDNA was tagged with one of either an N-terminal yellow fluorescent protein (YFP) or Luciferase (LUC) tag (YFP-CesA or LUC-CesA).
2.3.4.1 \( P35S::AtCesA \) expression in AtCesA\(^{irx} \) plants

Expression of wild-type \( AtCesA \) in an \( AtCesA^{irx} \) mutant background should restore mutant plants to a wild-type phenotype if the \( CesA \) transgene is functional and expressed at the appropriate temporal and spatial levels. Expression of \( P35S::LUC-AtCesA \) constructs in \( A4^{irx5-1} \) and \( A8^{irx1-1} \) plants were not sufficient to rescue the mutant phenotype (Figure 2.8). Three independent transgenic \( A4^{irx5-1} \) lines carrying the \( P35S::LUC-AtCesA4 \) construct showed no recovery of stunted growth or collapsed xylem vessels. Similarly, \( A8^{irx1-1} \) plants carrying the \( P35S::LUC-AtCesA8 \) construct showed no recovery from the mutant phenotype. Lack of complementation appears to be correlated to low transgene expression levels in the transgenic \( A4^{irx5-1} \) lines (Figure 2.9 A) compared to endogenous \( CesA \) expression in wild-type plants, and may be due to 35S-driven expression in the \( A8^{irx1-1} \).
Figure 2.8. *AtCesA*<sup>irx</sup> plants transformed with *P<sub>35S</sub>::LUC-AtCesA* constructs do not show recovery of a wild-type phenotype. *P<sub>35S</sub>::LUC-AtCesA4* is expressed in the *AtCesA4<sup>irx5-1</sup>* mutant background (C, G), and *P<sub>35S</sub>::Luc-AtCesA8* in *AtCesA8<sup>irx1-1</sup>* (D, H). Transgenic lines and non-transformed *AtCesA<sup>irx</sup>* (B, F) plants exhibit stunted growth and collapsed xylem compared to wild-type plants (A, E). Scale bar represents 50 μm.
Figure 2.9. Real-time PCR analysis of transcript abundance in RNA from stems of 15-day-old plants. \textit{AtCesA}^{irx} plants were transformed with endogenous \textit{AtCesA}s under the 35S promoter. (A) \textit{AtCesA}4 expression was measured in wild-type (WT), \textit{AtCesA}4^{irx5-1} (A4-irx), and \textit{A4}^{irx5-1}.\textit{P35S::LUC-AtCesA}4 stems (#3, #4, and #5). (B) \textit{AtCesA}8 expression was measured in wild-type (WT), \textit{AtCesA}8^{irx1-1} (A8-irx), and \textit{A8}^{irx1-1}.\textit{P35S::LUC-AtCesA}8 (35S-L-A8) stems. Expression levels are relative to the control gene, \textit{AtUBQ5}. Error bars represent standard error of the mean, n represents 3 biological replicates.
2.3.4.2  \( P_{AtCesA}:AtCesA \) expression in \( AtCesA^{\text{irx}} \) plants

Proper temporal and spatial expression of the \( AtCesA \) transgenes should be achieved when expression is regulated by the native \( AtCesA \) promoters. Indeed, \( A4^{\text{irx5-1}} \) plants transformed with \( P_{AtCesA4}:LUC-AtCesA4 \), \( A7^{\text{irx3-1}} \) plants transformed with \( P_{AtCesA7}:YFP-AtCesA7 \), and \( A8^{\text{irx1-1}} \) plants transformed with \( P_{AtCesA8}:LUC-AtCesA8 \) all showed varying levels of complementation. Transgenic \( A4^{\text{irx5-1}} \) and \( A7^{\text{irx3-1}} \) plants did not show the stunted growth phenotype and had open, normal-shaped xylem vessels (Figure 2.10). Transgenic \( A8^{\text{irx1-1}} \) plants exhibited only partial complementation as evidenced as growth remained stunted, but microscopically had fewer collapsed xylem (Figure 2.10). Varying degrees of complementation could be the result of transgene expression levels. Real-time PCR analysis revealed different levels of transgene expression in the transgenic lines (Figure 2.11). Levels of \( AtCesA7 \) in the \( A7^{\text{irx3-1}} \) \( YFP-AtCesA7 \) transgenic plants were 25\% greater than endogenous \( AtCesA7 \) levels in wild-type plants. Comparatively, levels of \( AtCesA4 \) in \( A4^{\text{irx5-1}} \) \( LUC-AtCesA4 \) transgenic plants and levels of \( AtCesA8 \) in \( A8^{\text{irx1-1}} \) \( LUC-AtCesA8 \) transgenic plants were only 7\% and 31\% of endogenous gene expression levels, respectively. Two additional lines of \( A7^{\text{irx3-1}} \) expressing \( P_{AtCesA7}:YFP-AtCesA7 \) had \( AtCesA7 \) transcript levels that were significantly lower than wild-type levels and showed little to no complementation of the mutant phenotype (data not shown).

2.3.4.3  Double-transgenic wild-type plants

Wild-type plants of the Columbia (WT-C) or Landsberg (WT-L) ecotypes were transformed with two constructs to create double transgenic plants. The following transformations were recovered: WT-C transformed with both \( P_{35S}:LUC-AtCesA7 \) and \( P_{35S}:YFP-AtCesA7 \) (WTC-2xA73S), WT-C transformed with \( P_{AtCesA7}:YFP-AtCesA7 \) and \( P_{35S}:LUC-AtCesA7 \) (WTC-2xA73S/\( P_{AtCesA7} \)), WT-C transformed with \( P_{35S}:LUC-AtCesA4 \) and \( P_{35S}:YFP-AtCesA4 \) (WTC-2xA43S), and WT-L transformed with \( P_{AtCesA8}:LUC-AtCesA8 \) and \( P_{AtCesA8}:YFP-AtCesA8 \) (WTL-2xA83S). The T1 generation plants were grown alongside untransformed WT and \( AtCesA^{\text{irx}} \) plants under long-day growth conditions.
Figure 2.10. Phenotype of $AtCesA^{irx}$ plants transformed with wild-type $AtCesA$s under the control of native $AtCesA$ promoters. Images are of 25-day-old plants. Wild-type (A and F), $AtCesA^{irx}$ (B and G), $AtCesA4^{irx5-1}$ transformed with $P_{AtCesA4}::LUC$-$AtCesA4$ (C and H), $AtCesA7^{irx3-1}$ transformed with $P_{AtCesA7}::YFP$-$AtCesA7$ (D and I), $AtCesA8^{irx1-1}$ transformed with $P_{AtCes8}::LUC$-$AtCesA8$ (E and J). Arrow heads indicate open xylem in F, H, I, and J and collapsed xylem in G. Scale bar represents 50 μm.
Figure 2.11. Real-time PCR analysis of transcript abundance of AtCesA<sup>irx</sup> plants transformed with endogenous AtCesAs under native AtCesA promoters. A: AtCesA4 expression was measured in wild-type (WT), A4<sup>irx5-1</sup> (IRX5), and A4<sup>irx5-1</sup>-PA<sub>CesA4</sub>::Luc-AtCesA4 stems (PA4-L-A4). B: AtCesA7 expression was measured in wild-type (WT), A7<sup>irx3-1</sup> (IRX3), and A7<sup>irx3-1</sup>-PA<sub>CesA7</sub>::YFP-AtCesA7 stems (PA7-Y-A7). C: AtCesA8 expression was measured in wild-type (WT), A8<sup>irx1-1</sup> (IRX1), and A8<sup>irx1-1</sup>-PA<sub>CesA8</sub>::Luc-AtCesA8 stems (PA8-L-A8). Expression levels are relative to the control gene, AtUBQ5. Error bars represent standard deviation, n represents 3 biological replicates.
All of the WT-C double transgenic plants (WTC-2xA735S, WTC-2xA735S/PA7, and WTC-2xA435S) showed a severely stunted, \textit{AtCesA}^{irx}-like phenotype (Figure 2.12), whereas the WTL-2xA8PA8 double transgenic line exhibited only a mild reduction in stature (Figure 2.13). The WT-C double transgenic lines had a bushy appearance due to the growth of many axillary branches. Stems were extremely thin and weak, and the double WT-C transgenic plants produced fewer mature siliques and siliques devoid of any seeds. Additionally, these transgenic lines had collapsed xylem and all had reduced \textit{AtCesA} transcript abundance compared to wild-type plants (data not shown). The stems of the WTL-2xA8PA8 transgenic line were slightly thinner compared to wild-type stems, and had a reduced ability to grow upright, but did not have collapsed xylem and had elevated \textit{AtCesA8} transcript abundance (data not shown).

### 2.3.5 Visual profile of YFP-CesA7

Expression of \textit{PAtCesA7::YFP-AtCesA7} in homozygous \textit{A7}^{irx3-1} mutant plants restored the wild-type phenotype. This suggests that the YFP-AtCesA7 subunit is functional in the cellulose synthesizing complex. Using fluorescence spinning-disc confocal microscopy, the distribution patterns and movement of YFP-CesA7 was observed in a variety of cell types during different stages of plant growth (Figure 2.14-Figure 2.19). Expression was observed in many tissue types including seedlings, roots, leaf, and very young stem tissue (Figure 2.14). In the root tip, YFP-CesA7 particles appeared as small, dense puncta. It is interesting to note that all cells observed in the root tip contained YFP-CesA7 except the outer most layer of root cap columella cells (Figure 2.14). The YFP-CesA7 particles observed varied in size, shape, rate of movement, and abundance depending on tissue type and developmental stage. Compared to the puncta observed in root tips, the density of YFP-CesA7 particles in hypocotyl xylem was reduced and often a diffuse signal was observed (Figure 2.14). In the cotyledon and leaf tissue, large, fast moving (likely due to cytoplasmic streaming) puncta were observed in pavement cells and guard cells (Figure 2.14). Using a longitudinal section of very young stem tissue, where a great deal of cellular division and expansion is expected to be occurring, a high concentration of large, bright puncta were observed (Figure 2.14 D-E).
Figure 2.12. Wild-type (ecotype Columbia) plants transformed with two *AtCesA*-expression constructs show a severe *AtCesA*_{irx}~like phenotype. Wild-type (A) and *AtCesA4*_{irx5-1} (B) plants were grown alongside the T1 generation double transgenic plants, (C) WTC-2xA735S, (D) WTC-2xA735S/PA7, and (E) WTC-2xA435S. Images are of mature, dried plants. Notice the reduced presence of full-sized siliques on the double transgenic plants.
Figure 2.13. Wild-type (ecotype Landsberg) plants transformed with two AtCesA8 expression constructs show a mostly wild-type like phenotype. Wild-type (A, C) plants were grown alongside the T1 generation of double transgenic plants WTL-2xA8PA8 (B, D). Plants were 25-30 days-old. Arrow heads indicate. Scale bars represent 50 µm.
Figure 2.14. YFP-CesA7 expression patterns in young tissues of AtCesA7<sup>irx3-1</sup>-<br>PA<sub>t</sub>CesA7::YFP-AtCesA7 plants. (A) Root tip in a 5-day-old seedling. (B) Hypocotyl of a 7-day-old seedling. (C) Cells in cotyledons of a 5-day-old seedling. (D) A longitudinal section of the uppermost region of an inflorescence stem. The blue lines outline predicted cell edges based on the corresponding bright field image (not shown). (E) A magnification of the ring-shaped YFP-CesA7 particles observed in upper, developing stem tissue.

White arrow heads indicate xylem vessels. Coloured arrow heads are positional markers to compare YFP and the corresponding brightfield image.

Scale bars represent 20 μm (A-D), 5 μm (E).
The cells are believed to be a combination of pith parenchyma, immature xylem, and immature fibres. As seen in Figure 2.14-E, some of the YFP-CesA7 particles in this tissue were often observed to be contained in ring shapes.

A profile of YFP-CesA7 in stems was created to document the varied expression in the various cell types of stem tissue, during different stages of development (Figure 2.15-2.18). From these profiles it is apparent that CesA7 is highly expressed in a variety of cell types from those in young developing stem tissue, to the mature stem base. Specifically, in stem tissue, it appears that CesA7 is expressed in cells undergoing cell expansion (based on the position of cells near the stem tip, Figure 2.15), young xylem vessels which exhibit spiral cell-wall thickenings (Figure 2.15 and 2.16), fibres (Figure 2.17), pith cells (Figure 2.15-2.18), and potentially phloem, epidermal tissue, cortex, and endodermal cells (Figure 2.15-2.18). In the upper stem there was a clear association of YFP-CesA7 with developing xylem (Figure 2.15) which decreases near the stem base (Figure 2.18). Additionally, it can be seen that as interfascicular fibre abundance increases from stem tip to base, there is a greater density of diffuse YFP-CesA7 signal visible in fibres (Figure 2.17). The mid-stem region, between 5 and 10 cm from the base of an approximately 20cm-tall plant, appeared to contain fibres with the strongest YFP-CesA7 signal. Focusing on the plasma membrane of these developing fibres, the YFP-CesA7 signal covered the plasma membrane, often appearing as a sheet of YFP signal (Figure 2.17-F). Only occasionally were small, distinct particles visible. The particles observed (both small and large) appeared to be moving at an extremely fast rate. However, measuring particle velocity or tracking the directionality of particle movement was not possible using traditional kymograph analysis due to the depth of fibre cells within the tissue, resulting in significant interference of YFP signal from surrounding cells.
Figure 2.15. Cellular anatomy and YFP-CesA7 expression patterns in the upper stem of $AtCesA7^{irx3-1}$ : $P_{AtCesA7}$ : YFP-$AtCesA7$ plants. Toluidine Blue staining of a stem cross section (A) and longitudinal sections (B) was performed to observe cellular anatomy. (B) A collage of longitudinal sections was assembled to compare cell types. YFP-CesA7 signal in longitudinal sections of stem tissue (C-E). (C) A collage of longitudinal sections was assembled to compare expression in various cell types across the stem. (D) and (E) are magnified images of xylem elements.

White arrow heads indicate xylem vessels. Coloured arrow heads are positional markers to compare YFP and the corresponding brightfield image. Letter markings indicate general regions containing the following cell types: ep-epidermis, c -cortex, en-endodermis, fi-fibres, x- xylem, p/c-phloem/cambium, pi-pith.

Scale bars represent 100 $\mu$m (A, B), 25 $\mu$m (C), 5$\mu$m (D, E).
**Figure 2.16.** Cellular anatomy and YFP-CesA7 expression patterns in the upper middle stem of *AtCesA7<sup>inx3-1</sup>-PAtCesA7::YFP-AtCesA7* plants. Toluidine Blue staining of a stem cross section (A) and longitudinal sections (B and C) were performed to observe cellular anatomy. YFP-CesA7 signal in longitudinal sections of stem tissue (D-F). (D) A collage of longitudinal sections was assembled to compare expression in various cell types across the stem (from one edge to just before the opposite outer edge). (E) and (F) are magnified images of a xylem element (E) and fibre (F).

White arrow heads indicate xylem vessels. Coloured arrow heads are positional markers to compare YFP and the corresponding brightfield image. Letter markings indicate general regions containing the following cell types: ep-epidermis, c -cortex, en-endodermis, fi-fibres, x- xylem, p/c-phloem/cambium, pi-pith.

Scale bars represent 100 μm (A-C), 25 μm (D), 10μm (E), 5μm (F).
**Figure 2.17.** Cellular anatomy and YFP-CesA7 expression patterns in the lower middle stem of *AtCesA7*/*px3-1-PAtCesA7::YFP-AtCesA7* plants. Toluidine Blue staining of a stem cross section (A) and longitudinal sections (B and C) were performed to observe cellular anatomy. YFP-CesA7 signal in longitudinal sections of stem tissue (D-F). (D) A collage of longitudinal sections was assembled to compare expression in various cell types across the stem (from the outer edge to the centre pith). (E) and (F) are magnified images of a xylem vessel (E) and two fibres (F). The red box highlights an area that contains a saturated signal due to the high abundance of YFP-CesA7 at the plasma membrane. The arrows indicate very small individual particles.

White arrow heads indicate xylem vessels. Coloured arrow heads are positional markers to compare YFP and the corresponding brightfield image. Letter markings indicate general regions containing the following cell types: ep-epidermis, c -cortex, fi-fibres, x- xylem, pi-pith.
Scale bars represent 100 μm (A-C), 25 μm (D), 10μm (E), 5μm (F).
**Figure 2.18.** Cellular anatomy and YFP-CesA7 expression patterns near the base of the stem of $\text{AtCesA7}^{\text{inx3-1}}-\text{PAtCesA7::YFP-AtCesA7}$ plants. Toluidine Blue staining of a stem cross section (A) and longitudinal sections (B and C) were performed to observe cellular anatomy. YFP-CesA7 signal in longitudinal sections of stem tissue (D-F). (D) A collage of longitudinal sections was assembled to compare expression in various cell types across the stem (from the outer edge to the centre pith). (E) and (F) are magnified images of a xylem vessel (E) and fibres (F). (F) The blue lines highlight predicted cell edges based on the associated brightfield image (not shown).

White arrow heads indicate xylem vessels. Coloured arrow heads are positional markers to compare YFP and the corresponding brightfield image. Letter markings indicate general regions containing the following cell types: ep-epidermis, c -cortex, en – endodermis, fi-fibres, x- xylem, pi-pith.

Scale bars represent 100 $\mu$m (A-C), 25 $\mu$m (D), 10$\mu$m (E), 5$\mu$m (F).
2.3.6 Visualization of YFP-CesA8 in wild-type plants

Wild-type (Landsberg ecotype) transformed with both $P_{AtCesA8}::Luc-AtCesA8$ and $P_{AtCesA8}::YFP-AtCesA8$ (WT-L-2xA8PA8) were used to visualize the distribution of CesA8 in stem tissue. Compared to YFP-CesA7-expressing plants, the YFP-CesA8 signal was weaker in intensity and less abundant in all cell types of the stem. In particular, it was not detected in developing fibres, compared to the high levels of YFP-CesA7 detected in fibres. As seen in Figure 2.19, it was possible to detect YFP-CesA8 in xylem cells. The observed patterns and abundance of YFP-CesA8 compared to YFP-CesA7 could be due to expression as a result of the transgene insertion, the expression of YFP-CesA8 in a wild-type background (compared to the irx background in the case of the YFP-CesA7 plants), the native differences in expression and function of CesA8 compared to CesA7, or a combination of these factors.
Figure 2.19. YFP-CesA8 expression patterns in xylem of wild-type plants transformed with \textit{PAtCesA8::YFP-AtCesA8} (PA8-YFP-A8). YFP-CesA8 signal in longitudinal sections of stem tissue from transformed, PA8-YFP-A8, plants (A) as compared to untransformed wild-type (B) and \textit{AtCesA7\textsuperscript{irx3-1}}-\textit{PAtCesA7::YFP-AtCesA7} plants (C). In (A) the yellow arrow head indicates a YFP-CesA8 ring structure.

Coloured arrow heads are positional markers to compare YFP and the corresponding bright field image. Scale bars represent 10 μm.
2.4 Discussion

To date, *AtCesA4*, *AtCesA7*, and *AtCesA8* are the most well-characterized secondary cell wall cellulose synthases in the plant kingdom, and mutants have been shown to possess altered cell wall composition (Turner and Somerville, 1997; Zhong *et al.*, 2003; Ha *et al.*, 2002; Taylor *et al.*, 2003; Taylor *et al.*, 1999; Brown *et al.*, 2005; Bosca *et al.*, 2006). However, to-date there has not been a comprehensive review of the *AtCesA4*, *AtCesA7*, and *AtCesA8* mutants in a single, independent study. Nor has there been a uniform report on the changes to cell wall composition (Table 2.1), and there is no information pertaining to cellulose ultrastructure such as MFA, DP, and crystallinity. In an attempt to increase our understanding of the potential role(s) of each CesA in cellulose biosynthesis we characterized how each mutant manifests differences in secondary cell wall composition and cellulose ultrastructure. Our findings provide evidence to suggest that AtCesA4 may have a unique effect on cellulose ultrastructure, and that the composition of the CSC has a very strong influence on cellulose MFA, crystallinity, and DP. Herein we provide the first report of three secondary cell wall AtCesA mutants analyzed together including novel information about the cellulose ultrastructure of these mutants, allowing for important comparisons among the mutants.

2.4.1 Growth defects of *AtCesA*irx mutants manifest differently under varied day-length conditions

The reports on the growth characteristics of the *AtCesA8*irx1-1, *AtCesA4*irx5-1, and *AtCesA7*irx3-1 mutant plants are that they exhibit stunted growth and have weak stems, compared to wild-type plants (Turner and Somerville, 1997; Bosca *et al.*, 2006). However, from the work presented herein it is apparent that when grown under short-day conditions the stunted *AtCesA*irx phenotype is more severe. Additionally, we reveal pleiotropic effects of the *AtCesA*irx mutations on growth rate and reproductive rates.

The abundance of collapsed xylem in *AtCesA*irx mutants likely reduces water transport, which may in turn reduce growth potential resulting in overall stunting of both stem and leaves. Total glucose levels were significantly decreased in *AtCesA*irx plants compared to wild-type, which could signify a reduced photosynthetic capacity in the mutants, thus limiting the amount of growth possible. However, having not measured
other physical parameters such as soluble sugar content and rate of photosynthesis, 
this remains a putative explanation.

The amount of \( \alpha \)-cellulose generally decreased under short-day conditions (in \( \text{AtCesA}^{irx} \) stems, but not in wild-type), despite the fact that the total glucose content was 
equivalent between long and short-day plants, suggesting that glucose allocation may 
be influenced by the rate and extent of synthesis of the cell wall. Late-night cell wall 
biosynthesis has been suggested to occur in \( \text{Arabidopsis} \) seedlings (Harmer \textit{et al.}, 2000). The greater abundance of \( \alpha \)-cellulose produced in short-day wild-type plants 
corroborates night-time cellulose biosynthesis. Some diurnal regulation of secondary 
cell wall formation could also explain why the \( \text{AtCesA}^{irx} \) plants exhibit a greater 
reduction in cellulose biosynthesis capacity under short day growth conditions.

Day length conditions significantly affected cellulose microfibril angle (MFA) in 
\( \text{AtCesA}^{irx} \) and wild-type plants. Under long-day conditions the \( \text{AtCesA7}^{irx3-1} \) and 
\( \text{AtCesA4}^{irx5-1} \) plants had significantly larger MFAs (20°) compared to wild-type and 
\( \text{AtCesA8}^{irx1-1} \) (14°). Under short-day conditions, however, all lines had a similar MFA 
(approximately 20°). Although it has been proposed that more rapid growth rates result 
in a larger MFA (Barnet and Bonham, 2004), this was not observed here as the 
opposite effect was observed: the wild-type and \( \text{AtCesA8}^{irx1-1} \) plants produced cellulose 
microfibrils aligned at a smaller angle to the longitudinal axis of the cell under long-day 
conditions, when they grow at a more rapid rate compared to short-day conditions. 
Correlations between MFA and growth rate have only been reported for various tree 
species, and thus the contrasting results of this study, compared to those reported in 
Barnett and Bonham (2004) for example, could be due to inherent differences in 
cellulose biosynthesis and cell wall properties between trees and \( \text{Arabidopsis} \). Also, the 
differences in growth rate between long and short days may be very different from the 
growth rate differences suggested to affect MFA in trees. Further investigation to the 
variety of MFA in \( \text{Arabidopsis} \), and the effect of growth rate on MFA in \( \text{Arabidopsis} \), is 
required to fully understand why MFA increased from long to short days.

It is interesting that the mutants and wild-type plants had similar MFA and 
crystallinity under short-days, suggesting that day-length muted, to some degree, the 
effects of the \( \text{AtCesA}^{irx} \) mutations. Extending this idea, it may be inferred that rate of
cellulose biosynthesis has a very significant role on the cellulose properties, perhaps more significant than the AtCesA mutations in the case of MFA and crystallinity. Wasteneys and Fujita (2006) proposed that the rate of cellulose biosynthesis could affect DP and crystallinity, which would in turn affect microfibril function in cell expansion. Clearly there are some intriguing areas of research required to elucidate the role of day length and growth rate on cellulose biosynthesis and therefore cell wall structure and function.

Cell wall crystallinity and cellulose DP in the AtCesA$_{irx}$ mutants also appeared to be affected by day length growth conditions as there was a general decreasing trend in DP for mutant plants. It could be that day length conditions effect the rate of cellulose biosynthesis, which manifests differences more strongly in the mutants due to reduced cellulose biosynthesizing capacity. In general, there was a trend of increased cell wall crystallinity in plants grown under short-day conditions. Under the assumption that cellulose biosynthesis occurs at a slower rate under short days, it could be that a slow rate of synthesis permits the formation of more crystalline structures, in particular cellulose. Cellulose DP was generally lower in all of the AtCesA$_{irx}$ mutants when grown under short-day conditions, compared to long-days. This suggests that CesA-composition of the CSC and the rate of cellulose biosynthesis could influence DP.

Stunted growth may be the result of reduced longitudinal cell expansion, or fewer cell divisions, or both. The reasons for reduced primary growth as a secondary consequence of altered secondary cell wall biosynthesis are unknown. However, primary cell wall alterations in the CesA$^{mut10}$ mutant (Bosca et al., 2006) do suggest there is some developmental feedback occurring between primary cell walls and secondary cell walls. Further analysis revealed that short-day AtCesA$_{irx}$ plants have elevated growth rates compared to wild-type, such that they reproduce and mature when wild-type plants are still undergoing significant growth. This suggests that there could be secondary effects of the AtCesA$_{irx}$ mutations on developmental cues. To further elucidate the mechanism for reduced growth in AtCesA$_{irx}$ mutants it would be interesting to identify if fewer cell divisions occur or if the cells produced undergo reduced longitudinal expansion.
2.4.2 Increased lignin and hemicellulose content in AtCesA^{irx} mutants suggest compensatory feedback in response to secondary cell wall perturbations

Lignin values were calculated as a percentage of the total stem dry weight, and shown to be increased in all of the **AtCesA^{irx}** plants. A portion of the increase observed could be a perceived increase due to reduced cellulose constituting the dry weight. Also, it could be the result of an increase in lignin biosynthesis as a compensatory mechanism to maintain structural integrity of the cell wall. In long-day **AtCesA8^{irx1-1}** plants there is very little change in lignin content compared to wild-type plants, despite the fact that there was a 37% decrease in \( \alpha \)-cellulose. In comparison, **AtCesA4^{irx5-1}** plants had a 25% reduction in \( \alpha \)-cellulose but a 22% increase in lignin content. This suggests that changes in lignin biosynthesis could be affected not only by a reduction in cellulose content (mass balance), but general perturbations to cell wall properties, as the **AtCesA4^{irx5-1}** lines exhibited more severe perturbations in the form of altered MFA. Although **AtCesA8^{irx1-1}** plants clearly have an \( \alpha \)-cellulose deficiency, the cellulose network seems to be more wild-type-like in terms of MFA, and these plants had a smaller increase in lignin compared to **AtCesA7^{irx3-1}** and **AtCesA4^{irx5-1}** plants. The differences between the mutant lines could be the result of stronger positive feedback for lignin biosynthesis in response to greatly perturbed secondary cell walls. Similar compensatory responses have been observed in other systems. For example, in lignin-deficient poplar it was suggested that there was an increase in cellulose (Hu et al., 1999). RNAi-suppression of **korrigan** in poplars caused a slight decrease in \( \alpha \)-cellulose (10% below wild-type) and a concomitant increase in lignin (Maloney and Mansfield, 2010). Interestingly, ectopic lignification in the **eli** mutants of **AtCesA3** has been postulated as a defence response as a result of alterations to cell wall integrity (Cano-Delgado et al., 2003). However, a compensatory response is not always observed. In **Arabidopsis irx4** mutant plants (mutation of a cinnamoyl-CoA reductase gene) that have perturbed lignin biosynthesis, there was no increase in cellulose or hemicellulose content of secondary cell walls (Jones et al., 2001). It could be that feedback in response to secondary cell wall alterations varies depending on 1) mutant phenotype
severity, 2) the gene(s) mutated and therefore biosynthetic pathways affected, and 3) the developmental stage when problems manifest.

The hemicellulose network provides cross-linkages between cellulose microfibrils and lignin to create a strong network that imparts a great deal of structural integrity to the secondary cell wall (Cosgrove, 2005). A range of reports exist in the literature regarding the degree of change in hemicellulose content in AtCesA mutants including significant increases in the AtCesA7fra5 mutant (Zhong et al., 2003), whereas others report little to no change (Turner and Somerville, 1997; Brown et al., 2005). Herein we provide a more thorough report on the changes to hemicellulose content in stems from long- and short-day AtCesA8irx1, AtCesA7irx3, and AtCesA4irx5-1 plants.

The proportion of total stem dry weight constituted by hemicelluloses did not significantly change between long and short-days. This is likely a reflection of reduced overall growth under short-days. The general increase in hemicellulose content in all AtCesAirx plants is believed to be a pleiotropic effect of the cellulose defects. Similar to the alterations in lignin content, an increase in hemicelluloses in response to decreased and altered cellulose could be a compensatory response. Given that xylans are very prominent hemicelluloses in plant secondary cell walls (Mellerowicz et al., 2001; Brown et al., 2009), it is not surprising that xylose levels showed some of the greatest increases. Secondary changes in cell wall composition, in response to cellulose deficiencies, have been reported for other mutants. The proscrete1 mutant (mutation of AtCesA6), which also has dramatically reduced cellulose content, also exhibits a concomitant increase in hemicellulose content, and a slight change to pectin content (Fagard et al., 2000). Other non-CesA mutants with alterations to cellulose have exhibited changes to other cell wall components. For example, mutations of the Arabidopsis endo-1,4-β-glucanase, korrigan, cause a decrease in cellulose content in primary cell walls (Sato et al., 2001; Peng et al., 2000) and crystalline cellulose in secondary cell walls (Szjanowicz et al., 2004) and secondary effects such as altered cell wall pectin content (Sato et al., 2001; Peng et al., 2000; His et al., 2001), minor changes to hemicellulose content (Lane et al., 2001; Peng et al., 2000), increased starch (Peng et al., 2000), and decreased structural integrity of the cellulose-xyloglucan network (Nicol et al., 1998). Changes to hemicelluloses and pectin content in korrigan mutants
have been attributed to a compensatory response as a result of altered cellulose (Peng et al., 2000).

Widespread cell wall changes were also observed in AtCesA7mut10 mutant plants (Bosca et al., 2006). These plants had altered secondary cell walls, but also exhibited significant changes to the monosaccharide composition and xyloglucan fucosylation in primary walls of seedlings. The results presented in this chapter, and those from other reports, suggest that a compensatory increase in hemicellulose content is likely the result of a feedback response to cell wall perturbations to provide additional structural integrity to the cell walls of mutant plants.

2.4.3 Mutant-specific differences indicate the importance of a complete CSC and suggest CesA-specific functions in cellulose biosynthesis

The non-redundant nature of AtCesA4, A7, and A8 (Taylor et al., 2008) could be because 1) the CSC will only form properly when three unique CesAs are present, 2) each CesA, as part of a CSC and rosette, contributes to the production of cellulose with unique properties, or both of these factors. Current literature, thus far, indirectly supports hypothesis one whereby it has been shown that CesA interactions are limited when one CesA is not functional (Taylor et al., 2003; Atanassov et al., 2009), which could lead to impaired CSC formation. Presented herein, the array of changes to cellulose content and ultrastructure among the AtCesAirx mutants also provide support for hypothesis one, and some support for hypothesis two.

The differences between the mutant phenotypes may be explained, in part, by the type of mutation and how the mutations affect CSC form and function. The AtCesA7irx3-1 and AtCesA4irx5-1 mutants are thought to be null-mutants due to a truncation of the CesA protein, whereas AtCesA8irx1-1 is thought to be a reduced-function mutant due to an amino acid substitution at a conserved residue in the catalytic domain (Taylor et al., 2003; Figure 2.1, Table 2.1). The reduced function of AtCesA8irx1-1 enzymes was indirectly confirmed by the low levels of α-cellulose produced in AtCesA8irx1-1 plants; levels that were similar to those of the null AtCesA7irx3-1 and AtCesA4irx5-1 mutants. Under the assumption that CesA4, A7, and A8 are the only CesAs contributing to secondary cell wall-specific cellulose biosynthesis, it stands to
reason that cellulose biosynthesis in $\text{AtCesA7}_{irx3-1}$ plants is performed only by CesA4 and CesA8 subunits and only by CesA7 and CesA8 in $\text{AtCesA4}_{irx5-1}$ plants. Therefore, it is hypothesized that aberrant and incomplete CSCs exist in the null mutant plants (Figure 2.20). In contrast, the CSC structure in $\text{AtCesA8}_{irx1-1}$ plants may be more wild-type-like if the CesA8 subunit (a proportion of normal, or a modified form of the CesA8 subunit) is still synthesized and incorporated (Figure 2.20). As such, it was expected that there would be differences in the cell wall properties between the mutant lines, which could be attributed to the presence of a CSC or the unique functions of the remaining CesAs in each mutant background.

Compared to the null mutants, the $\text{AtCesA8}_{irx1-1}$ plants did not show a significant change in cellulose MFA, suggesting that a properly formed CSC could have a direct role in dictating MFA. The null mutants, $\text{AtCesA7}_{irx3-1}$ and $\text{AtCesA4}_{irx5-1}$, had similar alterations in MFA, however, the changes to other cellulose properties varied between these two mutant lines. In particular, the $\text{AtCesA7}_{irx3-1}$ mutants had greatly reduced $\alpha$-cellulose content and the lowest crystallinity. In comparison, $\text{AtCesA4}_{irx5-1}$ plants had near wild-type levels of crystallinity and more $\alpha$-cellulose than $\text{AtCesA7}_{irx3-1}$ plants. Combined, these results suggest that the presence of a functional AtCesA7 subunit may have a greater influence on total cellulose production and crystallinity than AtCesA4.
Figure 2.20. Hypothetical effects of AtCesA mutations on CSC formation and cellulose properties. Based on the alterations to cellulose content, MFA, crystallinity, and DP, the effect of each CesA mutation on CSC form and function was inferred. Predicted CSC structures in (A) wild-type plants, (B) AtCesA8irx1-1 plants, (C) AtCesA7irx3-1 plants, and (D) AtCesA4irx5-1 plants are presented. It is proposed that a fully-formed CSC provides optimal conditions for potential interactions with other factors that regulate MFA (A, B). In null-mutant plants, it is assumed that the remaining functional CesAs form aberrant CSCs (C, D), and it is proposed that CesA-specific functions influence CSC function and ultimately manifest altered cellulose properties (C and D). For example, perhaps AtCesA7 facilitates better CSC formation and higher crystallinity (C), and AtCesA4 creates cellulose chains of greater length (D).
Perhaps the aberrant CSCs formed in the *AtCesA7^irx3-1* mutant plants do not provide a suitable environment for synthesized glucan chains to form highly crystalline bonds (Figure 2.20 C). Also, these results could suggest that AtCesA7 is required at higher levels to form CSCs, such that the presence of CesA7 and CesA8 in *AtCesA4^irx5-1* plants permits the formation of more stable and more CesA-rich CSCs (compared to the CesA4-CesA8 CSCs in *AtCesA7^irx3-1* plants) which are able to synthesize greater amounts of cellulose (Figure 2.20 C, D). Although these are inferences about CSC form and function, the results presented herein suggest that CesA4, A7, and A8 subunits may be required at different levels in a CSC.

To date, there have been no successful experiments to determine the ratio of CesA4:CesA7:CesA8 proteins within active CSCs. However, gene expression analysis suggests that *AtCesA7* and *AtCesA8* are more highly expressed than *AtCesA4* (Appendix A). Perhaps CesA7 has a unique role compared to CesA4 and A8, such as acting as the initiation site for CSC formation. Zhong *et al.* (2003) found that the expression of a mutant form of AtCesA7 (*fra5*) caused a dominant negative effect, whereas expression of mutant form of CesA8 (*fra6*) did not, suggesting that CesA7 has a larger influence on cellulose biosynthesis. Anti-CesA antibody probing suggests near equal amounts of each CesA in stem total protein extracts (Atanassov *et al.*, 2009). However, (what are presumed to be) the same antibodies used on stem tissue prints suggest that there are higher levels of AtCesA4 in the xylem compared to AtCesA8 and AtCesA7 (Taylor *et al.*, 2003). Additionally, it appears as though more AtCesA4 and AtCesA8 are present in interfascicular cells compared to AtCesA7. These results must be interpreted with caution, as the visual differences could be the result of differences in antibody efficiencies, the region from which stem sections were taken, or potential masking of the epitope by CesA-protein interactions.

As mentioned previously, another putative reason for the requirement of all three CesA proteins (AtCesA4, A7, and A8) for proper cellulose biosynthesis could be that each CesA has a unique function that influences the final cellulose properties. For example, one proposed possibility is that one (or two) CesAs have specific roles in using a cellulose biosynthesis primer, sitosterol-β-glucoside, such that these primer-utilizing CesAs are required for proper cellulose biosynthesis by the other CesA
subunits (Doblin et al., 2002). However, if the remaining CesA subunits do not readily form hetero-CesA complexes in the mutant plants (Taylor et al., 2003; Atanassov et al., 2009), and priming of biosynthesis is required by one of the CesA subunits in a fully formed CSC, we would expect to see no cellulose produced, but this is not the case. From the results obtained herein, it appears that AtCesA7 is required for a more crystalline cell wall to be produced. This could be the result of CesA7 being required for proper CSC formation (as discussed above), or it could be an indication of an AtCesA7-specific role in producing higher ordered crystalline cellulose. Under long-day conditions, the cellulose DP of AtCesA4

mutants was the lowest of all the mutant lines. This may indicate that AtCesA4 has a unique role in producing long chains of cellulose, a role that may not be shared by AtCesA8 or AtCesA7.

Combined, these results could indicate that 1) a fully formed CSC influences MFA; 2) AtCesA7 may be required at a greater level for proper CSC formation; 3) CSCs containing AtCesA7-AtCesA8 are stable than AtCesA8-AtCesA4, allowing for increased crystalline cellulose to be produced; 4) AtCesA4 may have a role in producing cellulose chains with a greater DP than those of AtCesA8 or AtCesA7.

2.4.4 Reporter tags may effect CesA function

Complementation of the null AtCesA4

and AtCesA7

mutants was achieved by expression of the missing wild-type-CesA under expression control of the native AtCesA promoter, but not by the 35S promoter. This emphasizes the requirement for appropriate spatial and temporal expression of AtCesA4, A7, and A8 for proper cellulose biosynthesis. In AtCesA4

plants, very low expression of LUC-AtCesA4 (by PAtCesA4::LUC-AtCesA4) resulted in an almost fully-complemented phenotype. This result suggests that the Luciferase tag does not significantly interfere with AtCesA4 function in these plants, and it is interesting that a low threshold level of AtCesA4 transcript, and presumably protein, is required to facilitate normal cellulose biosynthesis and growth. In comparison, expression of YFP-AtCesA7 in A7

plants resulted in a complemented phenotype, but only when expression levels were excessive compared to endogenous AtCesA7 transcript levels. In the non-null mutant AtCesA8

expression of LUC-AtCesA8 under the native AtCesA8 promoter was not
sufficient to fully recover a wild-type phenotype as plants remained significantly stunted, but exhibited some recovery from the collapsed xylem phenotype. This is likely due to the continued presence of the mutant form of AtCesA8 in the \( \textit{AtCesA8}^{ix1-1} \) background, and that the mutant AtCesA8 protein likely successfully out-competes the LUC-AtCesA8 protein for positions within the CSC. LUC-AtCesA8 transcript abundance was relatively low compared to wild-type. Full complementation of the mutant phenotype can likely only be achieved by excessive expression of the wild-type \( \textit{AtCesA8} \). Atanassov \textit{et al.} (2009) reported that an N-terminal tag on AtCesA4 and AtCesA8 resulted in a non-functional protein, based on lack of mutant phenotype complementation by expression of a STREP-AtCesA4 or STREP-AtCesA8 fusion in the \( \textit{AtCesA4}^{ix5-1} \) and \( \textit{AtCesA8}^{ix1-1} \) mutants, respectively. Interestingly, there are multiple reports of successful complementation of the \( \textit{AtCesA7}^{ix3-1} \) mutant tagged-CesA7, including STREP-AtCesA7 (Atanassov \textit{et al.}, 2009), GFP-AtCesA7 (Gardiner \textit{et al.}, 2003), and a small epitope-tagged CesA7 (Taylor \textit{et al.}, 2004), suggesting that tagged AtCesA7 subunits function normally. Furthermore, this highlights the fact that the \( \textit{AtCesA7}^{ix3-1} \) mutant appears to be more easily complemented compared to the \( \textit{AtCesA4} \) and \( \textit{A8} \) mutants. If all three of AtCesA4, A7, and A8 are required in the CSC, then why do some tagged-CesA subunits seem more functional than others? Could these disparities in ease of complementation suggest that the CesA subunits have different roles within the CSC, such that tags may interfere with the function of AtCesA4 and A8 but not AtCesA7? The role of transgene expression levels on complementation cannot be ignored, however, the results presented herein, and those of others (Atanassov \textit{et al.}, 2009; Gardiner \textit{et al.}, 2003; Taylor \textit{et al.}, 2004), suggest that there may be some differences in the inherent role of each CesA in a CSC, such that complementation varies between CesAs when a tag is added to the N-terminus. These differences could be due to 1) CesA-specific functions, such that tags interfere more or less with CesA function; 2) CesA-CesA interactions, such that each CesA has a different ratio within a CSC, and these ratios are dependent on interactions which could be altered by an N-terminal tag; and 3) non-CesA interactions. Perhaps some of the CesAs within a CSC interact with other proteins during biosynthesis and a tag would interfere with these interactions thus making complementation more or less difficult. As the role of each CesA in CSC formation,
function, and the ratios of CesAs within a CSC are elucidated it may become clear why there are differences in complementation ability.

2.4.5 Exogenous expression of AtCesAs in wild-type plants causes a severe mutant phenotype

The expression of two AtCesA transgenes in wild-type Columbia Arabidopsis plants resulted in a severe AtCesA\textsuperscript{irx} phenotype (Figure 2.12). It is postulated that the mutant phenotype observed is the result of sense suppression, which was supported by reduced AtCesA transcript abundance in RNA from stem tissue of the mutant plants.

Interestingly, expression of AtCesA8 (wild-type Columbia cDNA) in wild-type Landsberg Arabidopsis plants (WT\textsubscript{L}-2xA8) did not cause the same severe mutant phenotype. In fact, the WTL-2xA8 plants looked similar to wild-type plants, although stems were thinner and slightly less upright. Additionally, sense suppression did not appear to occur, as AtCesA8 transcript remained at near wild-type levels. It has been noted by others that AtCesA mutants in the Arabidopsis Columbia ecotype appear more severe than those in the Landsberg ecotype (Taylor \textit{et al.}, 2008). It was postulated that the reduced severity of AtCesA\textsuperscript{irx} mutations in Landsberg plants is potentially the result of reduced signalling, and therefore feedback, in response to cell wall changes in Landsberg compared to Columbia (Taylor \textit{et al.}, 2008).

2.4.6 YFP-CesA7 expression patterns reveal a strong presence of AtCesA7 in fibres

AtCesA7 is believed to be strongly associated with secondary cell wall development (Turner and Somerville, 1997; Taylor \textit{et al.}, 2003; Zhong \textit{et al.}, 2003; Brown \textit{et al.}, 2005), although it has also been thought to also affect (directly or indirectly) primary cell wall formation (Bosca \textit{et al.}, 2006). Additionally, phylogenetic comparisons of the secondary cell wall AtCesAs suggests that AtCesA7 is less divergent than AtCesA4 and AtCesA8 (Nairn and Haselkorn, 2005; Roberts and Bushoven, 2007; McDonnell, this study and Chapter 3). A visual profile of YFP-CesA7 expression in Arabidopsis plants, from seedlings to mature stem tissue, suggests that AtCesA7 is expressed in almost all tissue types, at a variety of developmental stages.
Additionally, our results support a potential involvement of AtCesA7 in primary growth, and that AtCesA7 (or entire AtCesA7-containing CSCs) are frequently compartmentalized.

The presence of large, fast moving, CesA-containing compartments have been previously reported for YFP-CesA6 (Gutierrez et al., 2009; Paredez et al., 2006) and GFP-CesA3 (Crowell et al., 2009) (reviewed in Wightman and Turner, 2010) and YFP-CesA7 (Wightman and Turner, 2008). Co-localization of GFP-CesA3, YFP-CesA6, and YFP-CesA7 particles with Golgi-specific markers identified the ring structures (GFP-CesA3, YFP-CesA6) and large YFP-CesA7 puncta as Golgi-distinct compartments. Using drug treatments, it was determined that positioning of CesA-Golgi bodies near the plasma membrane is influenced by the microtubule array (Crowell et al., 2009), and their movement appears to be regulated somewhat by actin networks (Crowell et al., 2009; Wightman and Turner, 2008). As in these previous reports, the putative CesA-Golgi bodies observed herein (YFP-CesA7) were fast moving and often moved in non-linear trajectories (casual observation). As well, at times it would appear as though a compartment would pause within the cell (or at the periphery) and then continue moving, or disappear. Erratic movement and pausing of large CesA puncta was also observed by Wightman and Turner (2008) who postulated the pause events represented delivery of CSCs from Golgi compartments to the plasma membrane under sites of secondary cell wall thickening. The periphery-limited location of the YFP signal in the ring-shaped puncta suggests that the YFP-CesAs could be within the membrane of the compartment. Putative CesA-Golgi bodies observed herein were in most tissue types, although very rarely observed in the root tip. Large and small, with respect to the CesA-Golgi bodies observed, bright YFP-CesA7 particles were also observed in all cell types documented, but they did not have the same ring-like structure. Thus, the two groups of signal could represent different compartments, or different states of YFP-CesA7 within a compartment. The non-Golgi YFP-CesA7 compartments observed could be part of the MASC/SmaCC population of vesicles observed by Crowell et al. (2009) and Gutierrez et al. (2009). These putative CSC-containing vesicles were observed in the cytosol, are approximately 300 to 500 nm in diameter (personal estimation, based on images presented in the literature) and are believed to be involved
in regulating the distribution, and perhaps internalization of CSCs to and from the plasma membrane (Crowell et al., 2009; Gutierrez et al., 2009; Wightman and Turner, 2010). It is not clear if these compartments are completely distinct from the ring-shaped Golgi-CesA compartments, as some SmaCCs (and perhaps MASCs) have co-localized with a trans-Golgi network marker (Gutierrez et al., 2009). Some of the non-ring-shaped puncta observed in the research presented herein range in size from approximately 300 to 1000 nm in diameter, placing some of these puncta in a similar size range as SmaCCs and MASCs. Putative SmaCCs-MASCs observed herein were present in almost all tissues observed, therefore it cannot be speculated if they have a role in a particular developmental stage or tissue type. It is feasible that they are involved in regulating CSC delivery or internalization to recycle CSCs or reduce cellulose biosynthesis. However, further investigation is required to truly elucidate the role of SmaCCs and MASCs in cellulose biosynthesis. The most commonly observed puncta were: cytosolic, 300 to 1000 nm, bright, non-ring shaped puncta in all tissues (MASCs-SmaCCs); cytosolic, large, ring-shaped puncta in all tissues (CesA-Golgi bodies), and very small, bright puncta at the plasma membrane of fibres. The variety of YFP-CesA7 signal observed likely represents a dynamic population of active and non-active CSCs being transported to and from the plasma membrane. A single ring-like structure was observed in YFP-CesA8 expressing plants (wild-type Landsberg expressing \textit{PAtCesA8::YFP-CesA8}) alongside developing xylem. The reduced frequency of YFP-CesA8-Golgi bodies is likely due to the expression of YFP-CesA8 in a wild-type background.

On the plasma membrane face of many fibres, presumed to be undergoing secondary cell wall thickening, the YFP-CesA7 signal was often observed as a layer of small, dense, diffuse particles that appeared to be moving very quickly (although the trajectories and speed could not be recorded). The particles were quite small and diffuse, and resembled the small puncta observed by others, which have been described as CSCs in the plasma membrane (Paredez et al., 2006; Crowell et al., 2009; Gutierrez et al., 2009; Wightman and Turner, 2008). Fibres with dense, bright, diffuse YFP-CesA7 signal were most often found within the central region of a growing stem, believed to be where interfascicular fibres are maturing and developing thickened
secondary cell walls. To our knowledge, this is the first report of a secondary cell wall signal visualized in developing fibres, *in planta*. In contrast, at the base or tip of the stem, the plasma membrane of fibres was not found to be saturated with similar fine YFP-CesA7 puncta. The density and rapidly moving signal in developing fibres reiterates the fact that an extremely high abundance of CesA protein is likely to be involved in cellulose biosynthesis, that the production of secondary cell wall material is rapid, and that there is an extremely high turnover rate of CesA proteins. It is not known if CesA subunits behave similarly in xylem and fibres. Are the ratios of CesA subunits within a CSC similar in all cell types? Observing the significant abundance of YFP-CesA7 signal in fibres emphasizes that CesA7 has a prominent role in fibre secondary cell wall biosynthesis.

The characterization of *AtCesA8*<sup>irx1-1</sup>, *AtCesA7*<sup>irx3-1</sup>, and *AtCesA4*<sup>irx5-1</sup> growth and cell wall composition has revealed much new information with respect to cellulose biosynthesis. The results presented herein suggest that CSC formation and composition affect cellulose properties such as MFA, DP, and crystallinity, and that day-length further affects MFA in *Arabidopsis*. There appear to be CesA-specific roles in cellulose biosynthesis which can be detected by measuring changes to cell wall and cellulose ultrastructure.
CHAPTER 3 Identification of secondary cell wall-specific cellulose synthase genes from *Picea glauca* and conservation of function among CesA orthologs

3.1 Introduction

Cellulose, a β-1,4 linked homopolymer comprised of glucose, represents as much as 40% of the plant secondary cell walls (Barnett and Bonham, 2004; Mellerowicz et al, 2001). The genes coding for the enzymes responsible for cellulose biosynthesis in secondary cell walls, cellulose synthases (CesAs) have been studied in many plant species including *Arabidopsis* (Hamman et al., 2004), *Populus* (Djerbi et al., 2005; Kumar et al., 2009), *Eucalyptus* (Lu et al., 2008; Ranik and Myburg, 2006), rice (Tanaka et al., 2003), maize (Holland et al., 2000; Appenzeller et al., 2004), barley (Burton et al., 2004), potato (Oomen et al., 2004), and moss (Roberts and Bushoven, 2007) to name a few. However, comparatively little is known about CesAs from gymnosperm species. To date, there are only a few papers reporting the identification of CesAs in pine species (Nairn and Haselkorn, 2005; Krauskopf et al., 2005; Nairn et al., 2008). Thus, there is a pressing need to further identify CesAs, particularly those involved in secondary cell wall formation, in other economically important gymnosperms, such as spruce.

CesA proteins have a conserved structure containing a zinc-binding domain, eight transmembrane domains (two located at the amino terminus and six at the carboxyl terminus), a plant-conserved region, a class-specific region (CSR), and two highly conserved domains, A and B (Richmond, 2000; Doblin et al., 2002), and conserved motifs believed to be involved in the catalytic activity of CesAs: three aspartic acid residues (D) and a QXXRW motif. Evolutionarily, CesA gene families appear to have emerged prior to the divergence of gymnosperms from angiosperms. The cotton (*Gossypium hirsutum*) cellulose synthase 1 (*GhCesA1*) and *GhCesA2* were some of the first CesA genes to be identified in higher plants (Pear et al, 1996). With the advancement of genome and EST-library sequencing, large CesA families have emerged from other plants. For example, up to 10 CesA and 30 cellulose synthase-like (Csl) genes have been identified in Arabidopsis (Somerville et al., 2000), 17 CesA genes in poplar (Kumar et al., 2009), at least six in *Eucalyptus* (Ranik and Myburg,
2006), and 11 CesAs in moss (Roberts and Bushoven, 2007). Phylogenetic analysis of plant CesAs reveals a distinct separation of primary and secondary cell wall-specific CesAs (Roberts and Bushoven, 2007; Kumar et al., 2009). Additionally, for secondary cell wall cellulose biosynthesis, it has been postulated that three unique CesAs are required to produce cellulose, thus forming proper secondary cell walls. This has been clearly illustrated in Arabidopsis mutants (Turner and Somerville, 1997; Taylor et al., 2000; Taylor et al., 2003). Sequence homology suggests the same requirements exist in Populus (Djerbi et al., 2005), Eucalyptus (Ranik and Myburg, 2006), and Pinus taeda (Nairn and Haselkorn, 2005). Predicted CesA amino acid sequences, particularly the class-specific regions (CSRs) of CesA sequences, are highly similar among putative orthologs, but less similar between unique CesA family members (Ranik and Myburg, 2006; Joshi et al, 2004; Nairn and Haselkhorn, 2004; Samuga and Joshi, 2002). The CSR may dictate differences in function among CesAs within a plant, and has recently been used to aid in classification. Although orthology of CesAs has been inferred based on phylogenetic analysis, there is no evidence to suggest that CesAs from different plant species are functionally orthologous. Examining the conservation of function among CesA orthologs will provide information critical to understanding the evolution of cellulose synthases, and the specific functions of individual CesA family members in cell wall biosynthesis.

The objectives of this study were three fold. The first was to identify the secondary cell wall-specific cellulose synthase genes from spruce, Picea glauca. Second, to characterize the identified genes based on phylogenetic analysis and gene expression profiling. Third, we wanted to examine the degree of conservation of function among CesA orthologs by assessing the ability of spruce and poplar CesAs to complement the Arabidopsis CesA/αx mutants.

3.2 Materials and methods

3.2.1 Isolation of full-length CesA cDNA

A spruce EST and contig database (Genome BC) provided short sequences from the 3’ end of putative CesA cDNAs (based on sequence similarity to known CesAs).
These were identified as *PgCesA1*, *PgCesA2*, and *PgCesA3*. First-strand cDNA was synthesized from total RNA harvested from spruce xylem tissues. To amplify the full-length coding region of *PgCesA1* 5’ RACE (Rapid Amplification of cDNA Ends, FirstChoice RLM RACE kit, Ambion) was used in combination with a primer specific for the known 3’ end. To amplify the full-length coding region of *PgCesA2* and *PgCesA3*, PCR was used with reverse primers specific for the known 3’ end and forward primers specific for the 5’ end of known pine cellulose synthase sequences (*PitCesA2*, AY89650.1; *PitCesA3*, AY789652.1). The three full-length cDNAs were analyzed by DNA sequence analysis. Primer oligonucleotides are shown in Table 3.1.

*Populus trichocarpa CesA* gene sequences with homology to *Arabidopsis AtCesA4*, *AtCesA7*, and *AtCesA8* described by Kumar *et al.* (2009) were retrieved from the JGI website (http://genome.jgi-psf.org/Poptr1_1.home.html). The following genes models were used as search terms to retrieve the sequences from JGI: *PtiCesA4* – eugene3.00002636; *PtiCesA8-A* – gw1.XI.3218.1; and *PtiCesA7-A* – estExt_Genewise1_v1.C_LG_V12188. The full-length *PtiCesA* cDNAs were amplified and cloned using PCR (primers listed in Table 3.1) and ligated into the Gateway compatible pENTR-D/TOPO donor cloning vector (Invitrogen) to create pE-*PtiCesA4* (*PtiA4*), pE-*PtiCesA7A*, pE-*PtiCesA7B*, pE-*PtCesiA8A*, pE-*PtCesiA8B*. Clones were sequenced to confirm identity and accuracy.

### 3.2.2 RNA extraction and cDNA synthesis for gene expression

Tissues from 5-year-old *Picea glauca* trees grown in Vancouver, British Columbia, in pots, outside, were harvested in early May. Specifically, young-unexpanded needles, young-expanded needles, phloem/cambium, xylem, and roots, were collected and immediately frozen in liquid nitrogen. Total RNA was extracted using methods described by Kolosova *et al.* (2004). RNA was treated with TURBO DNase (Ambion) to remove DNA.
Table 3.1. Sequences of primers used for isolation, cloning, real-time PCR (RT), and screening.

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First-strand cDNA was synthesized from 1 μg of DNase-treated RNA using Superscript II Reverse Transcriptase (Invitrogen) with dT_{18} oligonucleotides.

3.2.3 Real-time PCR analysis

PgCesA gene expression was measured using quantitative real-time PCR (RT-PCR). RT-PCR reactions were set-up in triplicate for each sample with Platinum SYBR Green qPCR Master Mix (Invitrogen) and run on a Mx3000p real-time PCR system (Stratagene). The primers used to detect PgCesA and housekeeping control genes are listed in Table 3.1. Conditions for the RT-PCR reactions were 95°C for 10 minutes followed by 35 cycles of 95°C for 30 seconds, 59°C for 1 minute, and 72°C for 30 seconds. Relative gene expression was calculated using the equation described by Pfaffl (2001), \( \Delta \text{ct}_{\text{CesA}}^{\text{ct}_{\text{EIF5α}}} \), in which CesA gene expression is relative to that of the housekeeping control, PgEIF5α-1. Melting curve analysis, agarose gel electrophoresis, and DNA sequencing of the RT-PCR products were used to confirm that the RT-PCR amplicons represented the expected RT-PCR products.

3.2.4 Phylogenetic analysis

Several cellulose synthase amino acid sequences were obtained from GenBank (NCBI) and compared to the predicted PgCesA amino acid sequences (Table 3.1). Amino acid alignments and subsequent dendrogram were created using the MEGA software (http://www.megasoftware.net/). For the box-shade figure, a sequence alignment file created using ClustalW (http://align.genome.jp/) was submitted to the BoxSHADE server (http://www.ch.embnet.org/software/BOX_form.html) to create the shaded alignment image.
Table 3.2. A list of CesA sequences used for phylogenetic analysis and associated GenBank accession numbers. When available, relevant literature sources are listed.

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<td>AAY60845.1</td>
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<td>PpCesA8</td>
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3.2.5 Plant transformation vectors

For expression of \textit{PgCesA} cDNAs in various \textit{AtCesA}$_{irx}$ mutants, the \textit{PgCesA} cDNA fragments were cloned into the \textit{P35S::LUC-attR} or the \textit{P35S::YFP-attR} expression constructs (Subramanian \textit{et al.}, 2006) with either the 35S promoter (P35S) intact, or with the 35S promoter replaced by \textit{Arabidopsis} \textit{AtCesA} promoters (detailed below). The placement of \textit{PgCesA}s into these vectors resulted in a fusion of Luciferase (LUC) or yellow fluorescent protein (YFP) at the N-terminus of the cloned gene. For \textit{AtCesA} promoter driven expression, \textit{PgCesA} cDNAs were cloned into one of the following binary vectors (described in Chapter 2): \textit{PatCesA4::LUC-attR}, \textit{PatCesA7::YFP-attR}, or \textit{PatCesA8::LUC-attR}. The full length \textit{PgCesA1}, \textit{PgCesA2}, \textit{PgCesA3}, \textit{PtiCesA4}, \textit{PtiCesA7-A}, and \textit{PtiCesA8-A} cDNAs were amplified by PCR (primers are listed in Table 3.1) and ligated into the pENTR-D cloning vector (Invitrogen). Using LR Clonase II (Invitrogen), \textit{PgCesA} cDNAs were transferred to the desired destination expression vector to create the following binary vectors: \textit{P35S::LUC-PgCesA1}, \textit{PAtCesA8::LUC-PgCesA1}, \textit{P35S::LUC-PgCesA3}, \textit{PAtCesA4::YFP-PgCesA3}, \textit{PAtCesA7::YFP-PgCesA3}, \textit{PAtCesA7::YFP-PgCesA2}, \textit{PAtCesA8::LUC-PtiCesA8-A}, \textit{PAtCesA4::LUC--PtiCesA4}, and \textit{PAtCesA7::LUC-PtiCesA7-A}.

3.2.6 Plant growth and transformations

\textit{AtCesA8}$_{irx1}$ (Turner and Somerville, 1997), \textit{AtCesA7}$_{irx3}$ (Turner and Somerville, 1997), and \textit{AtCesA4}$_{irx5}$ (Taylor et al., 2003) \textit{Arabidopsis} plants were germinated on half-concentration MS medium (Mirashige and Skoog, 1962) with no sucrose, and grown under a 16-hour light/8-hour dark cycle. Seedlings were transferred to soil approximately seven days post-germination, and grown in a growth chamber at 21°C under a 16-hour light/8-hour dark cycle. Plants were transformed using a method modified from Clough and Bent (1998). \textit{Agrobacterium tumefaciens} GV3101-pMP90 (Hellens \textit{et al.}, 2000) was transformed with the binary vector using a freeze-thaw method. Cultures were grown overnight at 28°C in Luria-Bertani medium containing 50 \(\mu\)g L$^{-1}$ kanamycin (YFP vectors) or 50 \(\mu\)g L$^{-1}$ spectinomycin (Luc vectors), 25 \(\mu\)g L$^{-1}$ rifampicin and 25 \(\mu\)g L$^{-1}$ gentamycin. The overnight culture was centrifuged and resuspended in a 5\% (w/v) sucrose solution to an OD$_{600}$ of at least 0.8. Silwet L-77
(LEHLE Seeds) was added to each resuspended culture at a final concentration of 0.02% (v/v). Newly flowering plants (approximately 4 weeks old) were sprayed with the Agrobacterium solution using a fine mist spray nozzle. Sprayed plants were placed in a dark, humid environment for 16 to 24 hours and returned to light. A second spraying was done 5 days after the first spray in attempts to improve the transformation efficiency. After all spray treatments, plants were maintained as previously described, and seeds were harvested from mature, dried plants. Seeds were screened for transformations by germinating on half-concentration MS medium (no sucrose) with the addition of either 75 μg L⁻¹ kanamycin (YFP vectors), 50 μg L⁻¹ glufosinate ammonium sulfate (Luc vectors), or 25 μg L⁻¹ hygromycin (PAtCesA4::YFP vectors). Seedlings that successfully grew on antibiotics were grown in soil for further analysis. Transformed plants were confirmed by PCR of genomic DNA using gene specific primers (Table 3.1)

### 3.2.7 Complementation assay

In an attempt to investigate conserved functional homology between spruce and Arabidopsis cellulose synthases and poplar and Arabidopsis cellulose synthases the ability of PgCesAs and PtiCesAs to rescue an Arabidopsis CesA mutant phenotype was assessed. Homozygous AtCesAirx mutant plants expressing a PgCesA or PtiCesA were grown in soil under long-day conditions (16-h light/8-h dark). Transgene expression was measured in RNA extracted from stem tissues of approximately 20-day-old plants. To assess xylem morphology, stem bases (approximately 1cm from soil) from 20-30 day old plants were hand-sectioned using a double-edged razor blade. Sections were stained for 5 minutes in a 0.25% (w/v) Toluidine Blue solution, and rinsed for 5 minutes in water. Hand-sections were viewed through a Leica Light Microscope, and pictures taken with a Q-imaging camera.
3.3 Results

3.3.2 Spruce CesA gene isolation and sequence analysis

Three putative secondary cell wall-specific cellulose synthase cDNAs were isolated from reverse transcribed xylem RNA of white spruce, *Picea glauca*: *PgCesA1*, *A2*, and *A3*. For *PgCesA1* and *A3*, approximately 1 kb from the 3’ end of the final 3.2 kb cDNA was available from the Genome BC EST library. *PgCesA1* was isolated using primers designed against the known 3’ region and 5’ RACE PCR. In contrast, several rounds of 5’ RACE PCRs to isolate the *PgCesA2* and *PgCesA3* cDNA were unsuccessful. PCR performed with a forward primer designed to amplify the 5’ region of a pine CesA (*Pinus taeda*, *PitCesA3* or *PitCesA2*) in combination with a reverse primer designed against the known 3’ end of *PgCesA2* or *PgCesA3* was employed to isolate the full length *PgCesA2* and *A3* cDNAs. All three genes were isolated from xylem RNA, whereas it was difficult to isolate the sequences from RNA extracted from leader tissue. This suggested that the three isolated CesAs are more highly expressed in xylem tissues.

At the nucleotide level, the maximum identity shared between the three identified *PgCesAs* is between 59 and 72% (Table 3.3) which is comparable to the level of similarity between unique secondary cell-wall specific CesAs in other plants, for example AtCesA4-AtCesA8: 58%, AtCesA8-AtCesA7: 56%, and AtCesA4-AtCesA7: 65%. A comparison of the nucleotide sequences of *PgCesA1*, *A2*, and *A3* cDNA sequences with those of other known CesAs by a standard BLASTN search revealed a strong similarity (68 to 95%) to secondary cell-wall specific CesAs from a variety of plants including *Arabidopsis*, *Populus*, and *Pinus*. Among the highly studied *Arabidopsis* secondary cell wall-specific CesAs the highest similarities were found between *PgCesA1* and *AtCesA8* (69%), *PgCesA2* and *AtCesA7* (69%), and *PgCesA3* with *AtCesA7* (74%). As expected, the shared identity between the spruce CesAs and those from another gymnosperm, *Pinus taeda*, was high: 93% between *PgCesA1* and *PitCesA1*, 93% between *PgCesA2* and *PitCesA2*, and 95% between *PgCesA3* and *PitCesA3*. 
The predicted amino acid sequences of the three putative PgCesAs were aligned to determine the level of consensus and to identify conserved domains (Figure 3.1). All three CesA genes contain domains known to be conserved among cellulose synthases of higher plants. This includes a putative zinc-binding domain (Kurek et al., 2002), a region of variability also referred to as a hypervariable region (HVR) and a class specific region (CSR), the conserved catalytic motif consisting of four aspartate residues and the QXXRW residue sequence, and eight trans-membrane domains (TMD) (two at the N-terminus and six at the C-terminus). The CSR regions of PgCesA1, A2, and A3 only share between 25 - 35% identity.
Table 3.3. Amino acid residue shared identities of deduced PgCesA proteins with each other and the most similar CesAs from *Arabidopsis* (At) and *Populus trichocarpa* (Pti). Three comparisons are presented: entire amino acid sequences, the hypervariable region sequences (HVR), and the class-specific region sequences (CSR). The number in brackets refers to the percentage identity of the nucleotide sequences. Values in bold represent the highest similarities within one group (e.g.: PgCesA1 within *Arabidopsis* CesAs has the highest similarity with AtCesA8). Grey shading is to differentiate the comparisons between PgCesAs, AtCesAs, and PtiCesAs.

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**Figure 3.1.** Alignment of the predicted amino acid sequences of spruce (*Picea glauca*) putative secondary cell wall-specific cellulose synthases, PgCesA1, PgCesA2, and PgCesA3. Black shading indicates identical conserved residues, grey shading indicates non-identical residues with similar properties. The known, essential, conserved catalytic motifs are indicated below the sequence with an asterisk (D, D, D, QXXRW motif). The predicted zinc-binding domain, class specific regions I and II, and trans-membrane domains (TMD) are indicated above the sequence.
3.3.3 Phylogenetic analysis

The putative amino acid sequences of PgCesA1, A2, and A3 were compared to 44 other full-length amino acid sequences (Table 3.2). The multiple sequence alignment produced by ClustalW was used to create a dendrogram to view phylogenetic relationships (Figure 3.2). The dendrogram shows six major clades: three containing primary cell-wall specific cellulose synthases and three containing secondary cell-wall specific proteins. Each major clade contains CesAs from monocot and dicotyledonous plants, as well as those from gymnosperms. The three PgCesAs fall into unique clades, each clustered with one of the previously identified secondary cell wall-specific CesAs from Arabidopsis. Specifically, PgCesA1 groups with AtCesA8, PgCesA2 groups with AtCesA4, and PgCesA3 groups with AtCesA7. Conflicting results were obtained for PgCesA3: whole amino acid sequence similarity is highest with AtCesA7 (Table 3.3, Figure 3.2), but CSR similarity groups PgCesA3 with AtCesA4 (Table 3.3). Reviewing the alignment of the N-terminal regions only, the degree of similarity is highest between PgCesA3 and AtCesA7 (58%), followed by PgCesA2 with AtCesA7 (44%), and PgCesA1 with AtCesA8 (36%). Based on all the alignments, it is thought that PgCesA1 is orthologous to AtCesA8, PgCesA2 is likely an ortholog of AtCesA4, but PgCesA3 could be an ortholog of either AtCesA4 or AtCesA7.

The class-specific regions of PgCesA1, A2, and A3 share an extremely low level of similarity with each other (Table 3.3). The degree of similarity was not much greater when compared to potential orthologs from Arabidopsis or Populus. However, when compared to the CSRs of the Pinus taeda orthologs the similarities were significantly higher, between 75 and 95%. This suggests that the CSR conservation is limited to more closely related orthologs.

3.3.4 Expression profiling of PgCesA1, A2, and A3

Using quantitative real-time PCR, the expression levels of PgCesA1, A2, and A3 were measured in tissues expected to be undergoing varying levels of secondary cell wall biosynthesis. Transcript abundance of the translation initiation factor eIF5α was measured as a control for data normalization.
Figure 3.2. A dendrogram assembled from the alignment of the deduced amino acid sequences of PgCesA1, A2, and A3 with 48 other confirmed and putative full-length cellulose synthases (Table 3.2). The AtCesAs known to be involved in secondary cell wall biosynthesis are marked with a coloured star, and the putative spruce and poplar homologues are marked with a similarly coloured star. Genus-species names were abbreviated as follows: At – Arabidopsis thaliana; Eg – Eucalyptus grandis; Gh – Gossipium hirsutum; Os – Oryza sativa; Pir – Pinus radiata; Pit – Pinus taeda; Ptd – Populus tremuloides; Pti – Populus trichocarpa; Pp – Physcomitrella patens.
Expression was measured in six different tissues from three individual trees. Expression of all three \( PgCesA \) genes showed to be highest in xylem tissue (Figure 3.3). Expression was also relatively high in young needles (both expanded and unexpanded), as well as in root tissue. By contrast, low expression was found in old needles and phloem tissue. In all tissues the transcript abundance of \( PgCesA1 \) and \( A2 \) was consistently higher than that of \( PgCesA3 \). \( PgCesA1 \) and \( A2 \) were expressed at very similar levels within a particular tissue.

3.3.5 Ability of spruce CesAs to functionally complement \( Arabidopsis \) CesA\textsubscript{irx} mutants

To test if the function of the spruce CesAs is conserved with the function of the \( Arabidopsis \) orthologs, \( PgCesA \) cDNAs were expressed in one of the \( Arabidopsis \) ortholog-mutant plants. The \( Arabidopsis \) CesA\textsubscript{irx} mutants \( AtCesA4_{irx5-1} \), \( AtCesA7_{irx3-1} \), and \( AtCesA8_{irx1-1} \) were used. These \( AtCesA_{irx} \) mutants exhibit an obvious mutant phenotype consisting of stunted growth, rounded leaves, and weaker stems compared to wild-type plants (Figure 3.4 B). At the cellular level, the \( AtCesA_{irx} \) plants have collapsed xylem vessels. The \( PgCesAs \) were expressed in homozygous \( Arabidopsis \) mutant plants under the expressional control of the \( 35S \) promoter (\( P_{35S}::LUC-PgCesA \)), or an \( Arabidopsis \) CesA promoter (\( PA4::LUC-PgCesA \), \( PA7::LUC-PgCesA \), or \( PA8::LUC-PgCesA \)). All expression constructs resulted in an N-terminal Luciferase tag (LUC) or an N-terminal yellow fluorescent (YFP) tag on the PgCesA. Tagged versions of \( AtCesAs \) have been previously found to allow complementation of \( AtCesA_{irx} \) mutants (McDonnell, unpublished), and were used because of 1) the ease with which PgCesAs could be cloned into the vector using Gateway technology, and 2) the LUC tag could provide the opportunity to monitor protein expression via detection of.
**Figure 3.3.** Real-time PCR analysis of *PgCesA1*, *PgCesA2*, and *PgCesA3* transcript abundance in tissues of 5-year-old spruce trees. Average expression is relative to levels of a house-keeping gene, *PgelF5α*. Error bars represent standard error of the mean, n=3 (biological replicates). Tissue labels are as follows: ON, old needles; YN-C, young-unexpanded needles, YN-O, young-expanded needles; PH, phloem/cambium; XY, xylem; RT, roots.
**Figure 3.4.** *LUC-PgCesA1* does not rescue the *A8irx1-1* mutant phenotype under expressional control of the 35S promoter. A-G: Phenotype of plants. A and E: Wild-type (WT). B and F: *AtCesA8irx1-1* mutant. C, D, G: *AtCesA8irx1-1* plants transformed with the *P35S::LUC-PgCesA1* construct, expressing *LUC-PgCesA1* (*A1 #4, A1 #7*). White arrow heads mark regular xylem in the wild-type, and collapsed xylem in the mutant and transgenic plants. H: Real-time PCR analysis of transcript abundance. *AtCesA8* (white bars) expression in WT and *AtCesA8irx1-1* (IRX1), and *PgCesA1* expression (grey bar) in stem tissue of an *AtCesA8irx1-1 -P35S::LUC-PgCesA1* transgenic plant (*35S::PgA1*). Expression is calculated relative to the control gene. Error bars represent standard error of the mean, n=3.
3.3.5.1 *PgCesA1*

Two sets of transformed plants expressing the *LUC-PgCesA1* cDNA were recovered after multiple transformation attempts. The first set consisted of two lines transformed with *P35S::LUC-PgCesA1*, and the second set consisted of eight lines transformed with *PA8::LUC-PgCesA1*. None of the *P35S::LUC-PgCesA1* lines showed complementation of the mutant phenotype (Figure 3.4 C, D, G), and only one was selected for further real-time PCR analysis.

Expression of *PgCesA1* measured with real-time PCR revealed very low transcript abundance, compared to the level of *AtCesA8* in wild-type stem tissue (Figure 3.4 H), despite *PgCesA1* being regulated by the 35S promoter. The transcript abundance of *AtCesA8* in wild-type plants was nearly 15x higher than *PgCesA1*.

In contrast, it appears as though the *PA8::LUC-PgCesA1* construct complemented the *AtCesA8*ix1-1 mutant (Figure 3.5 A-F), and one line showed slightly greater height growth compared to wild-type plants after 21 days growth. The xylem vessels did not appear to be collapsed, suggesting that the expression of *PgCesA1* had rescued the cellulose-deficient phenotype of the *AtCesA8*ix1-1 mutation. However, leaf size and morphology appeared similar to the mutant plants (smaller and more round than wild-type leaves). The complemented line also produced more axillary stems. Expression levels of *PgCesA1* were very high (over 8x higher) compared to levels of *AtCesA8* in wild-type plants (Figure 3.5 G).

The remaining seven *AtCesA8*ix1-1 *PA8::LUC-PgCesA1* lines exhibited varying degrees of complementation of the *AtCesA8*ix phenotype (Figure 3.6) which appear to be related to varying levels of *PgCesA1* expression (Table 3.4). Expression of *PgCesA1* at levels exceeding those of *AtCesA8* in wild-type plants seems sufficient to rescue the mutant phenotype, however, levels significantly lower than those of *AtCesA8* (from 12% to 78%) resulted in partial or full complementation of the *AtCesA8*ix1-1 phenotype.
Figure 3.5. Complementation of the $A8^{irx1-1}$ mutant phenotype by LUC-PgCesA1 under expressional control of the native $AtCesA8$ promoter. A-F: Phenotype of plants. Wild-type (A, D), $A8^{irx1-1}$ mutant (B, E) and an $A8^{irx1-1}$ plant transformed with the $PA8::LUC$-$PgCesA1$ construct (C, F). Black arrow heads mark regular xylem in stems of a wild-type plant (D) and transgenic plant (F), and collapsed xylem in an $A8^{irx1-1}$ stem (E). Scale bar = 50 $\mu$m. G: Real-time PCR analysis of $AtCesA8$ (white bars) expression in wild-type (WT) and $A8^{irx1-1}$ (IRX1) stem tissue, and $PgCesA1$ expression (grey bar) in stem tissue of $A8^{irx1-1}$-$PA8::LUC$-$PgCesA1$ transgenic plant #6. Expression is calculated relative to the control gene, $AtUBQ5$, Error bars represent standard error of the mean, n=3.
Table 3.4. Phenotype survey of \( A8^{irx1-1}-LUC-PgCesA1 \) transformed lines. Eight \( AtCesA8^{irx1-1} \) lines expressing \( LUC-PgCesA1 \) were assessed for complementation of the \( AtCesA^{irx} \) phenotype based on xylem morphology (collapsed or not) and growth (stature). Using real-time PCR, \( AtCesA8 \) expression was measured in wild-type plants (Ler-WT) and \( AtCesA8^{irx1-1} \) plants, and \( PgCesA1 \) expression was measured in the transgenic \( AtCesA8^{irx1-1} \) plants (A1—6, 2, 3, 4, 5, 7, 8, 9). \( AtCesA8 \) and \( PgCesA1 \) expression levels were calculated relative to that of the control gene, \( AtUBQ5 \). Expression was then calculated as a percentage of the \( AtCesA8 \) values in wild-type plants. Plant A1-6 is imaged in Figure 3.5, plants A1-2, -3, -4, -5, -7, -8, and -9 are imaged in Figure 3.6.

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Figure 3.6. Additional $A8^{irx1-1}$ plants expressing $LUC-PgCesA1$ under the regulation of the $AtCesA8$ promoter show varying degrees of complementation. $AtCesA8^{irx1-1}$ transgenic plants (A1-2, -3, -4, -5, -7, -8, and -9) transformed with the $PA8::LUC-PgCesA1$ construct are compared to a wild-type plant (WT-Ler) and an $A8^{irx1-1}$ mutant plant. Stem cross sections were taken from the stem base (within 2 cm of the rosette) to compare xylem morphology. Scale bar = 150 μm.
**3.3.5.2 PgCesA3**

Multiple attempts were made to transform *AtCesA7*irx3-1 plants with *P35S::LUC-PgCesA3* or *PA7::YFP-PgCesA3*, and *AtCesA4*irx5-1 plants with *P35S::LUC-PgCesA3* or *PA4::YFP-PgCesA3*. However, only three transgenic lines were recovered: *AtCesA4*irx5-1 transformed with *P35S::LUC-PgCesA3*. None of the transgenic lines showed a complemented phenotype (Figure 3.7 C and F). Expression of *PgCesA3* in the three transgenic lines varied (Figure 3.7 G), but was not as high as *AtCesA4* expression in wild-type plants. Although *AtCesA4*irx5-1 - *P35S::LUC-PgCesA3* line #4 expressed *PgCesA3* at levels only slightly lower than *AtCesA4* in wild-type plants, complementation of the mutant phenotype was not observed. This implies that the 35S promoter does not express *PgCesA3* in the necessary cells and tissues at levels sufficiently high enough to complement the mutant phenotype, or that *PgCesA3* is not able to functionally complement the *AtCesA4*irx5-1 mutation. These results could also suggest that PgCesA3 is not able to function properly with the LUC tag attached at the N-terminus of the PgCesA3 protein.
Figure 3.7. *P35S::LUC-PgCesA3* is not sufficient to rescue the *A4*<sup>irx5-1</sup> mutant phenotype. A-F: Phenotype of plants. Wild-type (A, D), *A4*<sup>irx5-1</sup> mutant (B, E), and an *A4*<sup>irx5-1</sup> plant transformed with the *P35S::LUC-PgCesA3* construct (C, F). White arrow heads mark regular xylem in the wild-type (D), and collapsed xylem in the *A4*<sup>irx5-1</sup> mutant (E) and LUC-PgCesA3 transgenic plant (F). Scale bar represents 100 μm.

G: Real-time PCR analysis. *AtCesA4* expression (white bars) in wild-type (WT) and *A4*<sup>irx5-1</sup> (IRX5), and *PgCesA3* expression (grey bars) in stem tissue of *A4*<sup>irx5-1</sup> - *P35S::PgCesA3* transgenic plants (#1, #4, #3). Expression is calculated relative to the control gene, *AtUBQ5*. Error bars represent standard error of the mean, n=3.
3.3.5.3 \textit{PgCesA2}  
Multiple attempts to transform \textit{AtCesA7}\textsuperscript{irx3-1} with \textit{P35S::YFP-PgCesA2} or \textit{PA7::YFP-PgCesA2}, and \textit{AtCesA4}\textsuperscript{irx5-1} with \textit{P35S::YFP-PgCesA2} were unsuccessful. Several plantlets were able to successfully grow on antibiotic selection medium, but the transgene could not be detected in genomic DNA or cDNA by PCR screening. For this reason, the ability of \textit{PgCesA2} to functionally complement these mutants is unknown.

3.3.6 Ability of Poplar CesAs to complement \textit{Arabidopsis CesA}\textit{irx} mutants  
The \textit{Arabidopsis} \textit{AtCesA4}, A7, and A8 amino acid sequences share a higher degree of similarity with the poplar CesA orthologs compared to the spruce CesA orthologs, as evidenced by phylogenetic analysis (Figure 3.2) and amino acid sequence alignments (data not shown). The alignment scores between whole predicted amino acid sequences were previously reported by Kumar \textit{et al.} (2009) as 88\% between \textit{AtCesA4} and \textit{PtiCesA4}, 93\% between \textit{AtCesA8} and \textit{PtiCesA8-A} (93\%), and 93\% between \textit{AtCesA7} and \textit{PtiCesA7-A}. An alignment of just the predicted class-specific region sequences (formerly hyper-variable region II) revealed that the \textit{AtCesA4}, A8, and A7 CSRs are approximately 65\%, 71\% and 80\% similar to those of \textit{PtiCesA4}, A7-A, and A8-A, respectively. These similarities are approximately 20\% higher than the similarity between the \textit{PgCesA} and \textit{AtCesA} CSRs.

Based on sequence similarity, the following transformations were performed to conduct functional complementation assays: \textit{AtCesA4}\textsuperscript{irx5-1} plants transformed with \textit{PA}\textit{AtCesA4::LUC-PtiCesA4}, \textit{AtCesA7}\textsuperscript{irx3-1} plants transformed with \textit{PA}\textit{AtCesA7::LUC-PtiCesA7-A}, and \textit{AtCesA8}\textsuperscript{irx1-1} plants transformed with \textit{PA}\textit{AtCesA8::LUC-PtiCesA8-A}. \textit{The Luciferase (LUC)} tagged constructs were used for reasons described above (section 3.3.5). Real-time PCR analysis confirmed transgene expression at levels that varied compared to the abundance of endogenous \textit{AtCesAs} in wild-type plants (Table 3.5).
Table 3.5. Real time PCR analysis of LUC-PtiCesA transgene expression in complemented Arabidopsis AtCesA<sup>irx</sup> mutant lines. Expression of endogenous AtCesA4, A7, or A8 in wild-type plants (grey highlighting) is compared to that of the LUC-PtiCesA transgene expression, which was measured in AtCesA<sup>irx</sup> plants transformed with PtiCesA7A (#4, #P3), A8A (#P1), or A4 (#1) Expression is calculated relative to the control gene, AtUBQ5, using the equation 2<sup>(CTCesA-CTubq5)</sup> (delta CT).

<table>
<thead>
<tr>
<th>Line</th>
<th>Gene</th>
<th>Relative expression</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type</td>
<td>AtCesA7</td>
<td>2.18</td>
<td>0.20</td>
</tr>
<tr>
<td>AtCesA7&lt;sup&gt;irx3-1&lt;/sup&gt; - PtiCesA7A #4</td>
<td>PtiCesA7-A</td>
<td>0.24</td>
<td>0.12</td>
</tr>
<tr>
<td>AtCesA7&lt;sup&gt;irx3-1&lt;/sup&gt; - PtiCesA7A #P3</td>
<td>PtiCesA7-A</td>
<td>0.17</td>
<td>0.14</td>
</tr>
<tr>
<td>wild-type</td>
<td>AtCesA8</td>
<td>5.24</td>
<td>1.61</td>
</tr>
<tr>
<td>AtCesA8&lt;sup&gt;irx1-1&lt;/sup&gt; - PtiCesA8A #P1</td>
<td>PtiCesA8-1</td>
<td>0.01</td>
<td>0.02</td>
</tr>
<tr>
<td>wild-type</td>
<td>AtCesA4</td>
<td>0.48</td>
<td>0.50</td>
</tr>
<tr>
<td>AtCesA4&lt;sup&gt;irx5-1&lt;/sup&gt; - PtiCesA4 #1</td>
<td>PtiCesA4-A</td>
<td>1.22</td>
<td>0.85</td>
</tr>
<tr>
<td>AtCesA4&lt;sup&gt;irx5-1&lt;/sup&gt; - PtiCesA4 #2</td>
<td>PtiCesA4-A</td>
<td>7.32</td>
<td>0.94</td>
</tr>
</tbody>
</table>
As seen in Figure 3.8, the T1 generation transgenic plants exhibited varied complementation when compared to the wild-type growth phenotype, yet all lines had normal xylem. In particular, $A7^{irx3-1}$ mutant lines expressing $LUC$-$PtiCesA7$-$A$ seemed more complemented than the others. $A4^{irx5-1}$ mutant plants expressing $LUC$-$PtiCesA4$ had an intermediate complemented phenotype based on a somewhat stunted like stature, but rescuing of the collapsed xylem phenotype. $A8^{irx1-1}$ mutant lines expressing the $PAtCesA8::LUC$-$PtiCesA8$-$A$ construct, which had stunted growth similar to (but not quite as severe as) the $AtCes^{irx}$ mutants, did exhibit non-collapsed xylem.

3.4 Discussion

3.4.1 Three unique cellulose synthase genes identified in spruce

At the nucleotide level, the three isolated $PgCesA$ cDNAs are highly similar to cDNAs of CesAs from other gymnosperms, such as $Pinus radiata$ and $Pinus taeda$. The predicted amino acid sequences of $PgCesA1$, $A2$, and $A3$ revealed motifs that are conserved among all other known cellulose synthases (Roberts and Roberts, 2007) including the defining D, D, D, QXXRW motif found in $\beta$-glycosyltransferases (Pear et al., 1996), and believed to be important for catalytic function (Pear et al., 1996; Taylor et al., 2000; Doblin et al., 2002). The degree of similarity between the $PgCesA$ CSRs was lower than alignments with CSR sequences of other CesA orthologs, therefore the isolated $PgCesA1$, $A2$, and $A3$ are likely to be uniquely different from one another.

3.4.2 Evolutionary relationship of $PgCesA1$, $A2$, and $A3$ with other known cellulose synthases

A dendrogram of several plant cellulose synthases resulted in a clear separation of CesAs implicated in primary cell wall development from those implicated in secondary cell wall development. These findings are consistent with phylogenetic analysis previously reported (Ranik and Myburg, 2006; Kumar et al., 2009).
Figure 3.8. Complementation of *Arabidopsis AtCesA*<sup>irx</sup> mutants by LUC- *PtiCesAs*. 
Top: Plant morphology, bottom: stem cross sections to visualize xylem morphology. 
Left to right: Wild-type (WT) is compared to *AtCesA*<sup>7/irx<sup>3-1</sup> (IRX, representative of A4, A7, and A8 mutants), and the transgenic complemented lines *AtCesA*<sup>7/irx<sup>3-1</sup> – LUC- *PtiCesA7-A* (PA7::LUC-PtiCesA7-A), *AtCesA*<sup>8/irx<sup>1-1</sup> – LUC- *PtiCesA8-A* (PA8::LUC-PtiCesA8-A), and *AtCesA*<sup>4/irx<sup>5-1</sup> – LUC- *PtiCesA4* (PA4::LUC-PtiCesA4). White arrow heads mark regular xylem in the wild-type and transgenic plants, and collapsed xylem in *A8*<sup>irx1-1</sup>. Scale bar represents 100 µm.
Putative orthologs of PgCesA1, A2, and A3 from *Arabidopsis* (Taylor et al., 2003), *Populus* (Joshi et al., 2004; Kumar et al., 2009), *Pinus* (Nairn and Haselkorn, 2005), *Eucalyptus* (Ranik and Myburg, 2006), and rice (Tanaka et al., 2003) are strongly associated with secondary cell wall development. The grouping of CesAs based on primary or secondary cell wall biosynthesis suggests a strong conservation of developmental specific functioning. Within both the AtCesA7 and AtCesA8-containing clades there are two CesAs from *Populus trichocarpa* (PtiCesA7-A and B, PtiCesA8-A and B, respectively). At the nucleotide level PtiCesA7-A and –B are 94% identical, and there close proximity within the dendrogram confirms that they are likely gene duplicates (Kumar et al., 2009). Contrastingly, the distinct positions of PgCesA1, A2, and A3 in separate clades further supports that they are unique cellulose synthases.

Amino acid sequence similarity suggests that PgCesA1, A2, and A3 are orthologs of AtCesA8, AtCesA4, and AtCesA7, respectively. Other reports have indicated that alignment based phylogeny is consistent whether it is based on alignment of whole amino acid sequences or only the CSR sequences (Vergara and Carpita, 2001; Ranik and Myburg, 2006). While whole amino acid alignments placed PgCesA3 within the AtCesA7 clade, an alignment based on the CSR sequences places PgCesA3 with AtCesA4 (along with PgCesA2). The CSR sits within the putative catalytic domain, between the second and third conserved aspartate residues, and therefore homology of CSRs could implicate functional specificity. Nairn and Haselkorn (2001) suggested that the N-terminal region of AtCesA7-like CesAs was more conserved compared to N-terminal regions of other orthologous groups of CesAs, perhaps due to a specialized function or greater evolutionary divergence of AtCesA4 and A8 compared to AtCesA7. As evidenced by the amino acid alignment of PgCesAs with AtCesAs, the N-terminal regions are most similar between AtCesA7 and PgCesA3, followed by PgCesA2. It is interesting to note that in an un-rooted alignment of CesAs, including those of a moss (*Physcomitrella patens*), AtCesA7 is grouped with the moss CesAs, whereas AtCesA4 and AtCesA8 are in separate clades (Roberts and Bushoven, 2007). Again, this suggests that AtCesA7, and perhaps AtCesA7 orthologs, are less divergent compared to the other secondary cell wall-specific CesAs. Based on all alignment analysis, it seems most likely that PgCesA3 is an ortholog of AtCesA7. The grouping of PgCesA3
with AtCesA4 based on CSR alignments could be the result of less divergence between the PgCesA2 and A3 (compared to, for example, AtCesA7 divergence from AtCesA4).

3.4.3 *PgCesA1*, *A2*, and *A3* expression suggests involvement in secondary cell wall formation

The tissues harvested for gene expression analysis were collected in early May, during active growth and activity of the vascular cambium. The expression profiles of *PgCesA1*, *A2*, and *A3* further support the notion that they are involved in secondary cell wall biosynthesis as expression was by far the highest in xylem tissue. The layers of tissue collected for xylem RNA extraction consisted of newly formed, immature xylem elements as well as xylem actively undergoing secondary cell wall formation and mature xylem tissue. Compared to the expression levels in xylem, only moderate expression was observed in young needles (both unexpanded and recently expanded). Expression of the three putative secondary cell wall *CesAs* (*PitCesA1*, *A2*, and *A3*) was also detected in young needles of pine (Nairn and Haselkorn, 2005). The synthesis of secondary cell walls in developing xylem of actively growing young needles could explain the expression of *PgCesA1*, *A2*, and *A3* in these tissue samples. Moderately high levels of *PgCesA1*, *A2*, and *A3* were also detected in root tissue, which can be attributed to developing xylem elements in the roots. Compared to mature needles there should be more xylem undergoing secondary cell wall formation, and therefore greater cellulose deposition in young needles and developing root tissue. Indeed, expression of the *PgCesAs* was the lowest in old needles, further supporting the need for *PgCesA1*, *A2*, and *A3* in tissues that are actively developing xylem and therefore secondary cell walls. Although secondary cell walls are formed in phloem tissue the expression of *PgCesA1*, *A2*, and *A3* was low. In *Populus tremuloides*, expression of *PtrCesA1*, *A2*, and *A3* (orthologs of AtCesA8, A7, and A4, respectively) was found in developing phloem fibres based on *in situ* mRNA hybridization in stem sections (Joshi et al., 2004). In *Eucalyptus*, RT-PCR expression profiles of putative secondary cell wall *EgCesAs* revealed low expression in phloem tissue (Ranik and Myburg, 2006), consistent with our findings. Low, but detectable, expression of hybrid poplar *PtxtCesA1*, *A2*, and *A3* (orthologs of AtCesA8, A7, and A4, respectively) in phloem was also reported by Djerbi et al. (2004). In both *Eucalyptus* (Ranik and Myburg, 2006) and
hybrid poplar (Djerbi et al., 2004) the CesAs more highly expressed in phloem had homology to the primary cell wall CesAs of Arabidopsis. It is postulated that PgCesA1, A2, and A3 might be involved in cellulose biosynthesis of secondary cell walls of phloem fibres, but that other yet to be examined PgCesAs are involved in primary cell wall biosynthesis in phloem which would be expressed at higher levels.

RT-PCR analysis of PgCesA1, A2, and A3 transcript abundance shows that A1 and A2 are consistently more highly expressed than A3. Unequal expression between CesA family members, within a specific tissue, has been reported for other groups of cellulose synthases, including those from Arabidopsis (Brown et al., 2005), Populus (Samuga and Joshi, 2002), Pinus (Nairn and Haselkorn, 2005), and Eucalyptus (Ranik and Myburg, 2006). The biological implications for unequal gene expression, and if differences in transcript abundance are always conserved at the protein level, are not clear. Current reports of CesA protein abundance are ambiguous. For example, antibody labelling of Arabidopsis stem interfascicular fibres indicated higher abundance of AtCesA4 and A8, compared to those of AtCesA7 (Taylor et al., 2003). However, proteins from Arabidopsis stem tissue separated using SDS-PAGE and probed with CesA-specific antibodies showed higher levels of AtCesA8 monomers, followed closely by AtCesA7 and lower levels of AtCesA4 (Atanassov et al., 2009). Thus, it stands to reason that unequal gene expression levels likely represent some disparity in CesA protein ratios, but to what degree is not clear. Unequal expression, within a specific tissue or cell type, could be due to a few key factors, such as: 1) functional differences between individual CesAs dictate the need for unequal ratios; 2) the rate of protein turnover of individual CesAs varies; 3) cellulose synthesizing complexes require an unequal ratio of CesAs to function properly. Measuring protein levels within different tissues and cell types, and determining if individual CesAs have unique and specific functions, could explain the need for multiple CesAs and their unequal expression patterns.

The comparative levels of expression between PgCesA1, A2, and A3 do not follow the expression pattern of their AtCesA orthologs. In inflorescence stems of 21 day-old Arabidopsis plants it was found that the AtCesA7 transcript (PgCesA3 ortholog) was most abundant followed closely by AtCesA8 (PgCesA1 ortholog), and lower expression of AtCesA4 (PgCesA2 ortholog) (Appendix A). A similar expression profile
was reported by Brown et al. (2005) in mature Arabidopsis stem tissue. However, Hamman et al., (2004) reported highest expression of AtCesA4 in stem tissue. In our analysis, PgCesA2, the AtCesA4 ortholog, showed the highest expression. In *Eucalyptus* xylem tissue, Ranik and Myburg (2006) reported the highest expression of EgCesA3 (the AtCesA7 ortholog) followed closely by EgCesA1 (AtCesA8 ortholog). In hybrid poplar, PtxtCesA1 (AtCesA8 ortholog) was the most abundant, followed by PtxtCesA2 (AtCesA7 ortholog) and PtxtCesA3 (AtCesA4 ortholog) (Djerbi et al., 2004). In *Pinus taeda*, Northern blot analysis (Nairn and Haselkorn, 2005) and RT-PCR analysis (Nairn et al., 2008) showed highest to expression in xylem of PitCesA2 (AtCesA7 ortholog) and slightly lower levels of PitCesA1 (AtCesA8 ortholog) and PitCesA3 (AtCesA4 ortholog). It is clear that unequal ratios exist amongst all secondary cell wall CesA gene families. However, there is some variability between different species as to which CesA is the most highly expressed and which CesA has the lowest expression in woody tissues. Different ratios between secondary cell wall CesAs from Arabidopsis, *Eucalyptus*, pine, and spruce could be due to experimental differences including tissue type, and time of harvest, both of which can have a dramatic effect on gene expression levels. Biologically, the differences could be due to the divergence of function between the angiosperm and gymnosperm CesAs. Interestingly, AtCesA8, EgCesA1, PtxtCesA1, PitCesA1, and PgCesA1 all show high expression (either the highest or nearly highest). This could suggest that the level of requirement of the AtCesA8 orthologs could be more conserved compared to the AtCesA7 and AtCesA4 orthologs.

It is thought that cellulose biosynthesis in secondary cell walls requires the function of three distinct CesAs. In *Arabidopsis* this has been confirmed by mutant studies, revealing the need for AtCesA8, A7, and A4 to be functionally active for proper secondary cell wall formation. Based on sequence homology and gene expression profiling it is also thought that PgCesA1, A2, and A3 are the three subunits that would work in concert during secondary cell wall formation in spruce. However, the identification of additional PgCesAs, particularly primary cell wall-specific, is required to gain a complete understanding of the CesA gene family in spruce.
3.4.4 Significant conservation of function exists between CesA orthologs

Wild-type Arabidopsis plants develop thick secondary cell walls in xylem vessels and interfascicular fibres which can be easily observed in hand sections of stem tissue. The cellulose synthase irregular xylem (irx) mutants have provided critical information about the role of AtCesA4, AtCesA7, and AtCesA8 in secondary cell wall formation in Arabidopsis stems (Turner and Somerville, 1997; Taylor et al., 2000; Taylor et al., 2003; Gardiner et al., 2003; Ha et al., 2002). Arabidopsis plants homozygous for the AtCesA8\textsuperscript{irx1-1} mutation contain a point mutation in the AtCesA8 gene, resulting in an amino acid substitution of one of the conserved aspartate residues within the catalytic domain, resulting in collapsed xylem with irregular cell wall deposition and reduced cellulose (Taylor et al., 2000), and mutant plants tend to be smaller in stature. The AtCesA8\textsuperscript{irx1-1} phenotype is easily observed, and provides a method to screen for functional complementation when transformed with other CesAs. In order to functionally complement AtCesA8\textsuperscript{irx1-1} it was expected that the orthologous gene product would be functionally equivalent to AtCesA8, and also expressed at temporally and spatially appropriate levels compared to the endogenous gene in wild-type plants. The cauliflower mosaic 35S promoter (P\textsuperscript{35S}) is a constitutive promoter, however, in this study AtCesA8\textsuperscript{irx1-1} plants transformed with P\textsuperscript{35S}::LUC-PgCesA1 expressed PgCesA1 at significantly lower levels than the native AtCesA8 in wild-type plants, and neither complete nor partial recovery of the AtCesA8\textsuperscript{irx1-1} phenotype was observed.

Expression of PgCesA1 under the control of the endogenous AtCesA8 promoter (PA8) was hypothesized to provide proper spatial and temporal expression of a transgene, and therefore better conditions to examine functional orthology between CesAs. Eight transgenic lines were recovered, expressing PA8::LUC-PgCesA1 at levels from 1% to over 700% of the native AtCesA8 expression in wild-type plants. Such a range of transgene expression, despite all being driven by the AtCesA8 promoter may be explained by the position and copy number of the PA8::LUC-PgCesA1 fragment insertion within the genome of transgenic plants (Matzke and Matzke, 1998). Seven of these transgenic lines showed partial or complete recovery from the AtCesA\textsuperscript{irx} phenotype based on overall plant growth and xylem morphology. Compared to the P\textsuperscript{35S}::LUC-PgCesA1 transgenic lines it is clear that the AtCesA8 promoter is likely
providing critical temporal and spatial-specific expression that is required for \textit{LUC-PgCesA1} to complement the \textit{AtCesA8\textsuperscript{irx1-1}} mutation.

In addition to \textit{PgCesA1}, we determined that the poplar CesAs (fused to Luciferase), \textit{PtiCesA4}, \textit{PtiCesA7-A}, and \textit{PtiCesA8-A}, could at least partially complement the \textit{AtCesA4\textsuperscript{irx5-1}}, \textit{AtCesA7\textsuperscript{irx3-1}}, and \textit{AtCesA8\textsuperscript{irx1-1}} mutant phenotypes (all under the expressional control of native \textit{AtCesA} promoters), respectively. Although, the expression of \textit{LUC-PtiCesA8-A} in \textit{AtCesA8\textsuperscript{irx1-1}} and \textit{LUC-PtiCesA4} in \textit{AtCesA4\textsuperscript{irx5-1}} only resulted in a partial complementation of the mutant phenotype, as transgenic plants exhibited \textit{AtCesA\textsuperscript{irx}}-like stunted growth but had normal xylem morphology. \textit{PtiCesA8-A} transcript abundance was extremely low in these transgenic lines, which could have resulted in insufficient protein expression to fully complement the mutant phenotype. As well, the presence of the mutant \textit{A8\textsuperscript{irx1-1}} protein in these mutants likely inhibits \textit{PtiCesA8-A} from entering the CSC, thus maintaining some of the mutant phenotype. It should also be noted that the Luciferase tag attached to the 5'-terminus of \textit{PtiCesA8-A} could interfere with CesA function or the ability of \textit{LUC-PtiCesAs} to access the CSC, or both, which would cause only partial complementation to be achieved. However, the partial complementation suggests that \textit{PtiCesA8-A} and \textit{PtiCesA4} likely has a high degree of functional conservation with \textit{AtCesA8} and \textit{AtCesA4}, respectively. It would be relevant to determine if full complementation could be achieved with 1) higher expression of the \textit{LUC-PtiCesA} fragment, or 2) expression of a non-tagged form of \textit{PtiCesA} in the mutant background.

The results presented herein are the first report of \textit{AtCesA} orthologs capable of complementing an \textit{AtCesA\textsuperscript{irx}} mutation, and strongly support the idea that CesA-specific functions in secondary cell wall biosynthesis may have evolved prior to the divergence between angiosperms and gymnosperms. Recently, Maloney (2010) reported that a spruce KORRIGAN was capable of complementing the \textit{AtKOR\textsuperscript{kor1-1}} mutant. Combined with the results presented herein, this suggests that there could be a great deal of conservation of function among cell wall biosynthetic genes. It would be interesting to explore whether monocot CesA orthologs of \textit{AtCesA4}, \textit{A7}, and \textit{A8} share the same degree of functional conservation. The total abundance, crystallinity, microfibril angle, and degree of polymerization of cellulose in secondary cell walls of spruce tracheids, poplar vessels, and \textit{Arabidopsis} vessels and fibres are likely very different. However, it
is clear that the functions of orthologous CesAs are sufficiently conserved. Thus, the ability of spruce and poplar CesAs to permit proper cellulose biosynthesis to occur in *Arabidopsis* suggests that the cellulose requirements of secondary cell walls are likely to be greatly influenced by factors other than the CesA composition of CSCs. However, it must also be considered that the ratio of CesA subunits (e.g. CesA4:CesA7:CesA8) within a CSC may vary from species to species, and that such variety could dictate differences in cellulose properties.

It is highly likely that the cellulose synthase complexes in the plasma membrane consist of multiple individual CesAs which interact within the complex. As such, in order to functionally complement the *AtCesA*<sup>irx1</sup> mutants the orthologous spruce and poplar CesA subunits must be able to integrate into the complex and interact with neighbouring AtCesAs. Our results suggest that PgCesA1, PtiCesA8-A, PtiCesA7-A, and PtiCesA4 contain the domain(s) required for proper CesA-CesA interaction with AtCesA subunits. Kurek *et al.* (2002) implicated the zinc-binding domain in CesA-CesA interactions, and therefore complex assembly. Based on an amino acid alignment, the PgCesA1 and AtCesA8 zinc-finger region shows low similarity (approximately 48% identity and 67% similarity). Perhaps it is simply the presence of the zinc-finger region that allows integration into the complex and interaction with other CesAs. The central domain of CesA proteins (containing the catalytic site and CSR region) has been identified as necessary for proper CesA function based on mutant analysis (see Joshi and Mansfield, 2007 for review). Additionally, a chimeric protein consisting of a fusion between the AtCesA3 N-terminus with the central domain of AtCesA1 could functionally complement an *AtCesA1* mutant phenotype (Wang *et al.*, 2006), again illustrating the importance of the central domain in function. Our results suggest that the catalytic function of the AtCesA orthologs we tested are highly conserved with those of the AtCesA enzymes.

3.4.5 *PgCesA2* and *PgCesA3* may be distant orthologs of either *AtCesA4* or *AtCesA7*

The inability of *PgCesA3* to complement the *AtCesA4*<sup>irx5-1</sup> mutant could be due to several factors: 1) insufficient expression of *PgCesA3* in the transgenic plants; 2) *PgCesA3* is not a true ortholog of *AtCesA4*; 3) *PgCesA3* is a distant ortholog of *AtCesA4* or *AtCesA7*, 4) the 5'-terminal LUC tag on PgCesA3 results in poor function of
PgCes3, or 5) the 5'-terminal LUC tag on PgCesA3 restricts PgCesA3 from accessing the CSC. Expression of PgCesA3 in the recovered transgenic plants was lower compared to endogenous AtCesA expression in wild-type plants. As shown by the complementation of AtCesA8\textsuperscript{irx1-1} by LUC-PgCesA1 under expressional control of the native AtCesA8 promoter, it is likely that tissue and developmental-specific expression driven by an appropriate promoter is required to enable complementation. Hence, the plants recovered in this experiment, in which LUC-PgCesA3 expression is driven by the 35S promoter, do not provide enough evidence to conclude if PgCesA3 can functionally complement AtCesA4. A LUC-AtCesA4 fusion protein has been shown to be able to rescue the A4\textsuperscript{irx5-1} mutant phenotype (McDonnell, Chapter 2), illustrating that LUC-tagged CesAs can access the CSC, and function properly. However, it has not been overlooked that LUC-AtCesA4 may be a better fit in the CSC, with other AtCesAs, than LUC-PgCesA3. Thus it should be explored if a non-tagged form of PgCesA3 can complement the mutant phenotype. Additionally, testing the ability of PgCesA3 to complement the AtCesA7\textsuperscript{irx3-1} mutant phenotype may help resolve if PgCesA3 is a functional ortholog of AtCesA4 or AtCesA7.

The lack of transgenic plants carrying the PgCesA2 construct is puzzling, and will require further examination in order to conclude if PgCesA2 is functionally orthologous to one of the Arabidopsis CesAs.

In summary, herein we report on the identification of three unique cellulose synthase genes from spruce (Picea glauca). Sequence homology and expression profiles indicate that these PgCesAs are members of a secondary cell wall-specific CesA gene family. One gene, PgCesA1, was confirmed to be a true ortholog of AtCesA8 based its ability to complement the Arabidopsis AtCesA8\textsuperscript{irx1-1} mutant phenotype. Additionally, conservation of function between AtCesA4-PtiCesA4, AtCesA7-PtiCesA7-A, and AtCesA8-PtiCesA8-A was shown, suggesting that the catalytic function of CesA orthologs is highly conserved. This is the first example of cross-species CesA functional complementation. Despite extensive evolutionary divergence between spruce, poplar, and Arabidopsis there is clearly a very strong conservation of the primary functions among the AtCesA4, A7, and A8 orthologs. Together, the sequence homology results and functional complementation provide further evidence that CesA functions evolved prior to the divergence of angiosperms.
from gymnosperms. Further investigation is required to identify additional members of the spruce CesA gene family, and to confirm if additional PgCesAs are able to functionally complement their AtCesA orthologs.
CHAPTER 4 Measuring CesA-CesA interactions in planta

4.1 Introduction

The polysaccharide network in both primary and secondary cell walls is critical to the integrity, plasticity and morphology of plant cell walls, and hence cell wall function. Large particles, or groups of particles, within the plasma membrane of bacteria, algae, and plants have been observed and are believed to be a complex of cellulose synthase enzymes, responsible for producing cellulose. Rows of particles in the plasma membrane of bacteria, Acetobacter xylinium, that were found to be associated with ribbons of cellulose microfibrils were visualized in 1976 and believed to be a cellulose synthesizing complex (Brown et al., 1976). Similarly, linear arrays of particles were observed in the plasma membrane of the green alga, Oocystis (Brown and Montezinos, 1976). Later, groups of particles, sometimes forming a hexameric structure and called rosettes, were observed in the plasma membrane of plants (Mueller and Brown, 1980). The rosette structure has been postulated to be composed of six cellulose synthesizing complexes (CSCs), and current models hypothesize that six CesA subunits are bound together to form the CSC (Delmer, 1999; Doblin et al., 2002; Ding and Hemmel, 2006). Kimura et al. (1999a) used gold-labeled CesA-specific antibodies to reveal that rosette structures in the plasma membrane of bean plants, Vigna angularis, contain CesAs.

CesA proteins have a conserved structure containing a zinc-binding domain at the N-terminus, eight transmembrane domains (two located at the amino terminus and six at the carboxyl terminus), a plant-conserved region, a class-specific region, and two highly conserved domains believed to be part of the catalytic site (Richmond, 2000; Krauskopf et al., 2005). The zinc-binding domains of cotton CesA1 and A2 have been shown with an in vitro assay to interact in a 1:1 ratio, in addition to forming homodimers, suggesting that the zinc binding domain could regulate CesA interactions in vivo (Kurek et al., 2002). Mutant forms of AtCesA4 (AtCesA4irx5) were used to examine the interactions among CesA proteins and confirm that three CesA subunits were required for proper cellulose biosynthesis (Taylor et al., 2003). Depending on the severity of the mutation, various levels of association between the three secondary cell wall-specific CesA proteins were observed using immunoprecipitation and CesA-antibody labelling (Taylor et al., 2003). For example, the authors found that null mutations of AtCesA4
eliminated interactions between AtCesA8 and A7. In addition, compared to wild-type plants, a reduced level of AtCesA8 and A7 protein was observed, possibly the result of degradation of improperly formed CSCs due to the lack of CesA4 (Taylor et al., 2003). Using an epitope-tagged AtCesA7, Atanassov et al. (2009) isolated a large protein complex containing CesA7, CesA4, and CesA8 proteins from Arabidopsis stem protein extracts, believed to be a CSC unit, or a portion of a CSC.

The use of in vitro methods to study CesA-CesA interactions suggests that all the CesAs form homodimers and heterodimers (Taylor et al., 2000; Taylor et al, 2003; Atanassov et al., 2009). However, it is still unclear as to whether these interactions are representative of those that occur in planta, in tissues undergoing active cell wall formation. For this reason, we chose to adopt in vivo methods to study CesA-CesA interactions, specifically, the bioluminescence resonance energy transfer (BRET) assay and bimolecular fluorescence complementation (BiFC), reviewed in Figure 4.1. The BRET assay (reviewed by Subramanian et al., 2004 and 2006) relies on the excitation-emission of Luciferase (LUC) to excite yellow fluorescent protein (YFP). Interaction candidates are genetically fused to the LUC or YFP cDNA and simultaneously expressed in planta (by stable or transient plant transformations). If protein-protein interactions are occurring, the excitation-emission of LUC will excite YFP, resulting in a distinct emission spectrum that differs from non-interacting proteins. Interactions are therefore detected by measuring emission spectra from plant tissues transformed with the LUC and YFP expression constructs. Compared to FRET, which requires laser excitation of the donor molecule, BRET avoids interference from autofluorescence.

BiFC is an in vivo assay that provides an opportunity to visually detect protein-protein interactions (reviewed by Citovsky et al., 2008; Kerppola, 2008). Briefly, the BiFC assay involves reconstitution of YFP when two proteins, one tagged with the N-terminal fragment of YFP and the other tagged with the C-terminal fragment of YFP, interact resulting in the two YFP fragments coming together to form a functional, fluorescent YFP signal that can be visualized using fluorescence microscopy. Moreover, we wanted to determine if it was possible to detect whether interactions were tissue-specific and consistent.
Figure 4.1. BRET and BiFC assays. (A) In BRET, candidate proteins are genetically fused to either the Luciferase protein (L, LUC) or the YFP protein (Y, YFP). (B) The Luciferase substrate, coelenterazine (X) is added to the tissue and resulting Luciferase and YFP emission intensities are recorded using specific filters to detect the LUC emission (480 nm) and YFP emission (530 nm). If the candidate proteins are interacting, the resulting emission spectrum consists of both LUC and YFP, such that the ratio of YFP/LUC is generally greater than one. (C) In BiFC, the YFP gene has been separated into an N- and C-fragment, which can be genetically fused to two candidate proteins (D). If the two proteins are interacting, the YFP fragments can fuse together to form a functional, fluorescent YFP, which can be detected by fluorescence microscopy (E). Diagrams are modified from Xu et al. (1999) (BRET) and Citovsky et al. (2008) (BiFC).
The use of *in planta* methods to study CesA interactions has the potential to reveal some critical and novel information that has not been provided by *in vitro* studies about how CesAs come together to form a CSC. In this study BRET was employed in *Arabidopsis* plants and BiFC in *Nicotiana tabacum* leaf tissue to determine if AtCesA4, A7, and A8 subunits form homodimers and heterodimers *in planta*. The results suggest that various combinations of interactions do occur *in planta* but that they are not consistent between seedlings and stem tissue. Additionally, interactions appear to be weak and potentially transient. Further optimization of the BiFC assay is required to fully elucidate the complexity of CesA-CesA interactions in the CSC.

4.2 Materials and methods

4.2.1 Plant transformation vectors

*Arabidopsis thaliana* cellulose synthase (AtCesA) 4, 7, and 8 cDNAs were isolated using PCR and cloned into the pENTR (Invitrogen) vector for use in Gateway recombination reactions to produce BRET-CesA and BiFC-CesA expression constructs (cloning primers listed in Table 4.1). BRET destination expression vectors containing Luciferase (LUC), pPZPhRLUC-attR (*P*35S::*LUC*), and Yellow Fluorescent Protein (YFP), pBin19YFP-attR (*P*35S::*YFP*) are described by Subramanian *et al.* (2006). BiFC destination expression vectors pBIFP-2 (*P*35S::*NYFP*, contains the N-terminal fragment of YFP) and pBIFP-3 (*P*35S::*CYFP*, contains the C-terminal fragment of YFP) are described by Hu *et al.* (2002). LR Clonase II (Invitrogen) reactions between pENTR-CesA plasmids and BRET or BiFC destination vectors were performed according to manufacturer’s instructions to create BRET (*P*35S::LUC-CesA and *P*35S::YFP-CesA) and BiFC (*P*35S::NYFP-CesA and *P*35S::CYFP-CesA) expression vectors.

The BRET expression vectors were also modified to replace the 35S promoter with *AtCesA* promoters (*PA*4, *PA*7, and *PA*8). For *PA*4, *PA*7, or *PA*8 to the 5’-terminus and AvrII (*PA*4, *PA*7, *PA*8) to the 3’-terminus. Primers are listed in Table 4.1.
Table 4.1. PCR primers used for cloning, screening, and real-time (RT) PCR.

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</tr>
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</tr>
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<td>CACCGAGTCTAGGTTCACCTCCA</td>
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</tr>
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<tr>
<td>UBQ5RV</td>
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<td>RT</td>
</tr>
</tbody>
</table>
Cloned promoter fragments were subsequently digested from cloning vectors with Sdal/AvrII or HindIII/AvrII and ligated into the P35S::LUC-CesA backbone, which had the 35S promoter removed by digestion with Sdal/AvrII or HindIII/AvrII. LR Clonase II reactions were performed between the binary destination vectors and pENTR-CesA vectors to create PA4::LUC-CesA4, PA7::LUC-CesA7, PA8::LUC-CesA8. PcesA::YFP-CesA vectors were created in a similar fashion, however, using Clal and AvrII to remove the 35S promoter and replaced with the CesA promoter, followed by an LR Clonase II reaction with pENTR-CesA vectors to create PA4::YFP-CesA4, PA7::YFP-CesA7, and PA8::YFP-CesA8. The promoter-tag-gene fragments of all binary vectors were verified by DNA sequencing.

A binary construct containing the LUC-YFP fragment under the control of the AtCesA4 promoter (PA4) was also created as a comparative positive control to the P35S::LUC-YFP control. The LUC-YFP fragment was amplified by PCR adding an AvrII site to the 5’ terminus and a KpnI site to the 3’ terminus (primers in Table 4.1), and cloned into the pBLUNT cloning vector (Invitrogen). The YFP fragment was removed from the PAtCesA4::YFP binary vector by digestion of endogenous AvrII/KpnI sites. The LUC-YFP fragment was cut and isolated from the cloning vector by a AvrII/KpnI double digest, and subsequently ligated into the previously linearized PAtCesA4::YFP vector, to create PAtCesA4::LUC-YFP.

4.2.2 Plant growth and transformations

Wild-type (WT, Landsberg ecotype), AtCesA8IRX1-1 (Taylor et al., 2003), AtCesA7IRX3-1 (Taylor et al., 1999), and AtCesA4IRX5-1 (Taylor et al., 2003) plants were grown and used for transformations for BRET assays. Seeds were germinated on half-concentration MS medium (Mirashige and Skoog, 1962) with no sucrose under continuous light. Seedlings were transferred to soil approximately seven days post germination, and grown in a growth chamber at 21°C under a 16-hour light/8-hour dark cycle. Plants were transformed using a method modified from Clough and Bent (1998). Agrobacterium tumefaciens GV3101-pMP90 (Hellens et al., 2000) was transformed with individual binary vectors (listed above) using a freeze-thaw method. Cultures were grown overnight at 28°C in Luria-Bertani medium containing 50 µg L⁻¹
kanamycin (YFP vectors) or 50 µg L\(^{-1}\) spectinomycin (LUC vectors), 25 µg L\(^{-1}\) rifampicin and 25 µg L\(^{-1}\) gentamycin. The overnight culture was centrifuged and resuspended in a 5% (w/v) sucrose solution to an OD\(_{600}\) of at least 0.8. Silwet L-77 (LEHLE Seeds) was added to each resuspended culture at a final concentration of 0.02% (v/v). Newly flowering plants (approximately 4 weeks old) were sprayed with the Agrobacterium solution using a fine mist spray nozzle. Sprayed plants were placed in a dark, humid environment for 16 to 24 hours and then returned to the light. A second spraying was done five days after the first spray to increase the transformation rate. After all spray treatments, plants were maintained as usual and seeds were harvested from mature, dried plants. Seeds were screened for transformations by germinating on half-concentration MS medium (no sucrose) with the addition of either 75 µg L\(^{-1}\) kanamycin for YFP vectors, 50 µg L\(^{-1}\) glufosinate ammonium sulfate for LUC vectors (or both for double transformations). Seedlings that successfully grew on antibiotics were grown in soil for further analysis. Transformed plants were confirmed by PCR screening of genomic DNA using primers listed in Table 4.1).

4.2.3 BRET methods

BRET assays were performed as described by Subramanian et al. (2004 and 2006). Briefly, luminescence spectra were measured from seedlings (cotyledons alone, hypocotyls/roots alone, or whole seedlings) or stem cross sections. Seedlings were five to seven-days old, and individual plantlets were tested for BRET. For stem cross sections, stem segments spanning the lower 3cm to 5cm from the stem base from 21 to 30-day-old plants were excised and hand sectioned to produce transverse sections, which were then used as the tissue sample for BRET testing. All BRET assays were performed with tissue samples immersed in 180 µl water in a well of a white 96-well Optiplate (Perkin Elmer), with native coelenterazine (Invitrogen) added to a final concentration of 2 µM. Measurements were made using a PerkinElmer Victor\(^3\)V microplate reader equipped with emission filters to capture LUC emissions (486/10 nm) and YFP emissions (530/10 nm). After the addition of coelenterazine, a protocol was initiated consisting of a 5-second shake, a 5-second pause, followed by sequential 0.5-second emission readings through the blue and yellow filters. Emission readings were
repeated two to eight times. BRET calculations were performed as described by Bacart et al. (2008). To calculate the BRET ratio, YFP emission values were divided by LUC emission values (BRET = YFP \text{emission}/LUC \text{emission}). To correct for background emission, the ratio in untransformed tissue was subtracted from all ratios calculated for experimental samples (BRET_{corrected} = BRET_{test \, sample} - BRET_{untransformed}). The final ratios were multiplied by 1000 to be expressed as milliBRET units (milliBRET = BRET_{corrected} \times 1000). To measure YFP abundance, YFP fluorescence intensity signals were recorded after a single excitation through a 490nm laser, from which background signal from untransformed plants was subtracted (YFP abundance = YFP_{test \, sample} - YFP_{untransformed}).

4.2.4 BiFC in transiently transformed tobacco leaf cells

Tobacco leaf cells were transiently transformed with BiFC expression vectors (combinations of P_{35S}::NYFP-CesA and P_{35S}::CYFP-CesA) and control expression vectors (P_{35S}::LUC-YFP, P_{35S}::YFP-CesA7) following the methods of Sparkes et al. (2006). Briefly, leaves of 5-week-old tobacco (Nicotiana tabacum) were transformed by infiltration with Agrobacterium tumefaciens carrying the expression vectors. YFP fluorescence was detected 48 and 65-hours post-infiltration through a Leica DMI6000 inverted microscope with a Quorum Wave FX system, which has a modified Yokogawa CSU-10 spinning disk scan head (Yokogawa Electric Corporation). YFP was excited with a 491 nm laser and emissions passed through a 528/38 band filter (Chroma Technology). Images were acquired using a Hamamatsu 9100-13 EMCCD camera (Hamamatsu) controlled by Volocity software (Improvision).
4.3 Results

4.3.1 BRET in Arabidopsis seedlings and stem tissues

The use of BRET to detect protein-protein interactions in vivo depends on a few key factors: 1) that interacting proteins are within 10 nm, and ideally closer; 2) that interactions are stable and abundant enough to be detected within the timeframe of a BRET assay (generally 20 minutes); and, 3) the stoichiometry of interacting proteins facilitates Luciferase to be within 10 nm of YFP. With these considerations in mind it was decided to explore the use of various tissue types from which to measure BRET, and to measure BRET multiple times over 20 minutes.

4.3.1.1 Comparing controls and tissue types

A very strong positive control consists of the LUC protein directly fused to YFP, and constitutively expressed by the 35S promoter. Stably transformed Arabidopsis plants expressing such a control construct (P35S::LUC-YFP) were used as a positive control in the BRET experiments. To determine if the use of a P35S-driven positive control was appropriate for a comparison to potential CesA-CesA interactions, we compared the effectiveness of an AtCesA promoter-driven LUC-YFP fusion as a second positive control (PA4::LUC-YFP). To compare the two controls, the BRET signal was measured from various tissues of Arabidopsis plants, and the YFP expression pattern was observed using confocal fluorescence microscopy. As expected, there were varied YFP expression patterns between the controls observed by confocal fluorescence microscopy (Figure 4.2). P35S-driven expression produced abundant YFP signal, present in most tissues at all stages of development (Figure 4.2, A-C). In contrast, the PA4-driven YFP expression was surprisingly abundant in cotyledons but less so in young roots (compared to P35S::LUC-YFP). Additionally, in stem tissue, PA4-YFP was highly localized to xylem and fibres (Figure 4.2-G), whereas P35S-driven YFP was mostly found in pith parenchyma. LUC and YFP intensity, and BRET ratios agreed with the visual expression patterns observed: P35S::LUC-YFP had the highest intensities and BRET ratios in seedlings (Figure 4.3, A-B), but in stem cross sections the two controls exhibited similar BRET signals (Figure 4.3-C).
Figure 4.2. YFP expression patterns in BRET control plants observed using confocal fluorescence microscopy. YFP was visualized in cotyledons (A, D) and roots (B, E, and F) of 5-day-old seedlings and in longitudinal sections of maturing stem tissue (C, G). P35S::LUC-YFP transformed plants are imaged in A-C, and PA4::LUC-YFP in D-G. White arrow heads indicate xylem vessels.
Scale bars represent 50 μm (A, B, D) and 10 μm (C, E, F, G).
Figure 4.3. Varied expression and BRET of the LUC-YFP control. Comparisons are made between two control constructs: \textit{P}_{35S}::\text{LUC-YFP} (\textit{P}_{35S}::\text{L-Y}) and the \textit{AtCesA4} promoter-driven control, \textit{P}_{A4}::\text{LUC-YFP}. (A) Relative LUC and YFP emission intensity in five-day-old seedlings. (B) Average milliBRET in seedlings compared to the LUC-only negative control (\textit{P}_{35S}::\text{LUC-AtCesA4}). (C) Average milliBRET in stem cross sections, compared to two negative controls (\textit{P}_{35S}::L-A4 and \textit{P}_{A4}::L-A4). (D) LUC-YFP transcript abundance in stem tissue of the control lines. Transcript abundance is calculated relative to \textit{AtUBQ5}. Error bars are standard error of the mean. A and B, \textit{n}=16 (\textit{P}_{35S}) or 32 (\textit{P}_{A4}); C, \textit{n}=6 (all samples); D, \textit{n}=3 (all samples).
Transcript abundance of the LUC-YFP fusion gene appeared to be lower in PA4::LUC-YFP stem tissue as measured by real-time PCR (Figure 4.3-D), and was reflected in lower overall LUC and YFP emission intensities in these plants compared to P35S::LUC-YFP. Despite the reduced transcript abundance, LUC luminescence, and YFP emissions detected in PA4::LUC-YFP stem tissue, the BRET ratios were similar to those of P35S::LUC-YFP stem sections, which suggests that there are saturation limits to the amount of LUC-YFP signal necessary to measure significant BRET interactions.

From this assessment there were clear differences between the two control constructs in seedlings. Therefore, I chose to conduct BRET assays in both whole seedlings and stem cross sections to account for potential differences in spatial and temporal gene expression patterns.

4.3.1.2 CesA7-CesA7 interactions

AtCesA7irx3-1 mutants transformed with two AtCesA7-BRET constructs (P35S::LUC-A7 x PA7::YFP-A7) were used to detect homodimerization of CesA7. Abundant YFP signal had previously been detected in seedling tissue, thus five-day-old plantlets were chosen as a suitable tissue for measuring BRET interactions. Although LUC and YFP signal was detectable during BRET assays, the signals were very low, and BRET ratios were always equivalent to, or lower than, ratios of the LUC-only negative control (Table 4.2). Gene expression analysis suggested elevated A7 transcript abundance in the transgenic lines. However, this failed to translate into elevated active protein levels as evidenced by the low LUC and YFP emissions.

4.3.1.3 CesA7-CesA8 interactions

AtCesA8irx1-1 mutant plants were transformed with two BRET constructs (PA8::LUC-A8 x P35S::YFP-A7) to measure the interaction between AtCesA8 and AtCesA7. LUC and YFP emissions were measured, and BRET ratios subsequently calculated, in whole seedlings, cotyledons only, and roots only. In cotyledons, there was a slight but not always statistically significant increase in BRET ratios of LUC-A8 x YFP-A7 plants compared to the negative control (Figure 4.4, B-C). In whole seedlings the BRET ratio of LUC-A8 x YFP-A7 samples was significantly higher than that of the negative control. The ratio values, however, were extremely low.
Table 4.2. YFP, LUC, BRET, and transcript abundance in whole seedlings carrying two *AtCesA7*-BRET constructs. *AtCesA7*<sup>irx3-1</sup> plants transformed with *P*<sup>35S</sup>::*AtCesA7*-LUC and *PA7*:YFP-*AtCesA7* (*irx3*-P<sup>35S</sup>::*A7*-L x *PA7*:Y-A7) are compared to the BRET positive control (WT x *P*35s::L-Y) and a negative control expressing a LUC-construct only (*irx3*-P<sup>35S</sup>::*A7*-L). LUC and YFP abundance is average relative emission intensity units. MilliBRET is the average ratio of YFP to Luciferase emissions (x1000) after the addition of the Luciferase substrate, coelenterazine (note that milliBRET values are not calculated form the LUC and YFP abundance values, abundance is a separate test from BRET). *AtCesA7* (A7) transcript abundance is relative to *AtUBQ5*. Standard error of the mean is in parenthesis, n=8 except for transcript abundance where n=3.

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<tr>
<th>Line</th>
<th>Abundance</th>
<th>YFP abundance</th>
<th>MilliBRET</th>
<th>A7 transcript abundance</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT x <em>P</em>35S::L-Y</td>
<td>1091.2 (279.5)</td>
<td>110708.2 (16960.3)</td>
<td>1.18 (0.15)</td>
<td>0.15 (0.04)</td>
</tr>
<tr>
<td><em>irx3</em>-P35S::A7-L</td>
<td>40.0 (7.0)</td>
<td>1832.6(1100.2)</td>
<td>0.01 (0.03)</td>
<td>na</td>
</tr>
<tr>
<td><em>irx3</em>-P35S::A7-L x <em>PA7</em>:Y-A7</td>
<td>51.2 (8.7)</td>
<td>6587.8 (1343.7)</td>
<td>0.01 (0.03)</td>
<td>0.40 (0.00)</td>
</tr>
</tbody>
</table>
Figure 4.4. BRET measurements and transcript abundance in PA8::LUC-CesA8 x P35S::YFP-CesA7 seedlings. BRET measurements were made after the addition of the Luciferase substrate, coelenterazine, to seedling roots (A), cotyledons (B), and whole seedlings (C). Tissues of AtA8irx1-1 plants transformed with PA8::LUC-CesA8 and P35S::YFP-CesA7 (PA8::L-A8 x P35s::Y-A7) are compared to the positive control (P35S::L-Y) and the negative control (P35S::L-A8) plants and tissues. (D) Relative transcript abundance of AtCesA7 and AtCesA8 transcripts in PA8::L-A8 x P35s::Y-A7 seedlings compared to wild-type (WT) and AtA8irx1-1 seedlings. Transcript abundance is relative to that of AtUBQ5.

Seedlings for all tests were seven days old. Error bars are standard error of the mean, n=8 (A-C) or n=3 (D). Stars indicate statistical significance compared to the negative control (p<0.05, t-test).
AtCesA7 and AtCesA8 transcript levels were found to be significantly higher in whole seedlings of the A8\textsuperscript{irx1-1}-PA8::L-A8 x P35s::Y-A7 transgenic line, compared to the levels measured in untransformed wild-type and AtCesA8\textsuperscript{irx1-1} seedlings, suggesting that there was sufficient expression of the BRET constructs in the transgenic plants. Combined, these results suggest that a very weak or transient interaction between CesA7 and CesA8 might occur in seedlings.

4.3.1.4 CesA7-CesA4 interactions

Crossing of AtCesA7\textsuperscript{irx3-1}-PA7::YFP-A7 plants with AtCesA4\textsuperscript{irx5-1}-PA4::LUC-A4 plants produced double transgenic plants carrying both PA7::YFP-A7 and PA4::LUC-A4 constructs. As seen in Table 4.3, LUC and YFP emission intensities varied greatly among the transgenic lines. In particular, the LUC signal is quite low in all crosses but this is likely the result of low expression in the AtCesA4\textsuperscript{irx5-1}-PA4::LUC-A4 parental line. YFP-CesA7 signal was visible in the crossed lines, and resembled that of the original parent line (data not shown). Although there were no statistically significant differences between the BRET ratios of crossed plants and the negative control, the crossed lines showed a general trend of higher BRET in stem tissue compared to the negative control (Figure 4.5-A). Additionally, weak BRET interactions were also detected in seedling tissue. The variation is quite large in all samples evaluated, illustrating the difficulty in accurately measuring live protein-protein interactions \textit{in vivo}. Large variation could also be an indication of transient or unstable interactions. It must be noted that the double transgenic plants recovered from the AtCesA7\textsuperscript{irx3-1}-PA7::YFP-A7 X AtCesA4\textsuperscript{irx5-1}-PA4::LUC-A4 cross were not selected for A7\textsuperscript{irx3-1} and A4\textsuperscript{irx5-1} homozygosity. Therefore, the transgenic plants could express a combination of endogenous wild-type, transgenic, and \textit{irx} CesA4 and A7 proteins, which could impact BRET interactions and intensity.

4.3.1.5 CesA8-CesA8 interactions

Several lines of transgenic wild-type (Landsberg) plants were recovered from a double transformation with two CesA8-BRET constructs: PA8::LUC-AtCesA8 and PA8::YFP-AtCesA8. Although LUC and YFP signals were detected in stem tissues of these transgenic lines, there was no BRET signal to indicate interaction between the
tagged CesA8 subunits (Figure 4.6-A). However, when BRET ratios were calculated over time in seedlings of one of the double transgenic lines a weak BRET ratio was detected that was significantly greater than the negative control at more than 50% of the time points measured (Figure 4.6-B). These results imply a transient interaction between CesA8 subunits, one that might be stronger in seedlings than in stem tissue.
Table 4.3. LUC and YFP emissions of LUC-A4 x YFP-A7 transgenic plants. Transgenic plants are compared to the \textit{P35S::LUC-YFP} positive control ((P35S::L-Y) and the parental lines used to create the double transgenic plants (PA4::L-A4 and PA7::Y-A7). Double transgenic plants carry both the \textit{PA4::LUC-AtCesA4} and \textit{PA7::YFP-AtCesA7} constructs, and offspring from reciprocal crosses are shown (AtCesA4xA7 compared to AtCesA7xA4). Values represent relative intensity units, standard error of the mean in parentheses. Colouration is to highlight the different groups of plants tested (white – controls, light grey – crosses, dark grey-reciprocal crosses).

<table>
<thead>
<tr>
<th>Line</th>
<th>LUC emission</th>
<th>YFP emission</th>
</tr>
</thead>
<tbody>
<tr>
<td>P35S::L-Y</td>
<td>14728 (4096)</td>
<td>41502 (4903)</td>
</tr>
<tr>
<td>PA4::L-A4</td>
<td>17 (12)</td>
<td>-5000 (1880)</td>
</tr>
<tr>
<td>PA7::Y-A7</td>
<td>-3 (12)</td>
<td>4023 (393)</td>
</tr>
<tr>
<td>A4 x A7 #1</td>
<td>43 (11)</td>
<td>3506 (388)</td>
</tr>
<tr>
<td>A4 x A7 #4</td>
<td>19 (3)</td>
<td>1912 (689)</td>
</tr>
<tr>
<td>A4 x A7 #12</td>
<td>27 (8)</td>
<td>1882 (244)</td>
</tr>
<tr>
<td>A4 x A7 #14</td>
<td>76 (15)</td>
<td>2797 (635)</td>
</tr>
<tr>
<td>A7 x A4 #1</td>
<td>23 (5)</td>
<td>1753 (288)</td>
</tr>
<tr>
<td>A7 x A4 #3</td>
<td>51 (9)</td>
<td>2218 (419)</td>
</tr>
<tr>
<td>A7 x A4 #5</td>
<td>20 (6)</td>
<td>2828 (671)</td>
</tr>
</tbody>
</table>
Figure 4.5. BRET measured in stem tissue and seedlings of double transgenic plants carrying \textit{PA4::LUC-CesA4} and \textit{PA7::YFP-CesA7}. MilliBRET values of the \textit{P35S::LUC-YFP} positive control (L-Y) the \textit{PA4::LUC-A4} negative control (L-A4), and the crossed lines expressing LUC-A4 and YFP-A7 are presented. (A) BRET measured in stem cross sections. “C” and “A” lines are LUC-A4 x YFP-A7 plants. (B) Measurements from seedlings of the C14 line (LUC-A4 x YFP-A7). Error bars are standard error of the mean, n=8 (A-C) or n=3 (D).
Figure 4.6. BRET measurements in AtCesA8xA8 double transgenic plants. Plants expressing PA8::LUC-A8 and PA8::YFP-A8 (L-A8 x Y-A8) were compared to the P35S::LUC-YFP positive control (P35S::L-Y or LY) and the PA8::LUC-A8 negative control (PA8::L-A8 or L8). (A) BRET in maturing stem tissue from six individual L-A8 x Y-A8 lines (#21-27). (B) BRET measured over time (after substrate addition) in seven-day-old seedlings. Error bars are standard error of the mean, n=16 except for PA8::L-A8 (n=8). Stars in B indicate statistical significance compared to PA8::L-A8 (p<0.05, ANOVA followed by a Tukey’s post-hoc test).
4.3.2 BiFC in transiently transformed tobacco epidermal cells

The BiFC assay was employed in attempts to visually detect CesA-CesA interactions in planta. Leaves from five-week-old *Nicotiana tabacum* were transiently transformed with combinations of N-YFP-CesA and C-YFP-CesA constructs. If the two tagged proteins interact it would be possible for the N-YFP and C-YFP fragments to reconstitute a functional YFP protein, which can then be visualized using fluorescence microscopy. To verify that YFP-tagged AtCesAs could be visualized in transiently transformed leaf tissue, leaves were infiltrated with YFP-CesA7 and YFP-CesA4 constructs. It was determined that YFP-CesA signal could not be detected after 24 hours, but that 48 to 65 hours post-infiltration was an optimal time to visualize signal (Figure 4.7). In transformed leaf cells, the YFP-CesA signal appeared as bright, large, puncta that moved along the edges of the cell. Much of the signal at cell edges is thought to be due to vacuole displacement. Additionally, most of the movement was thought to be due to cytoplasmic streaming. This pattern of fluorescence is similar to that of YFP-CesA7 in leaf tissue of *Arabidopsis* (data not shown). As expected with localized infiltration of the tissue, fluorescently labelled cells were found in clusters surrounding the site of infiltration.

After infiltration with BiFC-tagged combinations of *AtCesAs*, leaf tissue was scanned for fluorescence as an indication of CesA-CesA interactions. In general, fluorescence observed in the BiFC tests was not greater than the background signal observed in negative controls (Figure 4.7 E-L). However, some weak fluorescence potentially indicating interaction was observed in the CesA4-homodimerization test (Figure 4.7, I-J), and the CesA8-homodimerization test (Figure 4.7, K-L). No signal above background was observed in the CesA7-homodimerization test or any of the AtCesA-heterodimer tests (e.g.: A4xA8, A4xA7).
**Figure 4.7.** BiFC assays in leaf epidermal cells of *Nicotiana tabacum*. YFP-fluorescence images are paired with the corresponding bright field image. Images were taken at 10x magnification (A, C, E, G, I, K) and 63x magnification (B, D, F, H, J, L). YFP-CesA7 (A-B) and YFP-CesA4 (C-D) were used as positive controls. Leaves infiltrated with the two BiFC constructs (P35S::N-YFP x P35S::C-YFP) were used as a negative control (E-F). CesA homodimer combinations are presented in panels G-L: CesA7xCesA7 (G-H), CesA4xCesA4 (I-J), and CesA8xCesA8 (K-L).

Scale bars represent 50 µm (A, C, E, G, I, K) and 50 um (B, D, F, H, J, L).
4.4 Discussion

4.4.1 Putative CesA homodimers and heterodimers detected in planta using BRET and BiFC

The BRET results presented herein suggest there could be interactions between CesA8-A8 in seedlings, and potential interactions between CesA7 and CesA4 (strongest in stem), and CesA7 and CesA8 (seedlings). BiFC results suggested homodimerization of CesA8, and potentially CesA4, but not CesA7, although all positive BiFC results were weak. Together these results suggest there may be interactions between some of the CesAs in vivo, but they are difficult to detect.

One limitation of the BRET and BiFC assays performed in this study is the potential interference of endogenous CesAs with the BRET- and BiFC-tagged CesAs. Previous experiments demonstrated that the YFP-AtCesA7, LUC-AtCesA4, and LUC-AtCesA8 fusions were functional in planta, based on full or partial complementation of AtCesA4irx mutant phenotypes by expression of these fusion proteins (Chapter 2). When YFP-AtCesA7 was expressed in AtCesA7irx3-1 mutants, under the expressional control of the native AtCesA7 promoter, the mutant A7irx3-1 phenotype was completely rescued, based on plant stature and the absence of collapsed xylem (Figure 2.1, Chapter 2). These results suggested that the YFP-AtCesA7 fusion protein was functional and thus likely accessing the CSCs and rosettes similar to wild-type AtCesA7 subunits. When LUC-AtCesA4 and LUC-AtCesA8 were expressed (under expressional control of the native AtCesA4 and AtCesA8 promoters) in the A4irx5-1 and A8irx1-1 mutant plants, respectively, only partial complementation of the mutant phenotype was achieved (Figure 2.1, Chapter 2). Partial complementation is likely due to low transgene expression in the case of LUC-A4 in A4irx5-1 plants. Partial complementation of the A8irx1-1 mutant phenotype by LUC-AtCesA8 is likely the result of interference of mutant AtCesA8 subunits with LUC-AtCesA8 subunits. As discussed in Chapter 2, the non-null nature of the A8irx1-1 mutation likely results in production of mutant AtCesA8 subunit, which would therefore compete with LUC-AtCesA8 for positions within the CSC and rosette. For this reason, despite partial complementation, it is believed that the LUC-AtCesA4 and LUC-AtCesA8 fusions are functional in planta. It has not been
overlooked, however, that partial complementation could also signify that tagged-CesA4 and CesA8 do not function at wild-type levels. Additionally, the interference that may result from the expression of two tagged-CesAs in one plant may also hinder function.

Some double-transgenic plants were created for BRET, but they were not always in a double-CesA mutant background. For example, the AtCesA8<sup>irx1-1</sup> mutants expressing LUC-A8 and YFP-A7 would contain endogenous CesA7 proteins, as well as mutant CesA8 proteins (Taylor et al., 2003), which could have competed with the LUC- and YFP-fused CesAs for positions within the CSC. Such interference would reduce any potential BRET signal. The same interference could occur in tobacco leaf cells, if AtCesAs are capable of interacting with NtCesAs. Also, expression of two AtCesAs in tobacco leaf could result in interactions that are not normally present in <i>Arabidopsis</i> due to the fact that only two of the three AtCesAs required for CSC formation are present in the transiently transformed leaf cells. These limitations must be considered if future BRET or BiFC experiments are to be performed to study CesA-CesA interactions.

<em>In vitro</em> assays performed by others have suggested that interactions occur between all of the secondary cell wall AtCesAs (A4, A7, and A8), although there are some conflicting results. Homodimerization of CesA4, A7, and A8, and heterodimerization between A4-A7, A7-A8, and A8-A4 has been reported using epitope-tagging purification and anti-CesA antibody labelling (Atanassov et al., 2009) and immunoprecipitation followed by anti-CesA antibody labelling (Taylor et al., 2000; Taylor et al., 2003). The proposed formation of AtCesA7 homodimers (Atanassov et al., 2009) does not agree with the yeast-two hybrid results of Timmers et al. (2009), and BRET and BiFC results presented here that suggested a lack of homodimerization of AtCesA7. Compared to the BiFC results of Timmers et al. (2009), our results suggest only very weak interactions, and fewer interactions. There were three key differences between the BiFC assay performed in this work, compared to that of Timmers et al. (2009): 1) here, 5-week-old leaves were infiltrated, whereas they used younger, 3-week-old tissue; 2) here, BiFC signal was measured 48 and 65 hours post-infiltration, whereas they looked at tissue 72-hours post-infiltration; 3) they included the start codon within the cloned and subsequently tagged CesAs, whereas herein we eliminated the start codon for the N-tagged fusions. These differences appear subtle, but could
explain some of the varied results. For example, the younger tissue used by Timmers et al. (2009) may have facilitated higher protein expression, thus enabling greater detection of interactions after infiltration.

Conflicting results regarding CesA-CesA interactions could also be attributed to the system employed to study interactions. *In vitro* systems are clearly limited by a lack of simulation of the natural environment in which proteins would interact. Perhaps there are other mechanisms *in planta* that regulate when, where, and if CesAs interact and that these are limited in *in vitro* systems. An interaction observed *in vitro* suggests that two proteins can physically interact but not necessarily that they always do interact *in planta*. As identified here there were clear differences between expression patterns among tissues. It is highly possible that CesA interactions vary temporally and spatially, which adds to the complexity of studying interactions using an *in vivo* system. Transient or unstable interactions could also be difficult to measure *in vivo*.

Interactions could also be affected by post-translational modifications. For example, the phosphorylation of serine residues within the N-terminal variable region appears to target CesA7 protein for degradation (Taylor et al., 2007). In fact, excess CesA7 produced in a transformed *AtCesA7*<sup>lux3</sup>-1 mutant line (over-expressing a tagged CesA7) was found to result in phosphorylation and subsequent degradation of the excess CesA7, likely as a mechanism to regulate the levels of active CesA7 or remove CesAs that are not part of a CSC (Taylor et al., 2007). Post-translational modifications may hamper the ability to measure CesA-CesA interactions by BRET in stably transformed *Arabidopsis* plants. If a tagged form of a CesA protein is not optimal for CSC formation and cellulose production, it is possible that the tagged-CesA will be degraded or prevented access to the CSC, thus reducing the potential BRET signal. Atanassov et al. (2009) noted that tagging of AtCesA4 and AtCesA8 did not result in fully functional CesAs (whereas tagging of AtCesA7 produced a functional enzyme). Some of the weak interactions detected and reported here could be the result of reduced function of tagged CesAs, and reduced production and placement of tagged-CesAs within functional CSCs.
4.4.2 Modifications to the CSC and rosette models

Current models propose that cellulose microfibrils are produced by a rosette terminal complex (or rosette) which may be composed of six lobes (six cellulose synthesizing complexes, CSCs), each of which may contain six individual CesA proteins (Scheible et al., 2001; Doblin et al., 2002). It has been suggested that that each CSC contains three types of CesA: $\beta$, $\alpha_1$, and $\alpha_2$, and that there are unequal ratios of each CesA (Doblin et al., 2002; Ding and Hemmel, 2006), as depicted in Figure 4.8A. Based on the proposed six-CesA composition of CSCs, and the idea that CSC-CSC interactions occur to form a rosette, Doblin et al. (2002) postulates that a CSC could contain three $\beta$-CesA subunits, two $\alpha_2$-CesA subunits, and one $\alpha_1$-CesA subunit, and that CesA-interactions within the CSC could consist of $\beta$-$\alpha_1$, $\beta$-$\alpha_2$, and $\alpha_2$-$\alpha_2$. Ding and Hemmel (2006) propose fewer interactions within a CSC, only $\alpha_1$-$\beta$ and $\alpha_2$-$\beta$, but that $\beta$-$\beta$ interactions may exist between CSCs to form a rosette.

Both models, then, only account for three CesA-CesA interactions: two heterodimers and one homodimer. Interaction studies (presented here and in the literature) do not agree with these models if it is assumed that each CesA is one of either $\alpha_1$, $\alpha_2$, or $\beta$ subunits, and that all three are required in a CSC. The in vivo results from Timmers et al. (2009) and presented herein suggest five possible interactions: A4-A4, A8-A8, A4-A7, A4-A8, and A7-A8. Thus, if the rosette models hold true, then more than one CesA must be able to fill the proposed $\beta$, $\alpha_1$, and $\alpha_2$ positions (Figure 4.8 B). Recently, a new model proposed by Carpita (2010) suggests that CesAs form dimers to produce a single glucan chain and dimer-dimer interactions also occur for CSCs and rosettes to form. This model may account for the large variety of interactions currently reported, using both in vitro and in vivo methods.
Figure 4.8. CSC composition revisited. Models of the CesA-composition of CSCs have been proposed (Ding and Hemmel, 2006; Doblin et al., 2002) suggesting combinations of CesA-CesA interactions within a CSC and between CSCs in a rosette (A). Here, it is proposed that CSC composition may be somewhat flexible; that a pool of CesA proteins could somewhat randomly associate to form complete CSCs during secondary cell wall formation (B). As such, it would be expected that a mixed population of CSCs may exist, and therefore CesA-CesA interactions will vary, perhaps depending on tissue type and developmental stage.

Models in A are based on those of Ding and Hemmel (2006), and Doblin et al. (2002).
AtCesA4 and A8 seem like suitable candidates to fill the $\beta$ position, as they appear to form homodimers and interact with all other CesAs. Perhaps A7 could fill the $\alpha_1$ position which does not form homodimers in the current models. It is possible that the designations change depending on cell type and developmental stage (Ding and Hemmel, 2006). However, it still remains to be resolved if CesA-CesA interactions are specific and, if so, if the specificity is required for proper cellulose biosynthesis. Perhaps the composition of the CSC is somewhat flexible, such that they can form by different combinations of CesA-CesA interactions. This would mean that CesA-CesA interactions are required for CSC formation (and possibly rosette formation), and function, but that the specificity of interactions is not a limiting factor for CSC formation. The CesA interactions within a CSC may therefore be more random than specific, and the composition of CSCs could vary greatly spatially and temporally within a plant.

The use of BRET and BiFC has provided some insight into CesA-CesA interactions in vivo, but has also generated more questions about the composition of CSCs. There is still a great deal of work required to clearly elucidate what interactions occur, if they are tissue- and development-specific, and the nature of the interactions. Does CesA8 always form homodimers? Do these homodimers then interact with CesA4 and CesA7? Optimization of the BiFC assay utilizing Arabidopsis double CesA mutants to avoid interference from endogenous CesAs will likely provide a great deal more information than has been achieved using BiFC in tobacco, or BRET in Arabidopsis.
CHAPTER 5  Conclusion

5.1 Thesis summary

In recent years, a substantial amount of information regarding the genes and proteins involved in cellulose biosynthesis of both the primary and secondary cell walls has become available in a variety of plants including trees, particularly poplar. From mutant analysis, it appears that AtCesA1, A3, and A6 are key CesA subunits involved in primary cell wall cellulose biosynthesis (Arioli et al., 1998; Scheible et al., 2001; Fagard et al., 2000). Additionally, AtCesA2 and A5 may be active, partially redundant forms of AtCesA6 (Desprez et al., 2007; Persson et al., 2007b). The roles of AtCesA10 and AtCesA9 are less well characterized, although AtCesA9 may play a role in secondary cell wall formation in seed coats (Stork et al., 2010). We know that a mutation in any one of AtCesA4, A7, or A8 causes aberrant cellulose production and secondary cell wall structure (Taylor et al., 2000; Taylor et al., 2003; Gardiner et al., 2003; Taylor et al., 2004), resulting in cell wall defects that presumably prevent xylem vessels from withstanding the pressure of water transport resulting in collapse, and potentially weakening the walls of supportive fibre cells in the stem (Zhong et al., 2003). However, the precise mechanism by which the cellulose synthase proteins and the cellulose synthesizing complex (CSC) affect cellulose properties such as abundance, crystallinity, microfibril angle, and degree of polymerization are still unknown.

From broadening our understanding of how plants regulate the inherent properties of cellulose in secondary cell walls, to the application for transgenic modifications of cellulose for downstream industrial applications, it is of interest to understand how the individual CesA protein subunits interact to form a fully functioning CSC and rosette, and ultimately how the CSC composition influences cellulose properties. This was the focus of the research presented in Chapter 2. The results obtained provided some support for our hypothesis, that each AtCesA has a unique role in cellulose production that could be manifested in altered cellulose properties, and additionally that the presence of a CSC influences cellulose ultrastructure independently of the abundance of cellulose biosynthesis.
The diversification of CesA genes into primary cell wall-specific or secondary cell wall-specific may have emerged prior to the divergence of gymnosperms from angiosperms. If this were true, it might be expected that CesA orthologs would have conserved functions. *In planta* evidence to suggest conservation of function between CesA orthologs has, however, not been determined. Elucidation of functional conservation may also lend information to understanding the composition of CSCs. Identifying CesAs from spruce and poplar, and determining if they are truly orthologous and have conserved functions with *Arabidopsis* was precisely the goal of the research presented in Chapter 3, and the results obtained supported our hypothesis: isolated spruce genes share similar conserved domains, exhibit the highest gene expression in tissues undergoing significant secondary cell wall formation, and both spruce and poplar CesAs are functionally orthologous to the *Arabidopsis* counterparts.

Based on phylogenetic analysis, there appears to be a great deal of conservation between *Arabidopsis* CesAs and those from trees such as poplar, *Eucalyptus*, and even gymnosperms such as pine and spruce. Thus, information obtained from studying *Arabidopsis* secondary cell wall formation may be useful for application in a tree model. Current models of CSC form and function do not address how or why multiple CesAs are required to synthesize normal cellulose. The capability of CesAs to interact as homodimers or heterodimers has been suggested by *in vitro* and *in vivo* experiments but further investigation using *in vivo* methods are required to accurately ascertain if specific CesA-CesA interactions occur in order for CSCs to form and functionally give rise to cellulose polymers. Studying such interactions (Chapter 4), however, only partially supported our hypothesis. We did observe that CesA homodimers and heterodimers may form, but were unable to determine confidently that the interactions are specific and consistent in particular tissues or during a defined developmental stage. However, the development of a system to visualize YFP-CesAs in live fibre cells suggested to us that revisiting the use of BiFC in *Arabidopsis* CesA mutant plants may provide a better model to study CesA-CesA interactions *in vivo*.

Combined, the results of the research presented in this thesis contribute to the current model of CSC composition and function.
5.2 A complete CSC influences the fundamental properties of cellulose

As presented in Chapter 2, the effects of the $AtCesA4^{irx5-1}$, $AtCesA7^{irx3-1}$, and $AtCesA8^{irx1-1}$ mutations go beyond decreased cellulose production. In addition to changes to cellulose properties (MFA, DP, crystallinity) the pleiotropy observed included changes to total growth, growth rate, and lignin and hemicellulose content. The secondary effects of altered cellulose, potentially on various developmental cues, appeared to vary depending on mutant phenotype severity and could be linked more specifically to the combined changes in cellulose crystallinity, DP, and MFA. Ultimately, the presence of a complete CSC appeared to dictate these fundamental cellulosic properties under long-day conditions. Our results suggest that a properly formed CSC does not have to contain all functional CesAs in order to produce cellulose with wild-type like properties. The phenotype of $AtCesA8^{irx1-1}$, then, can be solely attributed to reduced cellulose in secondary cell walls, and not aberrant cellulose structure. To that end, the more severe pleiotropy observed in the $AtCesA4^{irx5-1}$ and $AtCesA7^{irx3-1}$ mutants are likely a result of stronger feedback by other developmental processes in response to changes in cellulose properties, or simply in response to more deficient cell walls.

When a CSC is able to form even with a mutant CesA subunit, the resulting cellulose polymers are mostly of normal length (DP), the secondary cell wall is highly crystalline, and cellulose microfibrils have a normal MFA (under long day conditions). This strongly suggests that the purpose of the CSC, in addition to cellulose biosynthesis, is to facilitate a stable localized environment where newly synthesized glucan chains can crystallize. However, under long-day conditions, it does not appear that a high degree of crystallinity is correlated with a large DP. In contrast, it appears that the presence of AtCesA4 has a larger influence on DP than the presence of a fully formed CSC.

The link between cellulose MFA and other cellulose properties, however, is not quite clear. There are some suggestions that rate of movement of CSCs affects microfibril organization (Himmelspach et al., 2003; Wasteneys and Fujita, 2006) and thus influences MFA. It could also be that a fully formed CSC is required to interact with another protein or complex that dictates MFA. Disorganized orientation of microfibrils has been found in response to mutations in various proteins, all of which may affect MFA. These include kinesins (Zhong et al., 2002), COBRA (Roudier et al., 2005), and a
CesA-interacting protein (CSI-1) with unidentified function (Gu et al., 2010). In transgenic poplar with RNAi-suppressed KORRIGAN, cell wall crystallinity increased, and MFA was smaller, but a link between the two cell wall characteristics was not clearly defined (Maloney and Mansfield, 2010). Greater crystallinity (as found in wild-type and \( \text{AtCesA8}^{irx1-1} \)) may facilitate proper microfibril organization, and therefore a smaller MFA (as was found in wild-type plants grown under long day conditions). It is clear that the mechanisms that regulate cellulose MFA and DP require further investigation.

5.3 The function of AtCesA4, A7, and A8 orthologs is highly conserved

The ability of spruce and poplar AtCesA orthologs to complement the \( \text{AtCesA}^{irx} \) mutant phenotype suggests a very strong conservation of function among CesAs from evolutionarily divergent species. It is postulated that complementation of the \( \text{AtCesA}^{irx} \) lines is the result of meeting a threshold of functional CesA, which then permits CSC formation and sufficient cellulose biosynthesis. This is the first indication of inter-species CesAs capable of integrating with AtCesA subunits to form a CSC, permits normal cell wall formation as exhibited by normal growth phenotype and a non-collapsed xylem of the complemented lines. Additionally, this suggests that the CesA-specific functions are maintained, despite as low as 64% amino acid sequence similarity (PgCesA1 with AtCesA8).

There were subtle differences in the level of complementation observed, which may be attributed to slight differences in function among the orthologs. It was observed that even a low level of LUC-PgCesA1 transcript resulted in a complemented phenotype of \( \text{AtCesA8}^{irx1-1} \) plants. Conversely, low expression of the wild-type form of LUC-AtCesA8 or LUC-PtiCesA8-A in \( \text{AtCesA8}^{irx1-1} \) plants only partially complemented the mutant phenotype. This suggests that the LUC-PgCesA1 protein can readily access the CSC, or that the function of PgCesA1 is less affected by the LUC tag compared to LUC-AtCesA8 and LUC-PtiCesA8-A. Also, observing degrees of complementation could be a function of the level of CesA protein produced in the transgenic lines. Transcript abundance suggests some lines had elevated transgene expression, whereas others had low levels of gene expression, but how do these levels of transcript translate into active protein units? Post-translational modifications to CesAs, such as
phosphorylation (Taylor, 2007), may be at play, which could limit the amount of active AtCesA8 and PtiCesA8 in AtCesA8\textsuperscript{irx1-1} transgenic lines.

A combination of the mutant genotype and post-translational modifications targeting transgenic CesAs for degradation may explain why PtiCesA8-A only partially complemented AtCesA8\textsuperscript{irx1-1} but PtiCesA7-A and PtiCesA4 more easily rescued the \textit{AtCesA7}\textsuperscript{irx3-1} or \textit{AtCesA4}\textsuperscript{irx5-1} mutants, respectively (based on the observed number of complemented lines). As discussed previously, the AtCesA8\textsuperscript{irx1-1} mutant is believed to continue to synthesize mutant AtCesA8 proteins, whereas little to no AtCesA7 protein is produced in \textit{AtCesA7}\textsuperscript{irx3-1} mutants. Therefore, if there is a required threshold of CesAs in order for CSCs to form, and beyond that threshold CesA proteins are targeted for degradation, then the threshold will be more easily met in AtCesA8\textsuperscript{irx1-1} plants, whereas more transgenic CesA protein will be produced to meet the necessary CesA thresholds in \textit{AtCesA7}\textsuperscript{irx3-1}. Based on this threshold hypothesis, it is thus postulated that various populations of CSCs exist in the PgCesA1- and PtiCesA8-A- AtCesA8\textsuperscript{irx1-1} transgenic plants. The combinations of CSCs could be (using the PgCesA1 transgenic line as an example): CSC1: AtCesA4, AtCesA7, and non-functional AtCesA8. CSC2: AtCesA4, AtCesA7, PgCesA1. CSC3: AtCesA4, AtCesA7, non-functional AtCesA8, and PgCesA1.

Under the assumption that PgCesA1, PtiCesA8-A, PtiCesA7-A, and PtiCesA4 were properly integrated into CSCs within the transgenic plants, there are two generalized conclusions that can be made by these findings: The first is the structure of these AtCesA orthologs is sufficiently conserved to be integrated into the CSC. Although Kurek et al. (2002) determined that the zinc-binding domain of cotton CesA1 and A2 could form homodimers and heterodimers, Timmers et al. (2009) showed that mutations in conserved motifs of the zinc-binding domain did not eliminate CesA-CesA interactions \textit{in vitro}. Therefore, the zinc-binding domain may be sufficient but not essential for CesA proteins to putatively interact and become part of a CSC. This may be why PgCesA1, which shares about 40% similarity with the AtCesA8 N-terminal region zinc binding domain, is able to become part of the CSC. It was demonstrated with AtCesA1-AtCesA3 chimeric proteins that the catalytic domain was sufficient to permit entry into the CSC (Wang et al., 2006) suggesting that this region could be involved in CesA-CesA interactions required for CSC formation. Secondly, our results
indicate that the catalytic function and role within the CSC of these AtCesA orthologs is highly similar to that of AtCesA8, thus allowing for normal cell wall formation to occur.

This is the first report of functional conservation between CesA orthologs, one which suggests that CesA form and function has not diverged significantly since putative CesA-specific functions evolved, which likely occurred prior to the evolutionary split of angiosperms and gymnosperms.

5.4 **What is the ratio of CesAs within the CSC?**

The inability of CesA4, A7, and A8 to complement one another when one is mutated suggests that each CesA protein subunit has a unique function. It could be that a ratio of each CesA is required for proper CSC form and function, or that each CesA has a unique function in cellulose biosynthesis or dictating cellulose properties, or both. The alterations to cellulose structure in *AtCesA4*<sup>irx5-1</sup> and *AtCesA7*<sup>irx3-1</sup> plants were not always similar, despite both being null-mutant lines (Chapter 2). The present results suggest that the AtCesA7 enzyme may influence cellulose crystallinity more so than the other CesAs, and that AtCesA4 could have a unique role in producing long cellulose chains. The ability of a spruce and poplar CesA to take the place of AtCesA8, and poplar CesAs to substitute AtCesA7 and AtCesA4 suggests that CesA-specific functions for the AtCesA8, A7, and A4 orthologs are highly conserved. Thus, it seems possible that the following scenarios could be correct: a CSC must contain all three CesAs, the ratio of which is somewhat flexible, and that the uniqueness of each CesA is due to both interactions they facilitate and the cellulose properties they dictate.

Interestingly, Song *et al.* (2010) recently reported that in poplar xylem there are CSCs which putatively contain five different CesA subunits: PttCesA7-A, PttCesA7-B, PttCesA8-A, PttCesA8-B, and PttCesA4. This contradicts the previous dogma that implied a three-CesA-CSC, given that the A and B isoforms are so similar. However, these results could also support the idea that a flexible ratio of CesAs is required for CSC formation and function, one which can be met by three or more CesAs.

Various CSC models have been proposed, classifying the CesAs within the CSC depending on their interactions (Doblin *et al.*, 2002; Ding and Hemmel, 2006). Interaction studies suggest more possibilities than the current CSC model, and therefore if these models hold true, then redundancy in the CesA must exist (to fill the
\( \alpha_1, \alpha_2, \) and \( \beta \) positions, see Chapter 4, and Figure 4.8). Based on the CesA-CesA interactions putatively detected using the BRET and BiFC assays (Chapter 4), as well as those reported by Timmers et al. (2009), it is believed that AtCesA4 and A8 are suitable candidates to occupy the \( \beta \) position, as they appear to form homodimers and interact with all other CesAs. Perhaps CesA7 could fill the \( \alpha_1 \) position, which does not appear to form homodimers in the current models. However, this contradicts the idea that AtCesA7 is required at higher levels in the CSC than the other CesAs (Chapter 2), compared to the models that indicate lower levels of \( \alpha_1 \) compared to \( \alpha_2 \) and \( \beta \). Is it possible that CesAs can exist in the CSC without interacting with other CesAs? Clearly, there is a need to further investigate the composition of the CSC, and how CesA-CesA interactions are related to CSC composition.

5.5 Should the cell wall-specific classifications of CesAs be revisited?

It is clear from mutant studies that CesA4, A7, and A8 affect cellulose biosynthesis of secondary cell walls. Additionally, mutant studies have determined that AtCesA1, A3, and A6 (and the potential A6 redundancies A2, A5, and A9) have a clear role in primary cell wall cellulose biosynthesis. However, results from some of the work presented in this thesis, and observations by other groups suggest that there could be some dual functionality, suggesting that our current model of primary or secondary wall-specificity are not fully representative of the roles of CesAs in cellulose biosynthesis. Dual functionality may be subtle, in some cases, but it begs the question: do the roles of CesAs need to be re-evaluated?

From this thesis, observations of YFP-AtCesA7 expression \textit{in vivo} revealed a diverse (in terms of tissue type) expression pattern, including expression in primary cell-walled cells (Chapter 2). The YFP-AtCesA7 fusion was under expressional control of the native \textit{AtCesA7} promoter, thus expression should be temporally and spatially precise with respect to native developmental cues and events. Additionally, the fusion protein was expressed in the \textit{AtCesA7}\textsuperscript{irx3-1} mutant background, so there would be no competition with endogenous, functional CesA7 proteins. Therefore, the expression patterns observed are believed to be a true representation of where the CesA7 protein is normally present. In support of our observations, Bosca et al. (2006) also reported a potential role for CesA7 in primary cell walls. The authors identified an \textit{AtCesA7} mutant
plant, *mur10*, based on the changes to primary cell wall carbohydrates in leaves. This questioned whether the primary wall changes were a secondary effect of the *AtCesA7* mutation, or if *AtCesA7* has an active role in cellulose biosynthesis of primary cell walls. As reported in this thesis, the YFP-*AtCesA7* was found in primary-walled cells in roots, cotyledons, mature leaves, and stems. Compared to *AtCesA4* and *A8*, *AtCesA7* shares a higher degree of similarity to moss (*Physcomitrella patens*) CesAs, (Roberts and Bushoven, 2007). This may be an indirect indication that *AtCesA7* is less divergent compared to the other secondary cell wall-specific CesAs, perhaps retaining some function in primary cell walls.

Dual functionality in both primary and secondary cell walls may also be true for *AtCesA4* and *AtCesA9*. Under the expressional control of the native *AtCesA4* promoter, a LUC-YFP fusion protein was observed to be highly expressed in cotyledons (non-vein tissue), mature leaves, and primary walled tissues in the stem, although to a lesser degree compared to the YFP-*AtCesA7* fusion (Chapter 4). Also, *AtCesA9*, previously classified as primary wall-specific (Persson et al., 2007b; Desprez et al., 2007) was recently identified as a CesA required for secondary cell wall biosynthesis in seed coats (Stork et al., 2010). Is *AtCesA9* functioning with other primary wall-specific CesAs to produce secondary cell walls in seed coats? Persson et al. (2007b) hypothesized that CesA9, A1, and A3 form the functional CSCs in floral organs and pollen. In hybrid poplar, Song et al. (2010) isolated CSCs from developing xylem and found that both primary and secondary cell wall-specific CesAs were expressed. CesA-interactions reported in large complexes isolated by immunoprecipitation do not support the conclusion that combinations of primary and secondary cell wall CesAs interact (Atanassov et al., 2009; Wang et al., 2008; Song et al., 2010). If CesAs do have dual functionality in both primary and secondary walls, it could be that combinations of primary and secondary CSCs are present, as suggested by Song et al. (2010). Further investigation into the expression patterns of all CesAs (particularly using fluorescent tagging), and perhaps more in depth analysis of CesA-mutant phenotypes, may shed light on whether or not CesAs have overlapping functions in both primary and secondary cell wall cellulose biosynthesis.
5.6 Critical comments and future work recommendations

5.6.1 Understanding the secondary effects of AtCesA4\textsuperscript{irx5-1}, A7\textsuperscript{irx3-1}, and A8\textsuperscript{irx1-1} mutations

The reduced stature phenotype of the AtCesA\textsuperscript{irx} mutants, identified in Chapter 2, strongly suggests that there are secondary effects of these mutations on primary growth. In an effort to more clearly elucidate the role of CesAs in primary growth, or to clarify if the effects measured are purely a secondary response, it would be interesting to identify the physiological reasons for reduced growth in the mutants. Using light microscopy could provide images of the cellular structure in these mutants and, as such, may offer a means to precisely measure the cellular dimensions in elongating stem tissues, to clarify if reduced stature is the result of fewer cell divisions of if the cells produced undergo less longitudinal expansion.

5.6.2 Investigate CSC composition and function more thoroughly

It has not been overlooked that there are additional AtCesA4, A7, and A8 mutants that could be studied to determine if the changes in cell wall composition are affected by the type of genetic mutation. In particular, it would be interesting to study the effects of a null-AtCesA8 mutation, such as AtCesA8\textsuperscript{low2-1} (Chen et al., 2005) or AtCesA8\textsuperscript{irx1-5} (Brown et al., 2005) and determine how it compares to the non-null mutant presented herein and additionally with the null AtCesA4\textsuperscript{irx5-1} and AtCesA7\textsuperscript{irx3-1} mutants. In addition, I am curious to know if non-null mutations in AtCesA4 and A7 would produce a similar phenotype as the AtCesA8\textsuperscript{irx1-1} mutant. Under the assumption that CSCs form in AtCesA8\textsuperscript{irx1-1}, but have reduced cellulose biosynthesizing capacity, one might assume that an incomplete knockout of either AtCesA4 or AtCesA7 should have a similar phenotype as the AtCesA8\textsuperscript{irx1-1} mutants for the characteristics that are greatly affected by CSC structure, such as MFA. However, if the role of each CesA is different in the CSC, or if unequal ratios of each CesA are required for CSCs to form and function, the severity of the mutant phenotype in non-null A4 and A7 mutants might vary.
To better understand the effect of AtCesA4, A7, and A8 mutations on CSC form and function in secondary cell wall biosynthesis it would be interesting to compare the abundance and movement of CSCs in the various mutant backgrounds by utilizing YFP-CesA fusions. However, directly tracking the movement of CSCs in secondary cell wall forming tissues such as xylem or fibres, *in planta*, has not been possible due to interference from surrounding cells. Thus, it would be advantageous to utilize a system that could permit direct detection, such as that described by Yamaguchi *et al.* (2010). This system allows for cells in whole *Arabidopsis* plants to be induced to trans-differentiate into xylem vessels, including leaf and root epidermal cells, which would help eliminate interference. If this system could be applied to null-CesA mutant plants then it would be possible to study the effects of a CesA mutation on CSC patterning and movement. For example, the *AtCesA7*<sup>irx3-1</sup>-YFP-*CesA7* line crossed with *AtCesA4*<sup>irx5-1</sup> to produce a double mutant, but complemented with YFP-CesA7 so only one CesA is non-functional. These double-mutant-YFP-CesA7 plants could then be used to create the xylem trans-differentiation system, and subsequently used to determine CSC movement and patterns. Do CSCs move more slowly when one CesA is non-functional? Are they less abundant in the plasma membrane? Answers to these questions may provide information about the reasons for aberrant cellulose produced in CesA-mutants.

The establishment of a xylem-inducible system would also be useful for documenting CesA interactions. Although BiFC results presented herein and by Timmers *et al.* (2009) have been informative, it cannot be overlooked that the interactions observed could be confounded by 1) expression of AtCesAs in tobacco that contain functional, endogenous CesAs, which could interfere with AtCesA-AtCesA interactions; and 2) expression of secondary cell wall-specific AtCesAs in primary cell wall cells. Therefore, we cannot confidently assume that the interactions observed *in vivo* thus far are representative of those that occur in secondary cell wall synthesizing cells such as xylem vessels and fibres.

Alternatively, BiFC could be conducted and observed in *Arabidopsis* stem tissue. Based on the observation of YFP-CesA7 in longitudinal sections of mature and developing stem (Chapter 2) I suggest that a CesA-BiFC system be set up utilizing *AtCesA*<sup>irx</sup> mutant backgrounds. This would allow for interactions between CesAs to be
visualized *in planta*, in appropriate tissues, over developmental stages, and without interference from endogenous wild-type proteins. Additionally, such a system could be expanded to visualize the interactions between CesAs and other candidate proteins such as CSI-1, SUSY, and COBRA, for example.

### 5.6.3 Broaden our understanding of conservation of function among CesAs

From the results presented in Chapter 3 it is clear that PgCesA1 and poplar CesAs are functional orthologs of *Arabidopsis* CesA proteins. In order to determine the extent of functional conservation it would be worthwhile determining the cell wall properties of the *PgCesA*- and *PtiCesA*-complemented plants, and comparing them to the properties of *AtCesA*-mutant lines (Chapter 2). If the PgCesA and PtiCesA subunits are functioning identically to the orthologous AtCesA subunits, then wild-type like cell wall properties would be expected in complemented lines. The complementation results presented herein also elicit questions about how easily *AtCesA*\textsuperscript{irx} mutants can be complemented with other orthologous, and even non-orthologous CesAs. If the ability to complement is tightly linked to meeting required thresholds of CesA protein, then it could be hypothesized that orthologous and even non-orthologous CesAs could complement the mutant phenotype if they were temporally and spatially expressed at appropriate levels.

It was not resolved if PgCesA2 and A3 are functional orthologs of the secondary cell wall CesAs of *Arabidopsis*. Further attempts to transform the *AtCesA*\textsuperscript{irx} plants with PgCesA2 and A3 would be recommended. Also, it would be worthwhile to identify additional members of the *PgCesA* genes family to enable a more accurate comparison of homologous sequences.

### 5.7 Final conclusions and relevance

Understanding the cellular mechanisms of cellulose biosynthesis has been a challenging area of research since the first identification of rosettes, CSCs, and *CesA* genes. This is particularly true of the CesAs involved in the synthesis of cellulose in the secondary walls, which have the added complexity and limitations of visualization.
The work presented in this thesis further supports the complexity of the relationship between CesAs, CSCs, and the cellulose product.

The key findings of this thesis are as follows:

1. \textit{AtCesA}^{irx} mutants provide an invaluable system to study the role of each CesA in cellulose biosynthesis (Chapter 2).
2. A fully-formed CSC influences cellulose properties such as MFA (Chapter 2).
3. Day-length affects MFA in \textit{Arabidopsis}
4. Longitudinal stem sections are a good system for visualizing the expression pattern of YFP-fused CesAs and measuring CesA-CesA interactions (Chapter 2, 4).
5. The newly isolated spruce CesA genes \textit{PgCesA1, A2, and A3} are likely involved in secondary cell wall cellulose biosynthesis (Chapter 3).
6. \textit{PgCesA1} and poplar PtiCesA8-A, PtiCesA7-A, and PtiCesA4 are functionally orthologous to \textit{AtCesA8, A7, and A4}, respectively (Chapter 3).
7. CesA-CesA interactions occurring \textit{in vivo} may include homodimers of CesA8 and CesA4, but not CesA7, CesA4-CesA7 heterodimers in stem tissue, and CesA8-CesA7 heterodimers in seedlings (Chapter 4).

From these findings, it is speculated that:

8. \textit{AtCesA7} has a greater presence in CSCs, affecting cellulose crystallinity and abundance (Chapter 2, 3)
9. \textit{AtCesA4} may influence cellulose degree of polymerization.
10. The composition of the CSC may vary depending on tissue and developmental stage, such that the CesA-CesA interactions vary (Chapter 2, 3, and 4).

The conclusions drawn from this thesis have enhanced our understanding of cellulose biosynthesis and the information herein can be used to guide new research questions in the pursuit of elucidating how CesAs and CSCs are intricately involved in cellulose biosynthesis.
REFERENCES


Appendix A: AtCesA gene expression in mature stem tissue of Arabidopsis thaliana plants.

Transcript abundance of AtCesA4, AtCesA7, and A8CesA8 in wild-type plants (Landsberg ecotype) was measured using real-time PCR analysis. RNA was harvested from stem tissue of 21-day-old plants that had been grown under long-day lighting conditions (16 hours light). Expression levels are relative to the control gene, AtUBQ5. Error bars represent standard deviation, n represents 3 biological replicates.