MODIFICATION OF CELLULOSE BIOSYNTHESIS THROUGH VARIED EXPRESSION OF SUCROSE METABOLISM GENES IN TOBACCO AND HYBRID POPLAR

by

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Abstract

UDP-glucose, the precursor for cellulose biosynthesis, can be produced via the catalysis of sucrose by sucrose synthase (SuSy) or through the phosphorylation of glucose-1-phosphate by UDP-glucose pyrophosphorylase (UGPase). As such, these genes, together with sucrose phosphate synthase (SPS) which recycles fructose (an inhibitor of SuSy), are interesting targets for altering carbon allocation in plants.

In an attempt to alter cell wall biosynthesis in plants, targeted overexpression of SuSy, UGPase and SPS independently and in a pyramiding strategy was assessed in tobacco. All lines displayed enhanced growth and biomass production, and in the case of double and triple transgenics, there was an additive effect. Despite the increased growth rates, there was no consistent change in soluble carbohydrate pools. Furthermore, only the triple transgenics had constant changes in structural carbohydrates: with increased hemicellulose content and slight increases in cellulose. Collectively, these results support the role of SPS, SuSy and UGPase in maintaining sink strength, but suggest that the reallocation of carbon to cellulose production in tobacco may not be possible by overexpressing these genes.

In contrast, transgenic poplar overexpressing UGPase produced significantly more cellulose than wild-type trees. However, this was accompanied by a severe reduction in growth and the production of a salicylic acid glucoside (SAG) in significant quantities. The UDP-glucose generated by UGPase overexpression appeared to participate in both the synthesis of cellulose and SAG, suggesting that cellulose biosynthesis may be limited by the cellulose synthase complex.

Poplar transformed with SuSy and with SuSy × UGPase also had increased cellulose production. The trees were phenotypically normal, with only minor reductions in height growth in some lines. It appears that UDP-glucose may be channelled directly to the cellulose synthase complex by SuSy. The increased cellulose content was associated with an increase in cell wall crystallinity, but there was no change in microfibril angle, confirming the re-allocation to cellulose synthesis was not the result of tension wood formation, again supporting the hypothesis that the cellulose synthase complex is the limiting factor.

Clearly, it is possible to alter cellulose deposition in trees by augmenting sucrose metabolism to produce UDP-glucose, the precursor to cellulose biosynthesis.

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This thesis contains four manuscripts written with the intent of publication in peer-reviewed journals. Chapters 2 and 4 comprise manuscripts that have been published. Chapters 3 and 5 consist of manuscripts that will be submitted for publication. Author contributions to the work are as follows:

Chapter 2: Heather Coleman helped design the research, performed the research, conducted all data analyses, and prepared the manuscript. Margarita Gilbert assisted in the design of the research. David Ellis helped identify the research opportunity, assisted in design of research and edited the manuscript. Shawn Mansfield contributed to research opportunity identification and research design, edited the manuscript, and supervised the work.

Chapter 3: Heather Coleman helped design the research and performed the research, conducted all data analyses, and prepared the manuscript. Leigh Beamish and Anya Reid assisted in performing research. Ji-Young Park assisted in the design of the research. Shawn Mansfield contributed to research opportunity identification and research design, edited the manuscript, and supervised the work.

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Introduction

Background

Cellulose is the most abundant organic polymer in the world and is produced by plants at an approximate rate of 10^{11} tons per year (Preston, 1974). The majority is deposited in the stems of woody plants and is commonly utilized for fuel, timber, forage, fibre and chemical cellulose. It is for this reason that carbon partitioning to cellulose production is a key question for researchers globally (Haigler *et al.*, 2001). The biosynthesis of cellulose utilizes the products of photosynthesis, and competes for these resources with a number of other pathways including starch deposition and the non-cellulosic components of cell walls.

Carbon sequestration and the intrinsic value of the forest are becoming more important to the global community resulting in the increased preservation of forested land. Concurrently, the global population is growing and the demand for wood fibre is increasing. As such, there is a trend towards more intensive management and shorter crop rotations. While this shift will result in an increase in the amount of fibre supplied, the rapid growth of these trees will result in decreased wood quality.

The value of harvestable trees is directly related to the amount and quality of the cellulose fibres. The availability of carbohydrates from photosynthesis is not a limiting factor in cellulose biosynthesis despite having a crucial role in plant growth and survival. Therefore, by increasing the utilization of carbohydrates in cellulose production, more cellulose should be produced, and consequently fibre yield would increase. Sucrose and other translocatable carbohydrates produced during photosynthesis are transferred to sink tissues where they are converted to starch or cellulose. The creation of a carbohydrate sink in cellulose-producing cells should allow a greater portion of the photosynthates to be diverted to these cells, and therefore to cellulose production.

Cellulose Biosynthesis

Cellulose is the structural backbone of plant cell walls and plays a key role in cell shape and the morphology of plants. It is a linear polymer comprised of D-glucose

residues linked by β-1,4 glycosidic bonds such that every glucose is rotated by approximately 180°. The structural repeating unit is cellobiose, in contrast to many other glucan polymers where the repeating unit is glucose (Brown *et al.*, 1996). Because of its unique structure, cellulose chains can interact with each other, and assemble into microfibrils containing an estimated 36 strands (Reiter, 2002). These strands are aligned in a parallel fashion in cellulose I, the most prevalent natural cellulose type (Reiter, 2002). Microfibrils are often further associated into macrofibrils or bundles. This strand association is so precise that microfibrillar cellulose is essentially crystalline (Delmer and Amor, 1995).

The formation of cellulose into microfibrils is associated with the structure of the cellulose synthase complex, or rosette terminal complex. The rosette is an organized enzyme complex, hexagonal in structure, which produces cellulose microfibrils. It is believed to be composed of 6 units that contain a number of cellulose synthase subunits (possibly 6) that each produces a glucan chain (Delmer, 1999). The resultant chain from each unit associates with adjacent chains of the same rosette to form a microfibril. The cellulose synthase complex is large, with distinct transmembrane helices and a cytosolic component that is expected to be the location of the active site involving UDP-glucose (Brown and Saxena, 2000; Delmer, 1999).

Although the cellulose synthase complex has been identified visually, identifying the number and association of the cellulose synthase genes has been a slower process. The first cellulose synthase gene was cloned and isolated from *Acetobacter xylinum*, a bacterium that secretes large quantities of cellulose in long microfibrils (Saxena *et al.*, 1990). However, an additional 6 years passed before the first identification and isolation of a cellulose synthase gene in plants. The first identified were 2 genes highly expressed during the phase of cellulose synthesis in cotton fibre development (Pear *et al.*, 1996). Since then, cellulose synthases have been identified in many plants and all have been shown to share the D,D,D,QxxRW signature (Williamson *et al.*, 2002). The sequencing of the *Arabidopsis* genome has identified 10 cellulose synthase (CesA) genes and an additional 6 groups of cellulose synthase like (CSL) genes (Richmond, 2000). The more recently sequenced poplar genome has identified 18 predicted CesA genes (Djerbi *et al.*, 2005), which are thought to be 9 types of duplicated genes.

Sucrose Metabolism

Cellulose is produced from the precursor UDP-glucose which can be produced by a number of pathways (Figure 1.1; Table 1.1). Sucrose synthase (SuSy; EC 2.4.1.13) catalyzes the reaction of sucrose and UDP to UDP-glucose and fructose. The reaction in plants is reversible, and SuSy has been shown to act in the degradation of sucrose. This reaction provides energy for phloem unloading by providing substrate for respiration (Hanggi and Fleming, 2001), and cleavage by SuSy has also been positively correlated with sink strength in storage organs of potatoes, maize kernels, and pea embryos (Sun *et al.*, 1992; Zrenner *et al.*, 1995; Dejardin *et al.*, 1999).

Two types of SuSy have been proposed, one of which is found in the cytosol and one that is directly associated with the plasmalemma (Amor *et al.*, 1995; Carlson and Chourey, 1996). Soluble SuSy (S-SuSy) exists in high levels in the cytoplasm of non-photosynthetic tissues where its products are used in general metabolism and for the synthesis of storage polymers such as starch (Haigler *et al.*, 2001). Particulate SuSy (P-SuSy) is associated with the plasma membrane or cortical cytoskeleton. It is believed to play a major role in providing UDP-glucose to the cellulose synthase complex, in particular during high rate secondary wall cellulose synthesis (Haigler *et al.*, 2001). There is evidence that the synthesis of the secondary cell wall may not rely on the pool of UDP-glucose, but may actually have access to UDP-glucose channelled directly from particulate SuSy in the cell cortex (Amor *et al.*, 1995). This would allow for the conservation of energy normally required for hydrolysis, as well as ensure the availability of UDP-glucose to the cellulose synthase complex despite the demand from other pathways.

The second putative UDP-glucose pathway involves a number of enzymes with the final reaction being catalyzed by UDP-glucose pyrophosphorylase (UGPase; EC 2.7.7.9), which phosphorylates glucose-1-phosphate (Table 1.1). This reaction is magnesium dependent and is regulated by pyrophosphate concentrations. It has been suggested that the direction of the reaction is likely regulated by the cytosolic availability of its substrates (Nakano *et al.*, 1989; Sowokinos *et al.*, 1993). In source tissues, UGPase works in conjunction with SPS in the synthesis of sucrose (Kleczkowski, 1994), while in sink tissues, where it is predominantly found, it is a key enzyme in carbohydrate

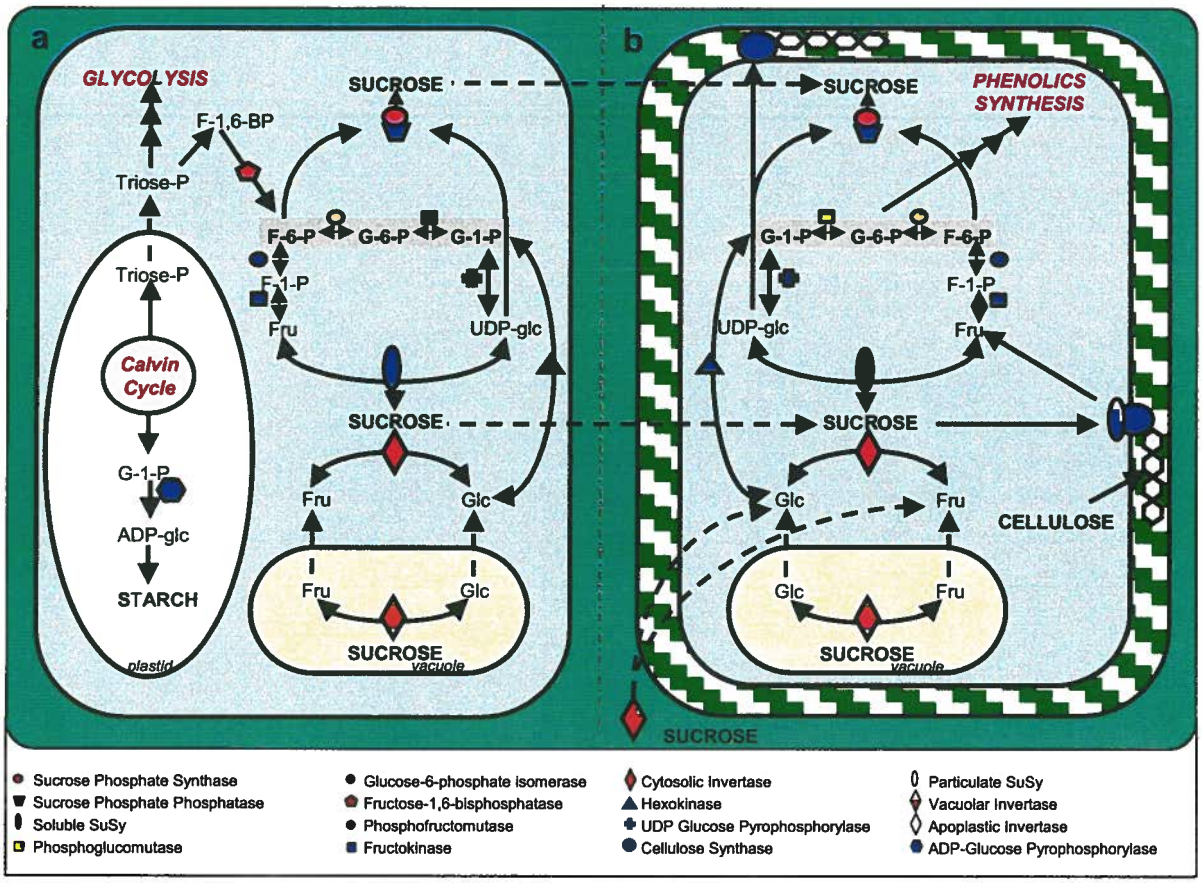

Figure 1.1. Schematic depicting sucrose metabolism from source (a) to sink (b).

Table 1.1. Enzymatic reactions catalyzed by UGPase, SuSy, SPS and SPP.

Gene	Reaction
UGPase	UTP + α-D-glucose 1-phosphate ↔ diphosphate + UDP-glucose
SuSy	UDP-glucose + D-fructose ↔ UDP + sucrose
SPS	UDP-glucose + D-fructose 6-phosphate → UDP + sucrose 6-phosphate
SPP	sucrose 6-phosphate + H_2O → sucrose + phosphate

biosynthesis. UGPase is thought to work in coordination with SuSy in the cycling between the hexose phosphate pool and sucrose.

Glucose-1-phosphate, along with glucose-6-phosphate and fructose-6-phosphate comprise the hexose phosphate pool. Phosphoglucomutase (PGM; EC 5.4.2.2), which reversibly converts glucose-1-phosphate to glucose-6-phosphate, and phosphoglucose isomerase (PGI; EC 5.3.1.9), which reversibly catalyses the reaction between glucose-6-phosphate and fructose-6-phosphate, maintain the equilibrium of the pool. Fructose-1,6-bisphosphate is an additional hexose phosphate, but is not normally considered to be a part of the hexose phosphate pool since in most organisms its conversion to fructose-6-phosphate by fructose-1,6-bisphosphatase (FPBase; EC 3.1.3.11) is irreversible. In plants however, there is an additional enzyme, phosphofructokinase (PFKase; 2.7.1.11), that catalyzes the pyrophosphate dependant reversible reaction between fructose-1,6-bisphosphate and fructose-6-phosphate, which is utilized in the production of sucrose.

An additional key enzyme in sucrose metabolism is sucrose phosphate synthase (SPS; EC 2.4.1.14). SPS works in coordination with sucrose phosphate phosphatase (SPP; EC 3.1.3.24) to synthesize sucrose from fructose-6-phosphate and UDP-glucose. This reaction is believed to play a large role in the recycling of fructose, which is a known inherent inhibitor of SuSy (Doehlert, 1987) and releases phosphate, which can then be recycled through triose-phosphate transporters into the chloroplast for subsequent photosynthetic reactions. As such, SPP has been implicated as having a substantial role in the regulation of the photosynthetic rate (Stitt *et al.*, 1987). The role of SPS in the synthesis of sucrose will also affect photosynthesis rate, as sucrose and other soluble sugars have a role in the regulation of photosynthetic gene expression (Koch, 1996).

It has been shown that cellulose biosynthesis is reliant on sucrose concentration rather than that of UDP-glucose, its immediate substrate. As such, invertase (EC 3.2.1.26) is another enzyme that may have a regulatory impact on the synthesis of cellulose. Invertase catalyzes the hydrolysis of sucrose into glucose and fructose. It is present in higher concentrations than the other sucrose metabolizing enzymes, and may play an important role in the hydrolysis of sucrose at all developmental stages (Canam *et al.*, 2008). Invertase works in coordination with SuSy and SPS in "futile

cycles" which allow for more efficient storage of sucrose in the vacuole and intercellular space (Nguyen-Quoc and Foyer, 2001).

Sucrose Synthase

Herbaceous Species

SuSy has been identified as having a key role in the metabolism of sucrose and as such has been employed in transgenic and mutant studies in numerous species including carrot (Tang and Sturm, 1999), maize (Carlson *et al.*, 2002; Chourey *et al.*, 1998), potato (Zrenner *et al.*, 1995; Bologa *et al.*, 2003), cotton (Ruan *et al.*, 2003), and tomato (Chengappa *et al.*, 1999; D'Aoust *et al.*, 1999).

The effect of reductions in SuSy has varying phenotypic effects on plants. In potato, the antisense suppression of SuSy resulted in no obvious phenotypic differences, but the tubers showed a pronounced decrease in dry weight. This was attributed to the enhanced levels of free glucose and fructose causing an increase in water uptake (Zrenner *et al.*, 1995). In carrot, both the tap roots and above ground portion of antisense plants were much smaller than those of the corresponding controls. In most lines the leaf-to-root ratio was unchanged, but in the line with the largest reduction in SuSy expression, there was a shift in the leaf-to-root ratio in favour of the leaves (Tang and Sturm, 1999). The downregulation of SuSy resulted in the accumulation of sucrose levels ranging from two- to four-fold in carrot tissue, while levels of fructose and glucose were correspondingly reduced to below 20% that of control levels. Both starch and cellulose were reduced to about 38% and 37% that of the control respectively, and UDP-glucose was reduced by up to 70% (Tang and Sturm, 1999). The maize endosperm mutant, *shrunken-1*, had shrunken seeds with collapsed endosperms, and showed a decrease in sucrose utilization along with increased glucose and fructose contents (Chourey *et al.*, 1998). In tomato, the dry weight was not affected, but there was a decrease in the number of flowers that set fruit in plants where SuSy activity was decreased to less than 10% that of the activity in the control plants. This suggests that SuSy, by controlling unloading capacity, determines the capability to set fruit (D'Aoust *et al.*, 1999). In cotton, the suppression of SuSy inhibited fibre initiation and elongation. When SuSy was decreased in the ovule epidermis by 70% or

more, the result was a fibreless phenotype. One line was also dwarfed and aborted all fruit by 10 days after anthesis (Ruan *et al.*, 2003). The transgenic fruit contained just as many seeds as the control fruit, but only 5.7% of these seeds were considered normal sized with approximately 70% reduction of fibre mass. The remaining seeds were unviable or stunted (Ruan *et al.*, 2003).

These studies provide insight into the role of SuSy in sink tissues. In some species and tissues, such as potato and maize endosperm, SuSy is involved mainly in the synthesis and storage of starch. Tang & Sturm (1999) surmised that SuSy is a major determinant of growth in carrot, and appears to be the major enzyme in sucrose cleavage supplying carbohydrates to metabolism. Plant size was greatly reduced as the downregulation of SuSy caused a decrease in the availability of carbon (Tang and Sturm, 1999). Furthermore, the maize and potato tuber studies suggest a role for SuSy in determining sink strength (Zrenner *et al.*, 1995). This is supported by the decrease in starch accumulation, which parallels the reduction in SuSy activity.

The *shrunken-1* maize mutant showed a 22% reduction in starch content relative to the control. More recently, this has been shown to be a secondary effect, with the real effect of the mutation being cell degeneration during the cell elongation phase (Chourey *et al.*, 1998). A supporting study illustrated a 99% reduction in SuSy activity resulted in 46% starch reduction. It was concluded that the two SuSy genes that had been identified in maize at the time of this study have different roles: one provides substrate for cellulose biosynthesis, while the other provides precursors for starch biosynthesis (Chourey *et al.*, 1998).

The study by Ruan *et al.* (2003) in cotton demonstrated a rate-limiting role for SuSy in the initiation and elongation of cotton fibre cells; SuSy can be used as a determinant for the sink strength of fibre cell development (Ruan *et al.*, 2003). This study also showed that the suppression of SuSy in the seed coat reduced fibre length, but if SuSy was also suppressed in the endosperm and embryo, the seed did not develop at all. This supports the role of SuSy in controlling plant cell and seed development (Ruan *et al.*, 2003).

Two concurrent studies employing antisense SuSy in tomato plants report the same findings with regards to sugars, starch and cellulose (Chengappa *et al.*, 1999; D'Aoust *et al.*, 1999). D'Aoust *et al.* (1999) found that fruit growing from flowers that

developed in the first week of flowering were smaller than those of the control from the same time period. Fruit set also decreased in the transgenic lines. The conclusion is that SuSy inhibition leads to a reduction in unloading capacity in the young fruit (D'Aoust *et al.*, 1999). Vacuolar invertase is the main sucrose metabolic pathway when SuSy is absent or inhibited. When the tomato fruit is young, it has small vacuoles thus when SuSy is reduced the fruit will grow more slowly. As the fruit from the transgenic plant gets larger and the vacuoles expand it will begin to grow as quickly as the fruit from control plants because there is an increase in invertase activity. This suggests that SuSy controls phloem loading into the initial fruits and that this unloading consequently determines the growth rate of the remaining growth phase (D'Aoust *et al.*, 1999).

Woody Species

In comparison to the numerous studies in herbaceous species, relatively little work has been carried out in woody species. A few studies of native gene expression (cited below) have been done, but to our knowledge, only one study has involved the use of transgenics (Konishi *et al.*, 2004).

Egger & Hampp (1993) studied the development of spruce needles throughout the transition from sink to source with respect to SPS, SuSy, and invertase. They found that SuSy was dominant in the very young needles when they are strong sinks, where it acts in coordination with invertase to import carbon in the form of sucrose. As the leaves mature, and gain photoassimilatory competence, the activity of both enzymes decreases and SPS begins to increase. A similar pattern was shown in maize leaves (Nguyen-Quoc *et al.*, 1990). In spruce needles, SuSy activity increased steeply in autumn and winter, along with that of SPS, and both enzymes are at their highest rates from October to February. This is similar to the trend observed in poplar stems (Schrader and Sauter, 2002), and is thought to be related to frost hardiness and the accumulation of osmotically active compounds (Guy, 1990). In late winter, the needles are filled with starch and high SPS and SuSy activities could be related to the mobilization of chloroplast starch for bud support, in addition to glycolytic turnover (Egger and Hampp, 1993). In poplar, SuSy shows particularly high activities in December and January when most of the starch has been converted in sucrose. The

activity of SuSy peaks shortly after SPS and is expected to work in coordination with SPS to maintain a sugar cycle where sucrose is synthesized by SPS and degraded by SuSy (Schrader and Sauter, 2002). This cycle is thought to regulate sucrose levels in response to changing temperatures.

Hauch and Magel (1999) examined the sucrose metabolism enzymes in *Robinia* trunk tissues throughout the year. In April and May, SuSy activity was at its highest in the differentiating xylem. This can be attributed to the high demand for cell wall material in this rapidly growing sink tissue. SuSy activity decreased as the tissue matured and was very low during the winter. The same trend was found in poplar, with SuSy activity being highest in the early summer, but only in the outer wood where cell differentiation takes place (Schrader and Sauter, 2002). In the main storage portion of the *Robinia* trunk, SuSy rates increased until autumn and then decreased, coinciding with starch accumulation. The transition zone from sapwood to heartwood also showed an increase from July to a maximum in September and November, and then decreased. The peak in SuSy activity coincides with increased activities of the enzymes involved in the phenylalanine pathway and with the accumulation of phenolic extractives. The conclusion was that a high amount of sucrose cleavage is required to provide the energy and substrates for the synthesis of these extractives (Hauch and Magel, 1999).

Magel *et al.* (2001) also studied non-structural carbohydrates and sucrose metabolizing enzymes in the trunk tissues of two *Juglans* species. In contrast to the study in *Robinia*, no season-dependent changes were seen in the radial distribution of non-structural carbohydrates. In Black Walnut, the content of starch strongly decreased with increasing tissue age. Sucrose pools were diminished in the transition zone, and both starch and sucrose were non-existent in the heartwood. Glucose started to accumulate at the transition zone and there were high levels in the heartwood. In contrast, in the *Juglans* hybrid, starch content was consistent throughout all living cells, and pools of sucrose decreased slightly towards the centre of the tree. Glucose and fructose levels were low in all tissues. Starch was deposited in the early sapwood until autumn, and decreased from November to February resulting in a significant increase in sucrose, fructose and glucose. Starch storage is correlated with the enhanced activities of SuSy and UGPase, while decreases in starch content and increases in soluble carbohydrates are correlated to an increase in SPS. An increase in sucrose activates

the enzymes of sucrolysis. In the wood of hybrids this increase is attributed to invertase, but in black walnut, as in many herbaceous plants, it is SuSy whose activity increases.

An additional study investigated the effects of carbohydrates and sucrose metabolism enzymes on the transition from early wood to late wood in Scots Pine (*Pinus sylvestris*) (Uggla *et al.*, 2001). Sucrose levels were highest in the phloem, with a steep decrease across the cambial zone to its lowest level in the developing xylem cells. Fructose and glucose showed the opposite pattern to sucrose, being present in low levels in the phloem and increasing towards the xylem. The peak amount was found in the zone of secondary wall formation. SuSy activity increased during the earlywood to latewood transition, and became predominant in the area of secondary wall formation. This is in agreement with SuSy having a role in both cellulose synthesis (Amor *et al.*, 1995) and lignin biosynthesis (Hauch and Magel, 1999). This is supported by the high activity of SuSy at the late stages of cell differentiation when cellulose deposition is decreasing and lignification occurs (Uggla *et al.*, 2001). The overexpression of a mutant mung bean (*Vigna radiata*) SuSy (S11E) in *Acetobacter xylinum* has been shown to enhance cellulose production (Nakai *et al.*, 1999).

SuSy upregulation has been studied in *Populus alba* using SuSy (S11E) and the 35S constitutive promoter (Konishi *et al.*, 2004). The overall morphology of the transgenics was unchanged, although a few lines were reported to have grown slightly better. SuSy expression was detected in both the leaves and stems, but was slightly higher in the leaves. It was also detected in both the soluble and microsomal fractions with higher expression in the soluble fraction. The study utilized a dual labelling system and provided evidence that at least some amount of sucrose is channelled directly to glucan formation via UDP-glucose. Overexpression of SuSy caused the glucose moiety of sucrose to be predominantly incorporated into the glucan backbone of xyloglucan in stem tissue. This allows the high-energy bond to be preserved and then used for polysaccharide synthesis. It has also been proposed that xyloglucan synthase may even form a putative synthase complex with SuSy (Konishi *et al.*, 2004). It appears that fructose is rapidly recycled to UDP-glucose by way of the hexose phosphate pool, or by SPS. Fructose inhibits SuSy (Doehlert, 1987) and may also be coupled with the negative signalling related to photosynthesis (Quereix *et al.*, 2001). Despite the

apparent channelling to glucan synthases, there was no change observed in cellulose or non-cellulosic polysaccharides. This is attributed to the high level of SuSy in leaf tissue relative to sink tissue, possibly retaining sucrose in the leaves and therefore never allowing it to reach the sink tissues.

UDP-Glucose Pyrophosphorylase

Herbaceous Species

UDP-glucose pyrophosphorylase (UGPase, EC 2.7.7.9) catalyzes the reversible reaction of glucose-1-phosphate and UTP to UDP-glucose and pyrophosphate. The reaction plays a large role in sugar accumulation (Sowokinos, 1990) and has the potential to restrict the flow of carbon to sucrose formation (Borokov *et al.*, 1996), due to the negative cooperativity for glucose-1-phosphate and UTP (Sowokinos *et al.*, 1993). However, it is unlikely that the role of UGPase is rate-limiting as it is generally present in very large quantities compared to other enzymes involved in sucrose metabolism (Jaing *et al.*, 2003). UGPase has been shown to directly affect cellulose biosynthesis, as in *Acetobacter xylinum*, cellulose negative mutants were complemented by UGPase and shown to mitigate cellulose accumulation (Valla *et al.*, 1989).

UGPase has been purified from numerous species including potato (Nakano *et al.*, 1989; Sowokinos *et al.*, 1993), soybean (Vella and Copeland, 1990), rice (Kimura *et al.*, 1992), and barley (Elling and Kula, 1994). Various isozymes seem to exist within the potato, which has both an acidic and a basic form. Transgenic studies that decreased UGPase activity with very little effect on growth and development support the theory of numerous isozymes (Borokov *et al.*, 1996; Spychalla *et al.*, 1994; Zrenner *et al.*, 1993). In *Dictyostelium discoideum*, two UGPase genes have been identified (Bishop *et al.*, 2002). A knockout of *udpgp1* had no phenotypic effect, while *ugpB* mutants were unaffected in early growth but unable to form viable spores. The lack of viable spores has been linked to an inability of the mutants to form cellulose (Bishop *et al.*, 2002). In barley, 11 cDNA encoding UGPase have been isolated, and differ only in the polyadenation site positioning. It has been suggested that they are likely encoded by the same gene as they are identical at the nucleotide level (Eimert *et al.*, 1996).

UGPase is believed to exist as a monomer, but a study of barley shows it can undergo reversible oligomerization as a form of regulatory mechanism and the monomer is the active form (Martz *et al.*, 2002). Two UGPase genes have been identified in poplar (Meng *et al.*, 2007). *UGP1* was shown to be upregulated by light and sucrose feeding, while both genes were upregulated by cold treatment.

Localization of the UGPase activity has been determined in a number of species. In the developing rice endosperm, UGPase is localized mainly in the cytosol (90%) with the remainder being found in the amyloplast and Golgi membranes (Kimura *et al.*, 1992). It has also been found in the microsome fraction of both rice and tobacco (Mikami *et al.*, 2001). In barley, cytosol and membrane fractions suggest that UGPase may bind to membranes (Becker *et al.*, 1995). UGPase shows high activity in the young seeds and vascular tissue of tomato, and is found in the phloem and epidermis hairs on petunia (Sergeeva and Vreugdenhil, 2002). In potato, the enzyme has been identified in roots, tubers, stolons, leaves and stems, but in varying quantities (Sowokinos *et al.*, 1993). The levels were highest in sink tubers and stolons, and the levels increased during tuber development and with sucrose feeding to detached leaves.

It has been shown in barley malt that inhibitory substrates exist in both the synthesis and pyrophosphorylysis reactions (Elling, 1996). In synthesis, UTP surplus has inhibitory effects. Pyrophosphate is a non-competitive inhibitor for glucose-1-phosphate and UTP. While inorganic phosphate is non-competitive inhibitor for glucose-1-phosphate and UTP, it has a much higher inhibition constant than pyrophosphate. UDP-glucose is a competitive inhibitor for UTP and a non-competitive inhibitor for glucose-1-phosphate. In synthesis, UGPase binds first to UTP and then to glucose-1-phosphate. In pyrophosphorylysis, UTP shows competitive product inhibition with UDP-glucose as a variable substrate.

A number of studies have examined the effect of pyrophosphatase on UGPase content. In potato, the overexpression causes a reduction in pyrophosphate content, with little effect on the plant growth (Farre *et al.*, 2001). The tubers were less dense, which was attributed to a 30-40% decrease in starch content. There was an increase in sucrose and glucose content, but no significant changes in fructose. UDP-glucose content increased significantly and there was a 40-60% decrease in glucose-6-

phosphate and 30-40% decrease in fructose-6-phosphate. Glucose-1-phosphate did not change appreciably. The removal of inorganic pyrophosphatase facilitated the enhanced conversion of glucose-1-phosphate to UDP-glucose. This is hypothesized to lead to improved sucrose and cell wall biosynthesis which may result in the accelerated sprouting phenotype observed in these plants (Farre *et al.*, 2001). This study reinforced the finding of a previous study that found the rate of sucrose degradation and starch content increased (20-30%) in growing potato tubers with pyrophosphatase overexpressed (Geigenberger *et al.*, 1998). This seemingly contrary report is related to the differing function of UGPase in source and sink tissues.

An investigation in developing potato tubers showed that the UGPase activity does not change with developmental stage (Appeldoorn *et al.*, 1997). In developing tubers UDP-glucose must be rapidly converted to allow the high net flux of sucrose into the tuber. While fructokinase activity increases, UGPase activity remains constant, suggesting that the presence of excess enzyme. Furthermore, it has been hypothesized that sucrose may regulate UGPase. This is consistent with the study by Spychalla *et al.* (1994), who showed an increase in mRNA in potato leaves incubated with high sucrose levels, as well as with the increased activity of the enzyme correlating strongly with sugar amounts accumulated during cold storage. UGPase activity is also regulated strongly by substrate availability (Vella and Copeland, 1990). It is also thought to be regulated by the ratio of SuSy to SPS activities (Kleczkowski, 1994). However, in transgenic maize where SuSy was downregulated, UGPase activity did not co-ordinately decrease (Carlson and Chourey, 1996). It has been suggested that the regulation of the gene is translational rather than transcriptional, as significant decreases in activity did not change growth and development (Kleczkowski, 1994; Zrenner *et al.*, 1993). However, a decrease in levels of inorganic phosphate has been connected to an increase in UGPase activity, which suggests transcriptional or post-translational control (Ciereszko *et al.*, 2001). Activities of AGPase, SPS, SuSy and invertase also increase in this situation and the effect may be related to phosphate-deficiency causing gene upregulation, or perhaps affecting the catalytic efficiency of the enzyme via protein turnover rate or post-translational mechanisms such as phosphorylation. Stress is also known to alter plant inorganic phosphate content and carbohydrate status. It is possible that the change in UGPase associated with

phosphate is related to the maintenance of plant nutritional status (Ciereszko *et al.*, 2001).

There have been a number of studies carried out on potato with decreased UGPase activity. Three significant studies resulted in contradicting results. The first showed that the activity of UGPase could be decreased by up to 96% without any detectable changes in sugar content or growth rates (Zrenner *et al.*, 1993). The other two studies showed that a decrease as low as 30-50% caused a decrease in sugar content (Borokov *et al.*, 1996; Spychalla *et al.*, 1994). The difference in findings could be attributed to differences in methods. Zrenner *et al.* (1993) utilized the entire coding region of a UGPase gene for the generation of the construct. Spychalla *et al.* (1994) used a 0.5 kb fragment from the 5' end of the cDNA and Borokov *et al.* (1996) used a piece of the *Ugp* genomic clone. The studies also employed different promoters with Zrenner *et al.* (1993) and Borokov *et al.* (1996) using the 35S cauliflower promoter and Spychalla *et al.* (1994) utilizing the patatin promoter. Zrenner *et al.* (1993) analyzed tubers that were still growing and acting as sinks, while the other studies looked at tubers stored at varying temperatures and were acting as source tissue. The conclusion from Zrenner *et al.* (1993) is that in sink tissues, only 4% of UGPase activity is necessary for normal plant growth. In mature tubers, it appears that the role of UGPase is more significant.

Woody Species

UGPase activity has been studied in a number of herbaceous species, but only preliminary work has been carried out in woody species, and there has yet to be any research evaluating transgenics. In a study on two species of *Juglans*, the distribution of UGPase activity was found to be similar to that of SuSy, but ten times higher (Magel *et al.*, 2001), with activity gradually decreased towards the transition zone and non-existent in the heartwood. UGPase activity also experienced seasonal fluctuations. In hybrid *Juglans*, activity increased during the summer and towards autumn, but was low in the winter. Activity in black walnut was similar, but the high autumn activity level was maintained through the winter and declined in the spring. UGPase is thought to play a crucial role in the metabolism of the products of sucrose cleavage, and it has a higher

activity than other sucrose metabolism enzymes. Based on the observation of the various sucrose metabolizing enzymes, Magel *et al.* (2001) presented the hypothesis that the formation of heartwood phenolics is enabled by the enzymes involved in sucrose metabolism as they facilitate the degradation of sucrose. At the same time and in the same location, the activities of enzymes involved in the phenolics pathways are also increased and utilize the products of sucrose degradation in the formation of heartwood extractives.

Sucrose Phosphate Synthase

Herbaceous Species

SPS is a key enzyme in regulating sucrose synthesis in plants. Protein association studies have shown evidence for a direct interaction between SPS and SPP, creating a metabolic channel from UDP-glucose and fructose-6-phosphate to sucrose (Echeverria *et al.*, 1997). SPS catalyzes the reaction from UDP-glucose and fructose-6-phosphate into sucrose-6-phosphate and UDP. Sucrose-6-phosphate phosphatase then acts to hydrolyse sucrose-6-phosphate into sucrose and a phosphate group. This specific and highly active phosphatase essentially draws the SPS reaction towards the production of sucrose, by altering the equilibrium of products. It is thought that SPS contributes to the control of flux into sucrose, as it contributes greatly to the biosynthesis of sucrose in both photosynthetic and non-photosynthetic tissues (Geigenberger *et al.*, 1999; Geigenberger and Stitt, 2000; Strand *et al.*, 2000). In addition to preventing the accumulation of fructose, a SuSy inhibitor, the reaction also releases phosphate, which has been suggested to have a substantial role in the regulation of photosynthetic rate through its cycling between the chloroplast and the cytosol (Stitt *et al.*, 1987). SPS has been shown to be under the regulation of a variety of mechanisms including enzyme abundance, allosteric control in which glucose-6-phosphate acts as an activator and phosphate as an inhibitor, and phosphorylation. SPS activity is also regulated by developmental, environmental and nutritional signals. Changes in irradiance can affect the transcription level, with an increase in light resulting in an increase in SPS mRNA levels followed by an increase in protein and activity (Klein *et al.*, 1993; Cheng *et al.*, 1996).

Transgenic plants have been used to examine the effects of SPS. In tomato, the overexpression of maize SPS caused an increase in the synthesis of sucrose, resulting in an increase in the sucrose to starch ratio in leaves, as well as an increase in photosynthetic capacity (Worrell *et al.*, 1991). This suggests that SPS has some effect on photosynthesis (Galtier *et al.*, 1993, Galtier *et al.*, 1995; Micallef *et al.*, 1995). In tobacco, the overexpression of a maize SPS caused the acceleration of flower development and an increase in flower numbers (Baxter *et al.*, 2003). Similarly, the overexpression of SPS resulted in an increased sucrose to starch ratio in *Arabidopsis* (Signora *et al.*, 1998) and improved fibre quality and yield in cotton (Haigler *et al.*, 2007). SPS overexpression in tomato resulted in a six-fold increase in SPS activity in leaf tissue, which resulted in slight changes in carbohydrate, but a marked increase in the sucrose to starch ratio (Worrell *et al.*, 1991).

There have been fewer studies involving the decreased expression of SPS, but a study in potato with decreased expression of SPS demonstrated the inhibition of sucrose synthesis and an increase in starch and amino acids (Krause *et al.*, 1998).

Recent studies of SPS overexpression in tobacco have revealed an increase in sink sucrose pools while there was not a concurrent increase in source sucrose pools. The plants were taller with increased biomass, but there were only minor changes in structural carbohydrates (Park *et al.*, 2008).

Woody Species

There is less known about SPS in trees than the body of knowledge in herbaceous plants and much of the research involves conifers. In spruce needles, SPS exhibits activity levels inverse to that of SuSy during the period directly following bud break (Egger and Hampp, 1993). SPS is negligible in the newest needles, but increases as the needles mature resulting in a change in the SPS/SuSy ratio as a tissue changes from a sink to a source. In autumn, the patterns of the two enzymes coincide, with both enzymes achieving their highest activity between October and February (Egger and Hampp, 1993). This is attributed to the role of these enzymes in metabolic acclimation to low temperatures. Further studies by the same group showed that SPS activity was low for the first 60 days after bud break; after the needle had achieved a

steady state dry weight/fresh weight ratio there was a steady increase in activity (Hampp *et al.*, 1994). This increase in the SPS/SuSy ratio is considered to be a measure of source or sink quality of a given tissue, and shows the transition point from the needle as a sink for carbohydrates to a source.

In spruce needles, SPS activity was higher during the period ending with bud break, due to an increase in protein levels and activation under metabolite control, high levels of glucose-6-phosphate and a low inorganic phosphate/glucose-6-phosphate ratio (Egger *et al.*, 1996). This period was also characterized by high rates of net photosynthesis, a large decrease in soluble sugars, and a steep rise in starch content. After bud break, net photosynthesis was greatly reduced (by about 75%), and SPS activity and protein level were decreased. There was also a reduction in the concentration of glucose-6-phosphate and an increased phosphate/glucose-6-phosphate ratio. During this period, sucrose synthesis was reduced in older needles and the carbon demand of the developing needles was met in part by the mobilization of starch from older needles (Egger *et al.*, 1996).

SPS activity in spruce can be regulated in an allosteric manner by glucose-6-phosphate and inorganic phosphate (Loewe *et al.*, 1996). Glucose-6-phosphate activates SPS by increasing its affinity for fructose-6-phosphate. Inorganic phosphate can inhibit this activation when the fructose-6-phosphate concentration is rate-limiting. The seasonal fluctuations in SPS activity correlate with fluctuations in fructose-6-phosphate and glucose-6-phosphate, which are both at higher concentrations in the winter. However, allosteric regulation of SPS seems to be species dependent, because various studies differing results (Loewe *et al.*, 1996).

In spruce, ATP-dependent phosphorylation did not play a major role in seasonal regulation of SPS activity, but the seasonal changes in activity are correlated with protein levels (Loewe *et al.*, 1996). High SPS activity in the winter indicates a high level of sucrose synthesis, which could be loaded into the phloem for export under mild winter conditions (Loewe *et al.*, 1996) or used for the formation of raffinose and stachyose, which are synthesized during cold acclimation (Egger *et al.*, 1996). The recycling of sucrose is necessary to recycle inorganic phosphate to accommodate the considerable rates of photosynthesis during the winter months. The role of SPS in cold

acclimated needles in correlation with photosynthetic activity and high levels of sucrose export have been supported by experiments in transgenic tomato (Galtier *et al.*, 1993).

In *Robinia*, SPS activity was low regardless of season, age, and physiological conditions (Hauch and Magel, 1999). Highest activities were in the middle sapwood (May) and inner sapwood (November), and decreased towards the bark and the sapwood/heartwood transition. Cold-adapted tissues show higher rates of SPS than samples harvested in the summer, which may be regulated allosterically by the seasonally increased pools of glucose-6-phosphate (Hauch and Magel, 1999). Exposure of living wood to decreased temperatures generally results in a decrease in starch content and a related increase in soluble carbohydrates which can act as cryoprotectants (Egger *et al.*, 1996; Sauter *et al.*, 1996; Schrader and Sauter, 2002). Increased SPS activities are directly related to the accumulation of sucrose over the winter.

Sucrose turnover dominates in the bark of *Robinia,* which shows a 10-fold higher protein content. Within the xylem, the radial distribution of sucrose-synthesizing activities is opposite to that of sucrose-cleaving activities, with SPS located in the mature middle and inner sapwood, versus the outermost wood where sucrose-cleaving activities dominate. In the spring, summer and autumn, the processes within the tree are more spatially defined. In the primary xylem, SPS increases in the spring coinciding with a decrease in starch content, indicating conversion from starch to sucrose (Hauch and Magel, 1999). Once the leaves begin to develop and supply sucrose to the tree, the level of SPS decreased. In the sapwood/heartwood transition zone, SPS levels are highest in autumn, suggesting that this region may be the source of sucrose required for the transition zone (Hauch and Magel, 1999).

In Scots pine sampled in the summer months, SPS activity was present in all tissues, but was highest in the phloem (Uggla *et al.*, 2001). The starch content in living bark decreases steadily from the onset of cambial activity until late summer in both Scots pine and Norway spruce (Egger *et al.*, 1996). This suggests that sucrose pools in the rays are partly derived from stored starch.

In hybrid poplar, SPS activities are high in autumn and winter, which correlates with sucrose content at this time and likely acts as a cryoprotectant mechanism (Schrader and Sauter, 2002). SPS is thought to play a role in the partitioning of

photoassimilates (Galtier *et al.*, 1993), in relation to the CO_2-fixation rate (Galtier *et al.*, 1995) and in relation to light/dark-changes (Stitt *et al.*, 1988). It also plays a role in the ripening of fruit, starch-to-sugar conversion in germination of some seeds, and in stress mechanisms in some plants in response to cold and water stress (Schrader and Sauter, 2002).

One study has looked at the overexpression of maize SPS in a poplar hybrid (*Populus tremula* × *P. tremuloides*), and the preliminary data showed an increase in total leaf carbohydrates and leaf starch content. There was also a significant increase in photosynthesis and higher growth rates (Mouillon and Hurry, 2001). Diurnal changes in leaf soluble sugar content were also noted, with an increase in the first part of the light period compared to controls.

Promoters

Enhanced Tandem Cauliflower Mosaic Virus Promoter

The enhanced tandem cauliflower mosaic virus promoter (2×35S) was originally designed to increase the efficiency of transcription obtained by the natural cauliflower mosaic virus 35S promoter. The 2×35S promoter contains two 35S promoters in tandem. When compared with the nopaline synthase (NOS) promoter, the 35S promoter showed a 10-fold increase in neomycin phosphotransferase (NPTII) transcript levels, while the 2×35S promoter showed a 100-fold increase in transgenic tobacco (Kay *et al.*, 1987).

Further experimentation on this promoter yielded even greater increases in transcript efficiency. A 40-base leader sequence from alfalfa mosaic virus (AMV leader) was utilized in combination with both the 35S promoter and 2×35S. Using β-glucuronidase (GUS) as a marker gene, the promoters were studied in protoplast suspensions of tobacco and white spruce transformed by electroporation (Datla *et al.*, 1993) . The 2×35S promoter consistently yielded expression levels 4 times that of the 35S promoter with the AMV leader. The promoter is constitutively expressed and yields expression levels of about 100-fold that of the natural cauliflower mosaic virus 35S (Appendix A).

4-Coumarate: Coenzyme A Ligase Promoter

The 4-coumarate:CoA ligase promoter was examined in a study evaluating the expression of the 4CL enzyme (Hauffe *et al.*, 1991). In tobacco transformed with GUS under the control of the 4CL promoter, it was found that activity was high in the primary xylem of axillary buds and developing leaf veins, but not observable in other cell types of young leaves or stems. The highest expression was found in xylem tissue during the differentiation of tracheary elements. In older stems, GUS activity was high in the secondary xylem, in the ray parenchyma cells. In the roots, GUS expression was observed in the vascular tissue, root hairs and subapical cells, while in immature flowers, GUS was expressed in the vascular tissue and developing nectaries. Expression was highest in developing seeds in a single epidermal cell layer and mature stigmas also showed high GUS expression. The 4CL promoter provides an interesting comparison with the 2×35S promoter due to its high expression in vascular tissues and the developing and secondary xylem (Appendix A).

Objectives

The objective of this research was to investigate the effect(s) of the constitutive (2×35S) and tissue-specific (4CL) overexpression of SuSy and UGPase in tobacco (*Nicotiana tabacum*) and hybrid poplar (*Populus alba* × *grandidentata*). The work examined the effects of each gene individually and in combination. In addition, in tobacco, these genes were combined with SPS to form double transgenics (SuSy × SPS and UGPase × SPS) as well as triple transgenics. The study focused on the effect of these genes on structural and soluble carbohydrates, as well as molecular characterization of the plants. These analyses permitted the testing of two hypotheses:

1. The tissue specific or constitutive expression of SuSy, UGPase, and SPS individually or in combination can alter the sucrose metabolism pathway, ultimately affecting plant growth rates.
2. The sucrose metabolism pathway can be altered in such a way as to affect the biosynthesis of cellulose, quantitatively and qualitatively.

As mentioned, limited transgenic work has been carried out in woody species, with only one study utilizing the 35S promoter and a modified SuSy gene (Konishi *et al.*,

2004), and one study examining the effects of the overexpression of SPS (Mouillon and Hurry, 2001). No work has yet been carried out with UGPase transgenic poplar, or with multiple genes from the sucrose metabolism pathway.

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Upregulation of Sucrose Synthase and UDP-Glucose Pyrophosphorylase Impacts Plant Growth and Metabolism[1]

Introduction

Cellulose, the most abundant organic polymer in the world, is deposited in the stems of plants and is extensively utilized for fuel, timber, forage, fibre and chemical cellulose. As such, how plants control carbon partitioning to cellulose biosynthesis is a key question for researchers globally (Haigler *et al.*, 2001). The availability of carbohydrates from photosynthesis is not generally a limiting factor in cellulose synthesis despite carbohydrates having other crucial roles in plant growth and maintenance. As such, the creation of photoassimilate sinks in cellulose-producing cells could partition a greater portion of the photosynthate to these cells, and therefore to cellulose production.

Cellulose synthesis, in contrast to starch, is essentially an irreversible sink (Haigler *et al.*, 2001). Cellulose is produced from the precursor UDP-glucose, which can be formed via two potential pathways. UDP-glucose can be derived from the cleavage of sucrose in a reaction catalyzed by sucrose synthase (SuSy; EC 2.4.1.13; Figure 1.1) yielding UDP-glucose and fructose. Alternately, UDP-glucose can be generated from the phosphorylation of glucose-1-phosphate in a reaction catalyzed by UDP-glucose pyrophosphorylase (UGPase, EC 2.7.7.9; Figure 1.1).

SuSy plays an important role in supplying energy for phloem loading by providing a substrate for respiration (Hanggi and Fleming, 2001). The reaction retains the energy of the glycosidic bond in UDP-glucose, conferring an energetic advantage over the hydrolysis of sucrose catalyzed by invertase. SuSy has been characterized as existing both in the cytosol and in association with the plasmalemma (Carlson and Chourey, 1996), with the latter membrane-associated form, hypothesized to provide UDP-glucose

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directly to the cellulose synthase complex (Amor *et al.*, 1995) through metabolic channelling. Direct channelling would facilitate the recycling of UDP into UDP-glucose, as well as ensure the availability of UDP-glucose to the cellulose synthase complex despite demands from other pathways. Salnikov *et al.* (2001) showed that during secondary wall formation in *Zinnea elegans*, SuSy is strongly recruited to the plasma membrane and is highly enriched beneath the secondary cell wall thickenings of differentiating tracheary elements. These findings support the putative relationship between SuSy and cellulose biosynthesis. SuSy activity has also been positively correlated with sink strength in storage organs of potatoes, maize kernels, and pea embryos (Dejardin *et al.*, 1999; Sun *et al.*, 1992; Zrenner *et al.*, 1995).

UGPase in contrast, utilizes the hexose phosphate pool and, in sink tissues where it is predominantly found, is considered a key enzyme for carbohydrate biosynthesis. UGPase can therefore serve as an extension of SuSy in cycling between sucrose and the hexose phosphate pool (Kleczkowski, 1994). Furthermore, UGPase plays an integral role in sugar accumulation, and has the potential to restrict the flow of carbon to sucrose formation in sink tissues (Borokov, *et al.*, 1996). In source tissues, UGPase works in conjunction with SPS, in the synthesis of sucrose (Kleczkowski, 1994). The apparent dual roles of UGPase in sink and source tissue makes UGPase an interesting target for the production of cellulose in sink tissues. By increasing the generation of sucrose in source tissues, while decreasing sucrose formation in sink tissues, there is the potential to selectively channel sucrose to sink tissue for use in carbohydrate biosynthesis.

Despite what is currently known about these two enzymes, few studies have been conducted to elucidate their role in sucrose metabolism and carbon partitioning to cellulose biosynthesis in plants. The purpose of this study was to investigate the effect of overexpressing SuSy and UGPase individually, and in combination, on cellulose synthesis and related pathways to determine the potential role of each enzyme in carbon allocation. Both genes were expressed under the control of a constitutive promoter, the double cauliflower mosaic virus (2×35S), and a vascular specific promoter, the parsley 4-Coumarate:CoA ligase (4CL; Hauffe *et al.*, 1991), in tobacco. Resultant analyses included quantifying soluble and structural carbohydrates and biomass accumulation. Although the results showed little increase in cellulose content

as a percentage of total mass, the upregulation of these genes caused dramatic variation in height growth and sucrose metabolism, thus affecting the total mass of the plant and therefore the overall amount of cellulose produced.

Methods

Cloning of UGPase and Plasmid Construction

UGPase was cloned from *Acetobacter xylinum* ATCC #23768 and inserted into the pBIN cloning vector with one of two promoters: the enhanced tandem CaMV35S constitutive promoter (2×35S) (Kay, *et al.*, 1987), or the vascular specific 4CL (*Petroselinum crispum* 4-Coumarate:CoA ligase) promoter (Hauffe, *et al.*, 1991). SuSy was cloned from *Gossypium hirsutum* (Perez-Grau, Genbank U73588) and inserted into pBIN under the control of the same two promoters. This plasmid also contained the NPTII gene under the control of the CaMV35S promoter.

Plant Transformation and Maintenance

Nicotiana tabacum cv Xanthi (tobacco) was transformed using *Agrobacterium tumefaciens* EHA105 (Hood, *et al.*, 1993) employing a standard leaf disk inoculation method. Binary plasmids were inserted into EHA105 using the freeze-thaw technique, and incubated overnight in liquid Murashige and Skoog media (Murashige and Skoog, 1962) supplemented with 3% sucrose (MS+3%) and 100μM acetosyringone. Leaf disks were cut and co-cultured with EHA105 for one hour at room temperature, blotted dry and plated abaxailly onto MS+3% supplemented with 0.1μM each α-naphthalene acetic acid (NAA) and 6-benzylaminopurine (BA) and solidified with 3% (w/v) agar and 1.1% (w/v) phytagel (MS+NAA/BA). After three days the discs were transferred to MS+NAA/BA supplemented with carbenicillin disodium (500 mg L^{-1}) and cefotaxime sodium salt (250 mg L^{-1}). Following three additional days of selective growth, the discs were transferred to MS NAA/BA containing carbenicillin, cefotaxime and kanamycin (25 mg L^{-1}). After two consecutive five-week periods on this media, shoot tips were isolated to solid MS+3% containing no antibiotics.

Plants were confirmed as transgenic by PCR screening of genomic DNA employing gene specific oligonucleotides: specifically, for UGPase UGP-1 (5'-atcgaggaattctgcctcgt-3') and UGP-2 (5'-tcgcaagaccggcaacaggatt-3') where used, while for SuSy SUS-1 (5'-ctcaacatcacccctcgaat-3') and SUS-2 (5'-accaggggaaacaatgttga-3') were employed. Genomic DNA was isolated using the Red Extract and Amp Kit (Qiagen).

All shoot cultures, including transgenic and non-transformed control lines, were maintained on solid MS+3% in GA-7 vessels at 22°C under a 16 hour photoperiod with an average photon flux of 40 µmol m^{-2} s^{-1}. Plants were maintained by transferring apical regions at four-week intervals.

Plant Growth

Primary transformed plants and control lines (T_0) were grown to maturity and selfed to generate T_1 lines of all the single transformants and the associated controls. The pods from each controlled cross were collected, and the seeds removed and sterilized by washing for 2 minutes in a 10% bleach solution, followed by a 1 minute rinse in sterile water. The seeds were washed in 70% ethanol for two additional minutes and then in sterile water for three consecutive 1 minute washes. Seeds were germinated on solid ½ MS with 2% sucrose and kanamycin (25 mg L^{-1}). The surviving seedlings were then PCR screened using the aforementioned primer sets. Seedlings were grown in GA-7 vessels prior to transfer into 7.5 L pots containing a 50% peat, 25% fine bark, 25% pumice soil mixture in the greenhouse, and covered with 16oz clear plastic cups for one week to aid in acclimation. Each line, transgenic and control, was represented by twelve individual plants (each from an individually selected seed). The greenhouse plants were harvested at the onset of flowering as indicated by the formation of flower buds. Plant height, from base to tip of the highest bud, was measured prior to harvest. Developmental stages of tissues were standardized by employing a plastichron index (PI), where leaf plastichron index PI=0 was defined as the first leaf greater than 5cm in length, and where PI=1 is the leaf immediately below PI=0. A portion of the stem from each plant spanning PI=5 to PI=15 was excised and immediately weighed for total stem fresh weight measurements and leaf biomass. This

same section was then dried at 105°C for 48 hours for dry weight determination, and retained for further analysis. Internode distance represents the average length between each internodes spanning PI =5 to PI =15. The lower section of the stem (below PI=15) was dried at room temperature for fibre quality analysis. All data were analysed using single variable ANOVA and Scheffe tests.

Production of Double Transformants

T_1 tobacco plants transformed with only one construct (*single transgene plants*) were grown to maturity in the greenhouse and selectively crossed with transformed lines expressing another construct to produce reciprocal *double transgene plants*. Double transgene plants are therefore defined as plants derived from the progeny of a controlled cross between a single SuSy transgene plant and a single UGPase transgene plant (under the regulation of the same promoter). The pods from each controlled cross were collected, and the seed removed and sterilized as described previously. The seeds were germinated on ½ MS with 2% sucrose and kanamycin (25 mg L^{-1}). Transformants were confirmed to contain both transgenes via PCR, using the same primers as used for single transformant confirmation.

Transcription Levels

Real time PCR was used to determine the transcript level of each transgene. Leaf and stem sections weighing approximately 100 mg were ground in liquid nitrogen, and RNA extracted using Trizol reagent (Gibco BRL, Gaithersburg, MD) according to manufacturers' instructions. Following extraction, 10 µg of total RNA was treated with 10 units of DNase I (Fermentas, Burlington, ON, Canada) in 6 mM $MgCl_s$. The reaction was incubated at 37°C for 30 minutes and then heat inactivated at 80°C for 10 minutes.

Following the addition of one volume of phenol:chloroform:isoamyl alcohol (25:24:1), the sample was briefly vortexed, and then centrifuged at 13,000 rpm for 5 minutes at 4°C. The upper phase was transferred to a fresh tube and 10 µL of 3 M sodium acetate and 200 µL of 100% ethanol added. The samples were incubated at -80°C for one hour and then centrifuged at 13,000 rpm for 15 minutes at 4°C. The supernatant was removed and the RNA pellet resuspended in 500 µL of 70% ethanol.

The sample was centrifuged at 13,000 rpm for 10 minutes at 4°C, the supernatant carefully removed and the pellet resuspended in 40 µL of RNase-free water.

Equal quantities of RNA (1 µg) were employed for the synthesis of cDNA using Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) and dT_{16} primers according to manufacturer's instructions. Samples were run in triplicate with Brilliant SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA) on an Mx3000P Real-Time PCR System (Stratagene). The primers for the RT-PCR analysis of UGPase were AU-RTF (5'-tggaagcaacccgcgtcatc-3') and AU-RTR (5'-gccaaggcccagcggttcc-3') and for SuSy were GS-RTF (5'-ccgtgagcgtttggatgagac-3') and GS-RTR (5'-ggccaaaatctcgttcctgtg- 3'). Conditions for the RT-PCR reactions were as follows: 95°C for 10 minutes, followed by 40 cycles of 95°C for 30 seconds, 62°C (64°C) for 1 minute, and 72°C for 30 seconds. Transcript levels (transcript copy number ug^{-1} total RNA) were based on standard curves derived from known concentrations of plasmid DNA run under the same conditions.

Enzyme Activity

Leaf and stem samples (approximately 1 g) were ground in liquid nitrogen with 1 mg of insoluble PVPP and four volumes of extraction buffer (50 mM HEPES-KOH pH 7.5, 10 mM $MgCl_s$, 1 mM EDTA, 2 mM DTT, 1 mM PMSF, 5 mM εAmino-n-caproic acid, 0.1% v/v Triton X-100, 10% v/v glycerol). The samples were centrifuged at 14,000 rpm for 20 minutes at 4°C. The extract was passed through a DG 10 desalting column (BioRad) pre-equilibrated with ice-cold extraction buffer without Triton X-100 and PVPP. Extracts were collected into pre-chilled vials and employed immediately. SuSy activity was assayed in the direction of sucrose breakdown, as described in Chourey (1981), using 50 µL of extract. The resultant fructose content was determined using a tetrazolium blue assay (Kennedy & White, 1983). This SuSy assay employs the appropriate controls without the supplementation of UDP to quantify inherent invertase activity, and therefore represents the breakdown of sucrose by SuSy. UGPase activity was determined as per Appeldoorn *et al.* (1997) using 100 µL of extract, and employing UGPase (Sigma) to develop a standard curve.

Soluble Carbohydrate and Starch Analysis

Soluble carbohydrates (glucose, fructose and sucrose) were extracted from ground freeze-dried plant material using methanol:chloroform:water (12:5:3) in a -20°C overnight incubation. The sample was centrifuged and the supernatant removed. The remaining pellet was washed twice with fresh methanol:chloroform:water (12:5:3) and all fractions pooled. Five mL of water was added to the combined supernatant and centrifuged to facilitate phase separation. The aqueous fraction was removed to a round bottom flask and rotary evaporated to dryness. The sample was resuspended in 3 mL of distilled water and analyzed using anion exchange HPLC (Dionex, Sunnyvale, CA) on a DX-600 equipped with a Carbopac PA20 column and an electrochemical detector.

The residual pellet was hydrolyzed using 4% sulfuric acid at 121°C for 4 minutes. The liberation of glucose represented starch content, and was directly quantified by HPLC employing similar conditions.

Determination of Cellulose and Holocellulose Content

Dried plant stem material was ground using a Wiley mill to pass through a 30-mesh screen, and then soxhlet-extracted with acetone for 24 hours. The extractive free material was used for all further analyses. Holocellulose and α-cellulose were determined using a modified microanalytical method developed by (Yokoyama *et al.*, 2002). In short, 200 mg of ground sample was weighed into a 25 mL round bottom flask and placed in a 90°C oil bath. The reaction was initiated by the addition of 1 mL of sodium chlorite solution (400 mg 80% sodium chlorite, 4 mL distilled water, 0.4 mL acetic acid). An additional 1 mL of sodium chlorite solution was added every half hour and the samples removed to a cold water bath after two hours. Samples were then filtered through a coarse crucible, dried overnight, and holocellulose composition determined gravimetrically. Fifty mg of this dried holocellulose sample was weighed into a reaction flask and allowed to equilibrate for 30 minutes. Four mL of 17.5% sodium hydroxide was added and allowed to react for 30 minutes, after which 4 mL of distilled water was added. The sample was macerated for 1 minute, allowed to react for an additional 29 minutes and then filtered through a coarse filter. Following a five-

minute soak in 1.0 M acetic acid, the sample was washed with 90 mL of distilled water and dried overnight. The α-cellulose content was then determined gravimetrically.

Fibre Quality Analysis

To determine the fibre length and coarseness, a portion of the lower stem of tobacco plants was cut into representative samples of approximate dimensions of 2 mm × 2 mm × 30 mm, and reacted in Franklin solution (1:1 30% peroxide and glacial acetic acid) with 3.6% sodium hypochlorite for 24 hours at 70°C. The solution was decanted and the remaining fibrous material was reacted in pure Franklin solution for an additional 48 hours at 70°C. The solution was decanted and the remaining material washed under vacuum with deionized water until a neutral pH was achieved. The samples were dried overnight at 105°C, and then resuspended in 10 mL of deionized water. Further dilution was used to obtain a count of 25-40 fibres per second on a Fibre Quality Analyzer (FQA). All samples were run in triplicate.

Microscopy

Tobacco stems were hand-sectioned using a double-edged razor blade. The samples were then fixed in FAA (formalin, acetic acid, alcohol), dehydrated through as series of acetone and xylene exchanges, and embedded in paraffin. Sections were cut to 10μm and mounted on glass slides, and images taken on a Leica microscope with a Q-imaging camera.

Results

Single Transformants

UGPase from *Acetobacter xylinum* and *Gossypium hirsutum* SuSy were inserted into the pBIN cloning vector under the regulation of one of two promoters: 2×35S and 4CL. Tobacco was transformed with one of the four resulting constructs (2×35S::SuSy, 2×35S::UGPase, 4CL::SuSy, or 4CL::UGPase) using an *Agrobacterium*-mediated transformation technology. The single transgene plants were PCR-confirmed as

transgenic, grown to maturity in a greenhouse, and the flowers bagged to produce selfed-T_1 seed. T_1 seed was then germinated on ½ MS with kanamycin (25 mg L^{-1}) to produce T_1 single transgene plants which were grown in the greenhouse until the onset of flower buds, measured, and a portion of the stem retained for further analysis.

Biomass Accumulation

At the onset of flower bud formation, all plants appeared phenotypically normal and displayed no observable pleiotropic effects. Of the lines analyzed, plants from at least two T_1 single trangene lines from each of the four constructs had statistically significant increases in height growth compared to the corresponding control lines (Figure 2.1A). There was also an increase in internode distance for all lines, however, at a α-value of 0.05 this difference was only significant in one line, 4CL::SuSy-3, while three additional lines (4CL::UGPase-4, 4CL::UGPase-5 4CL::SuSy-8) were significant at an α-value of 0.10 (Figure 2.1B). Despite the increased height growth and internode length, only one line, 4CL::UGPase-4, had a significant increase in total dry weight of the stem (Figure 2.1C).

Transcription Levels

Although transcript levels within single transgene lines were relatively constant, there was wide variation in transcript levels between lines transformed with either the UGPase or SuSy construct. In general, the transcript levels of genes under the regulation of the 4CL promoter were lower in the leaves but similar in the stems to that of the 2×35S plants, which is consistent with expectations for relative expression of the two promoters. Interestingly, 2×35S::UGPase-8 showed very low transcript levels while the other three 2×35S::UGPase lines displayed the highest transcript levels. Additionally, UGPase transcript levels were up to 10-fold greater than that of SuSy under the regulation of either promoter (Table 2.1).

Promoter activity also varied with tissue source (stem *vs* leaves) in the single transgene lines. In six of the seven lines evaluated, the transcript abundance in leaves of both transgenes under the regulation of the 2×35S promoter was equal to, or greater, than levels in stems. However, in line 2×35S::SuSy-17 transcript levels were reduced in

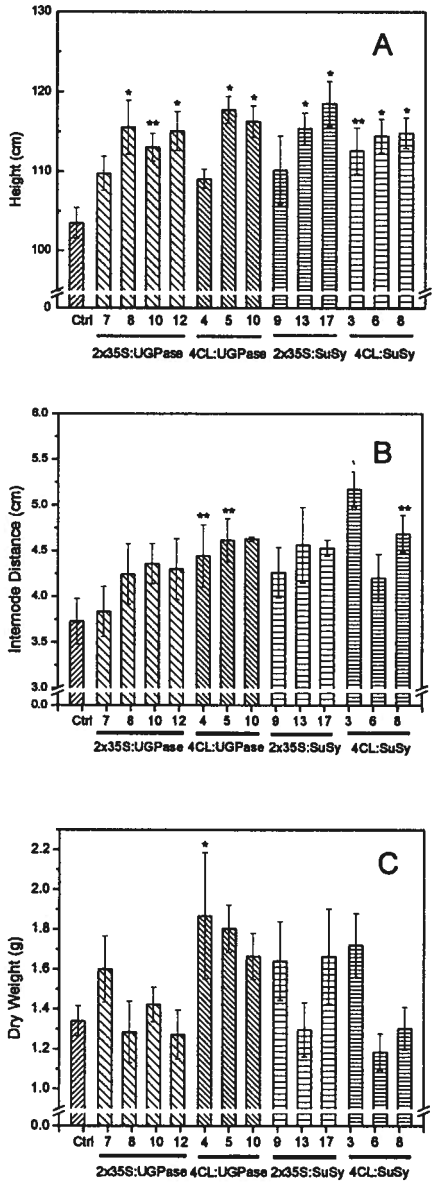

Figure 2.1. Plant height (A), internode length (PI = 5 to PI = 15) (B) and total plant biomass (C) in single transgene plants. Mean ±SE were calculated from 16 plants per transgenic line and 40 plants in the non-transformed control line. * Indicates significance at α=0.05. ** indicates significance at α=0.10.

Table 2.1. Transcript level and enzyme activity in leaf and stem tissue for single transgene and non-transformed control plants. Mean and SE calculated from 3 plants per line. nd = no transcripts detected

Line	UGPase Transcript Level x10^3 copy number μg^{-1} total RNA				UGPase Enzyme Activity Units g^{-1} fresh weight			
	Leaf		Stem		Leaf		Stem	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
2×35S::UGPase 7	1946.5	591.5	1254.0	466.3	0.304	0.227	0.253	0.150
2×35S::UGPase 8	6.0	0.8	2.9	0.9	0.175	0.109	0.119	0.087
2×35S::UGPase 10	1729.0	268.2	535.7	221.3	0.086	0.059	0.012	0.051
2×35S::UGPase 12	1834.6	42.1	382.5	61.3	0.012	0.010	0.043	0.021
4CL::UGPase 4	195.8	37.1	1116.4	172.5	0.031	0.002	0.232	0.063
4CL::UGPase 5	44.5	29.6	244.2	21.8	0.034	0.003	0.153	0.087
4CL::UGPase 10	68.8	53.6	153.5	36.2	0.042	0.001	0.007	0.009
Control	nd	-	nd	-	0.081	0.063	0.133	0.090
Line	SuSy Transcript Level x10^3 copy number μg^{-1} total RNA				SuSy Enzyme Activity μg fructose g^{-1} fresh weight			
	Leaf		Stem		Leaf		Stem	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
2×35S::SuSy 9	248.6	46.1	8.7	4.8	0.010	0.000	0.010	0.002
2×35S::SuSy 13	309.1	96.2	315.8	179.4	0.010	0.002	0.010	0.001
2×35S::SuSy 17	175.1	40.3	315.4	128.3	0.013	0.003	0.011	0.001
4CL::SuSy 3	nd	-	65.1	0.0	0.010	0.001	0.010	0.001
4CL::SuSy 6	nd	-	312.8	119.6	0.009	0.000	0.009	0.000
4CL::SuSy 8	4.1	3.3	83.0	35.7	0.008	0.001	0.009	0.001
Control	nd	-	nd	-	0.009	0.001	0.010	0.001

the leaves relative to the stems, although the difference was not significant. In contrast, the transcript levels of the 4CL promoter were consistently higher in the stems, although the difference between leaf and stem tissue in two of the 4CL::UGPase lines was not significant. Interestingly, no transcripts were detected in the leaves of two 4CL::SuSy lines, while all 4CL::UGPase lines had relatively high UGPase transcripts levels in the leaves.

Enzyme Activity

UGPase activity was determined using an indirect assay measuring the production of NADH. In the 2×35S::UGPase lines, the mean UGPase activity was generally increased in leaf material (Table 2.1), however, this increase was not significant. Two out of three 4CL::UGPase lines had decreased UGPase activity in the leaves compared to the controls, while one line (4CL::UGPase-10) showed an increase in UGPase activity in the leaf relative to the stem. UGPase activity in the 4CL::SuSy lines were similar to that of the control plants (data not shown).

SuSy activity was determined using a direct assay that quantified the production of fructose from the catalysis of sucrose, and considered (subtracted) any inherent invertase activity in the tissue. Single SuSy transgene plants showed slight increases in SuSy activity compared to controls. In contrast to UGPase activity in UGPase single transformed lines, SuSy activity in the single transgene lines, also did not show a tissue-specific response between the leaves and stems, regardless of the promoter used (Table 2.1).

Soluble Carbohydrates

In general, all single transgene lines had increased total soluble sugar content. This increase in total sugar content was not due to increases in sucrose, as no significant differences in sucrose levels between the controls and single transgenic lines were detected. However, as expected with the overexpression of genes encoding enzymes that degrade sucrose, significant increases in both glucose and fructose content were evident (Table 2.2). Further, in the control plants, soluble sucrose concentrations were higher than either glucose or fructose, while in all transformed lines

Table 2.2. Concentration of total soluble carbohydrates (μg mg^{-1} dry weight tissue) in stem tissue of single transgene and non-transformed control tobacco plants. Mean and SE calculated from 4 plants per line. Bold indicates significance at α=0.05.

Line	Glucose		Fructose		Sucrose		Total	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
2×35S::UGPase 7	7.57	2.97	5.52	2.75	6.23	0.74	19.32	6.36
2×35S::UGPase 8	7.19	1.93	6.95	3.03	4.74	0.19	18.88	4.77
2×35S::UGPase 10	6.20	1.42	6.21	1.37	4.24	0.86	16.64	3.59
2×35S::UGPase 12	6.10	2.11	6.38	2.38	3.59	0.60	16.07	4.91
4CL::UGPase 4	7.02	1.85	9.01	3.64	3.83	0.78	19.86	6.27
4CL::UGPase 5	7.99	0.34	10.05	1.14	5.08	0.24	23.12	1.24
4CL::UGPase 10	7.28	1.40	8.01	2.18	4.50	0.72	19.79	4.30
2×35S::SuSy 9	6.32	0.55	**7.54**	0.48	4.43	0.86	18.30	1.88
2×35S::SuSy 13	6.52	0.28	**7.93**	0.21	4.16	0.34	18.62	0.43
2×35S::SuSy 17	**6.91**	0.32	**8.43**	0.58	5.10	0.42	**20.43**	0.69
4CL::SuSy 3	6.33	0.26	6.36	0.78	5.05	1.17	17.74	0.14
4CL::SuSy 6	4.47	0.59	5.93	1.17	4.61	0.22	15.00	1.79
4CL::SuSy 8	4.20	1.00	4.10	0.70	3.53	0.56	11.83	1.98
Control	3.48	1.01	3.51	0.68	4.49	1.19	11.48	1.99

glucose and fructose levels were higher than sucrose levels. Furthermore, while the concentration of glucose and fructose in control plants were roughly equal, in both the 4CL::UGPase and 2×35S::SuSy lines there were higher fructose levels relative to glucose content.

Polymeric Carbohydrates

No significant differences in starch content were detected between control plants and single transgene plants (Figure 2.2A). Similarly, with the exception of the one line, 2×35S::SuSy-9, there were also no significant differences in cellulose content between control plants and single transgene plants (Figure 2.2B).

Fibre Quality Analysis

In the UGPase-expressing plants, no significant differences in fibre length and coarseness were detected; however, fibre length was generally decreased while fibre coarseness generally increased (Figures 2.2C & D). Similarly, two SuSy-expressing lines, 2×35S::SuSy-9 and 4CL::SuSy-6, had significantly shorter fibres relative to the controls, but were not different in terms of coarseness.

Double Transgene Plants

Biomass Accumulation

Single transgene plants were grown in the greenhouse through flowering and reciprocal crosses were made between 2×35S::UGPase and 2×35S::SuSy single transgene plants, and 4CL::UGPase and 4CL::SuSy single transgene plants. The double transgene progeny seed was collected, planted on selective media, and kanamycin resistant offspring were selected and confirmed for the presence of both genes using genomic PCR screening. The double transgene tobacco seedlings were then planted in the greenhouse and allowed to reach reproductive maturity prior to growth analysis and destructive harvesting.

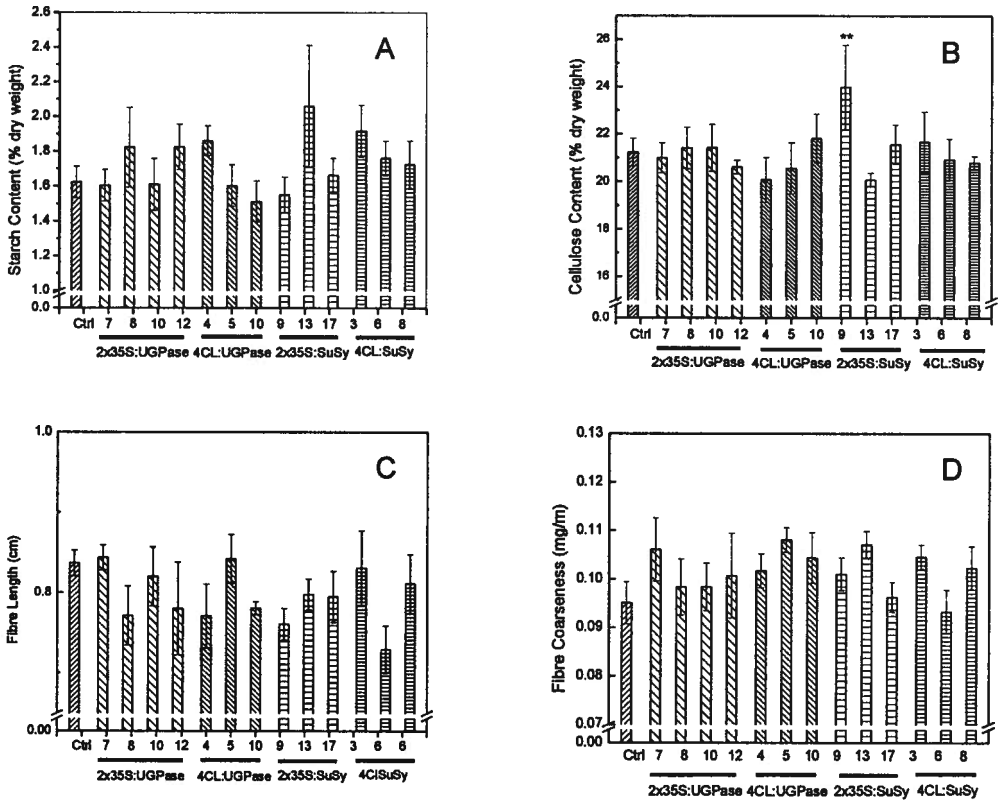

Figure 2.2. Starch (A) and cellulose (B) content, and fibre length (C) and coarseness (D) of single transgene lines and non-transformed control line. Starch and cellulose mean ±SE calculated from 5 plants per line. Fibre quality mean ±SE calculated from 4 plants per transgene line and 12 plants per non-transformed control. *indicates significance at α=0.05. ** indicates significance at α=0.10.

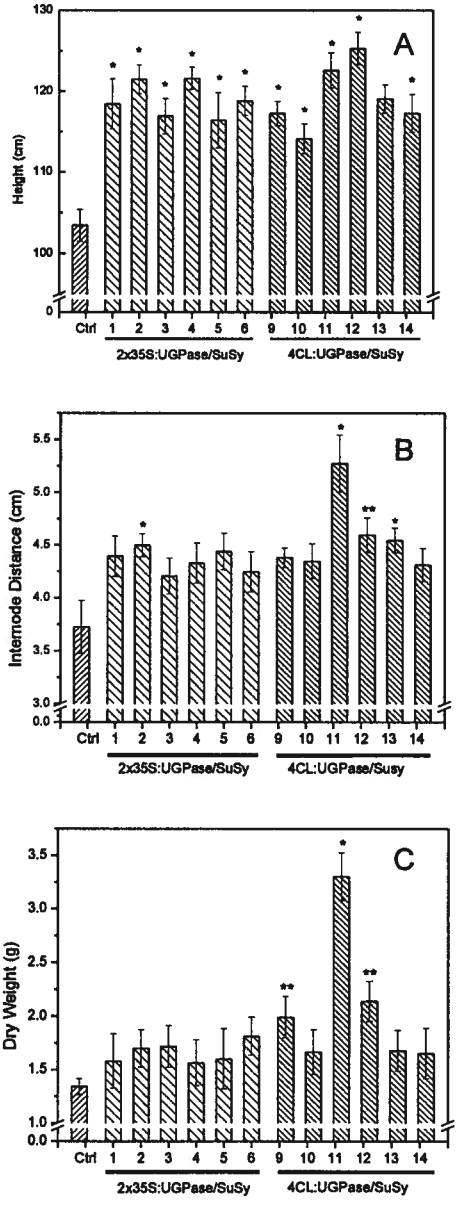

Figure 2.3. Plant height (A), internode length (PI = 5 to PI = 15) (B) and total plant biomass (C) in double transgene tobacco lines. Mean ±SE were calculated from 12 plants per transgenic line and 40 plants in the non-transformed control line. * indicates significance at α=0.05. ** indicates significance at α=0.10.

All of the double transgene lines showed statistically significant increases in height growth, when compared to the corresponding non-transformed control plants (Figure 2.3A). There was also a significant increase in internode length in many (2×35S::UGPase/SuSy-2 and 4CL::UGPase/SuSy-11, 4CL::UGPase/SuSy-12, and 4CL::UGPase/SuSy-13) of the double transgene lines evaluated, with all other double transgene lines showing increases in absolute values (Figure 2.3B). In addition, plant biomass was clearly increased (Figure 2.3C) in the double transgene lines relative to the non-transformed control, particularly in the lines under the control of the 4CL promoter, in which half of the lines (4CL::UGPase/SuSy-9, 4CL::UGPase/SuSy-11, and 4CL::UGPase/SuSy-12) showed significant increases in biomass. One line (4CL::UGPase/SuSy-11) had over 2 times the biomass of the control line.

Transcription Levels

Similar to single transgene lines, there was wide variation in both UGPase and SuSy transcript levels in the lines expressing two transgenes. Transcription levels were, however, correlated with transcript levels of the parental lines. For example, 2×35S::UGPase/SuSy-1 and 2×35S::UGPase/SuSy-2 are reciprocal crosses and had similar relatively low transcript levels for both genes compared to the other 2×35S double transgene lines (Table 2.3). The transcript level detected in the stem of these lines correlates well to the low transcript levels detected in the stem of associated parental lines, 2×35S::SuSy-9 and 2×35S::UGPase-8, both of which had very low transcript levels. In general, the transcript levels of the double transgene lines were slightly lower than their corresponding parental lines.

Similar to single transformed lines, transcript levels in 4CL-regulated double transformed lines were significantly higher in the stems than in the leaves and 4CL transcript levels were generally lower than with the 2×35S promoter. Additionally, UGPase transcript abundance was shown to be up to 200-fold that of SuSy expression.

Enzyme Activity

The 2×35S double transformed lines showed a consistent increase in UGPase activity in the leaf tissue, with one line (2×35S::UGPase/SuSy-6) having a significant

Table 2.3. Transcript level and enzyme activity of UGPase and SuSy in leaf and stem tissue of double transgene and non-transformed control tobacco plants. Mean and SE calculated from 3 plants per line. Bold indicates significance at α=0.05. nd = no transcripts detected.

	UGPase Transcript Level x10^3 copy number μg^{-1} total RNA				UGPase Enzyme Activity Units g^{-1} fresh weight			
Line	Leaf		Stem		Leaf		Stem	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
2×35S::UGPase/SuSy-1	10.8	6.6	8.3	12.6	0.329	0.270	0.142	0.198
2×35S::UGPase/SuSy-2	2.1	2.6	20.2	8.1	0.208	0.201	0.195	0.227
2×35S::UGPase/SuSy-3	4126.5	1818.6	3695.6	1888.9	0.330	0.277	0.170	0.189
2×35S::UGPase/SuSy-4	756.7	402.9	843.5	115.7	0.204	0.202	0.035	0.079
2×35S::UGPase/SuSy-5	506.4	136.4	566.0	328.8	0.338	0.349	0.230	0.079
2×35S::UGPase/SuSy-6	693.1	479.4	969.1	619.8	**0.461**	0.040	0.346	0.127
4CL::UGPase/SuSy-9	15.7	3.1	402.9	67.9	**0.291**	0.063	0.186	0.029
4CL::UGPase/SuSy-10	10.1	11.1	220.5	124.3	**0.361**	0.015	0.297	0.109
4CL::UGPase/SuSy-11	4.5	1.0	124.9	39.4	0.081	0.084	0.203	0.036
4CL::UGPase/SuSy-12	30.4	25.4	193.5	172.1	0.233	0.042	0.116	0.080
4CL::UGPase/SuSy-13	29.9	19.9	228.5	41.9	**0.371**	0.021	0.292	0.055
4CL::UGPase/SuSy-14	14.8	9.9	233.5	61.1	**0.606**	0.246	**0.450**	0.020
Control	nd	-	nd	-	0.081	0.063	0.133	0.090
	SuSy Transcript Level x10^3 copy number μg^{-1} total RNA				SuSy Enzyme Activity μg fructose g^{-1} fresh weight			
Line	Leaf		Stem		Leaf		Stem	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
2×35S::UGPase/SuSy-1	48.2	35.5	nd	-	0.016	0.006	0.010	0.002
2×35S::UGPase/SuSy-2	89.8	39.4	57.1	19.4	**0.016**	0.002	**0.013**	0.001
2×35S::UGPase/SuSy-3	529.8	144.1	1210.6	339.0	**0.022**	0.005	0.009	0.005
2×35S::UGPase/SuSy-4	194.2	94.9	183.4	92.4	**0.015**	0.001	0.011	0.004
2×35S::UGPase/SuSy-5	316.4	38.2	158.4	76.8	0.013	0.002	0.013	0.002
2×35S::UGPase/SuSy-6	326.6	60.6	139.4	100.5	0.010	0.000	0.013	0.004
4CL::UGPase/SuSy-9	21.1	21.1	70.8	5.0	0.009	0.010	0.013	0.010
4CL::UGPase/SuSy-10	nd	-	46.7	14.4	0.009	0.008	0.009	0.009
4CL::UGPase/SuSy-11	nd	-	36.2	24.5	0.009	0.009	0.009	0.008
4CL::UGPase/SuSy-12	3.7	3.1	23.1	9.0	0.007	0.008	0.007	0.003
4CL::UGPase/SuSy-13	nd	-	13.4	13.4	0.008	0.009	0.010	0.008
4CL::UGPase/SuSy-14	3.8	3.8	32.5	21.5	0.011	0.007	0.007	0.007
Control	nd	-	nd	-	0.009	0.001	0.010	0.001

increase in UGPase activity relative to the control (Table 2.3). However, no significant differences were seen in UGPase activity in the stem of the 2×35S::UGPase/SuSy lines. Interestingly, despite the desire to have a xylem directed expression pattern with 4CL, four of the six 4CL::UGPase/SuSy lines had significantly elevated UGPase activity in the leaves, while only one of the six lines had significantly higher activity in the stems. In fact, line 4CL::UGPase/SuSy-14 had the highest UGPase activity detected and this activity was in the leaves, not the stems. The UGPase activity of this transformed line was also the highest detected in the stem as well.

SuSy activity in double transgene plants was substantially increased in a number of lines relative to the non-transformed control (Table 2.3). The leaves of the 2×35S double transgene lines had a large increase in SuSy activity with half the lines (three out of six lines) showing a significant difference from the controls. In contrast to the leaves, the SuSy activity in the stem tissue remained relatively consistent with that of the control plants with only one line (2×35S::UGPase/SuSy-2) having a significant increase relative to the control. The leaves and stems of the 4CL lines did not show the same increase in SuSy activity as observed in the 2×35S lines.

Soluble Carbohydrates

While no significant differences in total soluble sugar content were detected in the 2×35S double transformed plants, five of the six 4CL double transgene lines had significant increases (α<0.10) in total soluble sugars (Table 2.4). Similar to the single transgene lines, this increase in the 4CL lines was attributed to elevated glucose and fructose concentrations and not elevated sucrose levels except in one line (4CL::UGPase/SuSy-14) where total sugars as well as glucose and fructose were significantly increased. While only 3/6 and 2/6 2×35S double transformed lines had elevated glucose and fructose levels respectively, 6/6 and 5/6 4CL double transformed lines had elevated glucose and fructose levels, respectively.

Polymeric Carbohydrates

No significant differences compared to the controls were detected in either starch or cellulose content (Figure 2.4A & B). Three of the five 2×35S lines and one 4CL line,

Table 2.4. Concentration of total soluble carbohydrates (μg mg^{-1} dry weight tissue) in stem tissue of double transgene and non-transformed control tobacco plants. Mean and SE calculated from 5 plants per line. Bold indicates significance at α=0.10.

Line	Glucose		Fructose		Sucrose		Total	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
2×35S::UGPase/SuSy-1	**6.00**	1.06	**5.48**	0.97	3.57	0.46	15.04	2.38
2×35S::UGPase/SuSy-2	5.20	0.56	5.03	0.51	3.83	0.39	14.06	1.34
2×35S::UGPase/SuSy-3	**6.60**	0.30	**5.98**	0.25	3.66	0.35	16.24	0.37
2×35S::UGPase/SuSy-4	**5.16**	1.94	4.45	1.66	4.81	0.26	14.43	3.63
2×35S::UGPase/SuSy-5	2.86	0.76	2.83	0.84	3.15	0.26	8.84	1.64
2×35S::UGPase/SuSy-6	4.44	1.04	4.36	1.00	3.26	0.42	12.07	2.32
4CL::UGPase/SuSy-9	**5.42**	0.71	4.89	0.68	4.83	0.50	15.14	1.83
4CL::UGPase/SuSy-10	**6.67**	0.65	**5.68**	0.70	5.45	0.89	**17.80**	1.08
4CL::UGPase/SuSy-11	**7.83**	0.73	**6.79**	0.81	4.84	0.49	**19.46**	1.66
4CL::UGPase/SuSy-12	**7.36**	0.87	**6.39**	0.81	5.39	0.36	**19.13**	1.77
4CL::UGPase/SuSy-13	**7.45**	0.91	**6.07**	0.68	5.03	0.35	**18.55**	1.75
4CL::UGPase/SuSy-14	**10.35**	0.74	**7.56**	0.84	**8.35**	0.95	**26.26**	1.66
Control	3.48	1.01	3.51	0.68	4.49	1.19	11.48	1.99

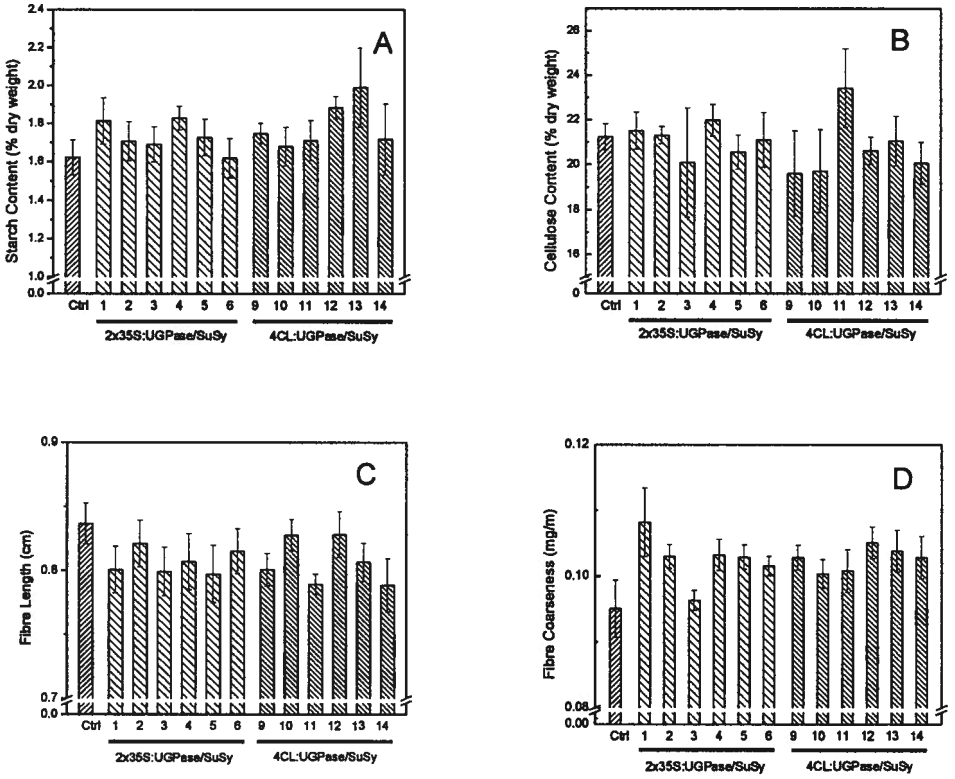

Figure 2.4. Starch (A) and cellulose (B) content, and fibre length (C) and coarseness (D) of stem tissue of double transgene lines and non-transformed control line. Starch and cellulose mean ±SE calculated from 5 plants per line. Fibre qualities mean ±SE calculated from 4 plants per transgene line and 12 plants per non-transformed control.

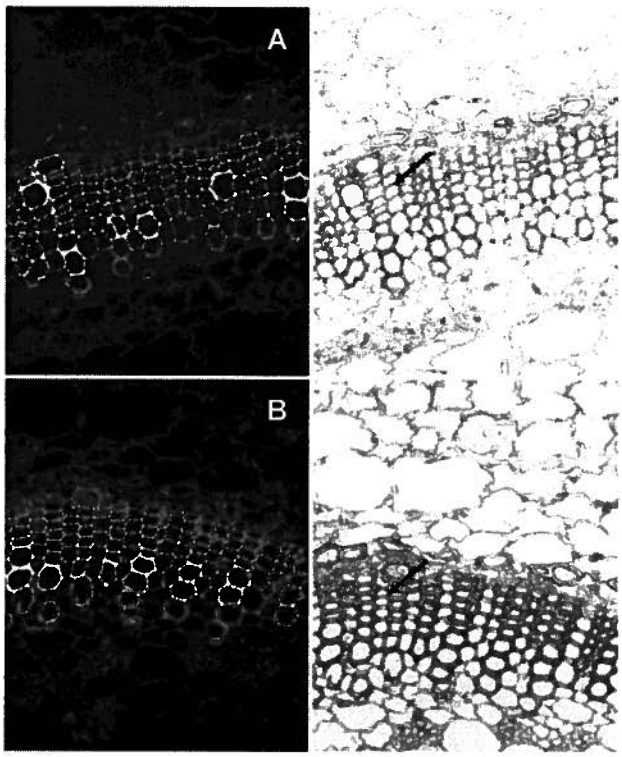

Figure 2.5. Light and UV-fluorescence microscopy of control (A) and transgenic (4CL::UGPase×SuSy 12) (B) tobacco. Arrows depict the observed increased cell wall thickening in transgenic lines.

4CL::UGPase/SuSy-11, showed an increase in cellulose content. However, the remaining 4CL lines had reduced cellulose content (Figure 2.4B).

Fibre Quality Analysis

As with the single transgene lines, fibres were consistently shorter and had increased cell wall coarseness in the double transformed lines relative to the controls, although these differences were significant in only one line (Figures 2.4C & D).

Discussion

This study investigated the effects of employing two promoters, a tissue-specific (4CL) and a ubiquitously expressed (2×35S) promoter, to overexpress exogenous SuSy and UGPase genes in tobacco. As expected, the expression profiles of the genes and enzyme activity varied amongst the four constructs (2×35::UGPase, 4CL::UGPase, 2X35::SuSy, 4CL::SuSy). The highest expression in leaf tissue was observed in single transgene plants under the control of the 2×35S promoter, where expression was equal to or higher than in the stems. In contrast, expression controlled by the 4CL promoter was generally higher in the stems. This is consistent with previous work which showed 4CL to be principally expressed in the xylem and tracheary elements in young tobacco (Hauffe *et al.*, 1991), while the 2×35S gave a more constitutive expression patterns (Kay *et al.*, 1987). The double transgene lines also showed similar patterns of expression, but expression was generally not as highly as in single transgene plants.

Neither the single nor double transgene plants showed any visible phenotypic differences when compared to the non-transformed controls. However, biomass measurements of plants suggested that the overexpression of UGPase and SuSy, both individually and in combination, has a significant impact on plant height growth in tobacco. Most single transgene lines and all of the double transgene lines showed significant increases in height growth. Furthermore, each of the double transgene lines had at least one parental line that also showed significant increases in height compared to the non-transformed controls. An increase in height growth was also reported with the overexpression of SuSy in poplar under the control of the CaMV35S promoter

(Konishi *et al.*, 2004), and similarly the increased height growth was not evident in all transformed lines. These findings corroborate SuSy antisense suppression studies which have shown reduced plant size and leaf number in carrot (Tang and Sturm, 1999), and significantly reduced fruit size in tomatoes (D'Aoust, *et al.*, 1999).

While there is some evidence for the role of SuSy in sink tissue, limited work has been conducted in elucidating a function for UGPase in the same tissue. The current study provides evidence and suggests that UGPase plays a role in the strength of sink tissues as demonstrated by the increased accumulation of biomass in both 2×35S and 4CL lines. There is evidence, although indirect, that UGPase may possess a similar role in poplar, as it was recently found to be upregulated during late cell expansion and secondary cell wall formation (Hertzberg *et al.*, 2001). However, in growing potato tubers, a reduction in UGPase activity (up to 96%) by antisense suppression caused no change in carbohydrate metabolism (Zrenner *et al.*, 1993). Similarly, in Arabidopsis, antisense suppression of UGPase (30% reduction) did not confer a phenotypic effect under normal light conditions (Johansson, 2003). However, Johansson (2003) attributed these findings to the existence of multiple UGPase isoforms.

In addition to a general increase in height due to the overexpression of SuSy and UGPase individually, when the data for single and double transgene line are compared, it is apparent that the double transgene lines were generally taller than the single transgene lines. Lines 2×35S::UGPase/SuSy-2 and 2×35S::UGPase/SuSy-4 and lines 4CL::UGPase/SuSy-11 and 4CL::UGPase/SuSy-12 were all significantly taller than either of their single transgene parental lines (2×35S::UGPase-8 and 2×35S::SuSy-9; 2×35S::UGPase-12 and 2×35S::SuSy-17; 4CL::UGPase-5 and 4CL::SuSy-3; 4CL::UGPase-5 and 4CL::SuSy-3). It is plausible that the combined overexpression of SuSy and UGPase affects the plants' ability to more effectively utilize photosynthates and produce more biomass. As SuSy has previously been identified as a marker of sink strength (D'Aoust *et al.*, 1999), it is not surprising that in combination with a second key enzyme in sucrose metabolism that a greater increase in hexose sugar sink strength is prevalent, and consequentially that plant growth is affected. As the direction of UGPase has been hypothesized to be affected by the availability of substrates, UGPase has the potential to utilize the UDP-glucose produced by SuSy and directly incorporate it in the production of hexose phosphates (Kleczkowski *et al.*, 2004). This

could facilitate the observed increased plant growth, and explain the lack of increase in cellulose content. Alternately, if there is a quantifiable supply of glucose-1-phosphate, UGPase could be effective in producing UDP-glucose. Fructose generated by SuSy-mediated catabolism of sucrose could be the indirect supply for glucose-1-phosphate (Kleczkowski *et al.*, 2004).

The single SuSy and UGPase transgene lines, as well as the double transgene lines had longer internodes. This suggests that the altered expression of these genes/enzymes does not affect plant development, but rather invokes either an increase in cell number and/or cell elongation in a given internodal region. Given there were no statistically significant changes in fibre morphology, the increased internodal distance, and consequently height, is the result of an increase in the number of fibres. Thus, plants displaying increased growth are likely producing fibres at a higher rate than control plants. This is further supported by the fact that the quantified fibre length tended to be slightly shorter, while fibre coarseness (wall thickness) was generally slightly greater for the transgenic plants in both single and double transgene lines. The increased height growth in the transgenic plants resulted in the formation of reaction-type fibres, analogous to tension and compression wood fibres in angiosperms and gymnosperms, respectively. Compression wood cells, in particular, are well known to be shorter in length and posses thicker cell walls as a result of growth stresses (Zobel and van Buijtenen, 1989). These results are further supported by microscopic sectioning (Figure 2.5).

Previous studies involving the antisense suppression of SuSy in potato (Zrenner *et al.*, 1995), carrot (Tang and Sturm, 1999), and tomato (D'Aoust *et al.*, 1999) have all demonstrated significant changes in the soluble carbohydrate content in sink tissue. Similarly, *shrunken 1*, a maize endosperm SuSy mutant, (Chourey and Nelson, 1976) was shown to possess elevated soluble sugar concentration in sink tissue. The compromised SuSy activity showed a direct increase in localized sucrose levels (2-4 fold). The altered phenotypes (substantially reduced dry weight) in both potato and maize were hypothesized to be related to the hexose concentration, and an associated increase in water influx (Tang and Sturm, 1999). The upregulated SuSy activity in the current study did not demonstrate an increase in the concentration of sucrose in sink

tissue. However, there was a significant increase in both fructose and glucose, with fructose levels increasing more than that of glucose. This indicates that the plant was maintaining a basal concentration of sucrose in the stem, and that the upregulation of SuSy may only be associated with the increased degradation of sucrose and the consequential accumulation of glucose and fructose monomers. When SuSy activity is suppressed, there is a build up of sucrose, suggesting that SuSy is a limiting step in sucrose catabolism. In contrast, these findings imply that an increase in SuSy activity facilitates elevated rates of sucrose degradation, and consequentially allows more sucrose to be transported to the stem tissue, and ultimately eases the metabolizing cells accessibility to photosynthates.

Studies examining the effect of UGPase on soluble sugar content are limited. In potato tubers, the antisense reduction of UGPase (up to 96%) caused no changes in soluble sugars (Zrenner *et al.*, 1993), while a 30% antisense reduction in arabidopsis resulted in a decrease in sugar content (Johansson, 2003). In the present study, much like the results with SuSy transformed lines, tobacco overexpressing UGPase had increased glucose and fructose content, yet only minor changes in sucrose concentrations.

What is particularly interesting in both the UGPase and SuSy transformed lines is that the elevated levels of fructose and glucose were independent of the measured transcript levels or enzyme activities. These data suggest that only a small increase in either of these genes can have a dramatic effect on carbohydrate levels and that there may be other factors limiting the accumulation above the modest, yet significant changes seen in the present study.

In plants with suppressed SuSy activity, a consistent decrease in starch content has been shown: 62% in maize (Chourey and Nelson, 1976), 34-63% in potato (Zrenner *et al.*, 1995), and 26% in carrot (Tang and Sturm, 1999). In contrast, the upregulation of SuSy in tobacco did not show a significant increase in starch content, however, all 4CL::SuSy lines showed an increasing trend for starch accumulation. While the suppression of SuSy causes a profound effect on the production of starch, the increased activity does not directly alter starch content. A similar trend is found with cellulose content. In SuSy suppressed carrot plants, there was an associated decrease in cellulose content (Tang and Sturm, 1999). The suppression of SuSy in cotton also

caused a profound effect on the production of cellulose, resulting in an almost fibreless phenotype (Ruan *et al.*, 2003). While no consistent trends in cellulose content were obvious among the SuSy transgenic tobacco, one line (2×35S::SuSy-9) did have a significant increase in cellulose content. The results from the antisense work suggest that although SuSy may be critical and necessary for starch biosynthesis, as with carbohydrate levels, other factors become limiting as SuSy is overexpressed.

2×35S::UGPase and 4CL::UGPase transgenic tobacco lines showed no significant change in either starch or cellulose content. While this suggests that UGPase is not rate limiting in cellulose production, it does not address the fact that despite an increase in soluble sugars there is no concurrent increase in cellulose or starch. Other studies have shown that UGPase plays a role in providing substrate for starch (Johansson, 2003). UGPase is also known to play a role in the production of cellulose in yeast and bacteria, as demonstrated by the reduction of cellulose in UGPase deficient mutants (Daran *et al.*, 1995; Valla *et al.*, 1989). One difference in these bacterial studies and the present study is the availability of SuSy in plants, as this enzyme is not present in either yeast or bacteria (Kleczkowski, *et al.*, 2004).

Despite the common hypothesis that implicates SuSy in regulating sink strength in storage organs, there is clearly a limit to its capability. When suppressed, it has a profound effect not only on the soluble sugar content of the sink organ, but also on the storage and structural carbohydrates cellulose and starch. Conversely, its increased expression can be expected to potentially increase the availability of the hexose sugar pool. Our results show that in tobacco under the control of either the 2×35S or 4CL promoters, the overexpression of SuSy or UGPase significantly influences and regulates the growth of plants, but does not generally increase the partitioning of storage of sugars into starch or cellulose. Clearly, both SuSy and UGPase have the potential to increase overall plant growth, and thus increase total cellulose yield attainable from an individual plant.

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Altered Sucrose Metabolism Impacts Biomass Production and Flower Development in Tobacco[2]

Introduction

Plant breeders and biotechnologists are consistently pursuing improved plant productivity (biomass) as a target trait (White *et al.*, 2006), the impetus for which incorporates several factors, including crop yield, carbon capture and more recently increased biomass for biofuels production. Generally, plant productivity can be viewed as a process governed by resource allocation. Plants have the innate ability to adapt their resource allocation to match resource acquisition, acquire resources more effectively, or avoid deleterious conditions. This plasticity in resource acquisition and allocation can have a profound effect not only on the development and physiology of plants, but on the industrial utility of the plant matter.

Generating or breeding plant genotypes where resource allocation is directed to vegetative biomass and/or altered fibre properties, is therefore directly related to altering sink tissue. Increasing the transport of photoassimilate to the sink tissue, and the subsequent catabolism and use of sucrose within this tissue has the potential to disrupt source-sink relationships, and therefore stimulate plants to respond by increasing the mobilization of stored carbohydrates or to alter the photosynthetic machinery to compensate. In many plants, the primary sink exists as secondary cell walls, in the form of cellulose and lignin, the two most abundant polymers on earth. Thus, to create a stronger sink within cellulose producing cells could allow plants to metabolize photoassimilate more rapidly and consequently stimulate plants to alter growth rates.

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UDP-glucose is the immediate precursor molecule in the synthesis of cellulose and can be formed by two pathways: UDP-glucose pyrophosphorylase (UGPase; EC 2.7.7.9) catalyses the production of UDP-glucose from glucose-1-phosphate and UTP, while sucrose synthase (SuSy; EC 2.4.1.13) cleaves sucrose into fructose and UDP-glucose. The latter reaction has an energetic advantage over that of UGPase as it retains the glycosidic bond for use in cellulose formation. There is additional evidence for the direct association of SuSy with the cellulose synthase complex which would permit the recycling of UDP into UDP-glucose (Salnikov *et al.*, 2001). Furthermore, SuSy activity has been positively associated with sink strength in many species, including potato (Zrenner *et al.*, 1995), tomato (Sun *et al.*, 1992; D'Aoust *et al.*, 1999), carrot (Tang & Sturm, 1999) and tobacco (Coleman *et al.*, 2006).

Fructose is a known inhibitor of SuSy activity (Doehlert, 1987). However, plants employ sucrose phosphate synthase (SPS; EC 2.4.1.14) to aid in fructose recycling and limit SuSy inhibition. SPS synthesizes sucrose-6-phosphate from fructose-6-phosphate and UDP-glucose, and concurrently provides additional substrate for SuSy (Delmer, 1999). In non-photosynthetic tissue, SPS has dual-functionality: the re-synthesis of sucrose following cleavage during import, and the involvement in carbohydrate regulatory cycles involving starch degradation and sucrose re-synthesis (Geigenberger *et al.*, 1997; Stitt *et al.*, 1988). Furthermore, SPS has been identified as playing a role in diurnal carbohydrate allocation (Huber and Huber, 1996), flower development (Baxter *et al.*, 2003), fruit development (Laporte *et al.*, 2001) and cell wall growth and expansion (Haigler *et al.*, 2001).

In source tissues, UGPase can act in concert with SPS in the formation of sucrose (Kleczkowski, 1994), while in sink tissues it has the potential to restrict carbon flow to sucrose formation (Borokov *et al.*, 1996) as it works co-ordinately with SuSy in the cycling between sucrose and the hexose phosphate pools (Kleczkowski, 1994). The objective of this study was to investigate the effects of misregulating multiple genes involved in sucrose metabolism on plant growth and secondary cell wall cellulose biosynthesis.

Methods

Crossing (transformation)

Transgenic tobacco overexpressing UGPase and SuSy (Coleman *et al.*, 2006) and SPS (Park *et al.*, 2008) under the control of either the enhanced tandem CaMV35S constitutive promoter (2×35S) (Kay *et al.*, 1987; Datla *et al.*, 1993) or the vascular specific 4CL (*Petroselinum crispum* 4-Coumarate:CoA ligase) promoter (Hauffe *et al.*, 1991) were grown in the greenhouse until flowering. The transgenic plants were then crossed to produce plants harbouring differing combinations of two exogenous transgenes, including: UGPase × SPS and SuSy × SPS. The resulting seeds were planted on media containing the respective selective antibiotics and double transgenic plants confirmed by PCR screening of genomic DNA using the following primer sets: UGP-F (5'-atcgaggaattctgcctcgt-3') and UGP-R (5'-tcgcaagaccggcaacaggatt-3') for UGPase confirmation, SUS-1 (5'-ctcaacatcacccctcgaat-3') and SUS-2 (5'-accaggggaaacaatgttga-3') for SuSy confirmation and SPS-F (5'-ggctatcgttcaagatgcctctg-3') and SPS-R (5'-aggcctcgcaagggcaagta-3') for SPS confirmation.

The successful crosses were grown and multiplied in tissue culture to produce a minimum of 12 individually double transformed plants per line. UGPase × SuSy plants (Coleman *et al.*, 2006) were also crossed with SPS tobacco plants, and the resulting plantlets screened for the presence of all three genes. All shoot cultures, including transgenic and non-transformed control lines, were maintained on solid MS+3% in GA-7 vessels at 22°C under a 16 hour photoperiod with an average photon flux of 40 µmol m^{-2} s^{-1}. Plants were maintained by transferring apical regions at four-week intervals.

All transgenic combinations, along with appropriate non-transformed control tobacco plants, were transferred to a greenhouse into 7.5 L pots containing a 50% peat, 25% fine bark, 25% pumice soil mixture. Each multi-gene pyramiding combination had a corresponding control group as the growth periods were staggered. UGPase × SPS plants were planted on the 16th of December, 2005. SuSy × SPS plants were planted on the 23rd of December, 2005 and UGPase × SuSy × SPS plants were planted 4th of July, 2006. Following the formation of flower buds, the plants were harvested and biomass measurements taken. Plant height was measured from the base (root collar)

to the tip of the highest bud. The stage of tissue development was standardized using a leaf plastichron index, where the first leaf larger than 5cm was defined as PI=0 and the leaf immediately below was PI=1. Leaves corresponding to PI=4 and PI=5, and the associated stem section, were harvested for enzyme assays, RNA transcript analysis and soluble sugars content determination. The section of stem spanning PI=5 to PI=15 was retained for cell wall analysis, as well as stem dry weight determination. The leaves associated with this section were also used for leaf dry weight determination. All data was analysed using students t-test assuming unequal variance.

Transcription Levels

Real time PCR was employed to determine transcript abundance of each transgene. RNA was isolated from 100 mg liquid nitrogen ground samples of stem and leaf tissue of plants using Trizol reagent (Gibco BRL, Gaithersburg, MD) according to manufacturers' instructions. Ten µg of RNA was then treated with TURBO DNase™ (Ambion, Austin, TX) to remove residual DNA. One µg of DNase-treated RNA was used for the synthesis of cDNA using Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) and dT_{16} primers according to the manufacturer's instructions. Samples were run in triplicate with Brilliant SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA) on an Mx3000P Real-Time PCR System (Stratagene) to determine critical thresholds (Ct). The primers employed for RT-PCR analysis primers pairs: UGPase - AU-RTF (5′-tggaagcaacccgcgtcatc-3′) and AU-RTR (5′-gccaaggcccagcggttcc-3′); SuSy - GS-RTF (5′-ccgtgagcgtttggatgagac-3′) and GS-RTR (5′-ggccaaaatctcgttcctgtg-3′); SPS - AtSPS-F3 (5′-ccacagtggcaaagtgatgatggc-3′) and AtSPS-R4 (5′-tctgacctctccagtgatccc-3′). As a house-keeping control, the transcript abundance of *Actin-9* was employed for normalization using primers described previously: NtAct-RTF (5′-ctattctccgctttggacttggca-3′) and NtAct-RTR (5′-aggacctcaggacaacggaaacg-3′) (Volkov *et al.*, 2003). Conditions for the RT-PCR reactions were as follows: 95°C for 10 minutes, followed by 40 cycles of 95°C for 30 seconds, 62°C SuSy (65°C SPS, 60 °C UGPase, 55°C Actin,) for 1 minute, and 72°C for 30 seconds. Relative expression was determined according to Levy *et al.* (2004) using the following equation: $\Delta ct = 2^{-(ctUGPase\ or\ SuSy\ or\ SPS - ctNtActin)}$.

Enzyme Activity

Leaf and stem samples (1g f.w.) were ground in liquid nitrogen with 1 mg of insoluble PVPP and four volumes of extraction buffer (50 mM HEPES-KOH pH 7.5, 10 mM $MgCl_s$, 1 mM EDTA, 2 mM DTT, 1 mM PMSF, 5 mM εAmino-n-caproic acid, 0.1% v/v Triton X-100, 10% v/v glycerol). The samples were centrifuged at 15,000×g for 20 minutes at 4°C. The extract was passed through a desalting column (DG 10 – BioRad) pre-equilibrated with ice-cold extraction buffer without Triton X-100 and PVPP. Extracts were collected in pre-chilled vials and used immediately. UGPase activity was determined spectrophotometrically at 340nm as per Appeldoorn *et al.* (1997) using 100 µL of plant extract and a NADH molar extinction coefficient of 6.22 mM cm^{-1}. SuSy activity was assayed in the direction of sucrose breakdown (Chourey, 1981), using 50 µL of plant extract. The resultant fructose content was determined using a tetrazolium blue assay (Kennedy and White, 1983). This SuSy assay employs the appropriate controls without the supplementation of UDP to quantify inherent invertase activity, and therefore represents only the breakdown of sucrose by SuSy. SPS activity was determined according to Iraqi and Tremblay (2001) and Baxter *et al.* (2003). Total protein content of the extracts was determined using a Bio-Rad Protein Assay (Bio-Rad, Hercules, CA).

Soluble Carbohydrate and Starch Analysis

Soluble carbohydrates (glucose, fructose and sucrose) were extracted from ground freeze-dried tissue overnight at -20°C using methanol:chloroform:water (12:5:3) as previously described (Coleman *et al.*, 2006). The sample was centrifuged, the supernatant removed, and the remaining pellet washed twice with fresh methanol:chloroform:water (12:5:3). All fractions were then pooled. Five mL of water was added to the combined supernatant and centrifuged to facilitate phase separation. The aqueous fraction was rotary evaporated to dryness and re-suspended in 3 mL of distilled water. Soluble carbohydrates were then analyzed using anion exchange HPLC (Dionex, Sunnyvale, CA) on a DX-600 equipped with a Carbopac PA1 column and an electrochemical detector.

The residual pellet after soluble sugar extraction was then hydrolyzed in 4% sulfuric acid at 121°C for 4 minutes. The liberation of glucose, representing starch content, was directly quantified by HPLC under similar conditions.

Cell Wall Composition

Oven dried tobacco stem segment spanning PI=5 to PI=15 were ground using a Wiley mill to pass through a 40-mesh screen and soxhlet extracted with acetone for 24 hours. Lignin and carbohydrate contents were determined using a modified Klason method (Huntley *et al.,* 2003) on 0.2g of extract free tissue. Carbohydrate content was determined using HPLC (Dionex DX-600, Dionex, CA) equipped with an anion exchange PA1 column, a pulsed amperometric detector with a gold electrode and post-column detection. Acid insoluble lignin was determined gravimetrically, while acid insoluble lignin was determined using spectrophotometric analysis at 205 nm according to TAPPI useful method UM-250.

Results

Transcript Expression and Enzyme Activity

Transcript abundance of all three transgenes was measured in each plant, and expressed relative to β-actin. All transgenics (doubles and triple lines) clearly demonstrated substantial expression of the exogenous transgenes, while no detectable transcripts were apparent in the wild-type tobacco plants, as would be expected (Table 3.1). Transgene expression levels were variable among the plants and crosses, which is also expected, as each plant represents the selection of independent transformation events. Furthermore, the independently selected double transformed lines were not selfed to select for homozygous lines prior to reciprocal mating, nor were the number of transgene insertion events quantified by Southern analysis. Therefore, the selection of double, and later triple transgenic lines represents the products stemming from allelic variation in heterozygous, segregating selected parents, and as such variability in gene dose effect is not accounted for. This variability likely accounts for the inconsistencies

Table 3.1. Transcript level and enzyme activity in leaf and stem of UGPase x SPS, SuSy x SPS and UGPase x SuSy x SPS transgenic and wild-type tobacco lines. Average ± standard error (n = 5). Bold indicates significance at α=0.10.

	UGPase Transcript Level ΔCt x 10000		UGPase Enzyme Activity μMolNADH min^{-1} mg^{-1} protein	
	Leaf	Stem	Leaf	Stem
2×35S::UGPase x SPS A	**2.43 ± 0.49**	**7.05 ± 1.62**	**125.5 ± 6.9**	**464.9 ± 88.0**
2×35S::UGPase x SPS B	**131.44 ± 24.45**	**91.43 ± 11.81**	**214.0 ± 48.6**	**267 ± 74.1**
2×35S::UGPase x SPS C	**90.67 ± 54.00**	**186.53 ± 58.60**	**168.2 ± 21.8**	**418.7 ± 137.1**
4CL::UGPase x SPS A	**562.30 ± 47.51**	**732.56 ± 173.19**	**241.3 ± 70.5**	**439.7 ± 122.1**
4CL::UGPase x SPS B	**403.94 ± 60.19**	**520.03 ± 83.71**	**186.6 ± 33.6**	**779.0 ± 149.6**
4CL::UGPase x SPS C	**545.99 ± 74.91**	**709.20 ± 81.06**	**144.2 ± 11.4**	**879.8 ± 83.9**
Control A-C	n.d.	n.d.	89.7 ± 7.5	140.4 ± 17.3
	SPS Transcript Level ΔCt x 10000		SPS Enzyme Activity μg sucrose min^{-1} mg^{-1} protein	
	Leaf	Stem	Leaf	Stem
2×35S::UGPase x SPS A	**5.11 ± 1.59**	**5.54 ± 1.66**	1.42 ± 0.45	**9.16 ± 0.81**
2×35S::UGPase x SPS B	**7.20 ± 1.05**	**10.58 ± 2.46**	**2.73 ± 0.63**	**11.63 ± 2.27**
2×35S::UGPase x SPS C	**6.63 ± 2.09**	**11.86 ± 2.40**	1.03 ± 0.24	**10.66 ± 1.97**
4CL::UGPase x SPS A	**69.55 ± 14.33**	**31.72 ± 10.07**	**1.90 ± 0.10**	**12.91 ± 2.33**
4CL::UGPase x SPS B	**42.84 ± 22.04**	**16.08 ± 4.78**	**2.05 ± 0.51**	**9.71 ± 1.53**
4CL::UGPase x SPS C	**65.71 ± 22.66**	**62.34 ± 13.34**	2.36 ± 0.69	**24.94 ± 5.59**
Control A-C	n.d.	n.d.	1.24 ± 0.14	5.73 ± 0.97

	SuSy Transcript Level ΔCt x 10000		SuSy Enzyme Activity µg fructose min^{-1} mg^{-1} protein	
	Leaf	Stem	Leaf	Stem
2×35S::SuSy x SPS D	**0.20 ± 0.04**	**10.26 ± 9.53**	**1.53 ± 0.37**	7.19 ± 2.20
2×35S::SuSy x SPS E	**0.25 ± 0.05**	**0.05 ± 0.02**	**0.94 ± 0.17**	8.69 ± 3.42
2×35S::SuSy x SPS F	**53.67 ± 49.74**	**65.71 ± 61.61**	**1.03 ± 0.13**	18.83 ± 10.88
4CL::SuSy x SPS D	**24.98 ± 7.75**	**40.54 ± 10.07**	**1.09 ± 0.20**	9.04 ± 1.54
4CL::SuSy x SPS E	**42.09 ± 14.83**	**44.55 ± 18.33**	**4.49 ± 1.25**	7.22 ± 2.14
4CL::SuSy x SPS F	**24.80 ± 6.03**	**34.42 ± 8.16**	**0.74 ± 0.06**	5.32 ± 2.09
Control D-F	n.d.	n.d.	0.33 ± 0.13	6.78 ± 1.40
	SPS Transcript Level ΔCt x 10000		SPS Enzyme Activity µg sucrose min^{-1} mg^{-1} protein	
	Leaf	Stem	Leaf	Stem
2×35S::SuSy x SPS D	**0.58 ± 0.16**	**0.83 ± 0.19**	**3.96 ± 0.89**	25.52 ± 7.87
2×35S::SuSy x SPS E	**0.88 ± 0.05**	**0.52 ± 0.33**	**4.07 ± 0.71**	19.46 ± 5.21
2×35S::SuSy x SPS F	**1.01 ± 0.50**	**1.72 ± 0.50**	**9.56 ± 3.67**	**32.84 ± 5.67**
4CL::SuSy x SPS D	**22.78 ± 9.71**	**8.43 ± 2.61**	**5.42 ± 0.33**	**20.39 ± 5.43**
4CL::SuSy x SPS E	**15.91 ± 2.49**	**8.19 ± 1.32**	**4.30 ± 0.73**	**21.26 ± 5.35**
4CL::SuSy x SPS F	**21.34 ± 7.76**	**21.29 ± 6.12**	**7.71 ± 0.39**	**30.58 ± 9.47**
Control D-F	n.d.	n.d.	1.97 ± 0.34	6.36 ± 0.10

	UGPase Transcript Level ΔCt x 10000		UGPase Enzyme Activity µMol NADH min^{-1} mg^{-1} protein	
	Leaf	Stem	Leaf	Stem
2×35S::UGPase × SuSy x SPS G	**38.39 ± 22.48**	**0.31 ± 0.09**	**21.86 ± 5.25**	93.40 ± 11.41
2×35S::UGPase × SuSy x SPS H	**56.73 ± 31.17**	**73.31 ± 27.90**	**20.88 ± 4.60**	73.58 ± 10.68
2×35S::UGPase × SuSy x SPS I	**2.01 ± 0.66**	**1.05 ± 0.12**	**10.23 ± 1.08**	90.09 ± 18.59
4CL::UGPase × SuSy x SPS G	**98.89 ± 41.30**	**224.73 ± 179.13**	**11.04 ± 1.75**	112.73 ± 14.56
4CL::UGPase × SuSy x SPS H	**169.92 ± 54.57**	**404.41 ± 9.72**	**20.84 ± 3.60**	80.92 ± 14.53
4CL::UGPase × SuSy x SPS I	**177.12 ± 5.01**	**562.58 ± 210.13**	**11.66 ± 1.53**	**55.95 ± 4.84**
Control G-I	n.d.	n.d.	5.70 ± 0.36	84.70 ± 7.26
	SuSy Transcript Level ΔCt x 10000		SuSy Enzyme Activity µg fructose min^{-1} mg^{-1} protein	
	Leaf	Stem	Leaf	Stem
2×35S::UGPase × SuSy x SPS G	**56.52 ± 41.41**	**14.73 ± 5.77**	1.14 ± 0.61	**2.27 ± 0.65**
2×35S::UGPase × SuSy x SPS H	**0.75 ± 0.60**	**0.68 ± 0.27**	1.84 ± 1.24	**1.33 ± 0.19**
2×35S::UGPase × SuSy x SPS I	**10.81 ± 4.12**	**29.80 ± 1.93**	0.43 ± 0.31	**2.01 ± 0.20**
4CL::UGPase × SuSy x SPS G	**55.63 ± 14.85**	**72.51 ± 2.85**	0.53 ± 0.09	**2.52 ± 0.34**
4CL::UGPase × SuSy x SPS H	**15.11 ± 6.80**	**122.35 ± 54.89**	**1.10 ± 0.11**	**2.78 ± 0.80**
4CL::UGPase × SuSy x SPS I	**8.89 ± 1.46**	**110.06 ± 66.84**	**1.77 ± 0.49**	**6.56 ± 2.59**
Control G-I	n.d.	n.d.	0.52 ± 0.09	0.36 ± 0.06
	SPS Transcript Level ΔCt x 10000		SPS Enzyme Activity µg sucrose min^{-1} mg^{-1} protein	
	Leaf	Stem	Leaf	Stem
2×35S::UGPase × SuSy x SPS G	**69.95 ± 46.06**	**77.46 ± 25.25**	**0.75 ± 0.16**	3.66 ± 0.50
2×35S::UGPase × SuSy x SPS H	**63.70 ± 58.61**	**101.83 ± 37.83**	**0.57 ± 0.02**	2.06 ± 0.30
2×35S::UGPase × SuSy x SPS I	**40.44 ± 17.55**	**87.09 ± 20.63**	**1.14 ± 0.23**	**7.10 ± 1.56**
4CL::UGPase × SuSy x SPS G	**56.40 ± 24.31**	**121.33 ± 82.00**	**0.90 ± 0.08**	2.83 ± 0.86
4CL::UGPase × SuSy x SPS H	**41.95 ± 9.13**	**38.34 ± 19.79**	**1.60 ± 0.08**	**8.56 ± 0.79**
4CL::UGPase × SuSy x SPS I	**40.06 ± 15.43**	**342.67 ± 88.64**	**1.24 ± 0.27**	4.50 ± 2.09
Control G-I	n.d.	n.d.	0.43 ± 0.05	2.41 ± 0.67

in gene expression observed for any given transgene when comparing among lines within a cross, as well as when expression patterns of lines between crosses. Despite the variability in transgene expression, some generalities regarding the influence of promoter and tissues can be made. When the transgenes were under the control of the 2×35S promoter no clear differences were apparent when comparing tissue specificity. 4CL-driven expression appeared to be consistently higher in the stem tissue, when compared to the leaf samples with the exception of the SPS transcript level. This latter finding is consistent with the targeted expression of this promoter (Hauffe *et al.*, 1991). Interestingly, the 4CL promoter appears to drive expression of these genes to a greater extent in both tissues when compared to the 2×35S promoter.

In general, there is good agreement between enzyme activity and transcript abundance, as in all cases the activity of the sucrose metabolizing enzymes is substantially greater than the native enzyme activity present in the control tobacco plants (Table 3.1). Furthermore, as with the transcript abundance, activity of all three proteins is generally higher in the stem sections as compared to the leaf tissue, and when the transgenes were under the control of the 4CL promoter. In the UGPase x SPS lines increases of ~5- and ~4-fold where apparent in UGPase and SPS activity, respectively in the stem segments of the transgenic plants. Similarly, in the SuSy × SPS transgenic lines the SPS activity was shown to be as much as 4.5 times greater than control plants, while one line showed a 12-fold increase in SuSy activity. However, the mean increase in SuSy activity across plants in this cross is approximately 3 times higher than the corresponding SuSy activity native to the control plants. The triple transgenic lines also displayed similar increases in enzyme activity.

Soluble Carbohydrates and Starch

While variability exists among individual lines and crosses, starch content appears to be influenced by the overexpression of these three transgenes in the tobacco plants compared to the corresponding controls (Table 3.2). It is not possible to draw comparisons between different transgene combinations, as the greenhouse growth trials were conducted at different times during the year. However, comparisons

Table 3.2. Soluble carbohydrate and starch content (mg g^{-1}) in leaf and stem of UGPase x SPS, SuSy x SPS and UGPase x SuSy x SPS transgenic and wild-type tobacco lines. Average ± standard error (n = 5). Bold indicates significance at α=0.10.

Leaf	Glucose	Fructose	Sucrose	Total	Starch
2×35S::UGPase x SPS A	6.30 ± 2.14	5.24 ± 1.52	12.32 ± 2.85	23.86 ± 6.42	38.93 ± 10.71
2×35S::UGPase x SPS B	8.69 ± 2.27	5.60 ± 1.27	10.86 ± 2.13	25.14 ± 5.48	41.62 ± 6.72
2×35S::UGPase x SPS C	**6.34 ± 1.16**	5.11 ± 1.09	7.50 ± 1.18	**18.95 ± 2.58**	**36.84 ± 5.53**
4CL::UGPase x SPS A	9.08 ± 1.55	5.73 ± 0.66	11.94 ± 1.92	26.75 ± 1.10	54.93 ± 8.93
4CL::UGPase x SPS B	**8.49 ± 0.72**	4.48 ± 0.23	15.59 ± 2.00	28.57 ± 2.58	40.04 ± 10.40
4CL::UGPase x SPS C	**7.04 ± 1.45**	4.77 ± 0.83	10.55 ± 1.46	22.35 ± 2.38	41.82 ± 6.70
Control A-C	12.98 ± 1.11	6.23 ± 0.93	12.51 ± 2.04	33.21 ± 3.63	60.07 ± 7.70
2×35S::SuSy x SPS D	8.52 ± 1.91	7.20 ± 1.05	14.42 ± 2.60	30.61 ± 4.45	43.72 ± 7.96
2×35S::SuSy x SPS E	8.81 ± 0.56	6.45 ± 1.26	14.66 ± 4.19	29.93 ± 3.41	58.05 ± 2.77
2×35S::SuSy x SPS F	7.76 ± 2.47	8.22 ± 0.91	9.81 ± 2.42	25.79 ± 5.69	49.71 ± 5.28
4CL::SuSy x SPS D	10.14 ± 0.56	9.62 ± 0.65	9.16 ± 0.84	28.92 ± 1.27	60.16 ± 9.59
4CL::SuSy x SPS E	**5.93 ± 0.20**	8.15 ± 0.23	8.66 ± 1.45	**22.74 ± 1.63**	55.02 ± 12.47
4CL::SuSy x SPS F	9.47 ± 1.27	9.35 ± 1.38	8.16 ± 1.89	26.98 ± 1.40	53.31 ± 10.50
Control D-F	9.23 ± 1.21	11.34 ± 2.70	9.25 ± 1.21	29.83 ± 2.59	55.83 ± 1.72
2×35S::UGPase × SuSy x SPS G	41.97 ± 6.23	31.48 ± 1.69	77.64 ± 10.82	151.09 ± 7.66	66.97 ± 10.65
2×35S::UGPase × SuSy x SPS H	28.69 ± 3.77	17.38 ± 0.81	59.44 ± 3.91	105.51 ± 8.08	**45.85 ± 2.69**
2×35S::UGPase × SuSy x SPS I	28.61 ± 2.27	24.28 ± 2.67	70.93 ± 4.50	123.81 ± 9.38	**54.33 ± 7.89**
4CL::UGPase × SuSy x SPS G	33.84 ± 7.35	32.41 ± 2.12	63.32 ± 2.39	119.43 ± 13.10	61.63 ± 20.71
4CL::UGPase × SuSy x SPS H	26.44 ± 2.29	24.40 ± 0.77	71.29 ± 4.43	118.76 ± 7.99	**42.25 ± 10.00**
4CL::UGPase × SuSy x SPS I	35.95 ± 3.55	26.39 ± 1.63	68.01 ± 7.84	130.35 ± 11.08	**28.81 ± 0.12**
Control G-I	48.25 ± 5.51	27.45 ± 2.16	78.80 ± 3.34	148.01 ± 5.65	93.63 ± 7.26

Stem	Glucose	Fructose	Sucrose	Total	Starch
2×35S::UGPase x SPS A	52.30 ± 7.46	46.29 ± 5.89	24.95 ± 3.82	123.55 ± 12.61	18.84 ± 2.75
2×35S::UGPase x SPS B	39.90 ± 4.36	35.19 ± 6.00	24.80 ± 3.93	99.89 ± 11.40	15.34 ± 2.09
2×35S::UGPase x SPS C	47.21 ± 4.68	41.31 ± 5.78	31.57 ± 6.14	120.95 ± 12.48	13.03 ± 0.68
4CL::UGPase x SPS A	49.67 ± 7.75	42.77 ± 4.31	29.02 ± 2.47	121.46 ± 12.94	15.94 ± 1.37
4CL::UGPase x SPS B	50.93 ± 7.13	47.90 ± 4.21	23.53 ± 2.93	122.36 ± 8.64	14.63 ± 1.38
4CL::UGPase x SPS C	38.10 ± 8.40	37.92 ± 4.86	27.87 ± 5.90	103.90 ± 16.39	17.08 ± 1.82
Control A-C	53.78 ± 2.82	52.43 ± 5.09	21.28 ± 4.11	127.49 ± 7.49	16.85 ± 1.78
2×35S::SuSy x SPS D	29.46 ± 4.73	24.96 ± 4.30	26.41 ± 2.93	80.83 ± 9.27	15.43 ± 1.33
2×35S::SuSy x SPS E	**44.16 ± 7.56**	30.20 ± 4.87	24.67 ± 5.11	99.03 ± 16.43	**18.23 ± 1.47**
2×35S::SuSy x SPS F	36.37 ± 3.80	25.62 ± 2.88	22.53 ± 2.58	84.52 ± 7.27	**18.43 ± 0.60**
4CL::SuSy x SPS D	28.78 ± 8.63	19.69 ± 5.57	25.23 ± 1.04	73.70 ± 14.11	16.86 ± 1.35
4CL::SuSy x SPS E	25.17 ± 4.57	24.58 ± 2.96	24.95 ± 2.47	74.70 ± 5.93	**20.99 ± 1.49**
4CL::SuSy x SPS F	24.17 ± 3.71	19.72 ± 3.78	33.08 ± 9.11	76.98 ± 2.27	15.47 ± 2.49
Control D-F	26.00 ± 4.55	21.62 ± 2.75	24.03 ± 4.37	71.65 ± 9.76	14.22 ± 0.41
2×35S::UGPase × SuSy x SPS G	183.84 ± 14.52	156.42 ± 7.62	84.94 ± 5.86	381.78 ± 35.66	14.34 ± 1.22
2×35S::UGPase × SuSy x SPS H	84.29 ± 17.30	122.25 ± 27.29	46.96 ± 16.27	253.51 ± 28.31	13.34 ± 0.53
2×35S::UGPase × SuSy x SPS I	158.85 ± 22.64	134.62 ± 9.94	99.55 ± 11.24	362.06 ± 51.04	12.93 ± 1.41
4CL::UGPase × SuSy x SPS G	134.44 ± 6.96	107.04 ± 4.39	88.75 ± 6.62	322.54 ± 16.22	16.48 ± 3.30
4CL::UGPase × SuSy x SPS H	113.68 ± 34.83	139.79 ± 38.98	88.22 ± 18.36	341.69 ± 92.17	19.33 ± 0.32
4CL::UGPase × SuSy x SPS I	79.06 ± 6.74	146.14 ± 1.68	91.36 ± 10.45	316.56 ± 5.38	17.62 ± 1.15
Control G-I	196.53 ± 11.77	140.67 ± 5.65	97.30 ± 4.49	442.80 ± 9.24	16.91 ± 1.23

with the independent corresponding controls demonstrate common trends. In particular, it appears that starch accumulation in leaf tissue is reduced in the transgenic lines, while the starch content in the stem segments appears to be unaltered or slightly increased. Similar findings were observed previously in the stem of UGPase × SuSy double transgenics (Coleman *et al.*, 2006). Furthermore, the selection of promoter does not appear to affect starch metabolism trends.

Despite the significant overexpression, as evident by transcript and enzyme activity, of three major sucrose metabolizing enzymes, the overall total soluble carbohydrate levels, and more specifically sucrose contents were generally unchanged or only marginally altered (Table 3.2). In the double transgenics it appears that sucrose levels in leaf tissue are comparable to the control plants, while some lines appear to have elevated sucrose contents in the stem segments.

Plant Growth

Total plant biomass of the transgenic lines and corresponding controls plants was assessed by measured height growth, calliper, and total leaf dry weight at harvest. All transgenic lines had significantly increased height growth regardless of transgene combination. These findings are consistent with previously reported transgenic tobacco overexpressing UGPase and SuSy alone and in tandem (Coleman *et al.*, 2006) and SPS alone (Park *et al.*, 2008). In the current study, the UGPase × SPS double transgenic lines showed 23-31% increases in height growth compared to the corresponding control plants (Figure 3.1A), while the SuSy × SPS lines growth enhancement ranged from 18-48% over controls (Figure 3.2A). The triple transgene tobacco, containing upregulated UGPase × SuSy × SPS transgenes demonstrated increases in height growth ranging from 20-57% over controls (Figure 3.3A). These observed gains do not appear to be dominated by either the constitutively expressed or vascular-specific promoter.

Consistent with the observed increases in height growth, stem size, as determined by calliper measurements, was generally increased in all transgenic lines at harvest. The combined increase in height and calliper clearly indicated that the overall

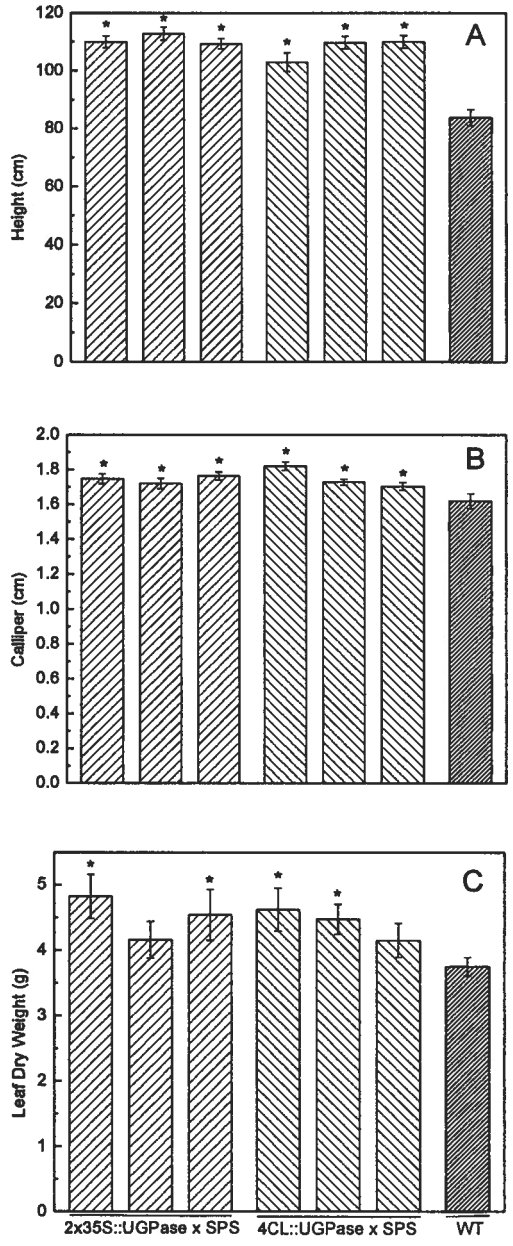

Figure 3.1. Biomass measurements of UGPase x SPS transgenic lines compared to wild-type tobacco plants: height (A), calliper (B), and leaf dry weight (C). *indicates significance at α=0.05.

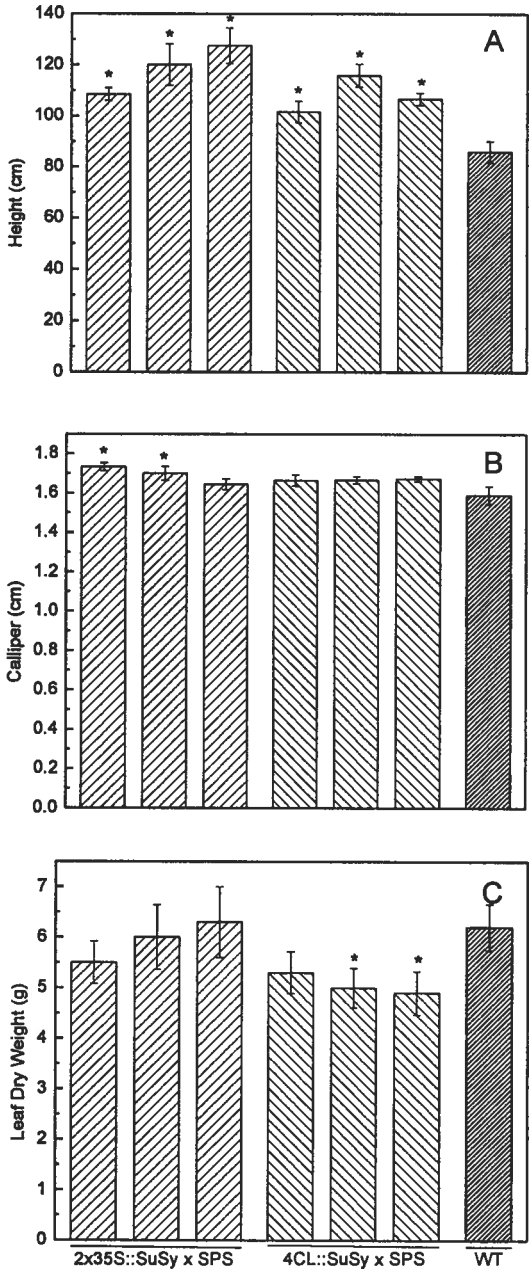

Figure 3.2. Biomass measurements of SuSy x SPS transgenic lines compared to wild-type tobacco plants: height (A), calliper (B) and leaf dry weight (C). *indicates significance at α=0.05.

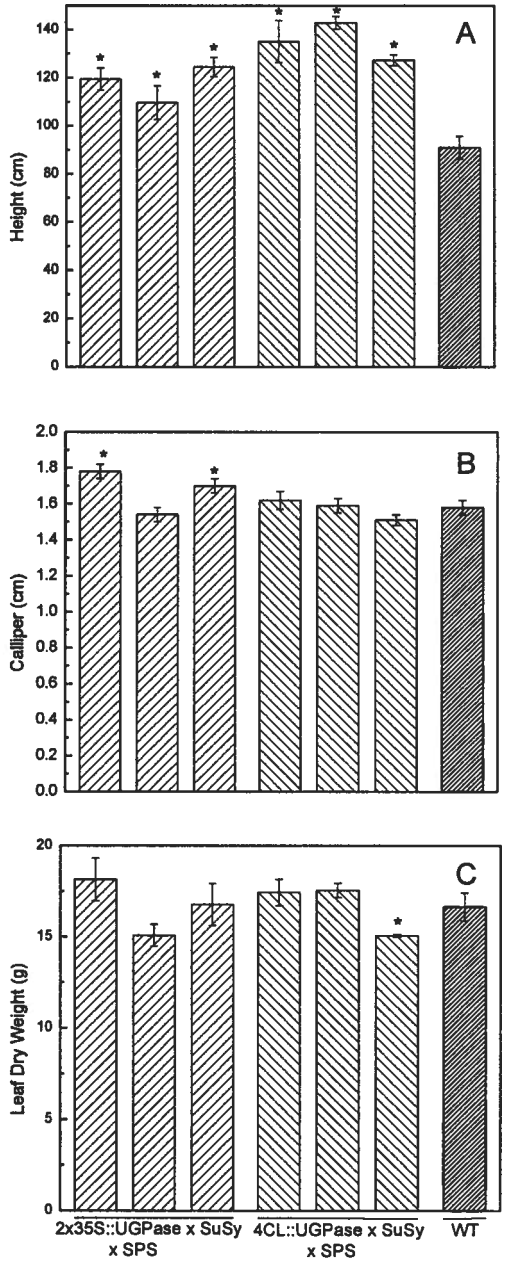

Figure 3.3. Biomass measurements of UGPase x SuSy x SPS transgenic lines compared to wild-type tobacco plants: height (A), calliper (B) and leaf dry weight (C). *indicates significance at α=0.05.

volume of plant biomass accrued during the growth trial by the transgenic lines was significantly greater than that observed in the corresponding control tobacco lines. The most significant increases in calliper were apparent in the UGPase × SPS double transgenics (Figure 3.1B). While increases in calliper were evident in the SuSy × SPS and UGPase × SuSy × SPS transgenics, not all lines evaluated consistently demonstrated improvements in this phenotype (Figures 3.2B & 3.3B). Total stem dry biomass (data not shown) were reflective of the combined growth traits. Again, promoter choice did not offer a selective advantage.

Total dry leaf biomass was also shown to be increased in the UGPase × SPS double transgenics lines compared to the corresponding control lines. However, similar significant changes were not apparent in the SuSy × SPS and UGPase × SuSy × SPS transgenics, consistent with the calliper measurements. However, most lines showed comparable leaf biomass as compared to the appropriate controls (Figures 3.1C, 3.2C, & 3.3C).

Interestingly, the parallel upregulation of multiple sucrose metabolizing genes altered time to set flower buds in many of the transgene combinations (Table 3.3). Initially, the extended time to flower development was thought to be responsible for the increase biomass accrual during the growth period. However, there was no change in timing of flower development in UGPase × SPS transgenics lines compared to the control plants, and these double transgenics displayed the largest and most consistent increases in plant biomass. Similarly, in a previous study (Coleman *et al.,* 2006), substantial increases in plant biomass were attainable by overexpressing a UGPase × SuSy transgene combination, without any change in timing to flower development. In contrast, the SuSy × SPS transgenic lines, and the triple upregulated transgenic lines, UGPase × SuSy × SPS, all showed extended days to floral developmental. In both of the latter two combinations, the length to complete flower development was increased by approximately 21 days, and these differences were independent of timing of the growth trial. In addition to the extended time for developmental completion, many of the 4CL lines showed foliar stipules and morphological alterations in flowers, while the 2×35S driven transgenics only showed similar abnormal morphological flower phenotypes (Figure 3.4).

Table 3.3. Time to flowering of UGPase x SPS, SuSy x SPS and UGPase x SuSy x SPS transgenic and wild-type tobacco lines. Average ± standard error (n = 12). Bold indicates significance at α=0.10.

	Days to Flowering
2×35S::UGPase x SPS A	50.2 ± 2.32
2×35S::UGPase x SPS B	50.0 ± 1.56
2×35S::UGPase x SPS C	51.8 ± 2.17
4CL::UGPase x SPS A	47.8 ± 1.19
4CL::UGPase x SPS B	47.7 ± 1.50
4CL::UGPase x SPS C	47.2 ± 0.89
Control A-C	51.7 ± 4.63
2×35S::SuSy x SPS D	57.3 ± 0.88
2×35S::SuSy x SPS E	**78.2 ± 4.98**
2×35S::SuSy x SPS F	**78.0 ± 3.37**
4CL::SuSy x SPS D	61.7 ± 4.18
4CL::SuSy x SPS E	**69.4 ± 4.29**
4CL::SuSy x SPS F	62.0 ± 7.29
Control D-F	56.2 ± 2.23
2×35S::UGPase × SuSy x SPS G	**35.3 ± 1.94**
2×35S::UGPase × SuSy x SPS H	**39.1 ± 2.09**
2×35S::UGPase × SuSy x SPS I	**39.6 ± 1.90**
4CL::UGPase × SuSy x SPS G	**37.3 ± 2.06**
4CL::UGPase × SuSy x SPS H	**51.4 ± 2.70**
4CL::UGPase × SuSy x SPS I	**48.4 ± 1.29**
Control G-I	30.4 ± 0.40

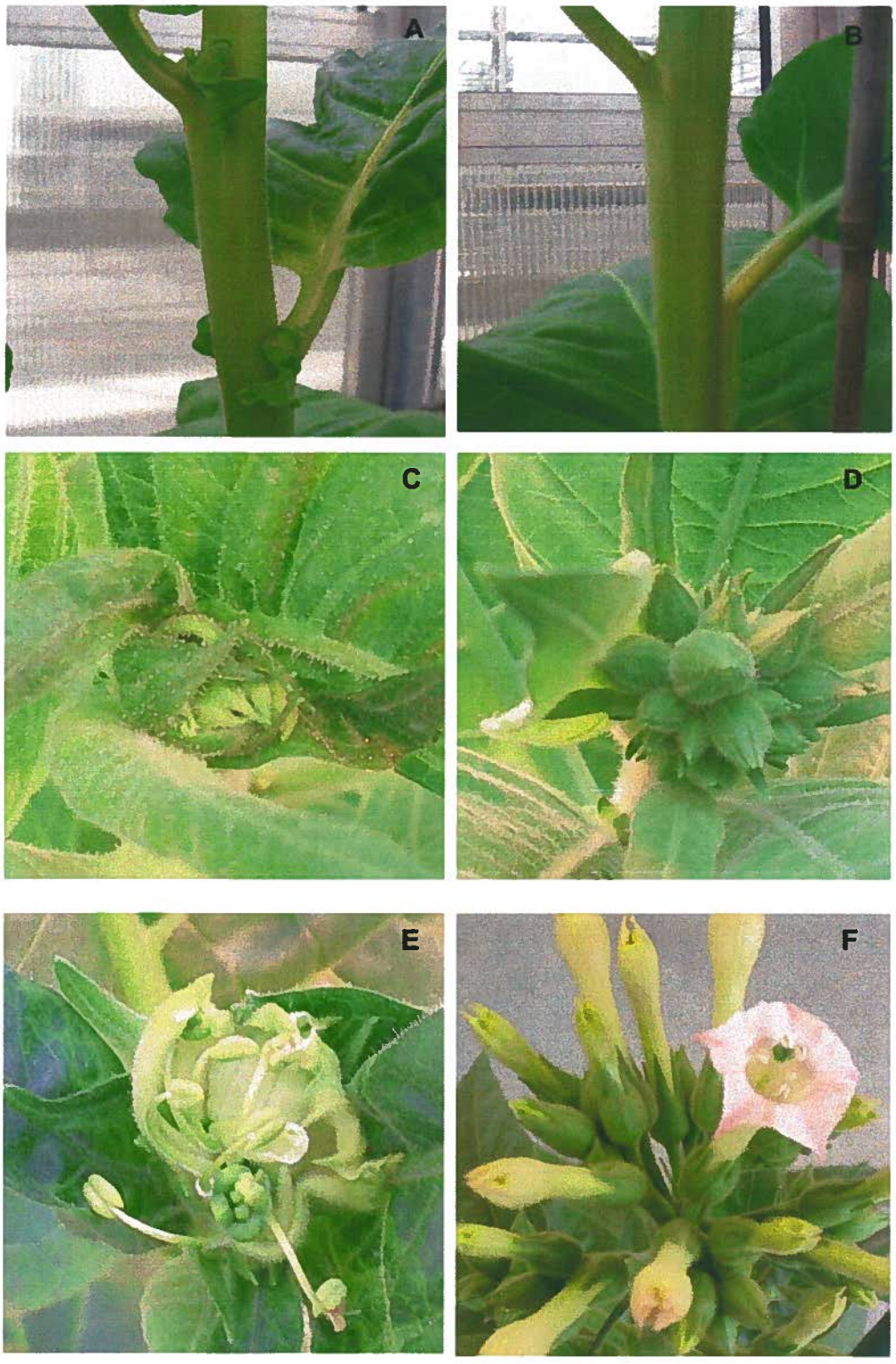

Figure 3.4. Morphological alterations of 4CL::UGPase x SuSy x SPS (A&C&E) plants relative to wild-type tobacco plants (B&D&F). A&B show foliar stipules on 4CL lines, C&D show altered reproductive bud and flower morphology.

Cell Wall Components

As a means to quantify carbon skeleton allocation, the stem segment spanning PI=5 to PI=15 was characterized for the changes in cell wall chemistry. The double transgenics did not show any measurable differences in wall chemistry (data not shown), and these findings concur with the previous observations of Coleman *et al.* (2006). In contrast, the triple overexpressing UGPase × SuSy × SPS transgenic lines showed statistically increased levels of total hemicellulose-derived sugars, represented by arabinose, rhamnose, galactose, mannose and xylose (Table 3.4). Additionally, these same plants appear to be synthesizing increased levels of cellulose, represented by liberated glucose monomers, and reduced levels of lignin. These latter two cell wall components seem to offset one another, showing an approximately 2% change in dry matter shift.

Discussion

Tobacco independently transformed with one of three key sucrose metabolizing enzymes, UGPase, SuSy and SPS, were generated under the regulation of two different promoters: a putative constitutive promoter (2×35S) and a vascular specific promoter (4CL). The ensuing single transgenic tobacco lines were then reciprocally crossed to produce double transgenic plants, which were subsequently employed to generate tobacco lines overexpressing all three sucrose metabolizing transgenes. In short, six transgenic combinations were employed for all growth and biochemical analysis, including: 2×35S::UGPase × SPS, 4CL::UGPase × SPS, 2×35S::SuSy × SPS, 4CL::SuSy × SPS, 2×35S::UGPase × SuSy × SPS, and 4CL::UGPase × SuSy × SPS. All six combinations clearly showed evidence of transgene expression, as determined by real time PCR evaluation. Correspondingly, the associated enzyme activity was significantly increased in either leaf or stem tissue of all lines relative to their corresponding non-transformed control plants.

All lines showed increases in height growth, which is consistent with previous reports of these genes in tobacco (Coleman *et al.*, 2006; Park *et al.*, 2008). The triple lines showed slightly higher percent increase in growth over the doubles. This is also

Table 3.4. Chemical composition (% dry weight) of cell wall of stem of UGPase × SuSy × SPS triple transgenic lines. Average ± standard error (n = 5). Bold indicates significance at α=0.10.

	Cellulose	Hemicellulose	Lignin
2×35S::UGPase × SuSy x SPS G	25.04 ± 0.92	**11.15 ± 0.29**	21.75 ± 1.32
2×35S::UGPase × SuSy x SPS H	25.94 ± 1.47	**11.25 ± 0.25**	20.70 ± 1.34
2×35S::UGPase × SuSy x SPS I	26.43 ± 1.96	11.64 ± 0.57	22.51 ± 0.34
4CL::UGPase × SuSy x SPS G	26.28 ± 2.25	**12.08 ± 0.57**	23.57 ± 1.36
4CL::UGPase × SuSy x SPS H	25.83 ± 0.66	**12.32 ± 0.39**	24.49 ± 1.80
4CL::UGPase × SuSy x SPS I	25.27 ± 0.81	**11.45 ± 0.47**	23.06 ± 0.12
Control G-I	24.07 ± 1.49	10.29 ± 0.21	25.49 ± 2.16

consistent with what was previously observed with UGPase × SuSy double transgenic tobacco plants (Coleman *et al.*, 2006) which displayed 10-48% increases in height growth, and performed better than plants transformed with only one of the genes (6-15% height increase). Furthermore, the extent of improved height growth in the current transgenics tobacco plants is consistent with the improvements attainable with SPS alone (Park *et al.*, 2007).

The increased growth observed in this study is consistent with other studies examining the misregulation of these three sucrose metabolizing genes individually in numerous other plant species. For example, the overexpression of a modified mung bean SuSy in *Populus alba* has been associated with slight increased height growth (Konishi *et al.*, 2004), while SuSy antisense studies have clearly shown reduced plant size in carrot (Tang and Sturm, 1999) and reduced fruit size in tomato (D'Aoust *et al.*, 1999). Overexpression of SuSy in tobacco resulted in increased height growth and internode length, despite no significant changes in fibre length or coarseness (Coleman *et al.*, 2006). In poplar overexpressing SuSy under the control of both the 2×35S and 4CL promoters, there was a slight reduction in height, but a significant increase in cellulose production (Coleman *et al.*, unpublished). SuSy has consistently been shown in previous studies, and in the current study, to strongly influence growth whether in height or in increased secondary growth.

The majority of studies investigating UDP-glucose pyrophosphorylase have focused primarily on downregulating enzyme activity, as evidence suggests that UGPase is present in ample supply in plants (Appeldoorn *et al.,* 1997, Magel *et al.,* 2001). However, the findings surrounding UGPase misregulation are inconsistent, ranging from no observed phenotypic effect in potato tubers despite a decrease in UGPase by 96% (Zrenner *et al.,* 1993) to substantial decreases in soluble sugar concentrations in potato tubers with a 30-50% reduction in activity (Borokov *et al.,* 1996; Spychalla *et al.,* 1994). In *Arabidopsis*, similar reductions in soluble sugars were observed when UGPase activity was reduced by ~50% (Johansson 2003), however, without observable plant phenotype. In contrast, when UGPase activity was upregulated in tobacco, increased plant biomass and changes in carbohydrate metabolism were observed, albeit without altering partitioning to cellulose (Coleman *et al.,* 2006). In addition, the overexpressing *Acetobacter xylinum* UGPase in poplar

resulted in severe reductions in height growth and biomass (Coleman *et al.*, 2007). The difference between these latter two studies using the same gene construct in two different species was not directly attributed to the activity of the UGPase gene, but rather to side effects in poplar in which a defence response appeared to be elicited by an increase in UDP-glucose (Coleman *et al.*, 2007). In addition, this also points to differences in sink strength between the two species, as alterations in UGPase activity appear to directly impact secondary growth in poplar, while in tobacco the impact is apparent on height and biomass accumulation.

SPS has also been shown to affect biomass production. Tomato overexpressing a maize SPS showed increased shoot growth (Galtier *et al.*, 1993), while antisense suppression of SPS resulted in a 50% reduction in tomato plant growth (Strand *et al.*, 2001). In rice, SPS has been identified as a targeted gene underlying a quantitative trait locus for plant height (Ishimaru *et al.*, 2004). These results were confirmed with the overexpression of a maize SPS gene in rice, which resulted in significantly taller plants at the early growth stage. In tobacco, the overexpression of an Arabidopsis SPS resulted in increased total dry biomass, attributed to both height growth and diameter growth (Park *et al.*, 2007). These plants also had increased internode length and fibre length, but only minor changes in soluble and structural carbohydrates. Consistent with the results of the current study, in SPS overexpressing tobacco, there was a slight decrease in lignin content attributed largely to a decrease in acid soluble lignin. However, in the case of the single SPS transgenics, there was also a reduction in cellulose and hemicelluloses which was attributed to a general reduction in secondary cell wall deposition related to increased growth rate (Park *et al.*, 2007). In potato, the overexpression of maize SPS resulted in a 20% increase in tuber weight and yield, along with increased tuber sucrose content (Ishimaru *et al.*, 2008). As the tuber is the main sink in potato, this corresponds well with the results seen in this study where the overexpression of SPS in conjunction with other genes results in an increase in sink biomass.

With the three sucrose metabolism genes being expressed in combination, it was anticipated that the effects would be additive, as seen with the UGPase × SuSy transgenic tobacco. However, SPS does appear to have the most significant effect on primary growth, and the addition of the UGPase and SuSy genes do not increase the

height gains achieved with SPS alone. However, the crossing of SuSy with the SPS plants added additional phenotypic variations in flower morphology and time to flowering.

Interestingly, the time to flowering in the case of tobacco transformed with SPS in combination with SuSy and in combination with both SuSy and UGPase is increased. Also, in the triple transgenic lines, the number of flowers per plant decreased greatly (Figure 3.4) with only one or two morphologically altered flowers per plant. This is inconsistent with previous findings in tobacco in which SPS overexpression resulted in decreased time to flowering and increased the number of flowers per plant (Baxter *et al.*, 2003). The decreased time to flowering was also seen in tomato overexpressing SPS (Micallef *et al.*, 1995). In both cases, the SPS was from maize. As well, in both cases there was an increase in sucrose to starch ratio in transgenic lines, which concurs with the current findings in triple transgenic lines. Sucrose content has been directly associated with flowering in some species (Lejeune *et al.*, 1993), however, the changes in intracellular pools manifested by the overexpression of these sucrose metabolizing genes does not appear to driving these substantial abnormalities in floral development. It is surprising that despite similarities in the sucrose and starch components in the plants, the opposite effect is seen in time to flowering and in number of flowers. It is possible that this is related to the type of SPS gene being used. Both previous papers (Baxter *et al.*, 2003; Micallef *et al.*, 1995) employed the maize Family B SPS gene originally employed by Worrell *et al.* (1991), while this study uses an Arabidopsis Family A SPS gene. B-Family genes in maize are found throughout the plant, but appear to be restricted almost exclusively to the anthers in wheat and barley (Castleden *et al.*, 2004). The effects of this gene appear to be consistent with changes seen as a result of the Arabidopsis Family A genes, with increased sucrose:starch ratios, but differences, include time to flowering, frequency of flowering and biomass production. UGPase and SuSy have also emerged as key genes involved in regulating, directly or indirectly, floral development. UGPase1 suppression has recently been shown to play a key role in pollen development in rice, where seed set is virtually eliminated (Woo *et al.*, 2008). SuSy activity has also been recently characterized in tobacco pollen tubes to occur in two distinct pools as soluble SuSy (S-SuSy) and

membrane associated SuSy (P-SuSy) forms, and is thought to play a role in extracellular matrix construction (Persia *et al.*, 2008).

There did not appear to be changes in cell wall chemistry, despite the substantial growth effects resulting from the misregulation of UGPase x SPS or SuSy x SPS. These results are consistent with what has previously been reported in tobacco expressing these three genes independently (Coleman *et al.*, 2006; Park *et al.*, 2008). Similarly, it was shown in UGPase x SuSy double transgenic tobacco, that there was no change seen in carbon allocation (Coleman *et al.*, 2006). In the present study, there does however appear to be a change, although minor, in the allocation of carbon skeletons to the various cell wall moieties in the triple transgenic lines. The UGPase x SuSy x SPS plants clearly show increased levels of the hemicellulose-derived carbohydrates and a 2% change in the balance of cellulose to lignin composition, with cellulose content increasing and lignin portion decreasing. Recently, it has been shown that cotton overexpressing a spinach SPS showed an increase in sucrose:starch ratio, and also had altered fibre qualities, with increased micronaire (wall thickness and diameter), maturity ratio and fibre length, all of which are consistent with increased deposition of fibre cellulose (Haigler *et al.*, 2007). In studies examining natural variation in wheat, increased SuSy activity was also correlated with an increase in CesA activity and cellulose production. This was associated with a decrease in water soluble carbohydrates (Xue *et al.*, 2007). Contrary to these results, in SuSy × SPS tobacco, there was a trend towards increased total soluble carbohydrates and increased starch in the stem. This could be associated with the regulatory role of the cellulose synthase complex in the production of cellulose. In tobacco, primary growth could take precedence over secondary growth, thus increased availability of sugars is stored and utilized for increased height growth, rather than for cellulose production as observed in cotton and wheat (Haigler *et al.*, 2007; Xue *et al.*, 2007). In triple transgenic tobacco, the addition increased UGPase activity further alters the sucrose metabolism, preventing increased starch storage and potentially increasing the plants ability to utilize sugars that would otherwise be stored for hemicellulose production. Interestingly, these same lines that have SPS and SuSy working in an apparent futile cycle (Nguyen-Quoc and Foyer, 2001) also have altered flower morphology.

In summary, the overexpression of UGPase, SuSy and SPS alone or in combination, clearly demonstrate an advantage in plant biomass accumulation in tobacco. As has previously been reported with these transgenes, there is an added advantage in employing a combined pyramiding strategy to alter sucrose metabolism. However, it is apparent from this study, that no single combination of two of these genes has an added advantage over any of the other combinations with respect to growth traits in tobacco. Pyramiding all three genes again has only slight, if any improvements, in growth traits, but does appear to challenge the normal cell wall chemical deposition. However, it is unknown whether this is true carbon re-allocation, or a simple a cellular wall response, and as such a chemical response, to alter growth patterns. Despite the slight advantage in altered cell wall chemistries, favouring carbohydrate deposition, the triple transgenic lines appear to have severely impaired floral development – a phenomena that warrants further investigation. Furthermore, this study suggests that choice of promoter did not, in these cases, offer an improved advantage in altering plant biomass accumulation.

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Overexpression of UDP-Glucose Pyrophosphorylase in Hybrid Poplar Affects Carbon Allocation[3]

Introduction

To maximize resource acquisition and to minimize exposure to deleterious phenomena, trees constantly monitor endogenous and environmental cues, and use this information to regulate resource allocation. Ultimately, these responses are controlled by gene expression patterns, resulting in the synthesis of a variety of metabolites, including carbohydrates, which can accumulate, participate in metabolite channelling and/or be polymerized into more complex macromolecules. Studies have shown that altered carbon partitioning can manifest changes in the chemical composition of plants by the altered regulation of genes involved in the synthesis of lignin or cellulose (Li *et al.* 2003, Canam *et al.* 2006). Despite these findings, how carbon partitioning occurs and the factors that regulate plants to produce more or less cellulose still remain largely unanswered. It may be possible to alter and/or regulate carbohydrate utilization by increasing the availability of precursor substrates such as UDP-glucose in the production of cellulose, or ADP-glucose in starch synthesis. The availability of soluble carbohydrates would therefore be a key component to altering and/or regulating carbohydrate utilization, thus by increasing available sugar metabolites in the sink cells (*i.e.* UDP-glucose), there may be the potential to augment cellulose production and content.

Two pathways have been identified that lead to the direct production of UDP-glucose. The first is based on the cleavage of sucrose by sucrose synthase (SuSy; EC 2.4.1.13) liberating fructose and UDP-glucose, while the second relies on the hexose phosphate pool and the active phosphorylation of glucose 1-phosphate by UDP-glucose pyrophosphorylase (UGPase, EC 2.7.7.9). UGPase is considered a key enzyme in

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carbohydrate biosynthesis and plays an important role in sucrose metabolism (Coleman *et al.*, 2006). In sink tissues, UGPase works in coordination with the sucrolytic enzymes (*i.e.* SuSy, fructokinase and SPS/SPP) in the metabolism of sucrose and hexose phosphates, while in source tissues, UGPase works closely with SPS in the synthesis of sucrose (Kleczkowski 1994). As such, the dual functionality of UGPase makes it an interesting target for altering cellulose production; it has the potential to increase the amount of sucrose in source tissues, and concurrently decrease sucrose in sink tissues, thereby modulating the solute potential gradient and facilitating sucrose channelling to sink tissues.

Previous investigations have focused primarily on downregulating UGPase enzyme activity, as evidence suggests that UGPase is present in ample supply in plants (Appeldoorn *et al.* 1997, Magel *et al.* 2001). However, the results of such studies are mixed, ranging from no observed phenotypic effect in potato tubers despite a decrease in UGPase by 96% (Zrenner *et al.* 1993) to substantial decreases in soluble sugar concentrations in potato tubers with a 30-50% reduction in activity (Borokov *et al.* 1996; Spychalla *et al.* 1994). In *Arabidopsis*, similar reductions in soluble sugars were observed when UGPase activity was reduced by ~50% (Johansson 2003). In contrast, when UGPase activity was upregulated in tobacco, increased plant biomass and changes in carbohydrate metabolism were observed, albeit without altering partitioning to cellulose (Coleman *et al.* 2006). Studies evaluating native UGPase gene expression patterns in poplar clearly demonstrate the onset of UGPase upregulation during late cell expansion and secondary cell wall formation, which is consistent with the theory that UGPase contributes in providing the immediate substrate for cellulose synthesis, and is co-ordinately up regulated with the cellulose synthase complex (Hertzberg *et al.* 2001).

The current study attempts to further elucidate the role of UGPase in secondary cell wall biosynthesis in hybrid poplar, and its effects on carbon partitioning. Poplar trees were transformed with UGPase from *Acetobacter xylinum* under the control of the constitutive (2×35S) promoter and were employed to evaluate changes in tree growth characteristics, biochemistry and cell wall chemistry. The Acetobacter-derived UGPase gene was chosen for these studies since it shows a high specificity for UDP-glucose (Brede *et al.* 1991), in contrast to the broader substrate specificity of plant-derived non-specific UDP-sugar pyrophosphorylases (Kotake *et al.* 2004).

Methods

Cloning of UGPase and Plasmid Construction

UGPase (M76548) was cloned from *Acetobacter xylinum* ATCC #23768 and inserted into the pBIN cloning vector under the control of the enhanced tandem CaMV35S (2×35S) constitutive promoter (Datla *et al.* 1993; Kay *et al.* 1987). Sequence analysis was used to confirm the proper insertion of the promoter and gene into the binary vector.

Plant Transformation and Maintenance

Hybrid poplar (*Populus alba* × *grandidentata*) was transformed using *Agrobacterium tumefaciens* EHA105 (Hood *et al.* 1993) employing a standard leaf disk inoculation. Binary plasmids were inserted into EHA105 using the freeze-thaw technique, and incubated overnight in liquid Woody Plant Media (WPM: McCown and Lloyd 1981) with 100 µM acetosyringone. Leaf disks were cut and co-cultured with EHA105 for one hour at room temperature, blotted dry and plated abaxailly onto WPM supplemented with 0.1 µM each α-naphthalene acetic acid (NAA), 6-benzylaminopurine (BA), and thiadiazuron (TDZ) and solidified with 3% (w/v) agar and 1.1% (w/v) phytagel (WPM 0.1/0.1/0.1). After three days the discs were transferred to WPM 0.1/0.1/0.1 supplemented with carbenicillin disodium (500 mg L^{-1}) and cefotaxime sodium salt (250 mg L^{-1}). Following three additional days, the discs were transferred to WPM 0.1/0.1/0.1 containing carbenicillin, cefotaxime and kanamycin (25 mg L^{-1}). After five weeks, shoots and callus material were transferred to WPM with agar and phytagel, 0.01 µM BA, carbenicillin, cefotaxime and kanamycin. Once individual shoots were visible, plantlets were transferred to solidified WPM with 0.01µM NAA and carbenicillin, cefotaxime and kanamycin to induce rooting. After two consecutive five-week periods on this media, shoot tips were isolated to solidified antibiotic-free WPM with 0.01 µM NAA.

Plants were confirmed as transgenic by PCR screening of genomic DNA employing gene specific oligonucleotides: specifically, UGP-F (5'-atcgaggaattctgcctcgt-3') and UGP-R (5'-tcgcaagaccggcaacaggatt-3').

All shoot cultures, including transgenic and non-transformed control lines, were maintained on solid WPM with 0.01 µM NAA in GA-7 vessels at 22°C under a 16-hour photoperiod with an average photon flux of 40 µmol m^{-2} s^{-1}.

Plant Growth and Biomass

Tissue culture plantlets were transferred into 7.5 L pots containing a 50% peat, 25% fine bark, 25% pumice soil mixture in the greenhouse, and covered with 16 oz clear plastic cups for one week to aid in acclimation. Each poplar line, transgenic and wild type, was represented by 12 clonally-propagated trees. The greenhouse trees were harvested after four months growth, at which time tree height, from base to tip, and stem diameter (10 cm above root collar) were measured.

Developmental stages of tissues were standardized by employing a plastichron index, where leaf plastichron index PI=0 was defined as the first leaf greater than 3cm in length, and where PI=1 was the leaf immediately below PI=0. Portions of the stem from each plant spanning PI=5 to PI=15, and from PI=15 to PI=25 were excised and dried at 105°C for 48h for dry weight determination, and retained for further analysis. Leaves were also collected in ten node groups (PI=6-15, PI=16-25) and analysed using an Area Meter (Li-Cor Environmental, Lincoln NE), and then dried at 105°C for 48 hours for dry weight determination. Developing xylem was scraped and flash frozen in liquid nitrogen for future analysis of enzyme activity, RNA transcript abundance, and soluble sugar analysis.

Transcription Levels

Real time PCR was used to determine transcript abundance of the transgene. Leaf and developing xylem samples weighing approximately 1 g (f.w.) were ground in liquid nitrogen, and RNA was extracted according to the method of Kolosova *et al.* (2004). One µg of RNA was used for the synthesis of cDNA using Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) and dT_{16} primers according to the manufacturer's instructions. Samples were run in triplicate with Brilliant SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA) on an Mx3000P Real-Time PCR System (Stratagene). The primers for the RT-PCR analysis of the *Acetobacter xylinum* UGPase

were AU-RTF (5'-tggaagcaacccgcgtcatc-3') and AU-RTR (5'-gccaaggcccagcggttcc-3'). Conditions for the RT-PCR reactions were as follows: 95°C for 10 minutes, followed by 40 cycles of 95°C for 30 seconds, 62°C for 1 minute, and 72°C for 30 seconds. Transcript levels were based on standard curves derived from known concentrations of plasmid DNA run under the same conditions. Transcript abundance of the two native poplar UGPase genes, as per Meng *et al.* (2007) was concurrently evaluated by RT-PCR using the following primers: PtUGP1F (5'-ggcttcttcagatttgcttctg-3'), PtUGP1R (5'-ccagtttcacaccagatttcac-3') and PtUGP2F (5'-gcaacttcagatctgcttcttg-3'), PtUGP2R (5'-tccaatttcacaccagattttg-3').

Additionally, the transcription abundance of key genes involved in lignin and cellulose production in the developing xylem were also quantified, including: phenylalanine ammonia-lyase, PAL-RT-FW (5'-aaaggtgccgaaattgccatgg-3'), PAL-RT-RV (5'-tgcagaaatcaagcccaaggag-3'); cinnamate 4-hydroxylase, C4H-RT-FW (5'-gtggggaattgctgagcttgt-3'), C4H-RT-RV (5'-cgcaacttcttctggatttca-3'); coumarate 3-hydroxylase, C3H-RT-FW (5'-atggcttcgttggatgtttc-3'), C3H-RT-RV (5'-atccataatagctctagtga-3'); caffeoyl CoA 3-O-methyltransferase, CCOMT-RT-FW (5'-tttgcatgcttcctgttggtga-3'), CCOMT-RT-RV (5'-aatgcagcccctcacttgatcc-3'); cinnamoyl CoA reductase, CCR-RT-FW (5'-atggtttactctatgtgcttctct-3'), CCR-RT-RV (5'-gctcctccttcacaaaccttaa-3'); cinnamyl alcohol dehydrogenase, CAD-RT-FW (5'-atgaagtggttggtgaggttgt-3'), CAD-RT-RV (5'-acaccgacaacatctccaactt-3'); ferulate 5-hydroxylase, F5H-RT-FW (5'-agctcgcagacgtggtgggtttag-3'), F5H-RT-RV (5'-gaaataaccagcaacctcagcatct-3'); caffeic acid 3-O-methyltransferase, COMT-RT-FW (5'-gccagtgcttcagttctaccaa-3'); COMT-RT-RV (5'-ggtcgagttcaatggctgtttt-3'); 4CL-RT-FW (5'-gcacctaaaactcaccatctctcc-3'), 4CL-RT-RV (5'-aaggtttttcgggatgtagatgtc-3'); sucrose synthase, SuSyPt-RT-FW (5'-ccatggattgctcttgctctgc-3'), SuSyPt-RT-RV (5'-gcaacacgcaaatcctcaacaa-3'); and cellulose synthase, CESA-RT-FW (5'-agagctgtgatcattatgcgactg-3'), CESA-RT-RV (5'-acccaagaaaatgcaaaccagatc-3'). The genes selected and employed for transcript analysis of the lignin branch of the phenylpropanoid pathway and cellulose biosynthesis were based on previously reported high levels (highest of each isoform) during EST expression profiling of the cambial zone and tension wood formation in poplar (Sterky *et al.* 2004). Critical threshold (ct)

values for all genes were quantified in triplicate and normalized to β-actin transcript levels.

Enzyme Activity

Leaf and developing xylem samples (approximately 1g f.w.) were ground in liquid nitrogen with 1 mg of insoluble PVPP and four volumes of extraction buffer (50 mM HEPES-KOH pH 7.5, 10 mM $MgCl_s$, 1 mM EDTA, 2 mM DTT, 1 mM PMSF, 5 mM εAmino-n-caproic acid, 0.1% v/v Triton X-100, 10% v/v glycerol). The samples were centrifuged at 14,000 rpm for 20 minutes at 4°C. The extract was passed through a desalting column (DG 10 – BioRad) and pre-equilibrated with ice-cold extraction buffer without Triton X-100 and PVPP. Extracts were collected into pre-chilled vials and used immediately. UGPase activity was determined spectrophotometrically at 340 nm as per Appeldoorn *et al.* (1997) using 100 μL of plant extract and a molar extinction coefficient of 6.22 mM cm^{-1}. Total protein content of the extracts was determined using a Bio-Rad Protein Assay (Bio-Rad, Hercules, CA).

Soluble Carbohydrate and Starch Analysis

Soluble carbohydrates (glucose, fructose and sucrose) were extracted overnight at -20°C from ground freeze-dried plant material using methanol:chloroform:water (12:5:3). The sample was centrifuged, the supernatant removed, and the remaining pellet washed twice with fresh methanol:chloroform:water (12:5:3) and all fractions pooled. Five mL of water was added to the combined pooled supernatant and centrifuged to facilitate phase separation. The aqueous fraction was removed to a round bottom flask and rotary evaporated to dryness. The sample was resuspended in 3 mL of distilled water and analyzed using anion exchange HPLC (Dionex, Sunnyvale, CA) on a DX-600 equipped with a Carbopac PA1 column and an electrochemical detector, as per Coleman *et al.* (2006).

The residual pellet was hydrolyzed using 4% sulfuric acid at 121°C for 4 minutes. The liberation of glucose represented starch content, and was directly quantified by HPLC employing similar conditions.

Cell Wall Compositional Analysis

Greenhouse grown plant stem material was ground using a Wiley mill to pass through a 40-mesh screen, and then soxhlet extracted with acetone for 24 hours. The extractive free material was used for all further analyses. Lignin content was determined using a modified Klason, where extracted ground stem tissue (0.2 g) was treated with 3 mL of 72% H_2SO_4 as per Coleman *et al.* (2006). Carbohydrate concentrations in the hydrolyzate were determined using high-performance liquid chromatography (HPLC) (Dionex DX-500, Dionex, CA) equipped with an ion exchange PA1 (Dionex) column, a pulsed amperometric detector with a gold electrode, and a Spectra AS 3500 auto-injector (Spectra-Physics, Los Angeles, CA). 20 µL of hydrolyzate was loaded on the column equilibrated with 250 mM NaOH and eluted with deionized water at a flow rate of 1.0 mL min^{-1}, followed by a post column addition of 200 mM NaOH at a flow rate of 0.5 mL min^{-1}. Each experiment was run in duplicate.

Determination of α-Cellulose Content

α-cellulose was determined from the extract free wood using a modified microanalytical method developed by (Yokoyama *et al.*, 2002). In short, 200 mg of ground extract-free wood was weighed into a 25 mL round bottom flask and placed in a 90ºC oil bath. The reaction was initiated by the addition of 1 mL of sodium chlorite solution (400 mg 80% sodium chlorite, 4 mL distilled water, 0.4 mL acetic acid). An additional 1 mL of sodium chlorite solution was added every half hour and the samples removed to a cold waterbath after two hours. Samples were then filtered through a coarse crucible, dried overnight, and holocellulose composition determined gravimetrically. Fifty mg of this dried holocellulose sample was weighed into a reaction flask and allowed to equilibrate for 30 minutes. Four mL of 17.5% sodium hydroxide was added and allowed to react for 30 minutes, after which 4 mL of distilled water was added. The sample was macerated for 1 minute, allowed to react for an additional 29 minutes and then filtered through a coarse filter. Following a five-minute soak in 1.0 M acetic acid, the sample was washed with 90 mL of distilled water and dried overnight. The α-cellulose content was then determined gravimetrically.

Monolignol Analysis

Thioacidolysis (Rolando *et al.* 1992) was employed to determine the lignin monomer ratios (syringyl:guaiacyl; S:G), using 10 mg of oven-dried extractive free wood and tetracosane as an internal standard (2 mL of 25 mg mL^{-1} in dichloromethane). The siylation reaction proceeded for a minimum of 2 hours, and gas chromatography was carried out on a ThermoFinnigan Trace GC-PolarisQ ion trap system with an AS2000 autosampler and a split/splitless injector. The GC was equipped with a 30 m, 0.25 mm internal diameter J&W DB-5 column. The GC conditions were as follows: initial injector temperature of 250°C, detector temperature of 270°C, and initial oven temperature of 130°C. Following a 2 µL injection, the oven remained at 130°C for 3 minutes and then ramped at a rate of 3°C min^{-1} to 260°C and held for 5 minutes.

Soluble Metabolite Analysis

Liquid nitrogen frozen developing xylem tissue was ground using a dental amalgam mixer, and extracted using a two-phase methanol:chloroform extraction as per Robinson *et al.* (2005). In short, 600 µL of methanol was added to 20 mg of frozen tissue to stop biological activity. The samples were vortexed and 40 µL of water, 10 µL of internal standard (10 mg mL^{-1} ribitol in water) and 10 µL of lipophilic internal standard (10 mg mL^{-1} nonadecanoic acid methyl ester in methanol) were added. The samples were then incubated for 15 minutes at 70°C with constant agitation, and then centrifuged for 5 minutes at 13000 rpm. The supernatant was removed, and 800 µL of methanol was added to the pellet, which was vortexed to resuspend the pellet, and then incubated at 35°C for 5 minutes with constant agitation. The sample was centrifuged for 5 minutes at 13000 rpm and then the supernatant removed and combined with the previous methanol supernatant. 600 µL of water was added to the pooled methanol extract, vortexed and centrifuged at 4000 rpm for 15 minutes. A 900 µL sample was removed from the upper potion (water/methanol) and dried at 40°C using a speedvac.

The pellet was resuspended in 50 µL of methoxyamine hydrochloride solution (20 mg mL^{-1} in chloroform) and incubated for 2 hours at 60°C with constant agitation. Following the addition of 200 µL of N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA), the sample was incubated at 60°C for 30 minutes with constant agitation. Samples

were left at room temperature overnight to allow the reaction to progress to completion, and then filtered through tissue paper to remove particulates. GC/MS analysis was carried out using a 30m, 0.25mm internal diameter Restek Rtx-5MS column using the following conditions: inlet temperature 250°C, injector split ratio 10:1, resting oven temperature 70°C, and GC-MS transfer line temperature of 300°C. Following a 1 µL injection, the oven remained at 70°C for 2 minutes and then ramped at a rate of 8°C min^{-1} to 325°C and held for 6 minutes.

Results

Transformed hybrid poplar trees regenerated from the AxUGPase-agrobacterium treated leaf explants were propagated from single shoots from individual unique explants, and represented independent transformed lines which were confirmed by PCR screening of genomic DNA to amplify a diagnostic fragment specific to the AxUGPase gene. From the independent transgenic lines 6 2×35S::UGPase transformants (based on high real time qPCR expression levels) and corresponding control (wild type) shoots were propagated *in vitro* as shoot cultures, and a minimum of 12 individual trees of each line were transferred into the greenhouse and grown for four months under 18 hour days supplemented with overhead lighting with a radiant flux density of 300 W m^{-2}. At harvest, all of the transgenic lines showed substantially altered growth characteristics, demonstrating significantly impaired height and diameter growth (Figure 4.1A & B). Furthermore, the trees displayed significantly reduced internodal length (Figure 4.1D), as well as decreased stem dry weight (data not shown).

Morphologically, the 2×35S::UGPase transgenic lines also displayed a greater abundance of significantly smaller leaves per stem, and consistently had elongated axial shoots at each leaf node (Figure 4.2). A quantitative evaluation of leaf characteristics confirmed that the 2×35S::UGPase leaves were 75% smaller, had reduced total leaf area per stem and reduced total leaf dry weight (Figure 4.1C). In addition, the trichomes appeared to be more highly concentrated in the 2×35S::UGPase leaves when compared to the wild-type poplar (data not shown).

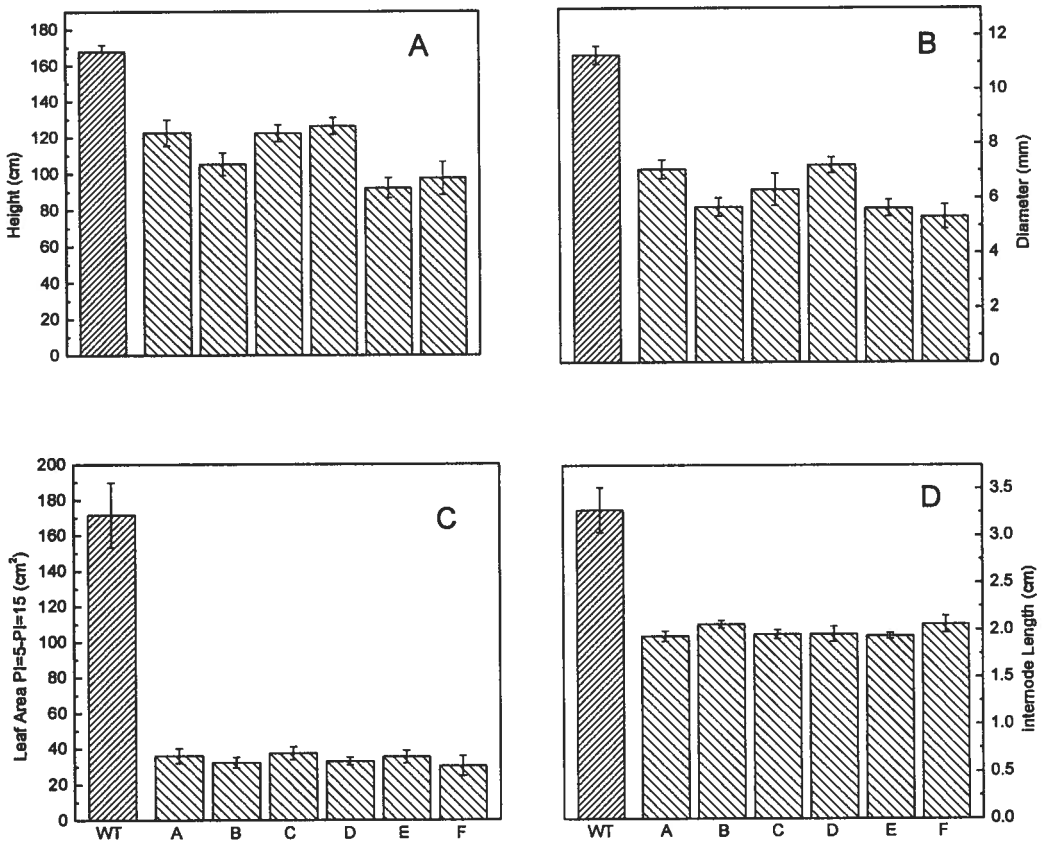

Figure 4.1. Plant height (A), diameter (B), leaf area (C) and internode length (D); for transgenic and wild-type trees. Mean (±SE) were calculated from 10 plants per line. All transgenic lines are statistically significant different from the control trees at α=0.05.

Figure 4.2. Image depicts axial shoot elongation and leaf size in a representative 2×35S::UGPase (right) and wild-type (left) poplar (inset is a close up of elongated axial shoots of transgenic poplar).

Transcription Levels

Real time quantitative PCR was used to evaluate transcript abundance of the exogenous UGPase gene in the transgenic lines (Table 4.1). All the 2×35S::UGPase transgenic lines had significantly higher transcript levels in the leaf material compared to the stem (developing xylem). Transcript abundance ranged from 68 to 173 copies μg^{-1} total RNA in leaf tissue and 2.1 to 27.9 copies μg^{-1} total RNA in the developing xylem, among the transgenic poplar trees. The expression of the two native poplar UGPase genes was also shown to be affected in most lines (Table B1), with smaller increases in PtUGP1 when compared to the observed increases in PtUGP2.

Transcript abundance of genes involved directly in cellulose and lignin biosynthesis in the developing xylem were also compared using real time quantitative PCR. PAL, 4CL, CST, CQT and COMT showed no change in the transgenic lines relative to wild type levels, while all other genes in the lignin biosynthetic pathway were up regulated (Figure 4.3). SAD, CAD, C3H, and F5H were increased between 2-3 fold relative to wild type levels, and C4H was increased to 4-fold the wild type level. CCR and CCoAMT showed the highest relative increases at over 6 times the abundance seen in wild-type trees (Table B2). Similarly, the CesA gene family was also shown to be up regulated approximately 4-fold over the corresponding wild type CesA transcript levels, while SuSy expression levels remained comparable to the wild-type trees.

Enzyme Activity

UGPase activity was determined using an indirect assay measuring the production of the reduced form of nicotinamideadenine dinucleotide (NADH). All of the 2×35S::UGPase transgenic trees showed consistent increases in UGPase activity in the leaf tissue compared to the wild-type trees (Table 4.1). However, in contrast, only one line (2×35S::UGPase line C) demonstrated a statistically significant increase in enzyme activity in the developing xylem, despite the slight general upward trend in activity in the other transgenic lines.

Table 4.1. Transcript level and enzyme activity in leaf tissue and developing xylem for transgenic and wild-type trees. Mean (±SE) were calculated from 3 plants per line. Bold denotes significant difference from control values at α=0.10.

	UGPase Transcript Level copy number μg^{-1} total RNA		UGPase Enzyme Activity μmolNADH min^{-1} mg^{-1}protein	
Transgenic line	Leaf	Developing Xylem	Leaf	Developing Xylem
Control			0.18 ± 0.05	0.71 ± 0.05
2×35S::UGPase A	99.47 ± 14.94	13.13 ± 12.10	**0.50 ± 0.10**	0.75 ± 0.01
2×35S::UGPase B	172.99 ± 15.17	20.79 ± 11.65	**0.34 ± 0.05**	0.80 ± 0.05
2×35S::UGPase C	89.81 ± 44.64	2.07 ± 1.23	**0.54 ± 0.12**	**1.69 ± 0.40**
2×35S::UGPase D	89.61 ± 48.89	19.24 ± 17.07	**0.47 ± 0.02**	0.68 ± 0.06
2×35S::UGPase E	67.51 ± 25.22	12.66 ± 11.13	**0.39 ± 0.08**	0.86 ± 0.18
2×35S::UGPase F	76.60 ± 42.27	27.92 ± 25.99	**0.84 ± 0.16**	0.82 ± 0.05

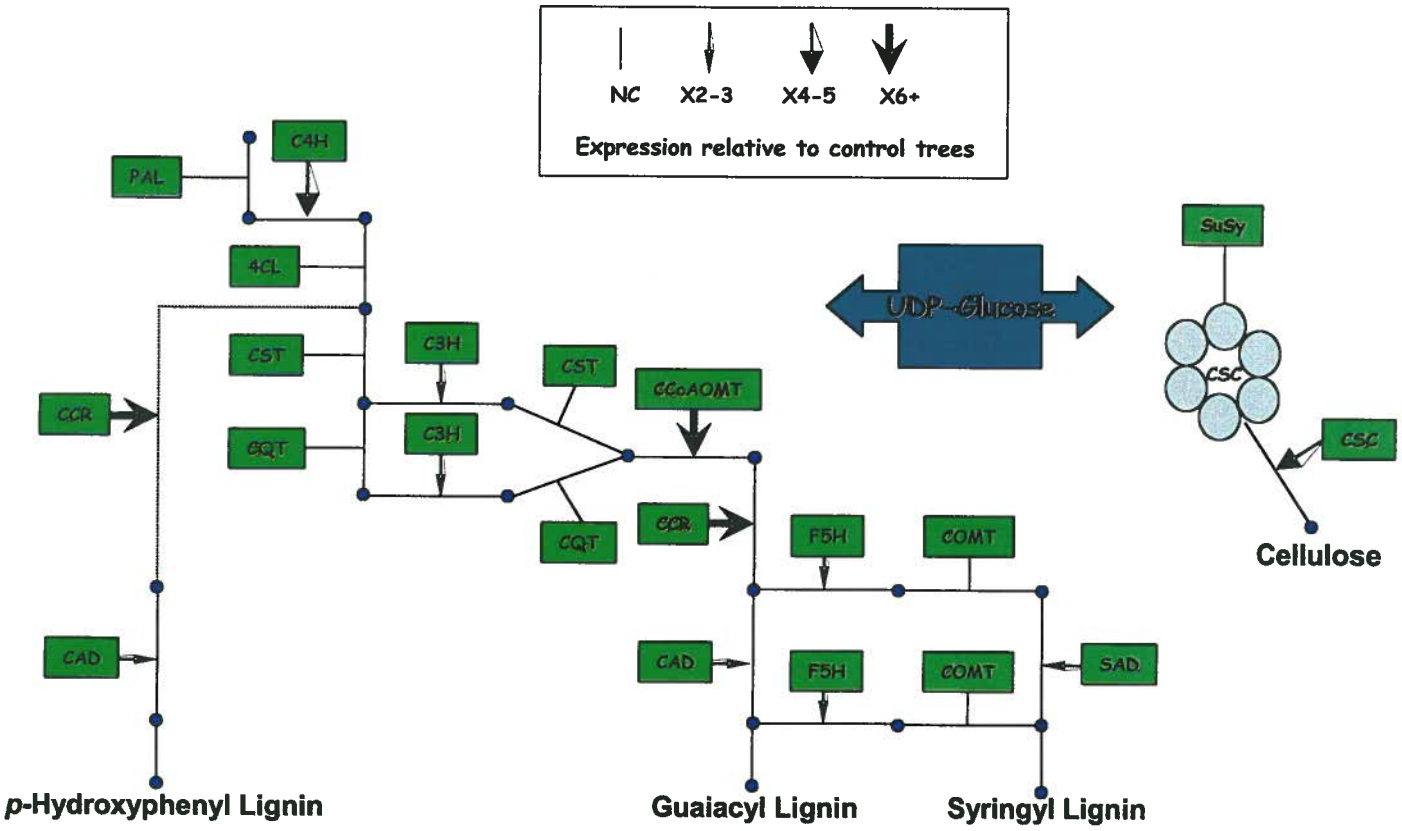

Figure 4.3. Schematic representation of the effect of overexpression of 2×35S::UGPase on the transcript abundance of the lignin biosynthetic genes in the developing xylem. All mRNA levels were calculated by qPCR from threshold cycle values and are relative to controls and normalized with respect to β-actin transcript abundance.

Soluble Carbohydrates

In general, all transgenic lines showed elevated levels of total soluble sugars in the leaf tissue, which was a result of a commensurate increase in all three soluble sugars quantified - sucrose, fructose and glucose (Table 4.2). The total soluble carbohydrate concentrations, as well as concentrations of each individual sugar, were significantly increased in the leaves of five out of the six 2×35S::UGPase transgenic lines evaluated relative to the controls. The exception was Line F, which despite having higher glucose and fructose levels did not have statistically significant higher levels of sucrose, and thus total carbohydrates. In contrast, significant increases in total soluble carbohydrates, as well as significant increases in glucose and sucrose, were only found in two 2×35S::UGPase lines (B and C) in the developing xylem.

Starch Content

Three of the six 2×35S::UGPase transgenic lines (A, B, and F) had significant increases in starch content in the leaves compared to starch levels in control leaves (Table 4.3). As well, five of the six 2×35S::UGPase transgenic lines (A, B, C, D and F) had significantly elevated starch in the developing xylem relative to control trees, with two lines (C and F) displaying three times the concentration of wild-type trees. And, although not statistically significant the sixth transgenic line (line E) did have higher levels of starch accumulation in the developing xylem, similar to the other transgenics.

Cell Wall Chemistry

The total cell wall carbohydrate content, as a measure of percent total dry weight, of the 2×35S::UGPase lines increased dramatically compared to the wild-type trees, where all six lines showed statistically significant changes in polymeric cell wall moieties. Wild-type hybrid poplar trees were shown to be composed of ~64.4% total carbohydrates, while the 2×35S::UGPase transgenic trees ranged from 69.2 to 73.1%. Although all cell wall carbohydrates (arabinose, galactose, glucose, xylose, mannose, and rhamnose) were shown to be elevated in the 2×35S::UGPase transgenic lines, the

Table 4.2. Total soluble carbohydrates (mg g^{-1}) in leaves and developing xylem of transgenic and wild-type trees. Mean (±SE) were calculated from 3 plants per line. Bold denotes significant difference from control values at α=0.10. Tissue (collected between PI=3-5) was collected from greenhouse plants between 10:00 and 12:00 am.

Leaf	Glucose	Fructose	Sucrose	Total
Control	7.49 ± 2.07	2.61 ± 0.58	49.90 ± 3.19	60.00 ± 2.57
2×35S::UGPase A	**18.79 ± 1.10**	**6.00 ± 0.75**	**68.96 ± 4.25**	**93.75 ± 5.60**
2×35S::UGPase B	**14.57 ± 6.29**	**4.93 ± 1.22**	**61.93 ± 3.95**	**81.42 ± 8.22**
2×35S::UGPase C	**17.54 ± 3.76**	**5.90 ± 1.61**	**66.33 ± 4.76**	**89.77 ± 6.79**
2×35S::UGPase D	**13.60 ± 3.11**	**6.59 ± 1.68**	**65.60 ± 6.63**	**85.79 ± 11.08**
2×35S::UGPase E	**24.35 ± 3.35**	**6.06 ± 1.83**	**64.45 ± 3.74**	**94.86 ± 2.56**
2×35S::UGPase F	**19.71 ± 4.45**	**6.89 ± 0.56**	51.30 ± 4.67	77.91 ± 9.12
Developing Xylem	Glucose	Fructose	Sucrose	Total
Control	3.61 ± 0.87	0.00 ± 0.00	23.71 ± 5.47	27.31 ± 6.12
2×35S::UGPase A	**8.65 ± 3.12**	0.00 ± 0.00	35.05 ± 7.90	43.71 ± 10.64
2×35S::UGPase B	**5.82 ± 0.97**	0.00 ± 0.00	**43.33 ± 1.93**	**49.15 ± 1.05**
2×35S::UGPase C	**13.19 ± 4.76**	0.00 ± 0.00	**61.95 ± 4.51**	**75.14 ±9.15**
2×35S::UGPase D	3.37 ± 0.51	0.00 ± 0.00	27.83 ± 6.70	31.20 ± 6.55
2×35S::UGPase E	4.38 ± 1.41	0.00 ± 0.00	40.52 ± 10.72	50.06 ± 15.77
2×35S::UGPase F	3.38 ± 0.53	0.00 ± 0.00	35.06 ± 6.36	38.44 ± 6.71

Table 4.3. Starch content (mg g^{-1}) in leaves and developing xylem of transgenic and wild-type trees. Mean (±SE) were calculated from 3 plants per line. Bold denotes significant difference from control values at α=0.05. Tissue (collected between PI= 3-5) was collected from greenhouse plants between 10:00 and 12:00 am.

	Leaf	Developing Xylem
Control	2.6 ± 0.1	8.3 ± 0.7
2×35S::UGPase A	**4.3 ± 0.5**	**18.3 ± 4.2**
2×35S::UGPase B	**4.2 ± 0.3**	**16.0 ± 4.0**
2×35S::UGPase C	1.3 ± 0.0	**27.6 ± 6.3**
2×35S::UGPase D	2.9 ± 0.4	**11.2 ± 0.6**
2×35S::UGPase E	2.4 ± 0.0	14.0 ± 6.4
2×35S::UGPase F	**3.7 ± 0.1**	**31.2 ±7.9**

Table 4.4. Chemical composition of stem material of transgenic and wild-type trees. Mean (±SE) were calculated from 3 plants per line. Bold denotes significant difference from control values at α=0.10.

	% Carbohydrates						% Lignin	
	Arabinose	Galactose	Glucose	Xylose	Mannose	Rhamnose	Acid Soluble	Acid Insoluble
Control	0.4 ± 0.0	1.0 ± 0.1	43.2 ± 0.2	17.7 ± 0.7	1.5 ± 0.1	0.6 ± 0.1	3.0 ± 0.0	20.7 ± 0.1
2×35S::UGPase A	**0.6 ± 0.0**	1.4 ± 0.1	**49.0 ± 1.4**	18.8 ± 0.7	**2.0 ± 0.1**	0.6 ± 0.0	**3.3 ± 0.1**	**15.4 ± 0.5**
2×35S::UGPase B	0.5 ± 0.0	**1.7 ± 0.2**	**50.6 ± 0.9**	18.0 ± 0.3	1.8 ± 0.1	0.5 ± 0.0	3.4 ± 0.1	**15.9 ± 0.5**
2×35S::UGPase C	**0.6 ± 0.0**	1.3 ± 0.3	47.7 ± 2.9	18.9 ± 1.2	1.8 ± 0.1	0.6 ± 0.0	**3.5 ± 0.2**	**16.4 ± 0.9**
2×35S::UGPase D	**0.6 ± 0.0**	1.3 ± 0.2	46.6 ± 2.1	19.6 ± 0.9	1.8 ± 0.2	0.7 ± 0.0	3.4 ± 0.1	**17.5 ± 0.7**
2×35S::UGPase E	**0.5 ± 0.0**	1.1 ± 0.1	**46.3 ± 0.8**	18.8 ± 0.6	1.9 ± 0.2	0.6 ± 0.0	3.2 ± 0.1	**17.6 ± 0.3**
2×35S::UGPase F	**0.5 ± 0.0**	1.2 ± 0.1	**48.8 ± 0.7**	17.4 ± 0.4	1.8 ± 0.1	0.5 ± 0.1	**3.2 ± 0.1**	**16.9 ± 0.7**

most significant changes were observed in glucose content (Table 4.4). The observed increases in glucose content is reflective of the overall increase in cellulose composition in all transgenic lines compared to the corresponding wild-type trees (Figure 4.4), as determined by α-cellulose quantification.

The total cell wall lignin content of the 2×35S::UGPase transgenic lines was concurrently shown to be significantly decreased. As measured by percentage weight of total dry mass, the total lignin was reduced from 23.7% of the dry weight in wild-type trees to a range of 18.7 to 20.1% in the 2×35S::UGPase transgenic lines. This represents a decrease of 12% to 21% across the lines. Furthermore, the drop in total lignin content appears to be directly related to a reduction in the acid-insoluble lignin moieties (Table 4.4).

Significant increases in the syringyl:guaicyl (S:G) ratio in the lignin was also observed in the stems of these 2×35S::UGPase transgenic lines (Table 4.5), with the % mol S concentration increasing from an average of 69.2% in wild-type poplar, to between 76.6% to 78.2% in the six 2×35S::UGPase lines with decreased lignin content. The increase in syringyl-based lignin monomers is proportionately related to a decrease in the guaiacyl monomers. Although significant, the change in the S:G ratio had a minor effect on the level of the *p*-hydroxyphenyl monomer (H-lignin) composition. However, H-lignin is only minor component in poplar trees relative to total lignin content.

Soluble Metabolite Analysis

Total soluble metabolites, extracted independently from leaf tissue and developing xylem, were evaluated by metabolite profiling with GC/MS analysis. Table 4.6 lists 18 (of ~280 compounds investigated) whose levels are changed two-times or greater in 2×35S::UGPase trees compared to wild type. In general, many of the identified compounds are associated with plant/tree defence or stress, including myo-inositol, galactinol, galactitol, and pinitol. As well, compounds related to carbon allocation, such as maltose and other carbohydrates were identified. However, a single compound was shown to dominant the pooling metabolites in the 2×35S::UGPase transgenic lines; salicylic acid 2-O-β-D-glucoside was dramatically increased in all

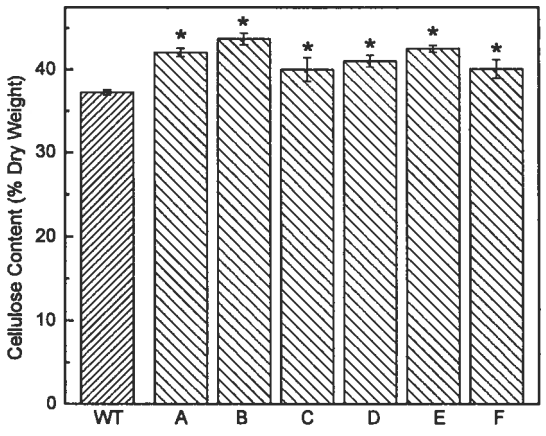

Figure 4.4. α-cellulose content of stem material from transgenic and wild-type trees. Mean (±SE) were calculated from 3 plants per line. Symbol denotes significant difference from control values at α=0.10.

Table 4.5. Syringyl, guaiacyl and p-hydroxyphenyl monomer contents (%) of transgenic and wild-type trees as determined by thioacidolysis. Mean (±SE) were calculated from 3 plants per line. Bold denotes significant difference from control values at α=0.05.

	p-Hydroxyphenyl	Guaiacyl	Syringyl
	Mean ± SE	Mean ± SE	Mean ± SE
Control	0.02 ± 0.01	30.78 ± 0.25	69.20 ± 0.26
2×35S::UGPase A	0.06 ± 0.02	**21.68 ± 1.25**	**78.26 ± 1.27**
2×35S::UGPase B	0.05 ± 0.01	**21.87 ± 0.65**	**78.08 ± 0.66**
2×35S::UGPase C	0.07 ± 0.02	**23.20 ± 0.23**	**76.73 ± 0.20**
2×35S::UGPase D	**0.08 ± 0.00**	**23.29 ± 0.39**	**76.63 ± 0.39**
2×35S::UGPase E	**0.11 ± 0.00**	**22.63 ± 1.08**	**77.26 ± 1.08**
2×35S::UGPase F	0.11 ± 0.01	**23.28 ± 1.08**	**76.61 ± 1.07**

Table 4.6. Metabolites identified in the developing xylem of 2×35S::UGPase hybrid poplar relative to levels in wild-type trees.

Fold change relative to wild-type poplar	Compound
232.6	Salicylic acid 2-O-β-D-glucoside
11.4	Maltose
5.8	Unknown
5.3	Myo-Inositol
5.1	Maltotriose methoxyamine
4.4	Galactinol
4.1	D-pinitol
3.0	Unknown
2.7	Galactitol
2.5	Unknown
2.5	Glucose
2.5	Galactose
2.3	Sucrose
2.2	Unknown
2.2	Sorbose
2.1	4-amino butyric acid
2.0	Fructose
2.0	Picein

transgenic lines with increases relative to wild-type poplar ranging from 175 to 270-fold (Table 4.6 and Table B3).

Discussion

This study investigated the effects of overexpressing the *Acetobacter xylinum* UGPase gene in hybrid poplar under the control of a constitutive (2×35S) promoter on tree growth and cell wall biochemistry. Transcript quantification revealed a much higher level of transcript abundance in the leaves of the 2×35S::UGPase lines relative to stem (developing xylem). Consistent with the observed elevated levels of transcription, the total UGPase enzyme activity followed a similar pattern in the leaves. The effect of the introduction of the exogenous UGPase transgene from *Acetobacter xylinum* on the transcript levels of the two native poplar UGPase gene was also examined, and an increase in both PtUGP1 and PtUGP2 was shown, in both leaf and developing xylem (Table B1). However, it was apparent that altered levels of expression of the two genes responded differently, with increases ranging from ~2-4-fold in developing xylem while a maximum 2-fold change was apparent in the leaf tissue. Meng *et al*, (2007), however, have shown tight post-transcriptional/ translational control for these two genes, and therefore, increases in transcript abundance, while expected with an increase in sucrose content, may not necessarily correlate to an increase in protein/enzyme activity.

The 2×35S::UGPase transgenic lines were shown to be substantially smaller than wild-type trees, with decreased height growth, diameter and stem dry weight (biomass). Morphologically, the 2×35S::UGPase trees, despite having a significantly greater number of leaves, had a reduced total leaf area and biomass compared to the corresponding wild-type trees. These same lines exhibited elongation of the axial shoots, which is consistent with weakening or loss of apical dominance resulting in changes in the auxin/cytokinin ratios. For example, in pea plants it has been shown that the removal of the apex resulted in the elongation of lateral shoots, with the exogenous application of NAA resulting in the retention of unaltered phenotype (Li *et al.* 1995). The same result was seen when the apex was removed from chick pea plants, or when exogenous cytokinins were applied (Turnbull *et al.* 2004).

To date, most of the work elucidating the function of UGPase is mixed and inconclusive, and has evolved from a limited number of studies downregulating UGPase activity. For example, reductions from 30 to 96% in UGPase activity by antisense suppression showed no change in plant biomass in either Arabidopsis or potato, respectively (Johansson 2003; Zrenner *et al.* 1993). In the case of Arabidopsis, Johansson (2003) speculated that the 30% reduction in Arabidopsis UGPase expression did not confer a phenotype as multiple UGPase isoforms exist permitting a compensatory control of pathway regulation (Johansson 2003). These studies imply that UGPase activity is abundant in the plant, and misregulation will likely have little or no effect on plant phenotype.

Contrary to this, the overexpression of transgenic UGPase in tobacco resulted in significantly improved growth rates, both under ubiquitous and tissue-specific promoters (Coleman *et al.* 2006). The improved growth characteristics were attributed to the potential role of UGPase in altering sink strength as it participates in the catabolism of sucrose into hexose phosphates. Although similar results were not observed in the current study investigating hybrid poplar, the differences may be attributed to the innate differences in mechanisms of carbon translocation, with tobacco employing an active mechanism (Burkle *et al.* 1998) and poplar using a passive system moving assimilate along a sucrose concentration gradient (Turgeon and Medville 1998). UGPase in source tissues tends to act in the formation of sucrose, providing the substrate to sucrose transporters for active loading from the leaves (Kleczkowski 1994). In poplar, photoassimilate accumulating in sink cells must be lower than the solute concentration in source cells, and this occurs primarily by temporary storage in starch, or permanent storage in structural carbohydrates such as cellulose. UGPase in sink tissue tends to function in the breakdown of sucrose, while in source cells it is thought to act in the synthesis of sucrose (Kleczkowski *et al.* 2004), thus creating the concentration gradient necessary for the passive flow of carbon skeletons required for active metabolism.

Altered UGPase activity in other plant species has also been shown to manifest changes in carbohydrate biochemistry, however, these changes are inconsistent and conflicting. In potato, Zrenner *et al.* (1993) showed no change in carbohydrate metabolism with a 96% reduction in UGPase enzyme activity, while other studies with less extreme reductions in UGPase activity showed significant decreases in stored

tuber carbohydrate concentrations (Spychalla *et al.* 1994; Borokov *et al.* 1996). Similarly, a 30% reduction in UGPase activity resulted in a decrease in soluble sugar and starch content in Arabidopsis (Kleczkowski *et al.* 2004). Consistent with these findings, tobacco plants overexpressing UGPase showed increases in stem glucose and fructose levels compared to non-transgenic control plants (Coleman *et al.* 2006). In the current study, significant increases in all soluble carbohydrates were observed in the leaf tissue of 2×35S::UGPase hybrid poplar. In the developing xylem, the 2×35S::UGPase transgenic lines showed significant, yet smaller, increases in only primarily glucose and sucrose.

These findings support the theory that UGPase activity can contribute to photoassimilate generation in source tissue and, in the case of symplastic loading plants like hybrid poplar, that this elevated activity in sink tissue can augment carbon allocation. In all of the 2×35S::UGPase transgenic lines, there was a significant increase in transcript, enzyme activity and consequently total soluble carbohydrate. Additionally, many of the transgenic lines showed increased levels of starch accumulation pointing to accumulation of storage carbohydrates resulting from the pooling of available soluble sugars. It appears that the expression of the exogenous AxUGPase in the leaf tissue is resulting in biosynthesis of carbohydrates in source tissue and is likely working co-ordinately with SPS in the synthesis of sucrose, as has been proposed by Kleczkowski (1994). Interestingly, the introduction of the exogenous UDP-glucose pyrophosphorylase from *Acetobacter* appears to have manifested an increase in transcript abundance of both endogenous poplar UGPase genes, and therefore it is difficult to ascertain if the observed effects are independent of the native gene/protein.

Despite not having measured metabolic flux, it appears that the increased biosynthesis of sucrose in the source tissue has resulted in an increase in the transport of photoassimilate to the sink tissue, as in all transgenic lines an accumulation of both storage and structural carbohydrates was apparent. Although changes in hemicellulose-derived carbohydrates were observed, the most dramatic increase in cell wall carbohydrate chemistry was observed in glucose concentrations, which is derived from cellulose. In these lines, increases in cellulose content ranged from ~2.8 to 6.5% (Figure 4.4). These findings are consistent with the theory that an increase in cellulose content would coincide with an increased supply of precursor to the cellulose synthase

complex (CSC) from elevated levels of soluble metabolites synthesized and likely transported to the sink (wall developing tissue). Furthermore, this is supported by the 4-fold increase in the secondary wall specific CSC gene expression levels in the transgenic lines. Confirmation of this theory and our results are also consistent with the microarray findings of Hertzberg *et al.* (2001) who showed that UGPase is upregulated during the period of late expansion and secondary cell wall formation. Similar changes in structural carbohydrates were not observed in tobacco plants transformed with the same constructs (Coleman *et al.* 2006), which can be attributed to a lack of any measurable change in stem sucrose content compared to the stem of the poplar.

In addition to the observed increases in carbohydrate composition in the 2×35S::UGPase transgenic trees, there was also an associated decrease in lignin content. This is not thought to be the result of a decrease in the rate of lignin deposition, but rather the result of a change in the ratio of cellulose to lignin. This was confirmed using real time PCR analysis of the lignin-branch of the phenylpropanoid biosynthetic genes toolbox, which showed that there was no decrease in the transcripts of any of the key lignin biosynthetic gene isoforms surveyed, rather in many of the genes there was an increase (Figure 4.3, Table B2).

Correlated to the change in the relative amount of lignin, was a change in lignin monomer composition in favour of syringyl units. These biochemical findings were supported by real time PCR evaluations, which demonstrated significant augmentation of ferulate 5-hydroxylase (F5H) consistent with the change in monomer composition of the cell wall lignin. These findings suggest that the elevated transcript abundance of lignin biosynthetic genes may be related to changes in sugar concentrations rather than a change in cellulose deposition. Sucrose has been shown to serve as a signal molecule regulating gene expression (Wiese *et al.* 2004), and consequently influences associated metabolic pathways and morphological development (Lunn and McRae 2003; Gibson 2005). A compelling body of evidence indicates that carbohydrates, particularly the hexose glucose, are essential sources of carbon skeletons and function as important signalling molecules (reviewed in Smeekens 2000; Rolland *et al.* 2002; Gibson 2005).

In Arabidopsis, a relationship between carbon availability and lignin accumulation has been established (Rogers *et al.* 2005), clearly demonstrating that metabolizable

carbohydrates positively influence the abundance of lignin. Further, concurrent transcriptome analysis lends support to the hypothesis that carbohydrates are not merely a source of carbon skeletons for lignification, but also function as a signal to enhance the capacity to synthesize these key cell wall macromolecules. Additionally, diurnal fluxes in lignin biosynthetic capacity were suggested to be modulated at the transcriptional level by at least three different stimuli: light, the circadian clock, and available hexose carbohydrates. The absolute abundance of these transcripts is shaped by the amount of available carbohydrates. The link between sugar signalling and lignification is particularly interesting, as carbohydrate-mediated changes in vegetative development have been well documented in dark-grown seedlings (Roldan *et al.* 1999; Baier *et al.* 2004).

Metabolic profiling was employed to investigate changes in "global" cell wall metabolism as a result of this single gene misregulation. In addition to the altered levels of soluble carbohydrates identified by HPLC, the 2×35S::UGPase transgenic poplar trees differentially accumulated several metabolites, many of which have been identified as products of sugar metabolism, such as galactose, maltose, and sorbose, or as metabolites commonly associated with abiotic or biotic stress (Table 4.5). Aside from being an integral component of cell wall hemicellulose, the monosaccharide galactose is also known to be a major carbohydrate participating in the formation of the raffinose family of oligosaccharides known to be elevated in response to stress, particularly in *Populus*. Maltose on the other hand is the immediate by-product of starch degradation, while sorbose is a precursor ketose monosaccharide involved in the biosynthesis of ascorbic acid, which has been shown to influence leaf development, growth and size (Chen and Gallie 2006), and may be responsible directly or indirectly for the alteration of leaf morphology in the current study. In addition, other stress-related compounds were identified including myo-inositol, galactinol, and pinitol. Myo-inositol and galactinol are the immediate precursors to the formation of raffinose family of oligosaccharides, while pinitol is a methylated cyclitol, derived from myo-inositol, thought to be an important osmolyte in plants responding to drought stress.

Most interesting was the extremely high quantities of a single compound, the glycoside of salicylic acid (salicylic acid 2-O-β-D-glucoside [SAG]), which was found at significantly elevated levels (up to a 230-fold increase compared to the wild-type trees)

in the developing xylem of all 2×35S::UGPase transgenics trees (Table 4.6 and Table B3). Salicylic acid 2-O-β-D-glucoside was also identified in the leaf tissue of all transgenic lines, where it was absent in the wild-type trees. The formation SAG is has been shown in tobacco to be catalyzed by a UDP-glucose:SA glucosyltransferase, which employs UDP-glucose as the sole glucose donor (Lee and Raskin 1999). Furthermore, it has been shown to be induced by the presence of salicylic acid, which has been shown to accumulate in tobacco leaves following TMV infections, resulting in an accumulation of SAG as a major product and glucosyl salicylate (GS) as a minor, less stable metabolite (Lee and Raskin 1998). Our results suggest that the accelerated generation of UDP-glucose, manifested by the overexpression of 2×35S::UGPase in hybrid poplar, resulted in the substantial accumulation of SAG. It is tempting to speculate that the allocation of UDP-glucose to salicylic acid may be a direct response of the inability of the cellulose synthase complex to effectively utilize the intracellular UDP-glucose channelled to the formation of cellulose, which was increased by ~6%. However, the accumulation of SAG, among other compounds, has also recently been observed in transgenic aspen overexpressing sucrose phosphate synthase (Hjältén *et al.* 2006), which should have provided another sink for UDP-glucose. Salicylic acid has been shown to act as a signalling molecule in local defence reactions and also in the induction of systemic resistance (Durner *et al.* 1997), and as such the increases in salicylic acid, and hence SAG and/or GS, may be the catalyst for the increases in other plant defence metabolites and may improve herbivore-plant interactions, as shown by Hjältén *et al.* (2006).

In summary, the overexpression of UGPase in hybrid poplar resulted in significant increases in soluble sugars, more-so in the leaves than the developing xylem. These increases in sugar appear to provide increased substrate to both cellulose and starch synthesis, resulting in changes in the chemical composition of the stem, with as much as a 6.6% increase in cell wall cellulose content being observed. Contrary to the results observed in tobacco, in hybrid poplar results suggest that the overexpression of UGPase under the control of a ubiquitous promoter can alter carbon partitioning to starch and cellulose. However, the alterations in sucrose metabolism, particularly in the symplastically loading plant *Populus*, appears to cause other down-stream repercussions within the plant, particularly with respect to carbohydrate

signalling and sensing, which can augment cell wall biosynthesis, as is evident in the altered lignin biosynthesis. In addition to changes in wood chemistry, the trees also produced more defence-related metabolites, which may explain the decreased energy directed to growth.

Supplemental Data

The data reported in this manuscript are supported by supplemental data, which is available in appendix B. Included in the supplemental data is transcript abundance of the native poplar UDP-glucose pyrophosphorylase genes in leaf and developing xylem tissue (Table B1), as well as transcript abundance of the cell wall biosynthetic genes involved in lignin and cellulose deposition for transgenic and wild-type trees (Table B2). Additionally, quantification of the fold change in salicylic acid 2-O-β-glucoside in the developing xylem of all transgenic 2×35S::UGPase hybrid poplar relative to levels in wild-type trees is available (Table B3).

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Overexpression of Sucrose Synthase and UDP-Glucose Pyrophosphorylase in Hybrid Poplar Affects Cellulose Partitioning and Ultrastructure[4]

Introduction

Sucrose is the primary translocatable carbohydrate in the majority of plants. As such its metabolism is vital to the regulation of photoassimilate in sink tissues (Asano *et al.*, 2002). Sucrose hydrolysis can be catalyzed by two enzymes: invertase, which cleaves sucrose into glucose and fructose, and sucrose synthase (SuSy), which catalyzes the formation of UDP-glucose and fructose from sucrose. Both SuSy and invertase have been shown to be tightly associated with the processes of phloem unloading, and SuSy has been repeatedly identified as playing a central role in modulating sink strength (Sun *et al.*, 1992; Zrenner *et al.*, 1995; Dejardin *et al.*, 1999). Furthermore, SuSy can be found in very high levels in companion cells (Nolte and Koch, 1993). SuSy has also been linked with the synthesis of both storage and structural carbohydrates, acting as the catalyst in the metabolism of sucrose which results in the liberation of the precursor for the generation of callose (Subbaiah and Sachs, 2001), cellulose (Amor, 1995), and mixed linkage β(1-3),(1-4)-glucans (Buckeridge *et al.*, 1999). Finally, SuSy expression has also been correlated with the development and deposition of secondary xylem in trees from the vascular cambium (Hauch and Magel, 1998; Hertzberg *et al.*, 2001).

SuSy has dual functionality, in that it provides the immediate precursor for cellulose biosynthesis and concomitantly recycles UDP which has been identified as an inhibitor of cellulose biosynthesis (Benziman *et al.*, 1983). It has been suggested that SuSy exists in two forms, a soluble (S-SuSy) and particulate (P-SuSy) form, with the

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latter being membrane bound and directly supplying UDP-glucose to the cellulose synthase complex for cellulose biosynthesis (Amor *et al.*, 1995). As such, the high energy bond is retained for use in the synthesis of polysaccharides. In maize, phosphorylation of SuSy has been shown to alter the enzyme from a membrane bound to a soluble form (Winter *et al.*, 1997, Komina *et al.*, 2002), but this phosphorylation is reversible (Winter and Huber, 2000).

A mutant form (replacement of Ser-11 by Glu-11) of the mung bean SuSy (S11E), which has a higher catalytic efficiency with respect to sucrose, was overexpressed in transgenic poplar (*Populus alba*) and investigated using a dual labelling system (Konishi *et al.*, 2004). Using this system, it was shown that only a fraction of the sucrose loaded into the phloem is directly used by the CesA complex associated with SuSy, thus conserving the high energy bond for use in polysaccharide synthesis. Altering the expression of SuSy has also been shown to cause changes in structural and storage carbohydrates in other species. For example, maize SuSy mutants displayed increased sucrose levels (Chourey & Nelson, 1976), while increased glucose and fructose levels were apparent in tobacco with SuSy overexpression (Coleman *et al.*, 2006). In wheat, natural variations in SuSy levels demonstrated a clear association between SuSy activity and increased cell wall polysaccharide levels (Xue *et al.*, 2007). Similar results have been observed in wheat roots, with high SuSy activity (caused by hypoxia) being associated with increased cellulose content (Albrecht & Mustroph, 2003). Studies investigating gene expression patterns in poplar have also identified SuSy as being associated with cellulose synthesis and with tension wood formation where increased cellulose deposition occurs (Hertzberg *et al.*, 2001; Andersson-Gunnerås *et al.*, 2006). As such, increasing the expression of SuSy appears to be a key target to accelerate or improve the production of cellulose within forest trees.

In addition to SuSy, UDP-glucose pyrophosphorylase (UGPase) acts as a source for the synthesis of UDP-glucose, the precursor to cellulose biosynthesis. UGPase phosphorylates glucose 1-phosphate producing UDP-glucose, while SuSy cleaves sucrose supplied to the cell. UGPase can be involved in the recycling of fructose produced by SuSy, which can act as an inhibitor of SuSy activity. In source tissues, UGPase works in conjunction with sucrose phosphate synthase in the synthesis of

sucrose (Kleczkowski, 1994), while in sink tissues, UGPase works with SuSy in the cycling of sugars between sucrose and the hexose phosphate pools (Borokov *et al.*, 1996). Overexpression of UGPase in poplar under the control of the 2×35S promoter resulted in significant increases in cellulose, but decreased biomass production (Coleman *et al.*, 2007) confirming the potential for altering cellulose synthesis by manipulating UDP-glucose pools.

This paper investigates the effect of misregulating SuSy and UGPase in hybrid poplar. SuSy was upregulated individually and in combination with UGPase, and the ensuing trees assessed for changes in transcript abundance, enzyme activity, biomass production, storage and structural polysaccharides, as well as cell wall crystallinity and microfibril angle. The results clearly show that SuSy can indeed modulate the production of cellulose, and its ultrastructural characteristics.

Methods

Plasmid Construction

SuSy was cloned from *Gossypium hirsutum* (Perez-Grau, GENBANK U73588) as previously described (Coleman *et al.*, 2006) and inserted into pBIN under the regulation of one of two promoters: the enhanced tandem cauliflower mosaic virus 35S constitutive promoter (2×35S) (Datla *et al.*, 1993; Kay *et al.*, 1987) or the vascular-specific 4CL (*Petroselinum crispum* 4-coumarate:CoA ligase) promoter (Hauffe *et al.*, 1991). UGPase was cloned from *Acetobacter xylinum* ATCC #23768 and inserted into the pBIN cloning vector under the control of the same two promoters. Sequence analysis was used to confirm insertion of the promoter and gene into the binary vector (Coleman *et al.*, 2006).

Plant Transformation and Maintenance

Hybrid poplar (*Populus alba* × *grandidentata*) was transformed using *Agrobacterium tumefaciens* EHA105 (Hood *et al.*, 1993). In short, wild-type and UGPase transgenic poplar (Coleman *et al.*, 2007) leaf disks were cut and co-cultured with EHA105 harbouring the 2×35S::SuSy or 4CL::SuSy transgenes. The explants

were plated on Woody Plant Media (WPM) (McCown and Lloyd 1981) supplemented with 0.1 μM each of α-naphthalene acetic acid (NAA), 6-benzylaminopurine (BA), and thiadiazuron (TDZ) and solidified with 3% (w/v) agar and 1.1% (w/v) phytagel (WPM 0.1/0.1/0.1). After three days the disks were transferred to WPM 0.1/0.1/0.1 supplemented with carbenicillin disodium (500 mg L^{-1}) and cefotaxime sodium salt (250 mg L^{-1}). Following three additional days, the disks were transferred to WPM 0.1/0.1/0.1 containing carbenicillin, cefotaxime and kanamycin (25 mg L^{-1}). After five weeks, shoots and callus material were transferred to WPM containing agar and phytagel, supplemented with 0.01 μM BA, carbenicillin, cefotaxime and kanamycin. Once individual shoots were visible, plantlets were transferred to solidified WPM with 0.01 μM NAA and carbenicillin, cefotaxime and kanamycin to induce rooting. After two consecutive five-week periods on this media, shoot tips were isolated to solidified antibiotic-free WPM with 0.01 μM NAA.

Plants were confirmed as transgenic using PCR screening of genomic DNA employing gene specific oligonucleotides: specifically, UGP-F (5′-atcgaggaattctgcctcgt-3′) and UGP-R (5′-tcgcaagaccggcaacaggatt-3′) were used for UGPase identification, and SUS-1 (5′-ctcaacatcacccctcgaat-3′) and SUS-2 (5′-accaggggaaacaatgttga-3′) were employed for SuSy confirmation.

All shoot cultures, including transgenic and non-transformed wild-type lines, were maintained on solid WPM with 0.01 μM NAA in GA-7 vessels at 22°C under a 16-hour photoperiod with an average photon flux of 50 μmol m^{-2} s^{-1} until out-planting to the greenhouse.

Growth Conditions and Biomass Measurements

Wild-type control trees and each transgenic line, represented by a minimum of 12 individual trees, were transferred into 7.5 L pots containing 50% peat, 25% fine bark, and 25% pumice soil mixture in the greenhouse, and allowed to acclimate under 16 oz clear plastic cups for one week. The trees were grown under 16 hour days supplemented with overhead lighting with a radiant flux density of 300 W m^{-2}. Following four months growth in the greenhouse, the trees were harvested and total height and stem diameter (10 cm above root collar) determined for each tree.

Tissue developmental stage was standardized using a plastichron index, where PI=0 was defined as the first leaf greater than 5 cm in length, and PI=1 is the leaf immediately below PI=0. Stem segments spanning PI=5 to PI=15 were retained for wood cell wall and chemical analysis. Leaves from PI=3 to PI=5 were frozen in liquid nitrogen and retained for RNA, enzyme and soluble carbohydrate analysis. Developing xylem was scraped and flash frozen in liquid nitrogen for analysis of enzyme activity, RNA transcript abundance, and soluble carbohydrate content.

Transcription Levels

Real time PCR was employed to determine transcript abundance of each transgene. Leaf and developing xylem samples (1 g f.w.) were ground in liquid nitrogen, and RNA extracted according to the method of Kolosova *et al.* (2004). Ten µg of RNA was then treated with TURBO DNase™ (Ambion, Austin, TX) to remove residual DNA. One µg of DNase-treated RNA was used for the synthesis of cDNA using Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) and dT_{16} primers according to the manufacturer's instructions. Samples were run in triplicate with Brilliant SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA) on an Mx3000P Real-Time PCR System (Stratagene) to determine critical thresholds (Ct). The primers employed for RT-PCR analysis of UGPase were AU-RTF (5′-tggaagcaacccgcgtcatc-3′) and AU-RTR (5′-gccaaggcccagcggttcc-3′), while the primers for SuSy were GS-RTF (5′-ccgtgagcgtttggatgagac-3′) and GS-RTR (5′-ggccaaaatctcgttcctgtg-3′). As a house-keeping control, the transcript abundance of transcript initiation factor 5A (TIF5A) was employed for normalization (Ralph *et al.*, 2006). The primers for TIF5A transcript quantification were TIF5A-RTF (5′-gacggtattttagctatggaattg -3′) and TIF5A-RTR (5′-ctgataacacaagttccctgc -3′). Conditions for the RT-PCR reactions were as follows: 95°C for 10 minutes, followed by 40 cycles of 95°C for 30 seconds, 62°C (64°C for SuSy, 55°C for TIF5A) for 1 minute, and 72°C for 30 seconds. Relative expression was determined according to Levy *et al.* (2004) using the following equation: $\Delta ct = 2^{-(ctUGPase\ or\ SuSy - ctTIF5A)}$.

Enzyme Activity

Leaf and developing xylem samples (1 g f.w.) were ground in liquid nitrogen with 1 mg of insoluble PVPP and four volumes of extraction buffer (50 mM HEPES-KOH pH 7.5, 10 mM $MgCl_s$, 1 mM EDTA, 2 mM DTT, 1 mM PMSF, 5 mM εAmino-n-caproic acid, 0.1% v/v Triton X-100, 10% v/v glycerol). The samples were centrifuged at 15,000×g for 20 minutes at 4°C. The extract was passed through a desalting column (DG 10 – BioRad) pre-equilibrated with ice-cold extraction buffer without Triton X-100 and PVPP. Extracts were collected in pre-chilled vials and used immediately. UGPase activity was determined spectrophotometrically at 340 nm as per Appeldoorn *et al.* (1997) using 100 µL of plant extract and a NADH molar extinction coefficient of 6.22 mM cm^{-1}. SuSy activity was assayed in the direction of sucrose breakdown (Chourey, 1981), using 50 µL of plant extract. The resultant fructose content was determined using a tetrazolium blue assay (Kennedy and White, 1983). This SuSy assay employs the appropriate controls without the supplementation of UDP to quantify inherent invertase activity, and therefore represents only the breakdown of sucrose by SuSy. Total protein content of the extracts was determined using a Bio-Rad Protein Assay (Bio-Rad, Hercules, CA).

Soluble Carbohydrate and Starch Analysis

Soluble carbohydrates (glucose, fructose and sucrose) were extracted from ground freeze-dried tissue overnight at -20°C using methanol:chloroform:water (12:5:3) as previously described (Coleman *et al.*, 2006). The sample was centrifuged, the supernatant removed, and the remaining pellet washed twice with fresh methanol:chloroform:water (12:5:3). All fractions were then pooled. Five mL of water was added to the combined supernatant and centrifuged to facilitate phase separation. The aqueous fraction was rotary evaporated to dryness and resuspended in 3 mL of distilled water. Soluble carbohydrates were then analyzed using anion exchange HPLC (Dionex, Sunnyvale, CA) on a DX-600 equipped with a Carbopac PA1 column and an electrochemical detector.

The residual pellet was then hydrolyzed in 4% sulfuric acid at 121°C for 4 minutes. The liberation of glucose, representing starch content, was directly quantified by HPLC under similar conditions.

Cell Wall Chemistry

Oven dried stem material was ground using a Wiley mill to pass through a 40-mesh screen and soxhlet extracted with acetone for 24 hours. Lignin and carbohydrate contents were determined using a modified Klason method (Huntley *et al.,* 2003) and 0.2 g of extract free tissue. Carbohydrate content was determined using HPLC (Dionex DX-600, Dionex, CA) equipped with an anion exchange PA1 column, a pulsed amperometric detector with a gold electrode and post-column detection. Acid insoluble lignin was determined gravimetrically, while acid insoluble lignin was determined using spectrophotometric analysis at 205 nm according to TAPPI Useful Method UM-250.

Determination of α-Cellulose and Holocellulose Content

Extract-free ground wood was used to determine holocellulose and α-cellulose contents according to the method of Yokoyama *et al.* (2002). In short, 100 mg of ground tissue was weighed into a 20 mL flask and placed in a 90°C heat block. The reaction was initiated by the addition of 0.5 mL of sodium chlorite solution (200 mg 80% sodium chlorite, 2 mL distilled water, 0.4 mL acetic acid). Additional 0.5 mL aliquots of solution were added every 30 minutes to a total volume of 2 mL. After 2 hours, the samples were removed to a cold water bath and filtered through a coarse crucible. Following overnight drying at 105°C, the holocellulose content was determined gravimetrically. 50 mg of dried holocellulose was then weighed into a reaction flask and allowed to equilibrate for 30 minutes. Four mL of 17.5% sodium hydroxide was added and permitted to react for 30 minutes, after which 4 mL of distilled water was added and the sample macerated for 1 minute. Following 29 minutes of reaction, the sample was filtered through a coarse crucible and placed in 1.0 M acetic acid for 5 minutes before washing with distilled water. α-cellulose content was determined gravimetrically following overnight drying at 105°C.

Crystallinity and Microfibril Angle

Microfibril angle and cell wall crystallinity were determined by X-ray diffraction using a Bruker D8 Discover X-ray diffraction unit equipped with an area array detector (GADDS) on the radial face of the wood section precision cut to 1.69 mm from the growing stem. Wide-angle diffraction was used in the transmission mode, and the measurements were performed with CuKα1 radiation (λ = 1.54 Å), the X-ray source fit with a 0.5 mm collimator and the scattered photon collected by the GADDS detector. Both the X-ray source and detector were set to theta = 0° for microfibril angle determination, while 2 theta (source) was set to 17° for wood crystallinity determination. The average T-value of the two 002 diffraction arc peaks was used for microfibril angle calculations, as per the method of Megraw *et al.* (1998), while crystallinity determined by mathematically fitting the data using the method of Vonk (1973). Two radii were taken from samples isolated 5 cm above ground on each tree, and these values were averaged for each tree.

Microscopy

Cross sections of stems (40 µm) from 5 cm above the ground were cut using a microtome. Sections were mounted on glass slides and visualized using a Leica microscope under UV fluorescence.

Histochemical examination of cellulose in the poplar stems was carried out using calcofluor staining. The samples were mounted in 10% KOH (w/v) and 0.1% calcofluor white (w/v) and then viewed after 5 minutes under bright-field illumination with a Leica DMR microscope equipped with a QICAM CCD camera (Q-imaging).

Histochemical examination of lignin was analysed using phloroglucinol staining achieved by mounting stem sections in a saturated solution of phloroglucinol in 20% HCl. Samples were viewed under dark-field illumination on the Leica DMR microscope.

Results

Transformed hybrid poplar trees regenerated from leaf tissue were propagated from single shoots from individual explants, and as such each line represents an individual transformation event. From the transformed lines produced, three 2×35S::SuSy lines and four 4CL::SuSy lines were selected for greenhouse growth trials and in-depth cell wall characterization, based on enzyme activity. Together with corresponding wild-type controls, 12 individual trees per line were transferred to the greenhouse. At harvest, two of three 2×35S::SuSy lines (lines 1 and 2) and one of four 4CL::SuSy (line 2) lines were slightly shorter than the corresponding wild-type poplar (Figure 5.1), while the other lines exhibited similar height growth. Only one line, 2×35S::SuSy-1, had a decreased calliper and leaf dry weight, while one 4CL::SuSy -1 had an increased calliper (Figure 5.1). All other lines displayed similar phenotypes to the wild-type trees. There was no change in stem weight relative to controls in any of the transformed lines.

Existing transgenic hybrid poplar trees expressing 2×35S::UGPase and 4CL::UGPase (Coleman *et al.*, 2007) were concurrently transformed with SuSy under control of the corresponding promoter (2×35S::UGPase×SuSy and 4CL::UGPase×SuSy). Trees regenerated from leaf explants were again propagated from single shoots from individual explants, and each line represents an individual SuSy transformation event. Despite several attempts, no 2×35S double transformed lines were recovered, which is likely due to the severe phenotype observed in 2×35S::UGPase overexpressing transgenic lines (Coleman *et al.*, 2007). However, four of 12 successfully transformed 4CL::UGPase×SuSy lines were selected, based on enzyme activity, and propagated for in-depth analysis. The double transformants were grown simultaneously with single gene transformed lines and controls trees in the greenhouse for four months. At harvest, two 4CL::UGPase×SuSy lines (lines 3 and 4) were significantly shorter than the corresponding wild-type poplar, and 4CL::UGPase×SuSy-3 also had decreased calliper. There were no changes in total dry weight (biomass) of leaf or stem (Figure 5.1).

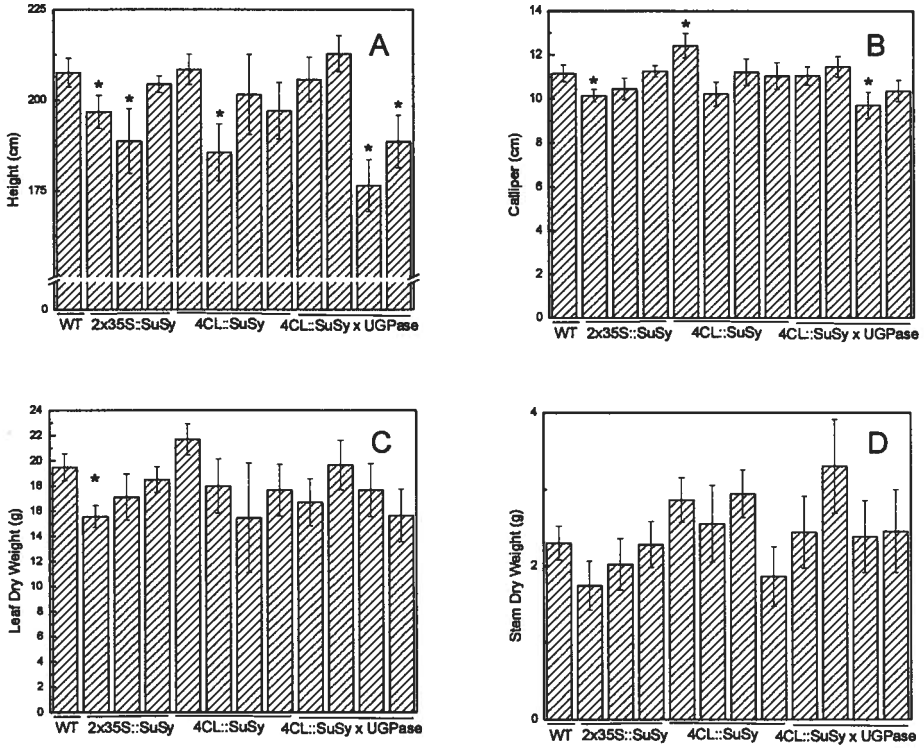

Figure 5.1. Biomass measurements of SuSy and SuSy × UGPase transgenic and wild-type poplar. Tree height (A), calliper (B), leaf dry weight (C) and stem dry weight (D). Mean (±SE) were calculated from 10 plants per line. * Indicates a significant difference between the transgenic and wild-type trees at α=0.10.

Transcript Level and Enzyme Activity

Quantitative real time PCR was used to measure transcript abundance of the SuSy and UGPase transgenes relative to actin (Table 5.1). All lines clearly showed the existence of the exogenous gene and its regulation. In general, transcript abundance was higher in leaf tissue than in the developing xylem of single gene transgenic lines. However, in the double transgenic lines, transcript abundance of SuSy was higher in leaf tissue than in the developing xylem, while transcript abundance of UGPase was higher in the developing xylem than in the leaf tissue. Correspondingly, SuSy activity, as determined by the breakdown of sucrose into UDP-glucose and fructose, was increased in developing xylem of all of the transgenic lines (Table 5.1). In some lines, as much as a 2.5-fold increase was apparent. Furthermore, two 2×35S::SuSy lines (lines 1 and 2) and one 4CL::SuSy line (line 1) also had increased SuSy activity in the leaf tissue.

All of the double transgenic lines showed increased SuSy activity in developing xylem, but not in the leaf tissue (Table 5.1). Enzyme activity ranged from 13.26 to 17.42 μg fructose mg^{-1} protein min^{-1} compared to 4.99 μg fructose mg^{-1} protein min^{-1} in the control trees, representing a 2.5- to 3.5-fold increase in transgene enzyme activity. UGPase activity was determined using an indirect assay measuring the production of the reduced form of nicotinamideadenine dinucleotide (NADH). All lines had significantly increased UGPase activity in the developing xylem; however, the changes were not as substantial as those observed in the SuSy activity levels (Table 5.1). Activity ranged from 0.52 to 0.65 μmol NADH min^{-1} mg $protein^{-1}$ compared to 0.42 μmol NADH min^{-1} mg $protein^{-1}$ in the control trees, representing a 1.25- to 1.5-fold increase in activity.

Soluble Carbohydrates and Starch Content

Changes in soluble sugar content were variable (Table 5.2). While there appeared to be an increasing trend in total soluble sugars (glucose, fructose and sucrose) in the transgenic lines, few of the changes were significant. In leaf tissue, 4CL::SuSy-3 and 4CL::SuSy × UGPase-2 had increased total soluble sugars. In the

Table 5.1. Mean transcript abundance and enzyme activity in leaf and developing xylem tissue for SuSy and SuSy × UGPase transgenic and wild-type poplar trees. Mean (±SE) were calculated from 3 plants per line. Bold denotes significant difference from control values at α=0.10.

	SuSy Transcript Level ΔCt		SuSy Enzyme Activity μg fructose min^{-1} mg^{-1}	
Transgenic line	Leaf	Developing Xylem	Leaf	Developing Xylem
Control	n.d.	n.d.	506.71 ± 45.66	4.99 ± 0.54
2×35S::SuSy 1	**2.50 ± 0.29**	**0.18 ± 0.05**	**1126.43 ± 178.08**	**8.18 ± 0.50**
2×35S ::SuSy 2	**2.34 ± 0.71**	**0.49 ± 0.23**	**845.45 ± 59.84**	**9.22 ± 1.17**
2×35S ::SuSy 3	1.32 ± 0.06	**0.14 ± 0.13**	455.90 ± 63.48	**11.95 ± 2.29**
4CL::SuSy 1	**0.59 ± 0.05**	**0.82 ± 0.53**	**788.67 ± 101.54**	**9.10 ± 0.34**
4CL::SuSy 2	**1.17 ± 0.57**	**0.12 ± 0.01**	541.56 ± 24.32	**8.06 ± 0.84**
4CL::SuSy 3	**0.99 ± 0.19**	**0.34 ± 0.15**	680.56 ± 74.44	**12.77 ± 1.34**
4CL::SuSy 4	**0.00 ± 0.00**	**0.02 ± 0.01**	581.24 ± 105.83	**7.61 ± 0.87**
4CL::SuSy x UGPase 1	**1.10 ± 0.20**	**0.27 ± 0.09**	679.99 ± 215.98	**16.70 ± 2.91**
4CL::SuSy x UGPase 2	**0.37 ± 0.10**	**0.20 ± 0.08**	1424.04 ± 121.50	**13.26 ± 2.79**
4CL::SuSy x UGPase 3	**0.30 ± 0.10**	**0.24 ± 0.08**	609.55 ± 202.16	**17.42 ± 0.39**
4CL::SuSy x UGPase 4	**1.14 ± 0.63**	**0.28 ± 0.09**	514.18 ± 90.41	**13.38 ± 2.29**
	UGPase Transcript Level ΔCt		UGPase Enzyme Activity μmolNADH min^{-1} mg^{-1} total protein	
Transgenic line	Leaf	Developing Xylem	Leaf	Developing Xylem
Control	n.d.	n.d.	0.15 ± 0.04	0.42 ± 0.03
4CL::SuSy x UGPase 1	**0.13 ± 0.01**	**0.26 ± 0.15**	0.12 ± 0.01	**0.65 ± 0.03**
4CL::SuSy x UGPase 2	**0.09 ± 0.02**	**0.24 ± 0.07**	0.12 ± 0.03	**0.58 ± 0.07**
4CL::SuSy x UGPase 3	**0.42 ± 0.04**	**3.46 ± 0.41**	0.13 ± 0.03	**0.52 ± 0.04**
4CL::SuSy x UGPase 4	**0.61 ± 0.09**	**2.35 ± 1.54**	0.12 ± 0.07	**0.54 ± 0.02**

Table 5.2. Total soluble carbohydrates and starch in leaf and developing xylem tissue of SuSy and SuSy × UGPase transgenic and wild-type trees. Mean (±SE) were calculated from 3 plants per line. Bold denotes significant difference from control values at α=0.10.

	Leaf mg g^{-1}		Developing Xylem mg g^{-1}	
Transgenic Line	Soluble Carbohydrates	Starch	Soluble Carbohydrates	Starch
Control	159.66 ± 12.68	0.76 ±0.15	54.04 ± 7.36	0.30 ± 0.05
2×35S ::SuSy 1	166.59 ± 11.75	0.83 ± 0.26	61.81 ± 3.36	0.28 ± 0.01
2×35S ::SuSy 2	191.24 ± 16.60	0.88 ± 0.31	105.22 ± 30.85	0.22 ± 0.05
2×35S ::SuSy 3	168.11 ± 16.87	0.68 ± 0.11	**134.27 ± 8.71**	0.26 ± 0.08
4CL::SuSy 1	185.51 ± 13.52	0.61 ± 0.13	**136.73 ± 21.27**	0.24 ± 0.00
4CL::SuSy 2	175.79 ± 14.73	**1.28 ± 0.13**	**91.52 ± 9.43**	0.39 ± 0.07
4CL::SuSy 3	**193.50 ± 8.74**	0.94 ± 0.35	**121.37 ± 9.48**	0.30 ± 0.04
4CL::SuSy 4	169.83 ± 13.48	0.90 ± 0.43	87.91 ± 20.98	0.31 ± 0.06
4CL::SuSy x UGPase 1	185.73 ± 17.24	0.73 ± 0.13	**128.29 ± 7.48**	0.23 ± 0.03
4CL::SuSy x UGPase 2	**203.97 ± 20.78**	1.12 ± 0.55	**93.98 ± 10.99**	0.29 ± 0.02
4CL::SuSy x UGPase 3	181.16 ± 22.26	0.92 ± 0.23	95.69 ± 38.44	0.33 ± 0.04
4CL::SuSy x UGPase 4	146.13 ± 16.62	0.44 ± 0.13	71.09 ± 7.89	0.24 ± 0.05

developing xylem, 2×35S::SuSy -3, 4CL::SuSy -1, 2, and 3 and 4CL::SuSy × UGPase-1 and 2 had significant increases in total soluble sugars.

Starch content was unchanged in the leaves of all but one of the single transgenic plants (Table 5.2). 4CL::SuSy-4 had increased leaf starch content (1.28 mg g^{-1}) relative to the control at 0.76 mg g^{-1}. There were no changes in the starch content of the developing xylem in any of the single transgenic lines. Furthermore, there were no significant changes in the starch content of leaves or developing xylem in any of the double transgenic lines.

Cell Wall Chemistry

Cell wall carbohydrate content was substantially altered in the transgenic lines. All lines showed increased glucose content, with all 2×35S::SuSy lines, 4CL::SuSy-1 and 4 and 4CL::SuSy × UGPase-1, 3, and 4 lines displaying statistically significant increases in glucose content. 2×35S::SuSy-3, 4CL::SuSy-1 and 4CL::SuSy × UGPase-1, 2, and 4 also had increased mannose levels. All lines showed decreases in arabinose content, while in general galactose, rhamnose, and xylose remained relatively unchanged when compared to the wild-type trees (Table 5.3). While there were no changes in overall lignin content, a number of lines showed significant changes in acid soluble lignin content, and all lines showed a trend towards decreased acid soluble lignin. Phloroglucinol staining confirmed the consistent level of lignin in the transgenics relative to wild-type trees.

The observed increases in glucose content, as determined by Klason analysis, were confirmed to be the result of an increase in cellulose content and not glucose-associated with hemicellulose biosynthesis, by α-cellulose quantification (Table 5.4). All lines were confirmed to have increased cellulose content, ranging from 2 to 6% by weight more than wild-type trees. As a mechanism to investigate if the changes in cellulose were related to a change in cellulose production and not to a formation of tension wood, crystallinity and microfibril angle of all transgenic and wild-type stems were determined. Crystallinity was increased in all but two transgenic lines ranging from 1 to 12%, while none of the transgenic lines showed any changes in microfibril

Table 5.3. Chemical composition of stem segments (PI=5 to PI=15) of SuSy and SuSy × UGPase transgenic and wild-type trees. Mean (±SE) were calculated from 3 plants per line. Bold denotes significant difference from control values at α=0.10.

	% Carbohydrates						% Lignin	
	Arabinose	Galactose	Glucose	Xylose	Mannose	Rhamnose	Acid Soluble	Acid Insoluble
Control	0.53 ± 0.03	1.00 ± 0.08	36.56 ± 1.05	15.49 ± 0.22	1.65 ± 0.05	0.50 ± 0.01	3.94 ± 0.26	20.15 ± 0.46
2×35S::SuSy 1	**0.36 ± 0.02**	0.93 ± 0.03	**40.97 ± 1.06**	15.33 ± 0.34	2.34 ± 0.36	0.55 ± 0.02	**2.72 ± 0.02**	20.68 ± 0.55
2×35S::SuSy 2	**0.43 ± 0.03**	0.99 ± 0.02	**40.71 ± 0.87**	15.82 ± 0.74	2.38 ± 0.38	0.51 ± 0.03	3.16 ± 0.32	20.92 ± 0.12
2×35S::SuSy 3	**0.41 ± 0.01**	1.03 ± 0.01	**41.01 ± 1.09**	16.03 ± 0.95	**2.41 ± 0.25**	0.51 ± 0.01	3.56 ± 0.43	22.50 ± 1.78
4CL::SuSy 1	0.47 ± 0.01	1.04 ± 0.05	**39.82 ± 0.86**	16.32 ± 0.89	**2.13 ± 0.12**	0.54 ± 0.02	**3.26 ± 0.16**	19.65 ± 0.33
4CL::SuSy 2	**0.37 ± 0.01**	1.03 ± 0.11	41.27 ± 2.49	15.16 ± 0.03	2.25 ± 0.42	0.49 ± 0.00	3.23 ± 0.30	19.55 ± 0.51
4CL::SuSy 3	0.42 ± 0.05	0.99 ± 0.04	39.32 ± 0.98	15.86 ± 0.22	2.22 ± 0.33	0.47 ± 0.03	3.48 ± 0.10	20.41 ± 1.20
4CL::SuSy 4	**0.40 ± 0.04**	1.03 ± 0.07	**39.75 ± 0.55**	15.78 ± 0.43	1.91 ± 0.12	0.51 ± 0.06	3.36 ± 0.13	20.74 ± 0.47
4CL::SuSy x UGPase 1	**0.36 ± 0.03**	1.18 ± 0.24	**42.33 ± 1.85**	14.82 ± 0.79	**2.25 ± 0.19**	0.54 ± 0.06	**3.14 ± 0.16**	19.57 ± 1.50
4CL::SuSy x UGPase 2	**0.36 ± 0.06**	0.95 ± 0.02	38.45 ± 0.37	15.74 ± 0.26	**2.41 ± 0.26**	0.51 ± 0.04	**3.17 ± 0.17**	20.83 ± 1.04
4CL::SuSy x UGPase 3	0.43 ± 0.08	0.91 ± 0.08	**40.80 ± 0.39**	14.36 ± 0.42	1.75 ± 0.18	0.48 ± 0.03	**3.05 ± 0.25**	20.28 ± 0.63
4CL::SuSy x UGPase 4	**0.39 ± 0.02**	0.96 ± 0.02	**40.37 ± 0.63**	15.95 ± 0.48	**1.98 ± 0.10**	0.51 ± 0.01	**2.68 ± 0.12**	20.69 ± 0.48

Table 5.4. α-cellulose content, cell wall crystallinity and microfibril angle (MFA) of stem segments (PI=5 to PI=15) of SuSy and SuSy × UGPase transgenic and wild-type trees. Mean (±SE) were calculated from 3 plants per line. Bold denotes significant difference from control values at α=0.10.

	α-Cellulose (% DW)	Cell Wall Crystallinity (%)	MFA (°)
Control	31.96 ± 0.07	51.40 ± 0.51	16.70 ± 0.35
2×35S::SuSy 1	**38.29 ± 1.22**	**56.60 ± 1.75**	15.66 ± 0.81
2×35S::SuSy 2	**36.61 ± 0.54**	**54.00 ± 1.00**	16.63 ± 0.87
2×35S::SuSy 3	**35.76 ± 1.20**	**56.40 ± 1.44**	16.63 ± 0.55
4CL::SuSy 1	**37.13 ± 0.04**	**53.40 ± 0.75**	16.43 ± 1.31
4CL::SuSy 2	**37.35 ± 0.87**	**53.25 ± 0.48**	19.08 ± 1.22
4CL::SuSy 3	**35.90 ± 0.47**	52.00 ± 1.00	18.21 ± 0.88
4CL::SuSy 4	**35.08 ± 0.46**	**54.00 ± 1.00**	17.30 ± 1.04
4CL::SuSy x UGPase 1	**37.43 ± 0.76**	**63.25 ± 2.17**	16.38 ± 1.60
4CL::SuSy x UGPase 2	**37.41 ± 0.87**	**56.80 ± 1.69**	17.37 ± 0.53
4CL::SuSy x UGPase 3	**33.84 ± 0.51**	**55.80 ± 1.83**	17.45 ± 0.93
4CL::SuSy x UGPase 4	**36.41 ± 0.81**	**60.00 ± 2.61**	17.52 ± 0.53

angle (Table 5.4). Microscopy and staining of stem cross-sections also demonstrate the lack of a G-layer commonly associated with tension wood formation, as well as an increase in cellulose content, as implied by calcofluor staining, in all transgenic lines (Figure 5.2).

Discussion

This study investigated the effects of overexpressing two exogenous genes, SuSy and UGPase (individually and in combination), on tree growth and cell wall chemistry and ultrastructure in hybrid poplar. Quantification of transcript abundance of the transgenes showed variable expression among lines and tissues. In general, the 2×35S::SuSy transgenic lines had higher expression in leaf tissue when compared to developing xylem, and similarly, higher transcript abundance than the 4CL::SuSy transgenic lines examined. Despite the tissue specificity of the 4CL promoter, there was no discernable difference in transgene expression in the developing xylem when comparing transcript abundance of 4CL::SuSy and 2×35S::SuSy transgenic lines. In the 4CL::UGPase×SuSy double transgenic lines, UGPase transcript level was higher in the developing xylem, while SuSy transcript abundance was higher in the leaf tissue.

The difference in exogenous transcript abundance between leaf and xylem tissue was also reflected in the enzyme activity, where SuSy was much more active in the leaf tissue, when compared to the developing xylem. These observations are consistent with the wild-type trees which inherently have an approximate 100-fold difference in SuSy activity between leaf and developing xylem tissue (Table 5.1). Furthermore, these findings concur with Le Hir *et al.*, (2005) who observed high levels of SuSy in leaf tissue of *Querus robur*, with undetectable levels in the stem. An obvious trend towards increased SuSy enzyme activity was apparent in all transgenic lines, with greater than two-fold increases being observed in the leaf tissue in some lines. Similar increases were observed in developing xylem, with greater than a 3-fold increase in some lines. In contrast, UGPase activity is inherently higher in the developing xylem, where as much as three times the activity per gram total protein is apparent. The observed

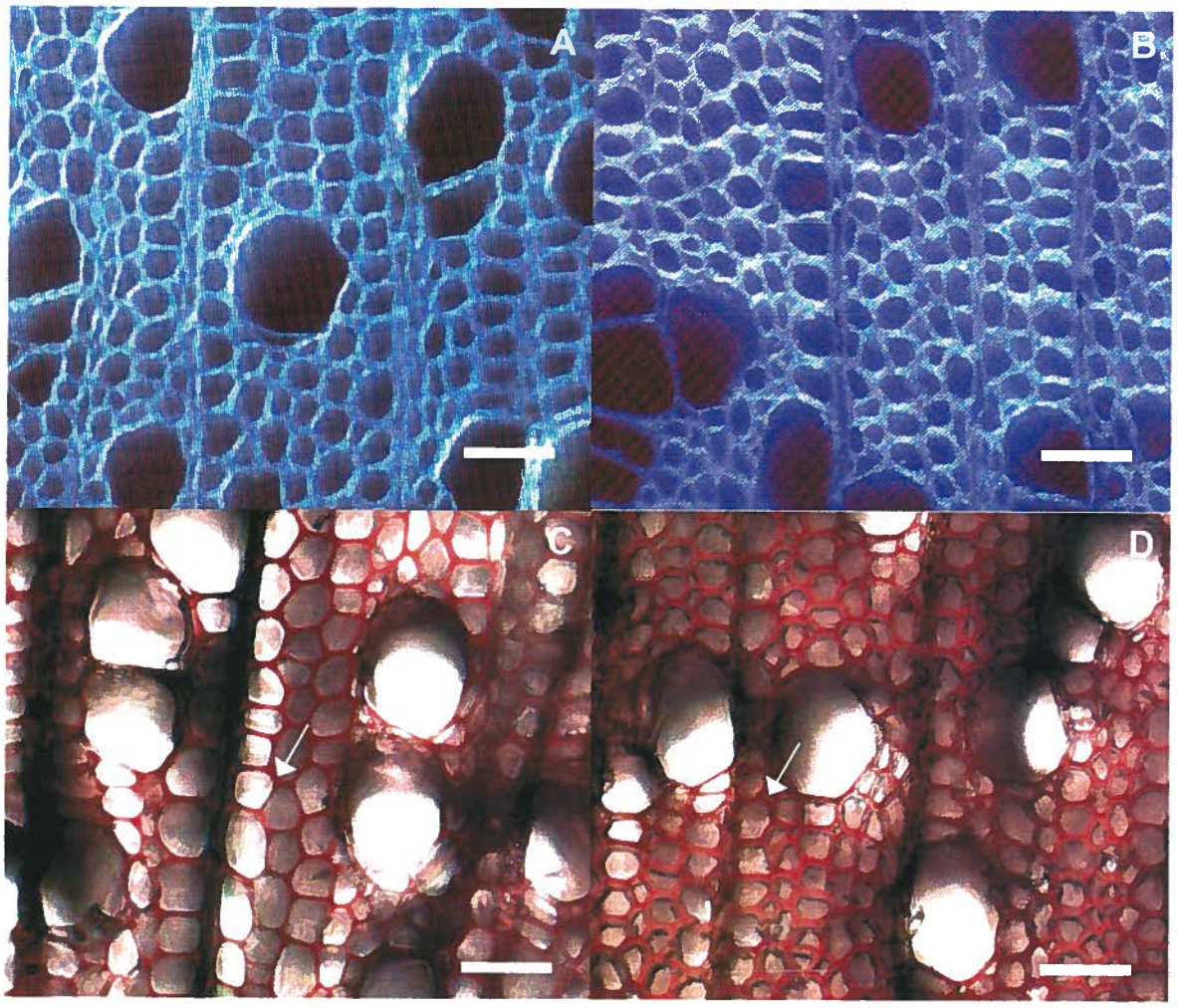

Figure 5.2. Calcofluor (A, B) and phloroglucinol (C, D) staining of wild-type (A, C) and 2×35S::SuSy transformed (B, D) poplar trees (Scale bars: 90 µm). Arrows depict the observed increased cell wall thickening in transgenic lines.

increase in UGPase activity was confined to the developing xylem, which may be reflective of tissue-specific targeting of the 4CL promoter.

An assessment of plant growth and architecture suggested that the overexpression of these targeted transgenes had no pleiotropic effect on the trees, as they appeared phenotypically normal. However, some lines appeared slightly shorter or had minor changes in calliper relative to corresponding wild-type trees. This is contrary to Konishi *et al.*, (2004) who reported an increase in height growth in some lines of *Populus alba* transformed with a modified mung bean SuSy under the control of the 35S promoter. In tobacco, the overexpression of the *G. hirsutum* SuSy under the control of two different promoters resulted in increased height growth (Coleman *et al.*, 2006). Similarly, in other plants the downregulation of SuSy has been shown to influence height growth. For example, using antisense suppression, Tang & Sturm (1999) generated carrot plants that were smaller and had fewer leaves, while D'Aoust *et al.*, (1999) created tomatoes with reduced fruit size and compromised sucrose unloading capacity. As SuSy is generally thought to exert control over sink strength, increased plant growth is consistent with its overexpression, while suppression of SuSy can be associated decreased growth.

Despite the apparent abundance of UGPase activity within plants, the overexpression of foreign *Acetobacter xylinum* UGPase in poplar and tobacco has yielded changes in plant growth and carbon allocation. In tobacco, plants were taller and some lines had increased internode lengths and stem dry weight (Coleman *et al.*, 2006), while in poplar, UGPase overexpression resulted in stunted growth and significant increases in cellulose content. The latter example implies that the generation of altered levels of UDP-glucose can facilitate the re-allocation of carbon skeletons derived from photosynthate towards cellulose deposition, as UDP-glucose is the immediate precursor to cellulose polymer biosynthesis (Coleman *et al.*, 2007). Unlike tobacco, the double transgenics in the current study did not show any additive effect as a result of the expression of UGPase along with SuSy, nor were any changes seen in 4CL::UGPase individual lines (Coleman, unpublished).

Previous studies evaluating SuSy and UGPase transgenic plants have demonstrated significant changes in soluble carbohydrate contents manifested by the misregulation of these genes. For example, a maize endosperm SuSy mutant had a 2-

to 4-fold increase in localized sucrose levels (Chourey & Nelson, 1976). Consistently, the overexpression of SuSy in tobacco resulted in an increase in stem glucose and fructose concentrations (Coleman *et al.*, 2006). This suggested that plants generally attempt to maintain a basal concentration of sucrose in the sink tissue (stem) despite the substantially increased metabolism of sucrose into glucose and fructose, which consequently pool. With increased SuSy activity in the sink tissue, an associated increase in sucrose catabolism exists, and therefore more sucrose can be translocated to the sink tissue. A comparison of wheat variants (*Triticum aestivum)* with differing water soluble carbohydrate concentrations, showed that SuSy transcript abundance (and enzyme activity) was inversely correlated with water soluble carbohydrate accumulation (Xue *et al.*, 2007). The same pattern was apparent with the transcript abundance of three cellulose biosynthesis subunits (TaCesA1, TaCesA4-like and TaCesA10). It was concluded that these gene families along with others, when present in lower abundance play a role in the accumulation of water soluble carbohydrates, which is associated with a decrease in sucrose hydrolysis and decreased polysaccharide accumulation. No associated change in height growth or stem strength was observed with a lower level of cell wall polysaccharides (Xue *et al.*, 2007).

UGPase has also been shown to affect sink strength in plants. Most studies have focussed on the downregulation of UGPase as it is thought to exist in abundance in plants (Appeldoorn *et al.*, 1997; Magel *et al.*, 2001). UGPase antisense reduction in potato tubers has resulted in conflicting results which range from no change in soluble sugars, despite a 96% reduction in activity (Zrenner *et al.*, 1993), to significant reductions in soluble carbohydrates (Borokov *et al.* 1996; Spychalla *et al.* 1994). In Arabidopsis, plants with decreased UGPase activity had lower soluble carbohydrate and starch contents (Kleczkowski *et al.*, 2004). Tobacco overexpressing UGPase on the other hand showed increased glucose and fructose contents, but only small changes in sucrose concentration and no change in cellulose production (Coleman *et al.*, 2006). Poplar overexpressing UGPase under the control of the 2×35S promoter had significant increases in all soluble carbohydrates in leaf tissue. In the developing xylem, glucose and sucrose were increased although less so than in leaf tissue (Coleman *et al.*, 2007). Native UGPase expression studies in poplar clearly show the coordinate upregulation of UGPase with late cell expansion and secondary cell wall

formation (Hertzberg *et al.*, 2001), as well as during the formation of tension wood (Andersson-Gunnerås *et al.*, 2006), which is known to be closely linked to cellulose production.

Although the SuSy and double transgenic trees did not generally show an increase in starch content, 2×35S::UGPase overexpressed poplar demonstrated increased starch accumulation in the developing xylem and leaf tissue of some lines (Coleman, *et al.*, 2007). These data concur with findings in Arabidopsis mutants with increased UGPase activity that were shown to have higher starch content when grown in both light and dark (Ciereszko *et al.*, 2005). However, when UGPase is expressed under the control of the 4CL promoter this effect is not duplicated (Coleman, unpublished), nor is it replicated when UGPase is expressed in combination with SuSy under the control of the 4CL promoter, as double transgenics did not show significant additive effects over single transgenics.

Contrary to the current results, Konishi *et al.* (2004) did not observe altered carbon allocation in poplar, as the xylem cellulose content of trees expressing the modified mung bean SuSy was unchanged. This discrepancy is likely a consequence of the origin of the exogenous gene and resulting differences in expression. Konishi *et al.* (2004) attributed the lack of changes in structural carbohydrates to the higher relative expression of SuSy in leaf tissue as compared to stem tissue. Furthermore, the expression of the mung bean SuSy was relatively higher in the soluble fraction than in the microsomal membrane fraction, and as such influenced the recycling of fructose. Subsequently, in feeder studies with these transgenic lines, labelled (H^3) fructose was shown to be incorporated into both cellulosic and non-cellulosic polymers at a greater rate than in control plants. In the current study, despite the inherently higher levels of SuSy activity in leaves, there did not appear to be an increase in SuSy activity in the leaves of the transgenic overexpressing poplar when compared to the wild-type control trees. In contrast, SuSy activity in the developing xylem was significantly increased, and this undoubtedly accounts for the increased cellulose production. Furthermore, the SuSy transgene employed in the current study has previously been shown to be strongly associated with cellulose formation in cotton and may associate more closely with the activity of the cellulose synthase complex in the cell wall (Amor *et al.*, 1995). This hypothesis is supported by evidence in pea, which has clearly shown that different

isoforms of SuSy are associated with the different metabolic fates of sucrose (Barratt, 2001).

In the hybrid poplar system employed herein, the elevated levels of cellulose deposition in the secondary xylem resulting from the overexpression of cotton SuSy was also mirrored with a significant change in the ultrastructural characteristic of the cellulose, as it had increased crystallinity. The higher crystalline nature of the cell wall was not associated with a commensurate increase in microfibril angle, as would be expected in the formation of tension wood (Joshi 2003). Intuitively, an increase in cellulose deposition would likely come at the expense or be mirrored by a change in ultrastructure, in this case crystallinity. Calcofluor staining also clearly shows that despite increased fluorescence associated with increased cellulose, there is no evidence of the G-layer that would appear in tension wood (Figure 5.2). An evaluation of the gene expression patterns surrounding tension wood formation identified many genes associated with its formation and the inherent higher degree of cellulose production. Both SuSy and UGPase were identified as genes that are significantly upregulated during tension wood formation (Andersson-Gunnerås *et al.*, 2006). SuSy was identified as one of the most highly expressed genes in tension wood, with ratios of 1.57 (PttSuS1) and 1.39 (PttSuS2) relative to their expression in normal wood. In an independent study, UDP-glucose pyrophosphorylase (PHUGP2) was shown to be present at a ratio of 2.31 relative to normal wood (Andersson-Gunnerås *et al.*, 2006).

The inconsistencies in biomass (*i.e.* height growth) results among investigations evaluating SuSy expression may be directly related to the observed differences in cell wall constituents (cellulose content). Increasing cellulose deposition, augmented by the misregulation of genes influencing key pathway steps, could manifest in altered tree growth as more energy and carbon skeletons are being directed or committed to cell wall deposition, as opposed to cell initiation or elongation. To this effect, trees with increased cellulose content have shown varying results. For example, we have previously shown large increases in cellulose content (2.8 to 6.5%) in UGPase transformed poplar, which were at the expense of biomass accumulation (Coleman *et al.*, 2007). These plants also had decreased lignin content. Furthermore, these trees were severely stunted, which was attributed in part to synthesis and accumulation of a salicylic acid glucoside, which has been shown in tobacco to be synthesized by a

salicylic acid glucosyltransferase that employs UDP-glucose as the sole source of glucose (Lee & Raskin, 1998). The slight reduction in height growth observed in the current transgenic SuSy trees is not as dramatic as that observed with the UGPase transgenics, and is perhaps due to the closer connection between SuSy (over UGPase) and the cellulose synthase complex proteins (CesA subunits) facilitating a direct metabolic channel for UDP-glucose to cellulose biosynthesis. Other studies, however, have shown marginal increases in cellulose content associated with an increase in height (Park *et al.*, 2004; Shani *et al.*, 2004; Hu *et al.*, 1999)

Similarly, in other plant species, the level of SuSy expression has been shown to be strongly associated with cellulose synthesis. Expression of the modified mung bean SuSy (S11E) in *Acetobacter xylinum* caused enhanced cellulose production by preventing the accumulation of UDP, which is known to inhibit cellulose formation in *A. xylinum* (Nakai *et al.*, 1999; Benziman *et al.*, 1983). In addition, carrot plants with suppressed SuSy showed decreased cellulose content (Tang & Sturm, 1999), while suppression of SuSy in cotton resulted in an almost fibreless phenotype (Ruan *et al.*, 2003). On the other hand, tobacco plants overexpressing UGPase showed no significant changes in starch or cellulose (Coleman *et al.*, 2006), despite an increase in soluble sugars.

Given that both SuSy and UGPase independently increased cellulose accumulation in poplar, an attempt to create double transgenics harbouring both genes, by pyramiding genes under the regulation of the vascular specific promoter, was attempted. To this end, gene stacking did not offer an advantage in diverting carbon skeletons from photosynthate to cellulose deposition, as additive effects were not apparent. The lack of additive influence is not consistent with our previous findings in tobacco which showed clear effects in height growth, but not carbon re-allocation to cellulose deposition (Coleman *et al.*, 2006). In the current case, it appears that in poplar, SuSy alone provides the largest effect in maintaining height growth and improving the level of cellulose deposition, while the additional effects of UGPase are not evident. This is not particularly surprising, as 4CL::UGPase on its own causes only minor changes in soluble sugars and does not appear to influence growth or cellulose content in poplar (Coleman, unpublished). Furthermore, under the regulation of the

2×35S promoter it was extremely difficult to obtain double transformants, as UGPase alone severely affected plant growth (Coleman *et al.*, 2007).

In summary, the overexpression of SuSy individually and in combination with UGPase in hybrid poplar resulted in increased cellulose production. While no apparent trend in soluble sugars is evident, the plants do indeed synthesize cellulose to a greater extent, thus providing evidence for a direct connection between sucrose supply (sink strength), its breakdown and cellulose deposition through the increased activity of SuSy. The overexpression of UGPase in combination with SuSy appears to manifest similar results, suggesting that SuSy is more closely related to cellulose synthesis, and consequently xylem deposition. These results together with the work of others clearly implicate SuSy as a strong component in the production of cellulose and in sink strength in poplar.

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Conclusions and Recommendations for Future Work

The molecular understanding of the biosynthesis of cellulose in plants and forest trees is still a relatively new area of study. The identification of UDP-glucose as the precursor to cellulose synthesis and the key substrate for the cellulose synthase complex has permitted the scientific community to make significant advances in understanding this vital pathway in recent years. For example, the knowledge of the pathway leading to the formation of UDP-glucose within plants has permitted the study of cellulose biosynthesis via the variation in expression of genes involved directly and indirectly in the formation of this precursor molecule.

Testing the hypothesis that altering carbohydrate metabolism to generate increased cellular pools of UDP-glucose should result in altered cellulose content by shifting carbon allocation towards cellulose biosynthesis was tested in two model plant species. In tobacco, the overexpression of SPS, UGPase and SuSy individually or in combination resulted in substantial increases in height growth, and consequently plant biomass. Furthermore, it was apparent that pyramiding genes to enhance the catalysis of sucrose to UDP-glucose offers a mechanism to increase growth additively over the ability of a single gene misregulation. However, consistent changes in either soluble or structural carbohydrates were not evident. Therefore, in tobacco, it appears that the reallocation of carbon skeletons from photosynthate to cellulose deposition may not be possible by targeting these sucrose metabolism genes and enzyme products.

In contrast, in poplar, the overexpression of UGPase and SuSy individually and in combination resulted in a significant increase in cellulose. However, in UGPase transgenics trees this was associated with reductions in growth, as well as a defence response which resulted in the formation of salicylic acid 2-O-β-salicylic acid-glucoside (SAG). The formation of this glucoside has been shown to be catalyzed by a UDP-glucose:salicylic acid glucosyltransferase in tobacco, where UDP-glucose acts as the sole donor. The results described in this thesis suggest that using the current system to increase cellular pools of UDP-glucose in poplar trees does indeed manifest in the formation of elevated levels of cell wall cellulose polymer. However, it appears that UDP-glucose formed in these targeted transgenics is excessive and the cellulose synthase complex becomes limiting. Ultimately, to maintain the homeostatic levels of

UDP-glucose near the plasma membrane, the excess UDP-glucose is mobilized from cell wall biosynthesis in the generation of the salicylic acid glucoside.

Similarly, in transgenic poplar overexpressing SuSy and SuSy × UGPase there is a significant increase in cellulose content. In these lines, while there was a slight reduction in height and biomass in some lines, the decrease was much less severe than in the UGPase transgenic lines. It appears that the close association of SuSy with the cellulose synthase complex (CSC) offers an avenue for the direct channelling of UDP-glucose to the CSC, and limits the availability of UDP-glucose to other cellular pathways. As such, the defence response induced in the UGPase transgenics was not apparent. Furthermore, there does not appear to be an additive gain in carbon partitioning to cellulose production by transforming poplar trees with both genes. The elevated cellulose content in the SuSy and SuSy × UGPase transgenic poplar was also associated with significant increase in crystallinity. This increase was not associated with a change in microfibril angle which would be expected in the case of tension wood formation, suggesting that the apparent change in cellulose ultrastructure is indeed a change in the mechanism of cellulose deposition as opposed to the induction of tension wood formation by the poplar trees.

The differences observed between tobacco and poplar, despite being transformed with the identical constructs has been attributed to two major factors. The first is the difference in phloem loading and unloading between the two species, with poplar being a symplastic loader and tobacco an apoplastic loader, and the second is the difference in sinks and in sink strength. In poplar, the stem is the major sink and as such is responsible for regulating much of the gradient in photoassimilate translocation, while in tobacco there are more numerous sinks including leaf and stem tissue, as well as reproductive bud formation.

The findings of this research clearly demonstrate that it is possible to alter cellulose metabolism in forest trees through the misregulation of key sucrose metabolism genes. It also suggests that the formation of UDP-glucose may not be the overall limiting factor in manipulating carbon allocation to cellulose biosynthesis. The formation of cellulose may also be highly limited by the CesA complex, thus encouraging the focus of cellulose research on these genes.

Future Work Recommendations

There are a number of interesting projects that should be developed and extended based on the findings of the work presented in this thesis. First of all, the gene encoding for UDP-glucose:salicylic acid glucosyltransferase has not yet been identified in poplar. This putative gene could be cloned and gene function confirmed in terms of glucose donor sensitivity. Once identified and cloned, transgenics verifying its catalytic function should be made. In addition, downregulating this gene in the over-expressing UDP-glucose pyrophosphorylase transgenics may offer an opportunity to increase the partitioning to cellulose without eliciting the defence response apparent in the existing transgenic UGPase poplar trees.

Within the array of successful poplar SuSy transformants, key lines (those that are not affected in growth and biomass accumulation) should be selected, and combined with misregulation efforts targeting key lignin biosynthetic genes (*i.e.* downregulation). For example, substantial industrial gains have been made in altering the lignin monomer composition and content in poplar using F5H or RNAi C3′H. By pyramiding SuSy transgenics with one (or both) of these two lignin genes, there is potential for substantial additive gains that could hypothetically manifest in an increased cellulose producing line with either reduced lignin production or altered lignin monomer composition in favour of syringyl lignin sub-units.

Ideally, as research progresses with the modulation of the CesA genes, there is the possibility that increased CSC activity will be attainable, and as such UDP-glucose could become the limiting factor in cellulose biosynthesis. Thus, pyramiding the SuSy or UDP-glucose pyrophosphorylase genes with a CSC modified tree with the ability to rapidly synthesize cellulose could provide increased substrate to an already improved system for further advancement.

Finally, in poplar, it would also be interesting to pursue the overexpression of SuSy under the control of a promoter that is more specific to the timing and location of cellulose biosynthesis. In particular, one of the secondary cell wall specific CesA promoters may facilitate the most useful and opportune expression of the SuSy gene in coordination with the CSC genes.

Appendix A - β-glucuronidase (GUS) Transformation and Staining

Methods

Construct Design

pBI-121 containing the 35S::GUS construct was modified by the replacement of the 35S promoter with either the 2×35S promoter (from pBI-426) or the 4CL promoter (from 99-G1-501). The resulting cassettes (2×35S::GUS::NOS and 4CL::GUS::NOS) were excised from pBI 121 as HindIII-EcoRI fragments and inserted into the pCAMBIA 1300 binary vector for use in plant transformation.

Transformation of Hybrid Poplar and Tobacco

Poplar and tobacco were transformed using *Agrobacterium* as previously described (Coleman *et al*, 2006 and 2007). In short, poplar leaf disks were cut and co-cultured with EHA105 harbouring the 2×35S::GUS or 4CL::GUS transgenes. The explants were plated on Woody Plant Media (WPM) (McCown and Lloyd 1981) supplemented with 0.1 μM each of α-naphthalene acetic acid (NAA), 6-benzylaminopurine (BA), and thiadiazuron (TDZ) and solidified with 3% (w/v) agar and 1.1% (w/v) phytagel (WPM 0.1/0.1/0.1). After three days the disks were transferred to WPM 0.1/0.1/0.1 supplemented with carbenicillin disodium (500 mg L^{-1}) and cefotaxime sodium salt (250 mg L^{-1}). Following three additional days, the disks were transferred to WPM 0.1/0.1/0.1 containing carbenicillin, cefotaxime and kanamycin (25 mg L^{-1}). After five weeks, shoots and callus material were transferred to WPM containing agar and phytagel, supplemented with 0.01 μM BA, carbenicillin, cefotaxime and kanamycin. Once individual shoots were visible, plantlets were transferred to solidified WPM with 0.01 μM NAA and carbenicillin, cefotaxime and kanamycin to induce rooting. After two consecutive five-week periods on this media, shoot tips were isolated to solidified antibiotic-free WPM with 0.01 μM NAA.

Procedures for tobacco were the same with the exception of the use of MS media (Murashige and Skoog, 1962) and the absence of TDZ in the media.

β-glucuronidase (GUS) Assays

GUS staining assays were performed as described by Jefferson (1987) using leaves, shoots, roots, and node sections from transgenic and control tobacco and hybrid poplar. Stems and nodes were hand sectioned, and leaves and roots were used directly.

Plant sections were vacuum infiltrated in acetone. Following the removal of acetone, the plants were incubated in an x-gluc solution (0.1% Triton X-100, 5% 1M $NaPO_4$ pH 7.0, 0.5% 100 mM Fe(CN), 0.05% x-gluc) overnight at 37°C. Prior to imaging, the samples were washed with 70% ethanol.

Sections were mounted on glass slides and visualized using a Leica DMR microscope equipped with a QICAM CCD camera (Q-imaging).

Results

Expression of the 2×35S and 4CL promoters was assessed using the GUS reporter gene. The resulting staining patterns in tobacco revealed very little difference between the two promoters (Figure A1). Both promoters were shown to be expressed in all tissues examined. In leaf tissue, 4CL expression was somewhat more restricted to the vasculature, but expression of the promoter was seen throughout the leaf tissue. Poplar results were similar to tobacco in that the promoters were expressed in all tissues examined (Figure A2). While 4CL again appeared to be somewhat more associated with vascular tissue, it was still apparent throughout the samples. Despite the significant differences seen by others (Hauffe *et al.*, 1991; Kay *et al.*, 1987) in the expression of 2×35S and 4CL, the results presented herein clearly demonstrate that both promoters effectively express throughout the plant, with no real significant differences between promoters.

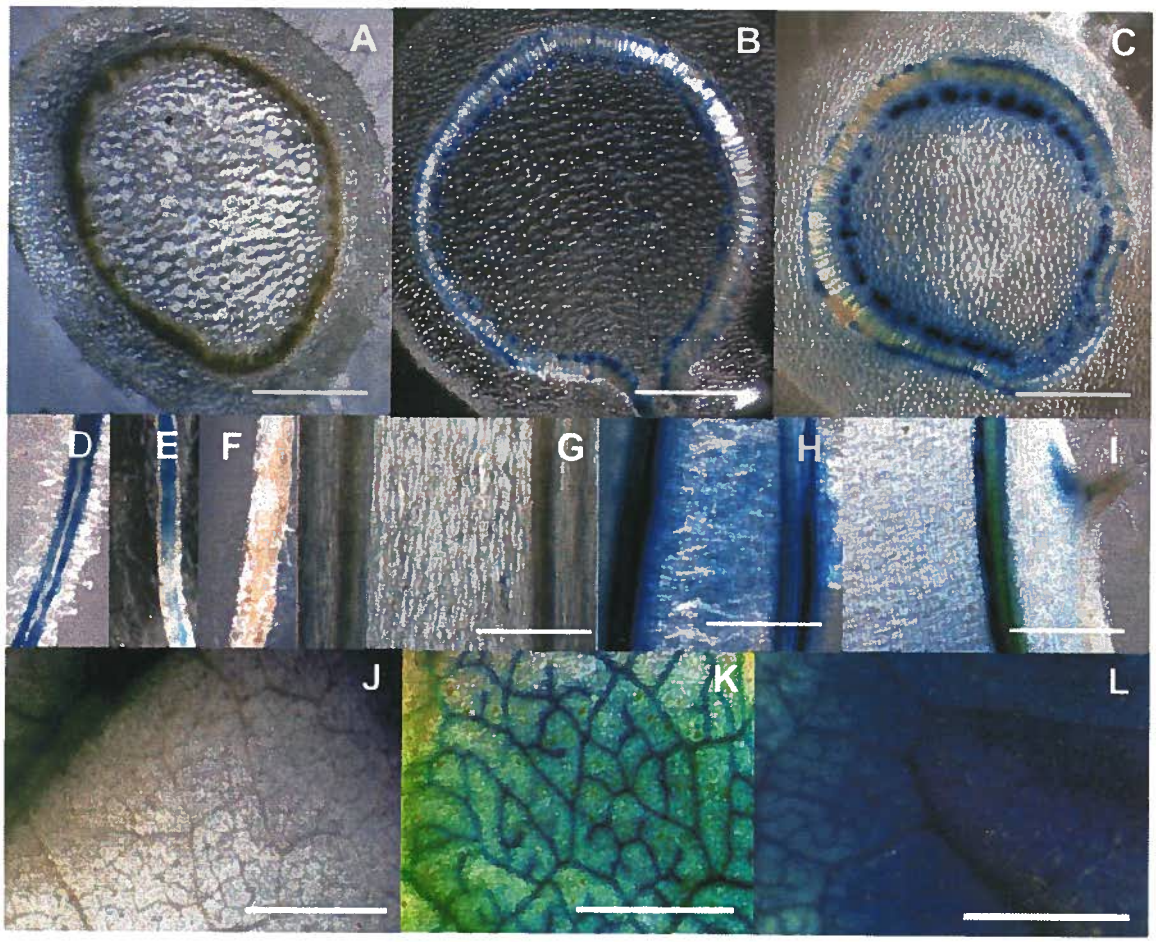

Figure A1. β-glucuronidase (GUS) staining in tobacco stem cross sections (A, B, C), roots (D, E, F), longitudinal stem sections (G, H, I) and leaves (J, K, L) of control (A, F, G, J), 2×35S (C, E, H, L), and 4CL (B, D, I, K). Scale bar: 1 mm.

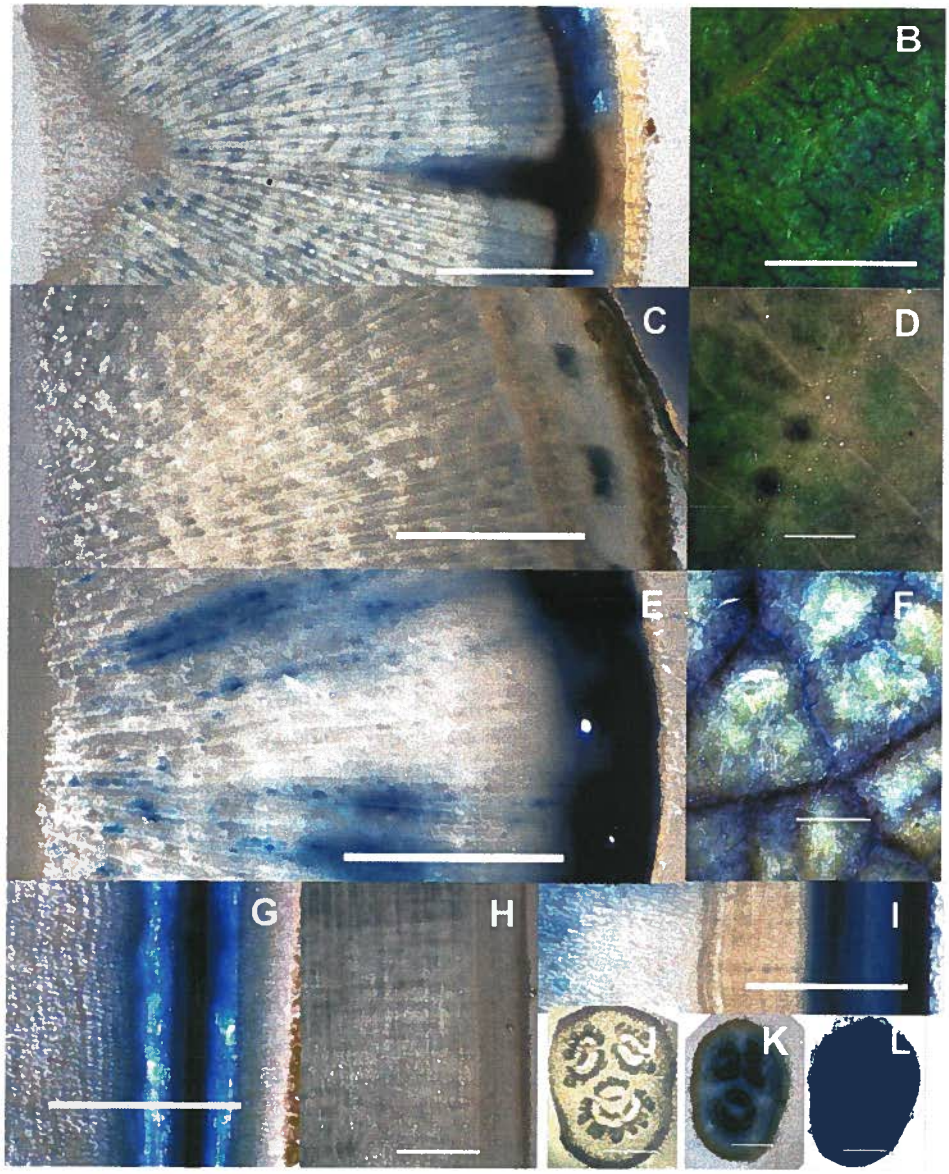

Figure A2. β-glucuronidase (GUS) staining in poplar stem cross sections (A, C, E), leaves (B, D, F), longitudinal stem sections (G, H, I) and petioles (J, K, L) of control (C, D, H, J), 2×35S (E, F, I, L) and 4CL (A, B, G, K). Scale bar: 1 mm.

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Appendix B – Supplemental Data Chapter 3

Table B1. Transcript abundance of the native poplar UDP-glucose pyrophosphorylase genes in leaf and developing xylem tissue for transgenic and wildtype trees. Mean (±SE) were calculated from 3 plants per line. Bold denotes significant difference from control values at α=0.10.

	Poplar UGPase1	Poplar UGPase2
Leaf	ΔCt	ΔCt
Control	9.08 ± 0.40	0.45 ± 0.01
2×35S::UGPase A	9.59 ± 0.27	**1.47 ± 0.06**
2×35S::UGPase B	9.38 ± 0.47	**1.19 ± 0.01**
2×35S::UGPase C	12.66 ± 2.76	**1.74 ± 0.14**
2×35S::UGPase D	**14.62 ± 1.03**	**2.15 ± 0.12**
2×35S::UGPase E	**12.19 ± 0.37**	0.99 ± 0.47
2×35S::UGPase F	**6.82 ± 0.44**	**0.62 ± 0.01**
Developing Xylem	Mean	Mean
Control	3.85 ± 0.43	0.48 ± 0.02
2×35S::UGPase A	**5.52 ± 1.25**	**1.99 ± 0.16**
2×35S::UGPase B	5.49 ± 0.10	**1.84 ± 0.12**
2×35S::UGPase C	4.28 ± 0.12	**1.06 ± 0.08**
2×35S::UGPase D	6.61 ± 0.37	**1.77 ± 0.17**
2×35S::UGPase E	**5.14 ± 0.17**	**1.80 ± 0.04**
2×35S::UGPase F	2.70 ± 0.08	**0.67 ± 0.01**

Table B2. Transcript abundance of the cell wall biosynthetic genes involved in lignin and cellulose deposition in the developing xylem tissue for transgenic and wild-type trees. Mean (±SE) were calculated from 3 plants per line.

Gene	2×35S::UGPase ΔCt ± Mean	Wild-type ΔCt ± Mean
PAL	1.51 ± 0.51	1.00 ± 0.06
C4H	3.94 ± 1.10	1.08 ± 0.28
SuSy	1.19 ± 0.09	1.03 ± 0.17
C3H	2.20 ± 0.42	1.01 ± 0.12
COMT	1.69 ± 0.38	1.07 ± 0.26
SAD	1.96 ± 0.22	0.86 ± 0.37
CCoAMT	8.85 ± 1.73	1.02 ± 0.17
CAD	2.18 ± 0.42	0.80 ± 0.03
CESA	4.11 ± 1.33	1.13 ± 0.40
F5H	1.61 ± 0.25	1.03 ± 0.16
4CL	1.18 ± 0.16	1.00 ± 0.00
CCR	6.14 ± 0.93	1.00 ± 0.00
UGP1	0.80 ± 0.19	1.05 ± 0.22
UGP2	2.34 ± 0.67	1.01 ± 0.10

Table B3. Fold changes in salicylic acid 2-O-β-glucoside in the developing xylem of all transgenic 2×35S::UGPase hybrid poplar relative to levels in wild-type trees.

	Salicylic acid 2-O-β-D-glucoside Fold Change
Control	1.0
2×35S::UGPase A	**221.4**
2×35S::UGPase B	**177.7**
2×35S::UGPase C	**239.6**
2×35S::UGPase D	**232.3**
2×35S::UGPase E	**270.2**
2×35S::UGPase F	**268.9**