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Site-directed mutagenesis and functional analysis of *Eucalyptus* sucrose synthase 1 (*EgSuSy1*) in *Arabidopsis thaliana*

by

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DECLARATION

I, Makobatjatji Mmoledi Mphahlele the undersigned, hereby declare that the thesis/dissertation submitted herewith for the degree ***Magister Scientiae*** to the University of Pretoria, contains my own independent work and has not been submitted previously by me for any degree at any other tertiary institution.

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PREFACE

Conventional breeding technologies, which are being used for the domestication of tree species, struggle to meet the current demand for wood products such as paper, pulp and timber. Future targets of developing cellulosic bio-fuels to mitigate the global demand for renewable energy will contribute to the increased demand of wood products. The strides made by biotechnology present opportunities for faster and sustainable genetic improvement of trees by targeting specific traits with accuracy to deliver on current and future wood product demands. Tree biotechnology will enable breeders to target important traits in trees for improvement, such as wood fibre with high cellulose content.

Desired improvements include the reduction of lignin in trees within physiological limits and changing its structural components for easy extraction and to increase of flux and partitioning of carbon into cellulose. These processes involve highly complex pathways with complex regulatory mechanisms, which are influenced directly and indirectly by internal and external stimuli. Therefore, there is a vital need to discover and functionally validate these mechanism and the components involved to unravel the complexities of wood development. However, a fundamental constraint is to increase cellulose deposition and simultaneously ensure that the balance of carbon allocation between other carbon utilising pathways is maintained under normal tree growth conditions. Therefore, understanding the assimilation of carbon in autotrophic (source) tissues, the partitioning of carbon into heterotrophic (sink) tissues and deciphering the pathways and genes involved is required to develop molecular strategies that will sustain this balance.

This dissertation focuses on sucrose synthase (SuSy) involvement in sucrose carbon allocation towards plants growth and development and more specifically, towards cellulose

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deposition. **Chapter One** reviews sucrose carbon metabolising enzymes and their regulatory mechanisms related to their potential role in increasing cellulose deposition. Focus was placed on the N-terminal serine phosphorylation domain of the sucrose synthase enzyme and its potential involvement in regulating SuSy sub-cellular localization to support cellulose biosynthesis. **Chapter Two** of this dissertation reports on the site-directed mutagenesis of the N-terminal non-catalytic serine phosphorylation domain of *Eucalyptus grandis* sucrose synthase 1 (*EgSuSy1*) gene. The unmodified and modified *EgSuSy1-S11A* were functionally characterized as potential candidates for allocation of sucrose carbon towards cellulose biosynthesis by overexpression in transgenic *Arabidopsis* plants. The objective of this research is to sub-localize sucrose synthase activity in the membrane fraction thereby allocating sucrose carbon towards cellulose biosynthesis. Our observation indicated that the *EgSuSy1* lines had more fibre development with slightly smaller secondary cell wall thickness, whereas *EgSuSy1-S11A* lines seem to maintain fibre development and secondary cell wall compared to the Col-0 control. No significant changes were observed in the percentage content of secondary cell wall sugars between Col-0, *EgSuSy1* and *EgSuSy1-S11A.*

The research findings presented in this dissertation represent the outcomes of the study undertaken from January 2007 to July 2009 in the Department of Genetics, University of Pretoria under the supervision of Prof. A.A. Myburg and Dr. S. Naidoo. Chapter Two is prepared in the format of a manuscript for a peer-reviewed research journal (e.g. *Tree Physiology*). Therefore, there is some degree of repetition between Chapter One and Chapter Two. The work from this study has not been presented orally or as a poster in any formal scientific meeting due to IP restrictions.

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CHAPTER 1

Literature Review:

Sucrose carbon allocation towards cellulose biosynthesis in plants

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1.1 Introduction

The land area, products and socio-environmental participation of the global forestry plantation industry is significant (FAO, 2007). However, conventional forestry tree breeding programmes are struggling to meet the demands for wood products such as pulp, paper and timber. Therefore, the added demand for wood products such as bio-fuels from cellulosic ethanol warrants accelerated tree improvement through biotechnological approaches. This will allow faster domestication of tree species into fibre and energy crops that are sustainable and suited to meet the global demand for conventional and future wood products.

Challenges facing the globe today include the development of sustainable renewable energy and reducing, or eliminating our dependence on fossil fuel based energy, which has detrimental effects on the environment. One of the potential resources for sustainable and renewable energy is fixed carbon in plant biomass. A number of plant species show potential in providing renewable energy in the form of fixed carbon (Huang *et al*, 2008). However, the production of renewable energy is a strict balance between the availability of carbon resources, the cost of conversion of carbon to usable energy (Huang *et al*, 2008), and the challenges of enzymatic and mechanical processing of plant material into biofuels (Kunz, 2008). Solving the practical problems of biofuel production is important, but the bottleneck is believed to be the availability of renewable, highly fermentable plant materials and the development of alternative fermentable plant species (Huang *et al*, 2008).

Carbon allocation in plants encompasses two processes, carbon flux - the rate at which carbon is allocated, and carbon partitioning - the proportion of carbon allocated to different plant organs, tissues and biopolymers in cells (Litton *et al*, 2007). Given these two processes, the challenge is for researchers to improve on one or both, to

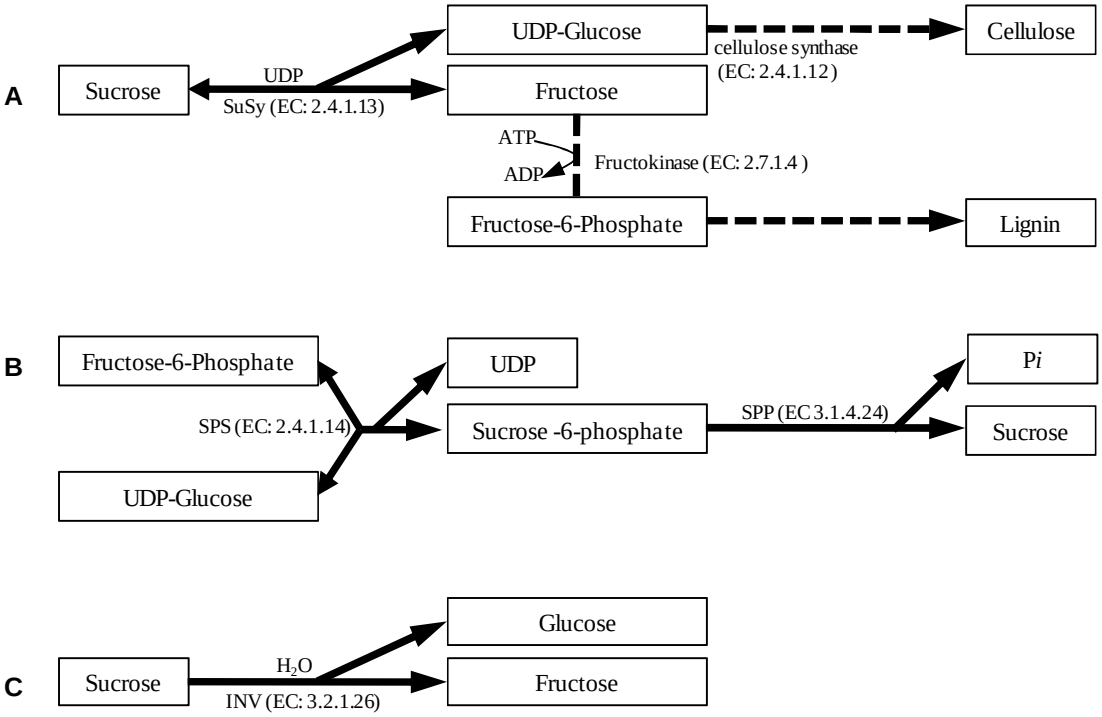
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ensure directed and increased carbon allocation into targeted plant species or tissues. To achieve this, particularly in trees, scientists have employed biotechnological approaches aimed at carbohydrate active enzymes (CAZymes) to understand the physiology of trees (Herschbach and Kopriva, 2002) and carbon allocation into cell wall polymers (Hu *et al*, 1999). CAZymes play an important role in cell wall biosynthesis and xylogenesis in woody trees such as poplar (Geisler-Lee *et al*, 2006). Genome sequence data suggest that CAZymes are more abundant in plants than any other organisms (Coutinho *et al*, 2003) and expression profile data suggest that CAZymes are more abundant in poplar compared to *Arabidopsis thaliana*, which is a herbaceous plant (Geisler-Lee *et al*, 2006).

Source tissues e.g. green leaves are photosynthetic (autotrophic) tissues, which are capable of assimilating atmospheric carbon into photosynthates, such as sucrose. These photosynthates are translocated via the phloem tissue to non-photosynthetic (heterotrophic) storage tissues also known as sink tissues, e.g. fruits, tubers, roots, and wood. In some cases, leaf tissue can serve as a temporary storage organ for starch as it makes a transition from a source to a sink tissue (Munoz et al, 2006).

Wood is an important sink of carbon from photosynthetic sucrose. Multiple CAZymes are involved in the metabolism of sucrose into sink tissues (Geisler-Lee *et al*, 2006). Sucrose synthase (SuSy: EC 2.4.1.13), a member of the glycosyltransferase 4 (GT4) family reversely converts sucrose into active UDP-glucose (UDPG) and fructose, which is activated to fructose-6-phosphate (F-6-P) by fructokinase (Figure 1.1A). UDPG and F-6-P are important substrate monomers for the biosynthesis of two major secondary cell wall polymers, cellulose and lignin, respectively. Unlike SuSy, invertase enzymes (INV: EC 3.2.1.26) hydrolyze sucrose into glucose and fructose to be utilized by plants (Roitsch and Gonzalez, 2004 - Figure 1.1C). The products of

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backbone. The cleavage reaction of sucrose by SuSy produced UDPG, and due to its ability to associate with the plasma membrane ensure the production of UDPG in the vicinity of the rosette complex supporting cellulose production (Amor *et al*, 1995). The association of SuSy with the plasma membrane is proposed to be regulated by the N-terminal serine phosphorylation domain (Winter *et al*, 1997; Zhang *et al*, 1999).

This review focuses on the biotechnological approaches directed towards sucrose metabolising enzymes specifically SuSy, and their anabolic and catabolic involvement in partitioning sucrose carbon into secondary cell wall synthesis and plant growth and development. Furthermore, it focuses on the regulatory properties of SuSy, and its potential role to alter carbon allocation into the glycolysis pathway for energy production or cellulose biosynthesis, which is a permanent store of secondary cell wall carbon and therefore a true carbon sink in woody trees (Lippke *et al*, 2010)

1.2 Wood as a sink tissue for carbon

Wood tissue is biochemically and physically complex and varied. It comprises of multiple xylem cell types responsible for structural integrity (fibres) and water transport (vessels) and phloem cell types responsible for nutrient transport in angiosperms (Plomion *et al*, 2001). In gymnosperms, water and nutrient transport and structural support are functions performed by treachery cells (Plomion *et al*, 2001). Secondary xylem fibre cells are important carbon sinks in woody plants, characterized by extensive secondary cell wall thickening which mainly consisting of cellulose, lignin, and hemicelluloses that are arranged in distinct layers and proportions resulting in varying physical and chemical properties (Plomion *et al*, 2001). Under bending tension, angiosperms form tension wood and gymnosperms for reaction wood, which are characterized by the deposition of a cellulose rich, gelatinous layer containing traces of hemicelluloses and lignin (Mellerowicz *et al*, 2008). This illustrated the

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ability of trees to alter carbon allocation into specific tissues under certain conditions. Tension wood formation in trees is correlated with increased expression of multiple CAZyme gene families including the SuSy GT4 family (Andersson-Gunneras *et al*, 2006). This correlation signifies the vital role CAZyme expression play in altering carbon allocation in trees.

1.3 Involvement of CAZymes in plant and wood development

CAZymes related genes are more abundant in tree species than in herbaceous species. These differences are required for regulating the complexities of carbon allocation and wood development in trees (Geisler-Lee *et al*, 2006). However, a herbaceous plant such as *Arabidopsis* is able to induce some form of secondary xylem formation under induced conditions of prolonged growth of the inflorescence stem into its second phase of development (Chaffey *et al*, 2002).

Differential or altered expression of CAZymes reveal differences in wood formation due to altered carbon allocation patterns and external stimuli (Andersson-Gunneras *et al*, 2006; Plomion *et al*, 2001). *Robinia pseudoacacia* (Black locust), which is generally described as a perennial tree with rapid growth rate in spring, illustrate this. SPS and SuSy showed marked differential seasonal expression in Black locust wood tissue zones from bark to inner heartwood with INV activity having limited contribution throughout the yearly seasons (Hauch and Magel, 1998). The overexpression of *Arabidopsis* SPS in tobacco resulted in increased plant biomass and fibre cell length relative to control plants (Park *et al*, 2008). The objective of this study was to increase the pool of sucrose in the plants to improve growth and fibre development. The overexpression of cotton SuSy in tobacco resulted in increased plant biomass with generally reduced fibre cell length (Coleman *et al*, 2006), whereas Coleman *et al* (2009) showed increases in crystalline cellulose production in hybrid

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poplar due to the overexpression of cotton SuSy. The objectives of these two studies were to investigate the role of SuSy in carbon allocation during secondary cell wall formation. Unlike, SPS, which is involved in the synthesis of sucrose, SuSy and INV are involved in sucrose cleavage into hexose sugars; therefore, the balance in activity of these key enzymes is vital for carbon allocation, wood fibre development and plant growth and development.

1.3.1 Sucrose metabolism by sucrose synthase and invertase

Photosynthetic sucrose is the predominant disaccharide by which carbon is transported and utilized by plants for energy/storage molecules (e.g. starch) and structural molecules (e.g. cellulose). Alternatively, sucrose can be synthesized *de novo* through the fructose salvage pathway (Figure 1.1B). The association of SuSy with the loading and unloading of phloem was investigated in maize and in citrus plants (Nolte and Koch, 1993) and both plants demonstrate clear association between SuSy and companion cells. Although the exact reasons for this association are unclear it is hypothesized to maintain sucrose levels in phloem tissue (Nolte and Koch, 1993) as it is transported to sink tissues (Lalonde *et al*, 2003). This suggests a similar mechanism of SuSy phloem loading and unloading in monocots and dicots (Lalonde *et al*, 2003). However, other SuSy functions are not related to sucrose metabolism, but involve the inter-compartmental signalling associated with voltage-dependent anion channel (VDAC) in the mitochondria (Sabbaiah *et al*, 2006). Maize plant SH1 and SUS1 SuSy isoform were shown to be partly localized with the mitochondria in association with its VDAC and the nuclei. Under prolonged anoxia, SuSy is released from the SuSy-VDAC complex and migrates to the nucleus where it is hypothesized to signal the induction of cell death of the root tip of maize seedlings (Sabbaiah *et al*, 2007; Sabbaiah and Sachs, 2001).

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This suggests diverse roles for SuSy in plant growth and development, of which some are not related to sucrose metabolism.

INVs are members of the β-fructosidase CAZyme family, which are involved in sucrose catabolism. INVs are classified into three general isoform categories distinguishable by pH, isometric point, and sub-cellular localization (Roitsch and Gonzalez, 2004; Winter and Huber, 2000). These categories are (A) insoluble acid cell wall, (B) soluble vacuole and (C) neutral/alkaline cytosol INVs (Roitsch and Gonzalez, 2004). The insoluble acid cell-wall INVs hydrolyze sucrose in the cell wall region for cell wall related pathways, the soluble vacuole INVs hydrolyse sucrose in the vacuole releasing it from temporary storage into the cytoplasm, whereas the neutral/alkaline cytosolic INVs hydrolyse sucrose in the cytosol for utilization for plant growth and development (Koch, 2004).

During the early phases of tomato fruit development, SuSy rather than INVs was shown to be vital in metabolizing imported sucrose in tomato fruits (Wang *et al*, 1993) and determining sink strength in potato (Zrenner *et al*, 1995). The opposite was true for carrots, were SuSy was shown to be responsible for facilitating plant growth (Tang and Strum, 1999a), whereas INVs were responsible for sucrose partitioning and determining sink strength (Tang *et al*, 1999b). INVs were also shown to signal stress responses (Roitsch and Gonzalez, 2004).

The cleavage activity of SuSy uses the energy stored in the sucrose glycosidic bond, whereas INV requires input energy in the form of ATP (Albrecht and Mustroph, 2003b; Zeng *et al*, 1999). The glycolysis pathway utilises F-6-P and when SuSy cleaves sucrose only one ATP molecule is required to convert fructose into F-6-P whereas when sucrose is cleaved by INVs, two ATP molecules are needed to convert glucose and fructose into F-6-P (Perata et al, 1997). This results in up-regulation of

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SuSy under hypoxic conditions to conserve energy (Albrecht and Mustroph, 2003b; Bieniawask *et al*, 2007; Bologa *et al*, 2003; Zeng *et al*, 1998). Even under anoxic conditions, SuSy remains active (Albrecht and Mustroph, 2003b). The roots of wheat plants under hypoxic conditions experience increased SuSy activity and reduced INV activity, resulting in increased cellulose and callose content (Albrecht and Mustroph, 2003a), but with no significant increase under anoxic conditions. These findings suggest SuSy as an enzyme important for plant growth and development and stress responses. Therefore, SuSy may not necessarily favour a particular cellular pathway. Nonetheless, it is an important regulator of sucrose carbon allocation.

1.3.2 Functions of sucrose synthase family members

Multiple SuSy family gene members are found within species (Bieniawask *et al*, 2007; Hirose *et al*, 2008). Possible functional redundancies have been identified in *Arabidopsis* (Bieniawask *et al*, 2007). SuSy family members have also been well characterised in a number of other plant species (Table 1.1). In some plant species, particularly SuSy family members may favour sucrose cleavage for energy production while other members favour sucrose cleavage for cell wall synthesis (Matic *et al*, 2004). *Zea mays* (*Zm*) *SUS1* mutant (*sus1*) plants exhibit lower starch production, whereas *ZmSH1* mutant (*sh1*) plants have disrupted cell wall structures (Chourey *et al*, 1998). Similar differences in functionality of SuSy family members were observed in pea (Barratt *et al*, 2001), and potato (Fu and Park, 1995), were different SuSy family members favour carbon allocation into different sink tissues. Other species show evidence of organ and tissue specific expression (Bieniawask *et al*, 2007; Hirose *et al*, 2008). This suggests distinct roles for SuSy gene members in allocating carbon in plant tissues and organs.

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Plant species	Common names	Genes	Reference
Arabidopsis thaliana	Thale cress	SUS1	Baud *et al*, (2004)
		SUS2	
		SUS3	
		SUS4	
		SUS5	
		SUS6	
Zea mays	Maize	SUS1	Duncan *et al*, (2006)
		SH1	
		SUS2	
Nicotiana tubacum	Tobacco	SuSy1	Matic *et al*, (2004)
		SuSy2	
Pisum sativum	Pea	Sus1	Barratt *et al*, (2001)
		Sus2	
		Sus3	
Beta vulgaris	Sugarbeet	SuSyI	Klotz *et al*, (2003)
		SuSyII	
Saccharum officinarum	Sugarcane	SuSyA	Schafer *et al*, (2003)
		SuSyB	
		SuSyC	
Solanum tuberosum	Potato	Sus1	Fu and Park, (1995)
		Sus2	
		Sus3	
		Sus4	
Oryza sativa	Rice	SUS1	Hirose *et al*, (2008)
		SUS2	
		SUS3	
		SUS4	
		SUS5	
		SUS6	

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of SuSy may be influenced differently depending on carbon allocation for specific roles such as growth and development in plants, which may be temporally and spatially varied.

1.3.3 Fructose salvage pathway and wood development

The fructose salvage pathway synthesizes sucrose *de novo* via a reversible reaction by SPS, followed by the irreversible hydrolytic reaction by SPP of sucrose-6-phosphate to sucrose and a free phosphate group (Figure 1.1B). SPS and SPP form an active enzyme complex and function in close association in the fructose salvage pathway (Echeverria *et al*, 1997; Lunn and MacRae, 2003). SPS and SPP are expressed in photosynthetic and non-photosynthetic tissues that metabolize sucrose. The overexpression of spinach SPS in cotton transgenic plants resulted in increased sucrose to starch ratio and increase cellulosic secondary fibre cell wall thickness (Haigler *et al*, 2007). The increase in cellulose production due to SPS expression follows a chain of substrate sharing, were SPS provides SuSy with sucrose and SuSy provides UDPG for cellulose biosynthesis (Figure 1.1A and B). This suggests the linked role of SPS and SuSy in cellulose biosynthesis. SPS activity modulates the fructose salvage pathway supporting increased cellulose deposition in secondary cell walls of cotton fibres, etiolated pea hypocotyls and *Zinnia elegans* cultured cells under normal conditions (Babb and Haigler, 2001).

The intermediate product of the fructose salvage pathway, F-6-P can be diverted towards multiple pathways, such as (i) glycolysis, (ii) the pentose phosphate pathway, (iii) GDP-mannose biosynthesis, or (iv) N-acetyl-glucosamine biosynthesis (Andersson-Gunneras *et al*, 2006). Excess F-6-P is diverted to N-acetyl-glucosamine biosynthesis and sucrose biosynthesis during tension wood formation, whereas in normal wood formation F-6-P is diverted to lignin and hemicellulose biosynthesis, via

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the pentose phosphate and shikimic biosynthesis pathway (Andersson-Gunneras *et al*, 2006). The versatility of F-6-P carbon flux during wood formation was suggested to represent a connection between cellulose and lignin biosynthesis (Andersson-Gunneras *et al*, 2006; Hu *et al*, 1999). Better understanding of the mechanisms involved in sucrose degradation and synthesis pathways will enable manipulation of carbon allocation strategies in commercially important sink tissues.

1.4 Sub-cellular localization of sucrose synthase

SuSy was long thought to be a cytosol-associated protein. However, it was shown to have membrane association capabilities, putatively supporting cellulose production in cotton fibres (Amor *et al*, 1995) and recently bacterial polysaccharides (Curatti *et al*, 2008). Sucrose synthase was also shown to be an actin binding protein (Winter *et al*, 1998). Further studies indicated that high sucrose concentrations and phosphorylation influence ZmSUS1 association with the plasma membrane and actin association (Duncan and Huber, 2007). The role of actin in intracellular translocation of SuSy via the cytoskeleton still needs further investigation.

The association of SuSy with the plasma membrane is proposed to optimise the channelling of UDPG “directly” from sucrose towards cell wall polymer biosynthesis. This association also reduces competition for UDPG from other cytosol-linked pathways and ensures rapid turnover of UDP in the vicinity of the plasma membrane, because UDP competitively inhibits cellulose synthase (Ross *et al*, 1991). To date no evidence has been shown, whether membrane association of SuSy directly or indirectly assists the channelling of UDPG to the cellulose synthase rosette complex.

Membrane association of the ZmSUS1 isoform was shown to have domains that interact with the plasma membrane such as the N-terminal non-catalytic domain and a

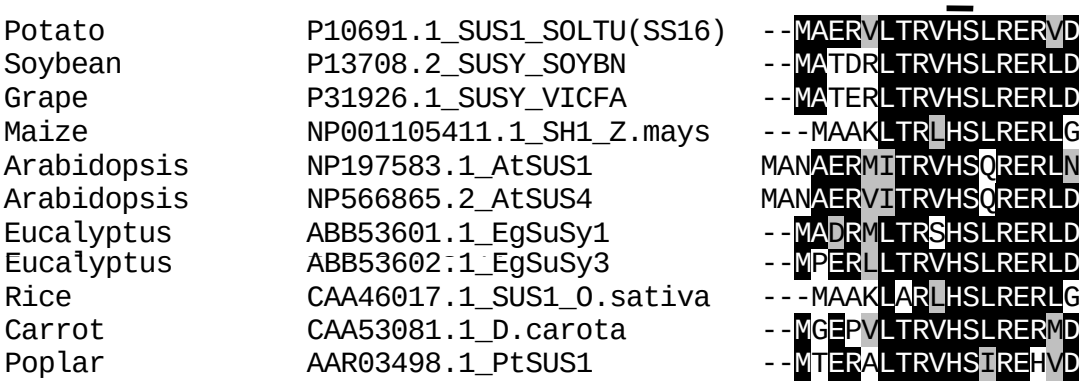
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C-terminal pleckstrin domain homologous to that in humans by site-directed mutation in these domains (Hardin *et al*, 2006). Duncan *et al* (2006) showed that homo-oligomers of ZmSUS1 associate with both the membrane and the cytosol fraction, whereas homo-oligomers of ZmSH1 another maize isoform are present predominantly in the cytosol fraction and homo-oligomers of ZmSUS2 another maize isoform associates only with the cytosol fraction (Duncan *et al*, 2006). ZmSUS1-ZmSUS2 hetero-oligomers confine ZmSUS1 to cytosol association and ZmSH1 associates with the membrane with lower affinity compared to ZmSUS1 (Duncan *et al*, 2006). These interactions add to the complexities involved in the regulation of the sub-cellular localization of SuSy family members. Other evidence suggest that phosphorylation/dephosphorylation of specific residues may influence the sub-cellular localization of SuSy (Winter *et al*, 1997).

1.4.1 Phosphorylation of sucrose synthase

SuSy is phosphorylated in monocots (Huber *et al*, 1996), and in dicots (Anguenot *et al*, 1999; Wienkoop *et al*, 2008; Zhang and Chollet, 1997) mediated by calcium dependent protein kinase (CDPK) on an N-terminal non-catalytic serine phosphorylation domain conserved among plant species (Figure 1.2). The sucrose cleaving activity of ZmSUS1 is enhanced by phosphorylation and low pH, causing conformational changes in the protein structure (Hardin *et al*, 2004). At low pH and/or the phosphorylation of ZmSUS1 and in the presence of sucrose, ZmSUS1 predominantly forms tetramers, which are important for promoting membrane association, but in the absence of only sucrose, it forms dimmers, which do not promote membrane association (Duncan and Huber, 2007). Adjacent to the serine residue there is a histidine residue (Figure 1.2). This may account for the seemingly interrelated low pH and phosphorylation properties as the two conditions introduce

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demonstrating the affect of phosphorylation on SuSy activity and sub-cellular localization.

SuSy showed increased activity in tomato fruit membrane fraction in the presence of Ca^{2+} inhibiting reagents (Auguenot *et al*, 2006). Cytosolic SuSy was heavily phosphorylated compared to membrane associated SuSy and its dephosphorylation led to the increased association with the membrane fraction (Winter *et al*, 1997). SuSy expression is altered during prolonged anoxia in an isoform-specific manner, were ZmSUS1 and ZmSH1 show preferential up-regulation under hypoxic and anoxic conditions, respectively (Zeng *et al*, 1998). ZmSH1 phosphorylation is increased in maize root tips under anoxic conditions, but during prolonged anoxia it is hypo-phosphorylated, inducing relocation into the microsomal membrane fraction were it assists in tolerance (Sabbaiah and Sachs, 2001). This is contrary to the suggestion of Duncan and Huber (2007), whereby phosphorylated ZmSUS1 associates with the membrane, which was supported by Hardin *et al* (2004), who proposed that a charged amino acid at S15 might promote membrane association. These results suggest that a mechanism involving protein phosphorylation/dephosphorylation and/or calcium signalling may in part control sub-cellular localization of SuSy isoforms, its activity and stress responses.

Phosphorylation of SuSy also affects its hydrophobicity due to conformational rearrangement of the protein (Hardin *et al*, 2004; Hardin *et al*, 2006). A hydrophobic probe, 4,4'-dianilino-1,1'-binaphthyl-5,5'-disulfonic acid (bis-ANS) showed increased association with dephosphorylated SuSy protein compared to phosphorylated ZmSUS1 (Winter *et al*, 1997) and for soybean SuSy (Zhang *et al*, 1999). The dephosphorylation of SuSy induces hydrophobic properties of the protein thereby promoting membrane association and the phosphorylation of SuSy induces

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hydrophilic properties of the protein thereby promoting cytosol association.

The *in vivo* control of maize SuSy distribution between the soluble and membrane phases is not solely due to changes in its phosphorylation as described above. ZmSUS1 is phosphorylated in both the soluble fraction and the membrane fraction, with hyperphosphorylation in the membrane fraction of the leaf sheath base and hyperphosphorylation in the soluble fraction of the leaf sheath apex (Hardin *et al*, 2004). Therefore, phosphorylation of ZmSUS1 may influence the intracellular distribution differently, dependent upon the particular region of the leaf under consideration. The influence of phosphorylation on ZmSUS1 membrane association may also depend on membrane lipids and/or protein composition, both of which are likely to vary among different organs or tissues studied, taking into account the pH and sucrose levels and the developmental stage and stresses of the organs and tissues studied. It is also important to note that different SuSy family members may be expressed at different times or in different organs. These factors need to be considered when studying SuSy in different plant species.

1.4.2 Phosphorylation and light/dark regulation

Phosphorylation plays an important role in 26S-proteosome mediated degradation of SuSy protein (Hardin *et al*, 2003). The 26S-proteosome-mediated degradation of SuSy is trigger by the CDPK phosphorylation of S15, which is followed by the CDPK phosphorylation of S170 (Hardin *et al*, 2003). Etiolated maize leaves show high levels of SuSy and these levels decrease in deetiolated leaves (Qiu *et al*, 2007). Qiu *et al* (2007) showed that SuSy degradation during deetiolation is selective, red/blue light dependent and is inhibited by free amino acids suggesting the redistribution of amino acids from SuSy to other proteins required for autotrophic metabolism. Qiu *et al* (2007) also showed that the degradation of SuSy during deetiolation does not involve

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26S-proteosome. These findings contradict those of Hardin *et al*, (2003) who showed the involvement of 26S-proteosome. The degradation mechanism proposed by Hardin *et al* (2003) may be used in maize leaves to maintain and regulated optimal levels SuSy proteins, whereas the mechanism proposed by Qiu *et al* (2007) may be used in response to extreme environmental changes. In addition to the role of phosphorylation influencing sub-cellular localization, activity, and stress responses of SuSy, it also influences the degradation SuSy protein. SuSy expression was shown to follow a diurnal rhythm in *Eucalyptus* (Solomon *et al*, 2008). Therefore, modification of SuSy phosphorylation/dephosphorylation could potentially influence its sub-cellular localization, activity, stress responses, and its degradation.

1.4.3 Sucrose synthase modification

The disruption of the N-terminal serine (S11) by site-directed mutagenesis of mung bean SuSy to glutamate (S11E) had a higher affinity to the cleavage reaction with the synthesis reaction remaining unchanged (Nakia *et al*, 1998). The glutamate residue is proposed to be phosphorylation insensitive but introduces a negative charge at the serine site thereby mimicking a phosphorylated wild-type serine residue (Nakai *et al*, 1999). Konishi *et al* (2004) showed that S11E had increased activity in the soluble fraction of the stem compared to the membrane fraction, consistent with the suggestion that the phosphorylation of SuSy promotes cytosol localization. This was in line with the suggestion that the functional properties of S11E mimic those of phosphorylated wild type SuSy, because of the charge introduced by glutamate (Hardin *et al*, 2004; Nakai *et al*, 1999).

Furthermore, the expression of S11E increased callose formation in cotton fibres (Konishi *et al*, 2001) and cellulose in *Acetobacter xylinum* (Nakai *et al*, 1999). In these experiments, the increase in callose and cellulose could have been due to the

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excess UDPG in the cytosol, diverted towards cellulose biosynthesis. Therefore, the increase in cellulose may be due to the production of excess UDP-glucose and not particularly the association of SuSy with the membrane as suggested by Amor *et al* (1995). These findings suggest defined changes in SuSy properties caused by the modification of N-terminal serine residue, linked to possible alteration in carbon allocation especially into cellulose.

Furthermore, the potato Sus1 gene was shown to have a single phosphorylation site at S11 (Sauerzapfe *et al*, 2008), in contrast, to SuSy proteins in other plant species which were shown to have multiple phosphorylation sites (Hardin *et al*, 2003; Zhang *et al*, 1999). Modification of potato Sus1 at the N-terminal serine residue to alanine (S11A) and expressed in yeast cells resulted in SuSy protein with higher affinity for the cleavage reaction. The N-terminal serine residue aspartate modification (S11D) of the potato Sus1 gene expressed in *Escherichia coli* had a higher affinity for the synthesis reaction compared to the potato normal Sus1 gene (Sauerzapfe *et al*, 2008). These experiments were performed to investigate the biocatalytic properties of phosphorylation insensitive potato SuSy recombinant proteins. The mung bean S11E has an affinity for the cleavage reaction whereas the potato S11D has an affinity for the synthesis reaction in *E. coli* cells. It must be noted that the differences observed in the activity of the potato S11A and S11D could be related to the host cells, whereas the differences observed in the activity of the mung bean S11E and potato S11D in *E. coli* could related to the modified amino acid. Furthermore, the potato S11A and S11D modification exhibited altered hexose substrate spectrum (D-psicose and L-fucose) and reduced enzyme activity (Sauerzapfe *et al*, 2008). The phosphorylation/dephosphorylation of the N-terminal serine residue possibly affects SuSy sub-cellular localization, activity, stress responses, protein degradation and its potential substrates. The N-terminal serine phosphorylation domain clearly plays a

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central role in the regulation of SuSy during plant growth and development and is therefore a potential biotechnology target to alter carbon allocation.

1.5 Conclusions

Sucrose synthase is an important sucrose metabolizing enzyme that generates substrates for storage, structural, stress response, and signalling molecules. Therefore, its activity and regulation plays a central role in channelling sucrose carbon to carbon utilizing cellular processes involved in growth and development in plants. The expression levels and activity of SuSy may be regulated differently depending on changing carbon requirements for growth and development in plants.

Sucrose synthase has been shown to associate with the cell membrane possibly to support cellulose biosynthesis suggesting a possible regulatory mechanism for sub-cellular localization. However, the direct mechanisms involved in the channelling of sucrose carbon into cell wall-related biosynthesis molecules are not fully understood. Hetero- and homo-oligomerization of SuSy with other family member proteins has been shown to influence sub-cellular localization of SuSy, as well as phosphorylation, pH and sucrose levels, which are varied in their sub-cellular distribution, dependent on the particular tissue, organ, stage of development and species considered.

SuSy was shown to be phosphorylated at a conserved N-terminal serine residue, which has been linked to the sub-cellular localization of the protein. Modification of this serine residue affects SuSy sub-cellular localization, enzyme activity, and its potential substrates. This review has underlined the complexities associated with the regulatory mechanisms of SuSy. Whether the default state of SuSy is phosphorylated or dephosphorylated is unclear, because this regulatory mechanism is provoked by responses to certain sucrose carbon requirements, which are affected by factors

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relating to growth, development, tissue and species specificities. However, the ability of SuSy to influence carbon allocation has been clearly validated.

This literature survey has reviewed SuSy activity and sub-cellular localization regulatory mechanisms that are potentially represented in the following two models. (A) Multiple SuSy family members are present in plants with possibly redundant or varied functions. These SuSy enzymes may direct sucrose carbon predominantly towards either cell wall-linked or cytosol-linked pathways. Serine phosphorylation or dephosphorylation could be linked to their activity within their respective localities. (B) The phosphorylation of SuSy is responsible for its sub-cellular localization and its activity levels in a direct or an indirect manner together with factors such as sucrose levels, pH levels and oligomerization with other family members to define it either as a membrane- or a cytosol-associated enzyme. These factors may also be involved in the proposed model A.

This study investigates the effect of constitutive expression of normal and modified SuSy protein in *Arabidopsis* and the effect of these constructs on the biology and secondary cell wall chemistry of *Arabidopsis* plants. The *Eucalyptus grandis* sucrose synthase 1 (*EgSuSy1*) gene, and a modified version of the gene, which introduces a neutral amino acid (alanine) to the N-terminal serine phosphorylation residue (S11A) were studied. The neutral amino acid is hypothesized to render the site insensitive to phosphorylation, thereby promoting membrane association, resulting in altered carbon allocation to cell wall related polymer synthesis, specifically cellulose. The effect of *EgSuSy1* and *EgSuSy1-S11A* on secondary cell wall biomass, secondary cell wall chemistry and changes in growth and development were measured.

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CHAPTER 2

Analysis of transgenic *Arabidopsis thaliana* plants overexpressing normal and modified *Eucalyptus grandis* sucrose synthase 1 (*EgSuSy1*) genes

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This chapter is prepared in the format of a manuscript for a peer-reviewed research journal (e.g. *Tree Physiology*). I generated the plant material and analysed all the relevant data related to the completion of this study. Mr Zhou isolated and sequenced the genomic and cDNA copy of the *Eucalyptus grandis* sucrose synthase 1 (*EgSuSy1* - DQ227993) gene. Mrs Jamie-Lee Sauer used this sequence data to amplify the full-length cDNA copy of *EgSuSy1* and cloned it into PCR8/GW/TOPO entry plasmid vector. This entry vector was made available to me for this research study. Prof. Shawn Mansfield (University of British Columbia) performed the cell wall chemistry analysis of the transgenic *Arabidopsis* plants and Dr. James Wesley-Smith (University of KwaZulu Natal) developed the transmission electron microscopy images and measurements of the secondary cell wall thickness for this study. Prof. Myburg provided the idea for the project, supervised the experimental and analysis work, and attained the funding for the project. Prof Myburg and Dr Naidoo, the co-supervisor critically read this research chapter and suggested changes to improve clarity and style.

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Abstract

The involvement of sucrose synthase (SuSy: EC 2.4.1.13) in the reversible conversion of sucrose to UDP-glucose (UDPG) and fructose enables UDPG and fructose to enter cytosolic and cell wall related metabolic pathways responsible for growth and development of commercially important tissues such as secondary xylem or wood. The sub-cellular localization of SuSy is thought to be regulated in part by a conserved N-terminal non-catalytic serine phosphorylation domain. The phosphorylation and dephosphorylation of this domain is proposed to affect the activity of SuSy protein in the cytosolic and membrane fractions, respectively by affecting its hydrophobicity. In order to determine the effect of the modified phosphorylation insensitive *EgSuSy1-S11A* and the unmodified (*EgSuSy1*) protein on carbon allocation, these constructs were overexpressed in *Arabidopsis* plants under the control of a double 35S CaMV promoter. The interfascicular fibre cells of *EgSuSy1* and *EgSuSy1-S11A* transgenic lines showed reduced lumen diameter and the plants had increased biomass attributed to the increased number of interfascicular fibre cells compared to Col-0 control plants. The secondary cell walls of interfascicular fibre cell were 33% thinner in *EgSuSy1* compared to Col-0, whereas *EgSuSy1-S11A* transgenic lines remained unchanged. Furthermore, no significant changes were observed in the cell wall chemistry of the inflorescence stem of Col-0, *EgSuSy1* and *EgSuSy1-S11A* lines. To conclude, *EgSuSy1* and *EgSuSy1-S11A* constructs affected plant biomass by altering its growth and development either than the composition cellulose in the cell wall chemistry.

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2.1 Introduction

Carbon allocation processes are vital for plant growth and development. These processes also have commercial importance for the forest products industry as they influence cellulose production and thus wood properties (Plomion *et al*, 2001). The carbon biomass of wood is comprised primarily of cellulose, lignin and hemicelluloses deposited in the secondary cell walls of xylem tissue (Plomion *et al*, 2001). This deposition may be associated with the increased number and differential expression of carbohydrate active enzyme (CAZyme) genes in trees relative to herbaceous plants (Geisler-Lee *et al*, 2006). These changes in expression profiles results in altered carbon allocation patterns in trees forming wood with altered chemical and physical properties (Andersson-Gunneras *et al*, 2006; Mellerowicz *et al*, 2008; Plomion *et al*, 2001).

Sucrose is the primary form by which carbon is translocated from source tissues (leaves) to sink tissues (wood) via the phloem vascular tissue (Lalonde *et al*, 2004; Winter and Huber, 2000). Sucrose synthase (SuSy: EC 2.4.1.13), a member of the glycosyltransferase 4 (GT4) CAZyme family, catalyzes the reversible conversion of sucrose and UDP to form UDP-glucose (UDPG) and fructose. This reaction allows photosynthetic and non-photosynthetic sucrose to enter multiple cellular metabolic pathways via UDPG and fructose (Winter and Huber, 2000). Different SuSy family members may divert sucrose carbon to different sink tissues (Barratt *et al*, 2001; Fu and Park, 1995; Haagenson *et al*, 2006; Matic *et al*, 2004).

Levels of SuSy activity have been correlated with starch synthesis (Dejardin *et al*, 1997), cell wall biosynthesis (Chourey *et al*, 1998; Coleman *et al*, 2009; Curatti *et al*, 2008; Nakai *et al*, 1999; Ruan *et al*, 1997; Salnikov *et al*, 2003,), and overall sink

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strength (Tang *et al*, 1999a; Zrenner *et al*, 1995). SuSy expression levels were also correlated with yield gains in rice (Counce and Gravois, 2006) and overall plant growth and development (Coleman *et al*, 2006).

Under bending tension, angiosperm tree species develop tension wood (Mellerowicz *et al*, 2008). Tension wood is defined by the deposition of a gelatinous layer with increased crystalline cellulose in secondary xylem fibre cell walls (Mellerowicz *et al*, 2008). This specialized development is correlated with increased expression of SuSy (Andersson-Gunneras *et al*, 2006). Coleman *et al* (2009) recently confirmed the increase in crystalline cellulose production in hybrid poplar trees when overexpressing cotton SuSy. Therefore, SuSy is a good candidate to alter sucrose carbon allocation into secondary xylem fibre cell wall polymers such as cellulose.

SuSy was initially thought of as a cytosol associated protein, but Amor *et al* (1995) demonstrated its association with the membrane fraction in cotton fibre cells that are actively producing cellulose. The association of SuSy with the cell membrane was proposed to reduce competition for UDPG between cytosolic and cell wall related biosynthetic pathways (Haigler *et al*, 2001; Konishi *et al*, 2001; Salnikov *et al*, 2001) and also to ensure rapid turnover of UDP, which is a completive inhibitor of UDPG (Ross *et al*, 1991). SuSy was also shown to associate with organelle membranes supporting growth and development (Etxeberria and Gonzalez, 2003; Persia *et al*, 2008; Sabbaiah *et al*, 2006). The regulatory mechanisms involved in the sub-cellular localization of SuSy protein have been the focus of research for the past decade (Anguenot *et al*, 2006; Duncan *et al*, 2006; Duncan *et al*, 2007; Nakai *et al*, 1998).

SuSy is phosphorylated at a non-catalytic N-terminal serine phosphorylation domain conserved among monocot and dicot species and mediated by a calcium dependent protein kinase (Huber *et al*, 1996; Zhang *et al*, 1999). Studies of the sub-cellular

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localization of SuSy have alluded to the involvement of the N-terminal serine phosphorylation domain. Anguenot *et al* (2006) demonstrated that a protein kinase inhibitor increased the membrane activity of SuSy in developing tomato fruits, whereas Winter *et al* (1997) demonstrated decreased membrane activity of SuSy due to a phosphatase inhibitor. Zhang *et al* (1999) showed that phosphorylation decreased the surface hydrophobicity of SuSy by influencing the conformation of the protein. The sub-cellular localization of SuSy was also shown to be affected by pH levels, sucrose levels (Hardin *et al*, 2004) and hetero- and homo-oligomerization with other SuSy family members (Duncan *et al*, 2006). These findings suggest increased activity of SuSy in the membrane fraction due to its dephosphorylation, perhaps supporting the observation by Amor *et al* (1995) of increased membrane activity of SuSy as it diverts sucrose carbon towards cell wall related pathways such as cellulose biosynthesis. The regulatory responses whether by phosphorylation, pH levels, sucrose levels or hetero/homo-oligomers of SuSy differed between SuSy family members (Duncan *et al*, 2006; Duncan *et al*, 2007) and environment exposure (Zeng *et al*, 1998; Bieniawask *et al*, 2007). SuSy family members respond differently to plant growth and development, and tissue specificities (Barratt *et al*, 2001; Fu and Park, 1995; Haagenson *et al*, 2006; Matic *et al*, 2004).

Site-directed modification of the conserved serine phosphorylation site results in altered properties of the SuSy protein. These include changes in the cleavage or synthesis reaction rate (Nakai *et al*, 1998; Nakai *et al*, 1999; Sauerzapfe *et al*, 2008) and alteration in its substrate spectrum (Sauerzapfe *et al*, 2008). This modification showed potential in altering carbon allocation patterns into cell wall related polymers such as cellulose (Konishi *et al*, 2001; Konishi *et al*, 2004; Nakai *et al*, 1999). The modification of the N-terminal serine residue of the mung bean SuSy to a charged amino acid, glutamate - S11E (Nakai *et al*, 1998) was proposed to mimic the

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properties of the phosphorylated wild type SuSy protein (Konishi *et al*, 2001; Nakai *et al*, 1999). Although S11E activity in transgenic poplar trees was demonstrated in both the membrane and the soluble fraction, there was a significant increase in activity in the soluble fraction of stem tissue compared to the membrane fraction (Konishi *et al*, 2004). This is consistent with the suggestion that phosphorylation of SuSy specifically at the N-terminal serine residue may in part influence its sub-cellular localization by promoting cytosolic association.

The relative phosphorylation and dephosphorylation of SuSy seem to vary spatially. This means that phosphorylation and dephosphorylation of SuSy differed from the apex to the base of a maize leaf sheath (Hardin *et al*, 2004). SuSy phosphorylation and dephosphorylation differed temporally within Japanese pear fruit. This means that the phosphorylation and dephosphorylation of SuSy changes from early development to the ripening stages of the fruit (Tanase *et al*, 2000). This suggests that phosphorylation may influence SuSy intracellular distribution differently, dependent upon the particular region or tissue under consideration, which may differ in membrane lipids and/or protein composition (Hardin *et al*, 2004).

Arabidopsis has been well characterized as model for lignin studies (Jones *et al*, 2001; Goujon *et al*, 2003) and secondary cell wall biosynthesis (Chaffey *et al*, 2002; Zhong *et al*, 2008). No study has reported the net increase of cellulose in *Arabidopsis* secondary cell walls as a function of carbon allocation influenced by the expression of SuSy. This study evaluated the effect of constitutive expression of *Eucalyptus grandis* sucrose synthase 1 (*EgSuSy1*) gene and a modified version of the gene with a phosphorylation insensitive neutral amino acid (alanine) at the N-terminal serine residue (*EgSuSy1-S11A*). We hypothesised that *EgSuSy1-S11A* will have increased intracellular distribution towards the membrane fraction, due to the absence of a

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charged residue at S11. Under this hypothesis, the aim was to determine whether the overexpression of *EgSuSy1* will increase the sink strength in *Arabidopsis* inflorescence stems by allocating sucrose carbon into secondary cell walls and whether *EgSuSy1-S11A* will enhance this allocation of sucrose carbon more specifically into cellulose resulting from the increased membrane association hypothesised by the site-directed modification. We showed that the *Arabidopsis* Col-0 plants overexpressing *EgSuSy1* and *EgSuSy1-S11A* had increased inflorescence stem biomass, due to increased number of interfascicular fibre cells. The interfascicular fibre cells had significantly reduced secondary cell wall thickness in *EgSuSy1* lines and reduced lumen sizes in *EgSuSy1* and *EgSuSy1-S11A* lines compared to Col-0. In addition, we showed that the percentage secondary cell wall sugars content remain unchanged in the *EgSuSy1* and *EgSuSy1-S11A* transgenic lines.

2.2 Materials and Methods

2.2.1 Plant materials and growth conditions

Immature xylem scrapings were obtained from a 4-year-old *Eucalyptus grandis* clone (kindly provided by Mondi Forestry Kwambonambi, KwaZulu Natal, South Africa). *Arabidopsis thaliana*, Columbia (Col-0) ecotype (Nottingham Arabidopsis Stock Centre – N1093), was transformed with the unmodified and modified *Eucalyptus* sucrose synthase 1 (*EgSuSy1*) gene. The Col-0 plants were grown under controlled light conditions of 16 hour light (1000 lum/sqf) and 8 hour dark (2 lum/sqf) at 22°C with relative humidity of 25% - light period and 45% - dark period (HOBO® Datalogger – BoxCar®) until flower development. The Col-0 plants were fertilized weekly with Multifeed-19:8:16 Classic (http://www.plaaskem.co.za/) as a soil drench. Identical growth conditions were used for transgenic and control plants. The ages of

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the plants refer to the period post transplantation, therefore excluding the two weeks on selection plates, unless specified otherwise.

2.2.2 Bacterial stains and vector materials

The bacterial lab strain *Escherichia coli* - DH5α was used for cloning and storing of glycerol stocks of vectors and plasmids at -80°C. *Agrobacterium tumefaciens* – strain LBA4404, was used for transformation of *Arabidopsis* plants. The Gateway® compatible pCR8/GW/TOPO entry vector (Invitrogen – Appendix A1), was used to TA clone the *EgSuSy1* and the modified *EgSuSy1* genes. The Gateway® compatible pMDC32 (Appendix A2) destination vector plasmid, with double 35S cauliflower mosaic virus (CaMV) constitutive promoter (Curtis and Grossnikklaus, 2003), was used for expression of the transgenes in Col-0 plants.

2.2.3 Phylogenetic analysis and site-directed mutagenesis of *EgSuSy1*

Molecular Evolutionary Genetic Analysis (MEGA 3.1) software was used to develop an unrooted phylogenetic grouping of SuSy full-length amino acid sequences from multiple species. The amino acid sequences were aligned using the MEGA 3.1 ClustalW pairwise multiple alignment tool. The phylogenetic tree was constructed using MEGA 3.1 Neighbour-Joining analysis at a bootstrap value of 5000 with the Poisson correction model, which included all substitutions with all gaps removed.

Total RNA was extracted from immature xylem tissue using a modified CTAB-based method (Chang *et al*, 1993) and this was followed by first strand cDNA synthesis using the ImProm-II™ Reverse Transcription System (Promega). Zhou *et al*, (2005, unpublished) used genome walking and rapid amplification of cDNA ends (RACE) to isolate a full-length cDNA copy of the *EgSuSy1* gene (GenBank ID, DQ227993), which was cloned into the pCR8/GW/TOPO entry vector. The Primer X automated

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Mutagenesis Primers	**Sequence**
Reference sequence	5' CATGTTGACTCGAAGCCAC**AGC**CTTCGCGAGCGTTTGGAC 3'
EgSuSy1-S11A-F	5' CATGTTGACTCGAAGCCAC**GCC**CTTCGCGAGCGTTTGGAC 3'
EgSuSy1-S11A-R	5' GTCCAAACGCTCGCGAAG**GGC**GTGGCTTCGAGTCAACATG 3'

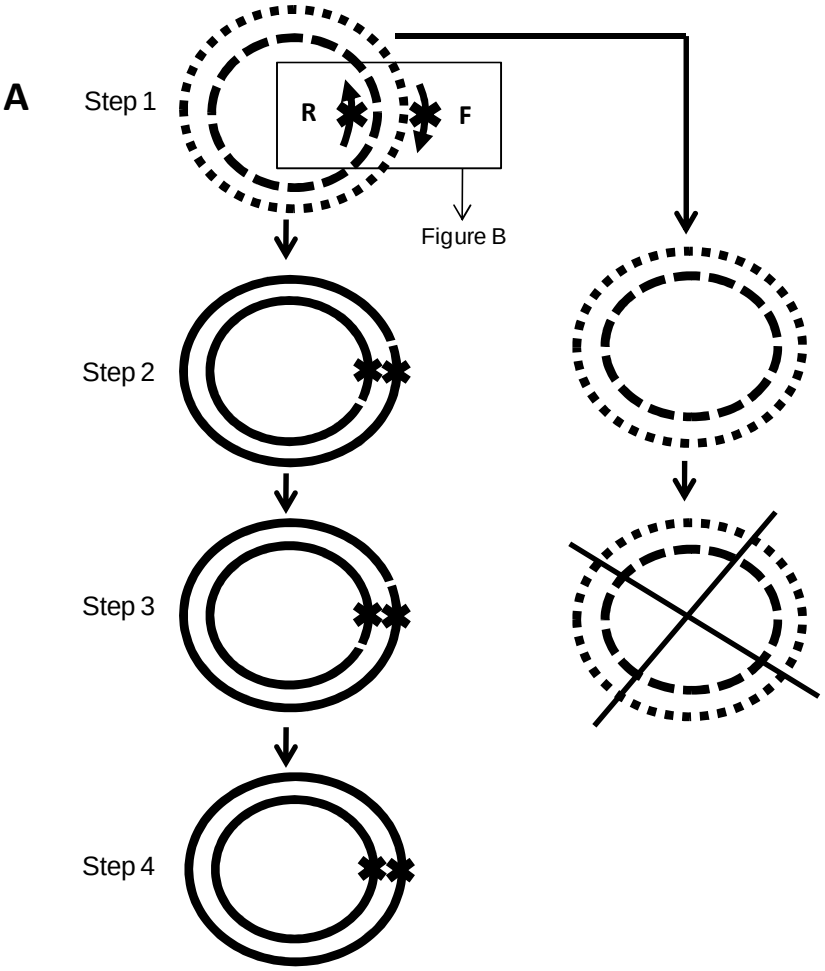
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T4 DNA ligase buffer and incubated at 16°C overnight. The re-circularized plasmids containing the modified *EgSuSy1* were transformed into competent DH5α *E. coli* cells, through heat-shock at 42°C for 50 sec in a water-bath and growth was observed under 50 µg/ml spectinomycin selection on Luria-Bertani (LB) media (1.0 g Tryptone, 0.5 g Yeast extract, 1.0 g NaCl, and 1.5 g Agar per 100 ml autoclaved media). Full-length DNA sequencing (Macrogen, USA) from discontinuous forward and reverse strand contigs was performed (Appendix B) on four colonies, one from each site-directed mutagenesis PCR sample to confirm the *EgSuSy1-S11A* modification and possible background PCR mutations.

The Gateway® LR clonase™ II Enzyme Mix kit (Invitrogen) was used to LR clone *EgSuSy1* and *EgSuSy1-S11A* from pCR8/GW/TOPO into the Gateway® compatible pMDC32 vector. The LR reaction contained 0.5 U of LR clonase enzyme, a 1:3 concentration ratio of entry vector (Figure 2.2A) and destination vector (Figure 2.2B), and 3 µl LR reaction buffer. The mixture was incubated at 16°C overnight. After incubation, 1 µl of Proteinase K (Invitrogen) was added to stop the LR clonase reaction. The LR clonase reaction mixture was used to transform competent *E. coli* DH5α cells by heat-shock. The LR reaction mix transformed *E coli* DH5α were incubated at 37°C overnight on LB selection media containing 50 µg/ml kanamycin to select for *E. coli* DH5α colonies transformed with the LR recombined plasmid (Figure 2.2C). The pMDC32-*EgSuSy1* and pMDC32-*EgSuSy1-S11A* constructs were subsequently transformed into the *Agrobacterium* LBA4404 strain under 50 µg/ml kanamycin and 50 µg/ml rifampicin selection on YEP media (1.0 g Peptone, 0.5 g NaCl, 1.0 g Yeast extract, and 1.5 g Agar per 100 ml autoclaved media).

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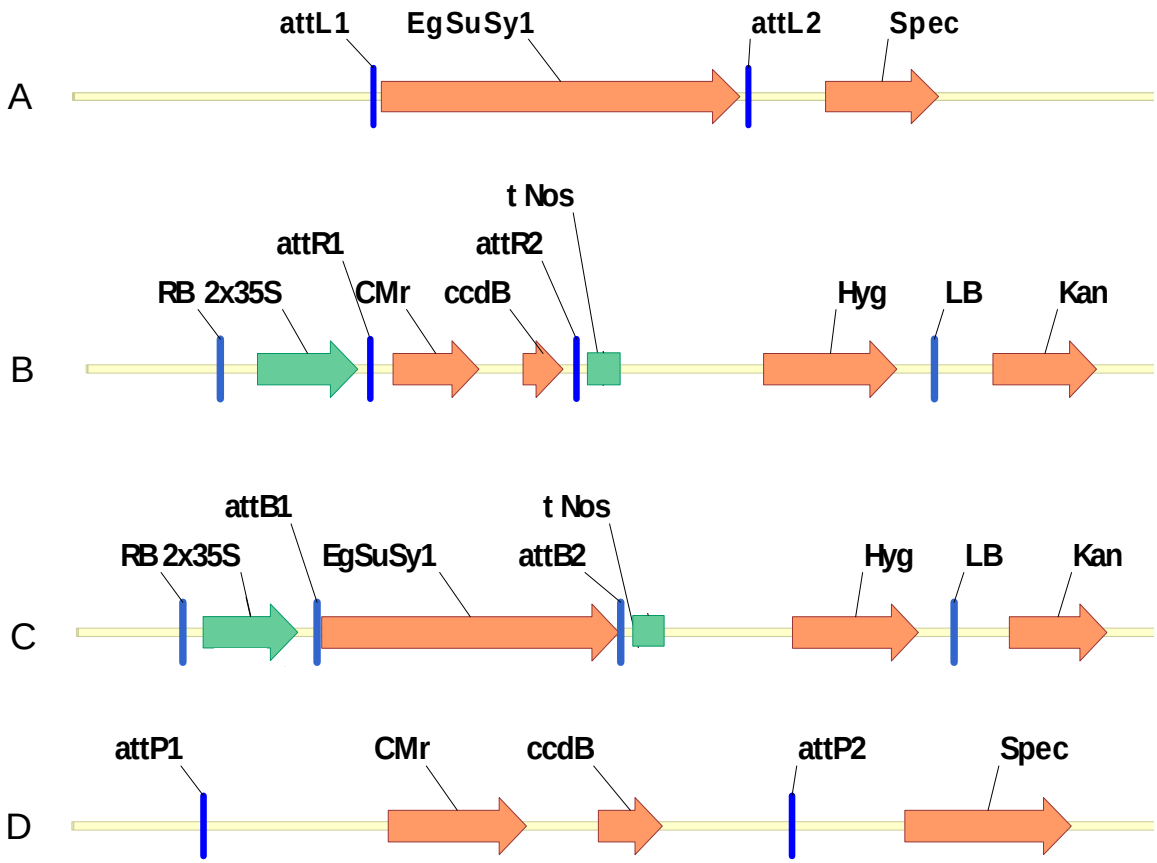
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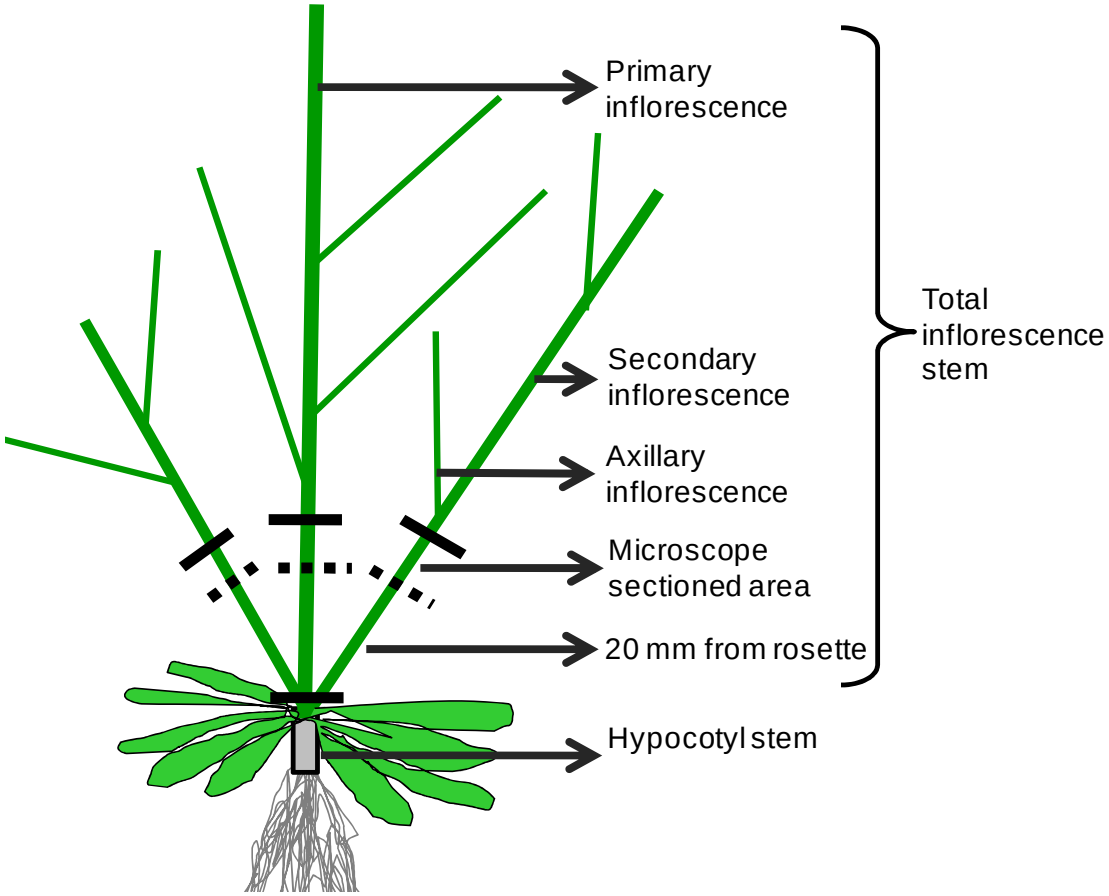
B

```
EgSuSy1-S11A-R      3′ GTACAACTGAGCTTCGGTGCGGGAAGCGCTCGCAAACCTG 5′
EgSuSy1-Strand-F  5′... CATGTTGACTCGAAGCCACAGCCTTCGCGAGCGTTTGGAC... 3′
EgSuSy1-Strand-R  3′... GTACAACTGAGCTTCGGTGTCGGAAGCGCTCGCAAACCTG... 5′
EgSuSy1-S11A-F      5′ CATGTTGACTCGAAGCCACGCCCTTCGCGAGCGTTTGGAC 3′
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contained 50 µg/ml kanamycin and 50 µg/ml rifampicin. The 200 ml culture was centrifuged for 5 min at 5000 rpm, followed by re-suspension of the pellet with 5% (w/v) sucrose to a total volume of 50 ml and OD_{600} ≈ 1.0 (NanoDrop® ND-1000 spectrophotometer). To facilitate effective infection of the *Agrobacterium*, the final dipping solution contained 0.05% (v/v) of surfactant Silwet-L77 (Lehle Seeds).

During floral dip transformation, only the immature flowers were submerged into the *Agrobacterium* solution for 15 to 20 sec. Contact of the *Agrobacterium* transformation solution with the rosette leaves was avoided, because this would lead to the rotting of the rosette leaves and prevent further development of the transformed plants. The plants were gently agitated to remove excess *Agrobacterium* and then placed on their side to avoid run-off of bacteria onto the rosette leaves. After leaving the plants overnight on their side in the dark, the plants were placed upright. The entire transformation procedure was repeated a week later to optimise transformation efficiency. Transformed seeds were collected at maturity by cutting the total inflorescence stems and drying at room temperature in brown paper envelopes.

2.2.5 Analysis of transgenic lines

Transformed seeds were dried for two weeks at room temperature and then stored at 4°C. Transformed seeds were sterilized in 70% (v/v) EtOH, with gentle agitation for 5 min followed with 15 min agitation in 10% (v/v) NaOCl with equal volume 0.1% (v/v) Triton X-100 solution. The transformed seeds were then rinsed three times under the laminar flow with dH_2O and re-suspended in 0.1% (w/v) agar. The resuspended seeds were plated evenly to avoid clumps on 0.7% (w/v) minimal agar plates, containing 1 mM KNO_3, 20 µg/ml hygromycin and 100 µg/ml cefotaxime. Plated seeds were placed under laminar flow overnight to settle. Selective growth on plates sealed with parafilm was allowed for a minimum of two weeks. The plates were

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incubated under continuous light at 22°C. Putative positive transgenic seedlings were transplanted onto Jiffy® pots (http://www.jiffypot.com) and then grown under similar growth conditions as mentioned above. The seeds of all the transgenic lines were advanced from generation one (T1) to T3 through selfing, and were sterilized and selected in each generation according to the above-mentioned procedures.

A PCR-based screening of T-DNA insertion into the genome of T1 plants was performed to screen for false positive transgenic plants from hygromycin selection plates. At week four on Jiffy® pots, genomic DNA was extracted from leaves of T1 plants using the Nucleospin® Plant II extraction kit (Macherey-Nagel). A standard PCR was performed to screen for pMDC32 T-DNA in T1 plants. The PCR protocol contained 20 ng genomic DNA, 1X ExSel PCR buffer with 25 mM $MgCl_2$ (Southern Cross Biotechnology), 0.2 mM dNTPs, 0.4 µM of each primer (EgSuSy1-Internal-F and pMDC32-R – Table 2.2; Appendix A3), and 0.8 U ExSel Taq polymerase (Southern Cross Biotechnology), adjusted to 20 µl total volume with dH_2O. The PCR program was as follows; 94ºC for 5 min initial step and then 30 cycles of 94°C for 30 sec denaturation, 62°C for 30 sec annealing and 72°C for 150 sec primer extension. T1 plants confirmed to contain the T-DNA insert were advanced to T2. The 5’ end of the full-length PCR product from genomic DNA was sequenced (Macrogen, USA) using the EgSuSy1-Internal-R primer (Table 2.2; Appendix A3) to confirm the S11A modification.

2.2.6 Reverse transcriptase (RT)-PCR analysis of transgenic lines

Fresh total inflorescence stems from 6-six-week-old Col-0 and T2 positive transgenic lines, with cauline leaves, flowers, and siliques (Figure 2.3), were collected into liquid nitrogen and stored at -80°C. Total RNA was extracted using the small-scale RNA isolation protocol using the Concert™ Plant RNA Reagent according to the

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manufacturer's specifications (Invitrogen). To eliminate residual genomic DNA contamination in the RNA samples, DNAse treatment was performed as follows: 20 U DNAse I (Roche), 40 U RNAse inhibitor (Promega), 1X reverse transcription buffer (Promega) and 5 mM $MgCl_2$ were added to the 20 µl dH_2O eluted total RNA sample and the reaction volume adjusted to 50 µl with RNAse free dH_20 (Fermentas). The reactions were incubated at 37°C for 30 min. Following the DNAse digestion, the RNA samples were purified using the RNeasy mini kit (Qiagen).

First strand cDNA synthesis was performed from total RNA using the ImProm-II™ Reverse Transcription System (Promega), using a Poly-dT primer anchored on the 3' end Poly-A tail of the RNA. The Poly-dT binding reaction was prepared from 0.2 µM total RNA, 10 µM Poly-dT primer, and the reaction volume adjusted to 5 µl with dH_20. The reaction was incubated at 70°C for 5 min, and place immediately on ice for 5 min. The synthesis reaction for the first strand cDNA contained the 5 µl Poly-dT bonded RNA as template, 20 U RNAse inhibitor (Promega), 0.5 mM dNTPs, 1X reverse transcription buffer (Promega), and 3 mM $MgCl_2$, in a total reaction volume of 20 µl adjusted with RNAse free dH_20 (Fermentas). The samples were incubated at 25°C for 5 min, 4°C for 60 min, and 72°C for 15 min. Genomic DNA contamination in the cDNA was tested by standard PCR using gene specific-primers (Table 2.2) for *AtACTIN2* (At3G18780), which flank an intron region.

2.2.7 Growth and development of transgenic lines

Growth rates of primary inflorescence stems (Figure 2.3) of Col-0 and T3 transgenic lines were determined from week 4 to week 6, followed by end-point analysis of fresh and dry weight of total inflorescence stems. At week 3, the primary inflorescence stem growth was initiated and subsequent measurements were taken at week 4, 5 and 6. Measurements of the stems were made using the ImageTool analysis software

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(http://ddsdx.uthscsa.edu/dig/itdesc.html), from images taken with a Canon EOS 350D digital camera calibrated with a standard 300 mm ruler placed beside the flattened and straightened primary inflorescence stems.

Table 2.2 Primer sequences used to target *EgSuSy1*, *EgSuSy1-S11A* and *AtACTIN2* from cDNA of T2 lines to confirm insertion events and cDNA purity.

Gene Primers	Sequence
EgSuSy1-Start-F[a]	5' ATGGCTGATCGCATGTTGAC 3'
EgSuSy1-Stop-R[a]	5' TTACTCGACAGCCGGAGGAA 3'
EgSuSy1-Internal-F[a]	5' GAACCTGACTGCCGTGTATG 3'
EgSuSy1-Internal-R[a]	5' GTCATGGAAGAGCTTAGCGGAGAG 3'
ACT2-F	5' TCTCGTTGTCCTCCTCACTT 3'
ACT2-R	5' AATCCAGCCTTCACCATACC 3'
pMDC32-F[a]	5' GTTTTCCCAGTCACGAC 3'
pMDC32-R[a]	5' GGATAACAATTTCACACAGG 3'

a - Primer position on vector plasmid are shown in Appendix A3

Growth rate and weight measurements were taken from five plants per line. At week 6, fresh stem weight analysis was performed from total fresh inflorescence stem (Figure 2.3) collected without siliques, flowers, and cauline leaves. The same samples were dried at room temperature for a week to determine dry stem weight.

2.2.8 Microscope analysis of secondary cell walls

Light microscopy analysis was performed on sections of primary inflorescence stems of 6-week-old Col-0 and T3 transgenic lines (Figure 2.3). Stems were embedded in Shandon Cryomatrix™ (Thermo Electron Corporation, USA) for 10 µm cross-sectioning using a Leica cryotome (model CM1100) followed by phloroglucinol-HCl staining (1% w/v phloroglucinol, Sigma) in HCl/ethanol (25/75, v/v, Speer, 1987). After staining, slides were viewed under a Zeiss light microscope and images were captured using the Axiovision 4.7.1 software at 20X and 40X magnification.

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Interfascicular fibre cell lumen diameters were measured from the widest part of each cell of 6-week-old Col-0 and T3 transgenic lines (Figure 2.15) using the ImageTool analysis software, calibrated using the measurement bar displayed by the Axiovision 4.7.1 software at 40X magnification.

Touluidine blues staining of the cross sections of the inflorescence stems regions (Figure 2.3) was done by placing the stem cross sections on class slides and allowing them to dry on a hot plate. A drop of toluidine blue stain was placed on the sections whilst they were in contact with the hot plate and after 15 sec the stain was washed off with running water and allowed to dry before being covered with a cover-slit for imaging under a light microscope.

The number of interfascicular fibre cells was obtained from counting the cells in each interfascicular region between the vascular bundle regions from the outer most layer to the last layer of complete thick walled fibre cells towards the pith area (Appendix G). To distinguish between the vascular bundle fibre cells and interfascicular fibre cells region a vertical file of interfascicular fibre cells closest to the phloem region was used as the end-point of the interfascicular region.

Transmission electron microscope (TEM) analysis was performed on 6-week-old T3 transgenic lines and Col-0 control plants inflorescence stems (Figure 2.3) to quantify secondary cell wall thickness of vascular bundle (vessels and fibre) cells (Appendix E) and interfascicular fibre cells (Appendix F). This analysis was performed at the University of KwaZulu Natal, Durban, South Africa.

2.2.9 Cell wall chemistry analysis of transgenic lines

A modified micro-Klason/HPLC analysis method (Huntley *et al*, 2003), was used to analyse changes in percentage total cell wall acid-soluble and acid-insoluble lignin

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content per 100 mg dry stem weight associated with the ectopic expression of *EgSuSy1* and *EgSuSy1-S11A*. In brief, 100 mg of freeze-dried stems were ground to pass a 40-mesh screen using a Wiley mill, followed by a Soxhlet extraction with acetone for six hours. The extracts were then hydrolysed with 72% (v/v) H_2SO_4 for 2 hours, and hydrolysed in 4% (v/v) H_2SO_4 for 1 hour at 121°C. The non-hydrolysed acid-insoluble lignin extracted components were measured gravimetrically, while the filtrate was analysed for acid-soluble lignin by absorbance at 205 nm according to the TAPPI Useful Method UM250.

The percentage total sugar composition of each sample filtrate, expressed in the anhydro-form, were determined by high performance anion-exchange chromatography on a CarboPac PA-1 column using a Dionex High Pressure Liquid Chromatography (HPLC) system (Dionex, Sunnyvale, CA, USA) equipped with a pulsed amperometric detector. Two technical replicates of 100 mg dry stem samples were measured for cell wall sugars. This analysis was performed at the University of British Columbia, Vancouver, Canada.

2.2.10 Reverse Transcriptase quantitative PCR (RT-qPCR) analysis of transgenic lines

Total fresh inflorescence stems from 6-week-old Col-0 and T3 positive transgenic lines were harvested without flowers, siliques and cauline leaves, placed directly into liquid nitrogen and then stored at -80°C (Figure 2.3). Samples were collected 8 hours into the light period and 4 hours into the dark period, representing the mid-times of the light (16 hours) and dark (8 hours) growth room photoperiods. Three biological replicates were collected per line (three *EgSuSy1* lines and three *EgSuSy1-S11A* lines) including Col-0 control plants; with two bulked individuals per biological replicate totalling 42 independent samples (Table 2.3). Total RNA was extracted and first

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Transgene	Lines	Bio. Rep. 1	Bio. Rep. 2	Bio. Rep. 3
Dark period				
N/A	Col-0	*A1*	*A2*	*A3*
	1A	*B1*	*B2*	*B3*
EgSuSy1-S11A	1C	*C1*	*C2*	*C3*
	1D	*D1*	*D2*	*D3*
	2C	*E1*	*E2*	*E3*
EgSuSy1	2D	*F1*	*F2*	*F3*
	2G	*G1*	*G2*	*G3*
Light period				
N/A	Col-0	*H1*	*H2*	*H3*
	1A	*I1*	*I2*	*I3*
EgSuSy1-S11A	1C	*J1*	*J2*	*J3*
	1D	*K1*	*K2*	*K3*
	2C	*L1*	*L2*	*L3*
EgSuSy1	2D	*M1*	*M2*	*M3*
	2G	*N1*	*N2*	*N3*

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Output raw data was initially analysed using the LightCycler® 480 1.5.0 software (Roche) to obtain crossing points (quantification cycle values; Cq) of the target and reference genes. Standard curves were generated using a serial dilution of 10^{-1}, 10^{-2} and 10^{-3} of bulked cDNA from a single biological replicate (i.e 14 samples) representing the light/dark period across all transgenic lines and Col-0.

The pre-analysed data with Cq values from LightCycler® 480 1.5.0 were imported into qBasePlus 1.2 (http://www.biogazelle.com/site/products/qbaseplus), for further analysis as outlined in Hellemans *et al* (2007). The target-specific amplification efficiency analysis of qBasePlus was used to analyse relative expression levels of target and reference genes. The parameters for qBasePlus quality control calculation for reference genes were set at 0.5 for the normalization stability (M) and 0.2 for the coefficient of variance (CV). These thresholds represent parameters for targeted reference genes with low heterogeneous expression (Hellemans *et al*, 2007). The technical reproducibility and negative control thresholds were set at 1.0 and 5.0 respectively. Output raw data from qBasePlus were exported into an Microsoft Office Excel 2007 worksheet, for display and statistical analysis of the RT-qPCR results.

2.11 Statistical analysis

Microsoft Office Excel 2007 data analysis tools, single factor ANOVA analysis at 0.05 significance level was performed to test the significance of variation between the measured phenotypes of Col-0, *EgSuSy1* and *EgSuSy1-S11A* transgenic lines. This was followed by performing a two-tailed student t-test assuming equal variance at 0.05 significance level, which is available in Microsoft Office Excel 2007. The t-test was used to determine the significant differences between Col-0 and *EgSuSy1*, Col-0 and *EgSuSy1-S11A* and *EgSuSy1* and *EgSuSy1-S11A.*

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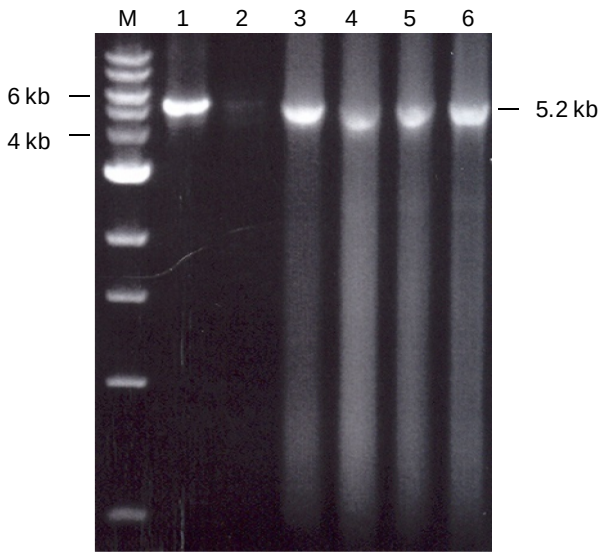
Gene names	Gene code	GeneBank ID	Primer sequence
Target			
Amylase 3	*AtAMY3*	At1G69830	5' CCTTGTAGTCTCTGCCTTCA 3' 5' AGCGGTCTTCTTCGACCATA 3'
Sucrose synthase 1	*AtSUS1*	At5G20830	5' GGTGATCAGGCTGCTGATAC 3' 5' CCTCAAGACGGTCAAGGTTC 3'
Sucrose synthase 4	*AtSUS4*	At3G43190	5' ATGGTGACAAGGCAGCTGAG 3' 5' AGCAAGAGGAACAGCTTGAG 3'
Sucrose phosphate synthase	*AtSPS*	At1G04920	5' ATGTGGCGAACATGTACGTG 3' 5' ACCGGTGAATCAACCTTGAG 3'
Cinnamoyl-CoA reductase 1	*AtCCR1*	At1G15950	5' GATTCTTGCGATGCTGAAGG 3' 5' TCCGGACGTTATCTCCTAGC 3'
Cellulose synthase	*AtCesA8*	At4G18780	5' GAGTGTCCTCGAGCTTCGAT 3' 5' CGACGACTCCGACCAAGTTA 3'
E. grandis sucrose synthase 1	*EgSuSy1*	DQ227993	5' CGGCCGAGCTTCTTGTAGAC 3' 5' AGCCGGAGGAACAGACTGTG 3'
Reference			
Ubiquitin 10	*AtUBQ10*	At4G05320	5' CAAGGAAGGTATTCCTCCGG 3' 5' GACCAGCAGAGGTTGATCTT 3'
Ubiquitin 5	*AtUBQ5*	At3G62250	5' GGTGGTGCTAAGAAGAGGAA 3' 5' TCGATCTACCGCTACAACAG 3'
Actin 2	*AtACT2*	At3G18780	5' TCATACGGTCAGCGATACCT 3' 5' CATCACGATTGGTGCTGAGA 3'
Elongation factor 1	*AtELF1*	At1G07920	5' GTTGAGATGCACCACGAGTC 3' 5' TCCTTACCAGAACGCCTGTC 3'
Tubulin 4	*AtTUB4*	At5G44340	5' GAGCGAACAGTTCACAGCTA 3' 5' GCTGCTTGCTTACACAGCTT 3'
Tubulin 5	*AtTUB5*	AtG20010	5' ACCTCACAGCCTCTGCTATT 3' 5' AACATAGCCGTGAACTGCTC 3'
Tubulin 6	*AtTUB6*	At5G44340	5' GTCAAGCGTCTGTGACATAG 3' 5' TCATACTCGCCTTCGTCATC 3'

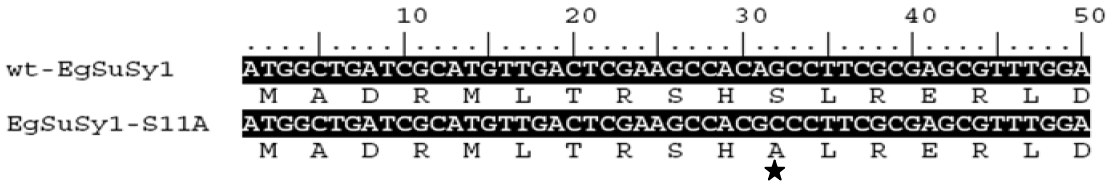
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92.7% consensus positions and 54.3% identical positions as determined by Invitrogen Vector NTI 9.1 - AlignX (Appendix H).

Codon-specific modification of the *EgSuSy1* transgene was performed to generate a phosphorylation insensitive N-terminal non-catalytic serine phosphorylation domain, which we hypothesised would cause the SuSy protein to have increased membrane association. The site-directed mutagenesis strategy was successfully used to modify the N-terminal non-catalytic serine residue of the *EgSuSy1* gene to alanine. The circularized pCR8/GW/TOPO-*EgSuSy1* plasmid (Figure 2.4), confirmed the expected plasmid size after the site-directed mutagenesis procedure. DNA sequencing of the 5'end of the transgene confirmed only one colony with the site-directed mutation of *EgSuSy1-S11A* (Figure 2.5) from the reactions in Figure 2.4. The success rate of the Quick-change® site-directed mutagenesis kit (Invitrogen) is reported at >80%. We observed lower success rates using the current modified method; specifically the Quick-change® method used 100 µM of primers and *Pfu Turbo* DNA polymerase, whereas we used 0.4 µM of primer and standard *Pfu* DNA polymerase (Section 2.2.3). Alignment of the conserved N-terminal non-catalytic phosphorylation domain of the predicted amino acid sequence confirmed the successful mutation of the conserved N-terminal serine residue (Figure 2.6). However, full-length sequencing of the *EgSuSy1-S11A* transgene subsequently revealed three background mutations E18K, L182P and Q232R introduced during the PCR site-directed mutagenesis protocol (Appendix H).

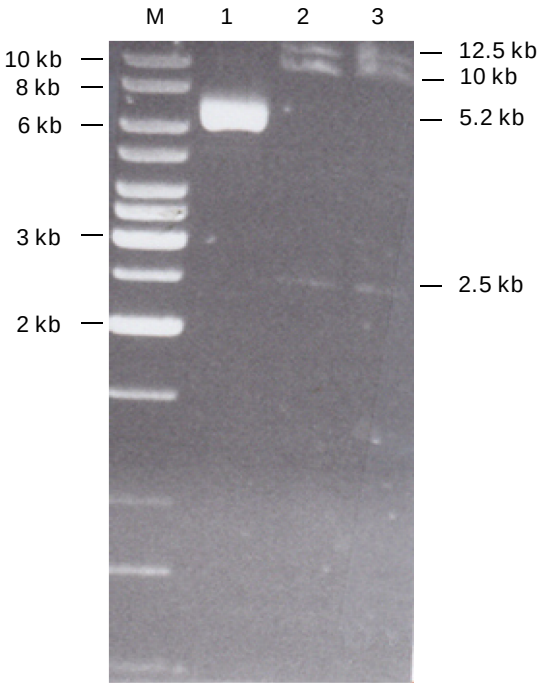
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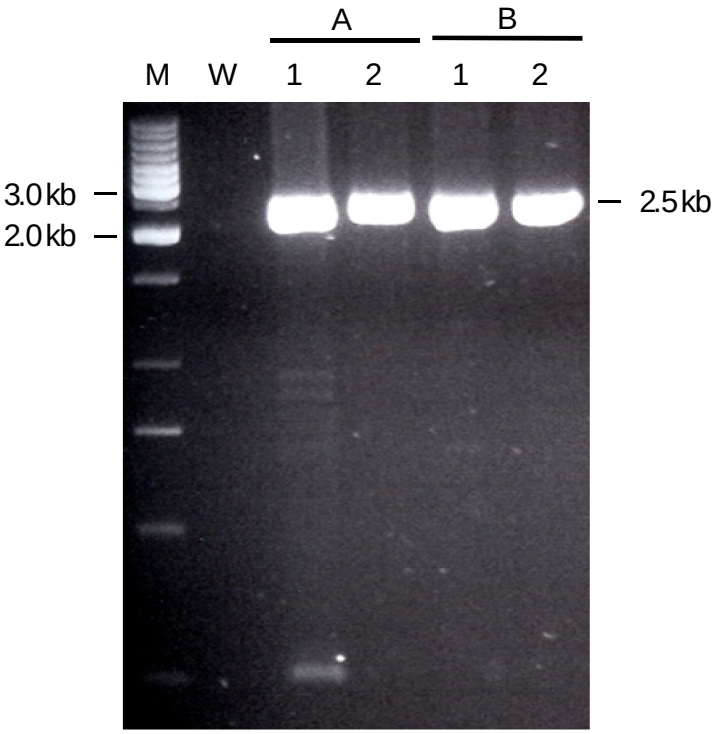


Potato	P10691.1_SUS1_SOLTU(SS16)	--MAERVLTRVHSLRERVD
Soybean	P13708.2_SUSY_SOYBN	--MATDRLTRVHSLRERLD
Grape	P31926.1_SUSY_VICFA	--MATERLTRVHSLRERLD
Maize	NP001105411.1_SH1_Z.mays	---MAAKLTRLHSLRERLG
Arabidopsis	NP197583.1_AtSUS1	MANAERMITRVHSQRERLN
Arabidopsis	NP566865.2_AtSUS4	MANAERVITRVHSQRERLD
Eucalyptus	ABB53601.1_EgSuSy1	--MADRMLTRSHSLRERLD
★Eucalyptus	EgSuSy1-S11A	--MADRMLTRSHALRERLD
Eucalyptus	ABB53602.1_EgSuSy3	--MPERLLTRVHSLRERLD
Rice	CAA46017.1_SUS1_O.sativa	---MAAKLARLHSLRERLG
Carrot	CAA53081.1_D.carota	--MGEPVLTRVHSLRERMD
Poplar	AAR03498.1_PtSUS1	--MTERALTRVHSIREHVD

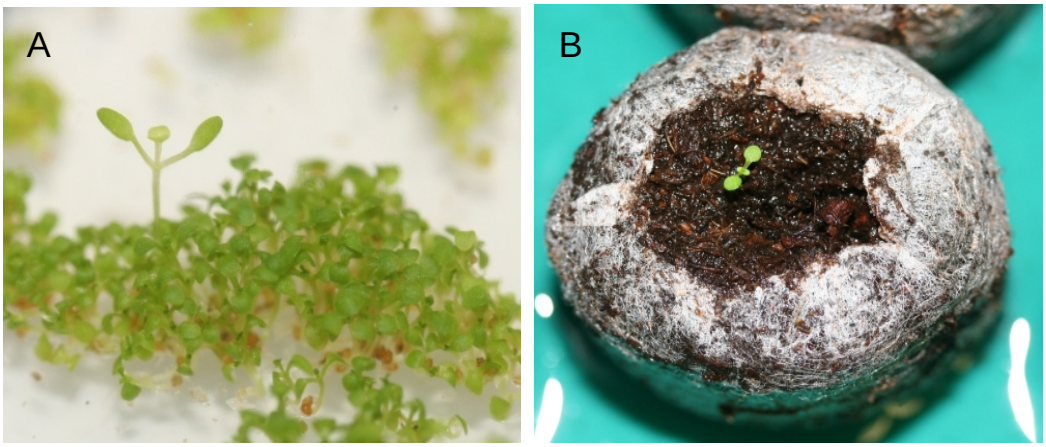
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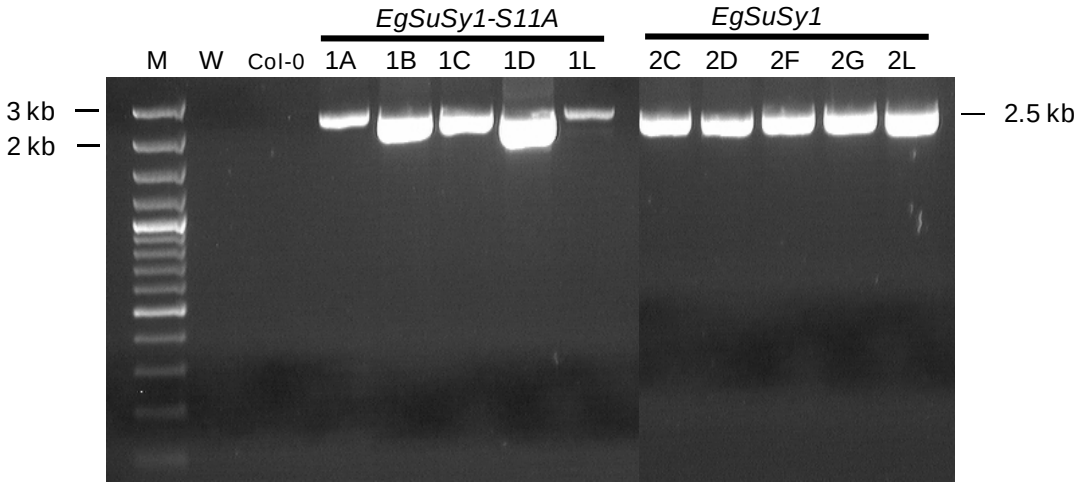


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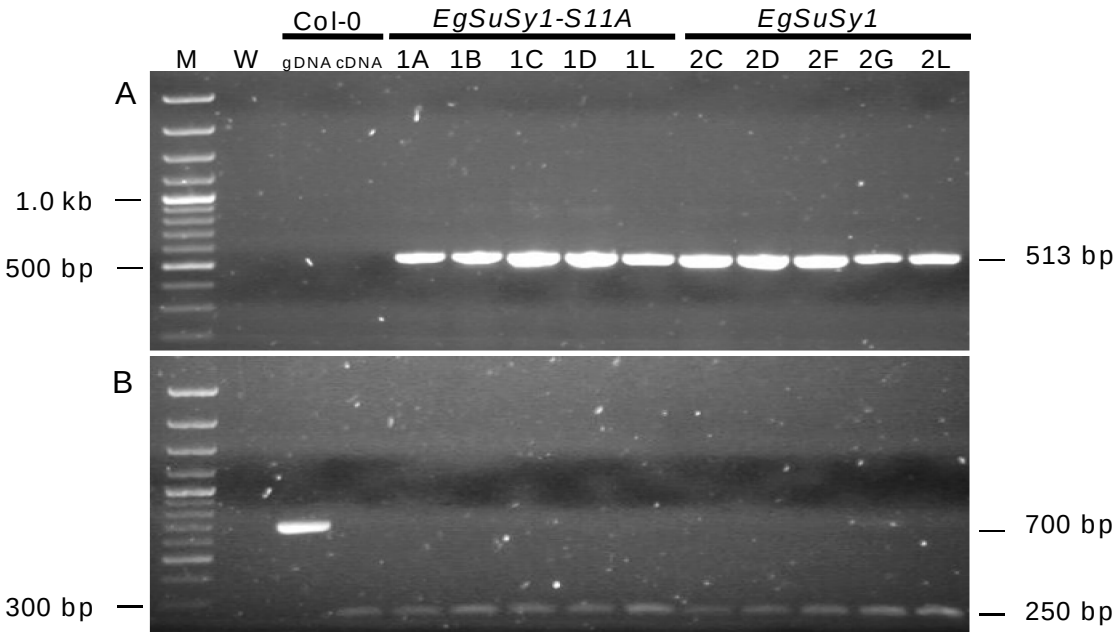


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2.3.4 Growth and development of transgenic lines

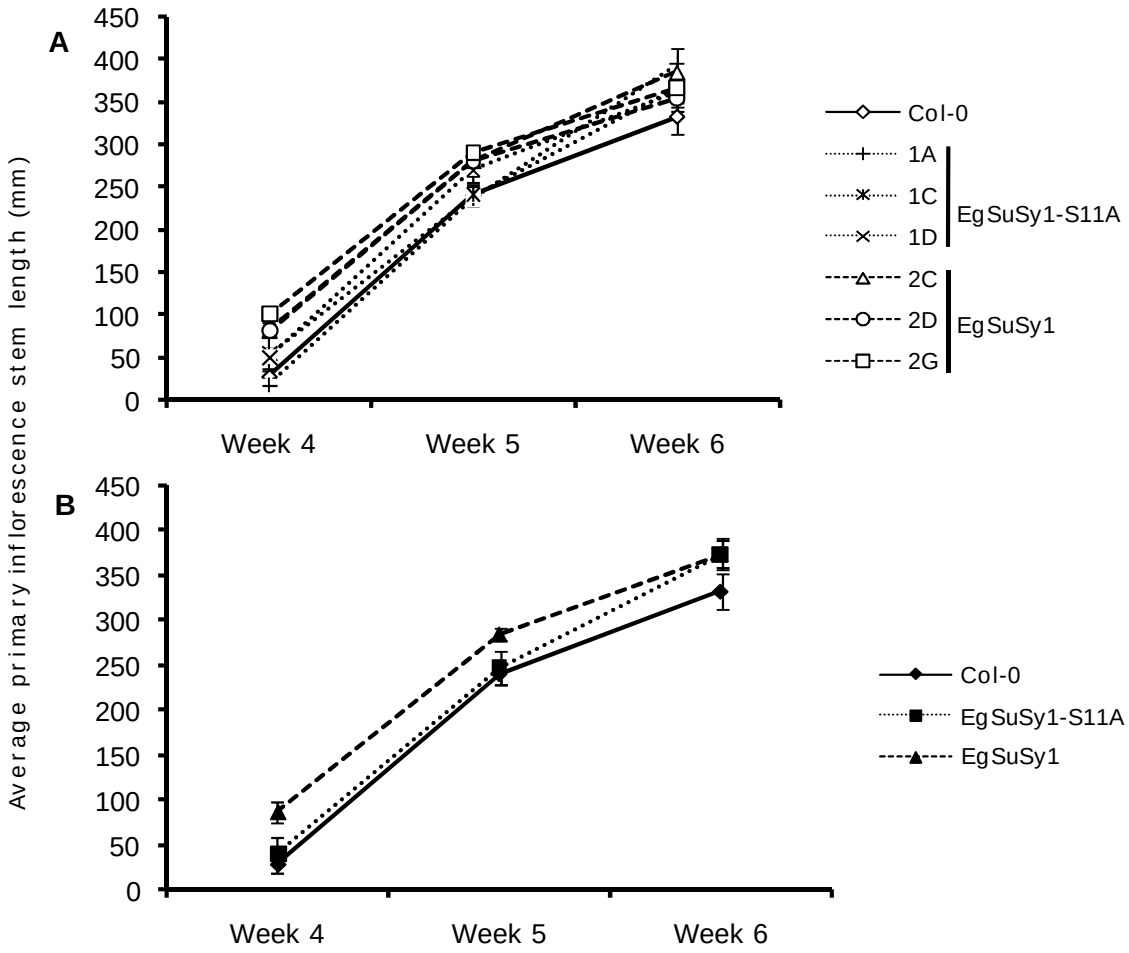
SuSy is a vital enzyme for growth and development in plants (Strum and Tang 1999). Therefore, growth and development related measurements were taken of T3 transgenic lines and compared to Col-0 control plants. Single factor ANOVA analysis of the primary inflorescence growth showed significant variation between Col-0, *EgSuSy1* and *EgSuSy1-S11A* lines at week four (p =9.70E-4) and week five (p = 0.00722), whereas at week six non-significant (p = 0.0625) variation was observed. The primary inflorescence stem growth average of *EgSuSy1* transgenic lines exhibited a 69% (p = 0.0032) initial growth increase at week four compared to Col-0, whereas *EgSuSy1-S11A* showed no significant difference in initial growth compared to Col-0 (Figure 2.12B). The average growth rate of *EgSuSy1* transgenic lines was similar to that of Col-0, but *EgSuSy1* lines had longer primary inflorescence stem from week four to week six (Figure 2.12B). The average growth rate of *EgSuSy1-S11A* transgenic lines increased between week five and six, resulting in a non-significant 10% (p = 0.154) increase compared to Col-0 (Figure 2.12B). *EgSuSy1-S11A* growth was significantly different to *EgSuSy1* by 54.5% (p = 0.0229) in week four and 12.8% (p = 0.0326) in week five, with no significant difference in week 6 (p = 0.951). The growth of the individual transgenic lines of *EgSuSy1* and *EgSuSy1-S11A* showed no significant increase compared to Col-0 (Figure 2.12A).

Fresh and dry weight measurements were analysed on the same plants at week six. Single factor ANOVA analysis showed no significant variation in the percentage dry weight (p = 0.0614) and fresh weight (p = 0.091) of total inflorescence stem between Col-0, *EgSuSy1* and *EgSuSy1-S11A* lines, whereas dry weight showed significant (p = 0.0111) variation. There was no significant difference in the average fresh weight of the transgenic lines compared to Col-0 (Figure 2.13D). The average dry weight

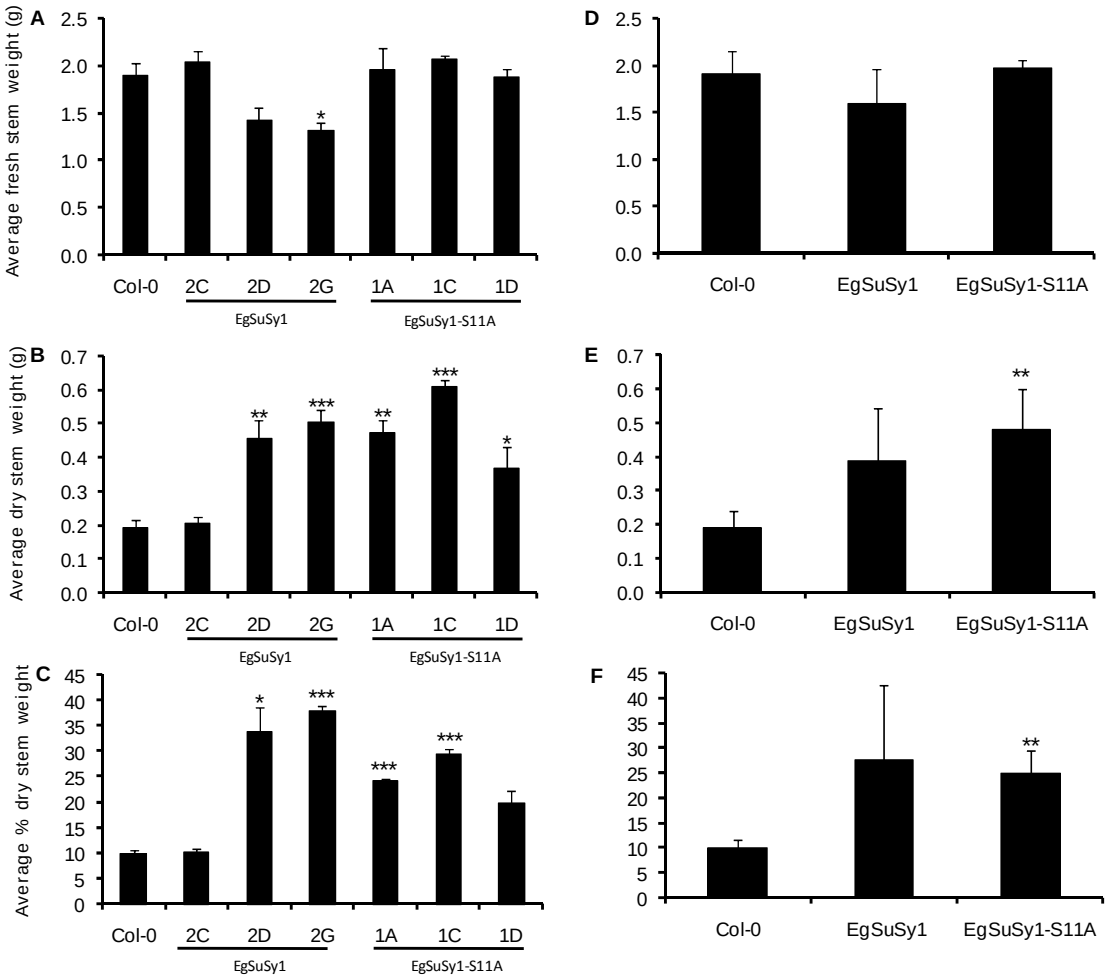
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analysis showed that *EgSuSy1-S11A* transgenic lines had 61% (p = 0.00673) more dry weight compared to Col-0 (Figure 2.13E). The *EgSuSy1* transgenic lines showed a 51%, but non-significant (p = 0.0630) increase in average dry weight compared to Col-0. Furthermore, *EgSuSy1-S11A* lines had a 60% (p = 0.00207) higher average percentage dry weight of total inflorescence stems compared to Col-0 (Figure 2.13F). Although, not significant the 64% (p = 0.0629) increase in percentage average dry weight of *EgSuSy1* lines compared to Col-0 (Figure 2.13F) was attributed to the 71% (p = 0.012) and 74% (p = 1.08E-5) increase in percentage dry weight of line 2D and line 2G, respectively (Figure 2.13C). Line 2C exhibits similar phenotype as Col-0 control plants. This introduces a higher standard deviation in the average dry and percentage dry weight measurements (Figure 2.13B and C). *EgSuSy1* and *EgSuSy1-S11A* lines showed no significant difference in dry weight (Figure 2.13E) and percentage dry weight (Figure 2.13F).

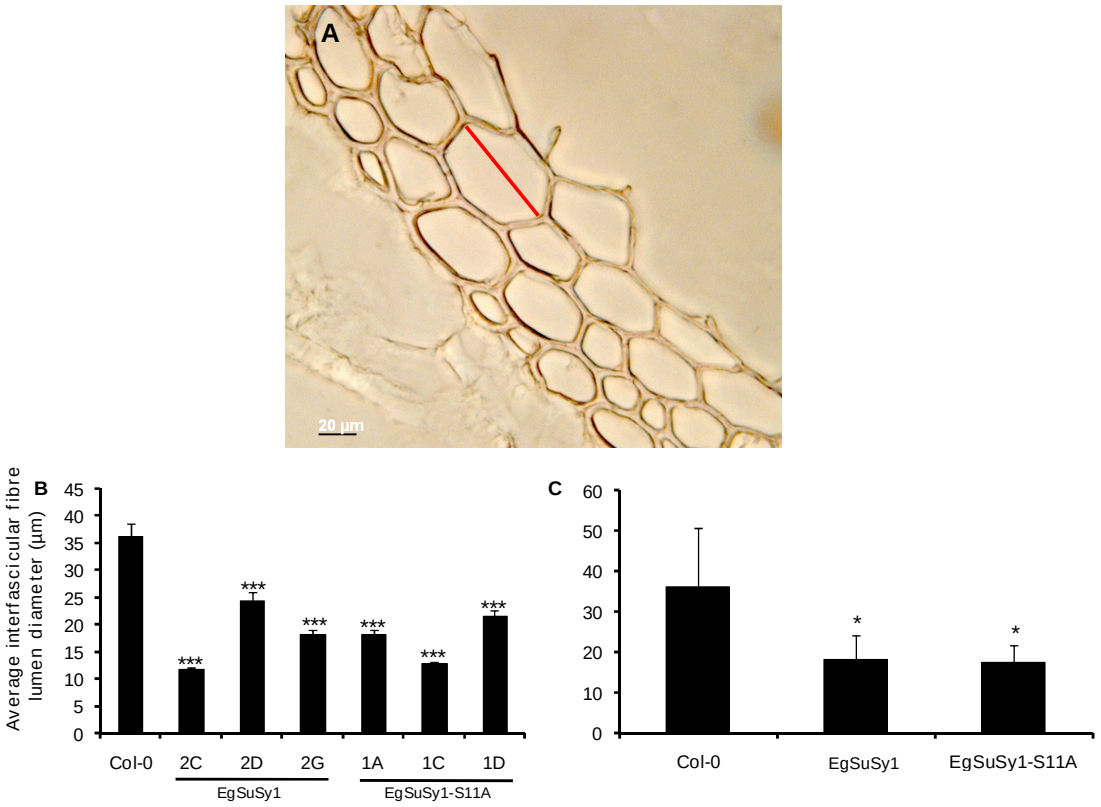
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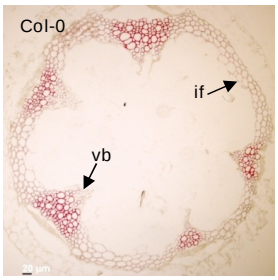
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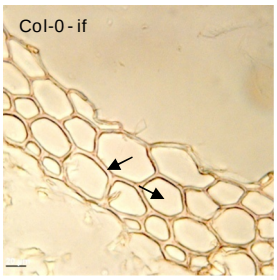


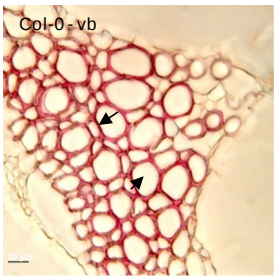
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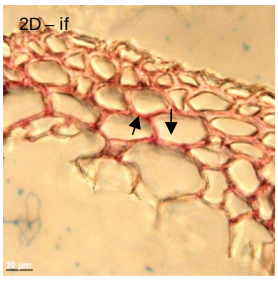
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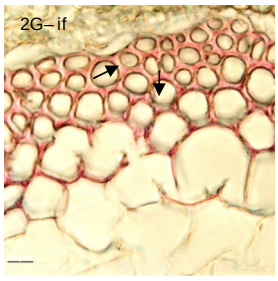


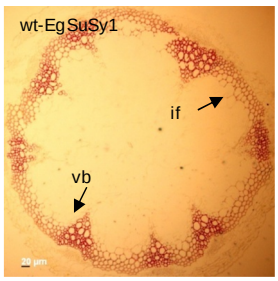




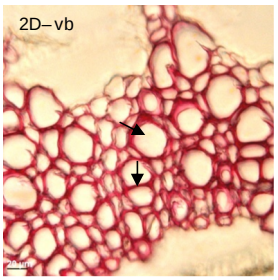


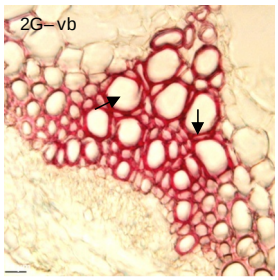


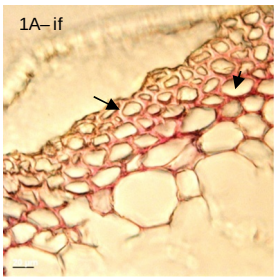


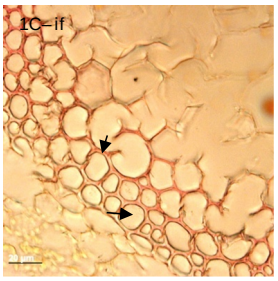


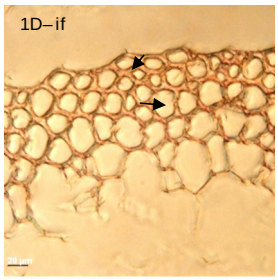


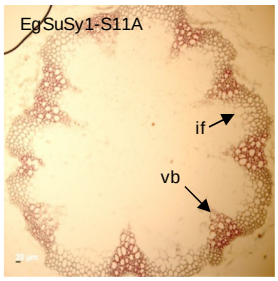


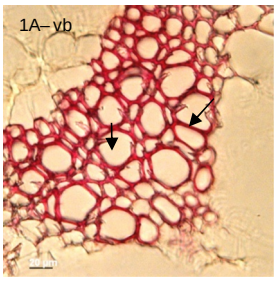


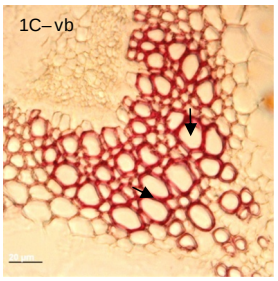


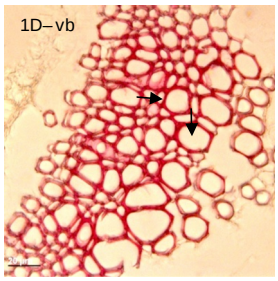












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The secondary cell wall thicknesses of vascular bundle vessels and fibre cells and interfascicular fibre cells of six-week-old transgenic and Col-0 plants were analysed using transmission electron microscopy (Appendix E and F). Single factor ANOVA analysis of the vascular bundle vessels cells secondary cell wall thickness showed significant (p = 0.00171) variation between Col-0, *EgSuSy1* and *EgSuSy1-S11A* lines. The average secondary cell wall thickness of vascular bundle vessels cells of *EgSuSy1* and *EgSuSy1-S11A* lines was not significantly difference from that of Col-0 and between each other (Figure 2.16D). Line 2C, 1A and 1D had significantly thinner vascular bundle vessels secondary cell walls compared to Col-0 (Figure 2.16C).

Single factor ANOVA analysis of the vascular bundle fibre cells secondary cell wall thickness showed significant (p = 8.47E-9) variation between Col-0, *EgSuSy1* and *EgSuSy1-S11A* lines. No significant difference was observed for the average secondary cell wall thickness of vascular bundle fibre cells of *EgSuSy1* and *EgSuSy1-S11A* lines compared to Col-0 and between each other (Figure 2.17D). However, all of the individual transgenic lines showed significantly thinner secondary cell walls in the vascular bundle fibre cells (Figure 2.17C).

Single factor ANOVA analysis of the interfascicular fibre cells secondary cell wall thickness showed significant (p = 1.38E-43) variation between Col-0, *EgSuSy1* and *EgSuSy1-S11A* lines. The average secondary cell wall thickness of interfascicular fibre cells of *EgSuSy1* transgenic lines was reduced significant (p = 0.00637) by 33% compared to Col-0 (Figure 2.18D). No significant difference (p = 0.128) was observed between *EgSuSy1* and *EgSuSy1-S11A* lines. A marginal significant increase of 6% (p = 0.0297) in interfascicular secondary cell wall thickness was observed in line 1D of *EgSuSy1-S11A* compared to Col-0 (Figure 2.18C).

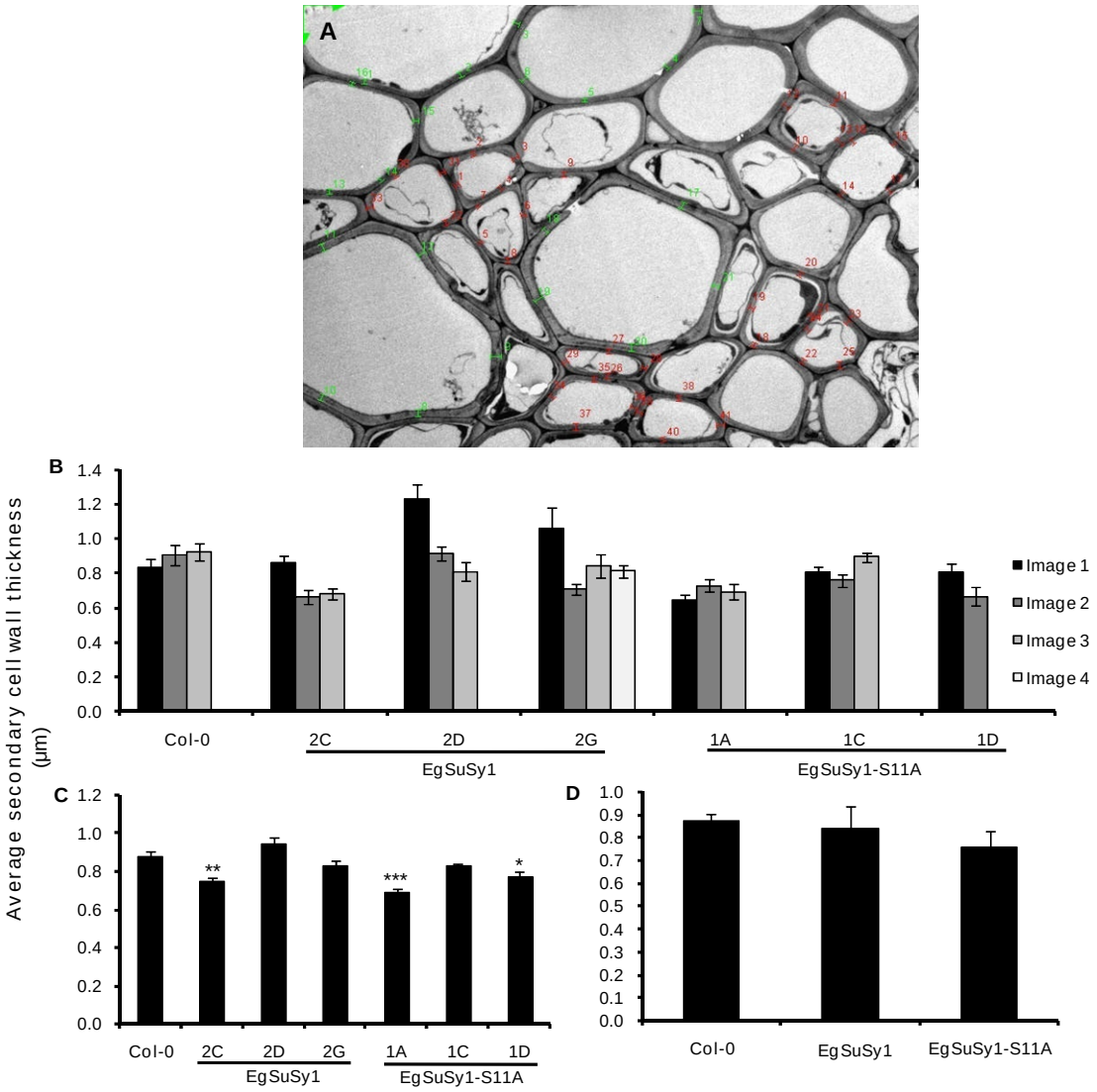
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Single factor ANOVA analysis of the number of interfascicular fibre cells showed significant (p = 1.02E-4) variation between Col-0, *EgSuSy1* and *EgSuSy1-S11A* lines. The average number of interfascicular fibre cells for *EgSuSy1* and *EgSuSy1-S11A* compared to Col-0, were increased significantly by 43% (p = 0.00145) and 30% (p = 0.0182), respectively (Figure 2.19C), but no significant difference was observed between *EgSuSy1* and *EgSuSy1-S11A* lines. This suggests more fibre development in *EgSuSy1* compared to *EgSuSy1-S11A* lines, which correlates to the observed increased growth (Figure 2.12B) and percentage dry weight (Figure 2.13F) in the *EgSuSy1* and *EgSuSy1-S11A* lines.

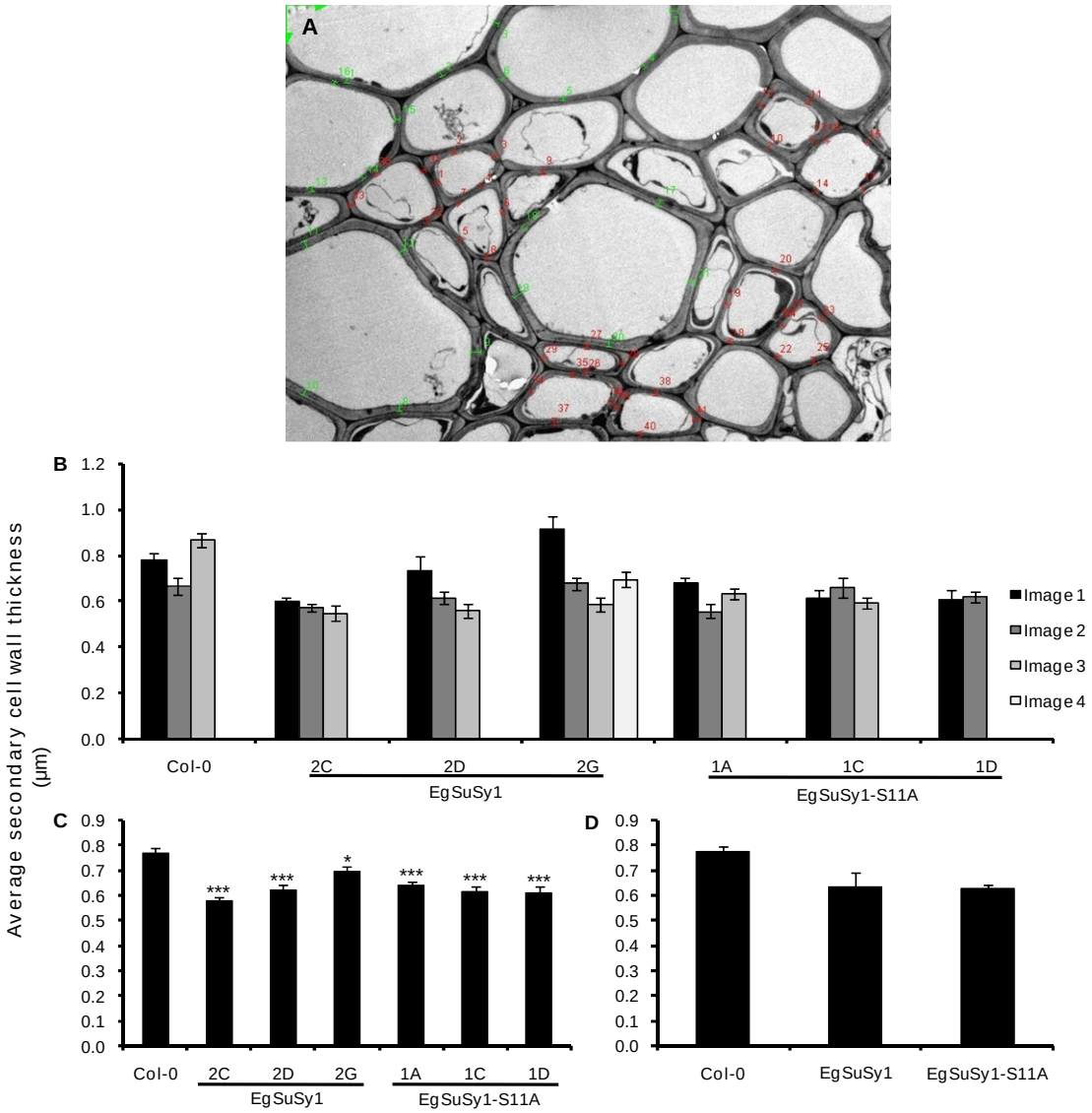
2.3.6 Cell wall chemistry analysis of transgenic lines

Cell wall chemistry analysis of the total inflorescence stems of *EgSuSy1* and *EgSuSy1-S11A* T3 transgenic lines was performed to determine whether net carbon allocation into secondary cell walls was altered. Single factor ANOVA analysis of the percentage mannose content showed significant (p = 0.00816) variation between Col-0, *EgSuSy1* and *EgSuSy1-S11A* lines, whereas as all the other sugars where not. No significant changes in percentage glucose and xylose were observed in *EgSuSy1* and *EgSuSy1-S11A* transgenic lines compared to Col-0 (Figure 2.20D). Similarly, fucose, arabinose, rhamnose and galactose showed no significant changes compared to Col-0 (Figure 2.20E and F). The percentage glucose was measured as an indirect indication of cellulose content. *EgSuSy1* transgenic lines has a 21% (p = 0.0111) and 25% (p = 0.00489) increase in average mannose content compared to Col-0 and *EgSuSy-S11A*, respectively (Figure 2.20E).

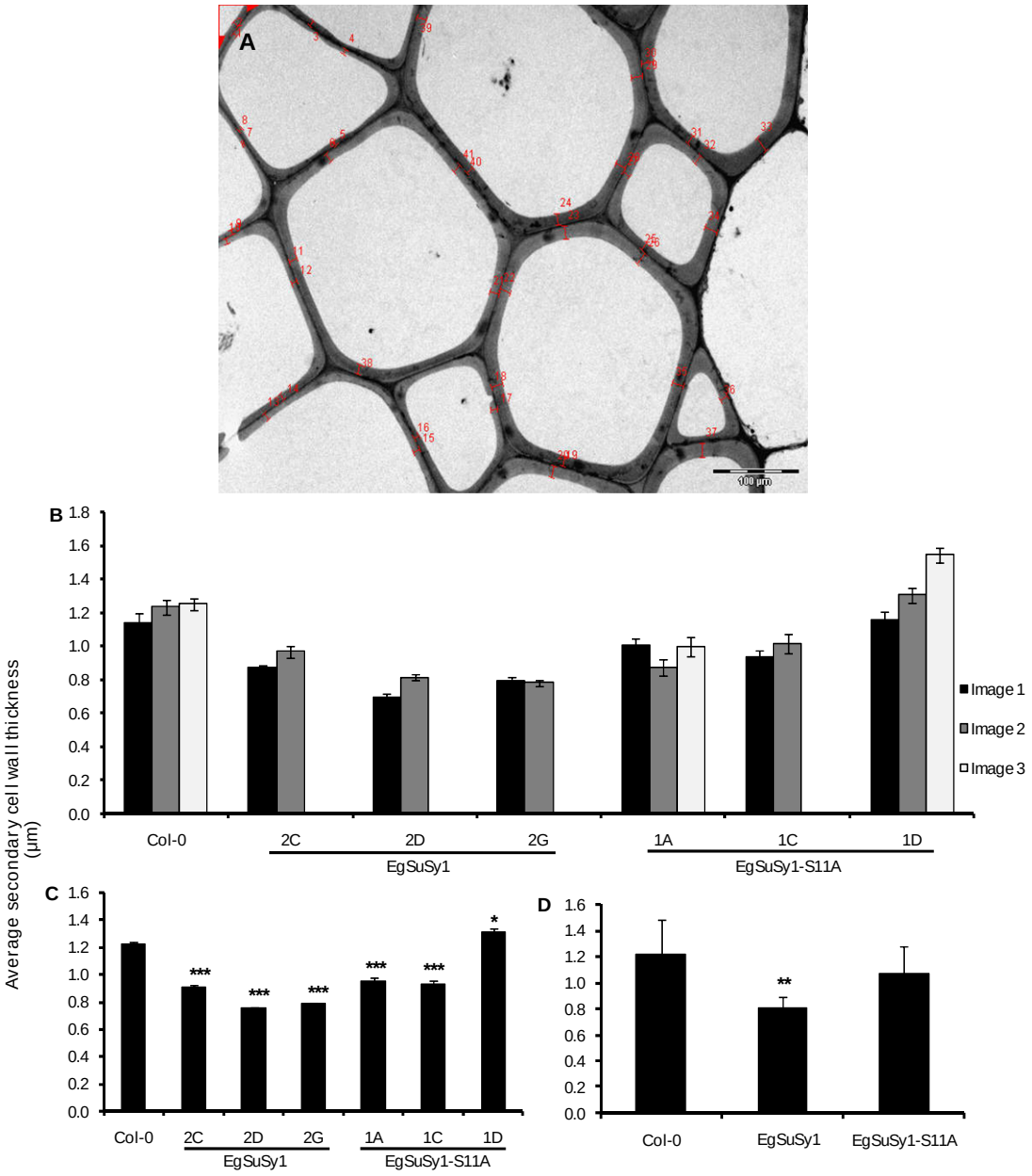
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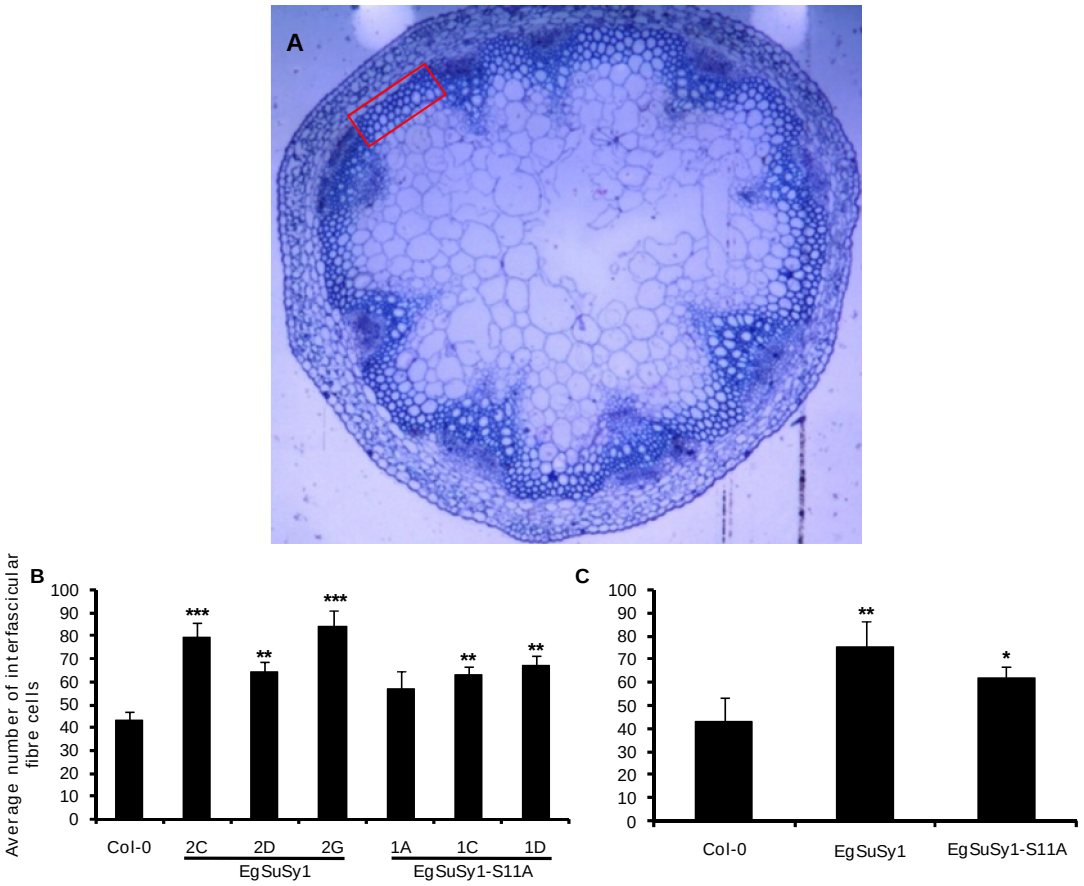
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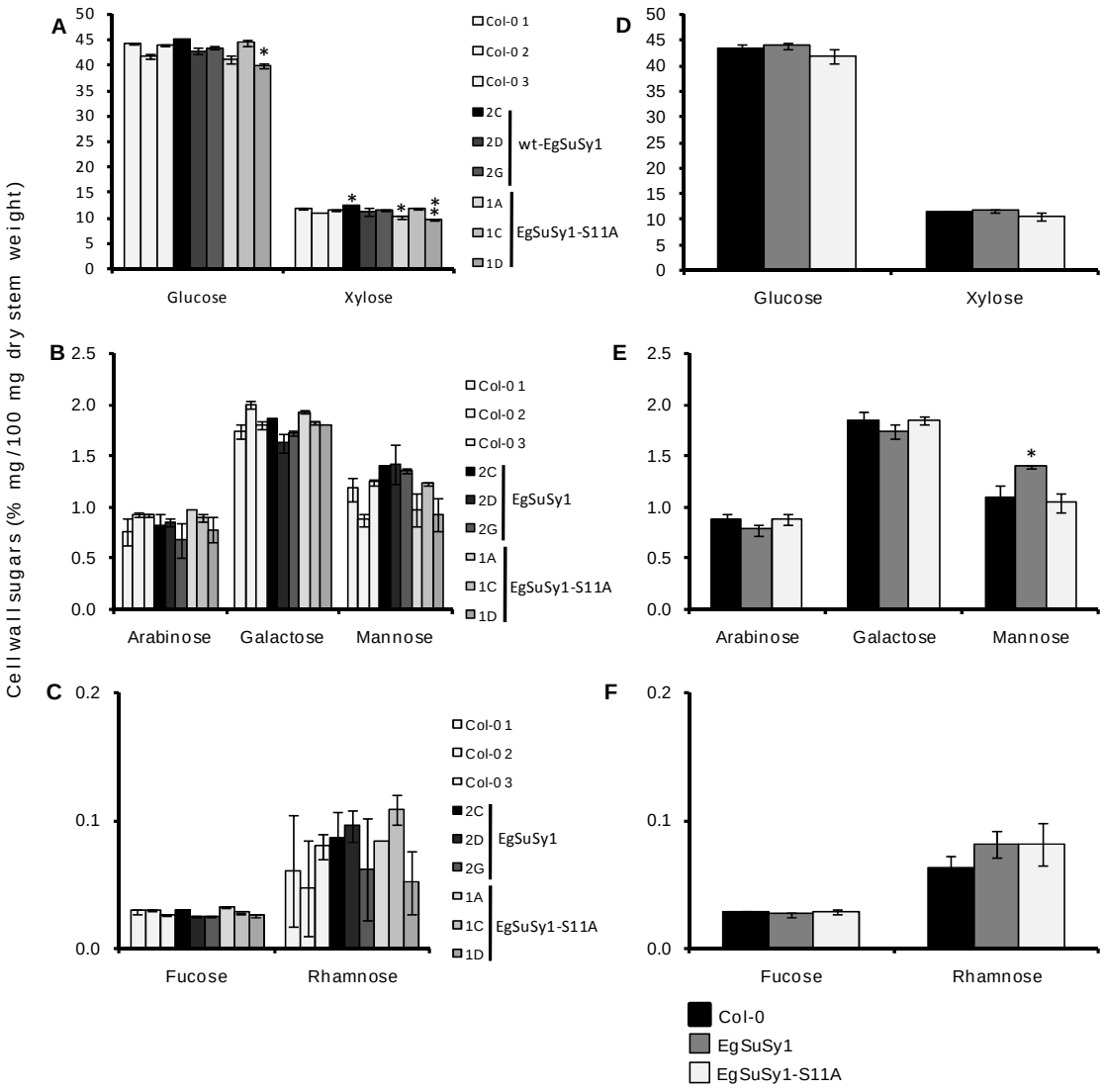
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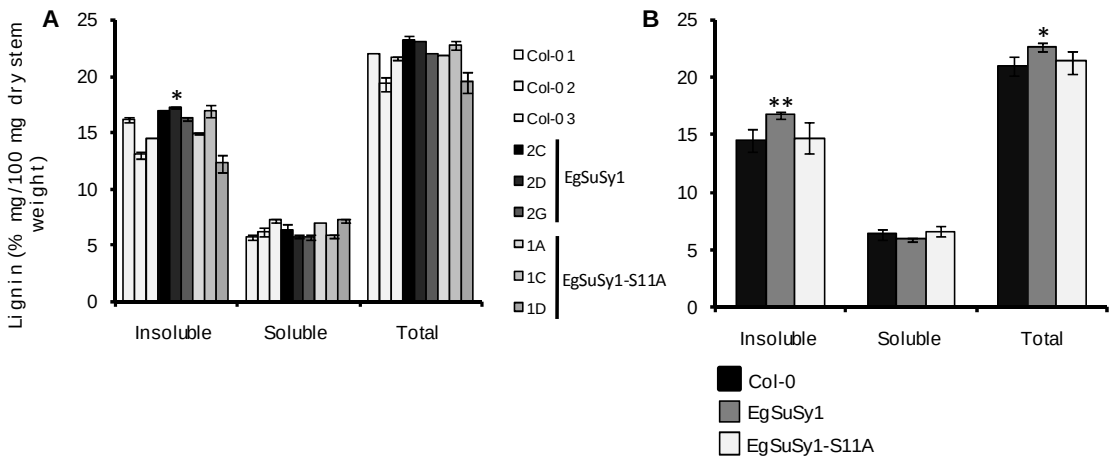
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found to have an average stability CV of 0.138 across the tested samples, across the light and dark period, as well as across the transgenic lines (Appendix C). In this study, the CV threshold was set at 0.2. The expected mean standard error of <0.2 for the standard curve data points was observed for all analysed target and reference genes (Table 2.5). This limit is recommended by the LightCycler® 480 V1.5.0 operation manual.

Table 2.5 The amplification efficiency values and the slope of the target and reference genes used for RT-qPCR analysis

Analysed genes	Efficiency (E)[b]	STD error (E)[c]	R^2 [d]
AtAMY3	2.079	0.022	0.987
AtCCR1	2.011	0.011	0.996
AtCesA8	2.187	0.03	0.992
AtSPS	2.048	0.129	0.775
AtSUS1	2.343	0.046	0.966
AtSUS4	1.974	0.08	0.81
EgSuSy1	2.143	0.019	0.992
AtTUB4[a]	1.945	0.01	0.996
AtTUB6[a]	1.928	0.01	0.997

a. Reference genes used in qBasePlus to attain normalization factor for each sample
b. Amplification efficiency of target genes during each thermal cycle
c. Standard error associated with the efficiency of the primers
d. Correlation of the three technical replicates of the three-series standard dilution to the regression line

The non-normalized expression profiles of the target genes showed extreme outliers that were consistent in specific samples across the tested genes (Appendix D). Since the expression of the reference genes in these particular samples did not follow a similar trend (Appendix C), the outliers were excluded from the results shown in (Figure 2.22). Sample K3 (Table 2.3) was excluded from the results in (Figure 2.22) because the three technical repeats for *AtTUB6* expression were unreliable. This affected the relative quantification by qBasePlus of the target genes in sample K3. The excluded samples represented one of the three biological replicates per transgenic lines and therefore standard deviations were calculated from the remaining two biological replicates per transgenic line.

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In a previous study, the expression levels of SuSy exhibited diurnal cycling in *Eucalyptus* (Solomon *et al,* 2008). Since the *Eucalyptus* transgenes are under the control of a double 35S promoter, we did not expect any diurnal cycling. However, it would be interesting to investigate the effect of the constitutive expression of *EgSuSy1* on the expression of *Arabidopsis* endogenous genes that respond to diurnal rhythms (Ma *et al,* 2001; Schaffer *et al,* 2001). Therefore, sample plant materials were collected in the light and dark periods for relative RT-qPCR analysis.

Relative expression between light and dark periods

There is a 79% (p = 0.00320) increase in expression of the *EgSuSy1* transgene in the light period compared to the dark period (Figure 2.22N). No significant (*p* = 0.458) difference in expression levels of *EgSuSy1-S11A* was observed in the light period compared to the dark period (Figure 2.22N). *AtSPS* (Figure 2.22H), *AtSUS4* (Figure 2.22I), *AtCCR1* (Figure 2.22J) and *AtSUS1* (Figure 2.22K) followed a similar trend in their relative expression as observed for *EgSuSy1* and *EgSuSy1-S11A* in the light and dark period (Figure 2.22). These observations seem to suggest responsiveness in expression levels of the endogenous genes comparable to the levels of *EgSuSy1* and *EgSuSy1-S11A* in the light and dark period. The expression levels of *AtAMY3* in Col-0, *EgSuSy1* and *EgSuSy1-S11A* transgenic lines in the light period was >95% (p = 8.03E-11) higher compared to the dark period (Figure 2.22M).

Relative expression of endogenous genes in transgenic lines compared to Col-0

The criteria used to select the individual transgenic lines was based on their relative growth. This resulted in the selection of line 1A, which had very low transgene expression levels (Figure 2.22G). The expression of *EgSuSy1-S11A* transgene in line 1A was demonstrated using RT-PCR (Figure 2.11A). The low level of expression

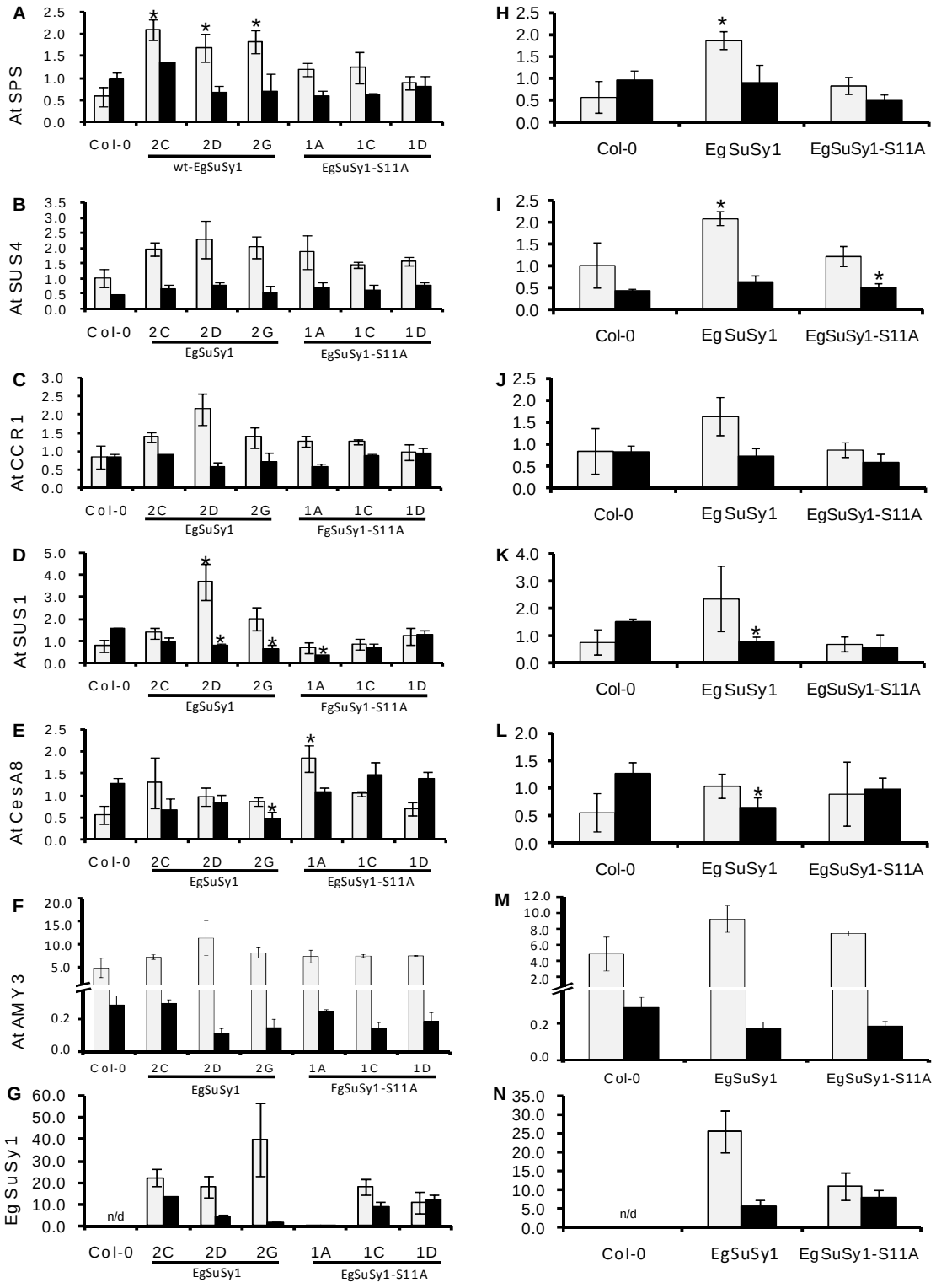
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may be attributed to positional effects of the T-DNA.

Single factor ANOVA analysis of the relative gene expression in the light period between Col-0, *EgSuSy1* and *EgSuSy1-S11A* lines showed significant variation for *AtSPS* (p = 0.00108), *AtCCR1* (p = 0.0361), *AtSUS1* (p = 0.0133), *AtSUS4* (p = 0.033) and *EgSuSy1* transgenes (p = 0.0137). In the dark period *AtCesA8* single factor ANOVA showed significant (p = 0.00178) variation between Col-0, *EgSuSy1* and *EgSuSy1-S11A* lines.

The relative expression of *AtSUS1* was decreased by 51% (p = 0.004453) in the dark period in *EgSuSy1* transgenic lines compared to expression in Col-0 (Figure 2.22K). *AtCesA8* expression was also significantly decreased by 49% (p = 0.0313) in the dark period in *EgSuSy1* transgenic lines compared to its expression in Col-0 (Figure 2.22L). However, the expression of *AtSPS* (Figure 2.22H) and *AtSUS4* (Figure 2.22I) in the light period of *EgSuSy1* transgenic lines were significantly increased by 69% (p = 0.00177) and 52% (p = 0.0377) respectively, compared to their expression in Col-0. Furthermore, *EgSuSy1* and *EgSuSy1-S11A* constitutive expression did not have a significant effect on the expression of *AtAMY3* in the light or dark period (Figure 2.22M). Although, not significant in all lines there seems to be a correlation in expression between the transgenes and the endogenous genes, except for *AtAMY3* and *AtCesA8* (Figure 2.22).

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2.4 Discussion

This study investigated the effect of constitutive heterogeneous over-expression of *EgSuSy1* and its modified version *EgSuSy1-S11A* in wild-type *Arabidopsis* plants. Phenotypic analysis of these transgenic lines included measurements of their growth rate, plant biomass, secondary cell wall thickness and the percentage content of lignin and secondary cell wall sugars. The relative expressions of endogenous genes linked directly and indirectly to the metabolic pathways involving SuSy were also determined.

Coleman *et al* (2006) constitutively expressed *AtSUS1* an ortholog of cotton SuSy in tobacco and reported increased biomass and growth of the transgenic plants, distinguishable by increased intermodal length in transgenic plants. However, these transgenic tobacco plants exhibited no change in the percentage cellulose content in secondary cell walls. Coleman *et al* (2009) constitutively expressed the same *AtSUS1* ortholog of cotton SuSy in hybrid poplar and reported, 2% to 6% increase in crystalline cellulose in xylem fibre cells and increased wood density. Konishi *et al* (2004) constitutively expressed a modified mung bean SuSy (S11E) in poplar and reported no significant changes in phenotype and cell wall polysaccharide deposition, but reported a higher expression of S11E in leaf tissue compared to stem tissue. S11E protein levels in the stem tissue were higher in the cytosolic fraction than the membrane fraction in the stem tissue. The over-expression of SuSy seems to increase cellulose deposition in trees species but increases biomass in herbaceous plants. The current study functionally analyzed the effect of *EgSuSy1* an ortholog of *AtSUS1* and its modified version *EgSuSy1-S11A* on carbon allocation towards cellulose deposition and growth and development in transgenic *Arabidopsis* plants.

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2.4.1 Modification of the N-terminal serine phosphorylation residue

Targeted manipulation of SuSy protein offers the potential to alter carbon allocation into carbon storing tissues and biopolymers of economical importance. The N-terminal non-catalytic serine phosphorylation domain of SuSy has been well characterized as a potential target for manipulation by site-directed mutagenesis (Nakia *et al,* 1998; Sauerzapfe *et al,* 2008). This conserved domain is believed to be involved in regulating the sub-cellular localization (Hardin *et al,* 2003; Hardin *et al,* 2004; Huber *et al,* 1996; Zhang and Chollet, 1997), pH modulation (Duncan and Huber, 2007; Hardin *et al,* 2004), and protein degradation (Hardin *et al*, 2003) of SuSy. It is also involved in modulating activity levels of SuSy and the direction of its reactions (Nakai *et al,* 1998; Sauerzapfe *et al,* 2008; Zhang *et al,* 1999). Therefore, manipulation by site-directed modification of SuSy at this site may alter the properties of the enzyme such that carbon allocation processes regulated by SuSy are affected. Despite several supporting observations there is no direct evidence suggesting that the phosphorylation or dephosphorylation of the N-terminal serine site influences the disassociation or association of SuSy with the membrane, respectively (Hardin *et al,* 2004).

The inhibition of SuSy phosphorylation increases its activity in the membrane fraction (Anguenot *et al,* 2006; Winter *et al,* 1997; Zhang *et al,* 1999). As mentioned previously, SuSy is affected by a multitude of factors. Therefore, the N-terminal serine phosphorylation site might not be the only factor that influences membrane association of SuSy. It was expected that the membrane activity of *EgSuSy1-S11A* would be higher than that of the unmodified form based on a study of the modified mung bean SuSy S11E (Konishi *et al,* 2004), which was predominantly associated with the soluble fraction in stem tissue. The proposed membrane association will be

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verified using green florescence protein (GFP) fusions of *EgSuSy1*, *EgSuSy1-S11A* and *EgSuSy1-S11E* recombinant proteins in transgenic *Arabidopsis* plants (ongoing parallel M.Sc. study by Mrs. Jamie-Lee Sauer).

The catalytic C-terminal region (amino acid 546 to 753) represents the conserved glucosyltransferase active region (Bateman *et al*, 2002; Baud *et al*, 2004). The observed PCR induced background mutations E18K, L182P and Q232R were not present in this region, or in the conserved N-terminal non-catalytic phosphorylation domain (amino acid 8 to 17, Appendix H). The mutation at position 18 changed a negatively charged polar acidic residue to a positively charged basic residue. The mutation at position 182 interchanged between two non-polar amino acids. The mutation at position 232 changed from an uncharged polar amino acid to a positively charged polar basic amino acid. Protein sequence alignment (Appendix H) of all dicot protein sequences represented in (Appendix I) showed that background mutation at position 18 is conserved for E and K is weakly similar to E. Position 182 is highly conserved for L and P is a non-similar amino acid. This mutation is significant in potentially affecting the function of the protein. Position 232 is highly conserved for Q the background mutation of R is weakly similar to Q. The intended properties of the site-directed mutation of S11A could be affected by these background mutations, however the extent of this is not known. The presence or absence of such effects will have to be confirmed in future by making a new construct that does not contain any background mutations.

2.4.2 Morphological characterization of transgenic lines

The growth and development of *Arabidopsis* at week 4 to 5 corresponds to leaf production, rosette growth and emergence of primary inflorescence stem, and from week 5 to 6 to growth and development of the inflorescence stem (Boyes *et al*,

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2001). *EgSuSy1-S11A* seemed to promote inflorescence-stem-specific growth and development, whereas *EgSuSy1* seemed to promote overall plant growth and development (Figure 2.12B). However, further measurements of other developing organs are needed to substantiate this.

The increase in biomass of the transgenic lines was evident by the increased average dry weight of 64% for *EgSuSy1* and 60% for *EgSuSy1-S11A* transgenic lines (Figure 2.13F). This increase in dry biomass most likely resulted from the increased numbers of interfascicular fibre cells of 43% for *EgSuSy1* and 30% for *EgSuSy1-S11A* (Figure 2.19), which also had reduced lumen sizes (Figure 2.14B). Coleman *et al* (2006) also observed increased fibre cell numbers resulting in the increased biomass of transgenic tobacco plants overexpressing cotton SuSy.

EgSuSy1 transgenic lines showed marginally reduced secondary cell wall thickness in the interfascicular fibre cells, while *EgSuSy1-S11A* transgenic lines remained unchanged compared to Col-0 (Figure 2.18C). In summary, *EgSuSy1* and *EgSuSy1-S11A* transgenic lines had increased inflorescence stem biomass, reduced interfascicular fibre lumen sizes and increased number of interfascicular fibre cells, and *EgSuSy1* lines also had thinner interfascicular secondary cell walls and significantly increased growth rates. This suggests a possible diversion of carbon from secondary cell wall thickness of interfascicular fibre cells towards increasing the number interfascicular fibre cells in *EgSuSy1* transgenic lines. In contrast, *EgSuSy1-S11A* lines seemed to have unchanged secondary cell wall thickness in the interfascicular fibre cells as Col-0 plants with higher number of interfascicular fibre cells.

2.4.3 Cell wall chemical analysis of transgenic lines

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Glucose molecules are the main component of the cellulose backbone, whereas xylose molecules form an important component of xyloglucan and xylan (Mellerowicz, *et al*, 2008). Cellulose, hemicellulose and lignin form a large component of secondary cell wall biomass (Plomion *et al*, 2001). The percentage content of glucose and xylose in the transgenic lines remained unchanged compared to Col-0 (Figure 2.20). The increase in percentage insoluble lignin (Figure 2.21) may be associated with the need for greater structural support of the faster growing *EgSuSy1* transgenic lines (Coleman *et al*, 2008; Leple *et al*, 2007), which accumulated biomass earlier than the control lines.

UDPG is a versatile substrate for multiple pathways linked to cell wall development and general plant growth and development. Therefore, the hypothesised increased in membrane activity of *EgSuSy1-S11A*, would increase the supply of UDPG to cell wall related biosynthesis particularly cellulose. The current results suggest that UDPG is not diverted towards cellulose biosynthesis at higher rates in the transgenic plants than control plants (Figure 2.20). UDPG in the *EgSuSy1* and *EgSuSy1-S11A* transgenic lines seems to be diverted towards pathways involved in increased growth and biomass (Figure 2.23). The increased biomass observed in the *EgSuSy1* and *EgSuSy1-S11A* transgenic lines is attributed to the increase in the number of interfascicular fibre cells (Figure 2.19C).

Due to the overexpression of *EgSuSy1*, the accumulation of F-6-P can be indirectly postulated since we did not measure F-6-P content. F-6-P is a precursor molecule for D-mannose (Figure 2.24), which was shown to be in surplus (Figure 2.20E). D-mannose is involved in the upregulation of *AtSUS1* expression via hexokinase modulation (Ciereszko and Kleczkowski, 2002). Therefore, the increased mannose content could be involved in the induction of the endogenous *AtSUS1* (Figure 2.22K),

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although not significant, but possibly to facilitate in the altered growth and development experienced by the *EgSuSy1* lines.

Interestingly, during tension wood development, trees exhibits increased SuSy expression (Andersson-Gunneras *et al*, 2006) and excess F-6-P is diverted towards glycoprotein synthesis (Andersson-Gunneras *et al*, 2006). The *EgSuSy1* transgenic lines seem to divert the excess F-6-P towards lignin biosynthesis (Figure 2.24). Similarly, in this study and in the study by Coleman *et al* (2006) in tobacco no significant changes in the secondary cell wall percentage sugars were reported in the transgenic plants, however, Coleman *et al* (2009) reported 2% to 6% increase in crystalline cellulose in secondary cell walls of hybrid poplar plants. This could be attributed to the fact that *Arabidopsis* and tobacco are herbaceous plants, while poplar as a woody plant has a stronger sink for carbon during wood formation. Therefore, the failure to observe increased percentage cellulose in *Arabidopsis* due to the overexpression of *EgSuSy1* and *EgSuSy1-S11A* could be attributed to the fact that *Arabidopsis* is a herbaceous plant and not woody like poplar.

2.4.4 Gene expression profiling of transgenic lines

The relative average increased expression of transgene *EgSuSy1* in the light period compared to the dark period (Figure 2.22N) suggests diurnal regulation of the promoter in *Arabidopsis*. Studies have demonstrated the effect of photoperiod on GUS expression under the control of the 35S promoter, demonstrating photoperiodic sensitivity of 35S promoter in tobacco (Schnurr *et al*, 2000). Other studies have shown selective expression of 35S promoter in alfalfa (Samac *et al*, 2004) and inactivity in reproductive organs of *Arabidopsis* (Ge *et al*, 2008) of GUS expression under 35S constitutive promoter. These findings warrant the investigation of new

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alternative constitutive promoters or strong tissue-specific promoters as investigated by Samac *et al* (2004).

Amylases are starch-degrading enzymes that enable the release of stored carbon for energy and development (Orzechowski, 2008). Therefore, *AtAMY3*, was profiled to observe whether the increased activity of *EgSuSy1* and *EgSuSy1-S11A* will result in greater demand for sucrose, thereby increasing the breakdown of starch to compensate the sucrose deficit. The non-responsiveness of *AtAMY3* to the constitutive overexpression of the transgenes (Figure 2.22F and M), possibly relates to its involvement in initial starch breakdown into smaller chains rather than transitory starch degradation (Yu *et al*, 2004). Furthermore, long daylight periods induce faster starch breakdown, this is consistent with the increased expression of starch degrading enzymes (Lu *et al*, 2005). Therefore, increased expression of *AtAMY3* in light period is evident of this, because these transgenic plants were grown in a long-day photoperiod. Recently it was shown in transgenic potatoes that overexpression of SuSy does not affect the activity of enzymes involve in starch metabolism (Baroja-Fernandez *et al*, 2009)

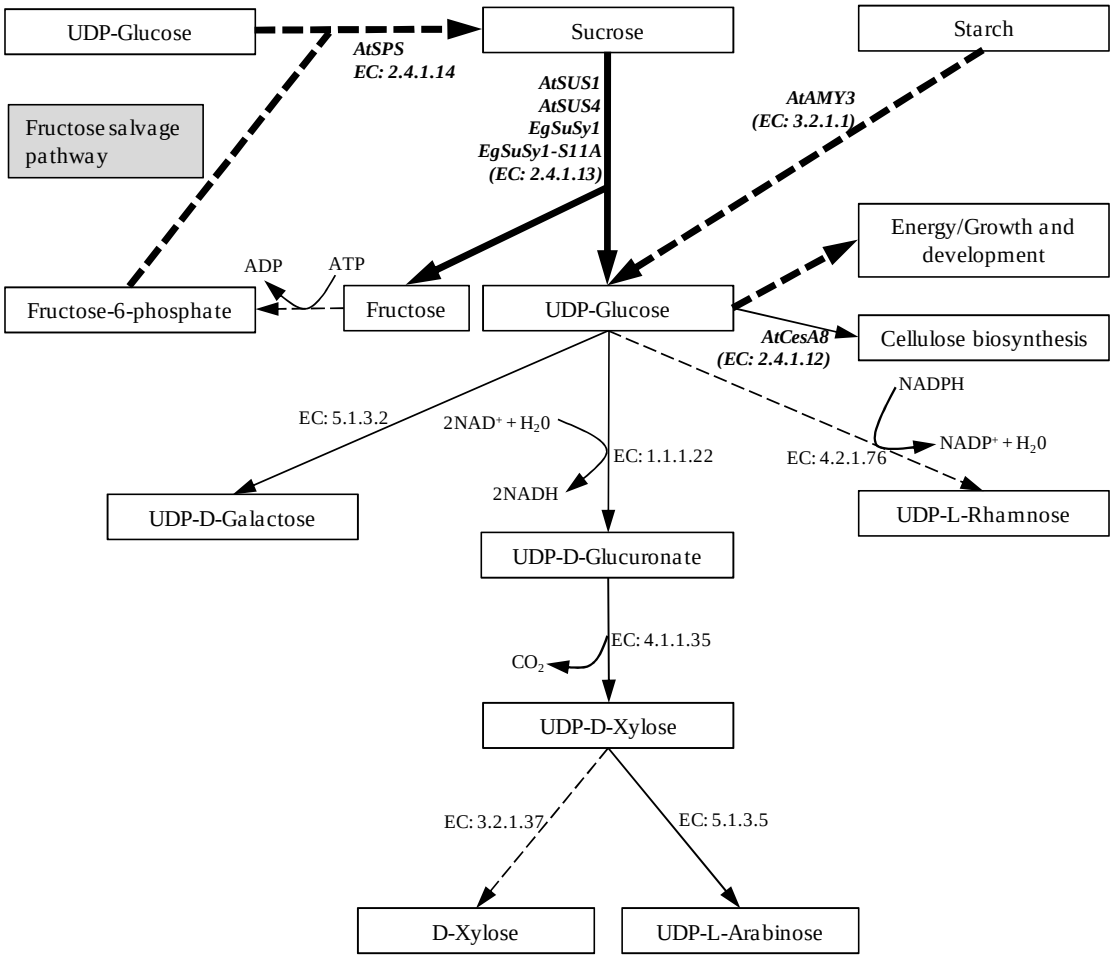
Arabidopsis secondary cell wall associated cellulose synthase *AtCesA8* in association with two other secondary cellulose synthase enzymes, *AtCesA4* and *AtCesA7*, are involved in the deposition of cellulose in the secondary xylem fibre cells (Taylor *et al*, 2003). *AtCesA8* was used as an indirect indicator of the regulation of cellulose deposition in the secondary cell wall. The unchanged percentage content of glucose in the secondary cell wall, (Figure 2.20D), was supported by the relative expression of *AtCesA8* (Figure 2.22L), which showed no significant changes in its relative expression compared to Col-0 that could suggest differences in the cell wall chemistry.

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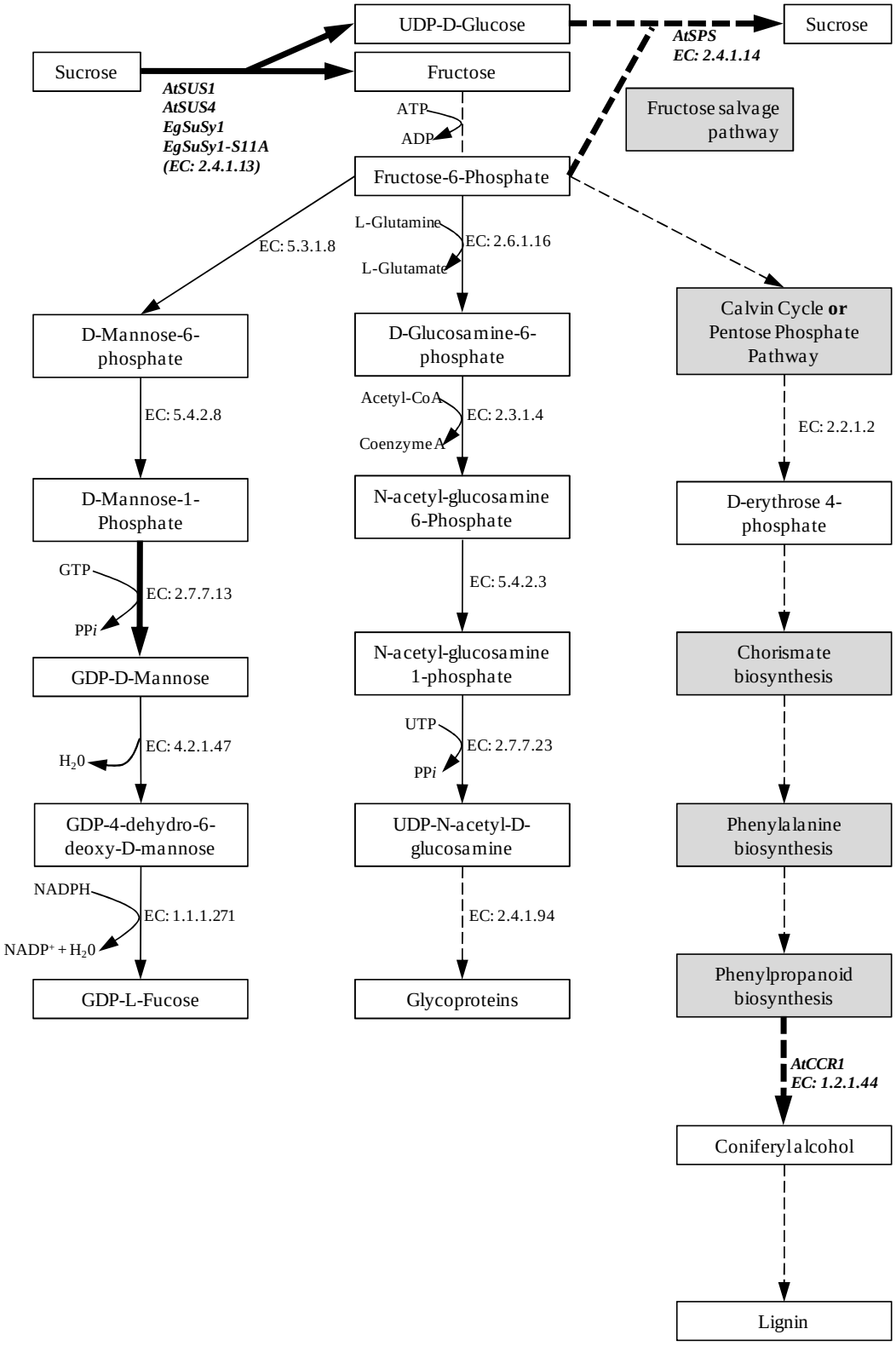
Lignin is an essential component of secondary cell walls for structural integrity and cinnamoyl-CoA reductase 1 (*AtCCR1*) enzyme is essential for lignin biosynthesis (Jones *et al*, 2003). The *EgSuSy1* transgenic lines seem to divert excess F-6-P towards lignin biosynthesis (Figure 2.24), supporting the 7% increase in percentage lignin content (Figure 2.21C).

Arabidopsis SPS (*AtSPS*) is involved in the fructose salvage pathway in which it *de novo* synthesizes sucrose from F-6-P and UDPG to sucrose and phosphate (Lunn and MacRae, 2003). The overexpression of SPS alters plant growth and development (Park *et al*, 2008). Therefore, its increased expression could also relate to the changes in growth and development of *EgSuSy1* transgenic lines (Figure 2.22H). The endogenous *AtSUS1* and *AtSUS4* are involved in cell wall formation under normal condition and maintaining normal growth and development under hypoxic conditions (Bieniawask *et al*, 2007). The overexpression of *EgSuSy1* and *EgSuSy1* in *Arabidopsis* resulted in significant changes to the growth and development of the transgenic lines (Figure 2.23). These endogenous genes *AtSUS1, AtSUS4, AtSPS* and *AtCCR1* are involved in growth and developmental processes. This could explain the seemingly correlated expression pattern with the transgenes (Figure 2.22). The relative expression profiles of these endogenous genes seemed to share similar patterns with the transgenes

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2.5 Conclusions

The overexpression of *EgSuSy1* in *Arabidopsis* resulted in increased growth and biomass formation. These transgenic lines had more interfascicular fibre cells, which had marginally reduced secondary cell walls thickness and lumen sizes. The overexpression of the modified *EgSuSy1-S11A* transgene similarly resulted in more biomass and interfascicular fibre cells that had reduced lumen sizes. However, *EgSuSy1-S11A* transgenic lines had unchanged secondary cell wall thickness in the interfascicular fibre cells compared to Col-0. These findings suggest that *EgSuSy1* transgenic lines seems to have increased interfascicular fibre cells with thinner secondary cell walls, whereas *EgSuSy1-S11A* seems to have increased interfascicular fibre cells with unchanged secondary cell wall thickness compared to control plants. No proportional change in the secondary cell wall sugars of these transgenic lines was observed relating to the changes in secondary cell wall thickness. Fibre quality of these transgenic lines needs to be assessed to fully characterise their potential properties that may benefit the paper and pulp industry.

The findings of in this study are consistent with those of Coleman *et al* (2006) who showed increased growth and biomass due to increases in the number of fibre cells in transgenic tobacco lines overexpressing cotton SuSy. Coleman *et al* (2006) also reported unchanged secondary cell wall thickness, which was not observed in the current study. This is different to the results of Coleman *et al* (2009), who observed increased cellulose deposition and secondary cell wall thickness of fibre cells leading to increased wood density in hybrid poplar plants overexpressing cotton SuSy. This suggests greater sink strength by secondary cell walls of woody plants such as poplar than herbaceous plants such as *Arabidopsis* and tobacco. In conclusion, the overexpression of *EgSuSy1* resulted in transgenic *Arabidopsis* lines with increased

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biomass due to increased fibre cell numbers with reduced secondary cell wall thickness, and the modified version *EgSuSy1-S11A* resulted in increased biomass due to increased fibre cell numbers with unchanged secondary cell wall thickness.

As part of this study, efforts are underway to attain *EgSuSy1-S11E* transgenic plants to contrast *EgSuSy1-S11A* with a phosphorylation insensitive, but negatively charged amino acid (Glutamate) that mimics phosphorylation (Nakia *et al*, 1998; Nakia *et al*, 1999). The rationale for this is to characterise fully the N-terminal non-catalytic serine phosphorylation domain of *EgSuSy1* and its effect on the biology of transgenic *Arabidopsis.* Furthermore, the ability of *EgSuSy1-S11A* and *EgSuSy1-S11E* to associate with the membrane fraction will be investigated using GFP fusions in transgenic *Arabidopsis* plants (ongoing parallel M.Sc. study by Mrs Jamie-Lee Sauer).

2.6 Recommendations

The current study has validated *Arabidopsis* as a model plant for functional studies of secondary cell wall formation and carbon allocation related candidate genes (Chaffey *et al*, 2002). However, a concern about whether *Arabidopsis* is a good model for carbon allocation in secondary cell walls is raised. Research into *Arabidopsis*, specifically aimed at addressing these points are warranted. Many more studies aimed at modelling the altering of carbon allocation into important sinks such as cellulose in forestry trees (e.g. *Eucalyptus*) using *Arabidopsis* or poplar (Coleman *et al*, 2009; Jansson and Dauglas, 2007) model systems are needed to meet current and future demands for cellulose products. Ultimately, these approaches need to be verified in the intended system such as *Eucalyptus* as final confirmation, and deployment on a commercial scale.

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2.8 Summary

Current demands for wood products such as paper, pulp and timber are increasing rapidly. There is also an increasing demand to develop cellulosic bio-fuels as renewable energy sources to mitigate the uses of fossil fuels. Current technologies such as biotechnology present opportunities to genetically improvement trees by targeting important traits with accuracy such as improving wood fibre with high cellulose content. Such traits are regulated by complex pathways, which are influenced directly and indirectly by internal and external stimuli. These traits include increasing cellulose deposition by increase carbon allocation towards cell walls of secondary xylem tissues. This requires the understanding of pathways and genes involved in carbon assimilation in autotrophic (source) tissues and allocation into heterotrophic (sink) tissues. Sucrose synthase (SuSy: EC 2.4.1.13) play a role in the reversible conversion of sucrose to UDP-glucose (UDPG) and fructose thereby enabling UDPG to enter cytosolic and cell wall related metabolic pathways responsible for growth and development of commercially important tissues such as secondary xylem.

The conserved N-terminal non-catalytic serine phosphorylation domain of SuSy is thought to regulate its sub-cellular localization. The phosphorylation and dephosphorylation of this domain affects the hydrophobicity of SuSy protein thereby sub-localizing it in the cytosolic and membrane fractions, respectively. This study investigated the effect of overexpressing unmodified (*EgSuSy1*) protein and the modified phosphorylation insensitive *EgSuSy1-S11A* on allocating sucrose carbon towards cellulose biopolymer of secondary xylem cell walls. The two constructs were overexpressed in *Arabidopsis* plants under the control of a double 35S CaMV promoter. *EgSuSy1* and *EgSuSy1-S11A* transgenic lines had smaller interfascicular fibre cells

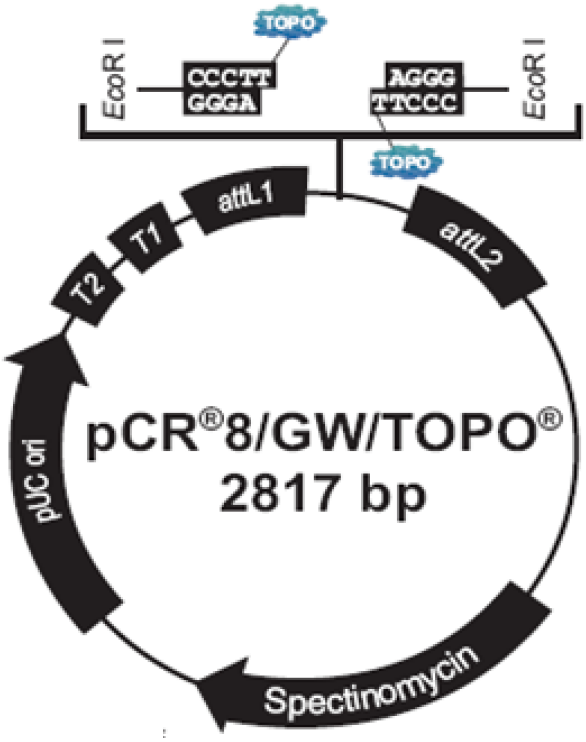
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lumen diameters compared to Col-0 control plants. The increase in biomass of the transgenic lines was attributed to the increase in the number of interfascicular fibre cells compared to the Col-0 control plants. The secondary cell walls of interfascicular fibre cells were 33% thinner in *EgSuSy1* compared to Col-0, whereas *EgSuSy1-S11A* transgenic lines remained unchanged. No significant changes were observed in the cell wall chemistry of the inflorescence stems of Col-0, *EgSuSy1* and *EgSuSy1-S11A* transgenic lines. The expression of *EgSuSy1* and *EgSuSy1-S11A* constructs affected the biomass of the transgenic lines by altering their growth and development, without diverting sucrose carbon towards cellulose deposition into secondary cell walls. We attributed this to the fact that *Arabidopsis* is a herbaceous plant and does not have a strong sink for carbon in the secondary xylem tissue as shown in woody plants such as poplar.

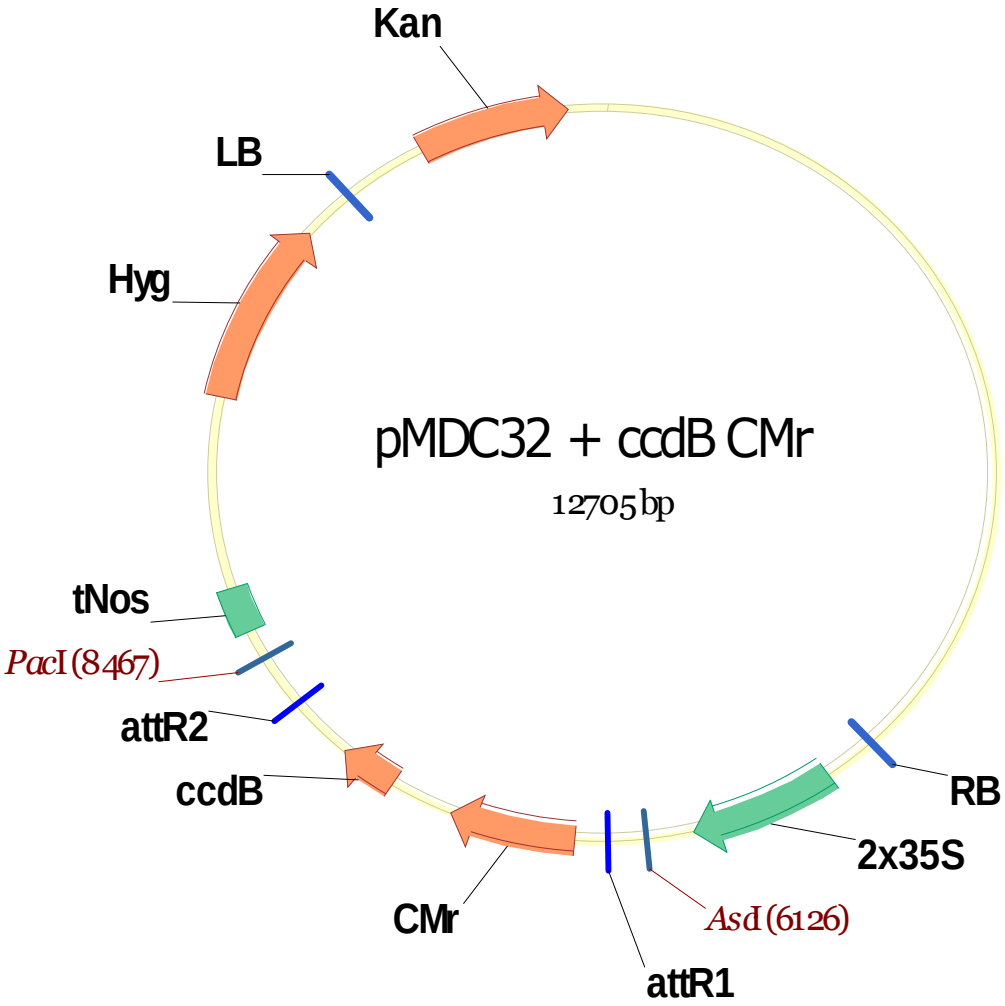
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APPENDICES

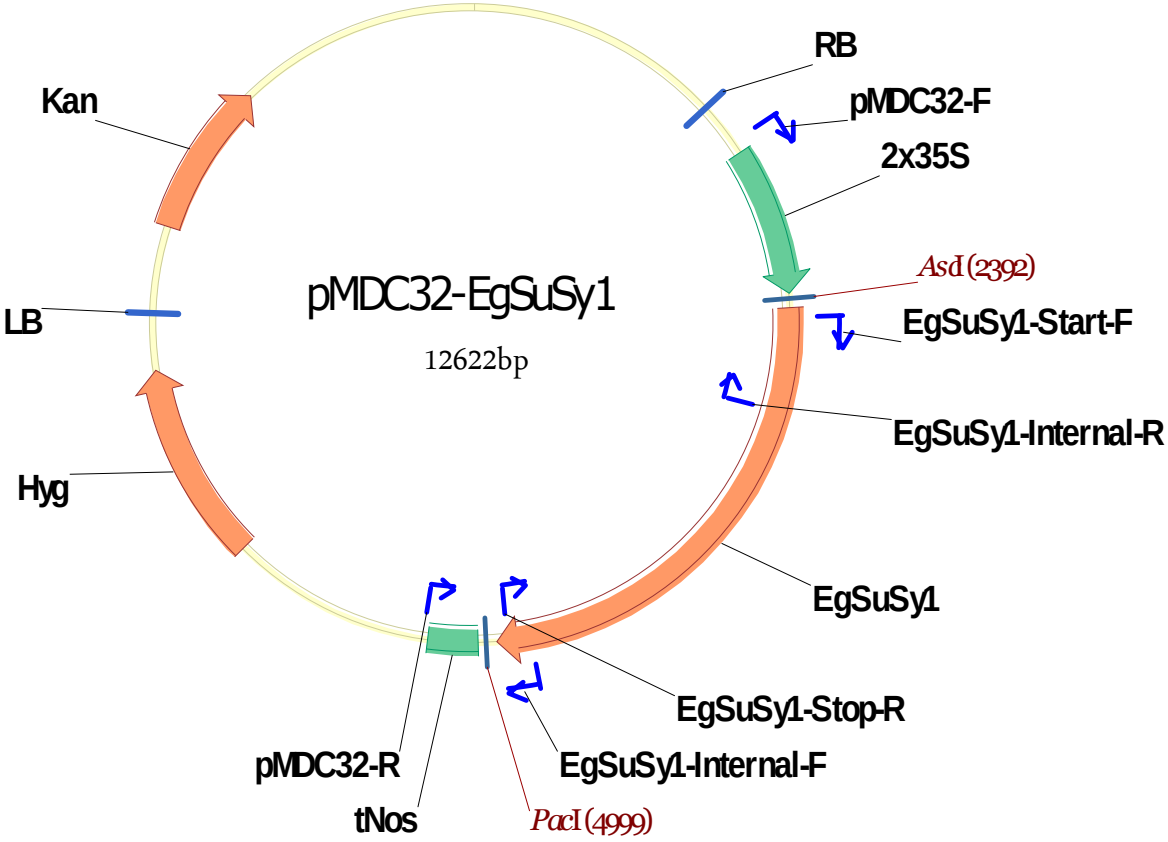
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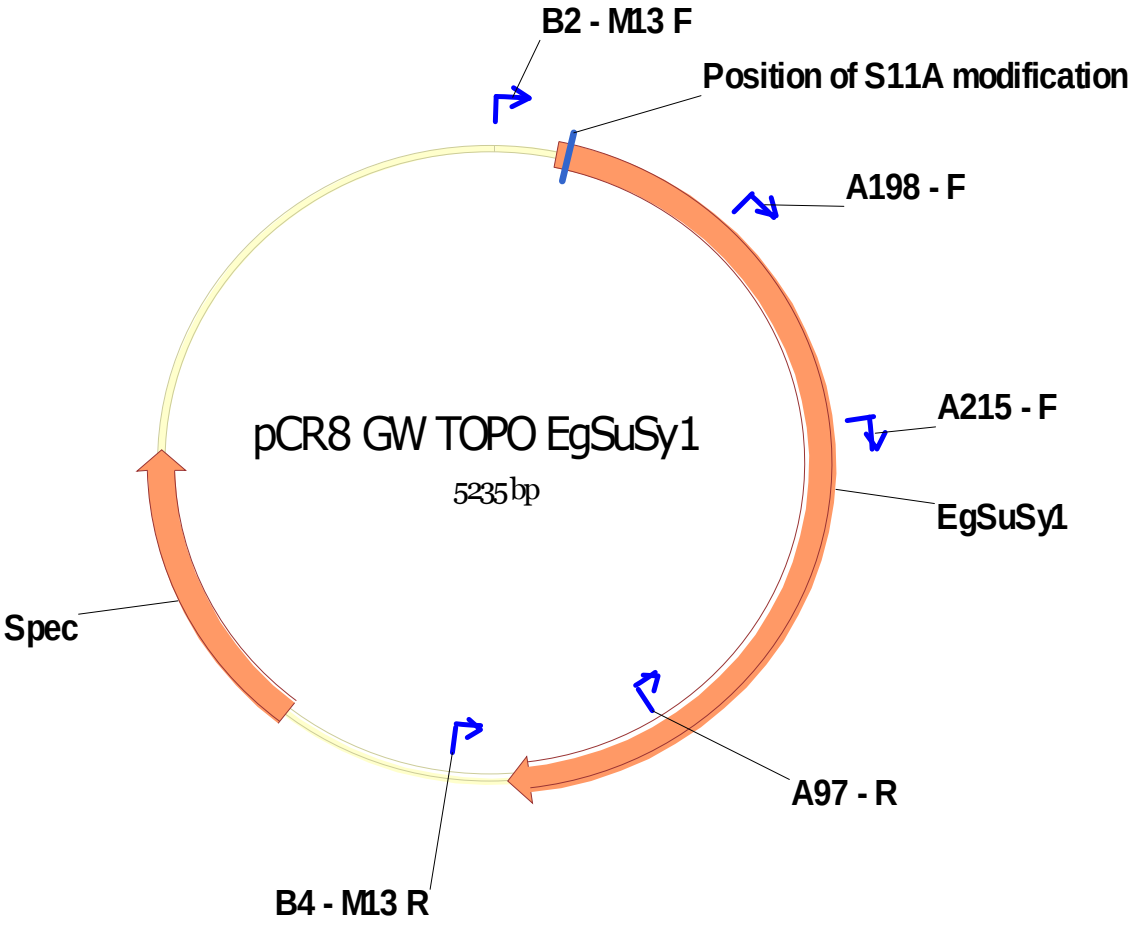
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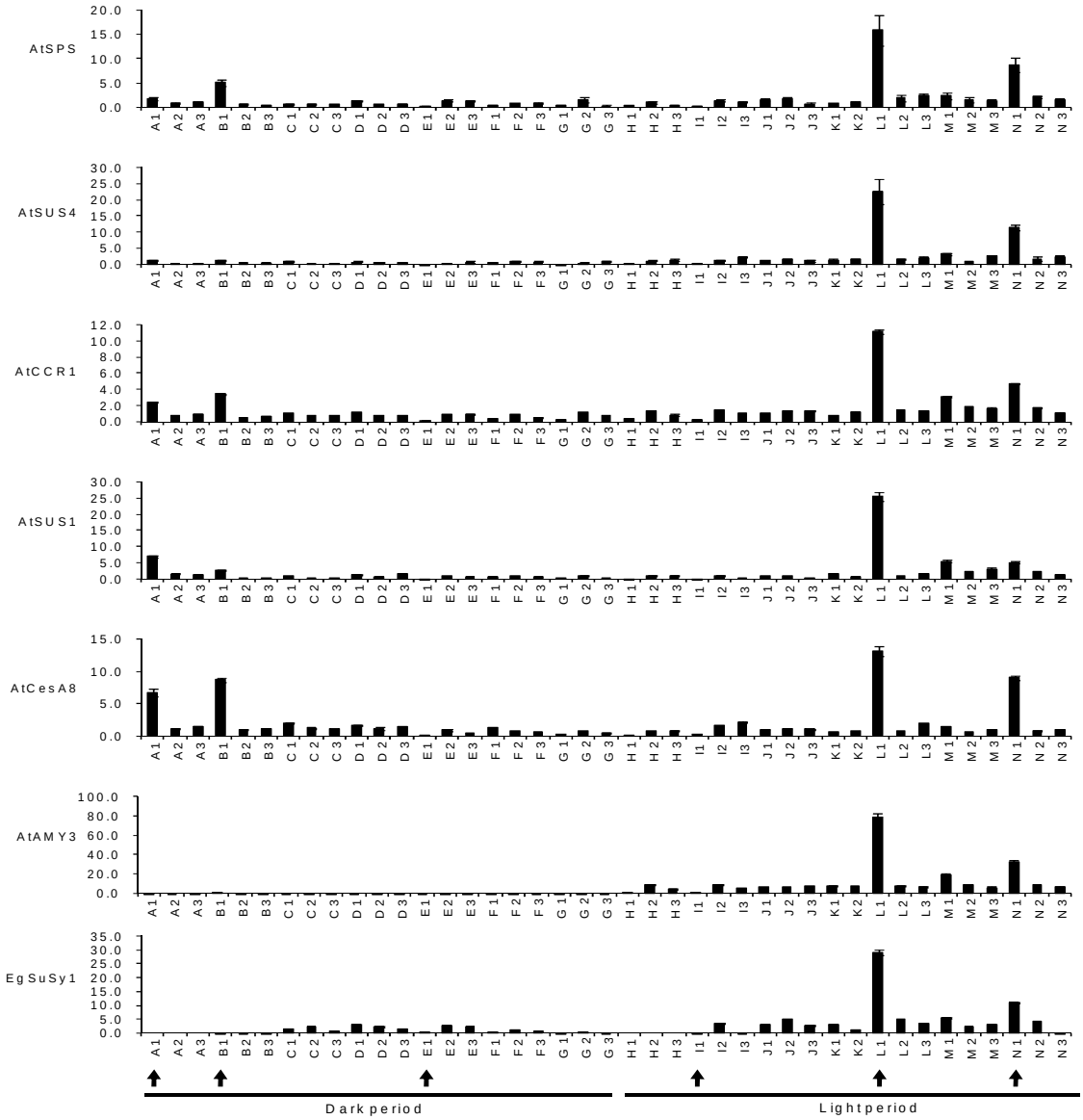
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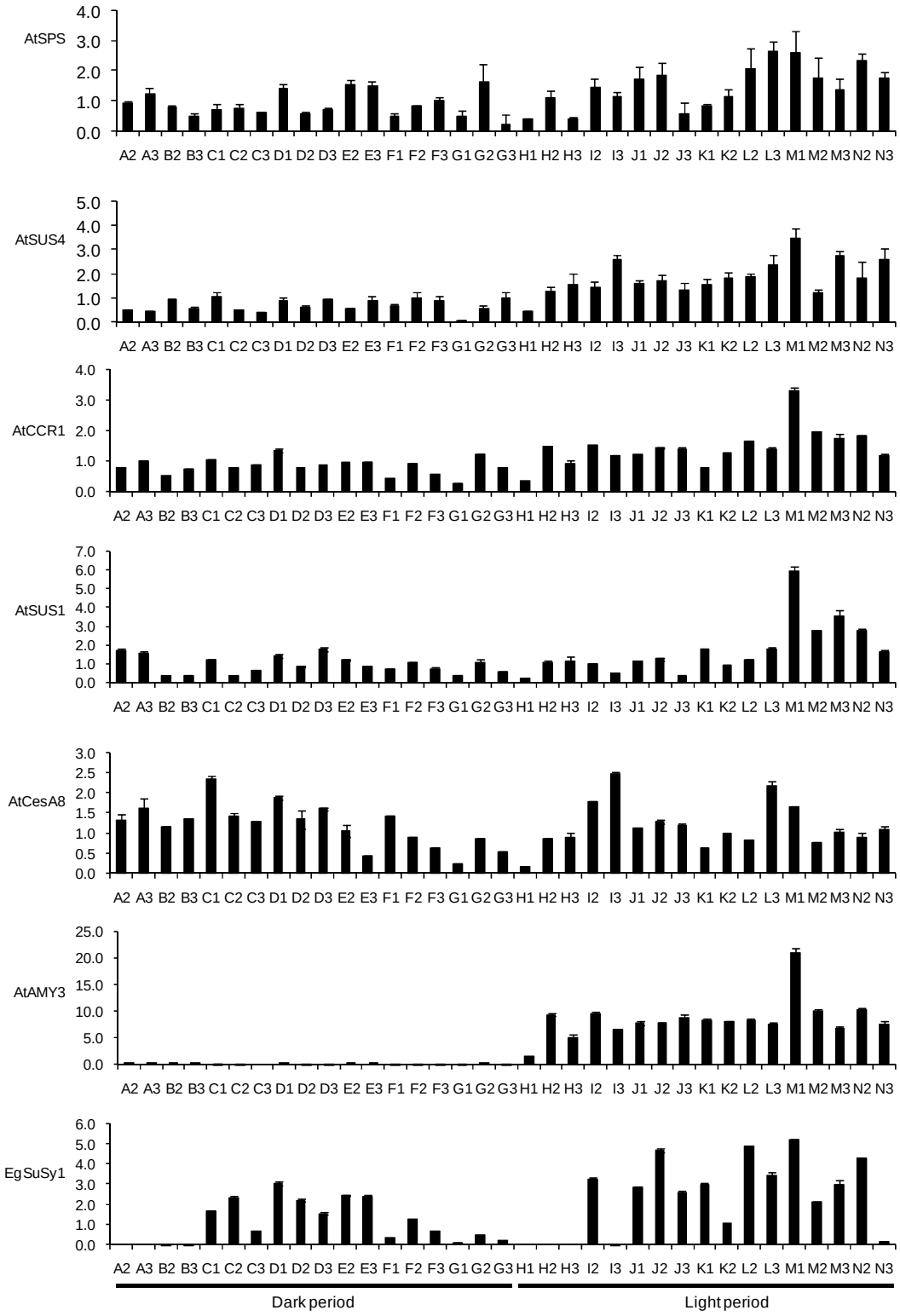
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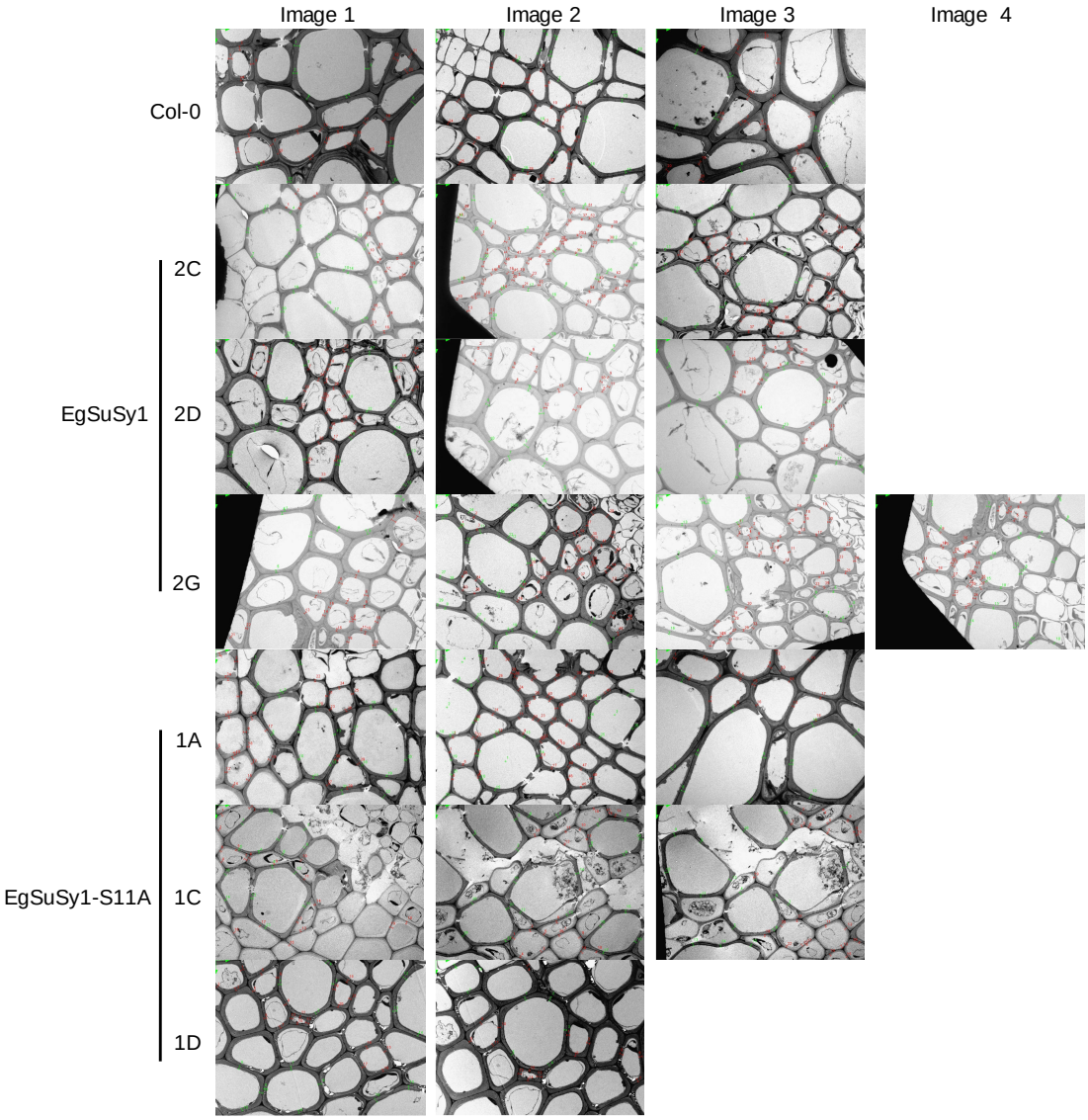
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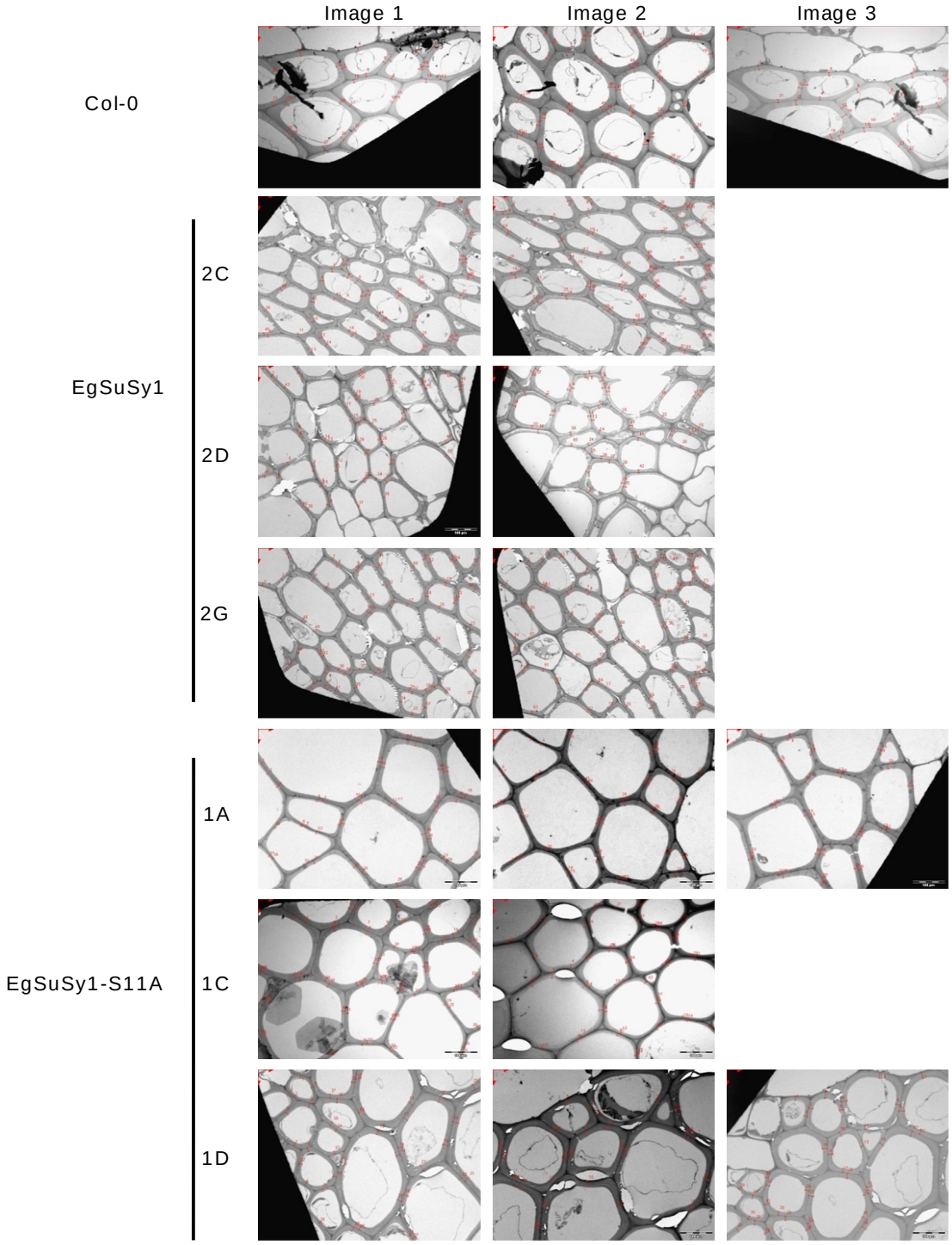
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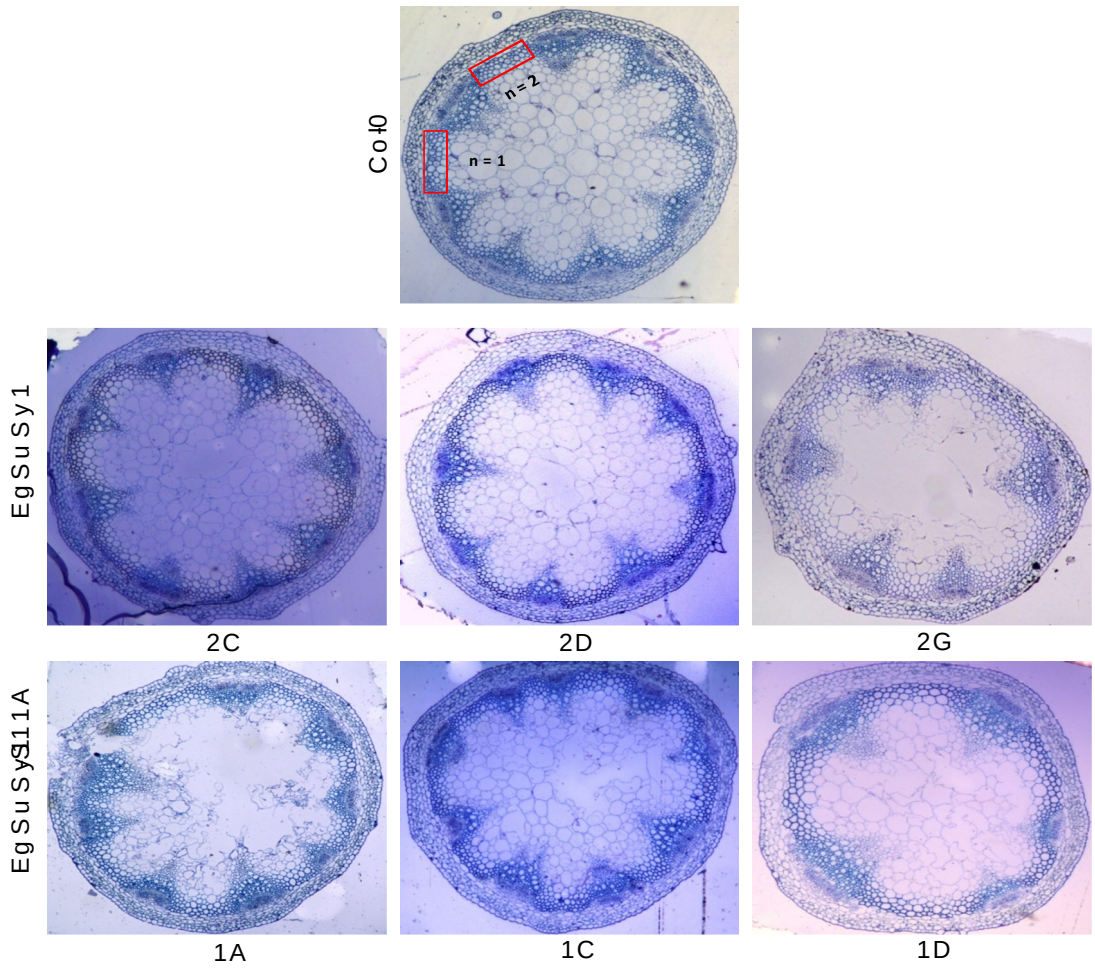
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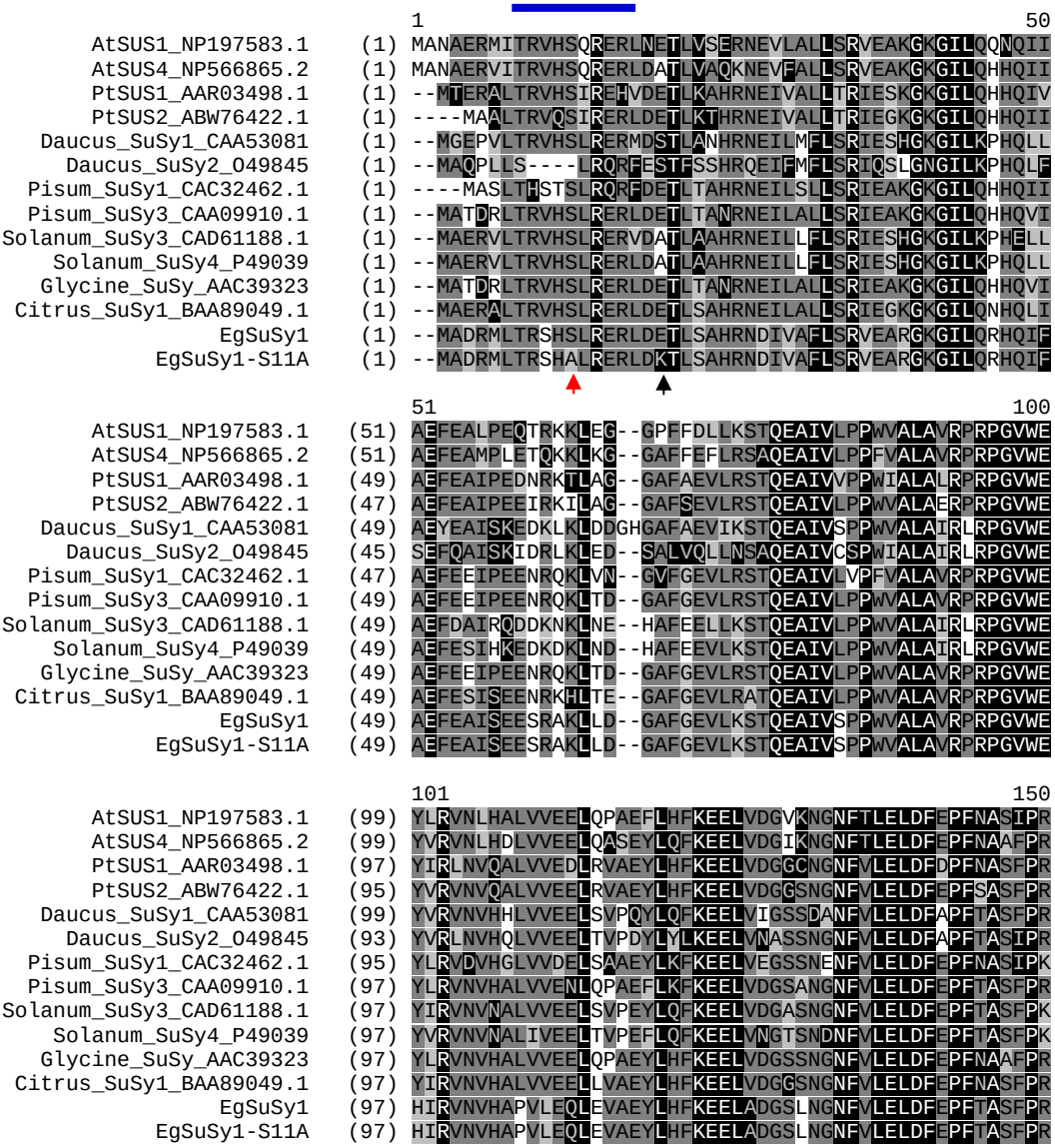
Number of interfascicular cell fibres in the interfascicular region (n) indicated by black line in the transgenic lines

n	Col-0	2C	2D	2G	1A	1C	1D
1	49	107	58	86	66	54	77
2	55	77	53	106	21	54	68
3	46	51	60	61	34	67	43
4	45	74	77	86	49	48	67
5	41	105	56	81	55	80	65
6	25	85	54		63	69	78
7	30	67	59		76	71	
8	53	64	92		89	70	
9						49	

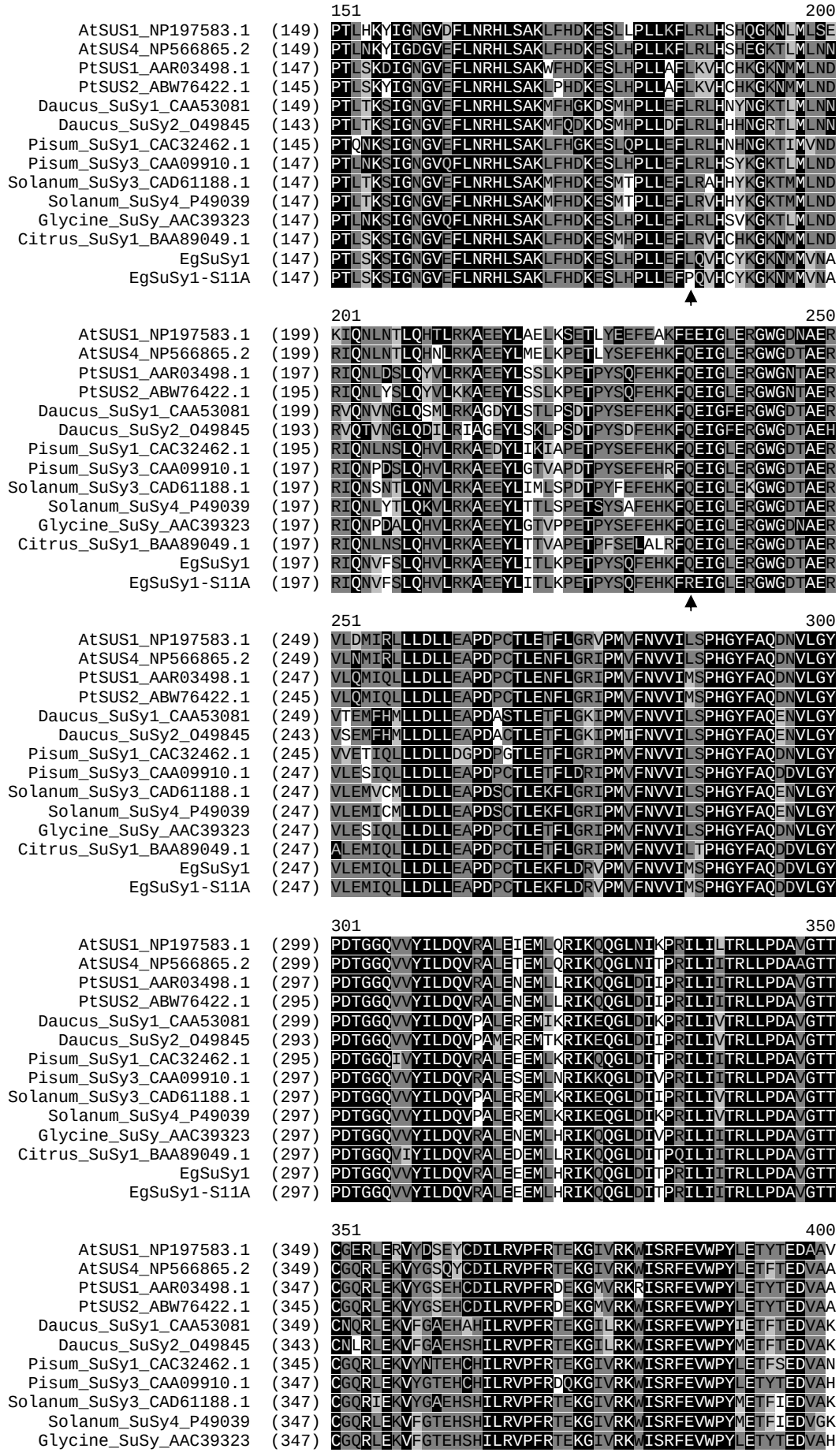
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Appendix H

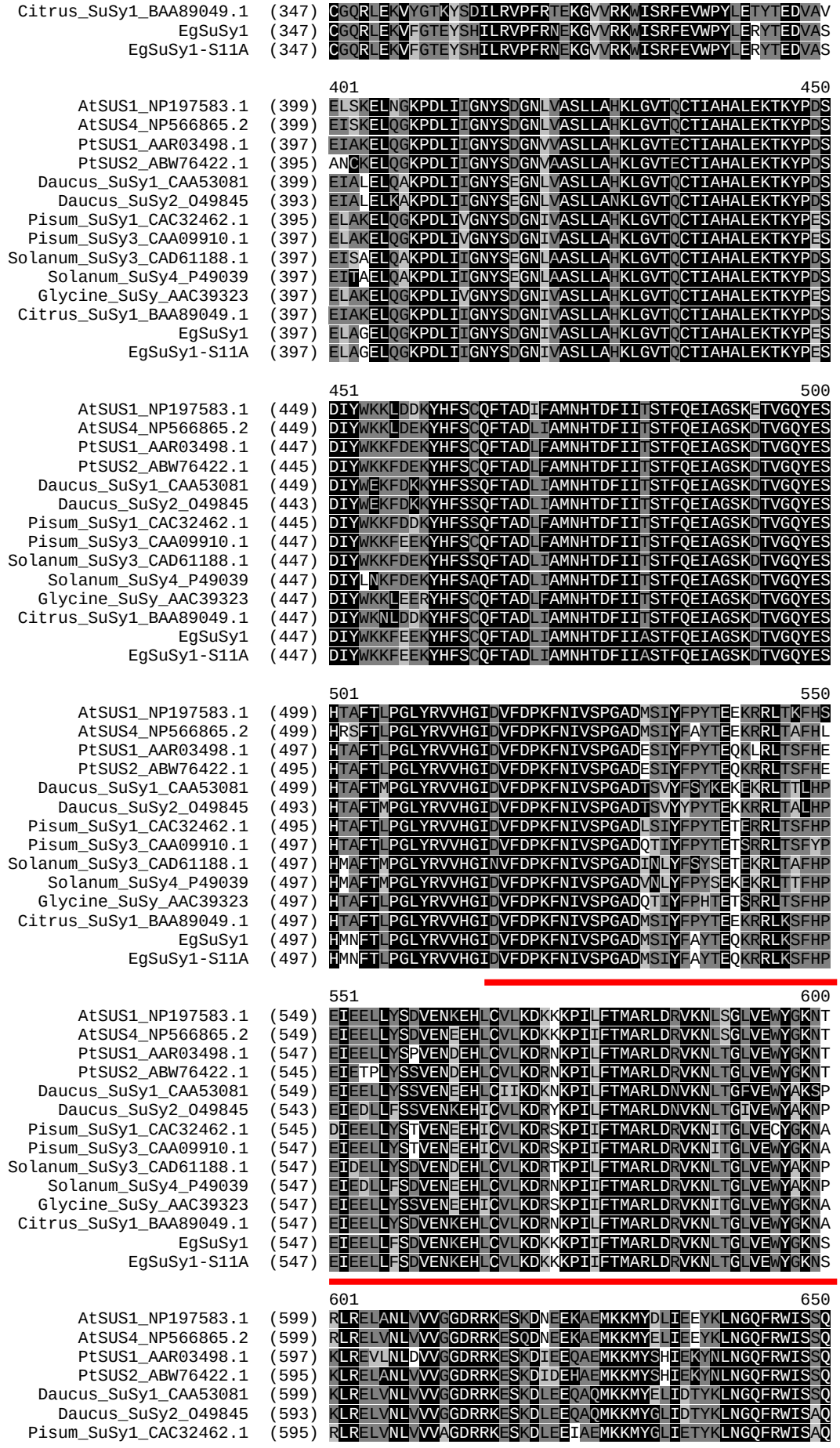
Full-length amino acid sequence alignment (Vector NTI 9.1 – AlignX, Invitrogen) of SuSy dicot group used in the phylogenetic tree (Appendix I) with *EgSuSy1-S11A*. Position 8 to 17 (blue line) represents the conserved N-terminal serine phosphorylation domain (LTRVH**S**LRER). The C-terminal catalytic domain from 564 to 753 (red line) represents the glucocyltransferase region (Baud *et al*, 2004). The red arrow indicates the serine phosphorylation residue. Background mutations indicated with black arrows and S11A with a red arrow.



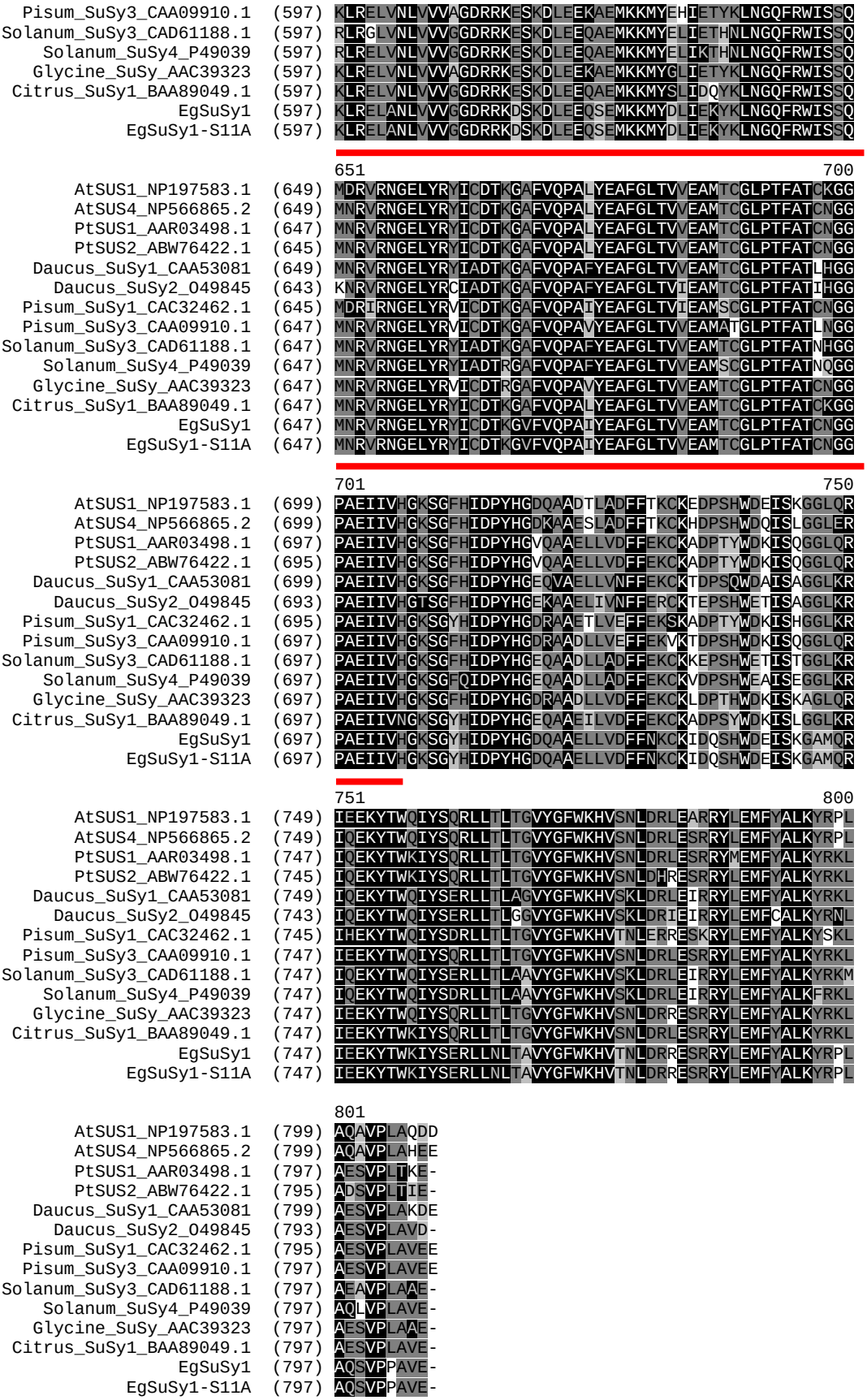
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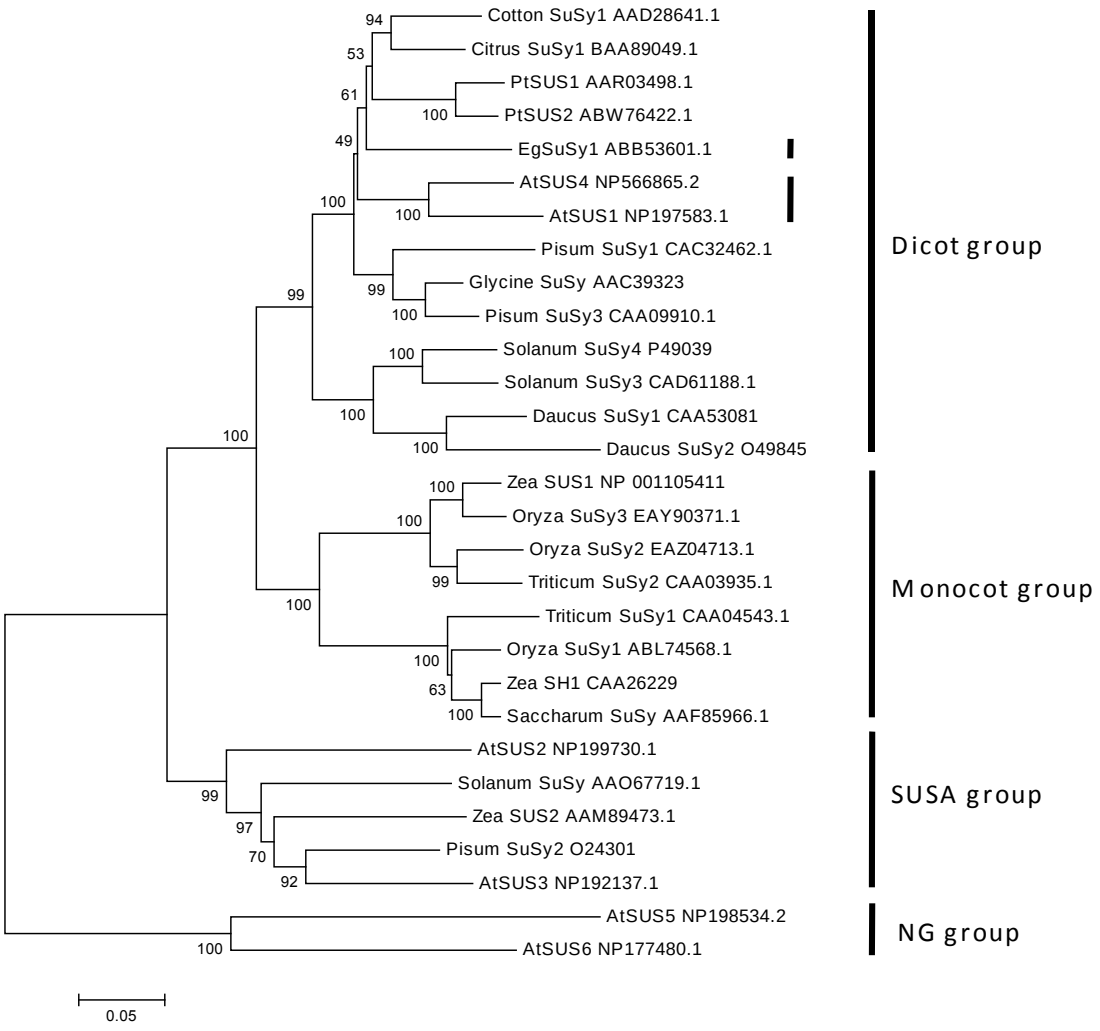
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