FUNCTIONAL GENOMICS OF PLANT CHITINASE-LIKE GENES

by

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Abstract

The *Arabidopsis chitinase-like1* (*Atctl1*) mutant, *pom1* is compromised in primary cell wall development, resulting in short roots when grown on high sucrose and shortened hypocotyls when grown in darkness. To better understand this phenotype and the evolution of *AtCTL1* and its homologue, *AtCTL2*, we obtained a large number of *CTL* sequences and determined the phylogenetic relationships among them. Since microarray analysis had suggested a change in auxin response or homeostasis in *pom1*, I used the auxin reporter *DR5::GUS* in the *pom1* background to assess changes in distribution. To assess whether the biochemical functions of *AtCTL1* homologues in *Arabidopsis* and other plants are conserved, I transformed *pom1* with *AtCTL2* and *CTLs* from poplar (*Populus trichocarpa* x *Populus deltoides* clone H-11) and from *Picea glauca* (spruce) and assessed rescue of the *pom1* phenotype. To further understand *CTL* expression and function, *Arabidopsis* and poplar *CTL promoter::GUS* fusions were also expressed in *Arabidopsis*, *PopCTL1* overexpressed in *Arabidopsis*, and *CTL* expression down regulated in poplar by RNAi. Our results indicate that *CTL* genes represent an ancient family encoding proteins of conserved biochemical function. In dicots, represented by Arabidopsis and poplar) duplicated *CTL* genes are differentially expressed in conjunction with primary and secondary cell wall development, respectively. Mutation of these genes results in improperly formed primary walls in certain cell types in the case of *AtCTL1*, and an impairment in the differentiation of vascular bundles for *AtCTL2*. Overexpression of *PopCTL1* in *Arabidopsis* seems to over stimulate the differentiation of vascular bundles, and our studies show that auxin distribution is altered in the *Atctl1* mutant. Down regulation of *PopCTL1* and *PopCTL2* in poplar appears to phenocopy aspects of these mutations, resulting in secondary cell walls that appear to have less deposition of lignin and an accelerated production of secondary xylem respectively. While specific biochemical function(s) of *CTL* genes were not studied, potential functions are discussed.

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Chapter 1 Introduction

1.1 Plant Cell Expansion

Plants grow through the organized processes of cell division, expansion, elongation, and differentiation. The radial pattern of tissues found in plant organs is first laid out during plant embryogenesis. Primary plant growth in seedlings then begins with cell division at apical meristems at both the root tip and the shoot tip. After cell division, new cells receive developmental information through biochemical gradients such as those provided by auxin and cytokinins, as well as cell-to-cell signalling. Plant cells use the turgor pressure they accumulate from absorption of water into their large vacuoles as the driving force behind cell expansion (Cosgrove 2005). Elongation is a specialized form of cell expansion, where plant cells undergo massive anti-isodiametric expansion to reach lengths many times greater than they are wide. The filamentous green algae *Nitella* for example, has internode cells that elongate to a length of 6 cm (average *Arabidopsis* cells are 20 - 100 um long), while remaining 0.1 cm wide! This regulation of this type of expansion is believed to be achieved by the properties of primary cell walls, which constrain and direct the nature of cell expansion, allowing for highly specific shapes and dimensions. Subsequent to elongation, cells undergo maturation where specialized programs of differentiation gives them different features and functions ranging from secretion of biochemicals to structural support through strengthened cell wall or nutrient storage in specialized plastids. A similar developmental progression takes place during secondary plant growth where cell division activity of the vascular and cork cambia creates new populations of cells that undergo expansion, elongation, and maturation, allowing the plant to extend radially after the basic plant form is produced by primary growth.

1.2 Mechanisms and mutants in plant cell expansion

To regulate the development of diverse shapes and sizes, plants use different mechanisms to control cell expansion and elongation. The main way that plants control and direct this turgor-driven cell expansion is through the constraining potential of the primary cell wall. This wall is composed mostly of aligned cellulosic microfibrils and cross-linked xyloglucans amid of a matrix of homogalacturonan and rhamnogalacturonan pectins that help determine physiological properties of the wall (Yong et al. 2005). The primary wall can be remodelled to allow the cell further expansion and differentiation until a secondary cell wall and lignin are added in some specialized cells. Interference with the production of primary cell walls results in varying severities of cell expansion mutant phenotypes that loose some aspect of cell expansion control (Dolan and Davies 2004; Lloyd and Chan 2004)

Synthesis of cellulose, which is a polymer of 1,4-glucan chains, is accomplished by cellulose synthases, which are composed of subunits termed CESA proteins, organized into plasma membrane localized complexes. *CESA1*, *CESA3*, and *CESA6*, are believed to encode protein subunits of the cellulose synthase complex involved in primary wall deposition (Gardiner et al. 2003). Mutants of these genes are *rsw1* (Arioli et al. 1998), *ixr1* (Scheible et al. 2001), and *prc1* (Fagard et al. 2000; Schindelman et al. 2001), all of which show reduced cellulose synthesis in primary cell walls, and perturbations of cell expansion in a number of different tissues. Other mutants showing a link between cellulose synthesis and regulation of cell expansion, include *cobra,* a mutant in a GPI anchored protein affecting the orientation of cell expansion (Schindelman et al. 2001), *kobito1* which has disorganized deposition of microfibrils (Pagant et al. 2002)*, knopf* with a mutated alpha-glucosidase (Gillmor et al. 2002)*,* the *pom1* mutant for a chitinase-like gene (Pagant et al. 2002; Zhong et al. 2002), and *botero1* which is involved in cytoskeletal organization mediating cellulose microfibril alignment (Bichet et al. 2001).

Other less well-defined mechanisms in cell expansion control are believed to be involved not directly in cellulose production, but at the level of signal production or perception. In addition to cellulose synthase like genes (*Csl*) and cellulases, *Arabidopsis thaliana* cell expansion mutants have implicated genes playing roles in coordination and communication in cell expansion, such those encoding N-glycosylases, membrane bound receptors, extra cellular matrix enzymes, and regulators of cell wall sugar composition, gibberellic acid (GA), ethylene regulation, auxin and cytokinin regulation, abscisic acid (ABA) regulation, and brassinosteroid regulation (Dolan and Davies 2004). Of particular interest are carbohydrate active enzymes (and not themselves cellulose synthases) involved in regulation of cell expansion such as those characterized by *korrigan,* a mutant in a gene encoding an endo-1,4-D-glucanase (Nicol et al. 1998), *rsw3* a mutant in a gene encoding an enzyme putatively processing N-glycans during ER quality control (Burn et al. 2002) and *rdh1,* a mutant in a gene encoding an arabinogalactan protein (Seifert et al. 2002). Expansins, a class of plant proteins that permit primary cell walls to expand during turgor-driven growth, are believed to disrupt non-covalent binding between cellulose and hemicellulose polysaccharides that constrain cell wall expansion, suggesting non catalytic interactions between wall components may also be important in development (Choi et al. 2003).

Arabidopsis contains over 800 genes or 3.3% of all of its genes, that are annotated as glycosidase or glycosyltransferase-related; far more than any other non-plant organism studied thus far (Coutinho et al. 2003). That a large number of mutants in wall synthesis have been reported and that such a large fraction of the *Arabidopsis* genome is involved in wall production and remodelling, supports the idea that the plant cell wall is a complex and dynamic structure, that is involved in complex processes such as control of morphogenesis in addition to providing structural integrity.

1.3 Secondary Cell Wall Formation in xylem development

Specialized plant cells such as xylem and fiber cells undergo an additional process of maturation after primary cell wall development called secondary wall formation, whereby additional cellulose layers and phenolic compounds are laid down inside the primary cell wall (Bourquin et al. 2002). There are different cell types within xylem tissue that carry out this program in different ways. Primary xylem is derived from procambium near the apex of the primary plant body and consists first of elongated protoxylem cells, which undergo secondary cell wall development and the formation of thick, lignified annular rings, followed by cell death. Metaxylem vessels are also dead at maturity, differentiate after protoxylem, and are characterized by scalariform, reticulate and pitted secondary walls impregnated with lignin (Fukuda 2004). Secondary xylem in angiosperms such as poplar consists of vessels similar to metaxylem as well as fiber and ray parenchyma cells, and is formed through the cell division and differentiation activity of a vascular cambium, which forms in stems at some distance below the apex at the onset of secondary growth (Israelsson et al. 2003).

Formation of secondarily thickened cells in the secondary xylem cells progresses through four broad phases of development: 1) Cell division from cambial cells, with some cells retaining capacity for continued division, 2) Radial and longitudinal cell expansion, 3) Secondary cell wall formation (cellulose and lignification continues/commences, and finally 4) programmed cell death, protoplast lysis, and selective cell wall digestion (Hertzberg et al. 2001). Selective cell wall digestion taking place in xylem tracheids and vessel elements creates pitted walls and pipelike cells with perforated end walls for the movement of water and minerals throughout the plant body. These stages of xylem development are depicted schematically in Figure 2B, below. Secondarily thickened cell walls are stronger and more impermeable to water, allowing xylem cells to function as vascular tissues which also provide the strength for which woody plants are known.

1.4 Mechanisms and mutants in xylem secondary cell wall formation

To form a secondary wall, a developing vessel or fiber cell must synthesize a large amount of additional cellulose. Since secondary cell walls are laid down inside the primary cell wall, perturbation of cellulose synthesis does not usually result in cell expansion phenotypes typical of defects in primary cell wall production. This is shown by analysis of the mutants *irx1* (Taylor et al. 2000), *irx3* (Taylor et al. 1999), and *irx5* (Taylor et al. 2003) which are null mutants in cellulose synthase genes encoding the CESA subunits involved in secondary wall formation, *CESA4*, *CESA7*, and *CESA8,* respectively. Rather than having isodiametric growth or broken walls, these mutants all show severe reduction in cellulose content of secondary cell walls have xylem vessels which collapse under the negative pressure inside. All three cellulose synthase subunits have been shown to be required for the formation of a cellulose synthase complex crucial for secondary cell wall production, and have also been shown to co-localize with microtubules which presumably orient cellulose microfibril production (Gardiner et al. 2003). Microtubule mutants such as *botero1* (Bichet et al. 2001) and *mor1* (Sugimoto et al. 2003) likely show cell expansion phenotypes because of microtubule involvement in primary cell wall development, masking potential cell wall defects specific to secondary cell wall synthesis. There are however mutants such as *fra3* (Zhong et al. 2004) where perturbations in cytoskeletal organization compromise secondary cell wall specific formation in fiber cells and do not have defects in primary cell wall expansion.

In addition to more extensive deposition of cellulose, secondary cell wall growth involves deposition of other secondary wall specific polysaccharides, proteins, and the complex phenylpropanoid polymer, lignin. Mutants showing reductions in lignin production often have xylem with compromised structural integrity (Anterola and Lewis 2002). Downregulation of lignin production may sometimes result in elevated

cellulose production (Anterola and Lewis 2002; Li et al. 2003), and conversely the downregulation of cellulose production has been shown to stimulate increased lignification (Cano-Delgado et al. 2003). Lignin production takes place as part of secondary cell wall development, but also during stress and pathogen responses mediated by jasmonate and ethylene production resulting from interference with cellulose synthesis in primary cell walls (Ellis et al. 2002)

1.5 Chitinase like gene involvement in development

Lipo-chitooligosaccharides (NOD factors) are chitin-like molecules that have long been known as growth promoting compounds used by nitrogen fixing bacteria such as *Rhizobium meliloti* in plant communication during the formation and maintainance of root nodules (Denarie et al. 1996). Purified NOD factors from *R. meliloti* and a wide range of taxonomically unrelated bacterial species can be applied to their host plant's roots where they trigger root hair deformations and often full nodule growth that is not inducible by any known class of plant hormone or hormone inhibitor. That similar lipo-chitooligosaccharide signals from diverse bacterial species can induce changes of plant development suggests convergent evolution, and hints that plants may use endogenous chitin-like signals in the regulation of their growth. Proteoglycans such as xylogen, a glycosylated protein involved in xylem formation, may provide plants with a regulatory link between such oligosaccharide signaling, and spatial/temporal control of development (Motose et al. 2004).

Strong support for this idea comes from studies of the temperature sensitive carrot mutant, *ts11*, which is arrested in cell expansion and embryo development at the globular stage when grown at 32°C, but grows normally when supplied with a bacterial NOD factor (De Jong et al. 1993). This same mutant was shown to be rescued by application of a glycosylated endochitinase enzyme secreted extracellularly in wild type carrot, suggesting the developmental function missing is the modification of an endogenous chitin-like molecule or proteoglycan to produce N-acetylglucosamine containing signals (De Jong et al. 1992; Kragh et al. 1996).

The importance of chitinase-like genes in development and cell expansion in the model plant *Arabidopsis thaliana* was first revealed by the discovery that mutations in a chitinase-like gene (*AtCTL1*) lead to cell expansion and other defects (Zhong et al., 2002), as first described for the *pom1* mutant. Eleven different *pom1* alleles of *AtCTL1* were described based on cell expansion defects (Benfey et al. 1993; Hauser et al. 1995). Under normal growth conditions, *pom1* plants are somewhat stunted in growth, however when grown in 4.5 % sucrose media, the seedlings show radial swelling of roots with greater numbers of root hairs (Figure 1), and etiolated hypocotyls grow to half the length of wild-type. These mutant plants also have isodiametric root epidermis and cortex cells, implying loss of cell expansion regulation (Figure 2). In the root, part of these observed differences might be accounted for by reduction of cell division, as the comparison of cell numbers in the plant's isodiametric growth zone show 20 in wild-type vs. 7 in *pom1* (Hauser et al., 1995).

Another 12 alleles of *pom1/Atctl1* have since been isolated in screens for phenotypes to unrelated to cell expansion, including *pom1-22* called *esr1* enhanced shoot regeneration in tissue culture (Cary et al. 2001) and *elp1*, ectopic deposition of lignin (Zhong et al. 2002). Characterization of the *esr1* mutation showed plant cells to be hyper responsive to cytokinins, having elevated shoot regeneration in *Arabidopsis* tissue culture. Additionally *Atctl1* mutants have malformed pith cell walls and a mosaic pattern of ectopic deposition of lignin throughout the pith and vascular tissues, totalling a 20% increase in total deposition of lignin (Zhong et al., 2002). This ectopic lignification correlates to a 50% increase in expression of genes encoding the lignin biosynthetic enzymes CCoAOMT, PAL, and CCR in the same tissues, in the absence of secondary cell wall thickening (Zhong et al. 2000). The *POM1* gene (At1g05850) was cloned on the basis of the *elp1* mutant phenotype and is predicted to encode a 321 amino acid protein with a predicted molecular mass of 35,556 Da. The gene was named *AtCTL1, CHITINASE LIKE 1*, because of the similarity of the predicted protein to chitinases (Zhong et al. 2002). A second *AtCTL* gene (At3g16920), *AtCTL2*,

annotated as having glycosyl transferase activity has been identified based on sequence similarity to *ATCTL1*, but until now has no described mutant phenotype (Hauser et al., unpublished). AtCTL2 has 66% amino acid identity to AtCTL1. Both AtCTL1 and AtCTL2 are predicted to be cell wall localized, based on N-terminal signal sequences (Zhong et al., 2002; M. Hauser, unpublished).

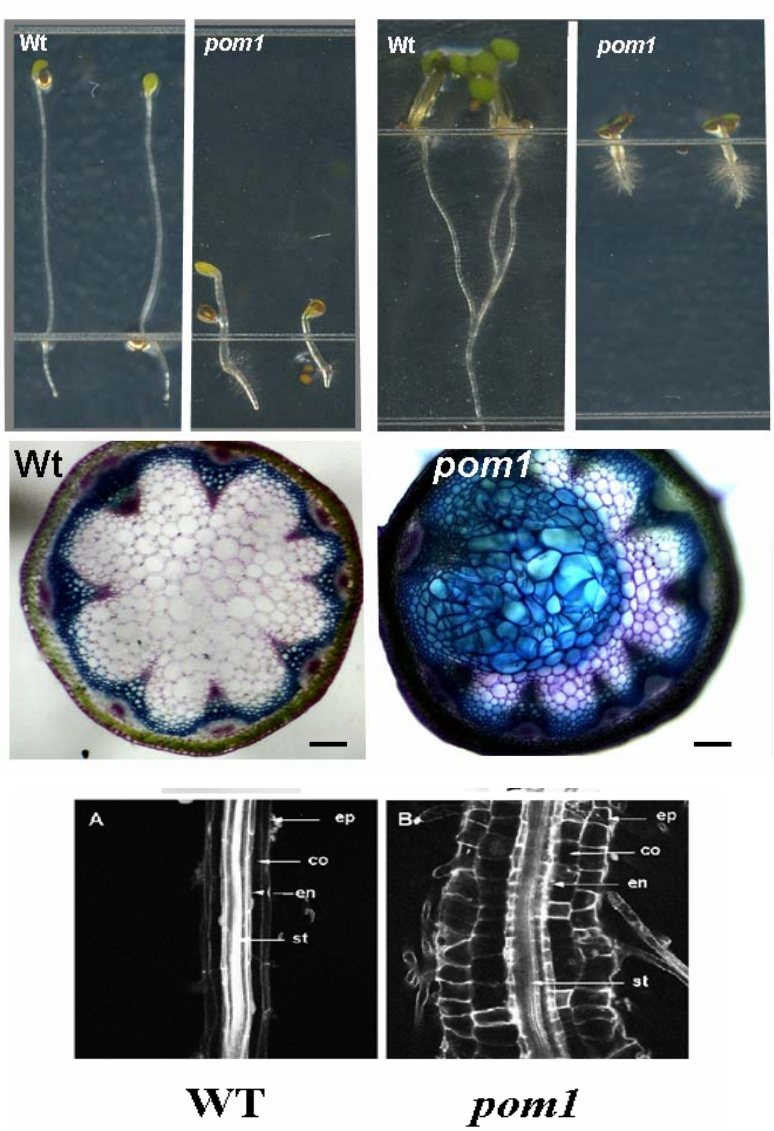

Figure 1: Wild type and *pom1* phenotypes of *Arabidopsis thaliana* (Columbia-0 ecotype). Plants were grown in the dark or on 4.5% sucrose for four days. Hand sections were taken from the bottom of 20 cm tall floral stems and stained with toluidine blue. Micrographs of root sections from root hair growth zones of seedlings grown on 4.5% sucrose are adapted from (Schneider et al. 1997). st, stele; en, endodermis; co, cortex; ep, epidermis. Scale bars 100 µm.

1.6 CTL expression in Arabidopsis and poplar

AtCTL gene expression patterns have been determined by microarray transcript profiling in *Arabidopsis*. Ehlting et al. (2005) profiled the expression of all *Arabidopsis* genes over the course of inflorescence stem development and interfascicular fiber differentiation. Data from this experiment on the expression of *AtCTL1* and *AtCTL2* are shown in Figure 2A. While *AtCTL1* is not differentially regulated, expression of *AtCTL2* is strongly upregulated, along with a large number of secondary cell wall biosynthetic genes (Ehlting et al., 2005), during stem development and fiber formation.

The first microarray–based gene profiling experiment in poplar used a 2,995-element EST array to profile to gene expression changes during secondary xylem differentiation (Hertzberg et al., 2001). Two ESTs encoding poplar chitinase-like proteins were present on the microarray (EST AI163580 and EST AI164688), and data from this study showed that one of chitinase-like mRNAs was upregulated during the late stages of xylem formation related to cell wall thickening and lignification (Figure 2B).

Work in the Douglas lab (unpublished) used PCR based cloning to isolate the full length cDNA clones corresponding to the two poplar chitinase-like genes from *P. trichocarpa* x *P. deltoides* hybrid clone H11. The poplar *PopCTL1* and *PopCTL2* genes encode proteins with 66% to 68% amino acid identity to *AtCTL1* and *AtCTL2*. Analysis of *PopCTL1* sequence using the PSORT program has shown the presence of a signal secretion peptide at the N-terminus, suggesting it is cell wall localized.

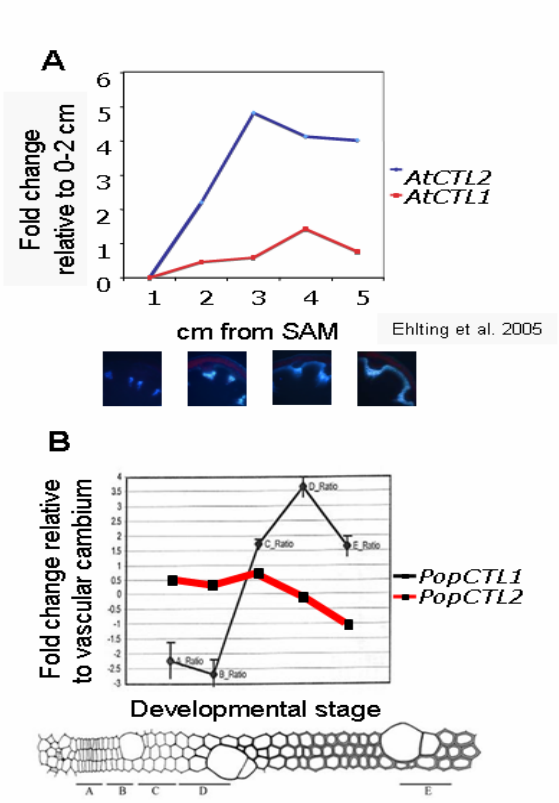

Figure 2: A) mRNA expression profiles of *AtCTL1* and *AtCTL2* using 26K gene longmer arrays during primary inflorescence development. RNA was extracted from sections illustrated in the UV photos of cross-sections taken at 1, 3, 5, 7 cm from SAM. B) mRNA expression profiles of *PopCTL1* and *PopCTL2* across developing xylem tissue in poplar trees. Samples were taken at stages A to E as shown and normalized by comparison to undifferentiated cambium. Cell division takes place in A, expansion and elongation in A, B, and C, secondary cell wall deposition (s1, s2, and s3 layers) in D and E, and programmed cell death in E. Adapted from Hertzberg et al. (2001).

1.7 Evolutionary and Phylogenetic considerations in functional elucidation

Phylogenetic reconstruction/comparison shows that *AtCTL1* and *AtCTL2* are members of a clade of chitinase-like genes distinct from a large number of other true chitinases in the *Arabidopsis* genome (M.T. Hauser, unpublished; Figure 3). Preliminary analysis of ESTs from the model non-vascular plant *Physcomitrella patens,* has shown it to also contain *CTL* sequences (B. Hamberger, unpublished) implying that plants possessed this family of genes for millions of years before evolution of vascular plants. It is also interesting to note that in all three plants with

fully sequenced genomes (*Arabidopsis*, poplar and rice), there are two *CTL* genes (B. Hamberger, unpublished), suggesting not only that two copies are sufficient for plant development, but that duplication is not tolerated. From a functional point of view, it is not clear why two *CTL* genes are apparently required in plants. While expression of *PopCTL1* during xylem development suggests a role there, the original CTL function may be in primary growth based on apparent conservation of *CTL* genes in a non-vascular plant (*Physcomitrella*). Identification and phylogenetic and functional comparisons between expected CTL proteins in other plant clades, should allow further hypothesis to be made with regard to *CTL* function and evolution in land plants.

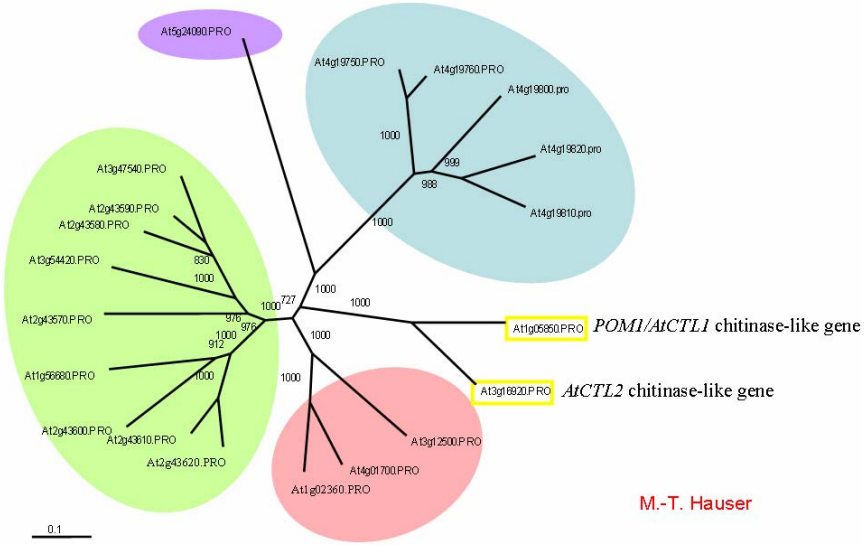

Figure 3: Phylogenetic alignment of all known *Arabidopsis* chitinases and chitinase-likes performed by M.-T. Hauser (unpublished). Branches are drawn to scale, with the scale bar showing 0.1 substitutions per site.

1.8 Predicting CTL function

Since *PopCTL1* is greatly upregulated in poplar secondary xylem, while *PopCTL2* is not differentially expressed across this developmental gradient, it seems likely that *PopCTL1* is the more important gene of the two in secondary wall formation or some other aspect of xylem development. Ehlting et al. (2005) and Figure 2 show that a similar *CTL* expression pattern exists in *Arabidopsis thaliana* with regards to *AtCTL1* and *AtCTL2* in floral stems. Consistent with this, other *Arabidopsis*

expression data mining studies (Brown 2005; Persson et al. 2005; Yokoyama and Nishitani 2006) also show that *AtCTL1* expression is correlated with expression of genes encoding primary cell wall cellulose synthase subunits, while *AtCTL2* expression is correlated with expression of genes encoding cellulose synthase subunits involved in secondary cell wall biosynthesis. This work suggests the hypothesis that CTL proteins in vascular plants have evolved two distinct functions, one associated with primary wall development, and one with secondary cell wall development, and that these two functions are conserved in different land plant lineages.

1.9 Thesis objectives and summary

The objectives of this thesis were to obtain functional information for poplar and *Arabidopsis CTL* genes, to test the hypothesis that conserved duplicated *CTL* genes are present in diverse land plant lineages, and to test the hypothesis that proteins encoded by duplicated *CTL* genes have distinct functions in primary and secondary growth and/or primary and secondary wall formation.

This thesis reports the results from bioinformatic analysis of *CTL* genes from a large number of plants having significant sequence information. This is followed by the comparative analysis of function between *AtCTL1* and *PopCTL2,* and *AtCTL2* and *PopCTL1,* as well as two *CTL* genes from *Picea glauca*. Information about *PopCTL* and *AtCTL* function and functional relationships between these *CTL* genes was obtained from Arabidopsis *pom1* phenotypic rescue experiments, analysis of the phenotype of an *AtCTL2* T-DNA insertion mutant, *CTL* overexpression, and analysis of *CTL promoter::GUS* expression in *Arabidopsis* for both *Arabidopsis* and poplar *CTLs*. To study *PopCTL1* and *PopCTL2* function *in vivo,* RNAi techniques were used to generate transgenic poplar lines with down regulated *PopCTL1* and *PopCTL2* expression, and the phenotypes of these lines investigated.

Chapter 2. Materials and Methods

2.1 CTL Phylogenetics, homologue discovery, and protein modelling

2.1.1 BLAST analysis and homologue discovery

The coding sequences of the *AtCTL1* and *AtCTL2* genes were submitted to the nucleotide-nucleotide BLAST function in the Genome BC Forestry Genomics EST database at http://treenomix0.forestry.ubc.ca/ to search for poplar, *Picea glauca*, and *Pinus taeda* CTLs before public release. To ensure all possible homologues were found, a number of different 200 bp long sequences from conserved regions of *AtCTL1* and *AtCTL2* were also submitted as queries against the EST database hosted by the Treenomix group for the Forestry Genome BC lab at http://treenomix0.forestry.ubc.ca/blast/index.html. Subsequent partial EST regions were resubmitted, allowing a number of contiguous fragments to be found. The CAP function within the BioEdit program (Hall 1999) was used on these fragments to form full length contigs. Contig sequences were analyzed for coding regions and predicted proteins were then aligned to *AtCTL1* and *AtCTL2* sequences for comparison. This was process was repeated using *Arabidopsis*, poplar, *Picea glauca and Physcomitrella patens CTLs* as queries of publicly available EST databases at: http://www.ncbi.nlm.nih.gov/, http://www.tigr.org/tdb/tgi/plant.shtml http://www.kazusa.or.jp/en/plant/porphyra/EST/

2.1.2 Tree construction using Phyml and Treeview

Translated *CTL* coding regions were aligned using the CLUSTALW Multiple alignment function in the BioEdit program (Hall 1999), then truncated to a strongly conserved region corresponding to amino acid 40 to amino acid 314 in *AtCTL1*. 32 putative CTLs were saved in FASTA format, along with CHI26, a family 19 chitinase from *Hordeum vulgare* (Song and Suh 1996). Phylogenetic predictions were generated by running these sequences through Phyml Version 2.4.4 (Guindon and Gascuel 2003) with 100 parametric bootstrap replicates, optimized

proportions of invariable sites, 4 categories of substitution rate, and optimized gamma distribution parameters; other parameters were left at their defaults. Finished, unrooted trees were then visualized using Treeview version 1.6.6 (Page 1996) available at http://taxonomy.zoology.gla.ac.uk/rod/rod.html

2.1.3 Protein modelling and trafficking prediction

To predict conserved protein domains, *Arabidopsis*, poplar, and *Picea glauca* CTL proteins were submitted to the NCBI protein domain database at (http://www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi?cmd=rps). Protein targeting prediction was accomplished by submission of *Arabidopsis*, poplar, and *Picea glauca* CTLs to the WOLF PSORT program at the website http://wolfpsort.seq.cbrc.jp/ (Horton et al. 2006).

2.2 General Nucleic Acid Methods

2.2.1 Plasmid DNA preparation and DNA sequencing

Plasmid DNA was prepared by alkaline lysis by use of Qiaprep spin Plasmid Miniprep kit or Midiprep kit (Qiagen), following the manufacturer's protocol. All DNA sequencing was carried out using M13 forward and reverse primers on PCR products cloned directly into pDRIVE (Quiagen). Sequencing chemistry was Big Dye 3.1 and reads were determined by automated Prism Cycle Sequencing with at the University of British Columbia Nucleic Acid and Protein Service unit.

2.1.2 DNA restriction, gel purification and ligation

Sequences to be digested were tested in silico using the Restriction Mapper software available at: http://www.bioinformatics.vg/biolinks/bioinformatics/Restriction%2520Analysis.shtml. All restriction reactions took place at 37 ˚C using Roche and New England Biolabs enzymes following manufacturer protocols for single, double, and triple digests. Complete or partial digests were run on 1% agarose gels and gel purified with Qiagen PCR purification kits or Minielute kits if under 10 kb in size, or Qiagen

QiaXII kits if larger than 10 kb. To concentrate the DNA, samples were vacuum dried for 5 min at room temperature and then assayed for quantity using a 1% agarose gel electrophoresis and EtBr staining and UV fluorescence imaging. Visual comparison of band intensity under UV allowed an estimate of molecular concentration. Vector and insert DNA was ligated in a ratio of 1 vector molecule to 3 insert molecules, using T4 ligase (Roche) and buffer following the manufacturer's protocols. Ligations were left for 48 h at 4 ˚C before transformation of half the volume into chemically competent DH5α *E. coli* cells.

2.1.3 Genomic DNA and total RNA isolation

Poplar and *Arabidopsis* tissue was disrupted by bead milling with 1 mm zirconia-silica beads and 0.09 g of fresh leaves in 2.0 mL Eppendorf tubes, then using a kit and following manufacturer's instructions from step 4 (Nucleon Phytopure Plant DNA Extraction Kit, Amersham). DNA pellets were eluted in 30 µl of distilled, filtered water, and then further diluted 1:15 in distilled water for PCR.

Poplar and *Arabidopsis* RNA was extracted by grinding 0.09 g of frozen leaf, whole seedling, or stem tissue, in liquid nitrogen then following a modified protocol of Qiagen's Rneasy kit developed by O. Schevchenko and A. Bruner (unpublished). Briefly this involved mixing 0.01 g of soluble polyvinylpyrrolidone (PVP-40,Sigma) per mL of Qiagen RLT buffer including beta mercaptoethanol. 500 µl of this was added to 0.1 g of tissue ground in liquid nitrogen within a 1.5 mL eppendorf micro centrifuge tube. This mixture was shaken for 1 minute, then vortexed at high speed for 30 seconds. 200 µl of Rnase free KoAC (pH 6.5) was then added, mixed by inversion, and then incubated on ice for 15 min. After incubation, tubes were spun at 12,000 rpm for 15 min at 4 ˚C, and the supernatant transferred to a clean 1.5 mL tube. 350 µl of 100% ethanol was added and mixed by pipetting, then this mixture was applied to pink Qiagen Rneasy columns and the Rneasy kit protocol followed from step 6 onward.

DNA and RNA quality and concentration was checked by analysis of 260 and 280 nm absorbance in a spectrophotometer, as well as direct visualization using EtBr UV fluorescence on a 1.5% agarose gel.

2.2.4 Primer design and PCR based genotyping

Primers were designed in the Bioedit program by visually selecting high G or C content sequences from desired regions and then testing them for primer dimers, optimal size, and annealing temperature in the virtual PCR program Amplifx, freely available at http://jullien.n.free.fr/article.php3?id_article=10.

PCR was performed in a Thermocycler PCR machine using the following protocol in a 50 µl volume plus a 20 ul volume of mineral oil:

Step 1. 94 ˚C for 2 minutes
Step 2. 94 ˚C for 30 seconds
Step 3. X ˚C for 30 seconds (annealing step that is reaction specific)
Step 4. 72 ˚C for Y seconds (template extension step that is reaction specific)
Step 5. Go to step two Z number of times (cycles number step that is reaction specific)
Step 6. 94 ˚C for 30 seconds
Step 7. X ˚C for 30 seconds (annealing step that is reaction specific)
Step 8. 72 ˚C for 7 minutes
Step 9. 12 ˚C for 1 hour

Arabidopsis DNA was used in a PCR reaction amplifying a 940 base pair region of the Actin2 gene using primers:

AtActin2F (CTGAGGCTGATGATATTCAACCAATCG) and,

AtActin2R (GATCCTTCCTGATATCCACATCACAC)

PCR was performed with 35 cycles at 55 ˚C annealing temperatures and 1 minute extension time. Plants were tested for transgenes using the information presented in Table 1.

Table 1: PCR protocols used in testing for transgenes in genomic DNA.

Transgene construct	Parameters	Primers used
AtCTL1 promoter with PopCTL1 coding region in pART27	58 ˚C, 1 min ext, 40 cycles	**PopCTL1.20** GTGCTACAACAAGGAAATGAGTCCCA **Han-R** CAACGTGCACAACAGAATTGAA
CaMV 35S promoter with AtCTL1 coding region in pCAMBIA 1305.1	57 ˚C, 1 min 15 sec ext, 35 cycles	**AtCTL1.3 Last Exon Fwd** CACTTGCATTCCAAGCTGCAATCTG **pCAMBIA1305.1 Nos Term Rev** GATTAATCATCGCAAGACCGGCAACAGG
CaMV 35S promoter with AtCTL2 coding region in pCAMBIA 1305.1	57 ˚C, 1 min 15 sec ext, 35 cycles	**AtCTL2.5 Last Exon Fwd** CTGCAACAGCGGATTCGATAACGATGAG **pCAMBIA1305.1 Nos Term Rev** GATTAATCATCGCAAGACCGGCAACAGG
CaMV 35S promoter with PopCTL1 coding region in pART27	58 ˚C, 1 min ext, 40 cycles	**PopCTL1.20** GTGCTACAACAAGGAAATGAGTCCCA **Han-R** CAACGTGCACAACAGAATTGAA
PopCTL2 promoter with PopCTL2 coding region in pCAMBIA 1305.1	57 ˚C, 1 min ext, 30 cycles	**PopCTL2.3 Last Exon Fwd** CTATGGGGATCAAGTTTGCGGCCAGG **pCAMBIA1305.1 Nos Term Rev** GATTAATCATCGCAAGACCGGCAACAGG
AtCTL1 promoter with GUS coding region in pCAMBIA 1305.1	52 ˚C, 1 min ext, 35 cycles	**AtCTL1.11B** GGGAGCTCACGCCATTGTTACTGTTGG **GUS Up** GGGTCCTAACCAAGAAAATGAAGGAG
PopCTL1 promoter with GUS coding region in pCAMBIA 1305.1	52 ˚C, 1 min ext, 35 cycles	**PopCTL1.19D** CCCATCTGAGCCTCAACGAAAa **GUS Up** GGGTCCTAACCAAGAAAATGAAGGAG
PopCTL1 150 base pair RNAi construct in pART27	54 ˚C, 45 sec ext, 35 cycles	**CAMV 35S Forward** CTGGCGAACAGTTCATACAGAGTC **CAMV 35S Reverse** GTGTTCTCTCCAAATGAAATGAACTTCC
PopCTL1 400 base pair RNAi construct in pART27	54 ˚C, 45 sec ext, 35 cycles	**CAMV 35S Forward** CTGGCGAACAGTTCATACAGAGTC **CAMV 35S Reverse** GTGTTCTCTCCAAATGAAATGAACTTCC
PopCTL2 150 base pair RNAi construct in pART27	54 ˚C, 45 sec ext, 35 cycles	**CAMV 35S Forward** CTGGCGAACAGTTCATACAGAGTC **CAMV 35S Reverse** GTGTTCTCTCCAAATGAAATGAACTTCC
PopCTL2 400 base pair RNAi construct in pART27	54 ˚C, 45 sec ext, 35 cycles	**CAMV 35S Forward** CTGGCGAACAGTTCATACAGAGTC **CAMV 35S Reverse** GTGTTCTCTCCAAATGAAATGAACTTCC

2.2.5 Reverse transcriptase PCR (RT PCR) and expression analysis

2 µg of total RNA was reverse transcribed in a 40 µl volume using Invitrogen Superscript II enzyme and following the manufacturer's protocol for first and second strand synthesis (Invitrogen).

Semi quantitative PCR was conducted on 2µl samples of cDNA in a 50 ul volume reaction (with an addition of 20 µl of mineral oil) to determine gene expression levels. As a control in *Arabidopsis* cDNA, *Actin2* expression levels were assayed by using the primers AtActin2F RT (CTTCCGCTCTTTCTTTCCAAGCTC) and AtActin2R RT (CCATCACCAGAATCCAGCACAATACCG) with 53 ˚C annealing temperature, 1 min extension time, and 27 cycles. As a control in poplar cDNA, TRANSLATION INITIATION FACTOR 5A (*TIF-5A*) expression levels were assayed by using the primers PopTIF-5A F RT (CTCATCTCACAACTGTGATGTTCCC) and PopTIF-5A R RT (CCATTTTCAGTCAAAAGACTCACAAAAC) with 53 ˚C annealing temperature, 1 min extension time, and 27 cycles. For both poplar and *Arabidopsis* housekeeping genes, optimal PCR sub saturation levels were determined to occur at 27 cycles. Primers were designed to selectively amplify cDNA despite gDNA contamination, and this was confirmed by testing these primers against genomic DNA negative controls. Table 2 lists the genes and conditions used to assay *CTL* mRNA levels in *Arabidopsis* and poplar.

2.3 Cloning procedures

2.3.1 Host bacterial strains, plasmids, and plant materials

All cloning was done in *Escherichia coli* strain DH5α which is a mutant for recombination and RNase enzymes, which was grown overnight in liquid LB broth at 37˚C and constant shaking at 200 rpm. Transformation of calcium chloride/rubidium competent cells was carried out as described in (Sambrook et al. 1982).

Table 2: Semiquantitative PCR parameters and primers used in gene expression analysis.

Gene expression tested	Semi-quantitative PCR conditions	cDNA specific primers
AtCTL1	52 ˚C, 1 min ext, 27 cycles	**AtCTL1.25RT** GATCTGCATTGCGGCTTGTAG **AtCTL1.26RT** GTTGCAACTCCATAGCCACAGGAC
AtCTL2	52 ˚C, 2 min ext, 27 cycles	**AtCTL2.11 RT Forward** GGGAG4CAAAACCTCATGCGGGTACG **AtCTL2.2** CCAGAGGTTATAAAACTTTCACTCAAG
PopCTL1	52 ˚C, 1 min ext, 27 cycles	**PopCTL1.25RT** GAAGCAAAACCTCATGTGGTTAT **PopCTL1.26RT** CAGTTTTGCCATAGTTGTAGTTCCA
PopCTL2	52 ˚C, 1 min ext, 27 cycles	**PopCTL2.25RT** CGTTGGCTGCAAAACCTCCTGTGGTTA **PopCTL2.26RT** CAGCTCCATAGTTATAGTTCCAGAAGAT

Agrobacterium strains used for transformation were GV3101 for *Arabidopsis* floral dip and EHA-105 for poplar leaf disc infection. It was found that 12 h of shaking at 200 rpm at 29˚C was enough to generate an OD_{600} of 1.8 in 2 mL of LB broth. The GV3101 strain used was found to be spectinomycin and streptomycin resistant, forcing pART27 selection to be carried out using kanamycin at a concentration of 100 µg/mL instead. Transformation of chemically competent *Agrobacterium* was carried out as described in (An 1987).

Arabidopsis plants were all ecotype Columbia-0. The *AtCTL1* knock out allele used was *pom1-9* an *Atctl1* EMS alllele containing a premature stop codon at amino acid 11 (M.-T.Hauser, unpublished). The *AtCTL2* knock out allele used was the T-DNA insertion line SAIL_319_A05 ordered from the Syngenta *Arabidopsis* Insertion Library (SAIL) collection available at http://www.arabidopsis.org/servlets/TairObject?type=organization&id=211883. It was confirmed to contain the T-DNA by BASTA selection and Thermal Asymmetric Interlaced-Polymerase Chain Reaction (TAIL-PCR) was used to amplify DNA fragments flanking the T-DNA left border from this transformed line (B. Hamberger,

person communication). RNA extraction and cDNA synthesis followed by semi-quantitative PCR (as outlined in Table 2) were used to confirm down regulation of *AtCTL2*.

Seeds for the *DR5::GUS* artificial auxin response promoter-GUS fusion in a Columbia-0 background were kindly provided by Dr. Thomas Berleth (University of Toronto, Toronto, ON). *DR5::GUS* in Col-0 was crossed with *pom1* to produce two lines of homozygous *DR5::GUS* in *pom1* mutant seeds (B. Hamberger, unpublished). Two lines of *AtCTL2::GUS* in Col-0*, PopCTL2::GUS* in Col-0, *CaMV 35S:AtCTL2* in *pom1*, and *PopCTL2:PopCTL2* in *pom1* seeds were also received from B. Hamberger (unpublished results) and included in the study.

The poplar clone used for transformation and all subsequent experiment was in clone 717-1B4, a hybrid of *Populus tremula* and *Populous alba* kindly provided by Dr. Shawn Mansfield. Plasmids used in this study were Qiagen's pDRIVE PCR cloning vector, CAMBIA's pCAMBIA 1305.1 (www.CAMBIA.org) , and RNAi vectors pHANNIBAL (Wesley et al. 2001) and pART27 (Gleave 1992). 150 bp and 500 bp *PopCTL2* RNAi constructs in pART27, as well as CaMV 35S:AtCTL2 and *PopCTL2:PopCTL2* constructs in pCAMBIA 1305.1 were received from B. Hamberger and were used for plant transformation. General plasmid maps are shown in Figure 4.

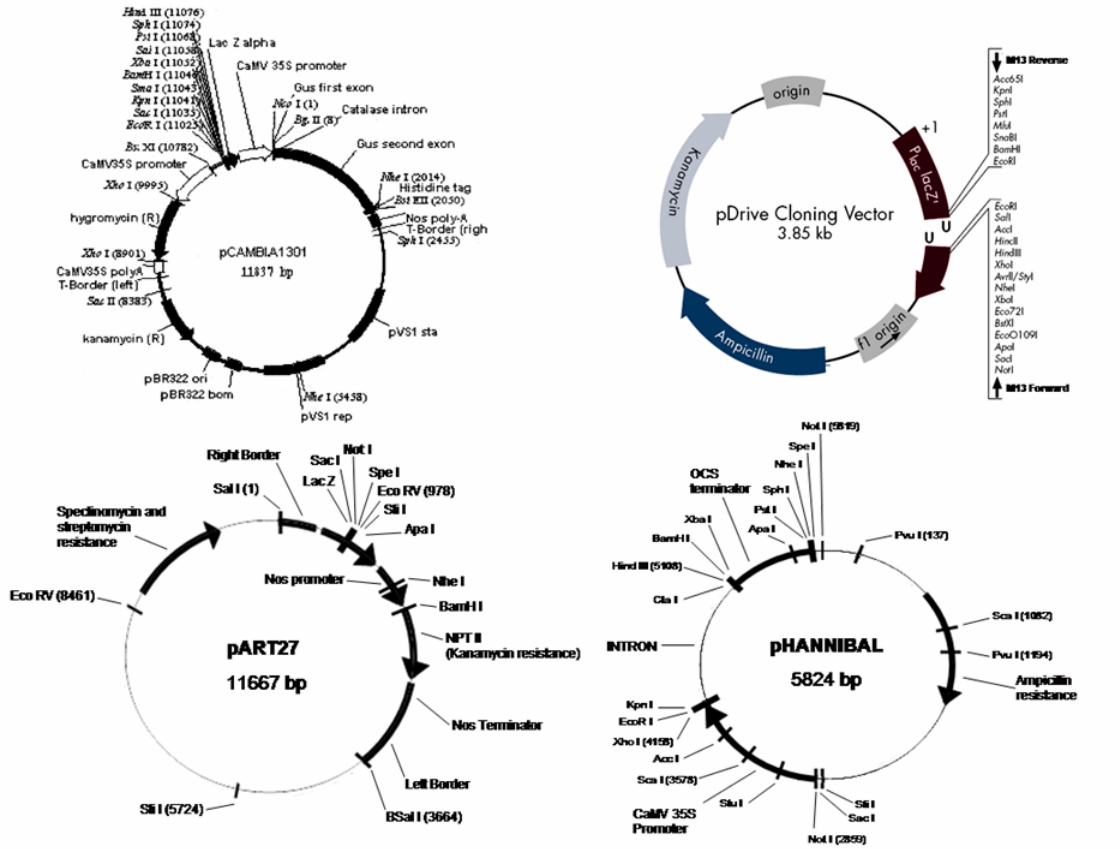

Figure 4: Plasmids used in all cloning and transformation procedures. Qiagen's pDRIVE was used to clone PCR products, which were then cut and ligated into either pHANNIBAL or pCAMBIA 1305.1. The binary plasmids pART27 and pCAMBIA 1305.1 were the final destination for cloned fragments which were intended for *Agrobacterium* mediated plant transformation.

2.3.2 Site directed mutagenesis, cloning, and sequencing of PCR products into the pDRIVE vector.

Site directed mutagenesis was employed to introduce appropriate restriction sites to *CTL* promoter and coding regions. The specific primers and conditions used are shown in Table 3. PCR products were run on a 1.5% agarose gel, along with a 1 kb DNA mass ladder (Invitrogen). No more than 10 seconds of UV light was used to visualize ethidium bromide stained nucleic acid, and appropriately sized bands were cut out and the DNA purified using either Qiagen PCR Purification Kits or Qiagen Minielute Purification kits. 4 ul of the resulting gel purified fragment was

Table 3: CTL promoters and coding regions, PCR conditions, and primers used for their amplification. Non-complimentary base pairs in primers are small letters, and restriction sites are underlined.

Region amplified	PCR conditions	Primers used
AtCTL1 promoter (955 bp)	55 ˚C, 1 min extension, 30 cycles	**AtCTL1.11B (with Sac I site)** GGgAGCTcACGCCATTGTTACTGTTGG **AtCTL1.12 (with Nar I site)** CAACTATgGcgCCAATACCAGCTAC
AtCTL1 promoter (957 bp)	55 ˚C, 1 min extension, 30 cycles	**AtCTL1.11B (with Sac I site)** GGgAGCTcACGCCATTGTTACTGTTGG **AtCTL1.12 (with Nco I site)** GCTTACAACCATgGAACAAATACCAGCTAC
AtCTL1 coding region (1645 bp)	52 ˚C, 1 min 30 seconds extension, 30 cycles	**AtCTL1.13B (with Nar I and Nco I site)** GTTGggcG*CcATGG*TGACAATCAGGAG **AtCTL1.14B (with Spe I site)** GAATAGAAAcTaGTATCATTCGCAAACC
PopCTL1 promoter (1722 bp)	55 ˚C annealing, 1 min 30 seconds extension, 30 cycles	**PopCTL1.11C (with Sac I site)** CATATAGAGCTCCAAGGGTAGTTGGCG **PopCTL1.12E (with Nco I site)** CATTTGGCCTCCATccTGGCTATGCCTG
PopCTL1 coding region (1794 bp)	54 ˚C annealing, 1 min 30 seconds extension, 30 cycles	**PopCTL1.13B (with Nar I site)** ggcGccATAGCCAAGATGGAGGCCAAATG **PopCTL1.14C (with Spe I site)** CACActAGTGACCCAATATACACTCTCTACATAG
PopCTL1 coding region (1797 bp)	50 ˚C annealing, 1 min 30 seconds extension, 30 cycles	**PopCTL1.15 (with Sac I site)** CGTACAGGCcTcgagAAGATGGAGGC **PopCTL1.14C (with Spe I site)** CACActAGTGACCCAATATACACTCTCTACATAG
PopCTL1 fragment of exon 1 (146 bp)	55 ˚C annealing, 45 seconds extension, 30 cycles	**Fwd54 (Xho I and Xba I sites)** GGGcTcgagtctagaGTAGTGGTAAATGGAGATGAGTC **Rev200 (Kpn I and Hind III sites)** gggGgTacCaagcTtGTAGGTCTGGAAAAAATCAGAA
PopCTL1 fragment of exon 1 (415 bp)	55 ˚C annealing, 45 seconds extension, 30 cycles	**PopCTL1.17 (Xho I and Xba I sites)** CAGctcgAGtCtAGATGGAGGCCAAATGGTGTTC **PopCTL1.18 (EcoR I and Hind III sites)** TACgaAttcaAGcTTTTGCTTCCAACATGCCCAAGGAAAGC

ligated into the linearized pDRIVE vector following the protocol included in the Qiagen PCR Cloning kit (Qiagen). 300 ng of pDRIVE plus PCR product was then sequenced using M13 forward or M13 reverse primers to get pair end reads covering the whole fragment. Where fragments were larger than 1000 bp, a fragment specific primer was used to achieve full coverage in the middle of inserts (generally priming 600 bp into the fragment)

2.3.3 Construction of *AtCTL1* promoter::*AtCTL1* coding region fusion construct in pART27

A number of pDRIVE plasmids containing either *AtCTL1* coding region in pDRIVE or *AtCTL1* promoter were screened by digestion with *Nar*I and *Bam*HI for clones with the insert having the *Bam*HI near to its 3' end. Positive *AtCTL1* coding region clones, had the excised insert gel purified and ligated to *Nar*I/ *Bam*HI pDRIVE molecules containing the promoter in the correct orientation. This promoter: coding region fusion was then cut out using *Bam*HI and a partial *Not*I digest, and ligated to a completely *Bam*HI and partially *Not*I digested pHANNIBAL vector backbone of around 3500 bp. This ligation added the OCS terminator to the 3' UTR of the *AtCTL1* coding region. Finally, the whole cassette was removed by *Not*I digestion and ligated into *Not*I cut pART27.

2.3.4 Construction of *AtCTL1* promoter::*PopCTL1* coding region fusion construct in pART27

A pDRIVE plasmid containing the *PopCTL1* coding region with the vector's *Sac* I restriction site in the 5' end was cut with *Nar*I and *Sac*I and ligated to NarI and SacI fragment of *AtCTL1* promoter. This promoter::coding region fusion was then cut out of pDRIVE using *Not*I and *Bam*HI, and ligated into the pHANNIBAL vector such that it replaced the CaMV 35S promoter and intron region bounded by these two enzymes. From pHANNIBAL, the cassette of *AtCTL1* promoter, *PopCTL1* coding region, and OCS terminator was then cut out by *Not*I digestion and ligated into *Not*I digested pART27.

2.3.5 Construction of *PopCTL1* coding region overexpression construct in pART27

pDRIVE plasmids with *PopCTL1* coding regions containing *Sac*I and *Spe*I sites, were screened by test digestion with *Bam*HI and *Spe*I. Plasmids with the vector *Bam*HI restriction site in the 3' end of the insert were first sequenced, then digested with *Xho*I and *Bam*HI, and the *PopCTL1* fragment was ligated into *Xho*I and *Bam*HI cut pHANNIBAL. From pHANNIBAL, the cassette of CaMV 35S promoter, *PopCTL1* coding region, and OCS terminator was then cut out by NotI and SpeI digestion and ligated into *Not*I and *Spe*I digested pART27.

2.3.6 Construction of *AtCTL1* coding region overexpression construct in pCAMBIA 1305.1

The *AtCTL1* coding region was cloned into the pDRIVE vector in an orientation with the vector's *Nhe*I restriction site in its 3 prime end. This clone was digested to completion with NcoI and *Nhe*I and run on a 1% agarose gel to yield a 1680 bp band. pCAMBIA 1305.1 was also digested with *Nco*I to completion, but only partially with *Nhe*I, allowing isolation of vector fragments of 9820 bp in size and only missing the GUSPlus coding region. Both digested insert and vector were purified in 40 ul of distilled water using a Qiagen QiaXII kit (Qiagen) and ligated together over night as described in 2.1.2.

2.3.7 Construction of *AtCTL1* and *PopCTL1* promoter::GUS fusions in pCAMBIA 1305.1

pDRIVE plasmids with *AtCTL1* coding regions containing sites were sequenced and digested with *Sac*I and *Nco*I. The resulting fragment was digested with *Sac*I and NcoI, gel extracted, and the resulting fragment was ligated into *Sac*I and *Nco*I digested pCAMBIA 1305.1.

pDRIVE plasmids with *PopCTL1* coding regions containing *Sac*I and *Nco*I sites, were sequenced and found to contain one of two allelic promoters both slightly divergent from the sequence reported in the published genome. One of these was

digested with *Sac*I and *Nco*I, and the resulting fragment was ligated into *Sac*I and *Nco*I digested pCAMBIA 1305.1.

2.3.8 Construction of *PopCTL1* RNAi constructs in pART27.

The 146 bp *PopCTL1* fragment was digested with *Xho*I and *Kpn*I and ligated into pHANNIBAL that had been cut with the same enzymes. This ligation product was sub cloned, and digested with *Xba*I and *Hin*dIII to receive the similarly digested 146 bp PopCTL1 fragment. This pHANNIBAL now contained two copies of the insert but in opposite orientations and separated by an intron. A cassette composed of the CaMV 35S promoter, OCS terminator, and these two inserts separated by an intron, was cut out by digestion with *Not*I and inserted into *Not*I digested pART27.

A nearly identical procedure was followed for 416 bp fragments, except *Eco*RI was used in place of *Kpn*I.

2.4 Arabidopsis germination, growth conditions, transformation, and selection

2.4.1 Seed sterilization, germination, transplantation, and growth conditions

Arabidopsis seeds were sterilized by a five-min incubation in 70% ethanol, followed by a 1 min spin at 13,000 rpm in an eppendorf micro centrifuge in discarded Qiagen Spin Miniprep columns. In the same column, seeds were then incubated in 100% ethanol for additional five min and again spun twice for one minute at 13,000 rpm to dryness. Seeds were then sprinkled on a sterile media of 2.35 g/L Murashi-Skoog salts (Sigma), 6 g/L agar (Sigma), 0.1% w/v sucrose and pH 5.7. Seeds were then stratified by placement at 4 ˚C for 72 h. After stratification, seeds were then placed in a light chamber where they received 18 h light per day at a constant 25 ˚C. After about two weeks of growth, plantlets developing true leaves were transferred to small pots filled with Sunshine mix number 5 thoroughly watered with All Purpose Miracle Grow at the recommended concentration. These pots were

covered with a transparent plastic dome, then transferred to a larger phytochamber where they received 18 h of light at constant temperature of 21 ˚C. Plants typically flowered one month after stratification.

2.4.2 *Arabidopsis* floral dip genetic transformation

Arabidopsis transformation was carried out using a modified floral dip protocol that allowed for up to five treatments per flowering plant and resulted in a high number of subsequent transformants (Martinez-Trujillo et al. 2004). After 12 h of darkness and humidity, dipped plants were transferred back to the phytochamber where they were allowed to further develop before another dip one week later. Siliques were harvested for seed as they opened while the plant continued to flower, and dry seeds were stored at room temperature in 1.5 mL eppendorf tubes.

2.4.3 *Arabidopsis* transgenic seed selection

Depending on the binary vector used, transgenic *Arabidopsis* seed was selected for by either 50 µg/mL of hygromycin B (Sigma) or kanaymicin sulfate (Sigma) supplemented in media used for the seed sterilization and germination protocol described above in 2.4.1. Positive transformants were transferred to soil after two weeks on the media and grown under a dome using the same protocol described in 2.4.1.

2.4.4. Seedling growth assays

To test for complementation of the *pom1* mutant phenotype, or changes in seedling growth rate in darkness or light, *Arabidopsis* seed from the T2 generation were plated in thin lines on grided square petri dishes containing only 2.35 g/L Murashi-Skoog salts (Sigma) and 6 g/L agar (Sigma), at pH 5.7 and stratified for 72 h at 4 ˚C. Seedlings tested for their sucrose enhanced phenotype were plated on similar media with 4.5% w/v sucrose added. Plates to undergo dark treatment were

carefully wrapped in tinfoil, and all plants were placed sideways in a growth chamber for four days under conditions of 18 h light per day at a constant 25 ˚C. Plants were imaged at the end of four days, using an Epson flatbed scanner.

2.5 Poplar growth conditions, transformation, and tissue culture

2.5.1. Agrobacterium mediated poplar transformation in tissue culture

Preparation of woody plant media (WPM) was accomplished by following the protocol described by McCown and Lloyd (1980). The following protocol kindly provided by Dr. Shawn Mansfield was then followed, using agro bacterium EHA-105 (Hood et al. 1993) and poplar clone 717-1B4.

Day 1

1. Overnight cultures of *Agrobacterium* containing large and small *PopCTL1* or *PopCTL2* RNAi constructs in pART27 were grown in liquid LB medium containing 25 ug/mL of rifampicin, 25 ug/mL of gentomycin, and 50 ug/ul of Kanamycin.
2. Filter papers were wrapped in tin foil and autoclaved. Media was made for each of the next 9 steps, including:
    - 2 litres of liquid WPM (step 4 and 5)
    - 25 petri dishes containing WPM .1/.1/.1 (0.1 µM of NAA, BA, and TDZ) agar (step 7)
    - 25 growth jars with WPM .1/.1/.1carb$^{500(ug/mL)}$cef$^{250(ug/mL)}$ (step 8)
    - 25 growth jars with WPM .1/.1/.1carb^{500}cef^{250}kan^{25} (step 9)

Day 2

3. *Agrobacterium* was grown overnight in WPM with 100µM filtered acetosyringone in ethanol.
4. Leaf discs were cut from 5 week old wild type 717 plants in sterile water using a #4 cork borer and placed immediately into liquid WPM in petri plates (~25 per plate).

Day 3

5. *Agrobacterium* grown overnight was diluted with WPM to an Optical Density of 0.1 – 0.2 at 600 nm absorbance.
6. 10ml *Agrobacterium* solution was placed in a 50ml falcon tube and co-cultivate with 25 discs for about ½ an hour with 75 to 100 rpm shaking at 28˚C.
7. Leaf discs were blotted dry with sterile filter paper and place abaxial side up (upside down) on WPM .1/.1/.1 in the dark.

Day 6

8. Leaf discs were moved to WPM .1/.1/.1 for callus growth and $carb^{500}cef^{250}$ antibiotics to kill *Agrobacterium* and again placed in the dark along with appropriate controls.

Day 9

9. Leaf discs were moved to WPM .1/.1/.1$carb^{500}cef^{250}kan^{25}$ in subdued light

Weekly

10. Culture jars were checked weekly for Agro or fungal contamination, and if found were transfered immediately to fresh WPM .1/.1/.1$carb^{500}cef^{250}kan^{25}$

5 Weeks

11. Distinct shoots or clumps from different discs were transferred to WPM0/0.01/0$carb^{500}cef^{250}kan^{25}$ to induce shoot formation.

9 Weeks

12. Elongated shoots were transferred to WPM0.01/0$carb^{500}cef^{250}kan^{50}$ for rooting.

13 Weeks

13. Rooted and good looking non-rooted tips were transferred to WPM0.01/0/0$carb^{500}cef^{250}kan^{75}$.
14. This was continued until a good number of shoots were rooting on both concentrations of kanamycin.

15. To clone lines and bulk up plant numbers, shoots were cut into sections with at least two nodes and put back into the rooting media.

2.5.2. Poplar growing conditions

An initial test trial took healthy wild type and *PopCTL2* 400 bp RNAi *in vitro* grown poplar plants of at least 5 cm in height and with well developed root systems, and planted them in 2 liter pots filled with well watered but unfertilized Sunshine Mix 5 soil. These were then grown for three months in a phytochamber with high air flow where they received water every second day,18 hours of light at constant temperature of 21 ˚C.

A more extensive trial transferred healthy *in vitro* grown poplar plants of at least 5 cm in height and with well developed root systems, and planted them in 1 liter pots filled with well watered Sunshine Mix 5 soil thoroughly soaked with All Purpose Miracle Grow at the recommended concentration. These plants were kept under transparent plastic domes at 18 h of light at constant temperature of 21 ˚C for one month as they became established. Healthy saplings were then transferred to a greenhouse in mid February where they received daily watering by flooding over a period of 3 to 10 months. After sectioning, poplars that were harvested for microscopy were transferred to a phytochamber with high air flow where they received water every second day,18 h of light at constant temperature of 21 ˚C . Only one branch was allowed to re-grow per tree and the rest were pruned with scissors. *PopCTL2* RNAi photographs in Figure 23 come exclusively from these re-grown trees.

2.6 Dissecting, light, UV fluorescence microscopy analysis

Thin hand sections were made using double edged Wilkinson Sword razor blades across and in transverse of *Arabidopsis* floral stems and hypocotyl/roots, or poplar stems. For increased resolution, a Leica vibratome was also used where possible

to cut 40 and 100 um thick sections of these tissues.

Both poplar and *Arabidopsis* sections were stained for 30 s with 1% toluidine blue in 1% sodium borate before addition of distilled water and immediate viewing. Lignified cell walls appeared turquoise coloured, while non-lignified walls stained different shades of purple. UV, bright and dark field illuminated cross and transverse sections were taken using a Zeiss Axioplan light microscope attached to a digital camera at the UBC Bioimaging facility and in the lab of Dr. Lacey Samuels.

Phloroglucinol staining of sections of *Arabidopsis* and poplar were performed as follows: samples were placed in a 1% phloroglucinol-HCl (1:1 water: EtOH solvent) solution for 10 min, and mounted in a 1:1 mixture of glycerol:6 N HCl and observed immediately. Sections were analyzed with bright and dark field illumination using a Zeiss Axioplan microscope at the UBC Bioimaging facility.

When observing large plant organs, GUS stained seedlings or thick poplar sections bright field microscopy using a Leica dissecting microscope and Spot32 camera and software were used in the UBC Bioimaging facility.

2.7 GUS expression analysis

GUS reagent solution was prepared by mixing a water based solution with concentrations of:

100 mM NaPO4, pH 7.0
0.1% Triton X-100
0.5% X-GLUC (bromochloroindoyl-b-glucuronide) in dimethyl formamide
2 mM potassium ferricyanide (K3Fe(CN)6)
2 mM potassium ferrocyanide (K4Fe(CN)6)

Whole seedlings, plant parts, or handcut floral stem or root sections were placed in the cold GUS solution, then put under full vacuum for 15 minutes as described in (Stomp 1992). Samples were incubated at 37 ˚C for 14 h or until a strong blue

colour developed. Large *Arabidopsis* organs were visualized using a Leica dissecting microscope and Spot32 camera and software, while thin GUS stained floral stem or root sections were visualized using a Zeiss Axioplan microscope and Q-Capture software at the UBC Bioimaging facility.

Chapter 3: Bioinformatic analysis of *CTL*s

3.1 Introduction

Bioinformatic approaches can allow gene discovery and predictions about gene function using electronically available sequence, expression, structural data and various tools. These *in silico* analyses can thus guide "wet lab" based work. For example, as previously mentioned, *AtCTL2* was discovered by sequence homology to *AtCTL1* using BLAST analysis in Genebank. These genes were both shown to be distinct from true chitinases by phylogenetic analysis (Figure 3; M.T. Hauser, unpublished)

Before I started my project, BLAST and MEGABLAST analysis against GenBank, using the coding regions of the *AtCTL1* gene yielded a number of homologous *CTL* sequences including most notably, *PintaCTL1* from *Pinus taeda*, both *Physcomatrella patens* homologues *PhypaCTL1* and *PhypaCTL2,* five new dicot *CTLs,* and five new monocot *CTLs.* (B. Hamberger, unpublished). Blast analysis of poplar EST databases had shown the presence of two poplar *CTL* genes, and analysis of the Genome BC Treenomix EST collection confirmed the presence of a second chitinase-like in poplar *PopCTL2* (B. Hamberger, unpublished).

While the biochemical function of *AtCTL1* or any CTL protein is not yet known, unpublished results on the recombinant protein expressed in *E. coli* indicates it does not have *in vitro* catalytic activity against a chitin substrate (C. Douglas, unpublished) This finding is supported by amino acid alignments amongst known chitinases and CTLs, suggesting the glutamate residues are important for hydrolytic activity in chitinases are not conserved in CTLs (Zhang et al. 2004).

As previously discussed, *AtCTL1* is implicated in primary cell wall development based on the *pom1* mutant phenotypes. A major function for *AtCTL2* has not been revealed by phenotypes in available *Atctl2* mutants (Brown 2005; Persson et al. 2005), although it has been predicted to be involved in secondary cell wall formation based on microarray analysis in developing *Arabidopsis* floral stems (Ehlting et al. 2005). Further, *AtCTL* expression data from the e-FP program from the University of Toronto (Vinegar et al. 2006) suggests wide expression of this gene. *AtCTL1* seems to be expressed at moderate levels in all *Arabidopsis* tissues at all stages of development except mature pollen, senescing leaves or sepals. Although also expressed in most tissues, *AtCTL2* is predicted to be most strongly expressed in inflorescences, roots, petioles, heart stage embryos, siliques and flowers, but has no expression in mature seeds, senescing leaves, meristems, or cotyledons. Primary cell walls are found in every cell of *Arabidopsis* so it makes sense to see near global expression of *AtCTL1* in growing plants, but this predicted pattern of *AtCTL2* expression does not lead to any clear predictions regarding function, aside from the high expression in inflorescence stems. To help clarify a relationship between expression and function, tissue and cell specific expression should be assayed, since *in silico* approaches only show organ specific data. It is likely that these genes are not expressed in all cell types within the organs described above and thus our picture of *CTL* expression is incomplete. The hypothesis that *CTLs* have distinct cell type-specific expression patterns is tested in Chapter 4 of this thesis.

A different approach to elucidate gene function using gene expression uses genes sharing similar expression patterns with our genes of interest. Dr. Bjoern Hamberger in the Douglas lab carried out a co-expression analysis by submitting *AtCTL1* and *AtCTL2* as queries to the GenExpress program (Segal et al. 2005) and identified the top 10 genes with most similar expression patterns. Reiteration of this process, by the same analysis of the 10 genes generated a network of genes that share expression patterns with *AtCTL1* and *AtCTL2* (Figure 5).

According to this analysis, *AtCTL1* is closely co-expressed with genes encoding KORRIGAN, COBRA, ACC oxidase and most interestingly, the primary cell wall cellulose synthases CesA3 and CesA5 (Cosgrove 2005), re-enforcing the idea that it is involved in primary cell wall development. *AtCTL2* on the other hand is co-expressed with *IRX1*, *IRX3*, and *IRX5*, genes encoding cellulose synthase subunits that have all been previously shown to be involved in secondary cell wall development (Taylor et al. 2000). Based on this analysis, and supporting expression data, we make the prediction that *AtCTL2* is a protein involved in secondary cell wall synthesis and possibly with cellulose production or modification in the layers of the secondary wall, while *AtCTL1* is involved in primary cell wall development. Similar predictions have been made for both genes by other groups working independently (Brown 2005; Persson et al. 2005).

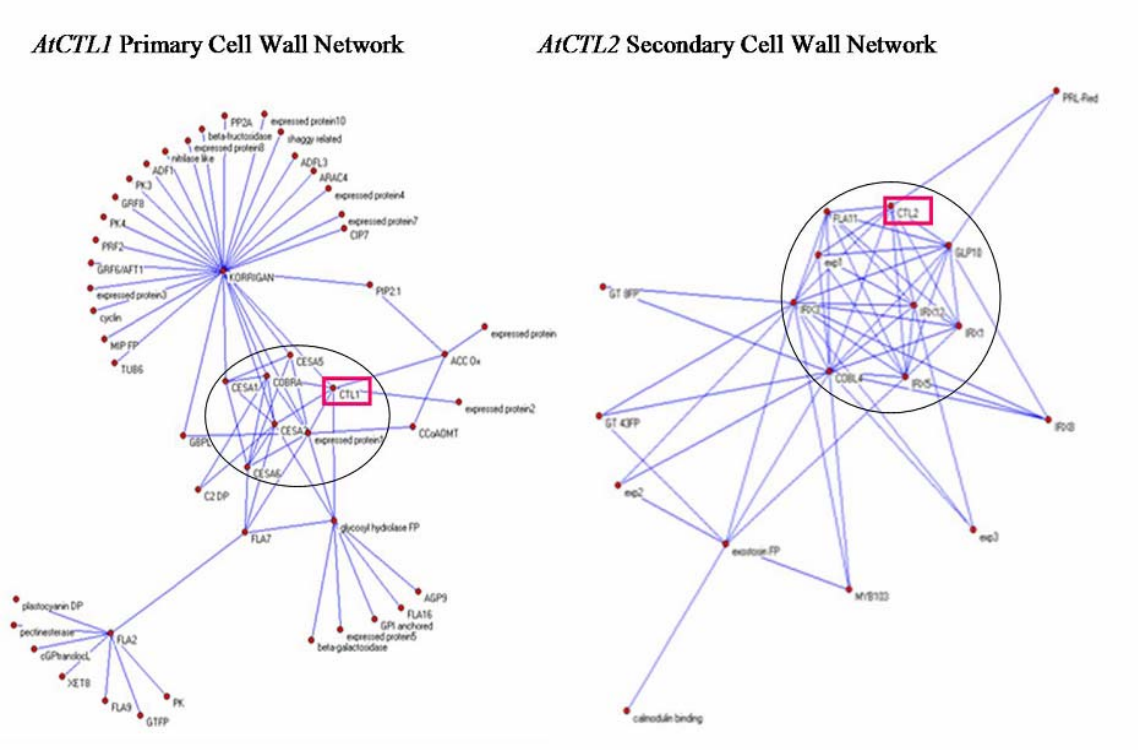

Figure 5: Co-expression analysis of *AtCTL1* and *AtCTL2*. The analysis was performed by Dr. Hamberger using using GenExpress (Segal et al. 2005). *CTL* genes are highlighted by a pink square and the immediate cellulose synthase co-expression network by a black circle.

Additional *CTL* gene discovery and phylogenetic analyses should help establish relationships between *Arabidopsis* CTLs with predicted functions based on *in silico*

analyses, and *CTL* genes found within other plant lineages, including poplar. This would also help to reveal patterns of *CTL* evolution and divergence amongst all plants and possibly other organisms. Finally, amino acid alignment of selected chitinases and CTLs from these lineages could shed light on potential biochemical properties of CTLs in relation to known chitinases.

3.2 Results

To increase the number of *CTL* sequences available for phylogenetic analysis, I screened public and private EST databases as listed in 2.1.1 using BLAST for possible *CTL* genes. Table 4 gives the name, species, publication source, and Genebank ID for each EST, as available. Two major phyla of land plants were discovered to contain *CTLs*; Bryophyta represented by *Physcomitrella patens*, and Spermatopsida represented by a number of gymnosperms and angiosperms. Of the other extant phyla of land plants, only Lycopsida represented by *Selaginella moellendorffii* has any appreciable amount of public genomic information, but only class I chitinases with greater similarity to the true *Hordeum vulgare* chitinase CHI26 than *CTLs* were found (data not shown). No *CTL* sequences were found in the single cell green algae *Chlamydomonas reinhardtii,* nor the multicellular red algae, *Porphyra yezoensis*, for which complete genome sequence information is available, suggesting that *CTL* genes encoding proteins with significant similarity to *Arabidopsis CTLs*, evolved after the evolution of the land plant habit.

Within the Spermatopsida, most species were found to contain a maximum of two *CTL* genes. *Gossypium hirsutum* was the exception and was found to contain at least three *CTLs*, including *GoshiCTL1* and *GoshiCTL2* which have been previously reported to be associated with secondary cell wall development (Zhang et al. 2004). That only two *CTLs* were usually observed in any single species is an especially important observation in *Arabidopsis*, poplar, and rice since these plants have fully sequenced genomes. Identification of two *Pinus taeda* and two *Picea glauca CTLs*, in addition to a *CTL* in *Cyclas rumphii* and *Ginko biloba*, was significant, since no Gymnosperm *CTLs* had been reported to this date.

Table 4: CTL genes/ESTs included in phylogenetic analysis and their Genbank ID.

Name	Species	Genebank ID and publication
HorvuCHI26	*Hordeum vulgare*	576565 (Leah et al. 1991)
PhypaCTL1	*Physcomitrella patens*	100362431
PhypaCTL2	*Physcomitrella patens*	67723837
PicglCTL1	*Picea glauca*	83392172, 83381286, 83390845, 70633925
PicglCTL2	*Picea glauca*	49061832, 70658804, 83380632, 83377852
PintaCTL1	*Pinus taeda*	67048305, 67048374
PintaCTL2	*Pinus taeda*	48936546, 48936546, 49451360
GinbiCTL	*Ginko biloba*	67051174, 67050807, 27918838, 66989050
CycruCTL	*Cyclas rumphii*	27915704, 27914102, 66986591, 66986572
OrysaCTL1	*Oryza sativa*	32979588
OrysaCTL2	*Oryza sativa*	51979696
SacofCTL	*Saccharum officinarum*	35023090, 35941565, 35012997, 35116451
ZeamaCTL	*Zea mays*	110530097, 76290204
HorvuCTL	*Hordeum vulgare*	16333065
TriaeCTL	*Triticum aestivum*	70969400, 110427949
AtCTL1	*Arabidopsis thaliana*	17226330 (Zhong et al. 2002)
AtCTL2	*Arabidopsis thaliana*	42564264
PopCTL1	*Populus trichocarpa*	3854865, 33452044, 33455768, 33455966,33456434
PopCTL2	*Populus trichocarpa*	46839267, 73870349
VinviCTL1	*Vitis vinifera*	110694702
VinviCTL2	*Vitis vinifera*	33407264, 110364977
GoshiCTL1	*Gossypium hirsutum*	32401252 (Zhang et al. 2004)
GoshiCTL2	*Gossypium hirsutum*	34016875 (Zhang et al. 2004)
GoshiCTL3	*Gossypium hirsutum*	31072749
SoltuCTL	*Solanum tuberosum*	39820453, 39825041, 39831061, 39834279
PissaCTL	*Pisum sativum*	37051095
BranaCTL	*Brassica napus*	56842897, 32501124
CitsiCTL1	*Citrus sinensis*	45449003, 46209309, 34519941, 34520272
CitsiCTL2	*Citrus sinensis*	21650175, 21650413, 21651525, 42477697
GlymaCTL	*Glycine max*	26048416, 26048212,
PontrCTL	*Ponc trifolia*	55286693, 55288819, 55293044, 55287218
MaldoCTL	*Malus domestica*	71818780
MedtrCTL	*Medicago trunculata*	83667010, 27403940

Many *CTLs* in non-fully sequenced genomes were represented by multiple ESTs, allowing *in silico* sequence contigs to be generated that represent predicted full length or near full length *CTL* sequences to be deduced I aligned the predicted amino acid sequences using a conserved 290 amino acid region from these 32 CTL proteins, excluding a variable N-terminal region of 23 to 47 amino acids pertaining to *AtCTL1* residues 1 to 48 (Figure 7). This alignment included the true *Hordeum vulgare* chitinase, CHI26. The alignments were used for phylogenetic

reconstructions, as described in 2.1.2. Predicted partial protein sequences used in this analysis are given in Appendix A.

As shown in Figure 6, the reconstructed CTL phylogeny grouped the proteins into clades that correspond to known land plant lineages, with separate Bryophytes and Spermatopsida clades, gymnosperm and angiosperm clades, and monocot and dicot clades. Within the dicot clade, there was support for a further phylogenetic distinction between *CTLs* potentially involved in primary vs. secondary wall formation. *AtCTL1* and *AtCTL2* were found within distinct dicot clades with moderate bootstrap support (73). Both cotton CTLs, *GhCTL1* and *GhCTL2* which were previously characterized as being involved with secondary cell wall development (Zhang et al. 2004) grouped with *AtCTL2*, while a new cotton *CTL* identified from EST data, *GhCTL3* was placed in the same clade as *AtCTL1* further suggesting differences in amino acid sequence may be useful in distinguishing CTL function. Functional relationships of poplar *CTLs* to other *CTLs* were more difficult to predict, since there was no support for a close relationship of *PopCTL2* with either *AtCTL1* or *AtCTL2. PopCTL1* grouped with *AtCTL2*, but was separated from *PopCTL2*. Lack of bootstrap support (38) for this topology makes the prediction of orthology to *AtCTL2* difficult.

Given that limited sequence information is available, gymnosperm *CTLs* were not as well represented in this analysis as angiosperm *CTLs*, but the two duplicated *CTL*s in *Picea glauca* and *Pinus taeda,* respectively were clearly in different clades. This was supported by a high bootstrap value of 95, suggesting independent evolution of two *CTL* genes within the gymnosperm lineage. Monocots also displayed this clear grouping of *CTLs* into distinct clades, with a very significant bootstrap value of 95 and rice representation in each indicating independent evolution after an initial duplication in monocotyledons. The topology of the reconstructed tree, with the monocot clade placed very close to the dicot clade containing *AtCTL1* suggests a possible origin of the monocot *CTLs* from a single ancestral gene common to monocots and dicots.

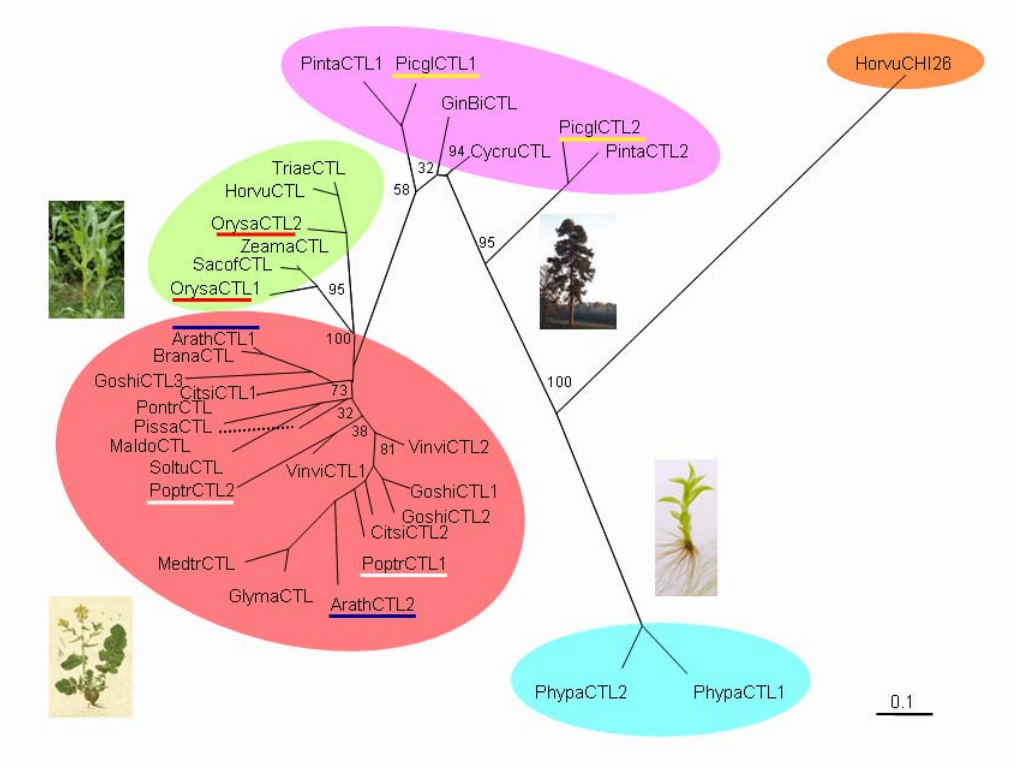

Figure 6: Phylogenetic analysis of plant chitinase like genes, based on alignment of conserved 290 amino acid region. A UPGMA tree was reconstructed from aligned protein sequences that were mined from NCBI and Genome BC EST libraries. Branches are drawn to scale, with the scale bar showing 0.1 substitutions per site. Major plant taxonomic divisions are indicated by coloured balloons and coloured bars highlight the position of poplar, rice, spruce and *Arabidopsis* CTL pairs. Bootstrap values are shown for major divisions. The outgroup is a barley (*Hordeum vulgare)* family 19 chitinase called CHI26/1cnsA. For this analysis, *AtCTL* and *PopCTL* were changed to *ArathCTL* and *PoptrCTL* respectively.

The obvious phylogenetic differences between gymnosperm and angiosperm *CTLs* are apparent in the amino acid alignment depicted in Figure 7. To qualitatively guide the alignment, data on amino acid conservation in chitinase evolution were included (Bishop et al. 2000). Focusing on the most highly conserved region between position 70 and 327 in CHI26, one can see that some amino acids important for chitinase function such as the catalytic glutamic acid at position 169, the tyrosine at position 200, and the asparagine at 201, are shared between the representative chitinase and chitinase likes.

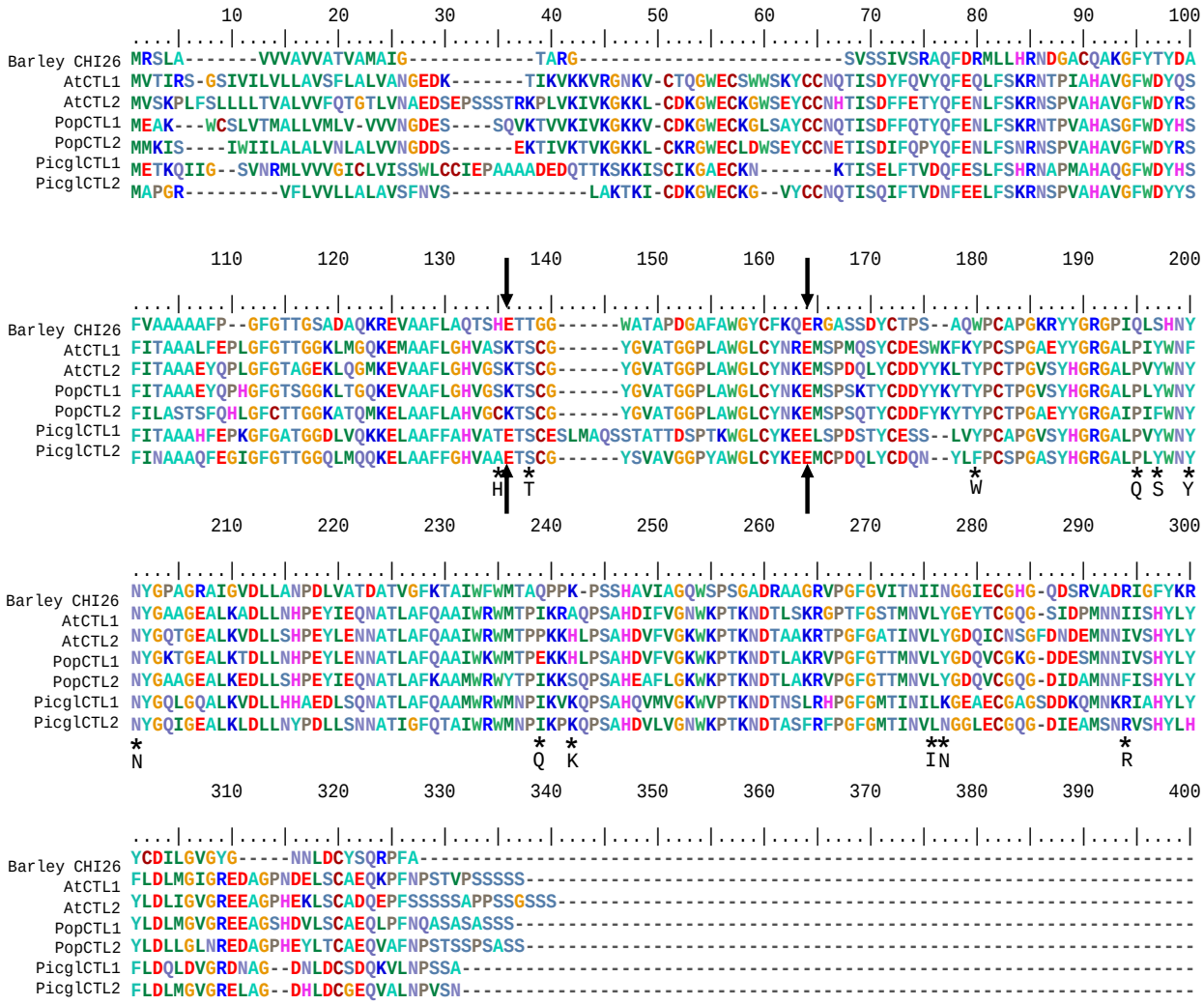

Figure 7: Primary structure alignment of *Arabidopsis*, poplar, and *Picea glauca* CTLs. Putative substrate binding residues (Bishop et al. 2000) are indicated with an asterix and the expected amino acid below it. The position of the two catalytic glutamic acids at residues 136 and 164 are indicated with arrows.

As previously noted (Passarinho and de Vries 2002; Zhang et al. 2004) substitution of a second conserved (acidic) catalytic glutamic acid residue at position 136 with a (basic) lysine residue likely abolishes chitinase activity. It is very interesting to note, that this substitution is seen in all *Arabidopsis*, poplar, and other angiosperm CTLs but not in *Picea glauca, Pinus taeda, Ginko biloba, Cyclas rumphii* or *Physcomitrella patens* CTLs. This suggests the latter species may retain CTLs with catalytic potential while angiosperm CTLs have lost that biochemical function. This idea is further supported by a similar pattern of amino acid residue retention in

predicted substrate binding sites shared between *Picea glauca* CTLs and the true chitinase CHI26 at positions 242, 276, and 294; positions with amino acid substitution in *Arabidopsis* and poplar CTLs. This finding suggests evolutionary relatedness of *Physcomitrella* CTLs and gymnosperm CTLs that that is not obvious in Figure 6.

To gain further possible insights into functional similarity between duplicated *CTL* genes, the gene structures of *Arabidopsis* and poplar *CTLs* were compared (Figure 8). Both sets of *Arabidopsis* and poplar *CTLs* have similar intron-exon patterns, with introns of similar sizes and locations in *AtCTL1* and *AtCTL2,* further suggesting evolution from a common ancestral gene. It was interesting to observe that *AtCTL1* has an intron of about 250 bp in its 5' UTR. Since none of the other genes shared this feature, this suggests recent acquisition of an intron in the *Arabidopsis* lineage.

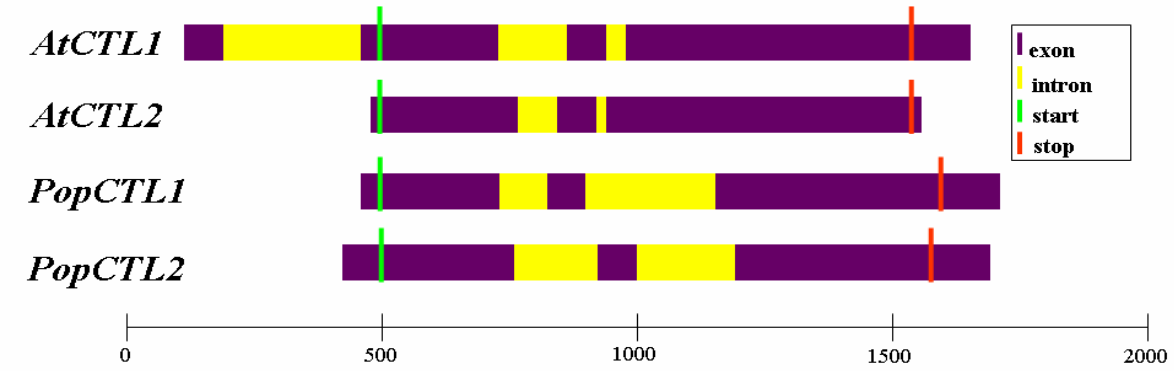

Figure 8: Gene structure comparison of genomic DNA representing *Arabidopsis* and poplar *CTL* genes. Models are drawn to scale in terms of nucleic acid base pairs.

3.3 Discussion

While chitinases were found in the single celled alga *Chlamydomonas reinhardtii,* and multicellular red alga, *Porphyra yezoensis* (data not shown) no homologous *CTLs* were observed. My analysis thus shows that *CTL* genes are likely to be a land plant specific family of genes. Furthermore, the presence of two *CTL* genes (three in cotton) appears to be a trait common to all land plant lineages examined. This suggests that two copies were found in early land plants that gave rise to

gymosperms and bryophytes. It further suggests that both modern angiosperm *CTLs* are derived from a single *CTL* after the divergence from gymnosperms. Evolution generally favors functional diversification of gene families following gene or genome duplication and this is supported in *Arabidopsis* by the observation that gene families tend to be about twice as big as in *Physcomitrella patens* (Rensing et al. 2002), and further by the observed retention of duplicated genes in poplar following a whole genome duplication event 60 million years ago (Tuskan et al. 2006). This trend is presumably more pronounced in plants like poplar which have undergone additional whole genome duplication events (Tuskan et al. 2006). However, the fully sequenced genome of poplar, like *Arabidopsis* and *Physcomytrella*, still only has only two CTLs.

The nearly universal trait of a two-member *CTL* gene family in land plants suggests that there emerged two CTL subfunctions as early as 400 million years ago, and that the loss of one copy has been generally selected against over time. Conversely, it shows that there has been no selection for gain of an extra gene product from a third or fourth copy of the gene. The only observed exception was the presence of two *AtCTL2*-like *CTLs* in cotton. Since this plant devotes a large amount of resources to secondary wall formation during cotton fiber differentiation over a short time, this could be an exceptional case in which there has been selection for a second *AtCTL2*-like gene.

The roles played by the duplicated *CTL* genes are hinted at by co-expression analysis done by the Douglas lab and two others independently (Brown 2005; Persson et al. 2005). This data strongly suggests that *AtCTL1* is involved in primary cell wall development, and that *AtCTL2* has a function in the formation of secondary cell walls. Expression patterns in developing secondary xylem in poplar seem to suggest the same division of primary and secondary cell wall development for *PopCTL2* and *PopCTL1* respectively (Figure 2). However, since *PopCTL1* did not clearly group with *Arabidopsis* CTLs in the phylogenetic reconstruction (Figure

6), and there were no unique commonalities in amino acid alignment (Figure 7) or gene structure comparison (Figure 8) between these genes, it is not possible on the basis of this data to make additional inferences about orthology. It is also difficult to speculate on evolutionary or functional relationships between characterized Angiosperm *CTLs* and *Physcomitrella CTLs*, but it is interesting to ask what the functions of two *CTLs* may be in a species which does not develop a specialized vascular system requiring secondary cell walls (Schaefer and Zryd 2001). *Physcomitrella* does share commonalities in hormone signaling (Decker et al. 2006) and primary cell wall development (Schipper et al. 2002; Lee et al. 2005), but without more knowledge of CTL function or expression in *Physcomitrella*, it is impossible to speculate whether the two PhypaCTLs serve the same function(s) as those in higher plants.

CTL genes are observed to exist in gymnosperms as well, a lineage that is estimated to have diverged from angiosperms about 300 million years ago (Kenrick and Crane 1997). Not surprisingly, gymnosperm CTLs appear quite different in amino acid sequence to Angiosperm CTLs, with PicglCTL1 having 43% amino acid identity to AtCTL1, relative to 58% for PopCTL2 to AtCTL1. A significant part of this difference arises from stronger homology to true chitinases and specifically, what appears to be an intact chitinase catalytic site, and a number of conserved residues in the putative substrate binding sites. While this does not mean that the gymnosperm CTLs do indeed retain chitinase activity, it does suggest that mechanisms in cell wall formation have functionally diverged in angiosperms relative to gymnosperms, such that distinct CTL proteins, are used. Interpretation is further complicated by the observation that of a number of genes with similarity to chitinases exist in diverse plant and animal species that lack an intact chitinase catalytic site, but still have a developmental function. One example is the NOD factor upregulated chitinase homologue *Srchi24*. While this gene is involved in plant nodule formation in *Sesbania rostrata*, was shown to lack hydrolytic activity on the NOD factor isolated from the bacterial symbiont due to the change in a catalytic glutamate into a lysine (Goormachtig et al. 2001). In a contrasting

experiment, mutation of either catalytic glutamate in a tobacco class 1 chitinase was shown to transform it into a chitin binding lectin-like molecule completely devoid of chitinase activity (Iseli-Gamboni et al. 1998) which strongly suggests a CTL without both glutamates is catalytically inactive against chitin, as we suggest. It will be interesting to test whether gymnosperm and bryophyte CTLs have chitinase activity, and to test whether they play the same role in wall development that CTLs seem to play in *Arabidopsis*.

Chapter 4 Functional analysis of *AtCTL1, AtCTL2*, *PopCTL1* and *PopCTL2*

4.1 Introduction

As discussed in Chapters 1 and 3, *Arabidopsis* and poplar CTLs have similar developmental expression patterns, and thus potentially similar developmental functions. *AtCTL2* and *PopCTL1* have similar expression patterns which associate with secondary wall formation and are thus putatively orthologous. Amino acid sequence alignments and phylogenetic reconstructions, showing conservation of two *CTL* genes in diverse taxa are also consistent with the prediction of subspecialized CTL function. In this chapter, I present experiments to test these hypotheses and help establish CTL function in primary and secondary wall development.

It has been previously suggested that mutants for *AtCTL2* have no observable cell wall phenotype although there is evidenced for alterations in non-cellulosic components of the wall (Brown 2005; Persson et al. 2005). This might be explained if secondary cell wall development is only partially compromised in these mutants. Presumably, only severely weakened secondary cell walls, or cells under heightened stress need the additional structural support provided by a normally thickened secondary wall before collapsing. However, even normally thickened vascular cell secondary walls may collapse under heightened stress as evidenced

by a study of tracheid structure under drought stress in pine needles (Cochard et al. 2004). It is also possible that partial functional redundancy exists between *AtCTL1* and *AtCTL2*, making an *AtCTL2* mutant phenotype difficult to observe. A knockout phenotype for a poplar gene orthologous to *AtCTL2* might show much more obvious phenotypes, since poplar makes a much larger commitment to secondary wall formation during secondary xylem formation. In this chapter, a poplar *AtCTL2* orthologue (e.g., *PopCTL1*) was downregulated to test for novel phenotypes not present or obvious in *Arabidopsis*, which does not rely intensively on vascular cambium activity to complete its life cycle.

In an effort to better characterize the *AtCTL1* phenotype, microarray experiments performed in our lab were performed comparing global gene expression profiles in wild-type vs. the *pom1* mutant in 4-day old etiolated seedlings. Genes differentially expressed (down regulated) in *pom1* included a significant number of genes related to auxin responses (B. Hamberger and C. Douglas, unpublished). This finding led to the hypothesis that auxin homeostasis, synthesis, transport or early steps in the signal transduction cascade may be affected by the mutation. Since auxin has been shown to affect patterning in root and inflorescences in *Arabidopsis* (Hobbie et al. 2000; Hamann et al. 2002) changes in auxin transport or function in the *pom1* mutant might help explain aspects of its phenotype. Auxin is also very important in secondary growth as a regulator of cambial activity and xylem formation (Uggla et al. 1996). As a way to further investigate this observation by indirectly assessing auxin distribution, a *DR5* synthetic auxin-responsive *promoter::GUS* fusion was crossed into the *pom1* background by B. Hamberger (unpublished), and in this chapter I analyzed *GUS* expression in these plants in parallel to *DR5::GUS* expression in wild-type plants.

Although *AtCTL1* and *AtCTL2*, as well as *PopCTL1* and *PopCTL2* have different expression patterns based on microarray expression profiling, little is known about the cell and tissue specific expression patterns of these genes. Also, since phylogenetic analysis did not generate a clear prediction of orthology between

CTLs in *Arabidopsis* and poplar, a comparison of these gene expression patterns in *Arabidopsis* using promoter::*GUS* fusions for promoters from *AtCTL1, AtCTL2, PopCTL1, and PopCTL2* might help to support putative orthology (e.g. by revealing common expression patterns).

Another approach to establishing whether proteins have common biochemical functions is phenotype complementation. To test conservation of CTL function, I transformed the *pom1* mutant with *Arabidopsis* and poplar *CTL* transgenes and tested for phenotype rescue. Since all *Arabidopsis* and poplar CTLs are predicted to have extracellular secretion peptide signals they should all be secreted to the cell wall where they could potentially replace the AtCTL1 (POM1) function. This could be further extended to the two *Picea glauca* CTLs included in this study, however *PicglCTL1* was not predicted by WOLF PSORT to have an extracellular targeting signal, while *PicglCTL2* was (data not shown).

In addition to mutant analysis to help determine gene function, overexpression can yield important clues about gene function. Overexpression of the *Arabidopsis* gene *IFL1*/*AVB1* results in an incredible change in vascular bundle formation and distribution in floral stems (Zhong and Ye 2004), while its null mutant results in reduced auxin transport down the inflorescence and is accompanied by reduced xylem and interfascicular fiber formation (Zhong and Ye 2001). Because they may have distinct developmental functions, deregulation of *AtCTL1* and *AtCTL2* expression through CaMV 35S promoter overexpression could result in contrasting phenotypes, which are different from loss of function (mutant) phenotypes..

In order to test these predictions, I carried out a series of experiments on *Arabidopsis* and poplar *CTL* genes in both plants, in collaboration and in parallel with my colleague Dr. Bjoern Hamberger. Dr. Hamberger generated the following materials and data used in my experiments;

- A homozygous *AtCTL2* T-DNA insertion line, in which the location of the insertion was verified.

- Construction of 35S::*AtCTL2* constructs and generation of transformants in the *pom1* mutant background
- Construction of *AtCTL2*::GUS fusions and generation of transformants in wild-type, and initial GUS expression analysis
- DR5::GUS lines in the *pom1* background and initial GUS expression analysis
- Generation of *PopCTL2* RNAi plasmids of two different insert sizes

All other materials and experiments in this chapter were generated and carried out by me, and the results shown are from my own experiments, in some cases using materials generated by Dr. Hamberger (as above).

4.2 Results

4.2.1 A knockout phenotype for *AtCTL2* mutant *Arabidopsis*

Plants from a homozygous line with a T-DNA insertion in *AtCTL2* (*Atctl2-1*) were grown on MS media in darkness and in light supplemented with 4.5% sucrose in an assay similar to that performed for the original assay used to identify the *pom1* mutation. No difference was observed between Col-0 and *Atctl2* root development on high sucrose or hypocotyls growth in the dark (data not shown). To confirm a null allele for *AtCTL2* in this line, semiquantitative RT-PCR was performed on RNA from this line, as described in Table 2. This confirmed that *AtCTL2* expression was completely eliminated from these plants (data not shown). *Atctl2* plants that were grown on soil had no obvious phenotypic difference from Col-0, except for a possibly thinner stems (data not shown). However, sectioning of the bases of these inflorescence stems and staining with phloroglucinol indicate that there was a reduction in number of vascular bundles (Figure 9). The mean number of bundles in *Atctl2* (n= 13) was 6.0 and mean stem diameter was 0.75 mm. Wild type plants (n= 14) had an average of 7.6 bundles and diameter of 1.01 mm. Statistical confidence was shown to be greater than 95% for both averages, using both a parametric (two tailed student t-test) and non-parametric Kriskal-Wallis tests (Figure 9). Three of the 13 plants displayed ectopic lignification in pith cells in

interfascicular regions, and increased formation of phloem fibers in a pattern similar to that seen in *pom1*, but there was no observed associated change in cell expansion. These cells displayed thickened secondary cells walls in cross section and appeared to occur in proximity to underdeveloped vascular bundles with interfascicular fiber development over the region between the protoxylem and the pith (Figure 10).

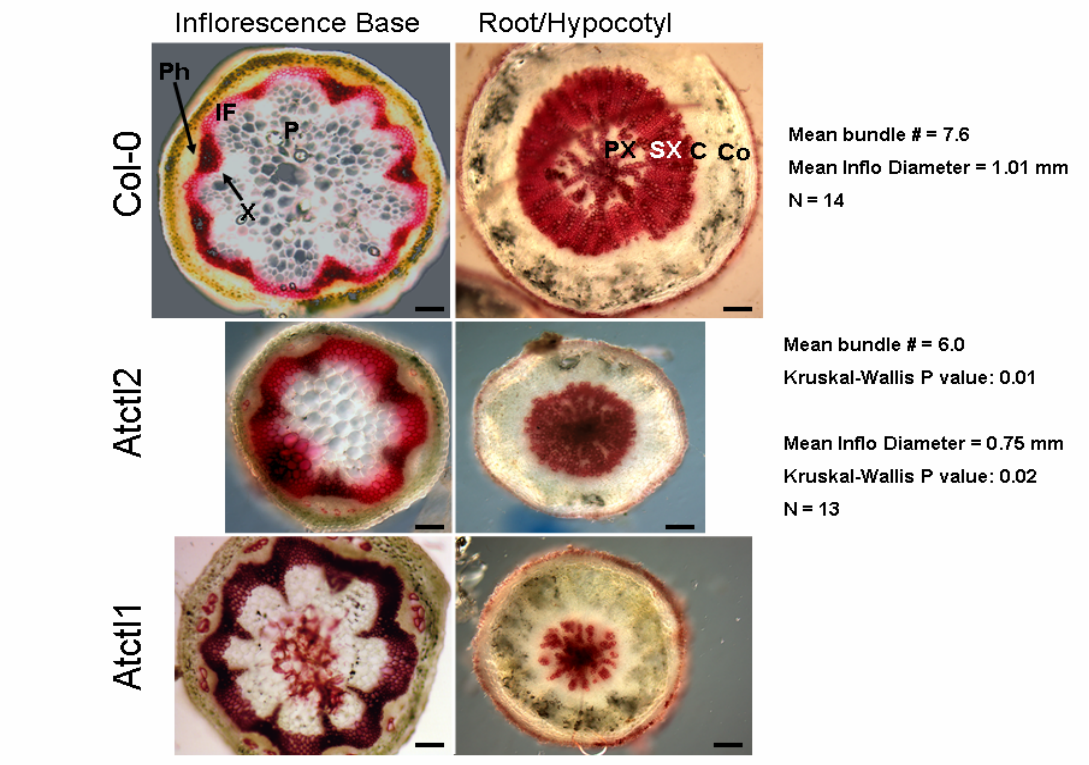

Figure 9: Sections of Col-0, *Atctl2*, and *Atctl1* root/hypocotyls and inflorescences taken at 1 cm from rosette level from 1.5 month old inflorescences. Sections were stained with phloroglucinol. Ph, phloem; X, xylem, P, pith; IF, interfascicular fibers, PX, primary xylem; SX, secondary xylem; C, cambium; Co, cortex. Scale bars: 100 um.

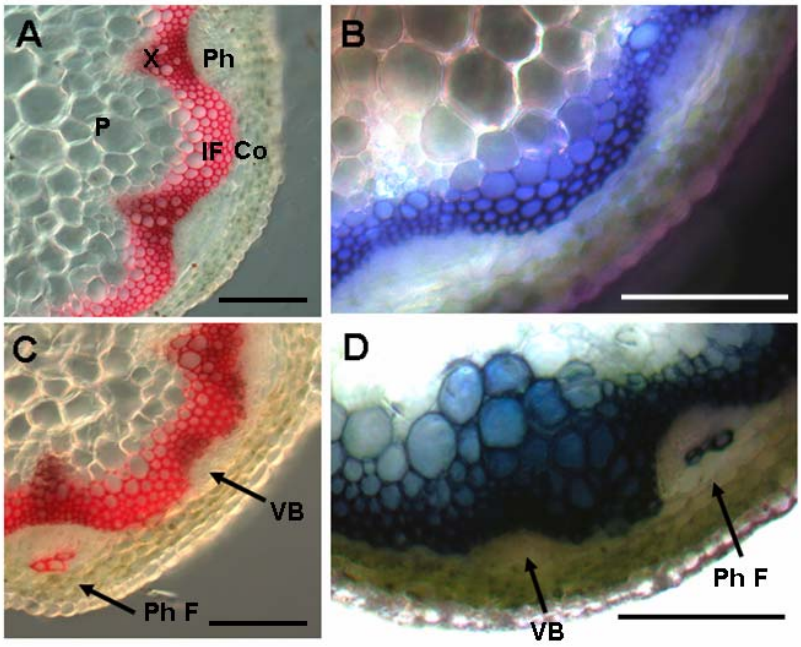

Figure 10: Handcut sections of Wt (A, B) and Atclt2 (C, D) inflorescences. Sections were taken at 1 cm above rosette level from 1.5 month old plants. A and B are Col-0, C and D are Atctl2 sections from different individuals. Sections were stained with phloroglucinol or toluidine blue. Ph, phloem; X, xylem, P, pith; IF, interfascicular fibers, Ph F, phloem fiber; VB, vascular bundle; Scale bars: 100 um.

4.2.2 Auxin involvement in the *pom1* phenotype as studied using the *DR5::GUS* reporter construct in Col-0 and *pom1*

In order to test the auxin responsiveness of *DR5::GUS* in the wild-type background, a proof of concept experiment was performed on 15 cm tall inflorescences by cutting halfway through the stem 1 cm above the level of rosette, followed by staining for GUS activity one week later as described in 2.7. Having severed four or more vascular bundles, GUS staining was observed above the wound site, but not below, confirming that auxin flow had been altered by the cut and that the transgene was working well (Figure 11). As a side note, it was interesting to observe that auxin accumulation correlated with ectopic deposition of lignin in the pith, cell expansion and division in the cortex, and what appeared to be greatly increased formation of xylem around the outside of the normal stele.

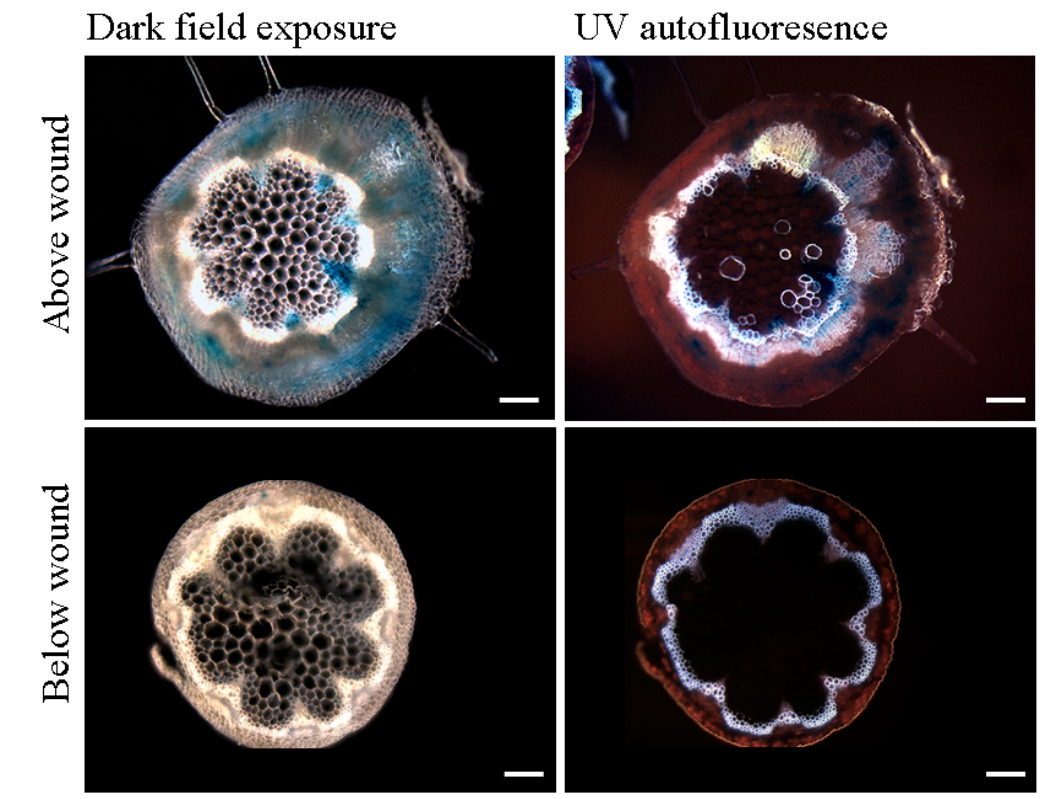

Figure 11: GUS assay testing activity of the DR5::GUS construct in Col-0. 25cm tall floral stems were cut with a razor blade, and sectioned above and below the site of wounding one week later. Photos were taken using both dark field and UV autofluoresence. Scale bars: 100 µm.

A seedling based assay was then carried out using the *DR5::GUS* reporter gene in both Col-0 and *pom1* backgrounds. Plants were grown on plain MS media, MS media supplemented with 4.5% sucrose, and in complete darkness for 4 days before staining for GUS activity. Results are shown in Figure 12.

DR5::GUS expression in Col-0 was observed throughout the cotyledons and roots, with local maxima at the root tip, SAM, and root/hypocotyl transition zone. *pom1* seedlings had a dramatically lowered *DR5::GUS* expression in roots, where only the above maxima and patchy GUS expression in cotyledons were visible. Under normal conditions, *pom1* plants appear to be reduced in either auxin production, translocation, or sensing since *DR5* is a synthetic promoter element sensitive to changes in all three (Ulmasov et al. 1997). High sucrose was observed to reduce GUS expression in cotyledons of both Col-0 and *pom1.* Sucrose also altered root

DR5::GUS expression in *pom1*, but not in Col-0. In sucrose-treated *pom1* roots, patchy *DR5::GUS* activity observed in small local maxima within the swollen roots, which suggests a defect in auxin distribution.

The *pom1* etiolated hypocotyls were observed to be characteristically short as expected, and, in contrast to Col-0, *DR5::GUS* expression was observed thoughout the hypocotyls. However, this since the *pom1* hypocotyl is much shorter, this could be interpreted as compression of the normal developmental gradient observed in Col-0.

To assess whether disruption of auxin distribution might be responsible for the *pom1* phenotypes later in development, I sectioned one month old plants at the base of the inflorescences and at the root/hypocotyl junction. Results are shown in Figure 13.

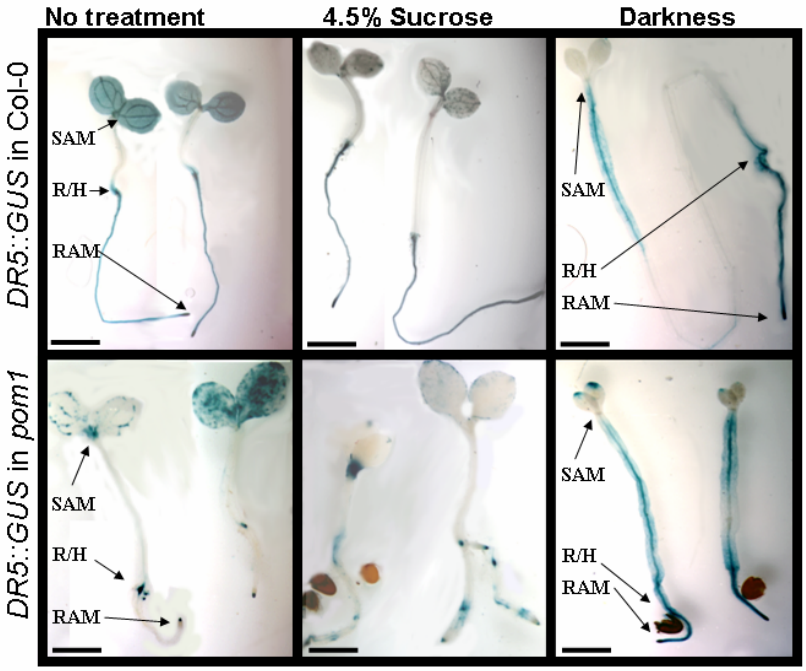

Figure 12: Comparison of DR5 driven GUS expression in 6 day old Col-0 or *pom1* seedlings grown in light, in darkness, or in light with 4.5% sucrose. All seedlings were stained for 14 hours at 37°C. Scale bars: 1 mm.

As predicted, GUS staining was observed in vascular bundles of Col-0, but was localized to the primary xylem and phloem at the base of the inflorescence and at the root/hypocotyls junction. In the *pom1* background, no GUS expression was detected in inflorescences or at the root/hypocotyl junction. This correlates with the reduced secondary xylem production seen at the root/hypocotyls junction and might partly explain the phenotype.

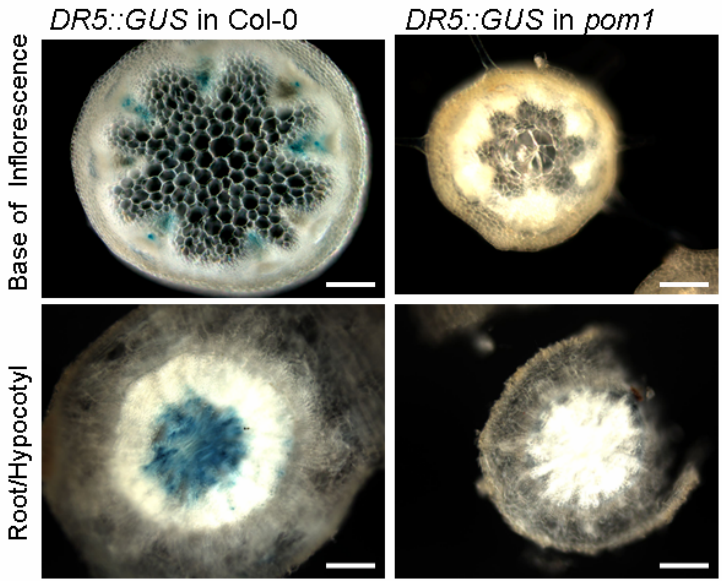

Figure 13: Comparison of handcut sections of floral stem and root/hypocotyl from *DR5::GUS* fusions in Col-0 or *pom1* backgrounds. One month old floral stems were sectioned at 1 cm from the base. Hypocotyls were sectioned from the region above the soil and below the rosette. Scale bars: 100 µm.

4.2.3 *Arabidopsis* and poplar CTL promoter ::*GUS* reporter constructs in Col-0

To compare *CTL* gene expression using an *in vivo* system, promoter::GUS constructs were prepared for *AtCTL1* and *PopCTL1* promoter regions as described in 2.3.7 and transformed into Col-0 *Arabidopsis* plants via floral dip. Selection and screening produced two lines for *AtCTL1::GUS* which displayed strong *GUS* expression, while multiple *PopCTL1::GUS* lines did not show any significant developmental expression in *Arabidopsis*. *AtCTL2* and *PopCTL2*, promoter::GUS lines were produced by Bjoern Hamberger, and two strongly expressing lines for each construct were included in this study.

Figure 14 shows the results of an initial assay for *GUS* expression using all four constructs. Grown for four days in the dark or in the light, *AtCTL1::GUS* plants displayed diffuse dark blue staining at the growing ends of the seedling, namely cotyledons and top and bottom of the hypocotyl and the entire root. Growth of these plants on a high sucrose media resulted in a reduction of cotyledon expression and did not affect root expression. Leaves showed a wound inducible response as shown in Figure 15. This pattern is strikingly similar to that of that directed by the *PopCTL2::GUS* construct, which showed much stronger expression and a slightly greater range of expression levels. A majority of *PopCTL2::GUS* seedlings accumulated a strong blue colour at local maxima part way up along the hypocotyl and this did not change with the addition of sucrose to the media.

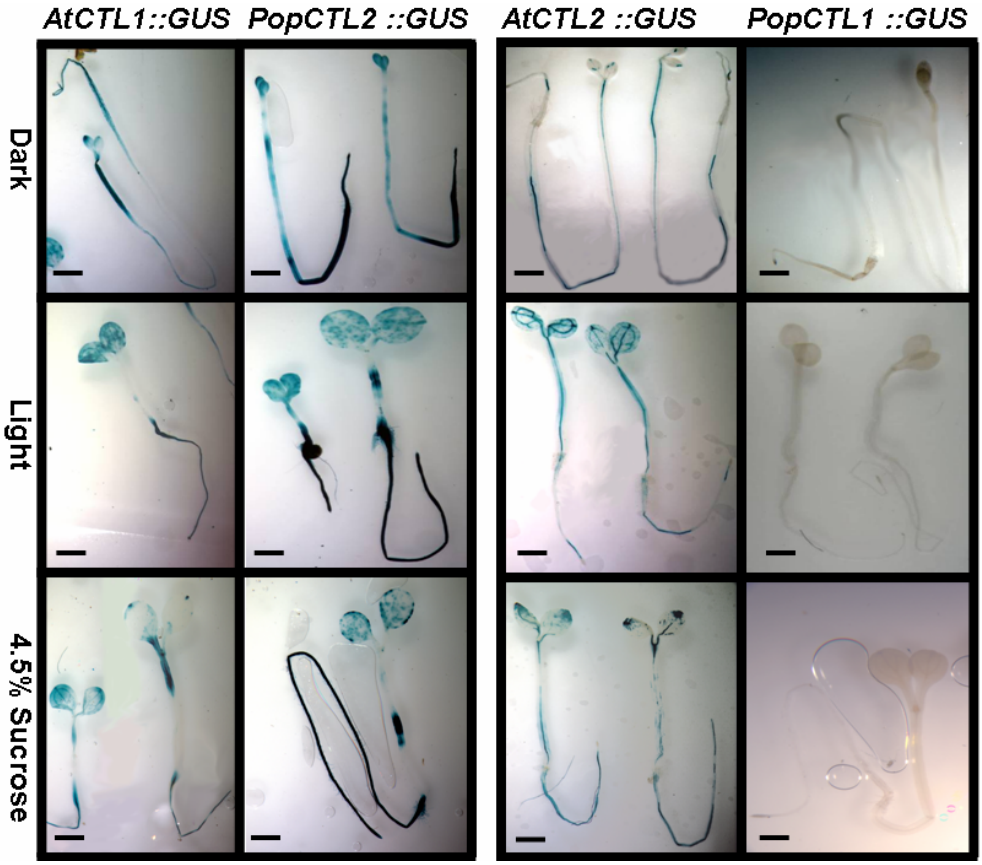

Figure 14: Comparison of *AtCTL1, AtCTL2, PopCTL1, PopCTL2 promoter::GUS* fusions in Col-0 at the 4 day old seedling stage grown in darkness, light or in light with 4.5% w/v sucrose. Scale bars: 1 mm.

In contrast to *AtCTL1::GUS* and *PopCTL2::GUS*, *AtCTL2::GUS* lines showed expression that co-localized with plant vasculature, including veins in the cotyledons, hypocotyls and root systems (Figure 14, 15). An interesting pattern observed in these plants was the discontinuous expression of *GUS* reporter along hypocotyl vasculature of etiolated seedlings, and similar expression along root vasculature of light grown seedlings.

Little or no *PopCTL1::GUS* expression was observed in multiple lines, although PCR based genotyping confirmed 6 independent lines assayed did indeed contain the construct (data not shown), and occasional GUS expression in trichomes and epidermal cells was observed in mature stems (see below).

To assay *CTL::GUS* expression patterns at the cellular level in mature plant tissues and in response to wounding, *CTL::GUS* plants were grown in soil and 15-cm tall inflorescences were harvested, sectioned and stained for GUS activity. Results are shown in Figure 16. Again, *AtCTL1* seems to have a similar pattern of expression to that of *PopCTL2*, with *GUS* expression in cortex, phloem, vascular bundle cambium, xylem and some pith cells near to vascular bundles. At the root/hypocotyls junction, both *GUS* constructs showed expression in the region

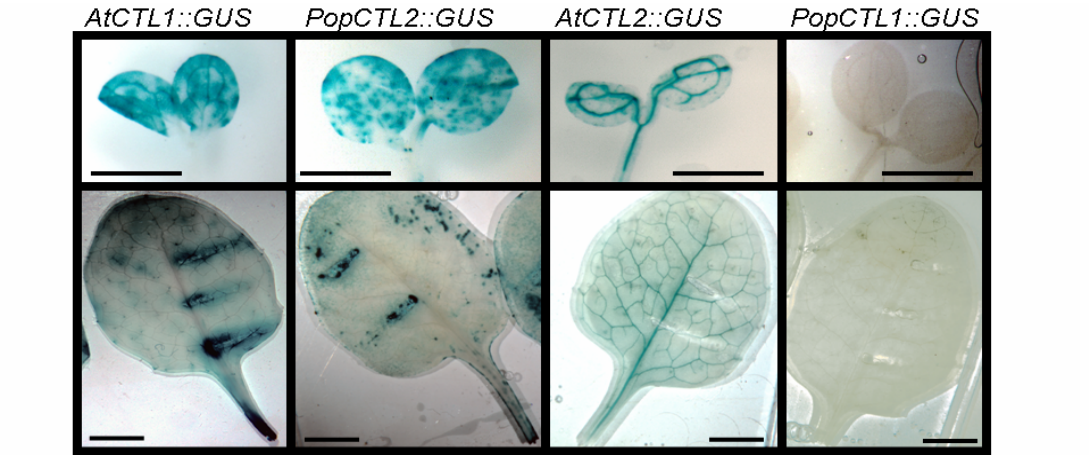

Figure 15: *DR5::GUS* expression in cotyledons and wounded leaves. Cotyledons from 6 day old *CTL::GUS* plants and leaves from 3 week old plants, one hour after wounding with forceps were stained with X-GLUC for 14 hours at 37°C. Scale bars: 1 mm.

corresponding to the vascular cambium, cortex, and some expression in xylem tissues, presumably xylem parenchyma cells.

In contrast, *AtCTL2::GUS* expression was confined to cells in which secondary cell walls develop. Inflorescences were observed to accumulate staining in xylem side of vascular bundles and in developing interfascicular fibers. Figure 16 shows that *AtCTL1* and *AtCTL2* seem to have overlapping expression only in the regions of vascular bundle cambium and developing xylem in inflorescences. In root/hypocotyls, the pattern of *AtCTL2* expression was very much more clearly localized to the secondary xylem at the root/hypocotyls junction, which may be another region of overlapping expression between *AtCTL1* and *AtCTL2*. No reproducible *PopCTL1::GUS* expression was observed and occasional expression was only seen sometimes in trichomes and epidermal cells.

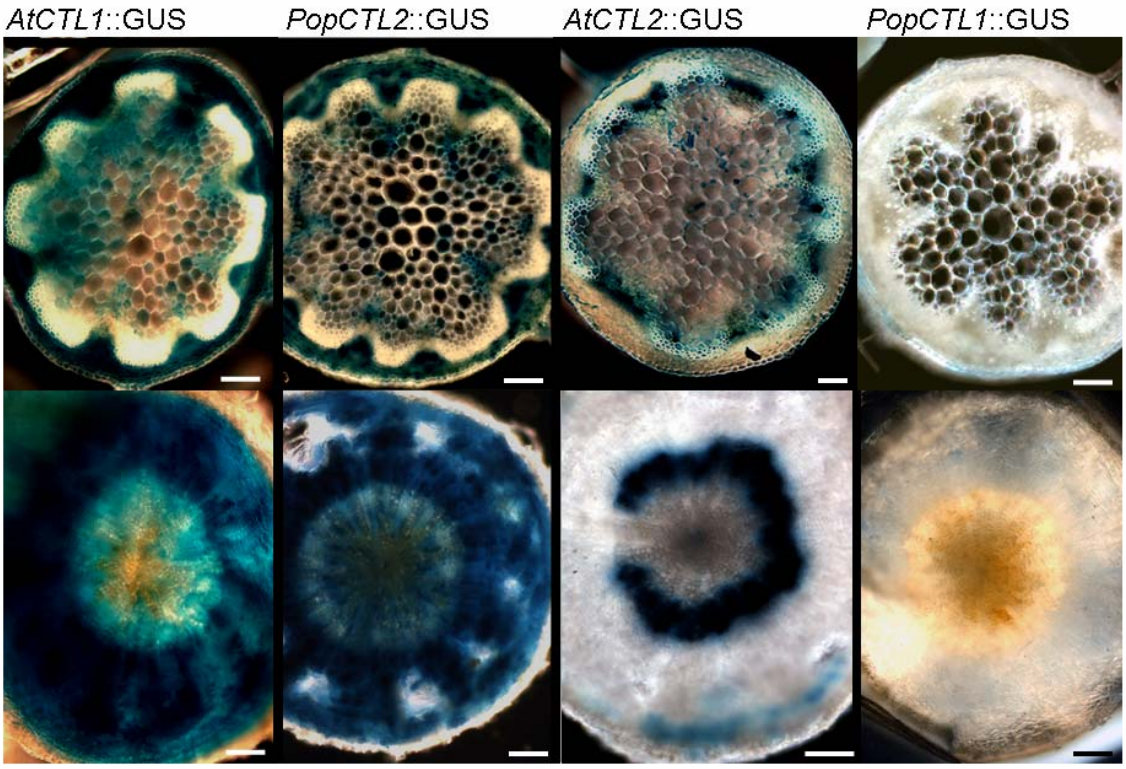

Figure 16: Comparison of root/hypocotyl and basal inflorescence sections from *AtCTL1*, *AtCTL2*, *PopCTL1*, *PopCTL2 promoter::GUS* fusions in Col-0 15 cm tall floral stems were sectioned at 1 cm from the bottom and both 1 5 cm from the shoot apical meristem. Hypocotyls were sectioned from the region above the soil and below the rosette. Scale bars: 100 µm.

4.2.4 *pom1* phenotype rescue using *AtCTL1*, *AtCTL2*, *PopCTL1*, and *PopCTL2* transgenes

Phenotype rescue assays were performed to test the ability of different *CTLs* under the control of different promoters to rescue the *pom1* phenotype when grown in darkness or on sucrose. Constructs were made as described in section 2.3.2 to 2.3.6 and transformed into the *pom1* mutant by floral dip. Two lines each of *35S::AtCTL2* and *PopCTL2::PopCTL2* plants were obtained from Bjoern Hamberger and included in this study.

Seed selection on either kanamycin or hygromycin plates produced 14 putative lines for *AtCTL1::AtCTL1*, of which genotyping showed 2 to be false positives (data

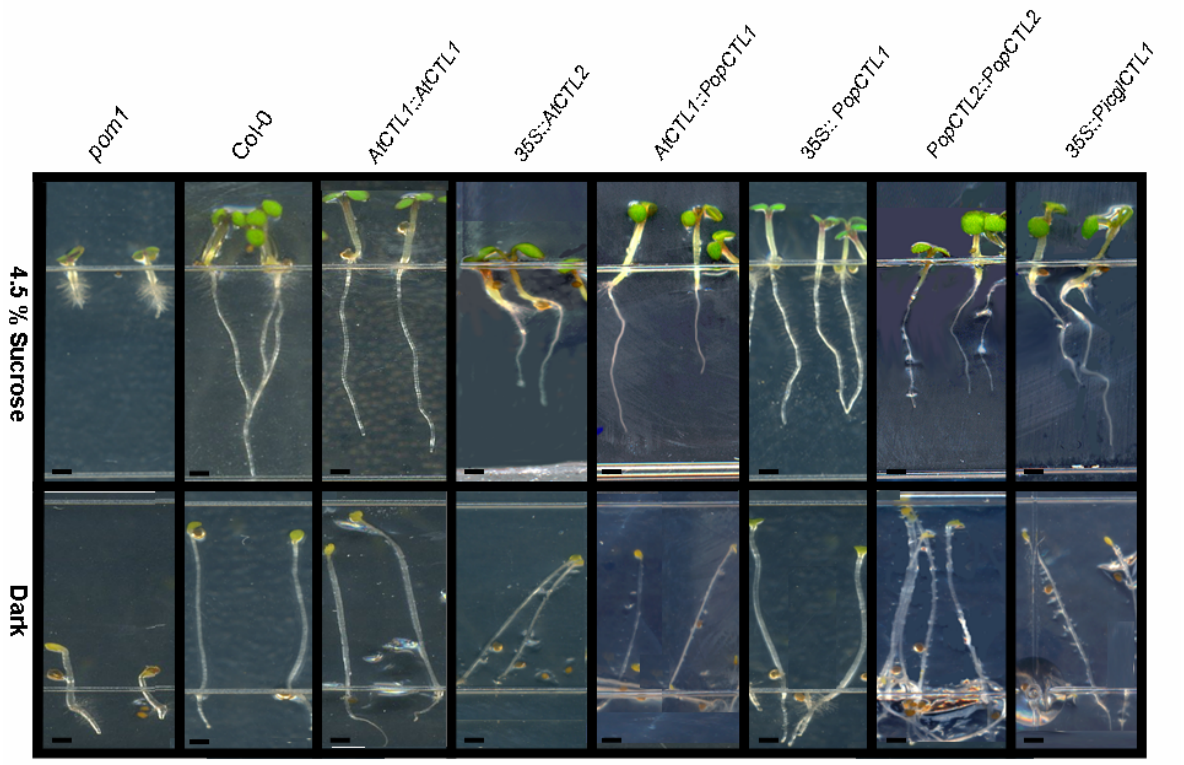

Figure 17: Phenotype rescue assays using 4.5% sucrose or dark on transgenic pom1 plants for four days. Promoter::coding region fusions tested were from left to right, *AtCTL1 promoter::AtCTL1*, CaMV35S::A*tCTL2*, CaMV35S::*PopCTL1*, *PopCTL2*::*PopCTL2*. Statistics were done on 10 seedlings per line. For segregating populations in transgenic lines, only long rescued roots or hypocotyls were included in this analysis. Scale bars: 1 mm.

not shown). All four *AtCTL1::PopCTL1* lines were genotyped by PCR as containing the rescue construct, and 9 of 10 lines proved to contain the *35S::PopCTL1* construct.

Seeds from two lines per construct were sterilized and plated on MS media, stratified at 4 degrees, and then allowed to grow vertically in a phytochamber for a period of four days. Plates were scanned on a bench-top scanner and measurements done in Adobe Photoshop Version 7.0. The results are displayed qualitatively in Figure 17 and quantitatively in Figure 18.

After four days of growth in the light on sucrose media, 30 Col-0 plants were found to have a mean root length of 0.77 cm. This was greater than the mean root length on sucrose for *pom1* (0.26 mm; n=29). *pom1* plants which contained the *AtCTL1::AtCTL1* construct were found to very closely match the mean root lengths of Col-0, which confirmed that this method of transgenic phenotype rescue works (Figures 17 and 18). Measurement of root lengths of *pom1* plants containing *35S::PopCTL1* constructs, *35S::AtCTL2* constructs, *PopCTL2::PopCTL2* constructs, likewise showed mean root lengths that were all well with one standard deviation of wild-type and at least 0.5 cm longer than *pom1* roots, showing that they are sufficient to substitute for the function of *AtCTL1*. I subsequently generated a spruce *CTL* complementation construct (*35S::PicglCTL1*). Interestingly, when introduced in to the *pom1* mutant background, this spruce *CTL* also was able to efficiently rescue the *pom1* phenotype (Figures 17 and 18).

All lines were also grown in darkness, and the phenotypes of etiolated seedlings observed (Figures 17 and 18). Col-0 plants had a mean hypocotyl length of 0.89 cm compared to 0.30 cm for *pom1* seedlings. Again, *pom1* plants which contained the *AtCTL1::AtCTL1* construct were found to very closely match the mean hypocotyl lengths of Col-0 with mean values of 0.88 cm for two independent lines. Measurement of etiolated hypocotyl lengths of *pom1* plants containing *35S::PopCTL1* constructs, *35S::AtCTL2* constructs, and *PopCTL2::PopCTL2*

constructs likewise showed means that were within 0.1 cm of Col-0 hypocotyl lengths, and at least 0.6 cm taller than *pom1* seedlings. Both tissue specific (*AtCTL1* and *PopCTL2* promoters) and constitutive, (CaMV 35S) regulatory regions were used in rescue the *pom1* mutation, and no obvious difference in the effectiveness of one or the other was observed.

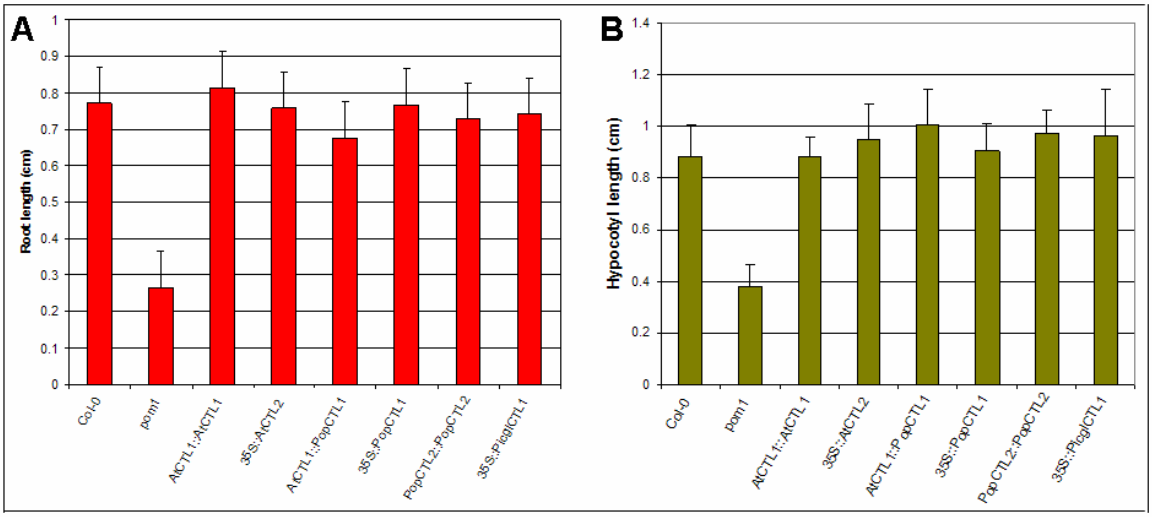

Figure 18: Measurements of transgenic *pom1* phenotype rescue. Plants were measured under conditions of 4.5% sucrose or darkness. Two lines with 10 plants each were measured; plants are labeled by their genotype or transgene in the *pom1* background.

4.2.5 CTL overexpression in *Arabidopsis*

In section 4.2.1 it was shown that elimination of *AtCTL2* function reduces the plant's ability to form vascular bundles and can create an ectopic lignification phenotype somewhat similar to that observed in *pom1*. An ideal way to confirm this phenomenon would be to test for increased numbers of vascular bundles by overexpression of the same gene. Such plants have been produced but not yet analyzed. Instead, overexpression and phenotype analysis of the putatively orthologous gene *PopCTL1* was accomplished by placing *PopCTL1* under control of a 35S promoter region in Col-0 plants. Selection on kanamycin produced 10 lines which were screened by PCR for the presence of the transgene, and 8 of which were (Figure 19A). To check three of the more promising lines for transgene expression, semiquantitative RT-PCR was performed first for the *Actin2* gene as a loading control, and then for the *PopCTL1* gene using specifications in Table 2.

Col-0, *pom1*, and all three transgenic lines showed similar levels of *Actin2* expression, but only lines 5 and 14 showed detectable levels of *PopCTL1* expression.

These two lines were then grown alongside Col-0 and *pom1* on MS plates in the light, with added sucrose, or in the dark, but there was no discernable difference between these *PopCTL1* expressing lines and Col-0 (data not shown). When grown on soil to maturity, these plants again showed no visible difference to Col-0, but sectioning of the floral stem when inflorescences were 25 cm tall and 1.5 months old showed several plants with an increased number of vascular bundles, from normal 8 in the wild type to as many as thirteen in overexpressing plants (Figure 19 and 20). The mean number of bundles in the 14 plants analyzed was 9.7, and it was found to be statistically significant by both parametric and non-parametric (Kruskal-Wallis) from the mean number of 7.6 in wild-type, with a p value of 0.01.

Bundles appeared varied in sizes and shapes, and seemed to be in various stages of development in the pith. Closer inspection showed that some large bundles were bifurcated into sub-bundles (Figure 19), a process rarely observed in wild-type plants of this age. This process might be analogous to the bundle bifurcation observed in transition from root stele to eustele in *Arabidopsis* seedlings (Busse and Evert 1999). Impaired bundle development in *PopCTL1* overexpressing plants seems to correlate with the bulbous, non-circular shape of the stems in cross section, suggesting deregulation of vascular bundle pattern can disrupt the normal shape of the inflorescence stem as it accommodates the extra tissues. This seems to be the case in *clavata* mutants, which have an enlarged apical meristem, produce many more vascular bundles than normal, and have flattened, axially distorted stems (Turner and Sieburth 2002). It is also possible that this distortion of stem shape is related to the unusual, parenchyma filled spaces observed between interfascicular fibers and cortex cells (Figure 19, 20). Root development was not extensively analyzed in these plants.

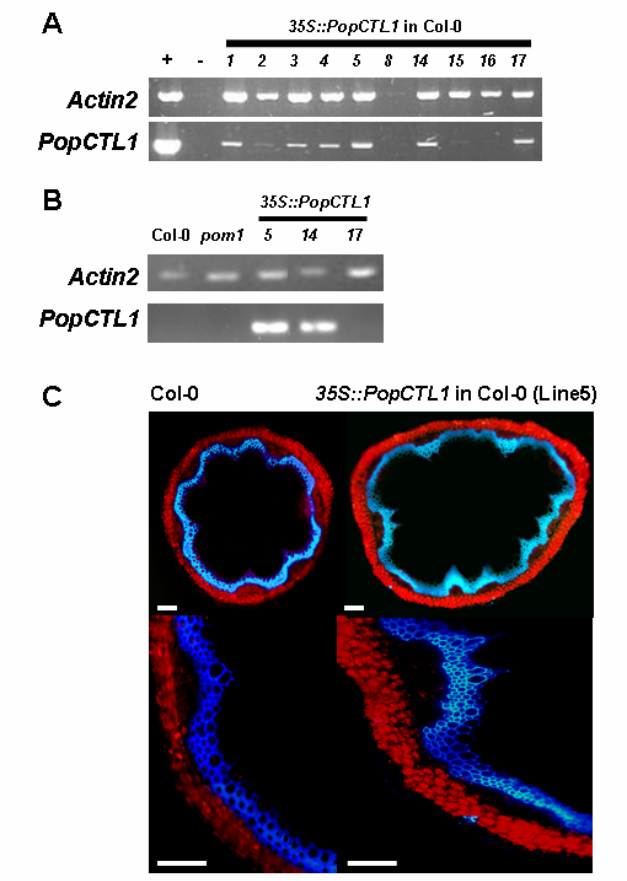

Figure 19: Expression of 35S::*PopCTL1* in Col-0. A) PCR based genotyping of transgenic lines B) Semi-quantitative RT-PCR based expression profiling of selected lines C) Phenotype of a representative PopCTL1 expressing line under UV autofluoresence. Scale bars: 100 µm.

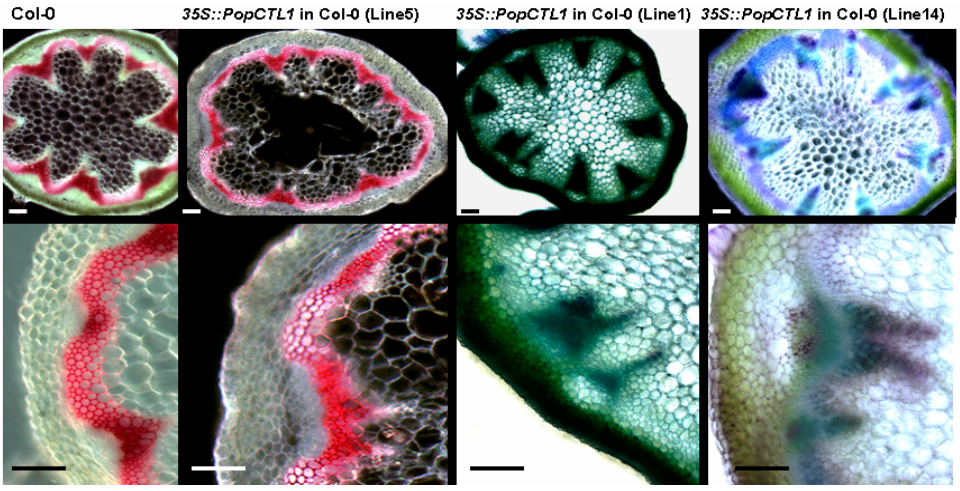

Figure 20: Hand sections of Col-0 and *35S::PopCTL1* plants. Sections were cut at 1 cm from soil and stained with either phloroglucinol or toluidine blue. Plants were 1.5 months old at the time of sectioning, but Lines 1 and 14 were sectioned earlier at three weeks of age. The hole in the pith of the *35S::PopCTL1* section is an artifact from sample preparation. Scale bars: 100 µm.

4.2.6 RNAi of *PopCTL1* and *PopCTL2* in transgenic poplar

A very important aspect of this thesis was to analyze CTL function in a system that undergoes dramatic secondary growth and might have forms of cell expansion and cell wall development that *Arabidopsis* does not normally have. To knock down *CTL* expression in *Populus alba X P. tremula* clone 717, I made RNAi constructs of two different sizes, homologous to the first exon in the coding regions of *PopCTL1* (see 2.3.8). A similar procedure was followed by Dr. Bjoern Hamberger for RNAi targeting the first exon of *PopCTL2*.

For transformation of these constructs into poplar, leaf discs were cut from 6 week old leaves and the protocol listed in 2.5.1 was followed. Over the course of one year in the tissue culture facility in Dr. Shawn Mansfield's lab at UBC, a large number of independent transformation events on separate discs were observed and cloned by tissue culture, of which 7 *PopCTL2* RNAi showed an enhanced organ regeneration phenotype (shown in Figure 23B) and similar to that reported in (Cary et al. 2001). Leaf discs giving this phenotype gave rise to 400 bp *PopCTL2* RNAi lines #A, E, F, I, J, and P as well as 150 bp RNAi line #H. No phenotypic differences from Wt were evident for *PopCTL1* RNAi shoots or roots in tissue culture.

When most lines had three individual plants which rooted successfully under kanamycin selection, these were transferred to soil in 1 liter pots, covered with plastic domes, and misted daily to reduce water stress. In total, five wild type survived soil transfer, in addition to plants representing 11 independent lines of *PopCTL1* 400 bp RNAi, 3 independent lines of *PopCTL1* 150 bp RNAi, 10 independent lines of *PopCTL2* 400 bp RNAi, and 3 independent lines of *PopCTL2* 150 bp RNAi.

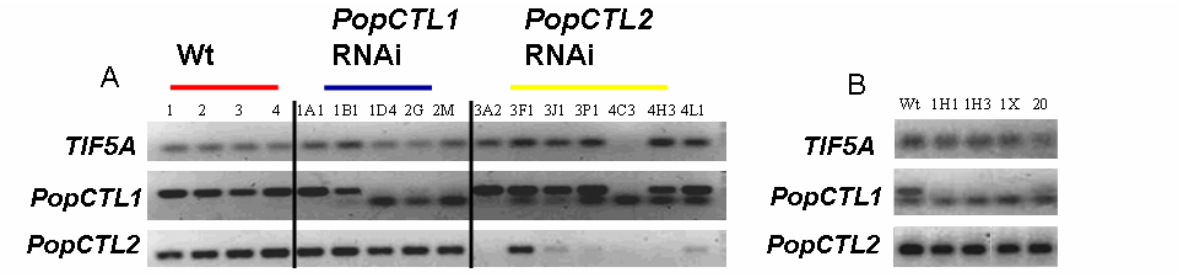

Figure 21: *CTL* expression in wild type and RNAi lines. A) Wild-type *PopCTL1* and *PopCTL2* RNAi 5 month old, greenhouse grown poplar trees. RNA was extracted from the 6th leaf from the first 0.5 cm long internode. A genomic DNA control was included to show primer specificity for cDNA. RNA from the housekeeping *TIF5A* gene was used as a control for normalizing sample amounts. *PopCTL1* RNAi 1A1, 1B1, and 1D4 contain 400 bp constructs, while 2G and 2M contain 150 bp constructs. *PopCTL2* RNAi 3A2, 3F2, 3J1, and 3P1 contain 400 bp constructs, while 4C3,4H3, 4L1 contain 150 bp constructs. B) Results of additional screening for *PopCTL1* RNAi knockdowns, from a second set of rooted transgenics. Smaller sized bands in *PopCTL1* reactions are primer dimers. Conditions and primers used are described in table 2.

After 5 months of growth in a greenhouse, RNAi mediated downregulation of *CTL* expression was monitored in individual trees by sampling the 6th leaf below the first 0.5 cm long internode, and isolating RNA for semi-quantitative RT-PCR analysis using the program in Table 2. Results are shown in Figure 21.

Of the lines tested and shown in Figure 21A, 1B1 had slight knockdown of *PopCTL1*, while 1D4, 2G, and 2M had much more dramatic and specific reduction in *PopCTL1* gene expression. *PopCTL2* appeared effectively downregulated in 3A2, 3J1, 3P1, 4H3, and 4L1, and the downregulation was specific to *PopCTL2*. To find additional lines with *PopCTL1* downregulation, RNA extraction was performed again on additional plants with results shown in Figure 21B. Lines 1H1, 1H3, 1X, and 2O were also significantly and specifically downregulated in *PopCTL1* expression. Wt plants did not show any appreciable fluctuation in *CTL* expression based on this semi-quantitative RT-PCR assay.

These *CTL* downregulated trees were measured for height and number of branches as shown in Figure 22. No clear trend was evident among trees based on height, but it is striking that about half of the RNAi lines with reduced *CTL* expression showed reduced apical dominance as evidenced by early branching

phenotypes. A number of *PopCTL1* RNAi trees broke at the base of the stem early in development and had to be staked for further upright growth, but this phenotype was not further investigated.

To assay possible anatomical phenotypes, 2 Wt plant and 4 *PopCTL1* downregulated RNAi lines were sectioned. In parallel, 2 Wt trees and 4 *PopCTL2* downregulated plants were cut to the base, transferred to a phytochamber and allowed to regrow one main shoot. The trees were hand sectioned in the middle of each internode, from internodes 1 through 25, whereas after internode 25 stems became very thick and section quality dropped off sharply. Hand sections were stained with toluidine blue (TEB) or phloroglucinol and viewed immediately in a dissecting microscope in the UBC Bioimaging facility or in the laboratory of Lacey Samuels. Figure 23 shows the results of this analysis.

Both TEB and phloroglucinol stains showed xylem, phloem fibers, and lignified pith cells very clearly as blue or red colours respectively, but TEB was also informative for its ability to stain pectin purple, while phloroglucinol was desirable for its ability to effectively diffuse through the thick woody sections. At very early stages of development (internode 2), it was possible to see major vascular bundles of protoxylem arranged in the vertices of a five point star eustele (Figure 23A). There was some variation observed in this pattern in *PopCTL1* downregulated line 1H1 and 2O, which seemed to have 6 and 4 vertices at internode two. Looking at Wt, it is possible to see there was some variation in rates of xylem development in Wt as well. Despite this, *PopCTL1* lines did all share what appears to be a reduction in red phloroglucinol staining of the xylem, evidenced especially well in internode 25 sections of 1D4 and 2O relative to Wt. This reduction in staining suggests a reduction in lignin content within these walls, and may be the cause for observed increased in breaking of *PopCTL1* downregulated xylem during sectioning.

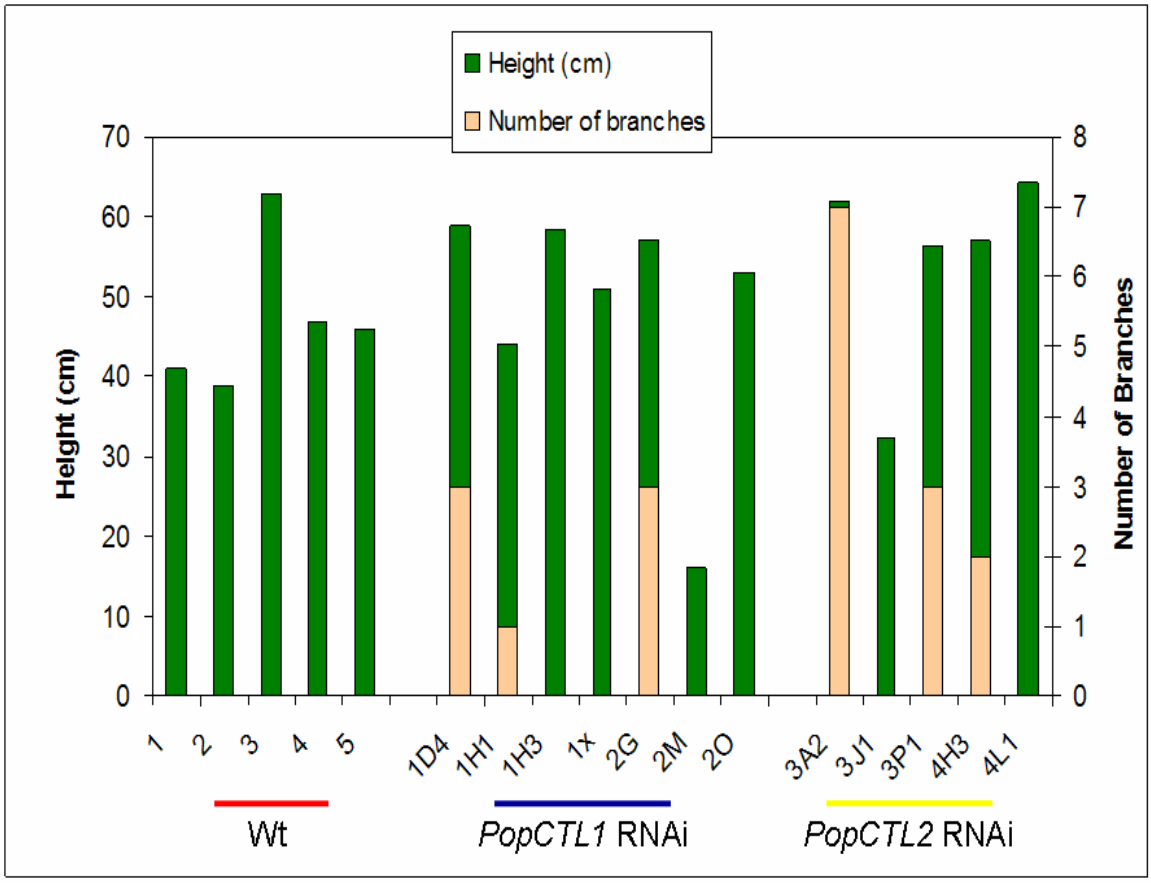

Figure 22: Greenhouse grown poplar tree heights and branch number. Trees were all grown for 5 months in soil within a greenhouse at the time of measurement. 2M died shortly after this data was recorded.

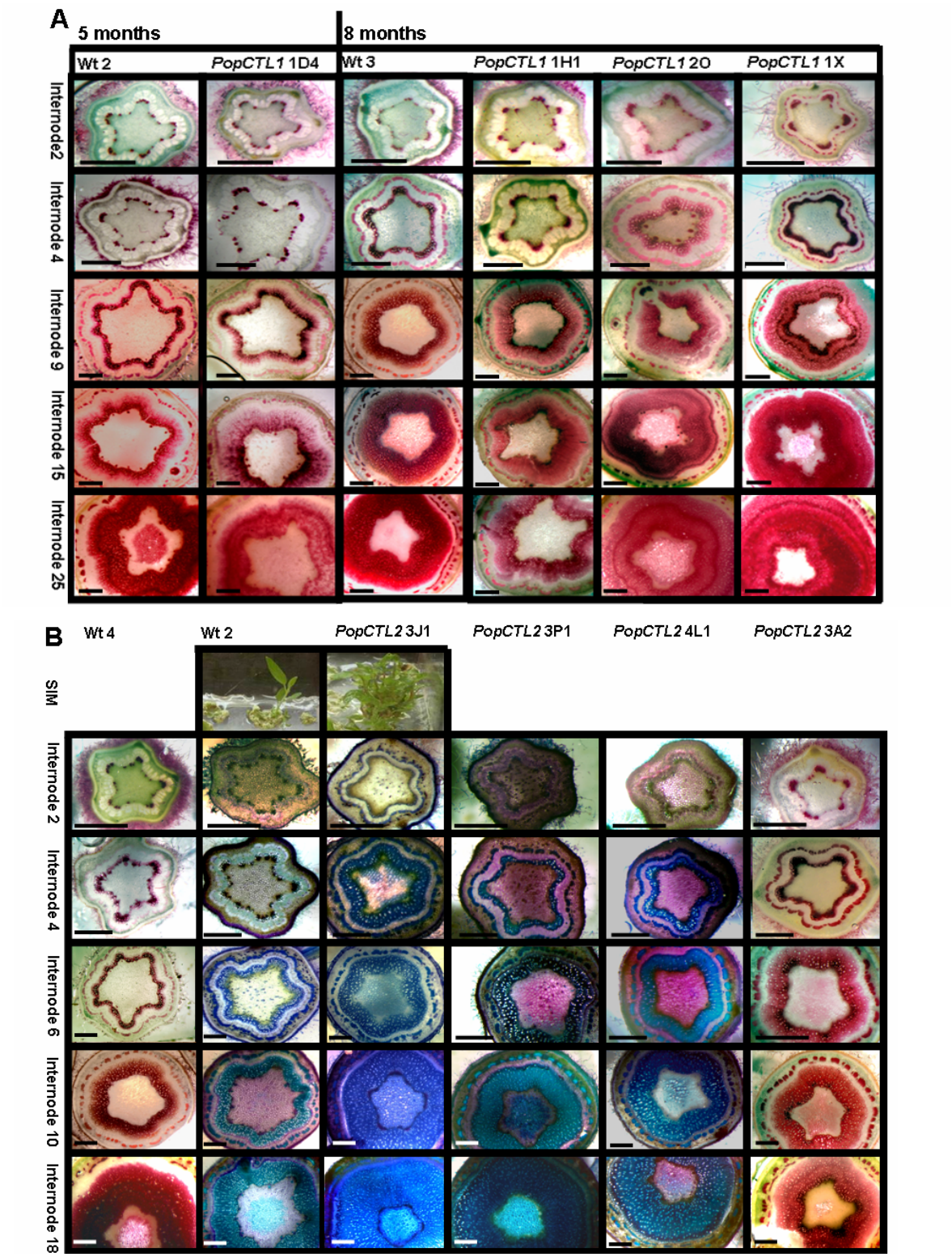

Figure 23: Handcut sections of wild type, A) *PopCTL1* RNAi and B) *PopCTL2* RNAi poplar. *PopCTL1* RNAi trees were grown in greenhouse and were 5 (Wt2,1D4) or 8 (Wt3,1H1,2O,1x) months old, while *PopCTL2* RNAi sections were derived from trees that were pruned at five months and regrown for two months in a phytochamber. SIM, shoot induction medium. Scale bars: 500 µm.

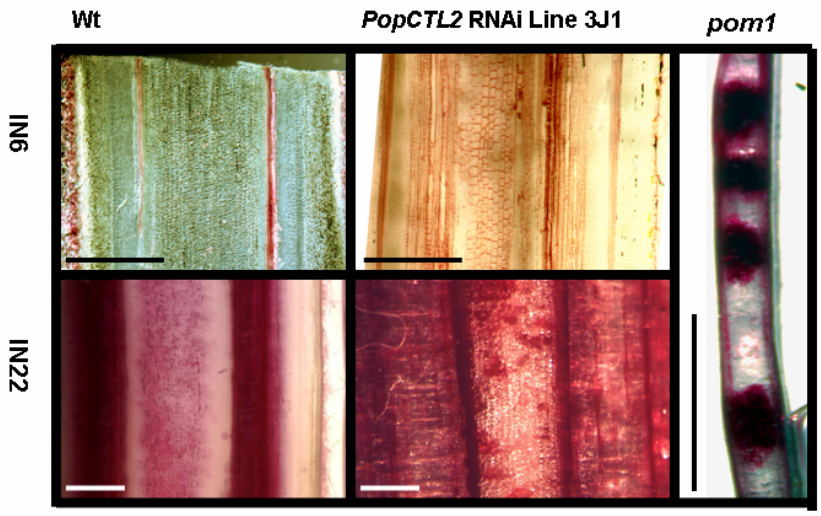

Figure 24: Longitudinal sections of Wt poplar and *PopCTL2* RNAi plant 3J1. Sections were stained with phloroglucinol The IN6 section from 3J1 was cut at a thickness of 40 micrometers using a vibratome. The ectopic pith lignification phenotype of *pom1* in an *Arabidopsis* inflorescence stem is shown for comparison. IN, internode. Scale bars: 1 mm.

Downregulation of *PopCTL2* seemed to be correlated with an accelerated program of secondary xylem formation, and staining for lignin in the pith, progressing to complete staining as seen in the extreme example at internode 10 of plant 3J1 (Figure 23B). Visible secondary growth began much earlier in this individual (as early as internode 2) than in wild type, beginning as a complete blue staining vascular cylinder in internode 2, followed by waves of lignification emanating from the secondary xylem and progressing to complete staining of the pith (shown in longitudinal section in Figure 24) which might be a poplar variation on ectopic deposition of lignin in the pith seen in *pom1* mutants. Other *PopCTL2* RNAi plants 3P1, 3A2, and 4L1 were less dramatically affected by the downregulation but did show this trend, suggesting a larger analysis with more individuals be done to increase our confidence in correlating phenotype to genotype.

4.2.7 Pith lignification in wild-type poplar

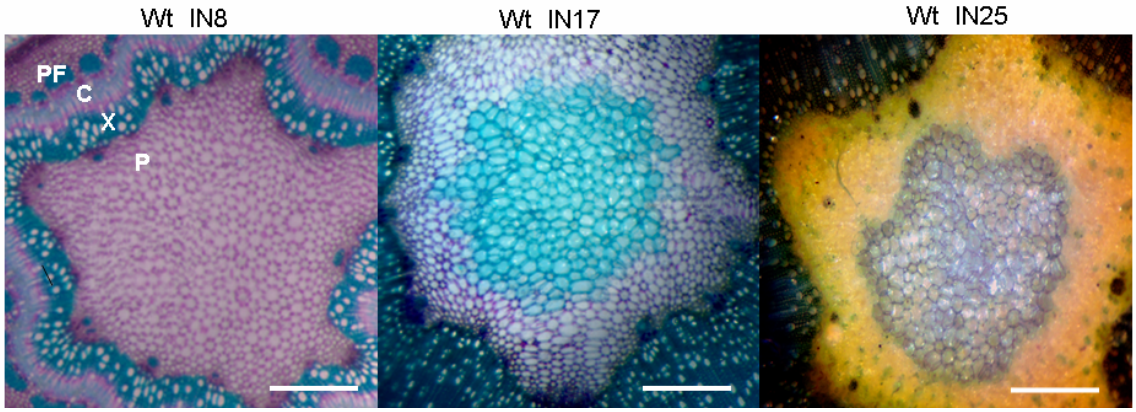

Figure 25: Sections of poplar Wt magnified 50 times showing pith details from different stages of growth. Sections were hand cut and stained with toluidine blue. PF, phloem fibers; C, cambium; X, xylem; P, pith. Scale bars: 500 µm.

While examining sections from multiple wild-type plants, a very exciting and unexpected discovery was the observation of an island of lignified pith cells in the center of the poplar stems. These cells also appeared to contain a significant amount of air after internode 20, as evidenced by a large number of bubbles after both types of stain and in this tissue only. Lignification, starch accumulation, cell death, and accumulation of air in pith cells have all been previously observed in different species of tree (Metcalfe 1979). In Wt2 (Figure 25) this was observed to begin as a few slightly expanded pith cells staining either blue in TEB or red in phloroglucinol at internode 17 and by internode 25 it was a strongly blue staining tissue containing highly reflective cell walls. Viewing these internodes in transverse sections with phloroglucinol stain (Figure 24), it is possible to see that this lignified pith island is a continuous tissue distributed vertically throughout the pith. Whether the cell wall of these cells are actually lignified and what the function of this phenomena may be, remains to be determined. In some individuals, this "lignification" of the pith was observed to accompany much greater changes in shape of pith cells, suggesting cell expansion and lignification both play a role in normal pith development of this tree species.

4.3 Discussion

A powerful test for characterizing the function of genes discovered by *in silico* and expression profiling approaches in poplar and other plants is complementation of mutations in potentially orthologous *Arabidopsis* genes. Rather than help determine functional orthology between poplar and *Arabidopsis CTL* genes however, the phenotype rescue experiments I performed showed that duplicated *CTL* genes in each species have apparently conserved biochemical functions despite significant differences in expression patterns, since all were able to complement the *pom1* (*Atctl1*) mutant phenotype. Conversely, assay of tissue specific expression patterns specified by *CTL promoter:*:*GUS* fusions did reveal strikingly similar patterns of *AtCTL1* and *PopCTL2* promoter driven expression in tissues developing primary walls, as predicted from expression profiling experiments (Hertzberg et al. 2001; Ehlting et al. 2005), suggesting possible similar roles in cell wall formation. It was not possible to draw the parallel conclusions with respect to *AtCTL2* and *PopCTL1*, since for unknown reasons the *PopCTL1* promoter region did not specify detectable developmental expression in *Arabidopsis*. However, the *AtCTL1* promoter::*GUS* results provided an independent confirmation that *AtCTL1* is expressed in a pattern consistent with primary wall formation or modification, while *AtCTL2* is expressed in cells undergoing secondary cell wall development, consistent with co-expression analyses carried out by Dr. Hamberger in the Douglas lab and others (Brown et al., 2005; Persson et al., 2005).

One prediction based on expression patterns was that *Arabidopsis Atctl2* mutant plants would show alterations in secondary wall formation. Instead, I observed a reduction in vascular bundle number in floral stems, and occasionally, ectopic lignification of the pith and enhanced phloem fiber formation, but no apparent defect in secondary wall formation, as observed by others (Persson et al. 2005). This might be interpreted as evidence that AtCTL2 activity is required for provascular cell type differentiation rather than directly in secondary wall formation. However, an alternative explanation for the *Atctl2* phenotype, in which no apparent

secondary cell wall defect was observed, could be partially overlapping expression between *AtCTL1* and *AtCTL2*, with residual AtCTL1 function in cells ungoing secondary wall development.

Because the mode of action of AtCTL1 that underlies the *Atctl1/pom1* loss of function phenotype is still not understood, the Douglas lab used global gene expression profiling to compare gene expression in *pom1* mutant relative to wild type etiolated seedlings. This experiment showed that several auxin inducible genes are downregulated in *pom1* (B. Hambereger, unpublished), suggesting a change in auxin levels, distribution, or response. The *DR5::GUS* reporter gene construct provides a powerful way to indirectly assay IAA levels in plants, since the *DR5* promoter is highly responsive to auxin concentration (Ulmasov et al. 1997). Analysis of *DR5::GUS* expression patterns in wild-type and *pom1* seedlings showed that levels of auxin may be reduced in *pom1* mutants, and that the sucrose induced *pom1* phenotype appears to disrupt auxin flow in the root (Figure 11). Sectioning of roots at the hypocotyl junction in fully grown *pom1* plants revealed that *DR5::GUS* expression was low or absent in roots at later stages of plant development at the root-hypocotyl junction, correlating strongly with reduced secondary xylem formation at this location. These results are consistent with the microarray data showing alterations in auxin regulated gene expression in *pom1*, and suggest that some *ctl* mutant phenotypes could be due to alterations in auxin homeostasis or transport.

Auxin has been shown to affect vascular patterning in root and inflorescence stems in *Arabidopsis* (Hobbie et al. 2000; Hamann et al. 2002) and to stimulate the formation of xylem fibers and vessels in both *Arabidopsis* and poplar. Auxin is also a well known stimulant of cell elongation in stems, while it inhibits cell elongation in roots (Taiz and Zeiger 1998). Its presence in a tissue has been shown to antagonize endogenous cytokinin induced cell division, and thus promote apical dominance in plants. One major way that auxin is believed to regulate cell development is through the regulation of auxin inducible genes, achieved by

stimulation of ubiquitination and degradation of negative regulators of Auxin Response Factor (ARF) transcription factors (Leyser 2001).The apparent changes in auxin levels and distribution based on *DR5::GUS* expression in *pom1* may help explain the reduced number of vascular bundles formed in *Atctl2* plants, and might be explained if the *CTL* gene product is normally involved in binding or modifying a chitin-like molecule involved directly or indirectly in regulating auxin response or distribution. Studies done in a symbiotic root nodule producing species of clover show that the application of symbiotic *Rhizobium leguminosarum*, lipo-chitin oligosaccharides (NOD factors), O-acetylated chitin fragments, and flavonoid aglycones all produce a transient decrease in root auxin transport (Mathesius et al. 1998). If CTLs are involved in mediating a chemically similar signal molecule, absence of CTL activity may allow signal over accumulation and a resulting constitutive reduction in auxin transport. Why nodule formation would require reduced auxin transport is not clear, but it was shown that high levels are also needed for induction of cell division at the site of nodulation. Whether auxin production or distribution is altered in *Atctl2* or in *PopCTL1* overexpressing plants, or whether it directly plays a part in the phenotypes seen remains to be shown by *DR5:GUS* activity or direct assays on auxin levels in the mutant.

Poplar plants with down regulated *CTL* expression were generated in order to test *CTL* function during primary and secondary growth in poplar, but the results remained somewhat inconclusive about this point. Down regulation of *PopCTL2* seemed to phenocopy the ectopic deposition of lignin in the pith seen in *pom1*, and abnormal cell expansion may have been observed in some of these same cells, suggesting a link to primary cell wall development in poplar. Likewise what might be perturbation of secondary wall formation observed as putatively reduced lignification in secondary xylem of *PopCTL1* down regulated trees, may be evidence of a function in secondary wall formation, as predicted by the *PopCTL1* expression pattern which is associated with secondary wall formation during xylem development (Hertzberg et al. 2001). More detailed biochemical analysis of lignin and cellulose content and structure in the secondary cell walls in *PopCTL1* down

regulated lines is necessary to further characterize this potential phenotype.

The increased branching in both *PopCTL1* and *PopCTL2* downregulated poplar lines is consistent with the effect of the *pom1* mutation on auxin levels or distribution. This phenotype suggests reduced apical dominance, which has long been understood as a characteristic effect of reduced basipetal auxin flow (Taiz and Zeiger 1998), consistent with the apparent affect of the *pom1* mutation on auxin distribution. In trees, auxin is believed to be transported mostly through the vascular cambium where it promotes maintenance of cambial initials, radial expansion of cambial cells (in contrast to its effect in root), and reaction wood responses (Little and Savidge 1987). *PopCTL2* downregulation appeared to accelerate secondary xylem formation and lignification or phenolic compound accumulation in the pith, which could be the result of a build up of auxin due to reduced translocation similar to that seen in auxin transport inhibitor treated poplar (Junghans et al. 2004) and *Arabidopsis* (Mattsson et al. 1999). As *pom1* plants were reported to overproduce ethylene and be hyper-sensitive to cytokinins (Cary et al. 2001; Zhong et al. 2002), it is possible that the observed xylem phenotype in *PopCTL2* RNAi poplars is connected to similar changes in hormone response in these plants. Reduced shoot auxin production in a tissue culture environment could account for the increased organ regeneration observed in *PopCTL2* RNAi tissue culture, and that reported for *Arabidopsis* (Cary et al. 2001).

Sucrose could play a role in the enhanced xylem formation observed in *PopCTL2* downregulated poplars. The *pom1* phenotype was originally described on the basis of a conditional root expansion phenotype when plants were grown on high sucrose (Benfey et al. 1993; Hauser et al. 1995). Cells in *Arabidopsis* roots and the base of the inflorescence are normally exposed to very low levels of glucose and sucrose (Deuschle et al. 2006). This is interesting to consider when growing a plant on high sucrose since it is known that plants use sugars as carbon sources in metabolism, and are very sensitive to their presence, with evidence suggesting roles as developmental signaling molecules (Moore et al. 2003; Yang et al. 2004;

Avonce et al. 2005; Rolland et al. 2006). Glucose for example has crosstalk with auxin, cytokinin, ethylene, and ABA hormone signaling pathways through the activity of the glucose hexokinase receptor, HXK1 (Xiao et al. 2000; Moore et al. 2003; Rolland et al. 2006). Pectins are also known to be important in developmental regulation (Ridley et al. 2001) and their production might be altered along with the apparent reduction in cellulose content of *pom1* cell walls (Mouille et al. 2003). Sucrose is the major transport form of carbon in plants which flows through the phloem from sources of production to sinks such as root tissues where it is hydrolyzed into glucose and fructose (Taiz, 1998). Zebra-like (Malcolm Campbell, unpublished results) stripped patterns of cell expansion and lignification of the pith in *pom1* inflorescences as seen in Fig 24 might be linked to altered source-sink relationships or changes in sensitivity/response to sugars whose production and transport are regulated by light and the circadian clock (Rogers et al. 2005a). *pom1* mutants were shown to contain lower levels of cellulose in their cell walls, which would also suggest the possibility of channeling of excess carbon into other metabolites such as pectins. Whether auxin levels or transport are directly or indirectly reduced by the *pom1* mutation, auxin plays a role in specifying source-sink relationships, helping to prevent sucrose efflux causing cell specific buildup in target cells (Maitra and Sen 1987). If auxin transport is compromised in *PopCTL2* downregulated poplars , it is possible that in the primary cambial region below the sugar producing shoot apical meristem (SAM) in poplar, altered levels of auxin either directly, or through altered sensitivity to sucrose, lead to an accelerated growth of vascular tissue as seen in experiments with chemical transport inhibitors (Mattsson et al. 1999), and in auxin transport mutants such as *pin1* and *acl5* (Clay and Nelson 2005).

Our GUS experiments showed expression of *AtCTL1* preferentially in parenchyma cells of the stem, suggesting even at advanced stages of development, primary cell wall development or modification might be taking place within the pith and cortex of the plant. Exactly why these cells specifically loose control over primary cell wall growth remains to be shown, but ectopic lignification might be explained as a

response triggered by reduced cellulose in the primary cell wall of these abnormally expanded pith cells, similar to that observed in response to wounding and locally increased levels of auxin as shown in Figure 11 and reported elsewhere (Cano-Delgado et al. 2003). *pom1* mutants have been previously shown not to be significantly altered in transcription factors regulating lignin metabolism (Rogers et al. 2005b) but rather are known to have lower levels of cellulose in their cell walls (Mouille et al. 2003), are upregulated in genes responsive to oxidative stress, and are downregulated in a number of primary cell wall and auxin inducible genes (Rogers et al. 2005b).

Auxin is produced in young tissues such as the SAM and then transported basipetaly mostly through phloem and cambial parenchyma (Berleth et al. 2000). Based on our *AtCTL1::GUS* and *DR5::GUS* experiments, *AtCTL1* and *DR5* activity are similar, with GUS expression for both reporter constructs increasing near the SAM and the RAM. If AtCTL1 plays an important role in regulating primary wall development, and thus controlling cell expansion, improper expansion of *pom1* mutant cells near the SAM or the RAM where auxin levels appear highest, may lead to altered auxin transport in these tissues. Improperly formed cells might mislocalize the auxin efflux transporter, PIN1 for example (Galweiler et al. 1998) resulting in non-polar, blocked flow of auxin. If these cells accumulate higher than normal levels of auxin, this may too feed into expansion phenotypes, as increased levels of auxin or heightened auxin response tends to increase cell size (Jones et al. 1998).

Although nothing is known of auxin relations in *Atctl2* mutants, *AtCTL2::GUS* expression shows a pattern consistent with a role in secondary wall formation in primary and secondary xylem formation and interfascicular fiber formation. Phenotype rescue does however suggest that the AtCTL2 protein is functionally equivalent to AtCTL1 (i.e., *35S::AtCTL2* expression can rescue the *pom1* phenotype, Figure 17 and 18), suggesting that the functional diversification of *CTLs* in *Arabidopsis* is based only on differential regulation of the genes. This

suggests that, AtCTL2 is mediating the same process as AtCTL1, but doing so in cells undergoing secondary wall development. Auxin is very important in this process (Uggla et al. 1996), and is involved in vascular patterning, cell expansion and elongation, and xylem differentiation in the differentiation zone below the SAM (Turner and Sieburth 2002). Auxin transported through the developing inflorescence stem from young parts is likely involved in continued xylem and interfascicular fiber development as the stem matures. Based on the *Atctl2* mutant phenotype (reduced numbers of vascular bundles), AtCTL2 may play a role in mediating the process of vascular bundle formation and development, perhaps in response to auxin transported via the vascular system. Alternatively, mis-expression of these *CTL* genes could indirectly affect auxin regulated processes by altering the shape or function of cells involved in auxin transport, thus altering the auxin distribution during inflorescence stem development and leading to developmental anomalies.

One way to further test to role of AtCTL2 in vascular patterning is test the effect of *AtCTL2* overexpression. Such plants have been produced, but have not been analyzed at the time of writing. Instead, *PopCTL1* overexpressing Col-0 plants were produced and studied. The phenotype of these plants (Figures 19 and 20), production of extra vascular bundles and a mis-shapen stem, supports the idea that CTLs can play a role, direct or indirect, in vascular development, provascular cell fate and cambial cell proliferation. It will be interesting to assay for auxin distribution in *AtCTL2* and *PopCTL1* overexpression lines relative to wild type plants using the *DR5::GUS* reporter system to determine if alterations in auxin distribution coincide with the observed developmental anomalies.

Four other vascular patterning mutants *avb1* (Zhong and Ye 2004), *hca* (Pineau et al. 2005), *pin1* (Galweiler et al. 1998) and *clavata1* (Brand et al. 2000) show changes in the formation and distribution of vascular bundles. IFL1/AVB1 is a very interesting transcription factor, since its overexpression completely transforms *Arabidopsis* vascular bundles from a collateral into an amphivasal arrangement,

while its null mutant has reduced basipetal auxin flow, reduced formation of interfascicular and xylem fibers in vascular bundles (Zhong and Ye 1999; Zhong and Ye 2001). *hca* (enhanced cambial activity), apparently like *pom1*, shows reduced levels of auxin, and increased sensitivity to cytokinins. Phenotypically, it also shows the reduced secondary xylem formation phenotype present in *pom1* at the root/hypocotyls junction, and has premature and enhanced cambial cell activity leading to an altered pattern of vascular tissues. *pin1* mutants also have reduced auxin transport, and while there is no change in vascular patterning, there is an increase in the size of vascular bundles (Okada et al. 1991). c*lavata 1* mutants have enlarged apical meristems that produce an increased number of provascular initials in the apical meristem, resulting in both many more vascular bundles and a very distorted stem axial shape (Brand et al. 2000; Turner and Sieburth 2002). Overexpression of the transcription factor ATHB-8 also increases cambial cell proliferation in *Arabidopsis*, resulting in increased and accelerated primary xylem formation (Baima et al. 2001). Reduced auxin in the *acl5* mutant results in increased recruitment of cambial cells into xylem and phloem of vascular bundles (Clay and Nelson 2005). These alterations in vascular patterning show some commonalities with those observed in *PopCTL1* overexpressing plants, but further analysis will have to be done to elucidate the precise mechanism behind the increase in bundle numbers.

The biochemical function(s) of CTL proteins is still a mystery, although the fact that all poplar, *Arabidopsis*, and one spruce *CTL* gene are able to rescue the *pom1* mutation suggests that despite their somewhat divergent amino acid sequences, these genes share a common biochemical function somehow tied to cell wall development and auxin, sucrose, ethylene, and cytokinin signaling (Hauser et al. 1995; Cary et al. 2001; Zhong et al. 2002). Previously, it has been shown that mutation of a chitin hydrolyzing catalytic site can change a chitinase into a lectin-like protein (Iseli-Gamboni et al. 1998) and that there are non CTL examples of such modified chitinases which nonetheless have developmental activity within plants (Goormachtig et al. 2001). It seems very likely that wall localized CTLs play

a direct role in developmental signaling by reversibly binding or modifying a chitin-like oligosaccharide ligand and releasing or degrading an effector molecule in response to other developmental signals. CTL activity could also involve physical interactions with the cellulose synthase complex and newly formed cellulose polymers, making most *pom1* phenotypes secondary pleiotropic effects resulting from inappropriate cell wall development.

I have shown that genes encoding biochemically similar CTL proteins persist as differentially expressed units in at least two different plant species (*Arabidopsis* and poplar). This suggests that two copies exist in angiosperms for the purpose of separating similar processes during development by allowing differential expression to evolve in a duplicated gene (subfunctionalization). Whatever the biochemical function of CTLs is, the presence of *CTL* genes in all land plants sampled, including *Physcomitrella*, and the ability of a spruce *CTL* gene to complement the *pom1* phenotype shows that CTL proteins have maintained conserved biochemical function(s) since at least the divergence of gymnosperms and angiosperms, and possibly since the evolution of land plants.

Chapter 5 Conclusions and Future Perspectives

In this thesis I showed that *CTLs* are a small family of genes that appear to have evolved specifically in land plants. As far as this study was able to ascertain, there appear to have been two copies present in a common ancestor of the bryophytes and Spermatopsida, but that there was only one ancestral angiosperm *CTL* which duplicated again into two *CTL* copies in most plants.

While the two *CTLs* in *Arabidopsis* and poplar have different patterns of expression based on GUS analysis, and *PicglCTL*1 from *Picea glauca* has important differences in amino acid sequence, they all share a biochemical function which allows them to complement the *pom1* mutation. *In vivo*, this suggests that *CTLs* are able might be able to complement each other's function in tissues where there

is overlapping expression, which appears to be vascular bundle cambium in the inflorescence and secondary xylem or xylem parenchyma in the root/hypocotyl. This idea helps explain why *Atctl1* and A*tctl2* phenotypes also show similar ectopic lignification of the pith and enhanced phloem fiber development, indicating that reduced total *CTL* expression in vascular bundles might be more important than either individual *AtCTL1* or *AtCTL2* expression. Production of *Atctl1/Atctl2* double mutants should allow better observation of what a plant looks like without any possible functional complementation.

My finds suggest that the observed patterns of *CTL* expression, rather than biochemical function dictate their biological roles. Consistent with the idea that regulation is important in CTL function, constitutive overexpression of *PopCTL1* resulted in altered vascular bundle formation and axial inflorescence shape. Transgenic plants for *AtCTL1, AtCTL2, PopCTL2, PicglCTL1*, and *PicglCTL2* overexpression have been made and await both expression analysis and phenotyping to independently confirm the enhanced bundle formation phenotype.

While our *Atctl1* microarray and *DR5::GUS* studies show that auxin production, transport, or response is reduced in *pom1*, studies of auxin levels in *Atctl2* mutants, *CTL* overexpressing plants and *CTL* downregulated poplar trees need to be pursued, and an explanation for altered auxin activity needs to be found. While alterations in auxin homeostasis as well as alterations in cell wall development, could help explain RNAi induced phenotypes in poplar. Further study into poplar CTL function will have to be done to be better able to make comparisons between species. Likewise, while *PicgluCTL1* was able to rescue the *pom1* sucrose induced phenotype, determining its function in that species is important to better understand evolution o fCTL biological function in land plants.

Finally, the precise biochemical activity of CTL proteins needs to be discovered. As CTLs were originally annotated to be involved in carbohydrate modification, and there are a number of oligosaccharide molecules implicated in cell expansion and

differentiation, it still seems likely that *CTLs* are part of glycoprotein complex regulating cell wall development. Investigation into hormonal regulation of *CTL* expression and protein function, as well as discovery of its predicted carbohydrate ligand and its mode of action are all work that remains to be done to fully understand this developing developmental story.

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Appendix A

Conserved portion of CTL protein sequences used to generate Figure 6.

```
            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                5         15         25         35         45         55
HorvuCHI26  ---------- ---------S VSSIVSRAQF DRMLLHRND- GACQAKGFYT YDAFVAAAAA
PhypaCTL1   KGCNK----- ---------T -SELFN-EMF EAMFKHRNDK -AAHAQGFWS YDGFIAAAKM
PhypaCTL2   DGC------- --------NT ISDLFTVQTF EDMFKHRNDR -AAHAAGFWT YDGFMAAAQM
CycruCTL    KICDRGWEC- K-GIYCCNKT ISEIFTVDQF ESLFSHRNA- PVAHAVGYWD YHSFITAAAQ
GinBiCTL    KICDRGWECK G--EYCCNKT ISQIFTVDQF ESLFSKRNS- PVAHAVGFWD YHSFITAAAQ
PintaCTL1   --------CA K-ATDCKNKT ISELFTVDQF ESLFSHRNA- PLAHAQGFWD YHSFITAAAH
PintaCTL2   KVCDKGWEC- K-GTYCCNQT ISEIFTVDNF EELFSKRNT- PVAHAVGFWD YYSFINAAAQ
PicglCTL1   --------CI K-GAECKNKT ISELFTVDQF ESLFSHRNA- PMAHAQGFWD YHSFITAAAH
PicglCTL2   KICDKGWEC- K-GVYCCNQT ISQIFTVDNF EELFSKRNS- PVAHAVGFWD YYSFINAAAQ
ArathCTL2   KLCDKGWECK GWSEYCCNHT ISDFFETYQF ENLFSKRNS- PVAHAVGFWD YRSFITAAAE
ArathCTL1   KVCTQGWECS WWSKYCCNQT ISDYFQVYQF EQLFSKRNT- PIAHAVGFWD YQSFITAAAL
PoptrCTL1   KVCDKGWECK GLSAYCCNQT ISDFFQTYQF ENLFSKRNT- PVAHASGFWD YHSFITAAAE
PissaCTL    KYCTQGWECN NWSIYCCNLT ISDYFQTYHF ENLFSKRNT- PVAHAVGFWD YHSFINAAAL
PoptrCTL2   KLCKRGWECL DWSEYCCNET ISDIFQPYQF ENLFSNRNS- PVAHAVGFWD YRSFILASTS
MedtrCTL    KLCDKGWECK GWSVYCCNET ISDYFQTYQF ENLFSKRND- PTAHASGFWD YRSFITAAAL
GlymaCTL    KVCDKGWECK GWSAYCCNET ISDYFQTYQF ENLFAKRNS- PVAHAVGFWD YRSFITAAAL
GoshiCTL1   KLCDKGWECK GWSQFCCNQT ISDYFRTYQF ENLFAKRNT- PVAHAVGFWD YHSFITAAAQ
GoshiCTL2   KLCDKGWECK GWSKYCCNHT ISDYFQTYQF EDLFAKRNT- PVAHAVGFWD YHSFITAAAQ
GoshiCTL3   KQCIQGWECS YWSKYCCNKT VSDVFQVYQF EDLFAKRNS- PVAHAVGFWD YHSFILAASI
OrisaCTL1   KICDKGWECS G-SKYCCNDT ITDFFKVYQF ENLFSKRNS- PVAHAVGFWD YQSFITAAAL
OrisaCTL2   KACDKGWECS G-SRFCCNDT ITDYFKAYQF EELFAHRNDR SLAHAAGFWD YHAFITAAAL
TriaeCTL    TACDKGWECS G-SRFCCNET ITDYFKAYQF EELFAKRNN- SLAHAAGFWD YKAFITAAAL
VinviCTL1   KVCDKGWECK GWSEFCCNLT ISDYFQTYQF ENLFSKRNS- PVAHAVGFWD YRSFILASAV
VinviCTL2   KVCDKGWECK GWSKYCCNQT VSDFFQTYQF ENLFAKRNT- PVAHAVGFWD YRSFITAAAV
CitsiCTL1   KLCDKGWECK GWSEYCCNQT ISDYFQTYQF ENLFAKRNT- PVAHAVGFWD YHSFITAAAL
CitsiCTL2   KVCIKGWECP TWSKFCCNET ISDYFQVYQF ENFFSKRNT- PVAHAVGFWD YQSFITATVK
SoltuCTL    KMCVKDWECN KLSKFCCNLT ITDYLDTDQF ELLFTKRNS- PVAHAVGFWD YGSFIRATAL
ZeamaCTL    KICNKGWECS G-SKYCCNDT ITDFFKVYQF ENLFAKRNT- PVAHAVGFWD YQAFITAAAL
MaldoCTL    KLCDKGWECK GWSKYCCNLT ISDYFQTYQF ENLFSKRNT- PVAHAVGFWD YQAFITAAAL
BranaCTL    KVCTQGWECV WWSEYCCNQT ISDYFQVYQF EQLFAKRNT- PIAHAVGFWD YQSFITAAAL
HorvuCTL    TACDKGWECS G-SRFCCNET ITDYFKAYQF EELFAQRNN- SLAHAAGFWN YQAFITAASL
PontrCTL    KVCIKGWECP TWSKFCCNET ISDYFQVYQF ENFFSKRNT- PVAHAVGFWD YQSFITATVK
SacofCTL    KICNKGWECS G-SKYCCNDT ITDFFKVYQF ENLFAKRNT- PVAHAVGFWD YQAFITAAAL
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             ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                 65         75         85         95        105        115
HorvuCHI26   FS--GFGTTG SADVQKREVA AFLAQTSHET TGGWATA--- ----PDGAFA WGYCFKQERG
PhypaCTL1    FEKDGFGMVG GEDVQKRELS AFFAHVAHET SCGWSGA--- ----KDGPTA WGLCYNQELA
PhypaCTL2    FEKDGFASVG GDDMQKRELA AFFAHVAHET SCGWSMA--- ----KDGPTA WGLCYNQELA
CycruCTL     YEGVGFGTTG GQLMQQRELA AFLGHVASET SCGYSVA--- ----VGGPLA WGLCYREEMS
GinBiCTL     YEGLGFGTTG GDKMQQKEIA AFLGHVASET SCGYSVA--- ----VGGPLA WGLCYKEEMS
PintaCTL1    YEPKGFGTTG GDIVQKRELA AFFAHIATET SCESLMAQAA STAPSDSPTK WGLCYKEELS
PintaCTL2    FEGIGFGTTG GQVMQQKELA AFLGNVAAET SCGYNVA--- ----TGGPTA WGLCYKEEMS
PicglCTL1    FEPKGFGATG GDLVQKKELA AFFAHVATET SCESLMAQS- STATTDSPTK WGLCYKEELS
PicglCTL2    FEGIGFGTTG GQLMQQKELA AFFGHVAAET SCGYSVA--- ----VGGPYA WGLCYKEEMS
ArathCTL2    YQPLGFGTAG EKLQGMKEVA AFLGHVGSKT SCGYGVA--- ----TGGPLA WGLCYNKEMS
ArathCTL1    FEPLGFGTTG GKLMGQKEMA AFLGHVASKT SCGYGVA--- ----TGGPLA WGLCYNREMS
PoptrCTL1    YQPHGFGTTG GKLTGQKEVA AFLGHVGSKT SCGYGVA--- ----TGGPLA WGLCYNKEMS
PissaCTL     FEPQGFGTTG NKTMQMMEIA AFLGHVGSKT SCGYGVA--- ----TGGPTA WGLCYNHEMS
PoptrCTL2    FQHLGFCTTG GKATQMKELA AFLAHVGCKT SCGYGVA--- ----TGGPLA WGLCYNKEMS
MedtrCTL     YQPLGFGTSG GKHGGQKEVA AFLGHVGSKT SCGYGVA--- ----TGGPFA WGLCYNKELS
GlymaCTL     YQPHGFGTTG GKTSGQKELA AFFGHVGSKT SCGYGVA--- ----TGGPLA WGLCYSKELS
GoshiCTL1    YQPHGFGTTG GKLQSMKEVA AFLGHVGSKT SCGYGVA--- ----TGGPLA WGLCYNKEMS
GoshiCTL2    YQPHGFGTTG EKLQNMKEVA AFLGHVGSKT SCGYGVA--- ----TGGPLA WGLCYNKEMS
GoshiCTL3    YEPLGFGTTG GKRMQMKEVA AFLAHVGAKT SCGDGVI--- ----DGGPLA WGLCFKREMS
OrisaCTL1    FEPLGFCTTG GKQMQMMELC AFLGHVGSKT SCGFGVA--- ----TGGPTA WGLCYNHEMS
OrisaCTL2    FEPRGFGTTG GKEVGMKEVA AFLGHVGAKT SCGYSVA--- ----TGGPLA WGLCYNHELS
TriaeCTL     YEPRGFGTTG GREMSMKEVA AFLGHVSAKT SCGYSLA--- ----DGGSLA WGLCYNHEMS
VinviCTL1    YQPLGFGTTG GKVMQMKELA AFLGHVGCKT SCGYGVA--- ----TGGPLS WGLCYNKEMS
VinviCTL2    YQPHGFGTAG GKLMQMKEVA AFLGHVGSKT TCGYGVA--- ----TGGPLA WGLCYNKEMS
CitsiCTL1    YQPHGFGTSA GKLMGQKEVA AFLGHVGSKT SCGYGVA--- ----TGGPLA WGLCYNKEMS
CitsiCTL2    YQPLGFGTTG TKLDKMKEIC AFLAHVGCKT SCGYGVA--- ----TGGPLA WGLCYNHEMS
SoltuCTL     YQPLGFGTTG GKKMQMKEIA AFLGHVGSKT SCGYGVA--- ----TGGPLA YGLCYNKEMS
ZeamaCTL     FEPQGFCTTG GKQMQMMELC AFLGHVGAKT SCGYGVA--- ----TGGPTA WGLCYNHEMS
MaldoCTL     FEPLGFGTTG GKLMQMKEIA AFLGHVGSKT SCGYGVA--- ----TGGPYA WGLCYNREMS
BranaCTL     FEPLGFGTTG GKLMGQKEMA AFLGHVASKT SCGYGVA--- ----TGGPLA WGLCYNREMS
HorvuCTL     FEPRGFGTTG GREMSMKEVA AFLGHVGAKT SCGYSLA--- ----TGGSLA WGLCYNHEMS
PontrCTL     YQPLGFGTTG TKLDKMKEIC AFLAHVGCKT SCGYGVA--- ----TGGPLA WGLCYNHEMS
SacofCTL     FEPQGFCTTG GKQMQMMELC AFLGHVGAKT SCGYGVA--- ----TGGPTA WGLCYNHEMS
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             ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                125        135        145        155        165        175
HorvuCHI26   ASSDYCTPS- ---AQWPCAP GKRYYGRGPI QLSHNYNYGP AGRAIGVDLL ANPDLVATDA
PhypaCTL1    PEKDYC-KTG D--LMYPCAP GAGYYGRGAF PLYWNYNYGP TGVALKQDLL HHPEILSQNE
PhypaCTL2    PMKDYC-KTG D--LLYPCAP GAGYYGRGAF PLYWNYNYGP TGKALKQDLL HHPEILAQNE
CycruCTL     PDQLYCAQNY ----LYPCAP GASYHGRGAL PVYWNYNYGQ IGEALKVDLL NHPEYLADNA
GinBiCTL     PDQLYCDPNY ----LYPCSP GASYHGRGAL PVYWNYNYGQ IGEALKVDLL THPEYLADNA
PintaCTL1    PDSTYCESS- ---LVYPCAP GVSYHGRGAL PVYWNYNYGQ LGQALKVDLL HHAEYLSENA
PintaCTL2    PDQLYCDQN- ---LLYPCAP GASYHGRGAL PIYWNFNYGP IGEALKLDLL TSPDMVSNNA
PicglCTL1    PDSTYCESS- ---LVYPCAP GVSYHGRGAL PVYWNYNYGQ LGQALKVDLL HHAEDLSQNA
PicglCTL2    PDQLYCDQNY ----LFPCSP GASYHGRGAL PLYWNYNYGQ IGEALKLDLL NYPDLLSNNA
ArathCTL2    PDQLYCDDYY --KLTYPCTP GVSYHGRGAL PVYWNYNYGQ TGEALKVDLL SHPEYLENNA
ArathCTL1    PMQSYCDES- -WKFKYPCSP GAEYYGRGAL PIYWNFNYGA AGEALKADLL NHPEYIEQNA
PoptrCTL1    PSKTYCDDYY --KYTYPCTP GVSYHGRGAL PLYWNYNYGK TGEALKTDLL NHPEYLENNA
PissaCTL     PSQTYCDDYY --KLTYPCTP GAEYYGRGAI PIYWNYNYGA AGEALKVNLL DHPEYIEQNA
PoptrCTL2    PSQTYCDDFY --KYTYPCTP GAEYYGRGAI PIFWNYNYGA AGEALKEDLL SHPEYIEQNA
MedtrCTL     PDKFYCDDYY --KLTYPCSP GAAYYGRGAI PIYWNYNYGK IGEALKVDLL NHPEYIEQNA
GlymaCTL     PDKFYCDDYY --KLTYPCTP GAAYYGRGAI PLYWNYNYGK AGEALKVDLL NHPEYIEQNA
GoshiCTL1    PSKLYCDDYY --KYTYPCTP GVSYHGRGAL PIYWNYNYGE TGDALKVDLL NHPEYIENNA
GoshiCTL2    PSKIYCDDYY --KYTYPCTP GVSYHGRGAL PIYWNYNYGE TGEALKVDLL NHPEYLEDNA
GoshiCTL3    PSQDYCDDY- -YKYMYPCAP GAQYYGRGAL PIYWNYNYGA AGDGIKVDLL HHPEYLEQNA
OrisaCTL1    PKEDYCDKTN ---LQYPCVE GAEYYGRGAI PVFWNYNYGA AGDGIHEDLL HHPEYLEQNA
OrisaCTL2    PSQSYCDNSN E---LYPCVE GVEYYGRGAL PVYWNYNYGI IGQGIKQDLL NHPELLEQNA
TriaeCTL     PSQSYCDDSN E---LYRCAE GVEYYGRGAL PVYWNYNYGI VGKGIKQDLL NHPELLEQNA
VinviCTL1    PSKSYCDDF- -YKYTYPCTP GADYYGRGAL PIFWNYNYGA TGEALKVNLL DHPEYIEQNA
VinviCTL2    PSKSYCDDD- -YKYTYPCTP GVEYFGRGAL PIYWNYNYGE AGEALKVDLL NHPEYIEQNA
CitsiCTL1    PNQIYCDDD- -FKYTYPCTP GVSYHGRGAL PLYWNYNYGE TGEALKVDIL NHPEYIENNA
CitsiCTL2    PSQSYCDDS- -YKYTYPCTP GAEYYGRGAI PIYWNYNYGA TGEALKADLL SHPEYIEQNA
SoltuCTL     PSQDYCDDY- -FKLTYPCTP GARYYGRGAL PIYWNYNYGA IGDALKLNLL DHPEYIEQNA
ZeamaCTL     PDQTYCDKTY ---TQYPCVE GAEYYGRGAI PVYWNYNYGA AGDGIKADLL HHPEYLEQNA
MaldoCTL     PMQSYCDDY- -YKYIYPCSP GAEYYGRGAL PIYWNYNYGA AGDALKVDLL NHPEYIEQNA
BranaCTL     PMQSYCDET- -WKYKYPCSP GAEYYGRGAL PIYWNFNYGA AGEALKADLL NHPEYIEQNA
HorvuCTL     PSQSYCDDSN E---LYRCAE GVGYYGRGAL PVYWNYNYGI VGKGIKQDLL NHPELLEQNA
PontrCTL     PSQSYCDDS- -YKYTYPCTP GAEYYGRGAI PIYWNYNYGA TGEALKADLL SHPEYIEQNA
SacofCTL     PDQTYCDKTY ---TQWPCVE GAEYYGRGAI PVYWNYNYGA AGDGIKVDLL HHPEYLEQNA
```

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            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
               185        195        205        215        225        235
HorvuCHI26  TVSFKTAMWF WMT-AQPPKP SSHAVIVGQW SPSGADRAAG RVPGFGVITN IINGGIECGH
PhypaCTL1   TIAWQAAVWY WMTPAKTR-P SPHEIMIGKW VPTKNDTLAY RKPGFGMTIN VKASDVECGH
PhypaCTL2   TIAWQAAIWY WMTPAKTR-P SPHEVMIGKW VPTKNDTLAN RKPGFGMTIN IKASDVECGH
CycruCTL    TLAFQAAIWR WMNPIKPKQP SAHDVMVGKW IPTKNDTNSF RLPGFGMTIN ILDGDAECGK
GinBiCTL    TLAFSAAIWR WMTPIKRKQP SAHEVIVGKW VPTKNDTKSF RLPGFGMTIN ILDGDAECGK
PintaCTL1   TLAFAAAIWR WMTPMKVKQP SAHQVMVGKW VPTKNDTEAL RLPGFGMTIN ILKADAECGT
PintaCTL2   TIGFLTAMWR WMNPIKPKQP SAHDVFVGNW KPTKNDTESY RLPGFGMVIN VLNGGLECGK
PicglCTL1   TLAFQAAMWR WMNPIKVKQP SAHQVMVGKW VPTKNDTNSL RHPGFGMTIN ILKGEAECGA
PicglCTL2   TIGFQTAIWR WMNPIKPKQP SAHDVLVGNW KPTKNDTASF RFPGFGMTIN VLNGGLECGQ
ArathCTL2   TLAFQAAIWR WMTPPKKHLP SAHDVFVGKW KPTKNDTAAK RTPGFGATIN VLYGDQICNS
ArathCTL1   TLAFQAAIWR WMTPIKRAQP SAHDIFVGNW KPTKNDTLSK RGPTFGSTMN VLYGEYTCGQ
PoptrCTL1   TLAFQAAIWK WMTPEKKHLP SAHDVFVGKW KPTKNDTLAK RVPGFGTTMN VLYGDQVCGK
PissaCTL    TLAFQAAIWK WMTPVKKAQP SAHDAFVGNW KPTKNDTMGN RVPGFGATMN ILYGEGVCGQ
PoptrCTL2   TLAFKAAMWR WYTPIKKSQP SAHEAFLGKW KPTKNDTLAK RVPGFGTTMN VLYGDQVCGQ
MedtrCTL    TLAFQAALWK WMTPPEKHIP SPHDVFVGNW KPTKNDTLSK RVPGFGATIN VLYGDQVCDQ
GlymaCTL    TLAFQAALWQ WMTPPEKHLP SPHDVFVGNW KPTKNDTLSK RVPGFGATIN LLYGDQTCGQ
GoshiCTL1   TLAFQAALWR WMTPVKKHQP SAHDVFVGSW KPTKNDTLAK RVPGFGATMN VLYGDQVCGR
GoshiCTL2   TLAFQTAMWR WMTPMKKHQP SAHDVFVGNW KPTKNDTLAK RVPGFGTTMN VLYGDQVCGQ
GoshiCTL3   TIAFQAAIWR WMTPIKKNQP SAHDIFVGNW KPTKNDTEEK RGPTFGSTMN VLYGDYTCGQ
OrisaCTL1   TMAFMAAMWR WMTPMKKKQP SAHDVFVGNW KPTKNDTLAK RLPGFGATMN VLYGDQICGK
OrisaCTL2   TLAFEAAIWR WMTPMKRKQP SAHDVFVGNW KPTKNDTLSK RYPGFGATMN ILYGDLICGQ
TriaeCTL    TLAFEAAIWR WMTPMKRKQP SAHDAFVGNW KPTKKDTLSK RYPGFGATMN ILYGDAICGK
VinviCTL1   TLAFQAAIWR WMTPVKKSQP SAHDVFVGNW KPTKNDTLAK RVPGFGTTMN ILYGDQVCGQ
VinviCTL2   TLAFQAAIWR WMTPVKKQQP SAHDVFVGTW KPTKNDTLAK RIPGFGATMN VLYGDSVCGQ
CitsiCTL1   TLAFQAAMWR WMTPVKKHQP SAHDAFVGNW KPTKNDTLAK RVHGFGTTMN VLYGDQVCGK
CitsiCTL2   TLAFEAAIWK WMTPVKKSQP SAHDAFVGNW KPTKNDTLSK RGPNFGTTMN ILYGESVCGQ
SoltuCTL    TMAFQAAIWR WMNPMKKGQP SAHDAFVGNW KPTKNDTLSK RLPGFGTTMN ILYGDAVCGQ
ZeamaCTL    TLAFMAAMWR WMTPIKKSQP SAHDAFVGNW KPTKNDTLSK RLPGFGATMN ILYGESICGK
MaldoCTL    TLAFQAAVWR WMTPIKKSQP SAHQAFVGDW KPTKNDTLSK RFPGFGTTMN ILYGESVCGK
BranaCTL    TLAFQAAIWR WMTPIKKAQP SAHDIFVGNW KPTKNDTLSK RGPTFGTTMN VLYGEYTCGQ
HorvuCTL    TLAFEAAIWR WMTPMKRKQP SAHDAFVGNW KPTKKDTLSK RYPGFGVTMN ILYGDAICGK
PontrCTL    TLAFEAAIWK WMTPVKKSQP SAHDAFVGNW KPTKNDTLSK RGPNFGTTMN ILYGESVCGQ
SacofCTL    TLAFMAAMWR WMTPIKKNQP SAHEAFVGTW KPTKNDTLSK RLPGFGATMN ILYGESICGK
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                245        255        265        275        285
HorvuCHI26   GQDS-RVADR IGFYKRYCDI LGVG---YGN N--LDCYSQ- -RPF----
PhypaCTL1    G-EDPRMQSR ISHYLTFLRD TFQLDDP-GS NLD--CGLQG VIPLAY--
PhypaCTL2    G-DDPRMLSR ISHYLDFLQN KFQVQDP-GA NLD--CGLQG VVPLAY--
CycruCTL     G-DVEKMDNR ISHYLYFLDL MGVGRQYSGD NLD--CGQQ- -VALNPAS
GinBiCTL     G-DIEKMSNR ISHYLYFLDL MGVGRQFAGV NLD--CGQQ- -VPLNPSA
PintaCTL1    DSDDKQMNTR IAHYLDFLDH MDVGRENAGD NVD--CSEQ- -KVLNPSS
PintaCTL2    G-DIDAMNNR ISHYLYFLDL LGVGREQAGD NLD--CGQQ- -VPLNPVS
PicglCTL1    GSDDKQMNKR IAHYLYFLDQ LDVGRDNAGD NLD--CSDQ- -KVLNPSS
PicglCTL2    G-DIEAMSNR VSHYLHFLDL MGVGRELAGD HLD--CGEQ- -VALNPVS
ArathCTL2    GfDNDEMNNI VSHYLYYLDL IGVGREEAGP HEKLSCADQ- -EPFSSSS
ArathCTL1    GS-IDPMNNI ISHYLYFLDL MGIGREDAGP NDELSCAEQ- -KPFNPST
PoptrCTL1    G-D-ESMNNI VSHYLYYLDL MGVGREEAGS HDVLSCAEQ- -LPFNQAS
PissaCTL     G-DVDSMNNI ASHFLFYLDL LGVGRDKGGT HDVLTCAEQ- -RPFNPNT
PoptrCTL2    G-DIDAMNNF ISHYLYYLDL LGLNREDAGP HEYLTCAEQ- -VAFNPST
MedtrCTL     GSDNEAMSNI ISHYLYYLDL LGVGREEAGP NEILSCAEQ- -AAFKPTG
GlymaCTL     GSDNEAMNNI ISHYLYYLDL LGVGREEAGP NEVLSCAEQ- -AAFKPSG
GoshiCTL1    G-DVDTMNNI ISHYLSYLDL MGVGREEAGP HEVLTCEEQ- -KPFTVSP
GoshiCTL2    G-DSDSMNNM ISHYLYYLDL LGVGREEAGP HDMLTCEEQ- -EPFTVSP
GoshiCTL3    G-DIDPMNII ISHYLHYLDL LGVGREEAGP HEELSCAEQ- -KAFNPTP
OrisaCTL1    GY-IDDMNVI ISHYQYYLDL MGVGREHSGD NRD--CAEQ- -AAFNPSY
OrisaCTL2    GS-IDKMNVI VSHYQHYLDL MGVGSDKAGD NLD--CADQ- -VAFNPSS
TriaeCTL     GS-IDNMNGI ISHYQHYLDL MGVGAQHSGD NLD--CADQ- -VPFNPSS
VinviCTL1    G-DVDSMNNI ISHYQYYLDL LGVGREQAGP HENVTCAEQ- -IAFNPSY
VinviCTL2    G-DVDSMNNI VSHYQYYLDL MGVGREEAGP HEVLTCAEQ- -EAFNPSS
CitsiCTL1    G-DDESMNNM ISHYLYYLDL MGVGREEAGP NEVLSCGEQ- -EPFNPSS
CitsiCTL2    G-DIDAMNNI VSHYLYYLDL LGVGREQAGP NEELSCAEQ- -KAFNPNT
SoltuCTL     G-DVDSMNNI ISHYQYYLDL MGVGREEAGP HEVLNCAEQ- -KPFNPTA
ZeamaCTL     GY-VDAMNVI ISHYQYYLDL MGVGREHSGD NRD--CAEQ- -APFNPSS
MaldoCTL     G-DIDAMNNI VSHYQYYLDL MGVNRDEAGP HEVLTCAEQ- -VAFNPIq
BranaCTL     G-DIEPMSNI VSHYLYFLDL LGIGREDAGP NEELSCAEQ- -KAFNPAT
HorvuCTL     GT-TESMNAI ISHYQHYLDL MGVGVQHSGD NLD--CADQ- -VPFNPSS
PontrCTL     G-DIDAMNNI VSHYLYYLDL LGVGREQAGP NEELSCAEQ- -KAFNPNT
SacofCTL     GF-IDAMNTI ISHYQYYLDL MGVGREHSGD NRD--CAEQ- -LPFNPSS
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