INCREASING CELLULOSIC BIOMASS IN SUGARCANE

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Declaration

I the undersigned hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted at any University for obtaining a degree.

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Summary

Increased demand of petroleum, declining fossil fuel reserves, geopolitical instability and the environmentally detrimental effects of fossil fuels have stimulated research to search for alternative sources of energy such as plant derived biofuels. The main feedstocks for production of first generation biofuels (bioethanol) are currently sucrose and starch, produced by crops such as sugarcane, sugarbeet, maize, and cassava. The use of food crop carbohydrates to produce biofuels is viewed as competing for limited agronomic resources and jeopardizing food security. Plants are also capable of storing sugars in their cell walls in the form of polysaccharides such as cellulose, hemicelluloses and pectin, however those are usually cross-linked with lignin, making their fermentation problematic, and are consequently referred to as lignocellulosics. Current technologies are not sufficient to degrade these cell wall sugars without large energy inputs, therefore making lignocellulosic biomass commercially unviable as a source of sugars for biofuel production. In the present study genes encoding for enzymes for cellulosic, hemicellulosic and starch-like polysaccharides biosynthesis were heterologously expressed to increase the amount of fermentable sugars in sugarcane.

Transgenic lines heterologously expressing *CsCesA,* encoding a cellulose synthase from the marine invertebrate *Ciona savignyi* showed significant increases in their total cellulose synthase enzyme activity as well as the total cellulose content in internodal tissues. Elevation in cellulose contents was accompanied by a rise in hemicellulosic glucose content and uronic acid amounts, while total lignin was reduced in internodal tissues. Enzymatic saccharification of untreated lignocellulosic biomass of transgenic sugarcane lines had improved glucose release when exposed to cellulose hydrolyzing enzymes.

Calli derived from transgenic sugarcane lines ectopically expressing galactomannan biosynthetic sequences *ManS* and *GMGT* from the cluster bean (*Cyamopsis tetragonoloba*) were observed to be capable of producing a galactomannan polysaccharide. However, after regeneration, transgenic sugarcane plants derived from those calli were unable to produce the polymer although the inserted genes were transcribed at the mRNA level.

While the ectopic expression of *Deinococcus radiodurans* amylosucrase protein in the cytosol had a detrimental effect on the growth of transgenic lines (plants showed stunted growth through the 18 months growth period in greenhouse), contrastingly targeting the amylosucrase protein into the vacuole resulted in 3 months old transgenic lines which were having high maltooligosaccharide and soluble sugar (sucrose, glucose and fructose) levels in leaves. After 18 months growing in the greenhouse, the mature transgenic lines were morphologically similar to the untransformed lines and also contained comparable maltooligosaccharide and soluble sugar and starch amounts. The non-biosynthesis of galactomannan and amylose polysaccharides in the matured transgenic plants may be due to post-transcriptional protein processing and or protein instability, possibly explainable by other epigenetic mechanisms taking place to regulate gene expression in the at least allo-octaploid species of sugarcane under investigation in this study.

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Preface

The dissertation is presented as a compilation of five chapters. Chapters 1 and 2 are written with the aim to motivate why the study was carried out and to provide a literature background on the work presented in the experimental chapters. Chapters 3 and 4 represent the experimental work performed during the course of the PhD and Chapter 3 will be subject to a patent application to be filed and a manuscript, which will be submitted to the Plant Biotechnology Journal. Chapter 5 is the summary of this study and a model and future work will be discussed.

Chapter 1: General introduction and project aims

Chapter 2: The Plant cell wall-composition and potential use in bioethanol production

The literature review will cover higher plant cell wall architecture and the biosynthesis of lignocellulosic polysaccharides composites.

Chapter 3: Research article

Heterologous expression of the *Ciona savignyi* cellulose synthase in sugarcane increases cellulose contents and improves lignocellulosic biomass saccharification for biofuel production.

Chapter 4: Research article

Ectopic expression of sequences encoding *Cyamopsis tetragonoloba* galactomannan and *Deinococcus radiodurans* α-1,4-glucan chain biosynthetic enzymes leads to non-biosynthesis of the polymers mature in transgenic sugarcane lines.

Chapter 5: Summary, conclusion and future work

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List of abbreviations

ADP	adenosine 5'-diphosphate
AI	acid invertase
AIR	alcohol insoluble residue
AO	ammonium oxalate
ATP	adenosine 5'-triphosphate
Bp	base-pairs
BSA	bovine serum albumin
cDNA	complementary deoxyribo nucleic acid
CesA	cellulose synthase
CTAB	cetyltrimethylammonium bromide
CWI	cell wall invertase
DTT	dithiothreitol
EDTA	ethylenediaminetetra-acetate
Fru-6-P	fructose-6-phosphate
GC-MS	gas chromatography mass spectrometry
Glc-1-P	glucose-1-phosphate
Glc-6-P	glucose-6-phosphate
G6PDH	glucose-6-phosphate dehydrogenase
HEPES	4(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid
IPTG	isopropyl-b-D-thiogalactopyranoside
KOH	potassium hydroxide
MS	Murashige and Skoog
MSTFA	N-Methyl-N-(Trimethylsilyl)-triflouroacetamide
NAD^+	nicotineamide adenine dinucleotide
NADH	nicotineamide adenine dinucleotide
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NI	neutral invertase
OD	optical density
ORFs	open reading frames
PCR	polymerase chain reaction
PGI	glucose-6-P isomerase
PGM	phosphoglucomutase
Pi	inorganic phosphate

PPi	inorganic pyrophosphate
pSuSy	plasma membrane sucrose synthase
RNA	ribonucleic acid
RNaseA	ribonuclease A
RT-PCR	reverse transcription polymerase chain reaction
SDS	sodium dodecyl sulphate
SPS	sucrose phosphate synthase
SuSy	sucrose synthase
Tris	2-amino-2-hydroxymethylpropane-1,3-diol
UDP	uridine diphosphate
UDP-Glc	UDP-D-Glucose
UGD	UDP-Glucose dehydrogenase
UGPase	UDP-Glucose pyrophosphorylase

Chapter 1

General introduction

1.1 Motivation

High demand of petroleum, declining fossil fuel reserves, geopolitical instability and the environmentally undesirable effects of the use of fossil fuels has stimulated research to search for alternative sources of energy such as plant derived biofuels. The main feedstocks for production of one of these biofuels (bioethanol) are currently sucrose and starch, produced by crops such as sugarcane, sugarbeet, maize, and cassava (Abramson *et al*., 2010; Waclawovsky *et al*., 2010; Karp and Richter, 2011). First generation biofuels, produced from food crop sugars, are viewed as competing for limited agronomic resources and jeopardizing food security (Abramson *et al*., 2010; Waclawovsky *et al*., 2010; Karp and Richter, 2011). However not all sugars are stored in the form of sucrose and starch in plants, plant cell walls are also a rich source for fermentable sugars locked in lignocelluloses in the form of cellulose, hemicelluloses and pectin polysaccharides. Current technology is not sufficient to degrade lignocellulosic biomass to soluble sugars this is due to complex structure of plant cell walls. To be efficiently hydrolysed to simple sugars lignocellulosic biomass requires pre-treatment at high temperature with acid or alkaline solution therefore adding to the cost making it commercially unviable as a source of sugars for biofuel production.

The main objective of the second generation biofuel technologies is to enable the utilization of lignocellulosic biomass from plant cell walls to produce bioethanol (Pauly and Keegstra, 2010; Harris and DeBolt, 2010; Cook and Devoto, 2011). Lignocellulosic polymers provide structural integrity and protect the plants from pathogens. Consequently, they have evolved to be recalcitrant to enzymatic hydrolysis (Abramson *et al*., 2010; Harris and DeBolt, 2010). The resistant nature of lignocellulosic feedstocks to degradation is ascribed partly to the components of the cell wall limiting access to cellulolytic enzymes (Mosier *et al*., 2005; Abramson *et al*., 2010, Cesarino *et al*., 2013). When lignin, pectin and hemicellulose polysaccharides polymers have been partially removed, the cellulose still remains resistant to

hydrolysis by cellulases due to the crystalline nature of cellulose microfibrils (Himmel *et al*., 2007; Igarashi *et al*., 2011). In addition pre-treatment of lignocellulosic biomass at high temperatures and/ or with acidic or basic solutions to remove hemicellulosic, pectic and lignin polymers produces inhibitory products (furfural and hydroxymethylfurfural) which can subsequently inhibit hexose fermenting microorganisms and reduce the ethanol yield (Gámez *et al*., 2004; Klinke *et al*., 2004; Alvira *et al*., 2010). In order to efficiently utilize lignocellulosic biomass as a feedstock for biofuel production, it should be readily available, abundant and, most critically, be amenable to enzymatic hydrolysis and bacterial fermentation.

Sugarcane (*Saccharum* spp. hybrids) is mainly cultivated for its ability to accumulate sucrose at up to 50-60% of its dry weight in internodal tissues (Moore, 1995; Waclawovsky *et al*., 2010). This perennial C_4 grass has the ability to accumulate high lignocellulosic biomass and requires minimal light, water and nitrogen sources for its cultivation (Taylor *et al.,* 2010; Waclawovsky *et al*., 2010). The South African sugar industry produces 1.8 million tons of sugar and 8 million tons bagasse (lignocelluloses) from approximately 270 thousand hectares annually (http://www.sasa.org.za/sugar_industry/IndustryOverview.aspx). Sucrose derived from sugarcane has been successfully used in Brazil for the production of bioethanol (Goldemberg, 2007; Cesarino *et al*., 2012), however, this is viewed as a potential jeopardization of food security (Abramson *et al*., 2010; Waclawovsky *et al*., 2010; Karp and Richter, 2011).

Sugarcane bagasse, the residue produced after sucrose extraction, is an abundant lignocellulosic biomass containing highly fermentable sugars locked in cellulose, hemicelluloses and pectin polysaccharides (Carroll and Somerville 2009; Dias *et al*., 2012; Dias *et al*., 2013). The bagasse is estimated to be composed of approximately 40-50% cellulose, 25-35% hemicelluloses, 18-23% lignin, 2-3% ash and 0.8% wax (Sun *et al*., 2004; Masarin *et al*., 2011). Currently, bagasse is burned to generate of heat for sugar processing and electricity production (Waclawovsky *et al*., 2010). The sugarcane industry could add value by converting bagasse to bioethanol since it is readily available after sucrose extraction and may also share part of the infrastructure and therefore might reduce cost (Dias *et al*., 2012; Dias *et al*., 2013). The above traits and abundance of bagasse mean that sugarcane has a great potential to be an energy crop (Taylor *et al.,* 2010; Waclawovsky *et al*., 2010; Byrt *et al*., 2011).

Although a number of studies have been conducted in sugarcane to introduce new metabolic carbon sinks through production of novel sugars and biopolymers (Hamerli and Birch 2011; Basnayake *et al*., 2012; Bauer *et al*., 2012; Mudge *et al*., 2013), there are few studies on lignocellulosic biomass improvement in sugarcane (Jung *et al*., 2012; Jung *et al*., 2013). Recently, a transgenic modification of the lignin biosynthetic pathway in sugarcane has been shown to result in improved saccharification of lignocellulosic biomass without compromising the plant performance (Jung *et al*., 2012; Jung *et al*., 2013). Tailoring sugarcane plants with altered polysaccharides such as more highly soluble cellulose (paracrystalline cellulose) or water soluble hemicelluloses (e.g. galactomannan) may be another way of improving saccharification efficiency of sugarcane lignocellulose biomass.

Cellulose is composed of unbranched β-(1,4)-linked glucose chains that are biosynthesised at the plasma membrane (Delmer and Haigler, 2002; Saxena and Brown 2005). It is produced by a diverse group of organisms, including plants, bacteria, cellular slime moulds and one group of marine invertebrates, the urochordates (Brown, 2004; Kimura and Itoh, 2004). Genes encoding cellulose synthase (*CesA*) enzymes have been identified and functionally characterized in many of these organisms (Pear *et al.,* 1996; Ariola *et al*., 1998; Matthysse *et al.,* 2004). Heterologous expression of bacterial or native *CesA* gene(s) in cotton and potato respectively have been observed to result in transgenic plants with increased cellulose contents (Li *et al*., 2004; Oomen *et al*., 2004). These studies show that the biosynthesis of the cellulose biopolymer could be modified in crops plants by native or non-native genes, therefore opening a possibility of altering the lignocellulosic polysaccharide composition.

1.2 Aims of this study

The main aim of the present study was to increase cellulosic bagasse biomass in sugarcane plants with the emphasis on increasing cellulose, galactomannan and α-1,4-glucan polysccharides. This was done in the following three main studies:

1) *CsCesA,* encoding a cellulose synthase from the marine invertebrate *Ciona savignyi* and was express in sugarcane plants.

2) Isolate the galactomannan biosynthetic sequences *ManS* and *GMGT* from *Cyamopsis tetragonoloba* and heterologously express in sugarcane.
3) Isolate the α-1,4-glucan biosynthetic gene *dras* encoding amylosucrase from the bacterium *Deinococcus radiodurans* and express it in sugarcane plants

Heterologous expression of cellulose, galactomannan and α-1,4-glucans biosynthesis sequences in sugarcane could lead to high fermentable carbohydrates and increase the potential for the production of biofuels from the sugarcane crop.

1.3 Layout and aims of the chapters

CHAPTER 2

The Plant cell wall-composition and potential use in bioethanol production

Aim: The literature review will cover the differences in plant cell wall architecture, the genes involved in the biosynthesis of lignocellulose polymers and the interaction of these polymers in the cell wall.

CHAPTER 3

Heterologous expression of the *Ciona savignyi* cellulose synthase in sugarcane increases cellulose contents and improves lignocellulosic biomass saccharification for biofuel production.

Aim: In this chapter a cellulose synthase of a *Ciona savignyi* cDNA sequence (*CsCesA)* for cellulose biosynthesis construct will be developed and transferred to sugarcane for heterologous expression. The levels of total cellulose synthase enzyme activity and cellulose content of the transgenic plants will be assessed. The impact of increased cellulose amounts on metabolites, hemicelluloses, pectins and lignin polymers content will also be evaluated. Lignocellulosic biomass saccharification efficiency will be assessed to evaluate the impact of the change in cell wall polymer composition.

CHAPTER 4

Ectopic expression of sequences encoding *Cyamopsis tetragonoloba* galactomannan and *Deinococcus radiodurans* α-1,4-glucan chain biosynthetic enzymes leads to non-biosynthesis of the polymers in mature transgenic sugarcane lines.

Aim 1: In this chapter galactomannan biosynthetic sequences *ManS* and *GMGT* from *Cyamopsis tetragonoloba* constructs will be developed and transferred to sugarcane for heterologous expression. The integration of galactomannan biosynthesis genes into sugarcane genomic DNA and their expression at the mRNA level will be evaluated from transgenic sugarcane calli. The enzymes activities of galactomannan mannan synthase and UDP-galactose-dependent galactosyltransferase from transgenic sugarcane suspension culture cells will be assessed as will their galactomannan contents.

Aim 2: In this chapter the α-1,4-glucan biosynthetic enzyme encoding gene *dras* from *Deinococcus radiodurans* constructs will be developed and transferred to sugarcane for heterologous expression in the cytoplasmic and vacuolar compartments. The integration of α-1,4-glucan biosynthesis gene into sugarcane genomic DNA and their expression at the mRNA level will be evaluated from putative transgenic sugarcane calli. The enzyme activies of amylosucrase from the transgenic sugarcane will be assessed and also their α-1,4-glucan amounts.

CHAPTER 5

Summary, conclusion and future work

This chapter aims to discuss and summarize the observations of the previous chapters and to recommend future research on the topic.

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Chapter 2

The Plant cell wall-composition and potential use in bioethanol production

2.1 Plant cell wall lignocellulosic matrix

Plant cells are surrounded by a physical barrier, the cell wall, which provides shape, structural integrity and protection against environmental stresses (Carpita and Gibeaut, 1993; Somerville *et al.*, 2004). They are an interwoven matrix of polysaccharides (cellulose, hemicelluloses, pectins), proteins and lignin (Figure 2.1) (Carpita and Gibeaut, 1993). Growing cells are covered by walls, referred to as primary cell walls, however, after growth and cell division has ceased these primary cell walls become encased by secondary cell walls.

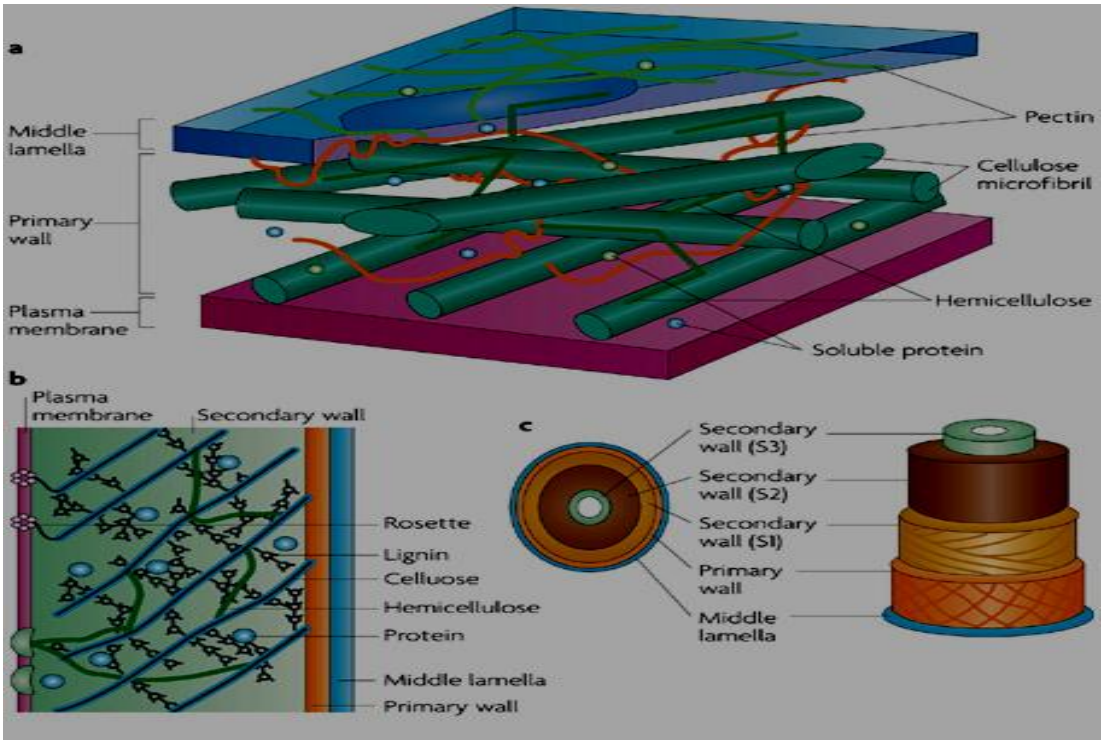

Figure 2.1: a) Cell wall structure containing cellulose microfibrils, hemicellulose, pectin, lignin and soluble proteins. b) Cellulose synthase enzymes are in the form of rosette complexes, which float in the plasma membrane. c) Lignification occurs in the S1, S2 and S3 layers of the cell wall.

The picture adapted from Mariam B. Sticklen (2008): Plant genetic engineering for biofuel production: towards affordable cellulosic ethanol. Nature Reviews Genetics 9, 433-443

Primary cell walls are further grouped as type I or type II based on their hemicellulosic polysaccharide composition (Carpita and Gibeaut, 1993, Carpita, 1996). Type I walls

are predominantly present in dicots and non-commelinoid monocots where xyloglucan is the main hemicellulose that is embedded in a pectinaceous gel cross-linked to structural proteins that form a matrix cover for cellulose (Carpita and Gibeaut, 1993). In type II cell walls, arabinoxylans and mixed-linkage (1,3;1,4)-β-glucans are the major hemicelluloses and are generally found in Poales (grasses, sugargane and sorghum) where the cell walls contain only minor amounts of pectic polysaccharides (Carpita, 1996; Scheller and Ulvskov, 2010). The secondary cell wall is deposited inside the primary walls as cell expansion and division approaches cessation and are largely composed of cellulose with glucuronoxylans (dicots) or arabinoxylans (monocots) as the major hemicellulosic polysaccharides (Mellerowics *et al.*, 2001; Turner *et al.*, 2007). In addition, cross-linking phenylpropanoid networks (lignin) are also deposited in the walls as the primary cells mature (Carpita, 1996).

2.1.1 Hemicellulosic polysaccharides

Hemicelluloses are a diverse group of polysaccharides that are characterized by a β-(1,4)-linked backbone of sugars in an equatorial configuration which are often substituted by side branches (Scheller and Ulvskov, 2010). They are synthesised in the Golgi lumen, then exported into the cell wall by exocytosis where they interweave and cross-link with cellulose microfibrils via hydrogen bonds (Figure 2.2) (Hayashi, 1989; Lerouxel *et al*., 2006). The hemicellulosic polysaccharides can be grouped into four main classes: xyloglucans, xylans (glucuronoxylans, arabinoxylans and glucurono-arabinoxylans), mannans (glucomannans, galactomannans and galactoglucomannans), and mixed-linkage β-(1,3;1,4)-glucans, which varies considerably between species and cell types (Pauly and Keegstra, 2008; Carpita and McCann, 2010; Carpita, 2011).

The biosynthesis of all hemicellulosic polysaccharides, except mixed-linkage β-(1,3;1,4)-glucans is divided into two main stages, the synthesis of the backbone by polysaccharide synthases and the addition of side chain residues in reactions catalyzed by glycosyltransferases (Perrin *et al*., 2001). The β-(1,4)-linked backbone has structural similarities with the cellulose β-(1,4)-glucan and this led to the assumption that *Cellulose synthase like* (*Csl*) genes may be involved in their biosynthesis (Richmond and Somerville, 2000; Richmond and Somerville, 2001; Hazen *et al*., 2002).

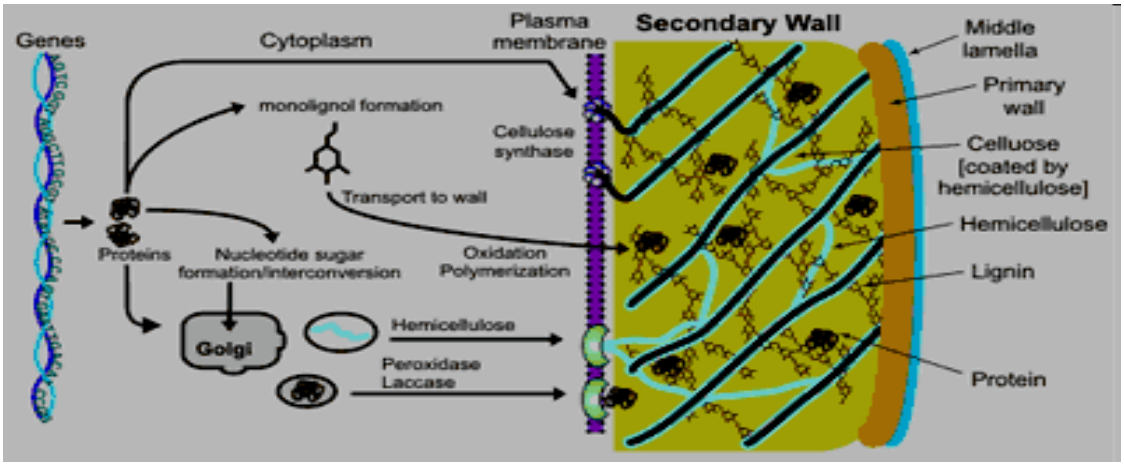

Figure 2.2: Cell wall structural polymers biosynthesis and their interaction in the walls.

The picture was adapted from www.ccrc.uga.edu

The hypothesis was later proven by Dhugga *et al* (2004), who heterologously expressed a mannan synthase (*ManS)* sequence in soybean from guar seeds which were undergoing galactomannan deposition in their endosperm to show that it was capable of *in vitro* synthesising β-(1,4)-mannan backbone and also it was a Golgi membrane associated protein. Furthermore, expression of *Csl* genes from rice and *Arabidopsis* in *Drosophila* cells, were shown to have the ability to produce proteins capable of synthesizing β-(1,4)-mannan backbone when GDP-mannose was present and also glucomannan when GDP-mannose and GDP-glucose substrate were available (Liepman et al, 2005).

The structural complexity of hemicellulosic polysccharides is due to the substitution of their β-(1,4)-glucan backbone with side chains (Perrin *et al*., 2001, Edwards *et al*., 2002). These are added by Golgi localised type II membrane glycosyltransferase enzymes (Reid *et al*., 1995; Edwards *et al*., 1999; Perrin *et al*., 2001). Detergent solubilised galactosyltransferases from galactomannan biosynthesizing fenugreek and guar developing endosperm have been shown *in vitro* to have the capability to add side chains to β-(1,4)-mannan backbones (Reid *et al*., 1995; Edwards *et al*., 1999; Edwards *et al*., 2002). The fucosyl residue on xyloglucan side chains is added by a fucosyltransferase and microsomal fucosyltransferase enzymes prepared from the pea epicotyls were shown to have the capacity to add fucose to tamarind non-fucosylated β-(1,4)-xyloglucans backbone (Perrin *et al*., 1999). In addition, peptide sequences from pea epicotyls led to the identification of Arabidopsis

fucosyltransferase sequences which, when expressed in mammalian cells, resulted in samples that had high fucosyltransferase activity (Perrin *et al*., 1999).

The biological importance of the hemicellulosic polysaccharides in plant cells is due to their interactions with cellulose, pectin, lignin and structural proteins (glycoproteins), which strengthen the cell walls (Scheller and Ulvskov, 2010). The hemicellulosic polysaccharides are strongly, non-covalently, interlinked to cellulose microfibrils via multiple hydrogen bonds (Hayashi, 1989; Pauly *et al*., 1999). In dicots and non-commelinoid monocots, xyloglucan is the main hemicellulose that is bound to the cellulose microfibrils while, in grasses, arabinoxylans, glucomannans and β-glucans play this role (Hayashi, 1989; Pauly *et al*., 1999; Carpita *et al*., 2001). Evidence of cellulose and xyloglucans forming crosslinks of 20 and 40 nm was revealed when rapidly frozen, deep-etched onion primary cell-walls were visually investigated by electron microscopy (McCann *et al*., 1990). Furthermore, the model of xyloglucan as a load bearing material was supported by sequential treatments of etiolated pea stems with a xyloglucan specific endoglucanse, 4N potassium hydroxide and non specific cellulases which demonstrated that the hemicellulosic polysaccharide xyloglucan is strongly linked to cellulose (Pauly *et al*., 1999). The interaction of hemicellulosic polysaccharides with cellulose in plants provides integrity, structural flexibility and strength, demonstrating that the plant cell wall is a complex interwoven structure rich in fermentable sugars for biofuel production.

2.1.2 Pectic polysaccharides

Pectins are a structurally complex family of galacturonic acid rich polysaccharides in plant cell walls (Ridley *et al*., 2001). These polysaccharides are present in all cell types but their abundance varies between cell type and species (O'Neill *et al*., 2004). The walls of dicots, gymnosperms and non-commelinoid monocots contain relatively high amounts of pectic polysaccharides while Poaceae grasses contain minor amounts of pectins (Willats *et al*., 2001; O'Neill *et al*., 2004; Mohnen, 2008). Pectic polysaccharides are generally divided into four structural classes: homogalacturonan (HG), rhamnogalacturonan I (RG I), rhamnogalacturonan II (RG II) and xylogalacturonan (XGA) (Willats *et al*., 2001; Mohnen, 2008; Caffall and Mohnen, 2009). The acid rich polysaccharides are considered to provide an environment for the deposition and extension of the cellulosic-glycan network and control of cell wall

permeability, elasticity and compressibility (Willats *et al*., 2001; Peaucelle *et al*., 2012).

Homogalacturonans is a linear homopolymer chain of (1→4)-*α*-linked-D-galacturonic acid, in which 70-80% of galacturonic acids are methyl esterified at the C6 carboxyl and may also be acetylated or substituted with xylose or apiose (Willats *et al*., 2001; Caffall and Mohnen, 2009). Homogalacturonans are the dominant pectin polysaccharide, representing approximately 65% of pectins in plants (Zandleven *et al*., 2007; Mohnen, 2008). RG-II is a complex structure that constitutes approximately 10% of pectic polysaccharides with twelve different types of sugar residues (Mohnen, 2008; Atmodjo *et al*., 2013). In spite of its structural complexity, RG-II has an HG backbone to which four structurally different oligosaccharide chains, denoted A, B, C, and D, are attached and its structure is conserved amongst plants (O'Neill *et al*., 2004; Mohnen, 2008). Rhamnogalacturonan I is different from RG-II and HG as it is composed of a disaccharide repeating backbone of (→α-D-Gal*p*A-1,2-α-l-Rha*p*-1,4→)$_n$, in which the galacturonic residues are highly acetylated at the O-2 or O-3 positions (Lau *et al*., 1985; Nakamura *et al*., 2002). The rhamnose residues of the backbone of RG I may also be substituted with *β*-1,4-galactan, branched arabinan, and/or arabinogalactan side chains (Caffall and Mohnen, 2009; Harholt *et al*., 2010; Atmodjo *et al*., 2013).

The biosynthesis of pectic polysaccharides is assumed to occur at the Golgi membranes, even though the possibility exists that the initial steps may take place in the endoplasmic reticulum (Harholt *et al*., 2010; Driouich *et al*., 2012; Atmodjo *et al*., 2013). Pectic polysaccharide biosynthetic enzyme activities have only been shown to be present in the Golgi vesicles (Geshi *et al*., 2000; Sterling *et al*., 2001; Geshi *et al*., 2004). Based on the structural complexity of the pectic polysaccharides, Mohnen (2008) predicted that 67 different glycosyltransferases, methyltransferases and acetyltransferases may be required for their biosynthesis. *Homogalacturonan glycosyltransferase* (*GAUT1*) was the first gene sequence to be identified in Arabidopsis plants; expression of the sequence in human kidney cell lines yielded proteins capable of synthesizing polygalacturonic acid in vitro (Sterling *et al*., 2006). Genes encoding enzymes involved in the biosynthesis of RG I and RG II pectic polysaccharides have yet to be identified.

The pectin RG-I backbone is assumed to be synthesized by two enzyme classes, galactosyltransferases and rhamnosyltransferase (Mohnen, 2008; Bar-Peled *et al*., 2012; Atmodjo *et al*., 2013). So far their activities have not been shown, which might be partially due to the commercial unavailability of the substrate donor UDP-Rha for demonstrating their biosynthesis biochemically (Bar-Peled *et al*., 2012; Atmodjo *et al*., 2013). The only gene predicted to be involved in the biosynthesis of RG-II polysaccharides side chains is *Rhamnogalacturonan II xylosyltransferase* (*RGXT4)* in Arabidopsis (Egelund *et al*.,2006; Liu *et al*.,2011). Mutation in *RGXT4* resulted in Arabidopsis plants with a 30% reduction in 2-*O*methyl-D-Xyl of RG-II and a 23% decrease in RG-II dimerization (Liu *et al*., 2011). These observations support the assumption that the *RGXT4* gene is involved in the biosynthesis of RG-II pectic polysaccharides.

The HG pectic polysaccharide backbone is covalently linked to RG I and RG II and is also thought to be covalently cross-linked to the hemicellulosic polysaccharide xyloglucan and to cellulose (Nakamura *et al*., 2002; Coenen *et al*., 2007; Marcus *et al*., 2008; Khodaei and Karboune, 2013). In soybeans and potato, pectic polysaccharides extracted with water or weak alkaline solutions cannot be separated using gel filtration or ion exchange chromatography (Nakamura *et al*., 2002; Khodaei and Karboune, 2013). However, treatment of these fractions with *α*-(1,4)-endo-polygalacturonase enzyme and structural analysis by NMR showed that they contain high galacturonic acid, rhamnose, and *α*-(1,4)-linked galacturonic acid flanked by RG-I fragments providing strong evidence that pectic polysaccharides are covalently bonded together by glycosidic linkages (Nakamura *et al*., 2002; Khodaei and Karboune, 2013). There is evidence to indicate that pectins and xyloglucans are covalently linked (Femenia *et al*., 1999; Thompson and Fry, 2000; Marcus *et al*., 2008). Femenia *et al*., (1999), digested a xyloglucan complex fraction isolated from cauliflower stem with *endo*-xylanase and *endo*-polygalacturonase, their observed a decresed in molecular weight of the xyloglucan moieties, this suggested xyloglucan was association with pectic polysaccharides. Similarly, pre-treatment of high xyloglucan accumulating tobacco and pea sections with pectate lyase to remove pectic homogalacturonan, resulted in an increased detection of xyloglucan by antibodies directed against the LM15 xyloglucan specific epitope in the sections (Marcus *et al*., 2008). These observations led the authors to conclude that pectin in plants cell walls is associated with the hemicellulosic polysaccharide xyloglucans.

2.1.3 Lignin biopolymers

Lignins are a highly complex structure of polyphenolic heteropolymers that is synthesized from three monolignol precursors, namely *p*-coumaryl, coniferyl and sinapyl alcohols. These are polymerized to give rise to phenylpropanoid lignin units: *p*-hydroxyphenyl (H units), guaiacyl (G units), and syringyl (S units) (Boerjan *et al*., 2003; Ralph *et al*., 2004; Vanholme *et al.*, 2012). The lignin biopolymers are predominantly deposited in the thickened secondary cell walls, after primary cell wall biosynthesis has ceased (Ralph *et al*., 2004; Vanholme *et al.*, 2010). Biosynthesis of these phenolic biopolymers can also be triggered by biotic and abiotic stresses as well as perturbations in cell wall structure (Boerjan *et al*., 2003; Caño-Delgado *et al*., 2003; Tronchet *et al*., 2010). Lignin composition varies amongst species, cell types, and individual cell wall layers and this variation can also be influenced by developmental and environmental signals (Campbell and Sederoff, 1996; Boerjan *et al*., 2003). The physiological function of lignification to the secondary cell wall is to provide additional strength, impermeability and defence against wounding and infection by pathogens (Jones *et al*., 2001, Vanholme *et al*., 2008).

The biosynthesis of lignin can be divided into two stages, the phenylpropanoid pathway (from phenylalanine to the hydroxycinnamic acids) and the monolignol pathway (reduction of the HCA-CoA esters into monolignols) (Goujon *et al*., 2003). The synthesis of the monolignols occurs in the cytoplasm and resulting units are transported to the cell wall where oxidation and polymerisation of the units to lignin polymers occurs (Vanholme *et al*., 2010; Bonawitz and Chapple, 2010). Phenylalanine ammonia lyases encoded by *PAL* genes deaminate L-phenylalanine to trans-cinnamic acid and this is followed by hydroxylation of trans-cinnamic acid to *p*-4-coumaric acid via cytochrome P450 cinnamic acid 4-hydroxylase (C4H) (Rasmussen and Dixon 1999; Humphreys and Chapple, 2002; Achnine *et al*., 2004). Analysis of microsomal proteins by subcellular fractionation and protein gel analysis of tobacco plants and suspension cells, revealed that PAL1 and C4H are co-localised in the endoplasmic reticulum (Rasmussen and Dixon 1999; Achnine *et al*., 2004). These results support the metabolic channelling model in the phenylpropanoid pathway, between trans-cinnamic acid to *p*-4-coumaric acid by these enzymes. The next step is the biosynthesis of CoA-thioesters when 4-coumaric acid CoA ligases (4CL) convert hydroxycinnamic acids to *p*-coumaroyl-CoA (Gross and Zenk, 1974;

Lozoya *et al*., 1988; Costa *et al*., 2005). The supression of *PAL*, *C4H*, and *4CL* genes in alfalfa, Arabidopsis, tobacco and poplar result in transgenic plants with decreased enzyme activities and lignin contents (Lee *et al*., 1997; Sewalt *et al*., 1997; Hu *et al*., 1999; Reddy *et al*., 2005). The reduction in lignin content was accompanied by a change in the relative proportion of monolignol units (H, G, S units), which varied between the plant species studied.

Activated *p*-coumaroyl-CoA is the precursor for the synthesis of monolignol units (Boerjan *et al*., 2003, Ralph *et al*., 2004; Bassard *et al*., 2012). Synthesis of monolignol H is thought to be catalyzed via two enzymatic reactions whereby cinnamoyl CoA reductase (CCR) reduces *p*-coumaroyl-CoA to *p*-coumaraldehyde and this, in turn, is reduced by cinnamyl alcohol dehydrogenase (CAD) to the monolignol *p*-coumaryl alcohol (Knight *et al*., 1992; Boerjan *et al*., 2003). Monolignol G synthesis is assumed to require, *p*-hydroxycinnamoyl-CoA-shikimate/quinate hydroxycinnamoyltransferase (HCT), *p*-coumaroyl-shikimate/quinate-3-hydroxylase (C3H), and caffeoyl-CoA-*O*-methyltransferase (CCoAOMT), CCR and CAD activities. The synthesis of the S unit needs all those enzymes and also ferulate 5-hydroxylase (F5H) and caffeic acid 5-hydroxyconiferaldehyde-*O*-methyltransferase (COMT) (Vanholme *et al*., 2010; Bonawitz and Chapple, 2010). Suppression studies of most monolignol pathway genes, with the exception of F5H, have led to decreased lignin contents and changes in the lignin monomer composition (Nakashima et al., 2008; Vanholme *et al*., 2010; Fu *et al*., 2011; Jung *et al*., 2013). Recently, transgenic sugarcane with suppressed COMT activity was shown to result in lines with reduced lignin contents and altered lignin monomer composition (Jung *et al*., 2012; Jung *et al*., 2013).

Subsequent to their biosynthesis, the monolignols are transported to the secondary cell wall by ATP-binding cassette (ABC) transporters where oxidation and polymerisation of the lignin monomers occurs (Vanholme *et al*., 2010; Alejandro *et al*., 2012, Banasiak *et al*., 2013). Oxidation is thought to be catalysed by laccases and/or peroxidases for polymerization into the lignin polymers to occur (Vanholme *et al*., 2010; Bonawitz and Chapple, 2010; Cesarino *et al*., 2013). Down-regulation of peroxidase encoding gene *Prx* has been shown in tobacco and aspen to lead to plants with reduced lignin levels (Blee *et al*., 2003; Li *et al*., 2003). Complementation of the Arabidopsis *lac17* mutant with the sugarcane *SofLAC* gene restored the lignin

contents (Cesarino *et al*., 2013). Taken together these studies show that laccases and peroxidases are involved in lignin polymerization. After the oxidative step it is assumed that the dehydrogenated phenolics may be coupled to form a (dehydro)-dimer creating a covalent bond between monolignol subunits (Boerjan *et al*., 2003; Vanholme *et al*., 2010).

After its biosynthesis lignin is deposited into a preformed network of polysaccharides, where it is thought to be crosslinked with hemicelluloses to form both covalent and non-covalent bond structures referred to as the lignin-carbohydrate complex (Balakshin *et al*., 2007; Balakshin *et al*., 2011). The cross-linking of lignin and hemicellulosic polysaccharides has been observed in maize and tree wood (Lapierre *et al*., 2001; Balakshin *et al*., 2011; Sipponen *et al*., 2013; Miyagawa *et al*., 2013). In maize, bran heteroxylan and stem hemicellulosic fractions isolated under mild alkaline conditions and subjected to depolymerization of lignins by thioacidolysis were shown to contain a noticeable amount of G and S lignin monomers and trace amounts of the H units (Lapierre *et al*., 2001; Sipponen *et al*., 2013). In addition, analysis of wood xylan-lignin fraction cross-linkages which are resistant to dissolution, have been shown to be benzyl ether, γ-ester and phenyl glycoside by one and two-dimensional nuclear magnetic resonance (Balakshin *et al*., 2011; Miyagawa *et al*., 2013).

2.1.4 Cellulose

Cellulose is the most abundantly biosynthesized biopolymer on earth as it is the major component of higher plant cell walls (Delmer, 1999). Primary cell walls contain approximately 20-30% cellulose, while secondary cell walls may be surrounded by up to 50% cellulose (Delmer and Haigler, 2002; Saxena and Brown, 2005). It is composed of β-(1,4)-linked glucan chains that form microfibrils through extensive inter and intramolecular hydrogen bonds and Van der Waals forces (Saxena and Brown 2005; Endler and Persson, 2011). The degree of polymerization (DP) of cellulose in plants primary cell walls is approximately 8 000, while the DP of secondary cell walls has been estimated to be between 10 000 and 15 000 (Brett, 2000; Brown, 2004). Cellulose is found in two crystalline forms namely cellulose I and cellulose II. The glucan chains in cellulose I are oriented parallel, while in cellulose II the chains are antiparallel (Kuga and Brown, 1988; Kuga *et al*., 1993). Cellulose is

produced by a diverse group of organisms, including plants, bacteria, cellular slime moulds and one group of marine invertebrates, the urochordates (Brown, 1996; Kimura and Itoh 2004).

In plants cellulose is synthesized at the plasma membrane by a large multimeric protein complex known as the rosette or the terminal complex (Brown, 1996). An immuno-gold label coupled to the antibodies directed to the catalytic region of cellulose synthase showed a rosette of approximately 20-30 nm in diameter to hold cellulose synthase (CesA) proteins in a study of *Vigna angularis* terminal complexes (Kimura et al., 1999). The rosette complexes have been proposed to hold six CesA proteins meaning that each rosette simultaneously synthesizes 36 microfibril glucan chains (Delmer, 1999).

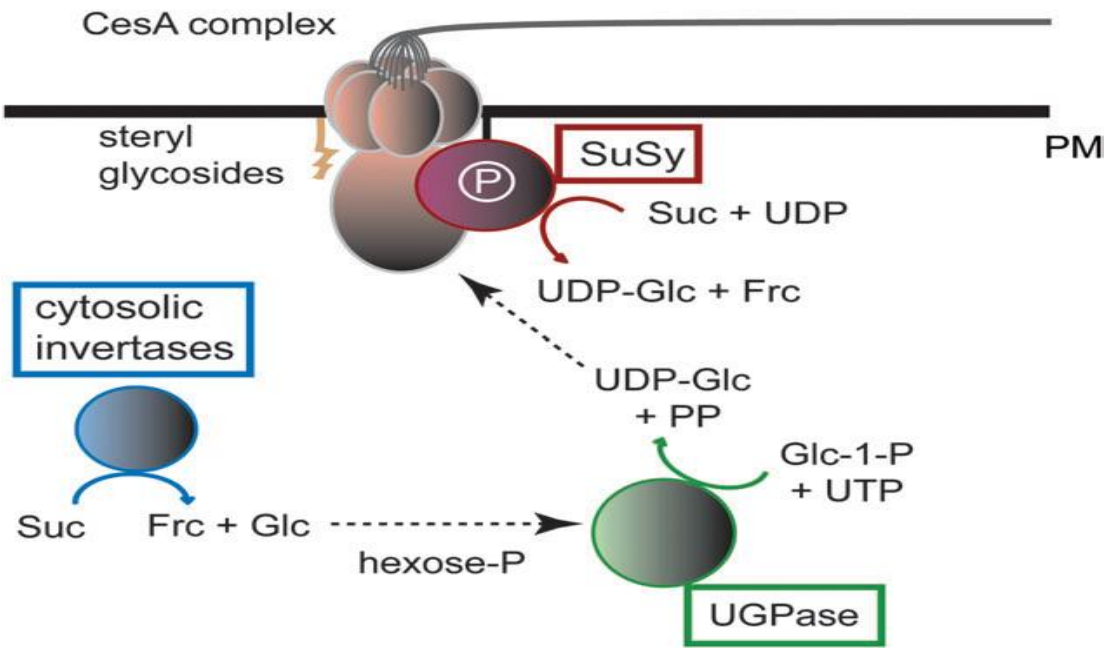

Figure 2.3: Model depicting carbon supply for cellulose biosynthesis. Cellulose protein complexes, depicted as a rosette-like structure and sucrose synthase (SuSy) or UDP-glucose pyrophosphorylase (UGPase) provides UDP-glucose for the synthesis of cellulose β-1,4-glucan chains. Invertases provide hexoses sugar (fructose and glucose) by hydrolysis of sucrose.
The picture was adapted from Anne Endler and Staffan Persson (2011). Cellulose Synthases and Synthesis in Arabidopsis. Molecular Plant 4, 199–211.

The genes responsible for cellulose synthesis in plants were first identified in cotton and expression of *CelA* sequence containing the UDP-glucose-binding domain was shown to have a high affinity for UDP-glucose as a substrate for cellulose synthesis (Pear *et al*., 1996). Mutation in the *CesA* genes of Arabidopsis, barley and rice, has been shown to lead to a reduction in the amounts of cellulose produced by these plants (Kokubo *et al*., 1991; Arioli *et al*., 1998; Taylor *et al*., 1999; Scheible *et al*., 2001; Tanaka *et al*., 2003). Similarly, antisense repression or virus induced silencing of *CesA* genes in Arabidopsis, potato and tobacco resulted in plants with reduced cellulose contents as compared to untransformed plants (Burton *et al*., 2000; Burn *et al*., 2002; Oomen *et al*., 2004). In contrast, over-expression of *StCesA3* sequence in potato was demonstrated to result in transgenic lines with increased cellulose contents (Oomen *et al*., 2004). Taken together these studies prove that *CesA* genes encode cellulose synthases.

UDP-glucose is presumed to be the main substrate for cellulose biosynthesis due to the *CesA* gene having a binding domain for UDP-glucose (Pear *et al.*, 1996). Biosynthesis of cellulose has been demonstrated in a number of plants in the presence of UDP-glucose using membrane and detergent solubilized extracts (Okuda *et al*., 1993; Kudlicka and Brown, 1997; Lai-Kee-Him *et al*., 2002). These studies strongly support a model whereby UDP-glucose is directly polymerized without any requirement for a primer molecule (Okuda *et al*., 1993; Kudlicka and Brown, 1997; Lai-Kee-Him *et al*., 2002).

UDP-glucose is synthesized by one of the two reversible enzymatic reactions catalyzed either by sucrose synthase (SuSy) or UDP-glucose pyrophosphorylase (UGPase) (Amor *et al*., 1995; Park *et al*., 2010). Plasma membrane bound SuSy isoforms have been identified in plants and it has been assumed that they channel UDP-glucose from sucrose to the cellulose synthase complex for cellulose synthesis (Salnikov *et al*., 2001; Albrecht and Mustroph, 2003; Salnikov *et al*., 2003; Persia et al., 2008, Fujii *et al*., 2010). Fujii *et al* (2010) were able to show that plasma membrane fractions from epicotyls of *Vigna angularis* contain granulates of 9.5–10 nm in size which contained the SuSy protein. The solubilized granulates were able to synthesize β-(1,4)-linked glucan chains *in vitro* in the presence of UDP-glucose or sucrose and UDP. Over-expression studies of cotton SuSy sequences in hybrid poplar and cotton resulted in transgenic lines with increased cellulose contents and

thickened secondary cell walls (Colemen *et al*., 2009; Jiang *et al*., 2012). The results from these studies support the assumption that SuSy is responsible for channelling UDP-glucose from sucrose for cellulose biosynthesis. However, antisense suppression or mutations in SuSy gene(s) have not always supported this assumption (Bieniawska *et al*., 2007; Barratt *et al*., 2009, Jiang *et al*., 2012). In Arabidopsis, single, double and quadruple SuSy mutant plants with reduced or eliminated SuSy enzyme activity were observed to be identical to the wild type plants, the UDP-glucose and cellulose contents were unaltered (Bieniawska *et al*., 2007; Barratt *et al*., 2009). In contrast to those studies, suppression of cotton and potato SuSy genes resulted in transgenic lines with reduced secondary cell wall thickness, decreased boll size, seed weight and decreased cellulose contents (Zrenner *et al*., 1996; Jiang *et al*., 2012). These studies indicate that SuSy is not the only UDP-glucose supplier for the synthesis of cellulose and that other pathways can compensate for the decreased activity.

UDP-glucose may also be biosynthesized via a cytosolic UDP-glucose pyrophosphorylase (UGPases) from Glc-1-P and UTP in plants (Kleczkowski *et al*., 2004; Park *et al*., 2010). When UGPase was over-expressed in hybrid poplar plants increases in the cellulose contents were observed (Coleman *et al*., 2007), however, transgenic tobacco plants over-expressing UGPase contained comparable cellulose contents to control lines (Coleman *et al*., 2006). Interestingly, in the same study while overexpression of either SuSy or UGPase alone was observed to have no impact on cellulose amounts, some plants overexpressing both SuSy and UGPase were shown to have elevated cellulose contents (Coleman *et al*., 2006).

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CHAPTER 3

Heterologous expression of the *Ciona savignyi* cellulose synthase in sugarcane increases cellulose contents and improves lignocellulosic biomass saccharification for biofuel production.

Summary

Bioethanol is usually produced through the fermentation of simple sugars derived either directly from crops such as sugarcane, or which are released following hydrolysis of starch. Lignocellulosic biomass from these plants has been identified as an alternative feedstock, which does not impact food security. Development of this second generation technology is complicated by the recalcitrance of lignocellulose to enzymatic digestion and is, therefore, reliant on the development of plants with enzyme-accessible cell wall polysaccharides. We report here on the heterologous expression in sugarcane of a cellulose synthase (*CsCesA*) cDNA sequence from the marine invertebrate *Ciona savignyi*. Cellulose synthase activity was increased (20 to 74%) as was the total cellulose content (13 to 28%) in internodal tissues. The elevation in cellulose was accompanied by increases in total soluble sugars (sucrose, glucose and fructose) of up to 12-25% and a reduction of 25-80% in starch concentrations. Hemicellulose and lignin contents were also altered, with the hemicellulosic polysaccharide monomers glucose and galacturonate increasing up to 56% and 53% respectively. Total lignin was reduced by 5-13%, S and G lignin monomer contents were also decreased by 24-73% in internodal tissues. Importantly, enzymatic saccharification of untreated lignocellulosic biomass of transgenic sugarcane lines had improved glucose release of between 5-39%.

3.1 Introduction

Declining fossil fuel reserves have stimulated research into renewable energy sources, such as plant-derived bioethanol. First generation biofuel technology relies on simple carbohydrates such as sucrose, or glucose derived from starch, as fermentable substrates and, therefore, competes for limited resources, possibly jeopardizing food security (Abramson *et al*., 2010; Waclawovsky *et al*., 2010; Karp and Richter, 2011). However not all sugars are stored in the form of sucrose and starch in plants, plant cell walls are also a rich source for fermentable sugars locked in lignocelluloses in the form of cellulose, hemicelluloses and pectin polysaccharides. The main objective of the second generation biofuel technologies is to enable the utilization of lignocellulosic biomass from plant cell walls to produce biofuels (Pauly and Keegstra, 2010; Harris and DeBolt, 2010; Cook and Devoto, 2011).

Sugarcane (*Saccharum* spp. hybrids) has been identified as a leading potential feedstock for biofuel production (Somerville *et al*., 2010). This perennial C_4 grass has the ability to accumulate high sucrose and lignocellulosic biomass and requires minimal light, water and nitrogen resources for cultivation (Taylor *et al.,* 2010; Waclawovsky *et al*., 2010). After sucrose extraction a larger amount of lignocellulosic bagasse is produced, currently the bagasse is underutilized for biofuels production due to its recalcitrant to enzymatic hydrolysis (Somerville *et al*., 2010; Jung *et al*., 2012; Jung *et al*., 2013).

Within the lignocellulosic composite cellulose is a rich source of glucose (Carroll and Somerville 2009), however, in the plant cell wall it cross-links with hemicellulosic polysaccharides and lignin (Vogel 2008). The interwoven matrix created by these three components renders lignocellulosic biomass recalcitrant to enzymatic hydrolysis by cellulases (Abramson *et al*., 2010; Harris and DeBolt, 2010). This is ascribed to both the phenylpropanoid polymer lignin as well as to hemicellulose polysaccharides being coated in cellulose, which limits access of cellulolytic enzymes (Mosier *et al*., 2005). Moreover, when lignin and hemicellulosic polymers have been partially removed, cellulose remains resistant to enzymatic hydrolysis; due to the crystalline nature of the cellulose microfibrils (Himmel *et al*., 2007; Igarashi *et al*., 2011). In addition pre-treatment of lignocellulosic biomass at high temperatures and/ or with acidic or basic solutions to remove hemicellulosic, pectic and lignin polymers

produces compounds (furfural and hydroxymethylfurfural) which can subsequently inhibit hexose fermenting microorganisms and reduce the ethanol yield (Gámez *et al*., 2004; Klinke *et al*., 2004; Alvira *et al*., 2010). In order to efficiently utilize lignocellulosic biomass as a feedstock for biofuel production, it should be readily available, abundant and, most critically, be amenable to enzymatic hydrolysis and bacterial fermentation.

Recently, a transgenic modification of the lignin biosynthetic pathway in sugarcane has been shown to result in transgenic lines with improved lignocellulosic saccharification efficiencies, yielding increases of the fermentable sugar glucose, with or without diluted acid pretreatment (Jung *et al*., 2012; Jung *et al*., 2013). These observations demonstrate that modification of the lignocellulosic biomass composite can reduce the recalcitrance of sugarcane feedstock without compromising plant performance. Tailoring sugarcane plants with altered lignocellulosic polysaccharide composition, such as more highly soluble cellulose (paracrystalline cellulose), would be a desired trait to improve saccharification efficiencies and/or increase cellulosic biofuel yield from bagasse.

Cellulose is composed of unbranched β-(1,4)-linked glucan chains (Saxena and Brown 2005). It is produced by a diverse group of organisms, including plants, bacteria, cellular slime moulds and one group of marine invertebrates, the urochordates (Brown et al., 1996; Kimura and Itoh 2004). Genes encoding cellulose synthases (*CesA*), have been identified and functionally characterized in many of these organisms (Pear *et al.,* 1996; Ariola *et al*., 1998; Matthysse *et al.,* 2004). In plants cellulose is synthesized at the plasma membrane by a large multimeric protein complex known as the rosette or the terminal complex (Brown, 1996). UDP-glucose is presumed to be the main substrate for cellulose biosynthesis due to the *CesA* gene having a binding domain for this activate sugar (Pear *et al.*, 1996). This is synthesized by one of the two reversible enzymatic reactions catalyzed either by sucrose synthase (SuSy) or UDP-glucose pyrophosphorylase (UGPase) (Amor *et al*., 1995; Kleczkowski *et al*., 2004; Park *et al*., 2010). Plasma membrane bound SuSy isoforms have been identified in plants and it has been assumed that they channel UDP-glucose from sucrose to the cellulose synthase complex for cellulose synthesis (Amor *et al*., 1995; Fujii *et al*., 2010).

Heterologous expression of bacterial or native *CesA* genes in cotton and potato led to increases in cellulose contents without compromising plant performance (Li *et al*., 2004; Oomen *et al*., 2004). Although a number of studies have been conducted in sugarcane to introduce new metabolic carbon sinks through production of novel sugars and biopolymers (Hamerli and Birch 2011; Basnayake *et al*., 2012; Bauer *et al*., 2012), studies for lignocellulosic biomass improvement especially cellulose has not been reported in sugarcane.

Here we show that heterologous expression of a cellulose synthase cDNA from the animal *Ciona savignyi* (*CsCesA;* Matthysse *et al.,* 2004) lead to the generation of transgenic sugarcane plants that have increased cellulose content. Moreover, the increased cellulose content resulted in plants with high hemicellulose glucose content, reduced lignin and improved lignocellulosic saccharification efficiency.

3.2 Materials

All chemicals and carbohydrate modulating enzymes were obtained from Sigma-Aldrich Fluka Chemical Company (St. Louis, MO, USA) or Roche Diagnostics (Basel, Switzerland), unless stated otherwise. Nucleic acid modifying enzymes were obtained from either Promega (Anatech Instruments, South Africa) or Fermentas (Inqaba Biotech, South Africa). Oligo nucleotides primers were purchased from Integrated DNA Technologies (IDT, Whitehead Scientific, South Africa) or Fermentas (Inqaba Biotech, South Africa).

3.3 Methods

3.3.1 Vector construction and polymerase chain reaction (PCR)

The *Ciona savingyi* cellulose synthase cDNA (*CsCesA*) was kindly provided by Professor William Smith (Neuroscience Research Institute and Department of Molecular, Cellular Developmental Biology, University of California). The cDNA was excised from the pTE3 plasmid (Matthysse *et al.,* 2004) by restricting using *Eco*RI and approximately 4.6 kbp was separated by gel electrophoresis and extracted using the GeneJET^{Tm} Extraction kit. The *CsCesA* sequence was then ligated in sense orientation to the constitutive tandem maize ubiquitin/ CaMV 35S promoter into the *Eco*RI site of the vector pUBI510 (Groenewald *et al.*, 2000) using T4 DNA ligase (Promega), resulting in the plant transformation construct pCel. pCel was electroporated into DH5 alpha *E. coli* competent cells using standard transformation procedures. Directional polymerase chain reaction (PCR) was performed using ubiquitin promoter forward primer: UBI Fwd: ATA CGC TAT TTA TTT GCT TGG and gene specific reverse primer: CsCesA Rev: TCT TCC GAA TAA CCC GAT TG, to select colonies with the start codon of *CsCesA* following the promoter of the expression vector. Amplification condition were as follows: 95°C for 5 min; 30 cycles (95°C for 30 sec, 52°C for 35 sec, 72°C for 1 min); 72°C for 5 min.

3.3.2 Sugarcane callus initiation and maintenance

Callus initiations were carried according to standard sugarcane transformation procedures (Bower and Birch 1992; Bower *et al*., 1996; Snyman et al., 1996). In brief,

freshly harvested sugarcane stalks (variety NCO310) and leaf rolls were surface sterilized using 96% ethanol (EtOH) and 0.5 cm sections were transferred aseptically to sterile MSC_3 media (containing 4.43 g/L MS basal medium, 20 g/L sucrose, 2.22 g/L gelrite gellan gum, 0.5 g/L casein enzymatic hydrolysate, pH 6.8 and 3 mg/L 2,4-dichlorophenoxyacetic acid). Calli were grown at 28°C in the dark and subcultured on freshly prepared MSC_3 media every 14 days.

3.3.3 Transformation of embryogenic calli by microprojectile bombardment

Actively growing embryogenic calli of eight to ten week age were subcultured on MSC_3 media four days before transformation. Four hours prior to bombardment, embryogenic calli were placed on MSC_3 osmoticum medium (MSC_3 containing 0.2 M mannitol and 0.2 M sorbitol). Prior to bombardment M17 (grade) 0.7 µm diameter tungsten beads (Bio-Rad) were sterilized in 96% ethanol before being coated with plasmid DNA. The tungsten-plasmid DNA mixture preparation contained 38.5 µg/µl tungsten, 1 µg/µl pCel, 1 µg/µl pEmuKN geneticin selection plasmid DNA containing the neomycinphosphotransferase encoding gene (*nptII*) driven by the maize Ubi-1 promoter, 963 mM $CaCl_2$, and 15 mM spermidine. The embryogenic calli were bombarded with the tungsten-plasmid DNA mixture under vacuum using 1200 KPa helium gas. After four hours the bombarded calli were transferred from MSC_3 osmoticum to MSC_3 media and grown at 28°C in the dark.

3.3.4 Geneticin selection and transgenic plant regeneration

Two days after bombardment the bombarded embryogenic calli were transferred from MSC_3 to the same medium containing additional 50 mg/ml geneticin (MSC_3G_{50}). After approximately ten to twelve weeks at 28°C in the dark, surviving calli were transferred to MSC regeneration medium and incubated at 28°C in the light. When plantlets reaches 3-6 cm in height they were transferred to sterile potting soil, hardened off and grown under greenhouse conditions of approximately 16 hour light and 26°C until maturity in the greenhouse.

3.3.5 Isolation of genomic DNA and identification of positive transformants by PCR

Genomic DNA (gDNA) was extracted from the geneticin selection surviving calli or mature sugarcane leaves from greenhouse as described (McGarvey and Kaper 1991). 400 µl of extraction buffer (50 mM Tris-HCl, pH 8.0, 1% cetyltrimethylammonium bromide, 0.7 M NaCl, 10 mM EDTA, 0.5% polyvinylpirrolidone, 0.1% β-mercaptoethnol) was added to 10-20 mg of finely ground tissue material, vortexed for 10 seconds and incubated for 1 h at 60°C. Chloroform (400 µl) was added to the samples, vortexed and centrifuged at 16 000g for 5 min. The aqueous layer was added to 1 volume of ice cold isopropanol, incubated on ice for 15 min and the precipitated nucleic acids were sedimented by centrifugation at 13 000g for 10 min. The precipitated nucleic acids were washed twice with 70% (v/v) ethanol, air dried and resuspended in 20 µl TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) buffer containing 20 µg/ml RNaseA. To screen for the integration of the pCel plasmid into the sugarcane genome, 1 µl of extracted gDNA was used as template in PCR reactions. The gene specific primer sets used were CsCesA Fwd: TTG CAA TGA GCA GGG ATA GA and CsCesA Rev: TCT TCC GAA TAA CCC GAT TG (amplified fragment ± 380 bp) and the PCR conditions were as follows: 95°C for 5 min; 30 cycles (94°C for 30 sec, 55°C for 35 sec, 72°C for 40 sec); 72°C for 5 min.

3.3.6 Total RNA extraction and first strand cDNA synthesis

Calli or internodal tissues derived from mature greenhouse grown sugarcane plants (internode 1-4 (younger internodes) and internodes 14-15 (mature internodes)) were used to screen for the expression of the *CsCesA* gene. Total RNA was extracted according to a method modified from Bugos *et al.* (1995). Internodal tissue was cut into small pieces directly into liquid nitrogen and ground into fine powder in an IKA® A11 basic analytical mill. Approximately 2 g internodal tissue was transferred to 50 ml centrifuge tubes containing 10 ml of homogenisation buffer (0.1 M Tris-Cl (pH 7.5); 1 mM EDTA; 0.1 M NaCl; 1% (w/v) SDS) and 10 ml of 25:24:1 (v/v/v) phenol:chloroform:isoamyl alcohol. This was vortexed for 1-2 min and sodium acetate (pH 5.2) was added to a final concentration of 0.1 M. Samples were vortexed for 30 sec, incubated on ice for 15 min followed by centrifugation at 12 000g for 15 min at 4°C. The upper aqueous phase of the sample was transferred to a centrifuge

tube containing 2.5 volumes of 100% ethanol and incubated for 2 hours at -20°C to precipitate nucleic acids and centrifuged at 12 000g for 15 min. The precipitated nucleic acids were washed with 2 ml 70% (v/v) ethanol, followed by centrifugation at 10 000g for 5 min and the resulting pellet was briefly dried. The nucleic acid pellet was resuspended in 400 µl DEPC-treated water and digested with RNase free Deoxyribonuclease I (Fermentas) according to the manufacturer's instructions. The remaining nucleic acids were precipitated following addition of 2.5 volumes of ethanol. Precipitated total RNA was recovered by centrifugation at 12 000g for 20 min at 4°C and the pellet was washed with 1 ml 70% (v/v) ethanol, centrifuged again at 10 000g for 5 min and dried briefly. Total RNA pellets were resuspended in 100 µl DEPC-treated water, and stored at -80°C until required. First strand cDNA was prepared using 1 µg of total RNA extracted from internodal tissues and reverse transcribed using Revertaid^{TM} H minus First Strand cDNA synthesis kit (Fermentas, Inqaba Biotech, South Africa) according to the manufacturer's instructions. The cDNA was stored at -80°C until required.

3.3.7 Expression analysis of *CsCesA* genes in sugarcane by semi-quantitative RT-PCR

Forward and reverse primers for *CsCesA* and the 25S ribosomal RNA reference gene for normalization (TC122907: Fwd: TGAAAGCGTGGCCTATCGATCCTT; Rev: CGGTTGCTAGCTTGGATTCTGACT) genes were designed using Primer3 software (http://frodo.wi.mit.edu). The annealing temperature and number of cycles was optimized to ensure linearity for the quantification of transcript abundance. cDNA was diluted to 10 ng with double distilled water before used in the semi quantitative RT-PCR. The amplification conditions for *CsCesA* were as follows: 95°C for 5 min; 26 or 28 cycles (94°C for 30 sec, 55°C for 35 sec, 72°C for 40 sec); 72°C for 5 min. for 25S ribosomal RNA were as follows: 95°C for 5 min; 26 or 28 cycles (94°C for 30 sec, 54°C for 35 sec, 72°C for 40 sec); 72°C for 5 min. The amplicons were separated on 1% agarose gels and spot densitometry was conducted by ascertaining the integrated density value of each PCR fragment, using the auto background option of AlphaEase image-analysis software version 4.0.1 (Alpha Innotech, San Leandro, CA, USA).

3.3.8 Extraction and determination of soluble sugars (sucrose, glucose and fructose)

Ground tissue (100 ± 10 mg) from young (internodes 1-4) or mature (internodes 14-15) internodes was added to extraction buffer (30 mM Hepes 5 mM $MgCl_2$ and 80% v/v ethanol) and incubated at 80°C for 1 hour. The samples were centrifuged at 10 000g for 10 min and the extraction process was repeated three times. The resulting supernatants were pooled, vacuum dried in the Genevac® EZ2 personal evaporator (Genevac LTD, Ipswich, England), re-suspended in 1 ml desalted water and used immediately or stored at -20°C.

Soluble sugars were determined according to Bergmeyer and Bernt (1974). Glucose and fructose were determined as followed: 5 µl of extract was combined with 45 µl desalted water and added to 200 µl buffer A (150 mM Tris-HCl pH 8.1, 5 mM $MgCl_2$ 1 mM ATP and 1 mM NADP) and incubated for 10 min at room temperature before the absorbance was determined at a wavelength of 340 nm. Glucose concentrations were determined following addition of 0.5 U Hexokinase/Glucose-6-phosphate dehydrogenase (HK/G6PDH) and incubation for 30 min at room temperature and measurement of the samples at 340 nm. Following this 0.7 U phosphoglucose isomerase (PGI) was added and the samples were incubated for 30 min at room temperature whereupon the absorbance at 340 nm was determined again to elucidate fructose concentrations. Sucrose was determined as follows: The extract was diluted 20 fold in water and 5 µl of this was combined with 40 µl of buffer B (100 mM citrate pH 5, 5 mM $MgCl_2$) containing 10 U β-fructosidase was incubated for 30 min at room temperature and the background absorbance at 340 nm was determined. Buffer A (200 µl) containing 0.5U HK/G6PDH was added to the samples, incubated for 30 min and read as before. All readings were performed using a PowerWaveX spectrophotometer plate reader.

3.3.9 Extraction and determination of starch concentrations

The starch content was quantified from the remaining alcohol insoluble material from sugar extraction, 50 ± 1 mg was added to 5mM sodium acetate buffer pH 4.8 and incubated at 100°C for 1 hour. The samples were cooled to room temperature, 5 U amyloglucosidase (AMG) from *Aspergillus niger* (Fluka) was added or sodium

acetate for sample background glucose content was added into the samples and incubated overnight (16-18 hours) at 55°C and the reaction terminated by incubating the sample at 100°C for 15 minutes. Samples were centrifuged at 10 000g for 10 min, washed twice with 70% (v/v) ethanol and the supernatants were pooled and vacuum dried. Background glucose and glucose released by AMG was quantified according to the Bergmeyer and Bernt (1974) method as described above.

3.3.10 Extraction and determination of hexose phosphates and UDP-Glucose

Hexose phosphate and UDP-Glucose pools in the internodal tissues were extracted according to Stitt *et al*., 1989. Frozen tissue (500 ± 10 mg) was added to ice cold 10% $HClO_4$; samples were vortexed and incubated at 4°C with mixing for 20 min. Samples were centrifuged at 4°C for 2 min at 13 000g and the pellet washed with 2% $HClO_4$ and incubated for 15 min as before. The supernatants were pooled and neutralized with 5 M KOH, 1 M triethanolamine to pH 7-7.5 and incubated for 15 min at 4°C. Samples were centrifuged at 4°C for 5 min at 13 000g and the supernatant retained. This was applied to activated supelclean 100 mg ENVI-Carb SPE (Supelco) columns and the flow through, containing the hexose phosphates, was retained (Räbina *et al*., 2001). The column was washed with 3 ml water, 3 ml 25% acetonitrile and 3 ml 50 mM triethylammonium acetate (TEAA) buffer pH 7. The nucleotide sugars were eluted with 3 ml 25% acetonitrile and 50 mM TEAA buffer pH 7. The hexose phosphates and nucleotide sugars containing supernatants were frozen in liquid nitrogen and freeze dried. Samples were resuspended in 250 µl desalted water and used immediately or stored at -20°C.

To quantify hexose phosphates (Gluc-6-P, Fruc-6-P and Glu-1-P), 50 µl sample was added to the reaction buffer 235 µl (100 mM Tris-HCl pH 8, 5 mM $MgCl_2$, 0.25 mM NADP) and, after 15 min incubation, the background absorbance was determined at 340 nm. Gluc-6-P was measured by the addition of 0.7 U G6PDH after which the samples were incubated at room temperature for 15 min and the absorbances determined at 340 nm. This was followed by sequential addition of 0.7 U PGI and 0.2 U phosphoglucomutase (PGM) for the determination of Fruc-6-P and Glu-1-P respectively.

UDP-glucose was quantified as follows: 50 µl of extract was added to 225 µl buffer (100 mM Tris-HCl pH 8, 5 mM $MgCl_2$, 0.8 mM NADP, 4 U PGM and 4 U G6PDH) and samples were incubated for 10 min at room temperature before the background absorbance was determined at 340 nm. The reaction was initiated by adding 0.2 U UDP-glucose pyrophosphorylase and 15 mM sodium pyrophosphate where upon samples were incubated for 20 min at room temperature and the absorbance was determined as above.

3.3.11 Protein extraction

Crude protein extracts were prepared from young and mature internodal tissues. The ground tissues were homogenized in cold extraction buffer (100 mM Tris-HCl pH 7.2, 2 mM $MgCl_2$, 2 mM EDTA, 2% (m/v) PVPP, 5 mM DTT, 10% (v/v) glycerol and 0.0016 gm L–1 Complete protease inhibitor) and were continuously stirred for 15 min at 4°C. The samples were centrifuged at 4°C for 5 min at 12 000g, supernatants transferred to a Sephadex G50 spun column pre-equilibrated with extraction buffer (without PVPP) and were centrifuged at 1 000g for 2 min at 4°C. Crude desalted proteins were immediately frozen in liquid nitrogen and stored at -80°C.

The residual pellet from the crude extract was washed three times in extraction buffer (lacking PVPP) to remove residual soluble invertase activity and were used immediately to determine cell wall bound invertase activity (Albertson *et al*., 2001).

3.3.12 Cellulose synthase enzyme activity determination

Protein extracts were prepared from young and mature internodal tissues according to a modified method described by Schäfer *et al*. (2004). Internodal tissues were homogenized in ice cold extraction buffer (50 mM Tris-HCl pH 7.2, 10 mM $MgCl_2$, 2 mM EDTA, 5 mM DTT, 10% (v/v) glycerol and 0.0016 mg/l complete protease inhibitor) before being centrifuged at 4°C for 15 min at 5 000g. The supernatant was filtered through two layers of nylon cloth at 4°C and centrifuged at 4°C for 60 min at 100 000g. The resulting pellet was re-suspended in a buffer containing 50 mM Tris-HCl pH 7.5, 1% (v/v) Triton X100 and was incubated with reaction buffer containing 50 mM Tris-HCl pH 7.5, 8 mM $MgCl_2$, 8 mM $CaCl_2$, 20 mM cellobiose, 1 mM (UDP-glucose and 1µCi of UDP-D-[U-^{14}C] glucose). The reaction was incubated at room

temperature for 30 min before being stopped by the addition of 0.5 M NaOH and heat treatment at 65°C for 20 min. The samples were filtered through glass fiber filters, washed twice with desalted water, three times with 70% (v/v) ethanol and dried. The filters were submerged in 4 ml scintillation cocktail and the amount of radioactive label incorporated was measured in a scintillation counter (Beckman Coulter).

3.3.13 Protein determination

Protein contents were measured according to Bradford (1979) using the Bio-Rad microassay solution. Bovine serum albumin (Fraction V; Roche) was used as a standard.

3.3.14 Sucrose phosphate synthase activity assay

SPS activity was measured in internodal tissues according to the method of Baxter *et al*., (2003) under maximal (V_{max}) and limiting (V_{lim}) reaction conditions. Desalted crude protein samples (10 µl) were added to 100 µl of reaction buffer (50 mM HEPES-KOH pH 7.5, 20 mM KCl, 4 mM $MgCl_2$,) containing for the V_{max} assay: 12 mM UDP-Glc, 10 mM Fru-6-P, 40 mM Glc-6-P or, for the V_{lim} assay: 4 mM UDP-Glc, 2 mM Fru-6-P, 8 mM Glc-6-P, 5 mM KH_2PO_4. Reactions were incubated for 30 min at 35°C and the assays were stopped by heating at 95°C for 5 min followed by centrifugation at 16 000g for 5 min. To destroy un-reacted hexose phosphates, 100 µl of 5 M KOH was added to supernatant (100 µl) and heated at 95°C for 10 min. After cooling the samples, 200 µl of anthrone reagent (0.14% anthrone in 14.6 M H_2SO_4) was added to 50 µl of the sample and incubated for 20 min at 40°C. Absorbance was measured at 620 nm and the sucrose concentration was calculated from a standard curve with 0-200 nmol sucrose.

3.3.15 Sucrose synthase activity assay in the synthesis direction

SuSy activity was determined in the synthesis direction according to Schäfer *et al*., (2004). Desalted crude protein samples (20 µl) were incubated with assay buffer containing (100 mM Tris-HCl pH 7.0; 10 mM $MgCl_2$, 20 mM UDP-Glc, 0.2 mM NADH, 1 mM phosphoenolpyruvate and 0.45 U/ml pyruvate kinase/lactate dehydrogenase).

The assays were initiated by the addition of 10 mM fructose and NAD^+ production was monitored at 340 nm.

3.3.16 Sucrose synthase activity assay in the breakdown direction

SuSy activity was assayed in the breakdown direction according to Schäfer *et al*., (2004). Desalted crude protein samples (20 µl) were incubated with assay buffer containing (100 mM Tris-HCl pH 7.0; 2 mM $MgCl_2$, 400 mM sucrose, 2 mM NAD^+, 1 mM sodium pyrophosphate,4 U/ml PGM, 4 U/ml UGPase and 4 U/ml G6PDH). The assays were initiated by the addition of 2 mM uridine diphosphate (UDP) and NADH production was monitored at 340 nm.

3.3.17 Acid and neutral invertase activity assays

Acid invertase (AI) and neutral invertase (NI) activity assays were performed according to Rossouw *et al* (2010). AI assays was conducted by incubation of internodal tissues desalted crude protein extract (20 µl) for 3 h in a reaction buffer consisting (50 mM citrate phosphate pH 5.5 and 125 mM sucrose). The reaction was stopped by incubating the samples at 90°C for 2 min before being frozen in liquid nitrogen and stored at -80°C until required.

Neutral invertase (NI) activity was assayed by incubation of internodal tissues desalted crude protein extract (20 µl) for 2 h in a reaction buffer consisting (50 mM HEPES-KOH pH 7.5 and 125 mM sucrose). The reaction was stopped by addition of 5 µl 2 M Tris and 22 mM $ZnSO_4$ solution before samples were frozen in liquid nitrogen and stored at -80°C until required.

The reducing sugars resulting from activities of AI and NI were measured according to Huber and Akazawa (1986). In brief, the assay buffers consist of: 50 mM HEPES-KOH pH 7.5, 2 mM $MgCl_2$, 15 mM KCl, 0.4 mM NAD, 1 mM ATP, 4 U/ml HK, and 2 U/ml G6PDH. The background absorbance was determined at 340 nm after 5 min and the reaction was initiated by the addition of reducing sugars resulting from activity of AI or NI on sucrose and NADH production was monitored at 340nm.

3.3.18 Cell wall bound invertase activity assay

The CWI activity assay was determined according to a method by Albertson *et al*., (2001). The cell wall pellet suspension (200 µl) was added to McLIvaine's buffer pH 3.5, 0.5 M sucrose, three acid washed beads (2.5 mm diameter) and samples were incubated at 37°C with continuous shaking. Sample aliquots (40 µl) were removed at 0. 15 and 30 min and the reaction was terminated through addition of 80 µl 4 M imidazole pH 7.6 and boiled for 2 min. Samples were stored overnight at -20°C, thawed and centrifuged at 16 000g and the supernatant was retained. Glucose released was measured as above by monitoring the production of NADH.

3.3.19 UDP-Glucose pyrophosphorylase activity assay

UDP-Glucose pyrophosphorylase (UGPase) activity was determined in the internodal tissues according to Ciereszko *et al* (2001). Desalted crude protein extracts (20 µl) were incubated with the reaction buffer consisting of 100 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 0.8 mM NAD, 0.8 mM UDP-glucose, 4 U PGM and 4 U G6PDH. After 5 min the background absorbance was determined at 340 nm and the reaction was started with the addition of 0.1 mM NaPPi. NADH production was monitored spectrophotometrically at 340nm.

3.3.20 UDP-Glucose dehydrogenase activity assay

UDP-Glucose dehydrogenase (UGD) activity was determined in the internodal tissues according to Kärkönen *et al* (2005). Desalted crude protein extracts (20 µl) were incubated with assay buffer consisting of 100 mM Tris-HCl pH 8.4 and 5 mM UDP-glucose. The background absorbance was determined at 340 nm and the reaction initiated with 2 mM NAD^+. Reduction of NAD^+ was monitored spectrophotometrically at 340nm.

3.3.21 Cell wall analysis

3.3.21.1 Preparation of alcohol insoluble residue (AIR) and destarching

Approximately 2 g of young (internodes 1-4) and mature (internodes 14-15) internodal tissues was added to 80% (v/v) ethanol and incubated at 80°C for 1 h. The samples were centrifuged at 5000 g for 10 min and the supernatant discarded, the extraction process was repeated three times. The alcohol insoluble material (AIR) was then washed three times with acetone and vacuum dried in the Genevac® EZ2 personal evaporator (Genevac LTD, Ipswich, England) and stored in air tight tubes in a desiccator under vacuum until required.

AIR (1 g ± 10 mg) was mixed with 50 mM sodium acetate buffer pH 4.8 and incubated at 100°C for 1 h. After cooling to room temperature, 10 U amyloglucosidase from *Aspergillus niger* (Fluka) was added and incubated overnight (16-18 h) at 55°C and the reaction was terminated by incubating the sample at 100°C for 10 min. Samples were centrifuged 10 000g for 10 min and washed three times with 80% (v/v) ethanol. AIR was washed with acetone and vacuum dried and stored as described before.

3.3.21.2 Cellulose content measurements

The cellulose content was determined according to a modified Updegraff method (1969). In brief 10 mg ± 1 mg of destarched AIR was boiled in 1 ml acetic- nitric acid reagent (acetic acid: nitric acid: water: 8:1:2) for 30 min and samples were cooled to room temperature and centrifuged at 8 000g for 10 min. The cellulosic residue was washed three times in 8 ml desalted water and and then in 4 ml acetone before being vacuum dried. Cellulosic material was then completely hydrolysed in 500 µl 67% (v/v) sulphuric acid at 30°C for 30 min. The glucose content of the samples was determined by the anthrone method as described by Leyva *et al*. (2008). In brief, 150 µl of anthrone reagent (1% anthrone in concentrated sulphuric acid; freshly prepared) was added to 50 µl of diluted samples or glucose standard solutions and incubated at 4°C for 10 min. The samples were subsequently incubated at 100°C for 20 min and allowed to cool to room temperature for 20 min before the absorbance was

determined at 620 nm. The glucose concentrations in the sample were compared to a standard curve of glucose with concentrations from 0-10 µg.

3.3.21.3 Depectination of alcohol insoluble residue

AIR (100 mg ± 1 mg) was depectinated by treatment with 5 ml of 1% (w/v) ammonium oxalate and incubation at 100°C for 1 h. Samples were centrifuged at 3 500g for 5 min, the supernatant removed, and the ammonium oxalate extraction repeated with the supernatants being combined. The remaining insoluble residue was washed twice with 5 ml desalted water and all the supernatants pooled with the ammonium oxalate supernatant. The samples were neutralized with acetic acid to pH 7 and dialyzed for 24 h at 4°C in desalted water (water change four times) containing 0.1% (v/v) sodium azide. The dialyzed supernatant and depectinated alcohol insoluble residue were freeze dried and stored in air tight tubes in a desiccator under vacuum until required for further analysis.

3.3.21.4 Hemicellulosic polysaccharide extraction from depectinated alcohol insoluble residue

Extraction of the hemicellulosic fractions was conducted using depectinated alcohol insoluble material (70 mg ± 1 mg) and the samples were treated sequentially with increasing amounts of potassium hydroxide with sodium tetraborate (0.1 M KOH/ 20 mM $NaBH_4$, 0.5 M KOH/ 20 mM $NaBH_4$ and 4 M KOH/ 20 mM $NaBH_4$). Samples were added to 5 ml 0.1 M KOH/ 20 mM $NaBH_4$ and incubated for 24 h at room temperature with shaking to extract hemicellulosic material. These were centrifuged at 3 500g for 5 min and the alcohol insoluble residue washed twice with desalted water. The resulting supernatants were pooled, neutralized to pH 7 with acetic acid and dialyzed for 24 h at 4°C in desalted water (water change four times) containing 0.1% sodium azide. The extraction was repeated as above with 0.5 M KOH/ 20 mM $NaBH_4$ and 4 M KOH/ 20 mM $NaBH_4$. The dialyzed supernatant fractions of KOH and the remaining cellulosic alcohol insoluble residue were freeze dried and stored in air tight tubes in a desiccator under vacuum until required for further analysis.

3.3.21.5 Hydrolysis of pectic and hemicellulosic cell wall polysaccharides fractions

Two milligrams (± 0.1 mg) of freeze dried pectic and hemicellulosic fractions were hydrolyzed with 1 ml of 2 M TFA at 121°C for 2 hours. The samples were then cooled and centrifuged at 8 000g for 10 min and the TFA resistant residue washed twice with 2 M TFA. The resulting supernatants were pooled, and TFA evaporated in the Genevac® EZ2 personal evaporator. To remove the residual TFA, monosaccharides were dissolved in 1 ml methanol and this was evaporated as before. This process was repeated twice and, with the final wash, 1 µl of 2 mg/ml ribitol (0.0131 µmol) added as internal standard.

3.3.21.6 Monosaccharide derivatisation and GC-MS analysis

The pectic and hemecellulosic TFA hydrolysed monosaccharides and standards (ribitol) were derivatised according to the method of Roessner *et al* (2002). To the dried samples 80 µl methoxyamine HCl in pyridine (20 mg/ml) was added, the samples vortexed thoroughly and incubated at 30°C for 90 min with intermittent vortexing. To the samples 20 µl of alkane mixture was added for used as retention time standards followed by addition of 140 µl N-Methyl-N-(Trimethylsilyl)-triflouroacetamide (MSTFA) and incubated at 37°C for 30 minutes. Samples were left for a further 2 hours before injection.

The gas chromatography was conducted on a 30 m Rtx®-5Sil MS column (RESTEK) with Integra guard with an inner diameter of 0.25 mm and 0.25 mm film thickness, the system consisted of an AS 2000 autosampler, trace GC and the quadropole trace MS (ThermoFinnigan). One microlitre of samples was injected with a splitless injection and the flow rate was 1ml/min. The injection temperature was 230°C and the ion source temperature was set at 200°C. The temperature of the program was as follows: 5 min at 70°C, followed by a 1°C/min oven ramp to 76°C and a second ramp of 6°C/min to 350°C and the temperature equilibrated to 70°C before injection of the next sample. The mass spectra were recorded at two scans per sec with the scanning range of 500-600 *m/z*. The chromatograms and mass spectra were evaluated using Xcalibar software bundle version 1.2 (Finnigan Corporation 1998-

200) and eluting compounds were identified using NIST library (www.nist.gov). The resulting response values were calculated as described by Glassop *et al.* (2007). In brief, this was done by dividing the peak area of the compound by the peak area of standard (ribitol) and .

3.3.21.7 Determination of total uronic acids

Uronic acid contents were determined from destarched alcohol insoluble residue as described by Filisetti-Cozzi and Carpita (1991). Five milligram (± 0.1 mg) of destarched alcohol insoluble material was combined with 1ml sulphuric acid and samples incubated on ice for 5 min with constant stirring. Another 1 ml concentrated sulphuric acid was added and samples incubated on ice for a further 5 min before 500 µl of water was added and again incubated on ice for 5 min with constant stirring. Another 500 µl of water was added and incubated on ice for 5 minutes and finally samples were diluted with water to 10 ml and centrifuged for 10 min at 5 000g.

400 µl of hydrolysate (two tubes: sample and sample control) was mixed with 40 µl of 4 M sulfamic acid/potassium sulfamate solution (pH 1.6) and vortexed. To the samples 2.4 ml of 75 mM sodium tetraborate in sulphuric acid was added, vortexed vigorously and incubated for 20 min at 100°C. The samples were then cooled on ice for 10 min and 80 µl of *m*-hydroxydiphenyl added both to the sample and to the sample control (80 µl of 0.5% sodium hydroxide) and vortexed. After 10 min the absorbances were determined at 525 nm and the sample background was subtracted from samples control absorbance. Uronic acid concentrations were determined by comparison with a standard curve obtained from known concentrations (0-40 µg) of galacturonic acid.

3.3.21.8 Total lignin composition analysis

Klason lignin was determined by a modified method described by Huntley *et al*. (2003), destarched alcohol insoluble material (100 mg ± 1 mg) was added to 1 ml of 72% (v/v) sulphuric acid and incubated at 20°C for 2 hours with constant mixing. The samples were diluted with water (28 ml) and autoclaved at 121°C for 1 hour. Samples were cooled to approximately 60°C, vacuum filtered through a pre-weighed glass filter after which the glass filter was washed with 500 ml of water to remove

residual acids and sugars. The glass filters were dried overnight at 105°C and weighed to determine the Klason acid insoluble lignin. Klason acid soluble lignin was determined by measuring the filtrate absorbance at 205 nm.

3.3.21.9 Lignin monomer composition determination

The lignin monomer composition was determined according to Robinson and Mansfield (2009). In brief, 2 mg of destarched alcohol insoluble material was added to 200 µl of a dioxane solution (containing 2.5% boron trifluoride diethyl etherate and 10% ethanethiol) and incubated at 100°C for 4 hours with intermittent mixing every hour. The reaction was terminated by cooling on ice for 5 minutes and addition of 150 µl of 0.4 M sodium bicarbonate and vortexing. The samples were washed by addition of 1 ml water and 500 µl of ethyl acetate and vortexing. The ethyl acetate phase (150 µl) was transferred to a glass vial and air evaporated. The dried samples were washed three times with 200 µl of acetone and TMS derivatised by adding 500 µl ethyl acetate, 20 µl pyridine and 100 µl N,O-bis(trimethylsilyl) acetamine and incubated for 2 hours at 25°C.

Gas chromatography was conducted on a 30 m Rtx®-5Sil MS column (RESTEK) with Integra guard, an inner diameter of 0.25 mm and 0.25 mm film thickness. The system consisted of an AS 2000 autosampler, trace GC and the quadropole trace MS (ThermoFinnigan). One microlitre was injected with a splitless injection and the flow rate was 1.1ml/min with a 30 min solvent delay. The temperature of the program was as follows: initial hold at 130°C for 3 min, a 3°C/min ramp to 250°C and hold for 1 min to allow equilibration to the initial temperature of 130°C. The peaks were identified by mass spectrum ions of 299 m/z and 269 m/z for S and G respectively. The resulting response values were calculated as described by Glassop *et al*. (2007). In brief, this was done by dividing the peak area of the compound by the peak area of standard (ribitol) and then dividing by the mass of the tissue used.

3.3.21.10 Enzymatic saccharification of sugarcane lignocellulosic biomass

Micro-scale enzyme saccharifications were performed as described by Stork *et al*. (2009) with some modification. Destarched alcohol insoluble residue 40 mg ± 0.1 mg were placed in 5 ml tubes and mixed with 2 ml of buffer consisting of 50 mM sodium

citrate ph 4.8, 0.1% (v/v) sodium azide, 60 filter paper unit Celluclast 1.5L and 134 U/ml cellobiose (Novozyme). The samples were shaken at 100 rpm in a horizontal position at 50°C. The progress of the reaction was measured by taking 100 µl aliquots at 2, 18, 24 and 48 h and the reaction terminated by incubating the sample at 100°C for 15 minutes. The glucose released was quantified according to Bergmeyer and Bernt (1974). The composition of sugars released by enzymatic hydrolysis were qualitatively identified by GC/MS as before.

3.3.21.11 Histochemical and microscopic analysis

Transverse stem sections from the sixth internode of the sugarcane plants were prepared by hand cutting with a razor blade. Internodal sections for cellulose staining were performed with a modified method described by Zhong *et al*., (2007). In brief, sections were immersed in 2 ml 0.01% solution of Calcofluor white M2R for 30-50 sec, followed by two washes with 0.1 M Tris-HCl buffer, pH 8.1 at room temperature for 30 sec. The stained sections were immediately investigated with a UV fluorescence microscope.

For Mäule and Wiesner stainings, internodal sections were analysed with a modified method described by Guo *et al*., (2001) as follows: sections were immersed in 2 ml 1% (w/v) potassium permanganate solution for 5 min at room temperature and then washed twice in 5 ml 3% hydrochloric acid for 3 min. For Wiesner stainings, sections were immersed in 3 ml phloroglucinol-HCl solution (2 volumes of 1% (w/v) phloroglucinol in 95% (v/v) ethanol was added in 1 volume of concentrated HCl) for 5 min and then washed with 18% (v/v) HCl for 10 sec. The sections were then investigated within 20 min under a light microscope.

3.3.22 Statistical analyses

The student's t-tests (two-tailed) were performed to test for significant differences between group means using Statistica version 10 (StatSoft, Inc. 2004). The term significant is used to indicate differences for which $P < 0.05$.

3.4 Results

3.4.1 Transformation vector construction

In order to constitutively express *Ciona savignyi* cellulose synthase sequence in sugarcane, a 4.6 Kbp *CsCesA* cDNA fragment was ligated into the *Eco*RI site of a pUBI510 vector. The resulting construct designated pCel was verified by directional polymerase chain reaction (PCR) analysis and restriction enzyme digestion with *Eco*RI. A fragment of approximately 780 bp was amplified by PCR analysis using a forward primer that binds into the ubiquitin promoter of the pUBI510 vector and a reverse primer that is *CsCesA* specific (Figure 3.1 a). The enzyme digestion of pCel resulted in two fragments sizes of 5.4 and 4.6 Kbp, confirming the presence of *CsCesA* cDNA sequence in the pCel construct (Figure 3.1 b).

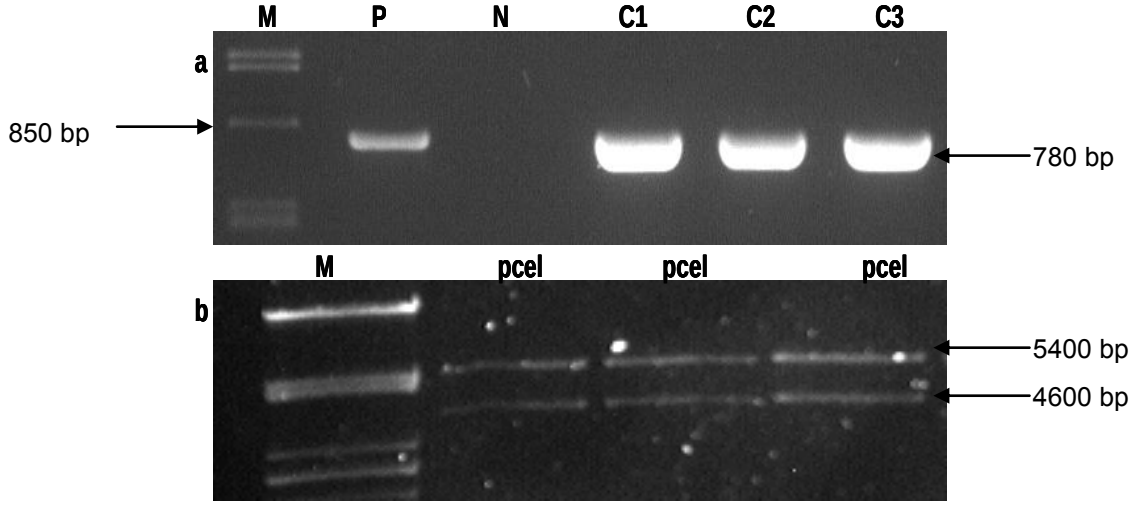

Figure 3.1: Analysis of bacterial cell by PCR for *CsCesA* and enzyme digestion of pCel expression vector.
(a) Bacterial colony PCR gel analysis for *Cs*CesA orientation in the pubi510 vector constructs.
(b) Restriction enzyme digestion of pCel constructs with *EcoRI.*
M: Marker DNA; P: Positive control; N: Negative control; C: Bacterial colony. pCel: *CsCesA* expression vector.

3.4.2 Transformation and confirmation of integration of *Ciona savignyi* cellulose biosynthesis sequence in sugarcane

Sugarcane embryogenic callus was biolistically bombarded with the pCel construct and pEmuKN selection vector containing the *npt-II* selectable marker gene. gDNA from callus of putatively transformed clones that survived geneticin selection was screened using PCR for the integration of the *CsCesA* sequence and 21 independent clones were found to contain the *CsCesA* sequence. Plants were regenerated from

the transgenic clones and transferred to soil in pots before being placed in a greenhouse. Mature transgenic plants were morphologically similar to the non transformed wild-type controls throughout the 18 months growth period in the glasshouse (Figure 3.2 a). Three independent transformed lines, pCel1.1, pCel6.2 and pCel8.3 were chosen for further investigation. PCR analysis of mature transgenic sugarcane lines confirmed the integration of the *C. savignyi* cellulose synthase sequence into their gDNA (Figure 3.2 b) and RT-PCR experiments demonstrated that the *CsCesA* sequence was transcribed (Figure 3.2 c).

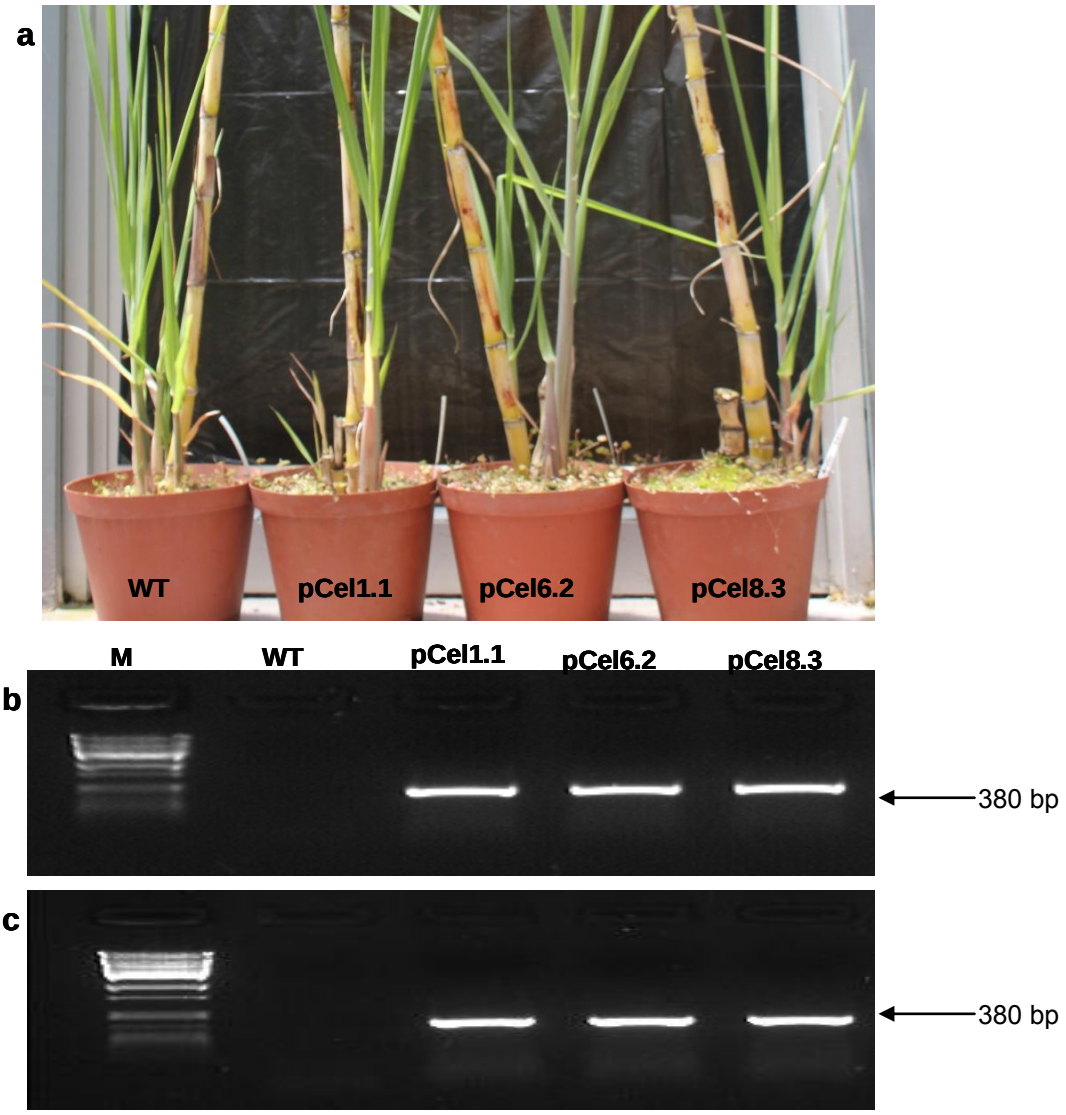

Figure 3.2: Phenotypic evaluation of transgenic sugarcane in comparisonwith wild-type and PCR analysis of *CsCesA* transgenic lines.

(a) Greenhouse grown wild-type (WT) and transgenic sugarcane lines (pCel).

(b) PCR gel analysis of integration of *CsCesA* in gDNA of mature sugarcane.

(c) RT-PCR gel analysis of *CsCesA* expression at mRNA level.

M: Marker DNA, WT, Wild-type; pCel1.1 pCel6.2 and pCel8.3, Transgenic sugarcane lines.

Further analysis of *CsCesA* transcript abundance in the mature transgenic sugarcane internodal tissues by semi-quantitative RT-PCR, showed that the younger internodal tissues (internode 1-4) had lower *CsCesA* steady-state transcript levels compared to the mature internodes (internode 14-15), with the exception of young internodes of pCel8.3Y which contained similar transcript levels in young and mature internodes (Figure 3.3 a).

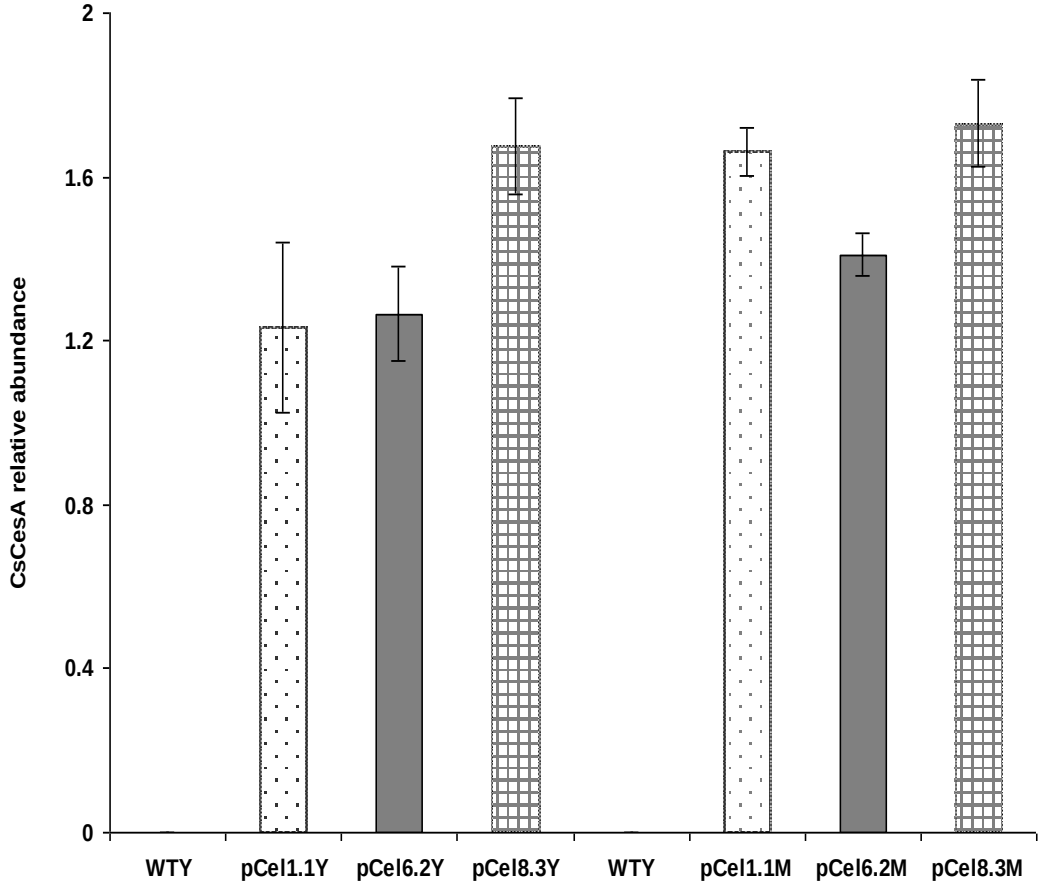

Figure 3.3: Analysis by RT-PCR of *CsCesA* expression level in transgenic lines internodal tissues. Transgenic lines: pCel1.1, pCel6.2 and pCel8.3. Y: internodes 1-4 and M: internodes 14-15.

3.4.3 Total cellulose synthase enzyme activity and cellulose content in mature transgenic sugarcane line.

Protein extracts prepared from internodal tissues were assayed for total cellulose synthase activity. The activity was significantly increased in the transgenic lines by between 20-74% in young and mature internodes (Figure 3.4 a). This correlated with increased cellulose contents in the transgenic lines (Figure 3.4 b) which increased between 18%-24% in younger internodes and between 17%-28% in mature tissues.

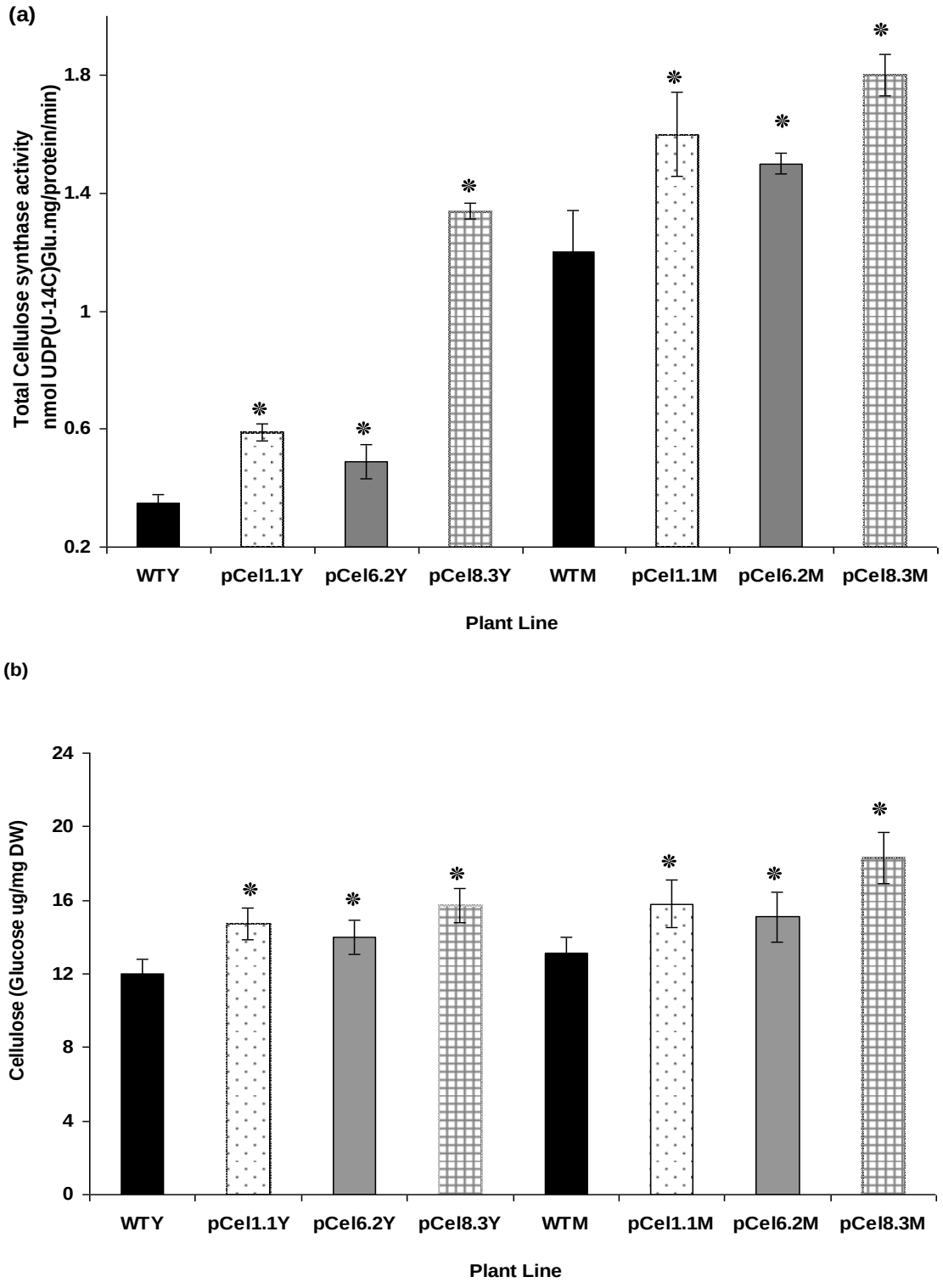

Figure 3.4: Analysis of total cellulose synthase enzyme activity and cellulose content in transgenic sugarcane in comparison with wild type control plants. Y; Younger internodes (internode 1-4); M; Mature internodes (internodes 14-15). WT; Wild-type sugarcane, pCel transgenenic sugarcane lines

(a) Total cellulose synthase enzyme activity in transgenic sugarcane internodal tissues.

(b) Total cellulose content in transgenic sugarcane internodal tissues.

Values are mean calculated from 3 plants per line. Mean values with * were determined by the t test to be significantly different ($P<0.05$) from the respective wild-type (WT) internodes.

Transverse section of mature internodes of the high cellulose accumulating pCel8.3 line and wild-type controls were histochemically stained with calcoflour solution. The intensity of the fluorescence approximately reflects the amounts of cellulose and this was visualized to be higher in the pCel8.3M internode (Figure 3.5 b).

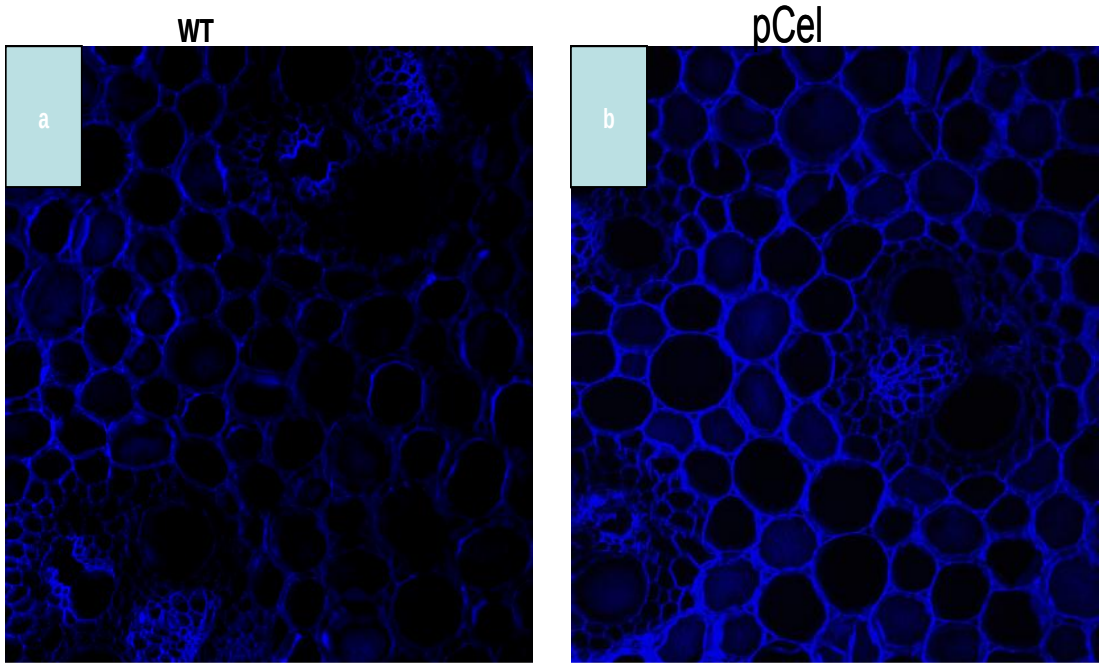

Figure 3.5: Microscopic and histochemical analysis of the transgenic line in comparison with the wild type control. Transverse sections of sugarcane internodal tissues were stained for cellulose with calcoflour stain.

(a) Wild-type control

(b) Transgenic line showing increased fluorescence in the cell walls.

WT: Wild type mature internode; pCel: Transgenic plant mature internode.

3.4.4 Effects of cellulose synthase (*CsCesA*) over-expression on soluble sugars pools and starch contents

Metabolites were extracted and their concentration determined. Glucose and fructose were slightly increased in both young and mature internodal tissues, while sucrose was elevated in the younger tissue (Table 1) of the transgenic lines expressing cellulose synthase from *C. savignyi*. The younger internodes of all transgenic lines contained generally more hexose phosphates and UDP-glucose (Table 3.1), however these differences disappeared in the mature internodes. In contrast to the increased soluble sugar pools, starch content was reduced in lines pCel1.1 and pCel8.3 (Table 3.2). Line pCel6.2 showed reduced starch levels in younger internodes but in mature internodes it was increased.

Table 3.1. Analysis of soluble sugars in the internodal tissues of transgenic and wild type sugarcane plants. WT; wild–type; pCel; Transgenic sugarcane lines Y; Younger internodes; M; Mature internodes. Values are means calculated from 3 plants per line. Values set in bold type were determined by the t test to be significantly different (P<0.05) from the respective wild type internodes.

	Concentration (µmol/g FW)				Concentration (nmol/g FW)			
Plant Line	**Gluc**	**Fruc**	**Suc**	**Total**	**Gluc-6-P**	**Gluc-1-P**	**Fruc-6-P**	**UDP-Gluc**
WTY	128 ± 1.1	71 ± 1.3	150 ± 2.1	349 ± 4.5	2.8 ± 0.04	1.5 ± 0.03	1.0 ± 0.05	0.006 ± 0.004
pCel1.1Y	**147 ± 0.9**	**81 ± 1.1**	**190 ± 1.6**	**418 ± 3.5**	**5.1 ± 0.05**	**3.5 ± 0.04**	**1.8 ± 0.03**	**0.018 ± 0.003**
pCel6.2Y	**145 ± 1.3**	**89 ± 1.0**	**169 ± 2.1**	**385 ± 4.4**	**3.5 ± 0.01**	**2.9 ± 0.03**	1.1 ± 0.02	**0.011 ± 0.002**
pCel8.3Y	**170 ± 1.5**	**98 ± 1.5**	**195 ± 1.2**	**443 ± 4.2**	**4.1 ± 0.03**	**3.0 ± 0.03**	**1.9 ± 0.04**	**0.017 ± 0.003**
WTM	98.1 ± 2.6	52 ± 1.3	389 ± 2.2	539 ± 6.1	0.8 ± 0.02	0.5 ± 0.02	0.3 ± 0.03	0.008 ± 0.004
pCel1.1M	**159 ± 1.5**	**68 ± 1.0**	391 ± 1.9	**618 ± 4.4**	0.7 ± 0.03	0.6 ± 0.03	0.4 ± 0.07	0.010 ± 0.006
pCel6.2M	**198 ± 2.4**	**70 ± 1.1**	399 ± 2.3	**667 ± 5.8**	1.0 ± 0.03	0.7 ± 0.01	0.3 ± 0.05	0.009 ± 0.005
pCel8.3M	**161 ± 1.6**	**78 ± 1.5**	393 ± 2.1	**632 ± 5.2**	0.9 ± 0.03	**1.0 ± 0.02**	**1.3 ± 0.05**	0.008 ± 0.005

Glu**, Glucose**; Fruc**, Fructose;** Suc, Sucrose; Gluc-6-P, **Glucose-6-phosphate;** Gluc-1-P**, Glucose-1-phosphate;** Fruc-6-P**, Fructose -6-phosphate;** UDP-Gluc**, Uridine-diphosphate glucose.**

Table 3.2. Analysis of starch content in internodes of mature sugarcane plants. WT; Wild type sugarcane. pCel; Transgenic sugarcane lines. Y; Young internodes. M; Mature internodes. Values are means calculated from 3 plants per line. Values set in bold type were determined by the t test to be significant different (P<0.05) from the respective wild type internodes.

Plant Line	**Starch** (mg/g DW)
WTY	0.128 ± 0.004
pCel1.1Y	**0.026 ± 0.002**
pCel6.2Y	**0.048 ± 0.005**
pCel8.3Y	**0.096 ± 0.003**
WTM	0.092 ± 0.006
pCel1.1M	**0.072 ± 0.004**
pCel6.2M	**0.127 ± 0.003**
pCel8.3M	**0.041 ± 0.003**

3.4.5 Influence of the cellulose synthase (*CsCesA*) over-expression on enzymes involved in sugar and cell wall metabolism

Sucrose phosphate synthase (SPS) and sucrose synthase (SuSy) are the main enzymes involved in sucrose metabolism in sugarcane. SPS was assayed under both excess and limiting substrate conditions and the transgenic lines had increased activity in the internodal tissues (Table 3.3). Similar to SPS enzyme activity, SuSy activity was also elevated in both internodal tissues when assayed in the sucrose synthesis direction.

The activities of sucrose catabolic enzymes (acid invertase (AI), neutral invertase (NI), cell wall invertase (CWI), and SuSy (assayed in the catabolic direction)

decreased in the mature internodes of sugarcane. The CWI and SuSy activities were increased in both young and mature internodes of the transgenic lines, with the exception of CWI in the mature internodes of the pCel6.2M line. However, NI and AI enzyme activities of the high cellulose accumulating transgenic lines were unchanged.

UDP-glucose pyrophosphorylase (UGPase) and UDP-glucose dehydrogenase (UGD) are responsible for providing precursors for sucrose and cell wall polysaccharide biosynthesis. Their activities were significantly increased in the younger internodal tissues of the transgenic lines. However, while UGPase activity was increased in older internodes, UGD activity in pCel1.1M and pCel8.3M was unaltered.

Table 3.3: Analysis of enzyme activity in the internodal tissues of transgenic and wild type sugarcane plants involved in sucrose metabolism. Values are means calculated from 3 plants per line. Values set in bold type were determined by the t test to be significantly different (P<0.05) from the respective wild type (WT) internodes.

	Activity (nmol/min/mg protein).								
Plant Line	**SPS E**	**SPS L**	**SUSY S**	**SUSY H**	**AI**	**NI**	**CWI**	**UGPase**	**UGD**
WTY	2.33 ± 0.3	2.66 ± 0.3	53 ± 1.1	50 ± 1.7	38 ± 1.4	30 ± 1.4	18 ± 1.7	219 ± 3.7	194 ± 10
pCel1.1Y	**3.85 ± 0.4**	**2.90 ± 0.3**	**86 ± 1.2**	**69 ± 1.4**	42 ± 2.8	33 ± 2.1	**28 ± 1.6**	**291 ± 3.9**	**355 ± 15**
pCel6.2Y	**3.89 ± 0.4**	**3.00 ± 0.4**	**67 ± 1.5**	**63 ± 1.1**	40 ± 3.6	29 ± 3.7	**31 ± 1.3**	**396 ± 2.5**	**244 ± 12**
pCel8.3Y	**4.40 ± 0.6**	**2.87 ± 0.4**	**71 ± 1.4**	**89 ± 1.6**	41 ± 1.8	32 ± 3.0	**28 ± 1.8**	**629 ± 3.2**	**317 ± 13**
WTM	5.84 ± 0.6	5.32 ± 0.6	12 ± 0.9	9.0± 0.6	8.2 ± 0.9	12 ± 0.2	8.0 ±1.2	193 ± 2.4	171 ± 12
pCel1.1M	5.89 ± 0.7	5.55 ± 0.8	**17 ± 0.5**	**19 ± 0.8**	9.5 ± 1.0	13 ± 0.1	**18 ± 0.7**	**321 ± 2.6**	184 ± 15
pCel6.2M	**6.79 ± 0.6**	**6.33 ± 0.7**	**20 ± 0.7**	**17 ± 0.9**	7.5 ± 0.9	11 ± 0.4	11 ± 1.9	**483 ± 2.3**	**280 ± 17**
pCel8.3M	5.96 ± 0.5	**6.52 ± 0.5**	**19 ± 0.4**	**16 ± 0.6**	8.3 ± 0.8	12 ± 0.1	**17 ± 0.9**	**281 ± 2.4**	174 ± 15

SPS E, **sucrose phosphate synthase (excess substrate);** SPS L; **sucrose phosphate synthase (limiting substrate);** SuSy S, **sucrose synthase (synthesis direction);** SuSy H, **sucrose synthase (hydrolysis direction);** AI, **acid invertase;** NI, **neutral invertase;** CWI, **cell wall invertase;** UGPase, **UDP-glucose pyrophosphorylase;** UGD, **UDP-glucose dehydrogenase.**

3.4.6 Cell wall composition is altered in sugarcane *CsCesA* expressing lines.

The present study focuses on increasing the cellulose content of sugarcane. The influence of increased cellulose content on pectin and hemicellulose monosaccharide sugar composition was evaluated by sequential extractions of the destarched cell wall preparations with ammonium oxalate (AO) and increasing amounts of potassium hydroxide (KOH). The cell wall monosaccharide composition in the internodal tissues of transgenic plants was altered due to an increase in glucose, galacturonic acid and galactose (Figure 3.6 a; b). The younger internodal tissues had a substantial increase of glucose 56%, 51% and 48% in the lines pCel1.1Y, pCel6.2Y and pCel8.3Y

respectively, while the mature internodal tissues had a moderate rise of between 12-17% (Table 3.4). Galacturonic acid was elevated in the AO fraction between 25-53% in the younger and 17-22% in the mature internodal tissues. However there were no changes in the amounts of mannose, rhamnose, arabinose and xylose. Due to the increase in galacturonic acid contents in the cell wall, the total uronic acid content was evaluated. The transgenic lines had significant increases in the total uronic acid content by between 34-53% in both young and mature internodal tissues (Figure 3.7).

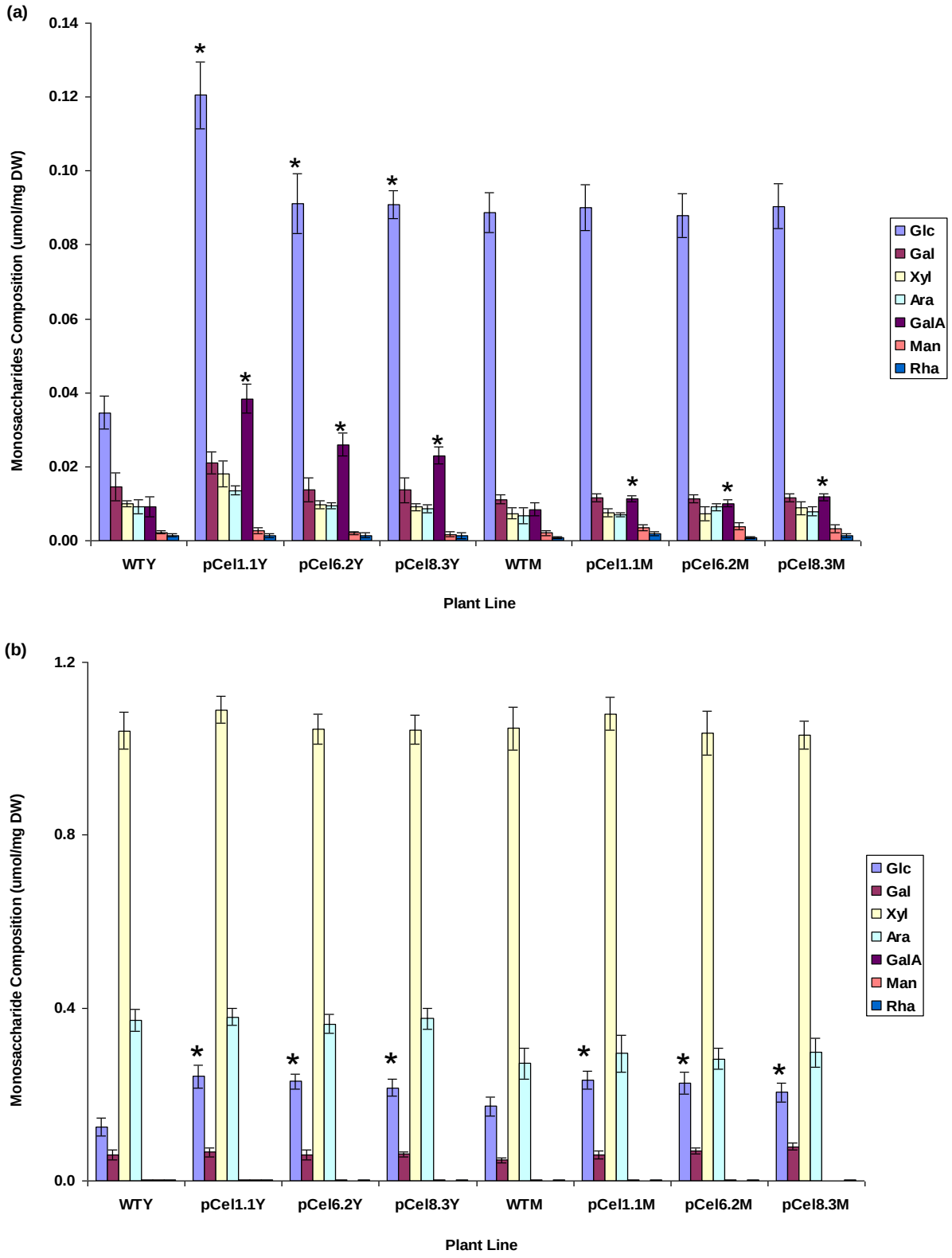

Figure 3.6: Analysis of internodal tissues sugar monomer composition of the transgenic lines in comparison with the wild-type control.

(a) Monosaccharide sugar composition in the ammonium oxalate fractions.

(b) Monosaccharide sugar composition in the potassium hydroxide fractions.

Y; Younger internodes (internodes 1-4); M; Mature internodes (internodes 14-15). WT; Wild-type sugarcane, pCel transgenic sugarcane lines. Values are mean calculated from 3 plants per line. Mean values with * were determined by the t test to be significantly different ($P<0.05$) from the respective wild-type (WT) internodes.

Table 3.4: Composition of hemicelluloses and pectin sugars monomers in internodal tissues of transgenic in comparison with the wild type sugarcane plants cell wall. WT; Wild type sugarcane. pCel; Transgenic sugarcane lines. Y; Young internodes. M; Mature internodes. Values are mean calculated from 3 plants per line. Mean values set in bold type were determined by the t test to be significantly different (P<0.05) from the respective wild-type (WT) internodes.

	(nmol/mg DW)							
Plant Line	**Mannose**	**Rhamnose**	**Galacturonic acid**	**Galactose**	**Glucose**	**Arabinose**	**Xylose**	**Total sugars**
WTY	3.4 ± 0.7	4.8 ± 0.5	12.1 ± 0.6	74.6 ± 3.8	158 ± 3.7	380 ± 4.1	1052 ± 23	1690 ± 33
pCel1.1Y	3.8 ± 0.8	5.0 ± 0.4	**16.8 ± 0.8**	69.8 ± 3.0	**363 ± 5.8**	385 ± 3.8	1107 ± 31	**1954 ± 40**
pCel6.2Y	2.9 ± 0.4	4.8 ± 0.7	**16.1 ± 1.2**	74.4 ± 3.5	**322 ± 3.9**	372 ± 3.4	1055 ± 30	**1848 ± 42**
pCel8.3Y	2.9 ± 0.6	4.8 ± 0.9	**26.0 ± 2.3**	75.0 ± 3.9	**306 ± 4.3**	279 ± 3.5	1052 ± 19	**1782 ± 34**
WTM	3.2 ± 0.7	2.6 ± 0.7	10.0 ± 1.0	58.6 ± 3.5	261 ± 4.1	279 ± 4.3	1055 ± 25	1663 ± 41
pCel1.1M	2.5 ± 0.5	2.4 ± 0.8	**12.9 ± 0.9**	65.5 ± 4.9	**315 ± 3.8**	297 ± 4.1	1084 ± 22	**1782 ± 38**
pCel6.2M	2.5 ± 0.4	2.4 ± 0.2	**12.1 ± 1.1**	**75.4 ± 3.6**	**301 ± 4.3**	288 ± 3.9	1041 ± 20	**1716 ± 34**
pCel8.3M	2.8 ± 0.8	2.5 ± 0.7	**12.9 ± 0.7**	**75.8 ± 2.7**	**296 ± 3.4**	302 ± 5.8	1038 ± 18	**1729 ± 33**

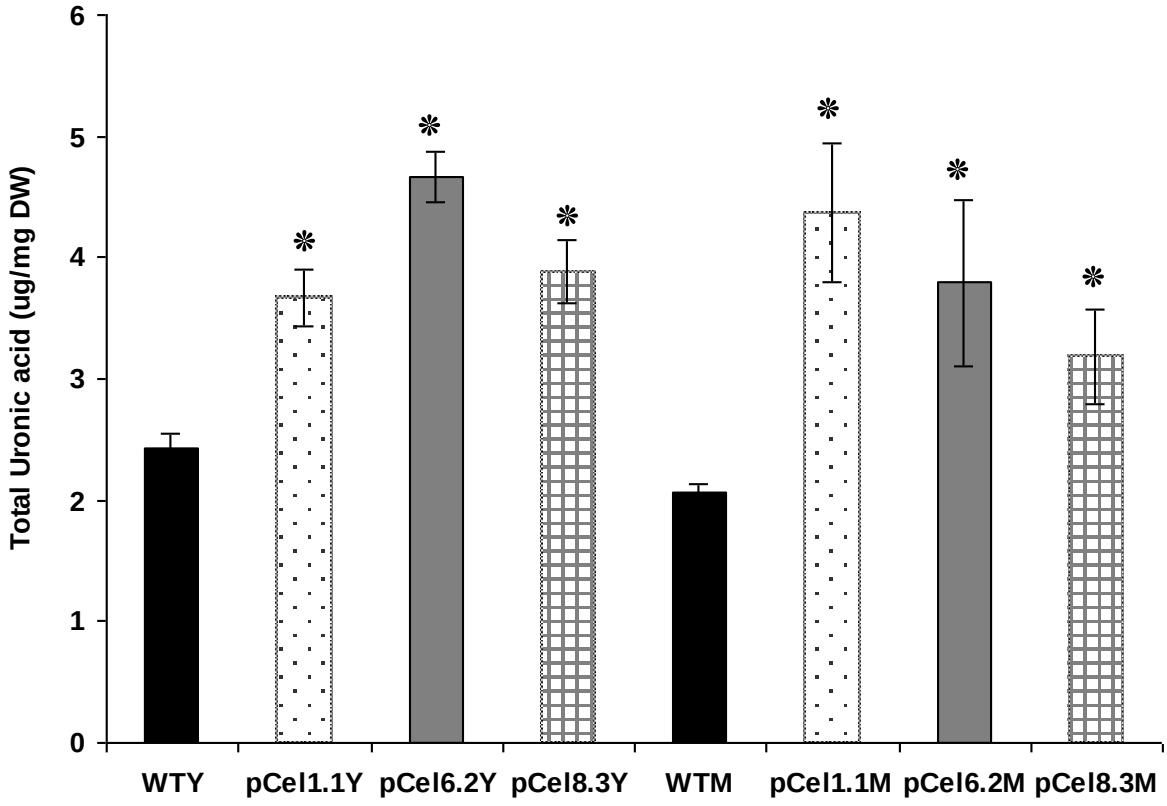

Figure 3.7: Analysis of total uronic acids in transgenic sugarcane and wild-type internodal tissues. Y; Younger internodes (internode 1-4); M; Mature internodes (internodes 14-15) WT; Wild-type sugarcane, pCel transgenenic sugarcane. Values are mean calculated from 3 plants per line. Mean values with * were determined by the t test to be significantly different (P<0.05) from the respective wild-type (WT) internodes.

Total lignin content and its monomer composition were also determined to examine the effect of increased cellulose on the lignin biosynthesis in the internodal tissues of transgenic plants. The total lignin contents of the transgenic plants was significantly reduced by between 5-13% (Table 3.5) Correspondingly, the lignin monomer composition between syringyl (S) and guaiacyl (G) units was also reduced (Table 3.5). This resulted in a higher S/G ratio of the younger and mature internodes of all transgenic plants with the only exception being the mature internodes of pCel6.2M which showed a lower ratio.

Table 3.5. Analysis of total lignin content and monomer unit composition in transgenic and wild type sugarcane plants. WT ; Wild type; pCel; Transgenic sugarcane lines. Y; Younger internodes; M; Mature internodes. Values are mean calculated from 3 plants per line. Mean values set in bold type were determined by the t test to be significantly different ($P<0.05$) from the respective wild-type (WT) internodes.

	Klason Lignin (mg/g DW)			Thioacidolysis (ug/mg DW)			
Plant Line	**Acid insoluble**	**Acid soluble**	**Total Lignin**	**G Lignin**	**S Lignin**	**S/G**	**Total yield**
WTY	0.16 ± 0.04	126 ± 3.5	127 ± 3.6	1.08 ± 0.07	0.70 ± 0.04	0.65	1.79 ± 0.11
pCel1.1Y	**0.10 ± 0.02**	**115 ± 3.3**	**115 ± 3.3**	**0.27 ± 0.06**	**0.21 ± 0.03**	0.77	**0.48 ± 0.09**
pCel6.2Y	**0.12 ± 0.04**	**120 ± 3.5**	**120 ± 3.5**	**0.61 ± 0.04**	**0.49 ± 0.05**	0.81	**1.10 ± 0.09**
pCel8.3Y	**0.09 ± 0.03**	**110 ± 4.2**	**110 ± 4.2**	**0.43 ± 0.05**	**0.29 ± 0.02**	0.66	**0.72 ± 0.07**
WTM	0.03 ± 0.00	170 ± 5.7	170 ± 5.7	1.09 ± 0.09	1.41 ± 0.34	1.28	2.49 ± 0.43
pCel1.1M	**0.01 ± 0.00**	**140 ± 3.8**	**159 ± 3.8**	**0.81 ± 0.02**	**1.13 ± 0.40**	1.40	**1.94 ± 0.42**
pCel6.2M	**0.02 ± 0.00**	**146 ± 6.7**	**161 ± 6.7**	**0.89 ± 0.07**	**1.01 ± 0.51**	1.13	**1.91 ± 0.58**
pCel8.3M	**0.01 ± 0.00**	**154 ± 4.7**	**155 ± 4.7**	**0.81 ± 0.07**	**1.07 ± 0.35**	1.32	**1.88 ± 0.42**

Histochemical analysis of the internodal tissues utilizing transverse section stained with phloroglucinol solution showed the vascular bundles of both wild-type and transgenic plants displayed a red colouration indicating no changes in H units (coniferaldehyde groups) of lignin (Figure 3.8 a and b). In contrast, staining with Mäuler solution showed a dark reddish brown colouration in the vascular bundles of the wild-type but a light brown colour in the transgenic plants, indicating a reduction in the S units of lignin (Figure 3.8 c and d).

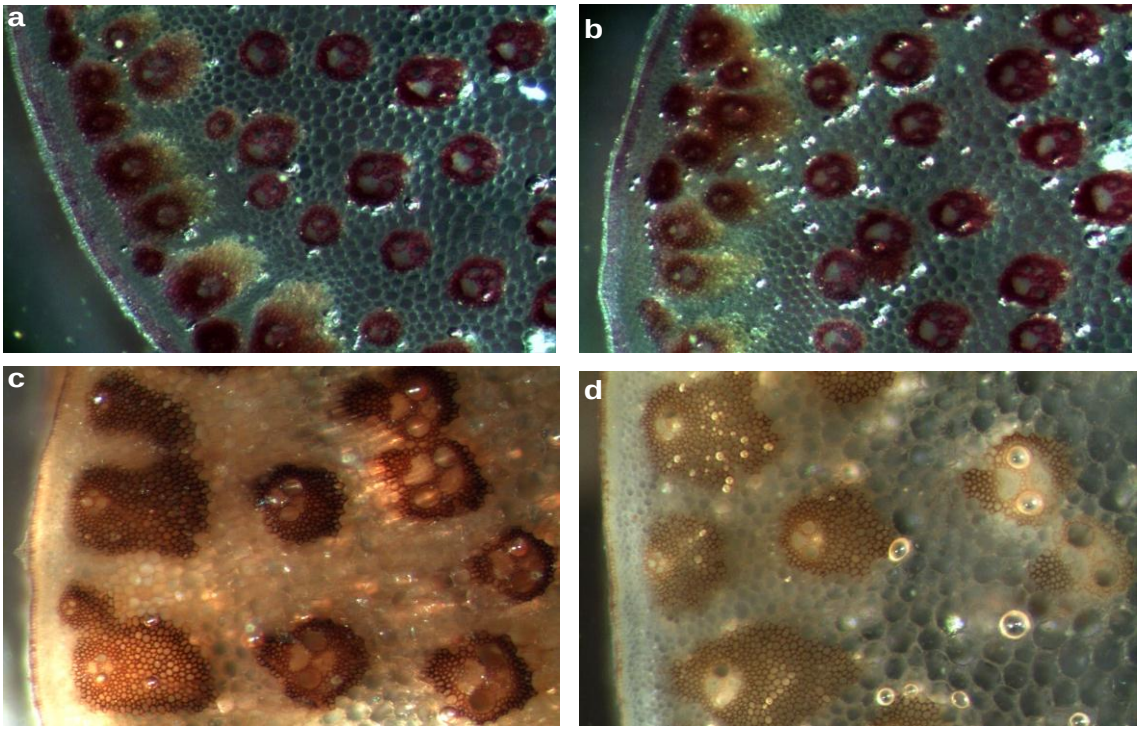

Figure 3.8: Microscopic and histochemical evaluation of sugarcane transgenic line in comparison with wild-type control. Microscopic observation of internodal tissue transverse sections, stained with phloroglucinol stain (a and b) and Mäule stain (c and d).
WT: Wild-type control a and c; pCel: Transgenic line b and d

3.4.7 The influence of increased cellulose and reduced lignin contents on enzymatic saccharification of untreated sugarcane biomass material.

Based on the alterations in the transgenic plant cell wall composition, the lignocellulosic biomass feedstock was examined for its susceptibility to saccharification by cellulosic enzymes. Internodal tissue from all transgenic lines showed significant improvement in their saccharification efficiencies of between 15-36% (Figure 3.9 a and b). In order to confirm that cellulose was hydrolysed to its monomer glucose, the sugars released by the enzymatic hydrolysis were analyzed by GC-MS (data not shown). As expected, the main sugar that was released by cellulase and cellobiase hydrolysis was glucose at more than 93% followed by trace amounts of xylose and arabinose in all transgenic internodal tissues. There was a positive correlation between cellulose contents and saccharification efficiency as demonstrated by performing a pairwise plot and linear regression analysis (Figure 3.9 c). However, glucose released after saccharification correlated negatively with lignin content (Figure 3.9 d and e).

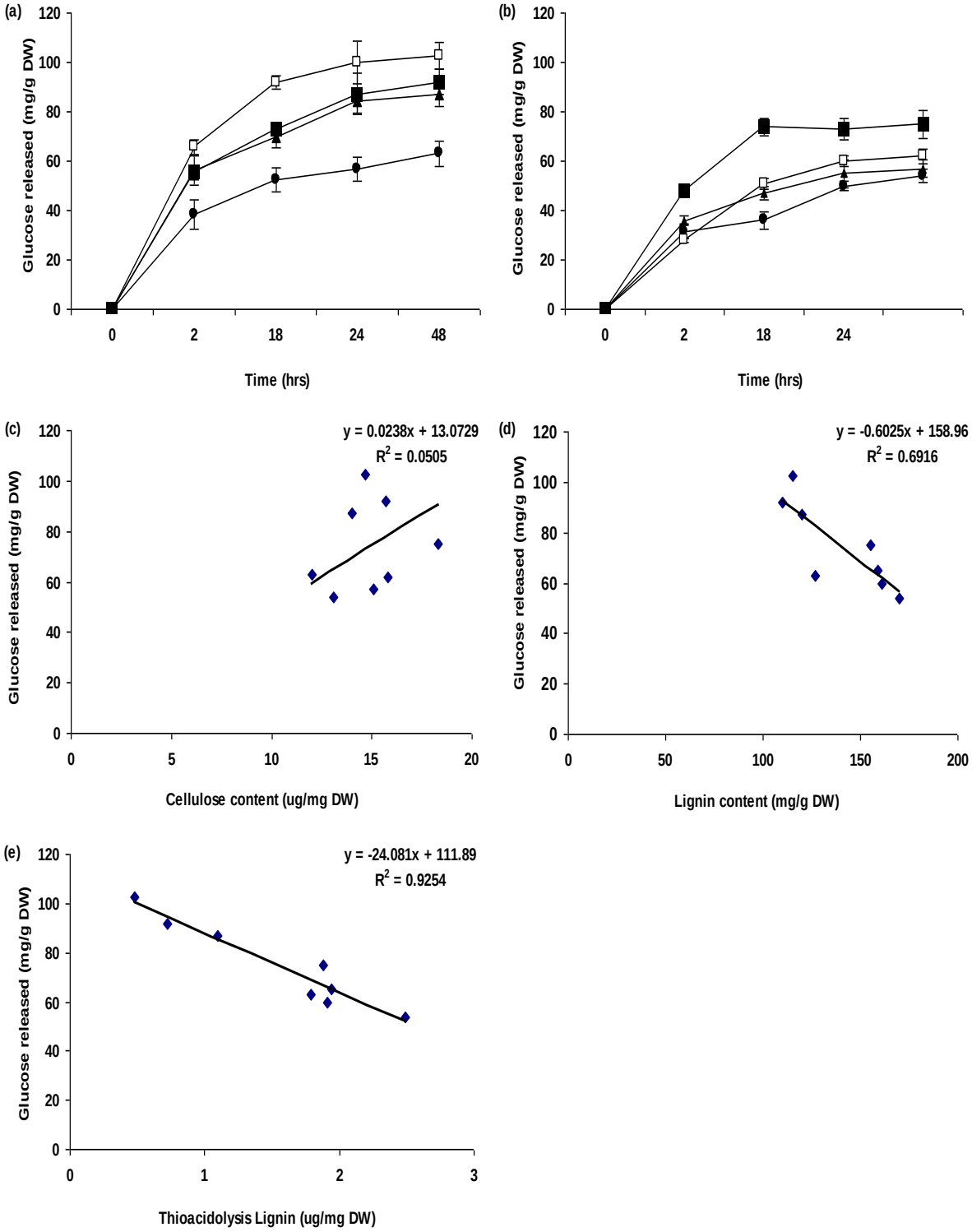

Figure 3.9: Evaluation of saccharification efficiency of transgenic sugarcane and wild–type plants. Wild type, ●; pCel1.1, □; pCel6.2, ▲; pCel8.3 ■;

(a) Saccharification of young internodal tissues.

(b) Saccharification of mature internodal tissues.

(c) Correlation of total cellulose content and glucose released during saccharification of lignocellulose biomass

(d) Correlation of total lignin content and glucose released during saccharification of lignocellulose biomass.

(e) Correlation of total lignin monomer unit content and glucose released during saccharification of lignocellulose biomass.

Values are mean calculated from 3 plants per line. Mean values with * were determined by the t test to be significantly different ($P<0.05$) from the respective wild-type (WT) internodes.

3.5 Discussion

Plants lignocellulosic biomass is an underutilized substrate for bioethanol production, this is due to recalcitrant to enzymatic hydrolysis. Cellulose is a major component of the lignocellulosic feedstock and a potential source of fermentable sugars. Development of sugarcane crop plants where cellulose can be accessed by hydrolytic enzymes is crucial for second generation bioethanol production. This study investigated the effects of heterologous expression of *Ciona savignyi* cellulose synthase (*CsCesA*) cDNA sequence in sugarcane on cell wall composition and fermentability. Quantification of *CsCesA* relative transcript abundance of the transgenes showed variable expression patterns amongst the transgenic lines and internodal tissues developmental stage (Figure 3.3). The differences in transcript abundances may be due to variable *CsCesA* copy number and integration position in the genome amongst independent transformants from particle bombardment (Bower *et al*., 1996).

The expression of *CsCesA* cDNA sequence resulted in increased total cellulose synthase (CesA) activity in both young (29-74%) and mature (20-33%) internodal tissues (Figure 3.4 a). Transcript abundance and the resulting enzyme activity levels in plants have been shown not always to correlate, something ascribed to control mechanism at translational and post translational levels (Gibon *et al*., 2004). The increases in total cellulose synthase activity was associated with concomitant rises in cellulose contents in young internodes of between 17-31% and between 13-28% in mature internodes (Figure 3.4 b). Overexpression studies of cellulose synthase cDNAs in plants have been reported previously (Li *et al*., 2004; Oomen *et al*., 2004). In potato, for example, over-expression of the native *StCesA3*, resulted in potato tubers with elevated cellulose contents of up to 200% (Oomen *et al*., 2004). Similarly, heterologous expression of bacterial cellulose synthase genes *acsA and acsB* from *Acetobacter xylinum* in cotton resulted in transgenic plants with higher cellulose contents (Li *et al.,* 2004). Although *CsCesA* expression has been shown to restore cellulose biosynthesis in cellulose deficient *Agrobacterium tumefaciens* this is, to our knowledge, the first report of an animal *CesA* sequence being expressed in plants (Matthysse *et al*., 2004). These observations demonstrate that heterologous expression of *CsCesA* sequence increased both total cellulose synthase enzyme activity levels and cellulose contents in the transgenic sugarcane plants.

To understand the impacts of increases in cellulose contents of the transgenic sugarcane lines on carbohydrate metabolism, the soluble sugars were determined. The concentrations of glucose, fructose and sucrose in internodal tissues vary according to the developmental stage of the tissue in sugarcane, with sucrose increasing as the internodes mature (Moore, 1995; Whittaker and Botha, 1997). The transgenic lines had slight increases in total soluble sugars (glucose, fructose and sucrose) of between 13-25% in younger internodes and 12-19% in the mature internodal tissues (Table 3.1). Carbon cycling between hexose and sucrose is a controlled process responsible for allocating carbon between respiration, synthesis of sucrose and production of cell wall polysaccharides (Whittaker and Botha 1997; Bindon and Botha, 2002; Fernie *et al*., 2002). Sucrose is thought to be the main source for the production of UDP-glucose the substrate of the cellulose synthase complex for the biosynthesis of cellulose (Amor *et al*., 1995). An increase in cellulose might, therefore, have been expected to decrease sucrose concentrations in the transgenic plants. In fact sucrose was slightly increased in young tissue, along with glucose and fructose. One explanation may be that, an increase in sucrose degradation by membrane pSuSy channels UDP-glucose to cellulose biosynthesis resulting in a positive feedback mechanism of sucrose unloading from source tissue (leaves) into sink tissues (internodes). McCormick *et al*., (2006) observed that a decrease in sugarcane internodal tissues sucrose levels stimulated a high photosynthetic rate in source leaves and this, in turn, promoted sucrose synthesis and transport from source to sink. Transgenic sugarcane lines accumulating large amounts (50-80% of total sugar) of a sucrose isomer were observed to have high leaf photosynthetic rates and it was suggested that the high demand for carbon stimulated photosynthesis (Wu and Birch, 2007). In the current study the photosynthesis rate was not measured in the leaves and so further investigation would be required to test if the same occurred in the transgenic plants developed in this study. After sucrose has been unloaded into the sink tissues it is degraded and re-synthesized to support cellulose biosynthesis. This cycling of sucrose is supported by the activity of the sucrose metabolizing enzymes SuSy, SPS, UGPase and CWI, the activites of all of which were elevated in the transgenic lines (Table 3.3). Moreover, UDP-glucose, a product of sucrose degradation by SuSy, as well as hexose phosphate pools (which are allosteric activators of SPS) were also increased (Table 3.1) (Reimholz *et al*., 1994 van der Merwe *et al*., 2010). The rise in hexose phosphates could also have activated UGPase enzyme which would lead to increased synthesis of UDP-glucose

from glucose-1-P (Kleczkowski *et al*., 2004). Under these conditions of high concentrations of hexose phosphates and UDP-glucose the biosynthesis of sucrose by SPS and /or SuSy would be favoured. Support fo this hypothesis comes from studies where reductions in sugarcane UGD and PFP activity resulted in transgenic lines with high hexose phosphates and UDP-glucose pools and a concomitant rise in sucrose levels (Bekker, 2007; Groenewald and Botha, 2008; van der Merwe *et al*., 2010). Furthermore, high sucrose levels of the transgenic lines were also observed to be accompanied by a rise in cellulose and fibre content (Bekker, 2007; Groenewald and Botha, 2008).

Similarly, in other plants species, elevations in sucrose concentrations have been showed to coincide with increases in SPS enzyme activity. For example, Babb and Haigler (2001) showed increases in SPS enzyme activity in cotton, zinnia and bean coincided with higher sucrose and the maximum deposition of cellulose in the secondary cell wall of these plants. Increase in SPS enzyme activity in cotton (Haigler *et al*., 2007), tobacco stem (Park *et al*., 2008) and poplar stem (Park *et al*., 2009) resulted in plants with elevated sucrose concentration and an increased cellulose content. Thus, it appears that in sugarcane like in other plants species cellulose biosynthesis may also be enhanced by the availibilty of sucrose.

However with maturation the sucrose contents of the transgenic lines is restored to the same concentrations as the wild type, despite the elevation of sucrose synthetic enzymes SPS and SuSy. Similar patterns of restorations in older suspension cells and mature internodes has been reported in transgenic sugarcane plants with reduced PFP and NI activity that showed high sucrose contents in the younger internodes (Rossouw *et al*., 2007; Groenewald and Botha, 2008; van der Merwe *et al*., 2010). The authors suggested that this may be controlled by an unknown sugar sensing mechanism that maintains basal levels of sucrose accumulation in the mature internodes of sugarcane. Our data are in agreement with previous studies that an increase in cellulose synthesis may be correlated with the availability of sucrose that provides the precursor UDP-glucose for cellulose synthesis (Amor *et al*., 1995; Babb and Haigler, 2001; Park *et al*., 2008; Coleman *et al*., 2009). These observations suggest that an increase of cellulose synthesis in the transgenic lines internodes is accompanied by synthesis and degradation of sucrose to meet the demands of flux redirection to cellulose synthesis

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The high cellulose accumulating internodal tissues had a reduced starch content of 25-80% in comparison to control plants (Table 3.2). In contrast to our findings transgenic poplar plants over-expressing cotton *SuSy* had invariable starch content to the wild-type controls despite increased cellulose contents (Coleman *et al*., 2009). However, Coleman *et al* (2010) showed that some tobacco transgenic lines with increased cellulose also had elevated starch levels. The increase in the starch contents in these transgenic lines was attributed to the availability of high hexose sugar pools. Although the hexose phosphate and hexose sugars pools were elevated in the transgenic lines, the starch contents were clearly reduced, therefore the results may indicate that carbon flux was redirected to cellulose synthesis in the transgenic sugarcane plants.

Sugarcane cell wall composition is estimated to consist of approximately 40-50% cellulose, 25-35% hemicelluloses, 18-23% lignin, and 1.6-7.5% extractives (Sun *et al*., 2004, Carroll and Somerville 2009; Masarin *et al*., 2011). The effects of an increase in cellulose in the transgenic plants on other cell wall components were investigated. The hemicellulosic and pectic polysaccharides total monosaccharide sugar composition of the transgenic plants extracted sequentially with ammonium oxalate (AO) and increasing amounts of KOH were altered, with an increase in the levels of glucose (12-56%), galacturonic acid (17-53%) and, galactose (22% pCel6.2M and pCel8.3M) (Figure 3.6 and Table 3.4) . However mannose, rhamnose, arabinose and xylose were invariable to the wild type control. The increases in hemicellulosic glucose may be due to the loosely bound mixed linked (1,3;1,4)-β-glucans polysaccharides that are part of the type II grass cell wall hemicellulose and are easily extracted with AO (Vergara and Carpita, 2001). Souza *et al* (2012) showed that glucose was the major monosaccharide in the sugarcane bagasse AO fraction, and further profiling of the extracted fractions with BG1 monoclonal antibody and lichenase enzyme specific for mixed linked (1,3;1,4)-β-glucans confirmed their presence. The mixed linked (1,3;1,4)-β-glucans are synthesised specifically using UDP-glucose by (1,3;1,4)-β-glucan synthase in grasses (Burton *et al*., 2006; Burton *et al*., 2010; Carpita and MCcann, 2010). The availability of UDP-glucose in the transgenic lines may, therefore, have promoted mixed linked (1,3;1,4)-β-glucans biosynthesis. Elevation of soluble sugars (sucrose, fructose, glucose) in transgenic poplar plants with high UGPase or SuSy activity was accompanied by increases in

cell wall hemicellulosic mononosaccharides composistion (arabinose, glucose, mannose and galactose) in some of the lines (Coleman *et al*., 2007; Coleman *et al*., 2009). The increases in hemicellulosic monomer composition were attributed to availability of the soluble sugars, which were also observed to be significantly high in the current study. Consistent with the previous studies, transgenic sugarcane lines with reduced UGD activity were observed to have high soluble sugars (sucrose, hexose phosphate and UDP-glucose) and concomitant rise in the hemicellulosic pentose sugars levels (arabinose and xylose; Bekker, 2007). Along with the increases in galacturonic acid, total uronic acid (glucuronic and galacturonic acid) contents were also elevated in the transgenic lines. Hemicellulosic and pectic polysaccharides are synthesised from UDP-glucuronic acid produced by the UGD enzyme or by the inositol oxygenation pathway (Tenhaken and Thulke, 1996; Loewus *and* Murthy, 2000). The enzyme activity of UGD and its substrate UDP-glucose were elevated in the high cellulose accumulating transgenic plants. Increases in uronic acids may be due to the increased availability of UDP-glucose which would be used by UGD to produce UDP-glucuronic acid, a precursor for the synthesis of cell wall structural polysaccharides. Transgenic sugarcane lines with reduced UGD activity were shown to have high uronic acid contents even when this pathway was significantly down regulated (Bekker, 2007). It was also shown that a reduction in the UGD pathway in sugarcane was compensated by the inositol oxygenation pathway, which utilizes glucose-6-phosphate as substrate to produce UDP-glucuronic acid. In the current study the rise in glucose-6-phosphate may, alternatively, have activated the synthesis of pectic or hemicellulosic polysaccharide precursors via the inositol oxygenation pathway. Our findings are consistent with the theory that an increased supply of sugar metabolite precursors may augment the biosynthesis and content of that particular pathway. To prove this hypothesis, metabolic flux studies need to be performed in the high cellulose accumulating transgenic lines to prove that the availability of soluble sugars influence the changes in cell wall sugar monomer composition.

We further measured lignin contents and its monomer composition in the transgenic lines and found that both soluble and insoluble Klason lignin contents were reduced by between 6-13% and 5-9% in young and mature internodes respectively (Table 3.5). The reduction in lignin was also mirrored by a decrease in lignin monomer (S and G units) content in the transgenic lines similar to what has been reported in other

studies where lignin was reduced (Fu *et al*., 2011; Ambavaram *et al*., 2011; Jung *et al*., 2012; Jung *et al*., 2013). These results are consistent with what was observed in transgenic sugarcane and switchgrass lines in which the *COMT* gene was repressed, which showed reduced lignin contents and lower S and G lignin monomer content (Fu *et al*., 2011; Jung *et al*., 2012). Reductions in lignin contents in a sorghum *COMT* mutant, aspen tree and switchgrass lines with suppressed *4CL* and *COMT* genes has been shown to result in plants with increased cellulose contents (Hu et al., 1999; Li et al., 2003; Dien *et al*., 2009). Similarly, overexpression of the Arabidopsis transcription factor (*SHN)* involved in the regulation of wax and cutin lipid accumulation in rice, also led to reduced lignin contents and increased cellulose levels (Ambavaram *et al*., 2011). Conversely, reduction in cellulose in transgenic aspen trees over-expressing a cellulose synthase A (*PtdCesA8*) was accompanied by an increase in lignin contents (Joshi *et al*., 2011). However, transgenic tobacco and poplar trees with more than 20% reduction in lignin were shown to have reduced biomass and growth (Kajita *et al*., 1997; Leplè *et al*., 2007; Voelker *et al*., 2010). The inconsistency in the results of all these studies indicates that changes in lignocellulosic components may have diverse and unpredictable impacts on the plant cell wall composition. The observations in the current study suggest that heterologous expression of *CsCesA* cDNA sequence diverts carbon away from the lignin pathway into the synthesis of cellulose.

The recalcitrant nature of lignocellulosic biomass for saccharification is associated with a number of factors including the lignin content, lignin monomer unit composition, hemicellulose content and the cellulose microfibril crystallinity (Chen and Dixon, 2007, Himmel, 2007; Dien *et al*., 2009; Fu *et al*., 2011; Jung *et al*., 2012). Reduction in lignin in several plants has been shown to lead to improved saccharification efficiency (Chen and Dixon, 2007, Himmel, 2007; Dien *et al*., 2009; Fu *et al*., 2011). In those studies the lignocellulosic biomass was pre-treated at high temperatures with acidic or basic solutions which have been reported to produce inhibitory products (furfural and hydroxymethylfurfural) that subsequently limits the availability of fermentable sugars, as well as inhibiting microorganisms for hexose fermentation resulting in reduced yields of bioethanol (Gåmez *et al*., 2004; Klinke *et al*., 2004; Chen and Dixon, 2007). In the current study untreated lignocellulosic biomass of young and mature internodes was exposed to a combination of the cellulosic hydrolysing enzymes cellulase and cellobiase. All tissues from the transgenic lines showed

increased saccharification efficiencies (Figure 3.9 a and b). Younger internodal tissues showed the highest increases of between 28-39% while the increases in mature internodes were between 5-28%. Reductions in lignin contents with concomitant changes in its monomer composition in transgenic sugarcane lines in which the *COMT* gene was repressed resulted in lignocelluloses with improved saccharification efficiencies even without pre-treatment (Jung *et al*., 2012; Jung *et al.,* 2013). As the transgenic lines in this study contain both increased cellulose as well as decreased lignin, the improved saccharification efficiency might be due either to the cellulose being more available, by the decreased lignin-cross-linking allowing improved cellulase contact, or to a combination of the two. We further analysed the relationship between saccharification efficiency and lignin contents of the transgenic lines using a pairwise linear regression analysis. There was an inverse correlation between saccharification efficiency levels and lignin contents in accordance with previous studies of mature and immature alfalfa fibers, switchgrass and mature sugarcane with suppressed lignin biosynthetic genes (Grabber *et al*., 2002; Chen and Dixon, 2007; Fu *et al*., 2011; Jung *et al.,* 2012; Jung *et al.,* 2013).

Second generation bioethanol production technology aims to use lignocellulosic biomass with reduced energy-input during pre-treament processes that are required for making carbohydrate polymers accessible for enzymatic hydrolysis and subsequent fermentation. The data in this chapter demonstrate that heterologous expression of a *CsCesA* cDNA sequence enhances total cellulose synthase enzyme activity and increases cellulose levels in the transgenic sugarcane plants. The elevation in cellulose contents of the transgenic lines results in increased hemicellulosic glucose contents and a reduction in lignin contents. Moreover, the untreated lignocellulosic feedstock biomass of the transgenic plants had improved saccharification efficiencies. Enhanced biomass and improved saccharification efficiency of the transgenic sugarcane plants promises to add value to sugarcane bagasse after sucrose juice extraction for the production of biofuels.

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CHAPTER 4

Ectopic expression of sequences encoding *Cyamopsis tetragonoloba* galactomannan and *Deinococcus radiodurans* α-1,4-glucan chain biosynthetic enzymes leads to non-biosynthesis of the polymers mature transgenic sugarcane lines.

Summary

This study was conducted to determine whether the hemicellulosic galactomannan and a α-1,4-glucan polymer from *Cyamopsis tetragonoloba* (guar) seed and *Deinococcus radiodurans* bacteria respectively can be produced in sugarcane. cDNAs encoding the galactomannan biosynthetic sequences mannan synthase (*CtManS*) and galactosyltransferase (*CtGMGT*) cDNA as well as the amylosucrase gene (*dras*) were successfully isolated, cloned into a sugarcane expression vector and transformed into sugarcane.

Heterologous expression of *CtManS* and *CtGMGT* sequences in sugarcane resulted in sugarcane callus cells that produce the galactomannan polymer. Mannan synthase and galactosyltransferase biosynthetic enzyme sequences were integrated into genomic DNA and also transcribed at mRNA level in transgenic sugarcane suspension culture cells and mature green house plants. However, the enzyme activities of mannan synthase and galactosyltransferase were not detected and consequently galactomannan polymer was not produced in the transgenic sugarcane lines.

Expression of the α-1,4 glucan biosynthetic DRAS protein to the cytosol or vacuole resulted in transgenic cells that were able to transcribe the sequence in both compartments. DRAS targeted to the cytosol was detrimental to plant development, regenerated transgenics lines had stunted growth after eighteen months of growth under green house conditions. However, when DRAS was present in the vacuole it resulted in transgenic plants which in the first three months had increased maltooligosaccharide, glucose, fructose and sucrose contents in the leaves. After 18 months in the greenhouse however differences between transgenic and wild-type lines were undetectable.

4.1 Introduction

Bioethanol is usually produced through the fermentation of simple sugars derived either directly from crops such as sugarcane, or which are released following hydrolysis of starch. Lignocellulosic biomass from these plants has been identified as an alternative feedstock for biofuels production, which does not impact food security (Abramson *et al*., 2010; Waclawovsky *et al*., 2010; Karp and Richter, 2011). Plants cell wall lignocellulosic structure is designed to give shape and to protect the plant against pathogen attacks. The cell wall lignocellulosic structure due to its interwoven nature is resistant to enzymatic hydrolysis (Abramson *et al*., 2010; Harris and DeBolt, 2010). The key objective of second geneneration biofuels production to be met is to make plant lignocellulosic cell wall polysachrides amendable to enzymatic degradion. To achieve this goal, cell wall lignocellulosic polysaccharides composites may be altered by increasing and/or a reduction in one of its composites by introducing highly soluble polysaccharides. Reduction of lignin content in plants cell wall has been shown in a number of studies to result in improved lignocellulosic hydrolysis (Hu *et al*., 1999, Dien *et al.*, 2009; Ambavaram *et al*., 2011; Fu *et al*., 2011; Jung *et al*., 2011; Jung *et al*., 2013). Decreased in ligning content in some the studies were observed to be compensated by an increase lignocellulosic glucose rich cellulose amounts (Hu *et al*., 1999, Dien *et al.*, 2009; Ambavaram *et al*., 2011). Therefore, altering lignocellulosic polymer composite may result in improved cell wall polysaccharides enzymatic hydrolysis.

Leguminous plants such as *Cyamopsis tetragonoloba* (guar) and fenugreek (*Trigonella foenum-graecum* L) seed endosperm cell wall contains high amounts of a water soluble hemicellulosic galactomannan (Edwards *et al*., 1989; Maier *et al*., 1993). Galactomannan polysaccharide from *C. tetragonoloba* is an attractive polymer for increasing cell wall lignocellulosic biomass, this is due to: 1) rich in hexose sugars that can be converted to biofuels, 2) the polymer is water soluble and 3) the sequences mannan synthase (*CtManS*) and UDP-galactose-dependent galactosyltransferase (*CtGMGT*) implicated in biosynthesis of galactomannan polymer have been identified and functionally characterized (Edwards *et al*., 1989; Reid *et al.,* 1995; Dhugga *et al.,* 2004). Therefore, introducing the galactomannan biosynthesis gene to other plants species may have the potential to increase biomass for biofuel production.

A number of bacteria are able to produce biopolymers (such as linear glucans) and sucrose isomers (Büttcher *et al*., 1997; Pizzut-Serin *et al*., 2005; Wu and Birch, 2005). The synthesis of the polymer does not require energetically expensive activated sugars, such as ADP-glucose or UDP-glucose, rather sucrose is the preferred substrate (Büttcher *et al*., 1997; Pizzut-Serin *et al*., 2005). Amylosucrases are transglucosidases which catalyse the synthesis of insoluble α-1;4-glucan polymers, sucrose isomers and simultaneously release fructose or soluble α-1;4-glucans from sucrose (Büttcher *et al*., 1997; Pizzut-Serin *et al*., 2005). Genes responsible for the biosynthesis of α-1;4-glucan polymers have been identified and characterized in *Deinococcus radiodurans* (*dras*) and *Neisseria polysaccharea* (*npas*) (Büttcher *et al*., 1997; Pizzut-Serin *et al*., 2005). Introducing these genes in high sucrose accumulating plants e.g. sorghum and sugarcane may be a way of increasing biomass and producing novel sugars e.g. turanose and trehalulose.

Sugarcane is an ideal higher plant bioreactor, owing to its C_4 photosynthesis carbon fixation pathway and hence large biomass efficient as compared to C_3 (ryegrass and bamboo) and some C_4 (sorghum and switchgrass) plants (Somerville, 2010, Taylor *et al*., 2010; Byrt *et al*., 2011). Sugarcane accumulates high sucrose levels in its internodal tissues and the larger amount of this sugar is transported from the cytoplasm and stored in the vacuole (Moore, 1995). Storage of sucrose into the vacuole promotes sucrose unloading from the source leaf tissue to the sink storage compartment (Moore, 1995; McCormick *et al*., 2006). Targeting of a bacterial sucrose isomerase protein into the vacuole of sugarcane resulted in transgenic lines with increase sucrose isomers in the internodal tissues without adversely effecting sucrose amounts (Wu and Birch, 2007). Therefore, targeting DRAS protein into sugarcane may result in the production of a α-1;4-glucan biopolymer, which could be utilized for the production of biofuels.

In this study galactomannan biosynthetic constructs containing sequences from *Cyamopsis tetragonoloba* (*CtManS* and *CtGMGT*) were developed for heterologous expression in sugarcane. Also constructs containing α-1,4-glucan biosynthetic *dras* gene from *Deinococcus radiodurans* were designed and manufactured to target the DRAS protein to the cytoplasm or vacoule in transgenic sugarcane.

4.2 Materials

All chemicals and carbohydrate modulating enzymes were obtained from Sigma-Aldrich Fluka Chemical Company (St. Louis, MO, USA) or Roche Diagnostics (Basel, Switzerland), unless stated otherwise. Nucleic acid modifying enzymes were obtained from either Promega (Anatech Instruments, South Africa) or Fermentas (Inqaba Biotech, South Africa). Oligo nucleotide primers were purchased from Integrated DNA Technologies (IDT, Whitehead Scientific, South Africa) or Fermentas (Inqaba Biotech, South Africa).

4.2.1 Methods

4.2.1.1 Isolation of galactomannan biosynthetic enzymes encoding sequences by reverse transcription polymerase chain reaction (RT-PCR) and construction of expression vectors

Total RNA and cDNA were extracted and synthesized as described in section 3.3.6. Galactomannan biosynthetic cDNA sequences were isolated by RT-PCR from *Cyamopsis tetragonoloba* (guar) seed endosperm cDNA. The guar mannan synthase (*CtManS)* 1965 bp and galactomannan UDP-galactose galactosyltransferase (*CtGMGT*) 1308 bp (accession number AJ938067) sequences (accession number AY372247) were used to design primers, *CtManS* Fwd: CCCGGGATGGCACGAGGTGCCTGCAACAAGT; and Rev: CCCGGGCTAATCCCTTTATCCATTCACAAAT: and for *CtGMGT* Fwd: CCCGGGATGGCCAAATTTGGTTCCAGAAACAAATC and Rev: *CCCGGG*CTATTATGCAGCAGGGTAACCAAAAGGTAAG. The RT-PCR amplification conditions for *CtManS* and *CtGMGT* sequences were as follows: 95°C for 5 min; 30 cycles (95°C for 30 sec, 56°C for 35 sec, 72°C for 2 min); 72°C for 5 min. The amplicons were separated on 1% agarose gel and extracted using the GeneJET^{Tm} Gel Extraction kit.

The *CtManS* and *CtGMGT* sequences were digested with *Sma*I then ligated in sense orientation with respect to the promoter into the *Sma*I site of the pUBI510 vector (Groenewald *et al*. 2000) using T4 DNA ligase (Promega). The resulting plant transformation constructs pUbi*ManS* and pUbi*CtGMGT* were electroporated into DH5

alpha *E. coli* competent cells using standard transformation procedures. Directional colony polymerase chain reaction (PCR) was performed using the ubiquitin promoter forward primer: UBI Fwd: ATACGCTATTTATTTGCTTGG and a gene specific reverse primer *CtManS* and *CtGMGT*, to select colonies ligated in sense orientation with respect to the promoter. Amplification conditions were as follows: 95°C for 5 min; 30 cycles (95°C for 30 sec, 56°C for 35sec, 72°C for 2 min); 72°C for 5 min.

4.2.1.2 Amylosucrase encoding sequence isolation by PCR and construction of a cytosolic and vacuolar targeted expression vectors

The *dras* sequence was isolated by PCR from *Deinococcus radiodurans* ATCC 13939 (American Type Culture Collection) genomic DNA (gDNA). In brief, *D. radiodurans* was grown for 48 hours at 30°C in nutrient broth (5 g/l peptone, 3 g/l meat extract and 10 g/l glucose) at pH 6.8 and 2 ml of the culture was centrifuged. The pellet was resuspended in buffer (Tris-HCl, pH 7, 10% v/v SDS and 20 ml/l pronase) and incubated for 1 h at 37°C followed by addition of 4 M NaCl and CTAB/NaCl solution (10 g CTAB and 4.1 g NaCl) and further incubated at 65°C for 10 min. One volume of chloroform/isoamyl alcohol (24:1) was added and the sample was centrifuged for 5 min at 10 000 g. The upper phase was added into the same volume of phenol/chloroform/isoamyl alcohol and centrifuged as before. The upper phase was added into 0.6 volumes of isopropanol, incubated for 1 hour at -80°C and centrifuged for 20 min. The nucleic acids pellet was washed three times in 70% (v/v) ethanol, dry and resuspended in buffer (Tris-HCl, pH 7 and RNase).

The 1935 bp sequence (Locus NP_294657.1) was used to design primers: Fwd: CGGGATCCATGCTCACGCCTGACCTCGC and Rev: CGGAGCTCCTATTACCCCCCCGCCACCA. PCR amplification conditions were as follows: 95°C for 5 min; 30 cycles (95°C for 60 sec, 62°C for 2 min, 72°C for 3 min); 72°C for 5 min. The amplicons were separated on 1% agarose gels and extracted as above.

The *dras* gene was digested with *Bam*H I and *Sac* I then ligated into pBluescript II KS plasmid treated with the same restriction enzymes. The resulting pKS-DRAS construct was electroporated into *E. coli* BL21 (DE3) competent cells using standard

transformation procedures. Colony PCR was performed using gene specific primers, Fwd: GCCTACCGCGACTATTTTCA and Rev: CCGCAAAGTCAGTGTCGTTA, Amplification condition were as follows: 95°C for 5 min; 30 cycles (95°C for 30 sec, 58°C for 35 sec, 72°C for 1 min); 72°C for 5 min.

The pKS-DRAS cloning plasmid was digested with *BamH* I and *Sac* I and the resulting *dras* gene was then ligated into the plant expression vector pU3Z treated with the same restriction enzymes, resulting in a cytosolic expression construct pU3Z-DRAS. Expression of the *dras* gene in the plant vector pU3Z is driven by the maize ubiquitin promoter of the *Ubi-1* gene (Vickers *et al*., 2005). The construct pU3Z-DRAS was electroporated into competent bacterial cells as described above. Colony PCR was performed as before using *dras* gene specific primers. pU3Z-DRAS was also treated with *BamH* I and *Sac* I restriction enzymes to screen for the presence of the *dras* gene.

The N-terminal pro-peptide (NTPP) of sweet potato sporamin for the vacuolar targeting of the *dras* gene was designed based on sugarcane or monocot preferred codon usage using the CUTG database (Nakamura *et al*., 1996). The NTPP-encoding sequence (5' GGT ACC ATG AAG GCC TTC ACC CTG GCC-CTG TTC CTG GCC CTG TCC CTG TAC CTG CTG CCG AAC CCG GCC CAC TCC CGC TTC AAC CCG ATC CGC CTG CCG ACC ACC CAC GAG CCG GCC GGA TCC 3') was treated with *Kpn* I and *Bam*H I and ligated into the pKS-DRAS plasmid treated with the same restriction enzymes, resulting in the pKSNTPP-DRAS plasmid and electroporated into competent bacterial cells as described before. Colony PCR was performed using NTPP specific forward primer, Fwd: CCGATCCGCCTGCCGACCACC and *dras* reverse primer, Rev: CGGAGCTCCTATTACCCCCCCGCCACCA. Amplification condition were as follows: 95°C for 5 min; 30 cycles (95°C for 30 sec, 55°C for 35 sec, 72°C for 2 min); 72°C for 5 min.

The pKSNTPP-DRAS was digested with the restriction enzymes *Kpn* I and *BamH* I and also with *BamH* I and *Sac* I to screen for the presence of the NTPP and the *dras* gene respectively. The vacoular expression construct was created by treating pKSNTPP-DRAS with *Kpn* I and *Sac* I, and the resulting fragment NTPP-DRAS was then ligated to pU3Z treated with the same restriction enzymes resulting in pU3ZNTPP-DRAS construct that targets the *dras* encoded protein to the vacuole.

4.2.1.3 Iodine staining of bacterial cells expressing the *dras* gene for the production of α-1;4-glucan polymers

Bacterial cells containing pKS-DRAS or pKSNTPP-DRAS plasmid were evaluated for their ability to produce α-1;4-glucan polymers. Single colonies were spread on solid LB agar plates containing 100 mg/ml ampicillin, 0.2 mM isopropyl-b-D-thiogalactopyranoside (IPTG) and 5% (w/v) sucrose and were incubated for 24 hours at 37°C. Bacterial plates where then exposed to iodine vapour for 3 min to screen for the production of α-1;4-glucans.

4.2.1.4 Sugarcane callus initiation, maintenance, transformation and plant regeneration.

Transformation of sugarcane was performed exactly as described in section 3.3.2, 3.3.3 and 3.3.4. Sugarcane embryogenic calli were transformed by microprojectile bombardment with pUbi*ManS*, pUbi*CtGMGT* and pEmuKN DNA (geneticin selection marker containing plasmid) for galactomannan biosynthesis.

Sugarcane embryogenic calli for α-1;4-glucan polymer biosynthesis in the cytosolic compartment were microprojectile bombarded with pU3Z-DRAS and pEmuKN DNA and for vacoular targeting of the amylosucrase, callus was bombarded with pU3ZNTPPDRAS and pEmuKN DNA.

4.2.1.5 Initiation and maintenance of sugarcane suspension culture cells

Sugarcane callus suspension cultures were initiated with callus from solid plates. The suspension cells mother cultures were initiated with 1 g callus in 50 ml of MSC_3 media for untransformed callus and the putative transgenic clones and also untransformed callus were inoculated in MSC_3G45 media containing geneticin. The callus suspension cell cultures were grown at 28°C with shaking at 170 rpm on an orbital shaker and subcultured at 14 days intervals. Once a week, optical densities (OD) were measured at 600 nm and the suspension cells were screened for contamination by inoculating aliquots in the yeast and bacteria growth media. Suspension cell mother cultures were then split into three replicates in order synchronize the growth cycles of the cultures. Cells were grown for approximately 14

days, harvested when reaching an OD of 1-1.5 and excess liquid media was removed. The suspension cells were analysed immediately or stored at -80°C until required.

4.2.1.6 Isolation of genomic DNA and identification of transformants by PCR

Genomic DNA (gDNA) was extracted from the geneticin surviving calli or suspension culture cells or matured sugarcane leaves or internodal tissues as described in section 3.3.5. Polymerase chain reaction analysis was performed using 10 ng gDNA as a template. Gene specific primer sets for mannan synthase (*CtManS)* primers Fwd: GGAGAAGCAATATGTAGAGGATT and Rev: ACCCTTTTGCAACAGATGATTTC; UDP-galactose galactosyltransferase (*CtGMGT*) primer set Fwd: GAGTTCAAGCTTCCGTTAAAGCG and Rev: CAGCCAGTACCCTTCGAAGTAGT.or UBI Fwd: ATA CGC TAT TTA TTT GCT TGG in combination with the gene specific reverse primer was used for PCR analysis. Amplification condition were as follows: 95°C for 5 min; 30 cycles (95°C for 30 sec, 60°C for 35sec, 72°C for 1 min); 72°C for 5 min.

Analysis by PCR for *dra* gene targeted to the cytosolic or vacoular compartment, gene specific primers were used, Fwd: GCCTACCGCGACTATTTTCA and Rev: CCGCAAAGTCAGTGTCGTTA. The amplification condition was as follows: 95°C for 5 min; 30 cycles (95°C for 30 sec, 58°C for 35 sec, 72°C for 1 min); 72°C for 5 min.

4.2.1.7 Total RNA extraction and expression analysis of galactomannan and α-1;4-glucan biosynthetic enzyme encoding sequences in sugarcane by RT-PCR

Total RNA extraction and first strand cDNA synthesis were performed as described in section 3.3.6 using callus that survived on geneticin, suspension culture cells and mature sugarcane leaves or internodal tissues. RT-PCR was performed using 2 µl from 20 µl cDNA (10 ng) synthesis reaction. Gene specific primers for the galactomannan biosynthetic enzymes encoding sequences *CtManS* and *CtGMGT* were used and the RT-PCR conditions were the same as above. Gene specific primers for the amylose biosynthetic enzyme encoding sequence *dras* were used for RT-PCR and PCR conditions were conducted as described above.

4.2.1.8 Isolation of membrane proteins from suspension culture cells and mature sugarcane internodal tissues

Membrane proteins were prepared from suspension cultured cells and mature internodal tissues according to a modified method by Dhugga *et al*., 2004. Finely ground tissue was homogenized in cold extraction buffer A (50 mM Hepes-KOH, pH 7.5, 5 mM $MgCl_2$, 4% (w/v) sucrose, 1% (w/v) glycerol, 5 mM mercaptoethanol, and 2 mM EDTA). The slurry was squeezed through one layer of Miracloth and then pressed through four layers of cheesecloth and the remaining residue cell wall debris washed three times with extraction buffer A. The resulting filtrates were centrifuged at 2 000g for 15 min at 4°C and the supernatant was transferred to ultracentrifuge tubes. The supernatant were centrifuged at 142 000g for 40 min in a fixed angle rotor at 4ºC. The resulting pellet of membrane proteins were resuspended in 100 ul buffer B (100 mM Hepes-KOH, pH 7.5; 10 mM DTT, 10% (w/w) glycerol and 1 mM EDTA or 1% (w/v) digitonin) using a glass homogeniser and immediately frozen in liquid nitrogen.

4.2.1.9 Mannan synthase enzyme activity determination

A mannan synthase enzyme activity assay was performed as described by Dhugga *et al*., 2004 with suspension cultured cell and internodal membrane proteins. In brief, 200 ug membrane proteins (or digitonin solubilized membrane proteins) were added to 100 mM Hepes-KOH, pH 7.5; 2.5 mM $MgCl_2$, 5 mM $MnCl_2$, 2.5 mM DTT, 6% glycerol (w/v) and 80 µM GDP-(^{14}C)-mannose in 50 ul reactions and incubated at 35ºC for 40 min. The specific activity of GDP-(^{14}C)-mannose in the reaction was 83 Bq/nmol. The reactions were stopped by adding 2 ml of 70% ethanol containing 2 mM EDTA followed by incubating the samples at 100ºC for 5 min and 10 ul galactomannans (0.2 mg/ml) were added to the reaction mixture subsequently. The samples were passed through glass microfiber filters (GF/A) and washed twice with 20 ml 70% ethanol. The filters were air dried at room temperature. The dried filters were submerged in 4 ml scintillation cocktail and the radioactivity incorporated was determined in a scintillation counter.

4.2.1.10 Galactomannan galactosyltransferase enzyme activity determination

Galactomannan galactosyltransferase enzyme activity was determined as described by Edwards *et al*., 1989 with suspension cultured cell and internodal membrane proteins. In brief, 200 ug membrane proteins (or digitonin solubilized membrane proteins) were added to 50 mM Tris-HCl, pH 7.5; 2.5 mM $MgCl_2$, 5 mM $MnCl_2$, 2.5 mM DTT, 80 µM GDP-mannose and 800 µM UDP-(^{14}C)-galactose in 50 ul reactions and incubated at 30ºC for 60 min. The specific activity of UDP-(^{14}C)-galactose in the reaction was 23 Bq/nmol. The reactions were stopped by adding 2 ml of 70% ethanol containing 2 mM EDTA followed by incubating the samples at 70ºC for 5 min and 10 ul galactomannans (0.2 mg/ml) were added in the reaction mixture. Samples were passed through glass microfiber filter discs (GF/A) and washed three times with 10 ml 70% ethanol. The filters were air dried at room temperature. The dried filters were submerged in 4 ml scintillation cocktail and the radioactivity incorporated was determined in a scintillation counter (Beckman Coulter).

4.2.1.11 Protein extraction from sugarcane tissues

Crude protein extracts were prepared from young and mature internodal or leaf tissues. The tissues were homogenized in cold extraction buffer (50 mM Tris-HCl pH 6.5, 2 mM DTT, 2 mM $CaCl_2$ and 0.0016 gm/l complete protease inhibitor) The samples were centrifuged at 4°C for 10 min at 10 000g, the supernatant transferred to Sephadex G50 spun columns, which had been pre-equilibrated with extraction buffer, and were centrifuged at 1 000 g for 2 min at 4°C. Crude desalted proteins were immediately frozen in liquid nitrogen and stored at -80°C.

4.2.1.12 Amylosucrase enzyme activity assays

200 ug desalted crude protein extract was added into 170 µl reaction buffer (100 mM citrate, pH 6.5 or 50 mM Tris-HCl, pH 7, 5% (v/v) sucrose and 0.1% (v/v) glycogen) and incubated at 30°C for 30 min. The reaction was terminated by incubating samples at 95°C for 5 min. Amylosucrase enzyme activity was determined by analyzing the fructose released by combining 180 µl of 50 mM imidazol buffer, pH 6.9, 2 mM $MgCl_2$, 1 mM ATP, 0.4 mM NAD, and 0.5 U hexokinase with 10 µl of the assay. The reaction was incubated for 15 min at room temperature and the

absorbance at 340 nm was determined, followed by addition of 1 U PGI and 0.5 U G6PDH enzymes. The change in absorption after 20 min was measured at 340 nm and the difference between the two absorbances was used to determine the activity.

4.2.1.13 In-gel amylosucrase enzyme activity assay

In-gel assays for amylosucrase enzyme activity were performed as followed, 200 ug proteins was mixed with 10 µl loading buffer (40% (w/v) glycerol, 250 mM Tris-HCl, pH 8.8, 0.02 % bromophenol blue) and separated by 7.5% polyacrylamide gel electrophoresis (375 mM Tris-HCl, pH 8.8) at 4°C with a constant current of 20 mA using 375 mM Tris-HCl, pH 8.8 and 200 mM glycerol as running buffer. After the bromophenol blue had run out of the gel the electrophoresis was stopped. Gels were then equilibrated in 100 mM sodium citrate, pH 6.5 five times for 20 min at room temperature. After incubating the gels at 37°C for 16 hours in buffer containing 100 mM sodium citrate, pH 6.5, 5% (v/v) sucrose and 0.1% (v/v) glycogen the incubation buffer was discarded and the α-1;4-glucans were detected with Lugol's solution.

4.2.1.14 Protein determination

Protein contents were measured according to Bradford (1979) using the Bio-Rad microassay solution. Bovine serum albumin (Fraction V) (Roche) was used as a standard.

4.2.1.15 Isolation of galactomannans from sugarcane plants

Galactomannans were extracted from the callus, callus suspension cultured cells and mature sugarcane internodal tissues by a modified method of Naoumkina *et al* (2008). In brief, 200 mg of finely ground sugarcane callus was hydrated in 10 ml of water at 70°C for 2 hours followed by an overnight hydration at 25°C. The samples were centrifuged for 30 min at 6 000g and supernatants were transferred to 50 ml tubes. The supernatants were treated with Fehling's solution added dropwise until no further blue precipitate was formed. The samples were centrifuged at 6 000g for 30 min, supernatants discarded and the pellets resuspended in 2 ml of water to which glacial acetic acid was added dropwise until the complex was dissolved. Ethanol was added to a final concentration of 66% (v/v) ethanol to precipitate the

galactomannans, centrifuged at 10 000g and followed by two washes with 70% (v/v) ethanol and the samples were freeze dried.

4.2.1.16 Isolation and determination of soluble sugars and starch

Maltooligosaccharides were isolated with a modified method according to Critchley *et al* (2001). In brief, 300 mg ground material from leaves and internodal tissues were added in 750 µl 0.7 M perchloric acid, mixed and incubated on ice for 30 min and centrifuged at 3 000g for 10 min at 4°C. Supernatant (500 µl) was added to 300 µl of 2 M KOH, 0.4 M MES, 0.4 M KCl and centrifuged at 20 000 g for 10 min at 4°C to remove the potassium perchlorate. 500 µl of the supernatant was added to 160 µl of 0.5 M MES, pH 5.6 and 500 µl of this added to 500 µl of water. 7.5 U β-fructosidase, 1 U glucose oxidase and 650 U catalase were added, samples incubated at 32°C for 2 hours to remove glucose and sucrose, and the reaction was further incubated at 70°C for 40 min to eliminate the glucose oxidase. Samples (50 µl) were incubated with 0.25 U maltase and 2 U amyloglucosidase at 37°C for 2 hours and the glucose released was measure as in section 3.3.8.

Soluble sugars (sucrose, glucose, and fructose) and starch were extracted and determined as described in sections 3.3.8 and 3.3.9.

Metabolites were extracted for GC/MS analysis as described by Glassop *et al* (2007). In brief, tissue samples (60 mg) were added to 350 µl of methanol containing 10 µl ribitol (0.2 mg/ml) as internal standard. Samples were then extracted at 70°C for 15 min, 350 µl water and 300 µl chloroform were added and the samples were vortexed. Polar and non-polar phases were separated by centrifugation at 12 000g for 10 min. Chloroform (300 µl) was added to the polar phase, vortexed and separated as before. The polar phases of the samples (60 µl) were dried and used immediately or stored at room temperature in a desiccator.

Soluble sugar extractions for analysis by HPLC, were performed as described by Peter *et al* (2007). Leaves and internodal tissues materials (50 mg) were extracted sequentially with 1.5 ml ethanol 80% (v/v) and 1.5 ml water respectively, each extraction was repeated twice. During extraction, samples were incubated at 80°C for

10 min, placed on ice for 2 min and centrifuged at 15 000g for 5 min. The supernatants were pooled and adjusted to 6 ml with water.

4.2.1.17 Seaman's hydrolysis of polysaccharides

Polysaccharides were hydrolyzed by adding 72% (w/w) sulphuric acid, vortexed, and incubated at 30°C for 45 min after which the sulphuric acid was diluted to 4% with water. The samples were autoclaved at 121°C for 1 hour and allowed to cool to room temperature. 25 µl of 0.5% bromophenomol blue was added and approximately 6 ml of 0.18 M $Ba(OH)_2$ solution was added slowly with rapid stirring. Neutralization was completed by the addition of 0.5-1 g of $BaCO_3$ until the indicator turned blue (pH 7-7.5). Samples were centrifuged at 5 000g for 15 min, supernatants collected and incubated at 4°C overnight. These were centrifuged at 5 000g for 15 min and lyophilized.

4.2.1.18 Hydrolysis of sugarcane alcohol insoluble residue

Alcohol insoluble residues (AIR) from sugarcane suspension culture cells and mature internodes of green house grown plants were hydrolyzed with 2M trifluoroacetic acid (TFA) as described in section 3.3.21.5.

4.2.1.19 Derivatisation and GC-MS analysis

Metabolites from sugarcane callus, callus suspension cells, leaves and internodal tissues, standards sugars (galactose, mannose, xylose, arabinose and glucose, turanose) and the internal standard ribitol were derivatised and analysed by GC-MS according to the method of Roessner *et al*., 2000 as described in section 3.3.21.6.

4.2.1.20 Soluble sugar identification by HPLC-PAD

Desalted samples (50 µl) were identified and quantified by HPLC-PAD (Bachmann *et al*., 1994). Soluble sugars were separated by a Ca/Na-moderated ion partitioning carbohydrate column (Benson BC-100 column, 7.8x300 mm; Benson Polymeric, Reno, NE, USA). The system was operated at 90°C and isocratically eluted with 0.005% (w/v) Ca/Na_2-EDTA at a flow rate of 0.6 ml/min. To confirm the identities of

sugars, samples were also analyzed by anion exchange chromatography using a CarboPac MA1 column (4x250 mm; Dionex, Sunnyvale, CA, USA) operated at 30°C and isocratically eluted with 0.6N NaOH at a flow rate of 0.4 ml/min.

4.2.1.21 Statistical analyses

Student's t-tests (two-tailed) were performed to test for significant differences between group means using Statistica version 10 (StatSoft, Inc. 2004). The term significant is used to indicate differences for which $P < 0.05$.

4.3. Results

4.3.1 Ectopic expression of galactomannan biosynthetic enzyme encoding sequences in sugarcane

4.3.1.1 Production of plant transformation constructs designed to lead to increased galactomannan biosynthesis

Full length coding regions of *C. tetragonoloba CtManS* and *CtGMGT* sequences were amplified by polymerase chain reaction (PCR) from cDNA isolated at 35 days after flowering from the endosperm of developing seeds. The amplified *CtManS* and *CtGMGT* sequences were 1965 and 1308 bp in length respectively and BLASTn analysis of sequence information obtained following the PCR confirmed that they were identical to the desired sequences present in the Genebank database (Figure 4.1). Both the *CtManS* and *CtGMGT* sequences were ligated in sense orientation with respect to the promoter of the plant expression vector pUbi510 (Figure 4.2 a and b).

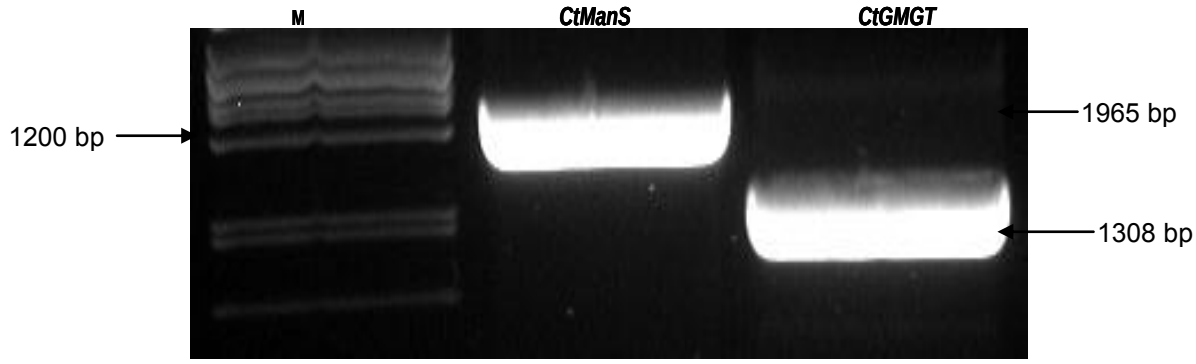

Figure 4.1: Isolation of galactomannan biosynthesis sequences from *Cyamopsis tetragonoloba* seeds endosperm by reverse transcriptase polymerase chain reaction (RT-PCR).
M: DNA Marker ; *CtManS:* Mannan synthase and *CtGMGT*: Galactomannan galactoslytransferase.

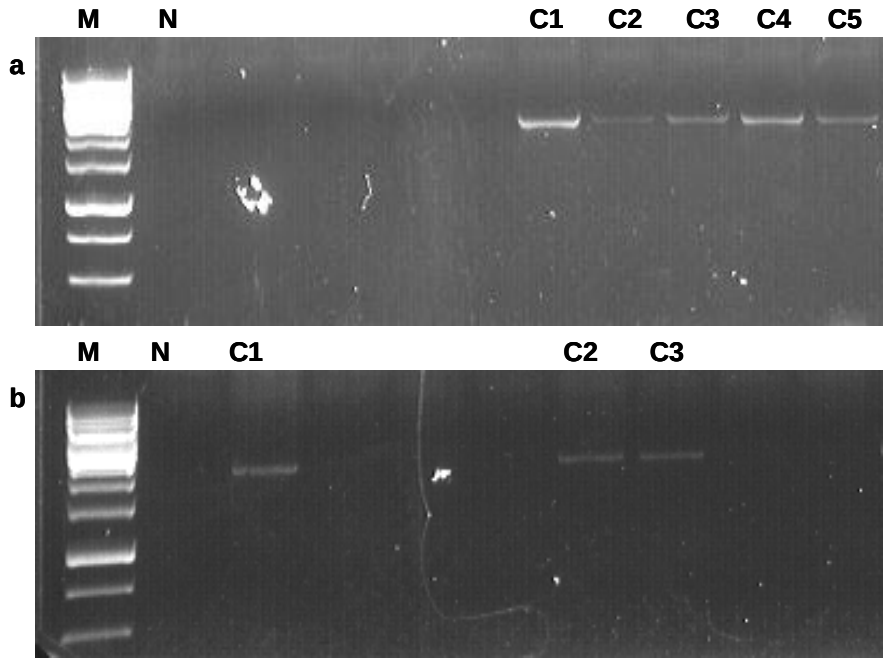

Figure 4.2: Directional polymerase chain reaction analysis of galactomannan biosynthesis sequence.

a) Mannan synthase sequence (*CtManS*), bacterial colonies containing *CtManS* sequence in the sense orientation with the promoter.

b) Galactomannan galatosyltransferase sequence (*CtGMGT*), bacterial colonies containing *CtGMGT* sequence in the sense orientation with the promoter the sense direction.

M: Marker DNA; N: Negative control; C: Bacterial colony.

4.3.1.2 Integration of galactomannan biosynthetic sequences into the sugarcane genome

Following biolistic transformation calli that survived geneticin selection were screened using PCR for the integration of pUbi*ManS* and/or pUbi*CtGMG* into the genome. Three independent clones designated GL1, GL9 and GL10 were found to contain both sequences (Figure 4.3 a) which were also transcribed to mRNA levels as shown by RT-PCR (Figure 4.3 b). Both the GL1 and GL9 clones were able to be regenerated into plants which were transferred to the greenhouse for further analysis.

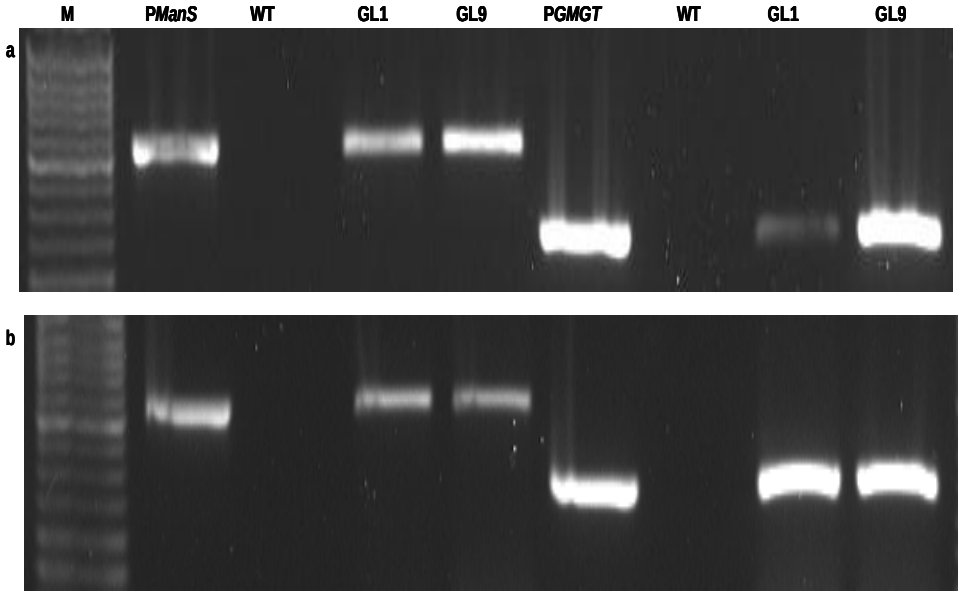

Figure 4.3: Analysis of sugarcane calli by polymerase chain reaction for *CtManS* and *CtGMGT* sequences integration at gDNA level and their expression at mRNA level.

(a) PCR gel analysis of *CtManS* and *CtGMGT* sequences integration at gDNA level.

(b) RT-PCR gel analysis of *CtManS* and *CtGMGT* sequences expression at mRNA level.

M: DNA maker; P*ManS:* Mannan synthase positive control; WT: Wild type control; GL1: Galactomannan Line1 and GL9: Galactomannan Line9

4.3.1.3 Galactomannan production in transgenic sugarcane calli

Callus growing on solid media plates were evaluated for the production of galactomannan polymers. Water soluble extracts were treated with Fehling's solution for the isolation of galactomannans. Transgenic lines GL1 and GL9 water soluble polymers were able to form a copper complex precipitate while the wild type calli did not. Hydrolysis with sulphuric acid of the precipitate followed by GC-MS analysis demonstrated the presence of mannose and galactose as the major constituents of this polymer (Figure 4.4).

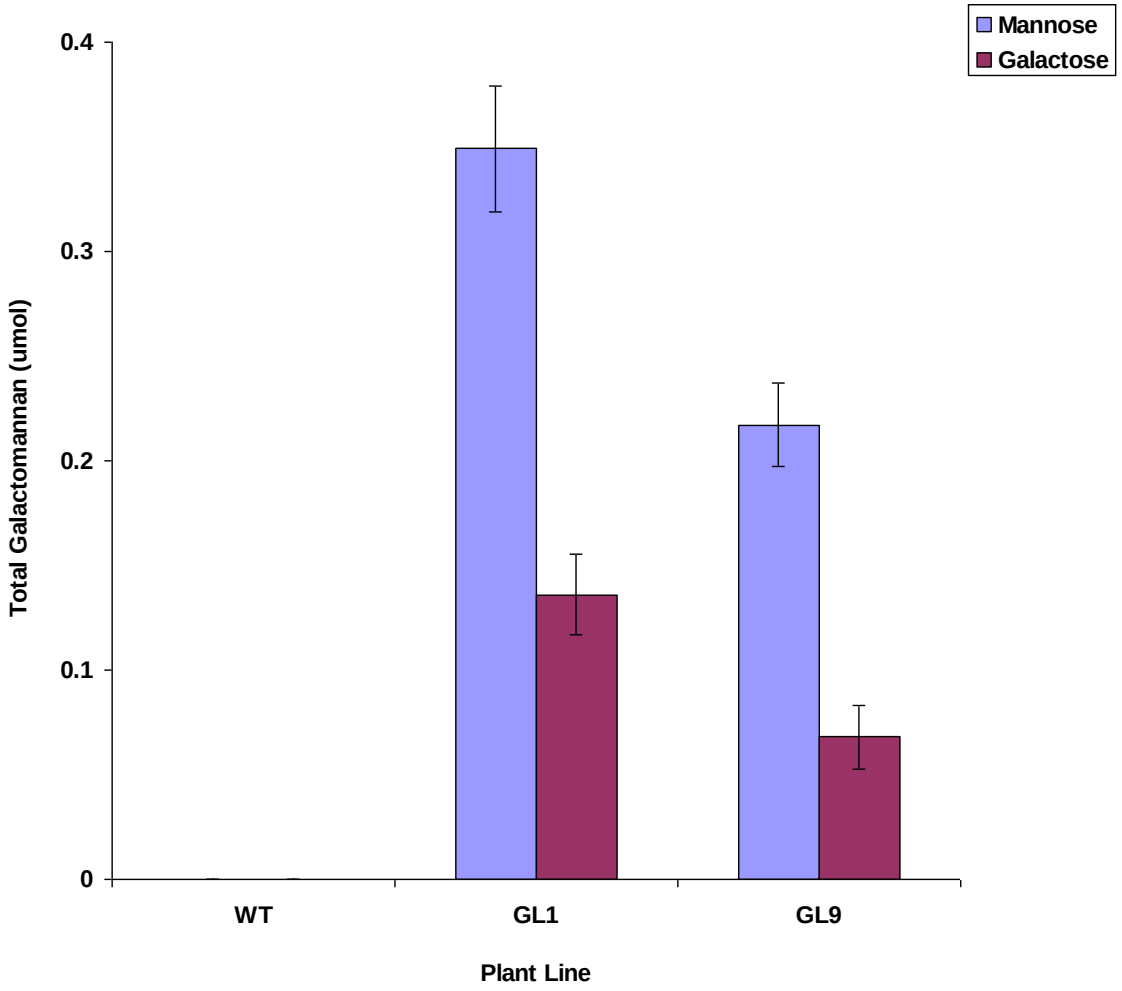

Figure 4.4: Level of mannose and galactose in transgenic sugarcane callus expressing galactomannan biosynthetic sequences.
WT: Wild type control; Transgenic lines: GL1 and GL9

4.3.1.4 Analysis of suspension culture cells and mature sugarcane plants expressing galactomannan encoding biosynthetic enzyme sequences by PCR and RT-PCR

GL1, GL9 and GL10 lines were further characterized to examine transgene expression in suspension cultures and mature regenerated plants grown in the greenhouse for eighteen months. Both sequences were integrated in the sugarcane genomic DNA and transcribed to mRNA in both suspension culture cells (GL1, GL9 and GL10) and mature transgenic sugarcane lines (GL1 and GL9; Figure 4.5 a, b and Figure 4.6 a, b).

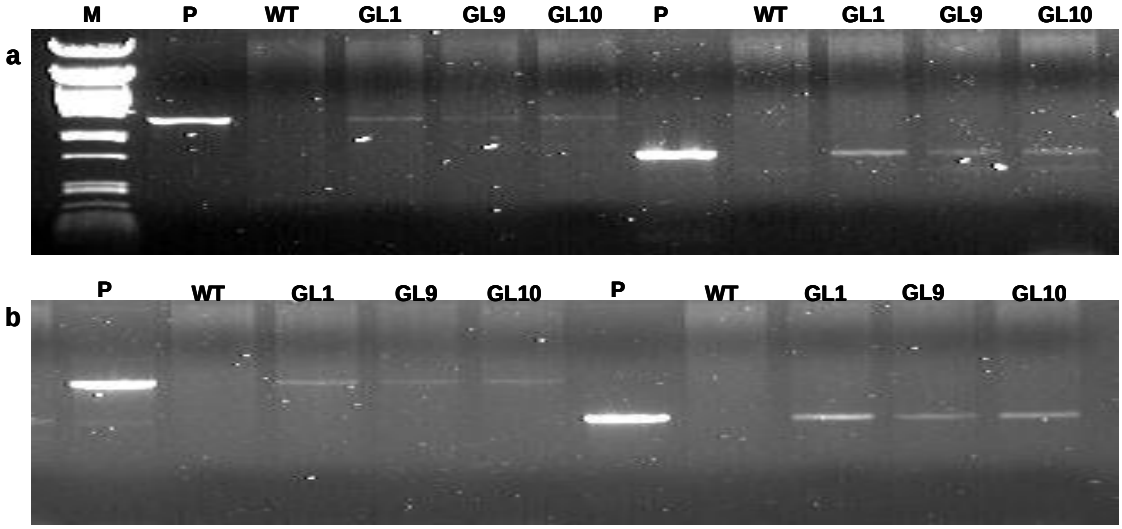

Figure 4.5: Polymerase chain reaction analysis of galactomannan biosynthetic sequences pUbi*CtManS* and pUbi*CtGMGT* in sugarcane suspension culture cells.

(a) PCR gel analysis of *CtManS* and *CtGMGT* sequences integration at gDNA.

(b) RT-PCR gel analysis of *CtManS* and *CtGMGT* expression at mRNA level.

P: Mannan synthase positive control; WT: Wild type control; Transgenic lines:GL1: Galactomannan Line1; GL9: Galactomannan Line9 and GL10: Galactomannan Line10

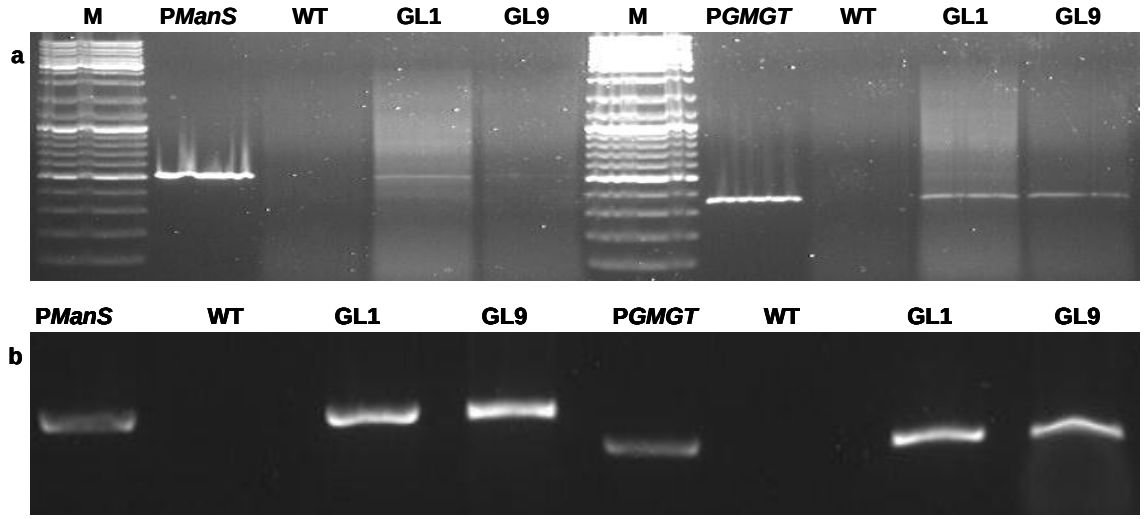

Figure 4.6: Polymerase chain reaction analysis of galactomannan biosynthetic Sequences pUbi*CtManS* and pUbi*CtGMGT* in mature transgenic sugarcane plants.

(a) PCR gel analysis of *CtManS* and *CtGMGT* sequences integration at gDNA.

(b) RT-PCR gel analysis of *CtManS* and *CtGMGT* sequences expression at mRNA level.

P*ManS:* Mannan synthase positive control; P*GMGT*; galactomannan galactosyltransferase positive control ; WT: Wild-type control; Transgenic lines:GL1: Galactomannan Line1; GL9: Galactomannan Line9

4.3.1.5 Determination of enzyme activities and galactomannan content in transgenic sugarcane lines

In order to demonstrate that presence of *CtManS* and *CtGMGT* in the genome also led to active proteins, mannan synthase and galactomannan galactosyltransferase enzymes activity were evaluated. Transgenic suspension culture cells (GL1; GL9 and GL10) and mature sugarcane (GL1 and GL9) lines had no detectable mannan synthase and galactomannan galactosyltransferase enzymes activities.

The presence of galactomannan was also determined. Isolation of galactomannan from water soluble polymers of sugarcane suspension cells and internodes tissue and treatment with Fehling's solution did not yield any copper complex precipitates. Furthermore, analysis of mannose and galactose amounts in cell wall alcohol insoluble residue (AIR) were evaluated by GC/MS. Transgenic suspension cell and internodal tissues had similar mannose and galactose sugar monomers as compared to untransformed wild-type suspension cells or internodal tissues (Figure 4.7 a, b).

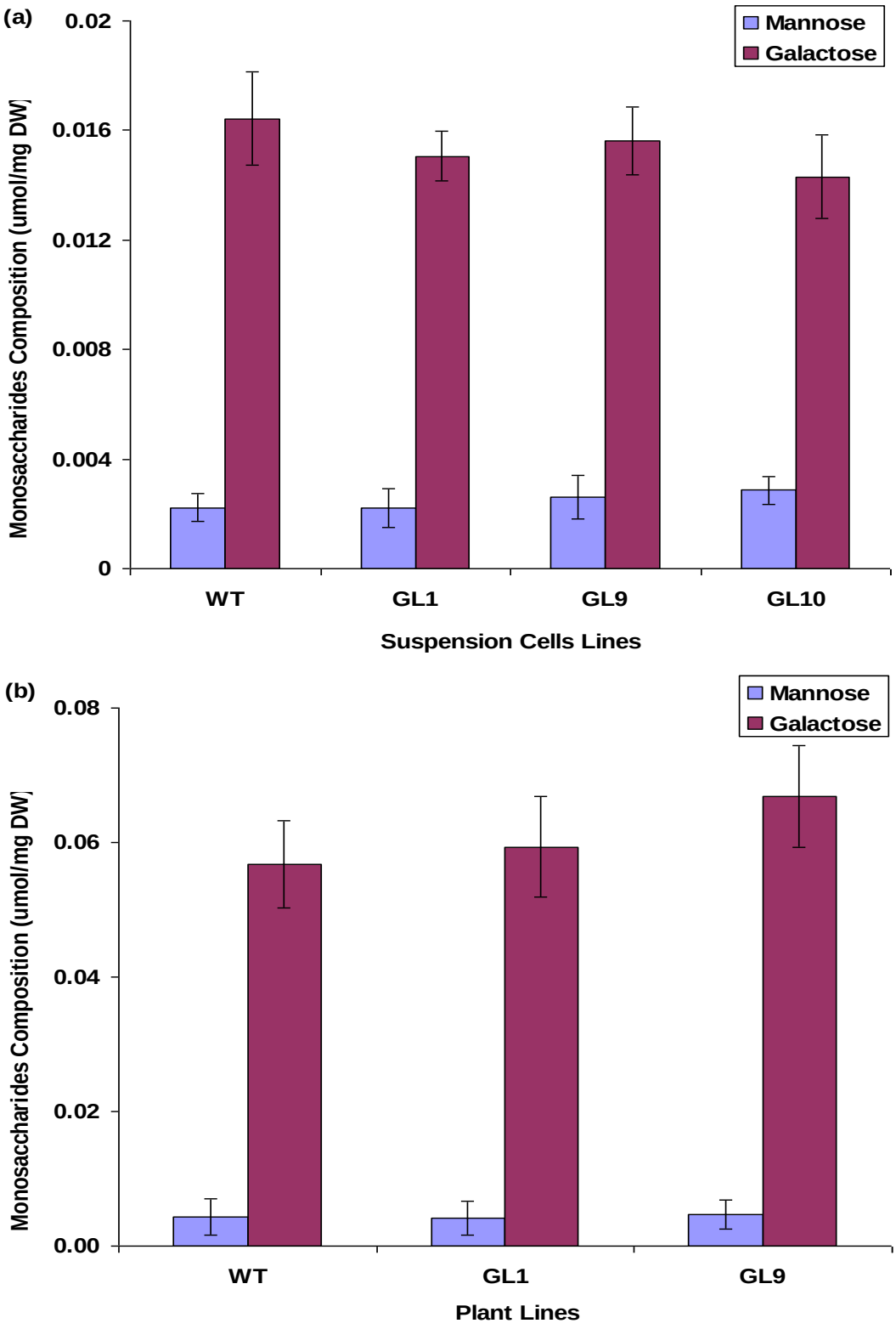

Figure 4.7: Analysis of cell wall mannose and galactose in transgenic sugarcane suspension cell and internodal tissues expresssing galactomannan biosynthetic sequences.

a) Suspension culture cells mannose and galactose content.

b) Internodal tissue mannose and galactose content.

WT: Wild type control; Transgenic lines: GL1; GL9 and GL10

4.3.2 Ectopic expression of α-1,4-glucan biosynthetic gene in sugarcane

4.3.2.1 Production of plant transformation constructs designed to lead to increased α-1,4-glucan biosynthesis

The coding region of the *Deinococcus radiodurans dras* gene was isolated from gDNA by PCR. The amplified *dras* gene was 1935 bp in length and BLASTn analysis of sequence information obtained following the PCR confirmed that it was identical to the desired sequence present in the Genebank database (Figure 8 a). The PCR product encoded an active protein as bacterial cells transformed with a plasmid allowing its expression produced linear glucan when grown on media containing sucrose (Figure 4.8 c and d). The functional *dras* gene was excised from pKSDRAS and pKSNTPPDRAS using *Bam*H I and *Sac* I and *Kpn* I and *Sac* I restriction enzymes respectively (Figure 4.8 b) and ligated into plant transformation vectors pU3Z allowing expression either in the cytosol or vacuole.

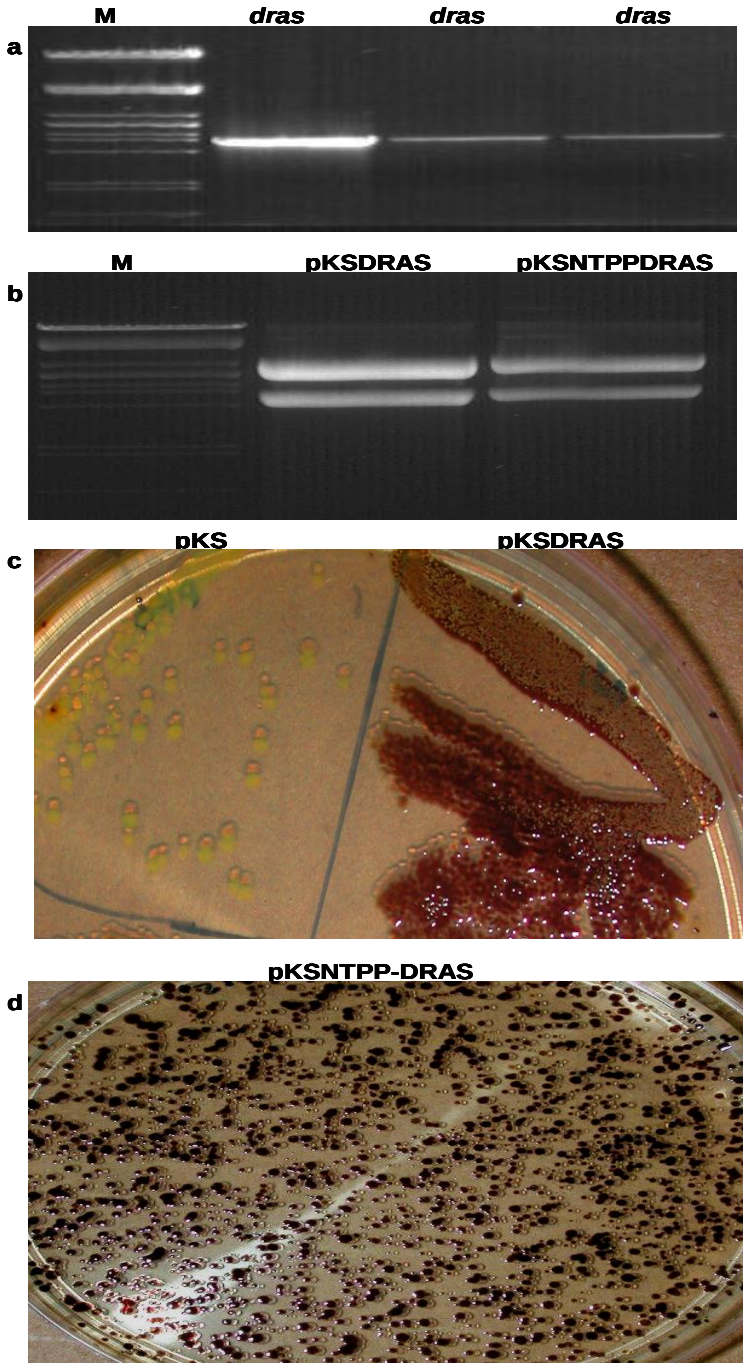

Figure 4.8: Isolation of α-1;4 glucan biosynthetic gene from *Deinococcus radiodurans* genomic DNA by polymerase chain reaction and iodine staining of bacterial cell expressing DRAS and NTPP fused DRAS protein for production of α-1;4 glucan chains.

a) PCR gel analysis of isolated *dras* gene.

b) Restriction enzyme digests of pKSDRAS and pKSNTPPDRAS plasmids with *Bam*HI and *Sac*I and *Kpn*I and *Sac*I respectively.

c) Iodine staining for α-1;4 glucan chain production in bacterial cells expressing DRAS protein.

d) Iodine staining for α-1;4 glucan chain production in bacterial cells expressing DRAS protein fused to NTPP.

M: DNA marker; *dras:* Amylosucrase gene; pKS: plasmid; pKSDRAS: plasmid containing amylosucrase *dras* gene; pKSNTPPDRAS: plasmid containing fused dras gene vacuole targeting sequence

4.3.2.2 Transformation of the *dras* gene into sugarcane and analysis of soluble and insoluble sugars in regenerated transgenic lines

Following transformation calli that survived geneticin selection were screened using PCR for the integration of *dras* gene into sugarcane genome. Two independently transformed lines (conferring cytosolic localisation designated ASL1 and ASL2) and three independently transformed lines (conferring vacuolar localisation designated spoASL1, spoASL2 and spoASL3) targeting the DRAS protein into the cytoplasm and the vacuole respectively were found to contain the *dras* gene (Figure 4.9 a; c). The gene was also transcribed at the mRNA level as shown by RT-PCR (Figure 4.9 b; d).

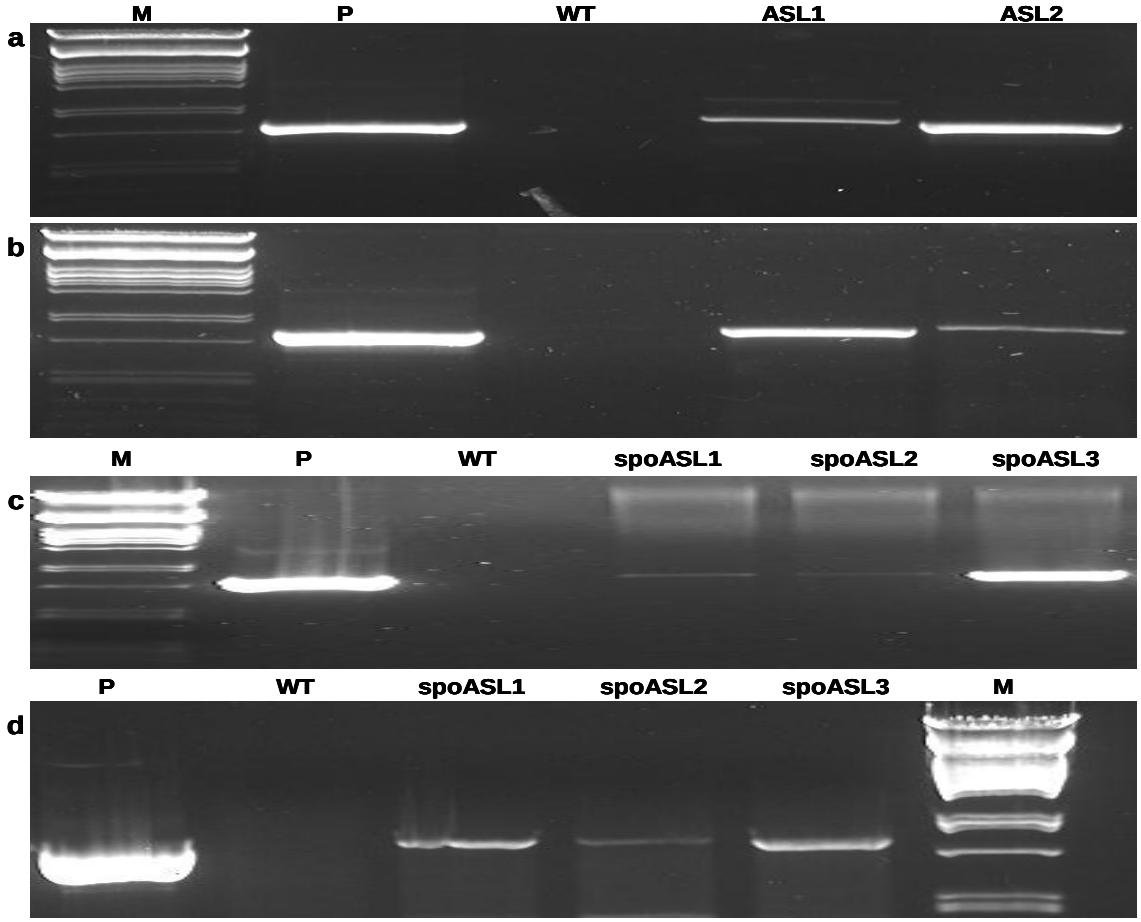

Figure 4.9: Polymerase chain reaction analysis of α-1;4 glucan chain biosynthetic gene in sugarcane calli.

a) PCR gel analysis of cytoplasmic targeted *dra* gene integration gDNA.
b) RT-PCR gel analysis of cytoplasmic targeted DRAS protein expression at mRNA.
c) PCR gel analysis of vacuole targeted *dra* gene integration in gDNA.
d) RT-PCR gel analysis of vacuole targeted DRAS protein expression at mRNA.

P: Positive control; WT: Wild type control; ASL1 and ASL2: Transgenic lines expressing DRAS protein in the cytosolic; spoASL1, spoASL2 and spoASL3: Transgenic lines expressing DRAS protein in the vacuole.

Transgenic calli were regenerated into plants and transferred to the greenhouse for further analysis. Ectopic expression of the DRAS protein into the cytosol was detrimental to plant development, as 18 month old plants had stunted growth (Figure 4.10 a; b, c). This resulted in the termination of the study.

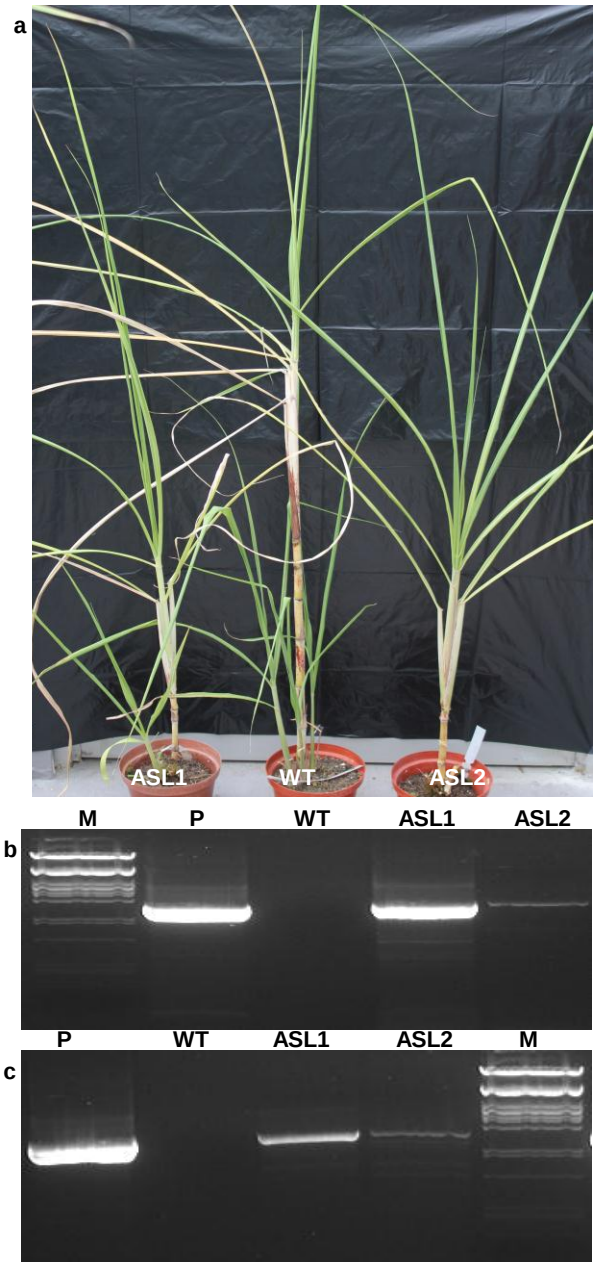

Figure 4.10: Phenotypic evaluation and analysis by PCR and RT-PCR expression of DRAS protein in the cytosol of mature greenhouse grown transgenic sugarcane plants

a) Phenotypic evaluation of mature greenhouse grown transgenic in comparison to wild type sugarcane plants
b) PCR gel analysis of *dras* gene integration in genomic DNA of sugarcane
c) RT-PCR gel analysis of Dras protein expression at mRNA in sugarcane

M: Marker DNA; P: Positive control; WT: Wild type; ASL: Transgeniclines expressing DRAS protein in the cytosol

Transgenic lines expressing the DRAS protein in the vacuole were observed to be taller than untransformed lines after 3 months growth in the greenhouse. The leaves of the transgenic lines had high maltooligosaccharide, sucrose, glucose and fructose contents (Figure 4.11).

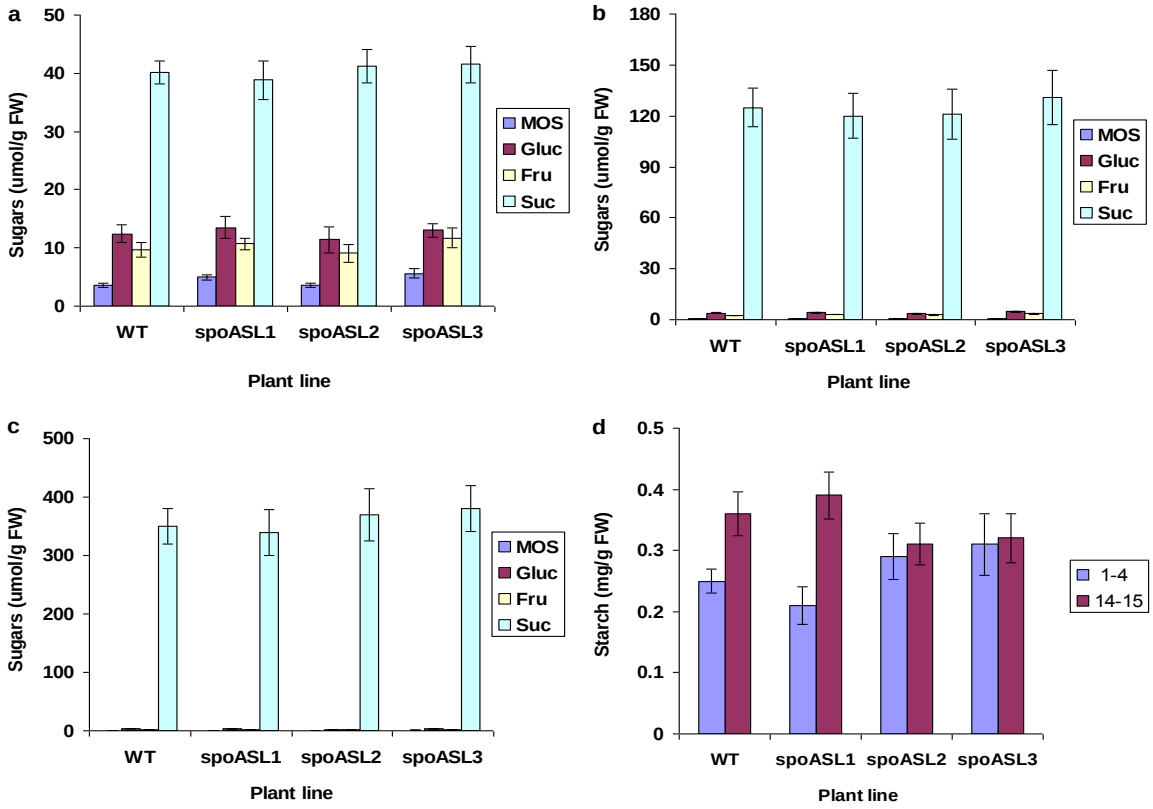

Figure 4.12: Quantification of soluble sugars and starch in the leaves and internodal tissues of transgenic lines expressing DRAS protein in the vacuole of mature greenhouse grown sugarcane plants.

a) Analysis of soluble sugars in leaf

b) Analysis of soluble sugars levels in young internodal (1-4) tissues

c) Analysis of soluble sugars levels in older internodal (14-15) tissues

d) Analysis of starch levels in young and old internodal tissues

WT: Wild type; spoASL: Transgenic lines expressing DRAS protein in the vacuole; MOS: Maltooligosaccharides; Gluc: Glucose; Fru: Fructose; Suc: Sucrose; 1-4: Internode 1-4; 14-15: Internodes 14-15. Values are mean calculated from 3 plants per line. Mean values with* were determined by the t test to be significantly different (P<0.05

However, after 18 months growth in the greenhouse the mature transgenic lines were morphologically similar and comparable in size to untransformed plants. Soluble sugars (maltooligosaccharides, glucose, fructose, sucrose, turanose, trehalulose) and starch content were evaluated in the transgenic lines. Soluble sugars and starch levels in the internodal and leaves tissues were observed to be similar to the wild type lines (Figure 4.12 a-d). The sucrose isomers turanose and trehalulose were also not detected with either GC/MS or HPLC analysis in leaves and internodal tissues of the mature transgenic lines.

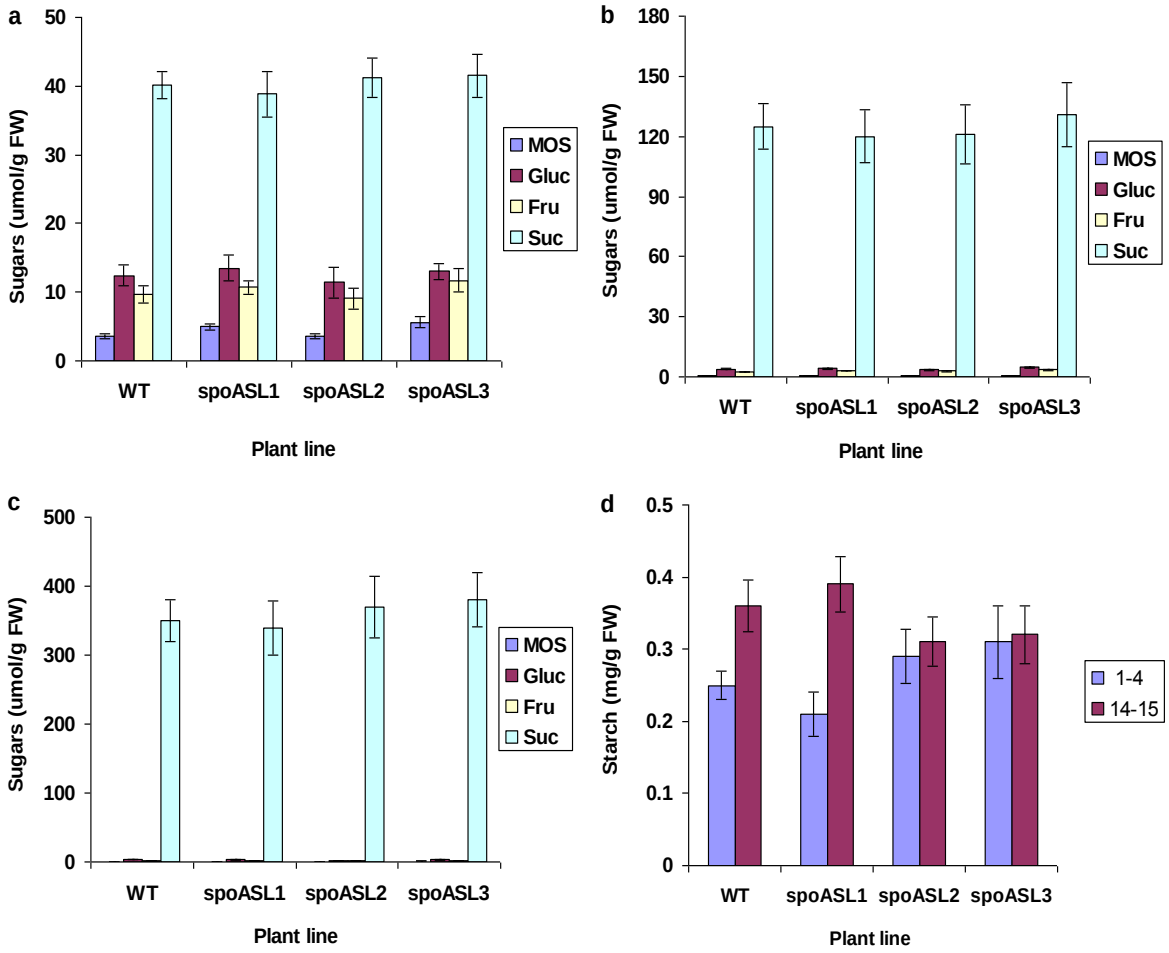

Figure 4.12: Analysis of soluble sugars and starch amounts in the leaves and internodal tissues of transgenic lines expressing DRAS protein in the vacuole of mature greenhouse grown sugarcane plants.

a) Analysis of soluble sugars in leaves

b) Analysis of soluble sugars levels in young internodal (1-4) tissues

c) Analysis of soluble sugars levels in older internodal (14-15) tissues

d) Analysis of starch levels in young and old internodal tissues

WT: Wild type; spoASL: Transgenic lines expressing DRAS protein in the vacuole; MOS: Maltooligosaccharides; Gluc: Glucose; Fru: Fructose; Suc: Sucrose; 1-4: Internode 1-4; 14-15: Internodes 14-15. Values are mean calculated from 3 plants per line. Mean values with* were determined by the t test to be significantly different ($P<0.05$

4.3.2.3 Analysis of amylosucrase enzyme activity in sugarcane plants expressing DRAS protein in the vacuole

Due to comparable soluble sugars, starch amounts and non-detection of turanose and trehalulose sugars between untransformed and transgenic lines, amylosucrase enzyme activity was evaluated. Amylosucrase enzyme activity was not detected in leaves and internodal tissues of mature transgenic lines and also performing in-gel enzyme activity assays, no α-1;4-glucan chains were detected (data not shown).

4.4 Discussion and Conclusion

Sugarcane bagasse is an underutilized substrate for bioethanol production due to its recalcitrance to enzymatic hydrolysis. Development of sugarcane where lignocellulosic polysaccharides can be increasingly accessed by hydrolytic enzymes is crucial for second generation bioethanol production. This study investigated the effects of heterologous expression of *Cyamopsis tetragonoloba* galactomannan (guar gum) biosynthetic (*CtManS* and *CtGMGT)* cDNA sequences in sugarcane. Galactomannan biosynthesis sequences were successfully isolated from developing endosperm of guar seeds and BLASTn analysis of the isolated sequences confirmed that the sequences were identical with the guar cDNA available in Genebank (Figure 4.1). The sequences were integrated into sugarcane gDNA and were observed to be transcribed at the mRNA level (Figure 4.3). Three independent transgenic clones GL1; GL9 and GL10 containing galactomannan biosynthesic sequences were generated by particle bombardment transformation.

Transgenic clones GL1; GL9 and GL10 calli were analyzed for the biosynthesis of galactomannans. Isolation of water soluble polymers, and treatment of these polymers with Fehling's solution resulted in a formation of a copper-polymer complex. Hydrolysis of the copper-polymer complex with sulphuric acid and subsequent GC/MS analysis, mannose and galactose were observed as major monosaccharides of this complex (Figure 4.4). Galactomannan is composed of mannose and galactose as major constituents of the polymer (Edwards *et al*., 1989) and isolation of water soluble galactomannan polymer by Fehling's solution and hydrolysis with sulphuric acid has been shown to result in mannose and galactose sugar monomers (Edwards *et al*., 1989; Naoumkina *et al*., 2008). These observations indicate that the isolated water soluble polymer in the calli of transgenic sugarcane clone GL1 and GL9 was galactomannan. Transgenic clone GL10 calli were also unable to regenerate plants, however plants were generated from galactomannan producing calli lines GL1 and GL9. Suspension culture cells expressing galactomannan cDNA's were evaluated for enzyme activity of mannan synthase and galactomannan galactosyltransferase. Enzyme activities of both galactomannan biosynthetic sequences were not detected in all transgenic sugarcane suspension culture cells. Consequently the suspension cell lines were unable to produce galactomannans even though the galactomannan biosynthetic sequences were transcribed at the mRNA level (Figure 4.5 a; b).

Moreover, complete acid hydrolysis of the cell walls of suspension cultured cells was observed to have comparable mannose and galactose sugar levels in the untransformed suspension cells confirming the non-biosynthesis of galactomannans (Figure 4.7 a). Similar to the suspension cultured cells results, mature transgenic sugarcane lines GL1 and GL9 were unable to biosynthesize galactomannans albeit *CtManS* and *CtGMGT* sequences of galactomannan biosynthesis being integrated into the gDNA and also transcribed at the mRNA level (Figure 6 a; b and Figure 7 b). There are only few studies of heterologous expression of guar sequences in plants, only the mannan synthase sequence (Ct*ManS*) has been expressed in *Glycine max* and *Medicago truncatula* (Dhugga *et al*., 2004; Naoumkina *et al*., 2008). Dhugga *et al* (2004) showed that expression of Ct*ManS* sequence in *G. max* suspension cells resulted in a functional ManS protein capable of *in vitro* synthesizing the 1,4-β-linked-mannan backbone and in mature transgenic seeds with increased amounts of mannose sugars. In contrast, heterologous expression of this sequence in *M. truncatula*, resulted in transgenic lines that were able to transcribe Ct*ManS,* and seeds with reduced mannose, galactose and galactomannan polymers (Naoumkina *et al*., 2008). However, mannan synthase enzyme activity was not reported in the previous study (Naoumkina *et al*., 2008). In both studies discussed above, no analysis of the cell wall constituents in other parts (e.g. stem and leaves) of the mature transgenic lines was reported and it is therefore difficult to assess the impact of expression of galactomannan biosynthetic sequences in these organs.

Linear α-1;4-glucan biosynthetic *dras* gene was successfully isolated from *D. radiodurans* and BLASTn analysis of the isolated gene was confirmed to be identical to the *dras* gene published in the Genebank (Figure 4.8 a). Bacterial cells expressing the *dras* gene were able to stain dark-brown when grown on sucrose as main carbon source when exposed to iodine vapours, even when the *dras* gene was fused to a sweet potato sporamin (NTPP) sequence, a vacuolar targeting sequence (Figure 8 c and d). Dark-brown staining of the cells is an indication of the production of α-1;4-glucan branched chains, it is due to the co-localization of the amylosucrase protein inside the bacterial cells and the glycogen branching enzyme (glgB) that adds side chains to the linear α-1;4-glucans produced by amylosucrase. Similar dark brown staining with iodine vapour was observed in *E. coli* cells expressing amylosucrase *AS* gene from *Neisseria polysaccharea* when grown on sucrose as carbon source (Büttcher *et al*., 1997). These results confirm that the isolated *dras* gene has an

amylosucrase enzyme activity even when fused to the NTPP vacuolar targeting sequence from sweet potato retains activity when expressed in *E. coli* .

Sugarcane embryogenic calli were transformed with the *dras* gene and calli that survived geniticin selection were screened by PCR and RT-PCR. Amylosucrase DRAS targeted to the cytosol was integrated into the sugarcane gDNA and was observed to be transcribed at the mRNA level in both calli and greenhouse grown sugarcane plants (Figure 4.9 a-b). Two independent transgenic lines ASL1 and ASL2 were regenerated and were observed to have stunted growth after 18 months growing in the greenhouse (Figure 10 a). Similar growth impediment has been observed in transgenic tobacco and sugarcane plants accumulating the sucrose isomer palatinose in the cytosolic compartment (Börnke *et al*., 2002; Wu and Birch 2007). Growth inhibition in the transgenic plants producing sucrose isomers is thought to be due to sugar signalling, osmotic stress or depletion of sugars. However, in this study the sucrose isomers trehalulose and turanose were not detected in dwarf transgenic sugarcane lines expressing the amylosucrase encoding *dras* gene (data not shown). Recently Wu and Birch (2011) showed that trehalulose and turanose sugars are hydrolyzed by sugarcane cytosolic SuSy and that these sugars have an inhibitory effect on NI enzyme activity. Consequently non-detection of these isomers might be due to hydrolysis of these isomers by cytosolic SuSy. The stunted growth in the transgenic lines may be suggested to be caused by the production of turanose and trehalulose (which is immediately degraded by SuSy) by DRAS protein in the cytosol compartment that has been showed to have adverse effects on plants growth and development when expressed in this compartment (Börnke *et al*., 2002; Wu and Birch 2007).

The amylosucrase encoding *dras* gene fused to a sweet potato sequence (NTPP) for vacuolar DRAS targeting was found to be integrated into the genome and transcribed in sugarcane calli (Figure 4.9 c-d). Transgenic sugarcane lines (spoASL1, spoASL2 and spoASL3) expressing the DRAS protein in the vacuole after three months growing in the greenhouse had increased levels of soluble sugars such as maltooligosaccharides, glucose, fructose and sucrose in the leaves (Figure 4.11). Increased sucrose and invert sugars amounts in DRAS expressing transgenic lines were not expected in leaves. Wu and Birch (2007) showed that plasma membrane vesicles isolated from transgenic lines leaves accumulating high levels of sucrose

isomers had 20-30% increases in sucrose transport into these vesicles. Furthermore, in sugarcane there is a strong relationship between sucrose concentrations in the source (leaves) and sink (internodes) tissues (McCormick *et al*., 2006; McCormick *et al*., 2008; McCormick *et al*., 2009). McCormick *et al*., (2006) observed that a decrease in internodal sucrose resulted in high photosynthesic rate in the leaves. Therefore, increased sucrose amounts in the transgenic lines leaves in the present study might be due to amylosucrase utilization of sucrose in the internodal tissues and in turn promoting sucrose synthesis in the source leaves. High glucose and fructose levels in the leaves may also be caused by hydrolysis of turanose and trehalulose in the vacuole by SuSy and SAI (Wu and Birch 2011). This assumption is supported by non-detection of sucrose isomers in the 3 months old transgenic leaves lines expressing the DRAS protein in the vacoule by GC/MS or HPLC analysis. The increases in maltooligosaccharides could be concluded to be due to the biosynthesis of α-1;4-glucan chains by amylosucrase activity in the vacuoles in the leaves of 3 months old transgenic lines.

However, after 18 months growing in the greenhouse the transgenic lines had similar maltooligasccharide, sucrose, glucose, fructose and starch contents in the leaves and internodal tissues as compared to untransformed plants (Figure 4.12 a-d). Moreover, amylosucrase enzyme activity was not detected in all transgenic lines during development, this may be explained by the sugarcane vacuole environment which is acidic and proteolytic (Gnanasambandam and Birch, 2004; Wu and Birch 2007). Enzyme activity of proteins targeted to sugarcane vacuoles in a number of studies has not been detected, even when the end product was biosynthesized (Wu and Birch 2007; Hamerli and Birch, 2011; Basnayake *et al*., 2012, Mudge *et al*., 2013). However in the present study α-1;4-glucans and sucrose isomers in the mature transgenic lines were not detected, even though the *dras* transgene was transcribed at the mRNA level.

Transgene silencing in plant is thought to be activated by invasive or replica DNA sequences, or by transcript mechanisms that detect mRNA that exceed a threshold, aberrant, or double-stranded mRNA (Baulcombe and English 1996; Kumpalta *et al*. 1998; Fagard and Vaucheret, 2000). Gene silencing in polyploidy sugarcane has been observed in a number of studies (Hansom *et al*., 1999; Ingelbrecht *et al*., 1999; Wei *et al*., 2003; Birch *et al*., 2010; Hamerli and Birch, 2011). In sugarcane silencing

is thought to be developmentally, copy number independent, promoter specific, transcriptional and posttranscriptional regulated (Ingelbrecht *et al*., 1999; Wei *et al*., 2003; Mudge *et al*., 2009; Birch *et al*., 2010, Moyle and Birch., 2013). Expression of reporter genes (*GUS* and *luc* gene) or fluorescent protein (*GFP*) in transgenic sugarcane and tobacco respectively, were observed to result in active proteins which diminish with development in these plants (Hamilton et al., 2002; Wei *et al*., 2003; Mudge *et al*., 2009; Birch *et al*., 2010). In contrast to the present study, mRNA was not detected in these transgenic plant lines. Hamilton *et al*., (2002) shown that the silenced transgene in tobacco contained two classes of GFP short interfering RNA (GFP siRNA), corresponding to both sense and antisense strands of GFP gene. The presences of siRNA were later shown to be a result of mRNA degradation by Dicer-like proteins (Tang *et al*., 2003; Qi *et al*., 2005). The inability to biosynthesize galactomannans and α-1;4-glucans and non-detection of enzyme activities in the transgenic lines transcribing these sequences at the mRNA level might be due improper protein processing post-transcriptional and protein instability in the acidic vacoule, possibly explainable by other epigenetic mechanisms taking place to regulate gene expression in the at least allo-octaploid species of sugarcane under investigation in this study.

In conclusion, the data presented demonstrated that heterologous expression of galactomannan biosynthetic (*CtManS* and *CtGMGT)* sequences in transgenic sugarcane callus led to the biosynthesis of galactomannan and resulted in high fermentable hexose (mannose and galactose) sugars. Similarly, heterologous expression of vacuole targeted DRAS protein resulted in plants which were able to produce α-1;4-glucan polymer. However with development the production of both polymers were inhibited in mature sugarcane plants. These data leads us to hypothesize that the biosynthetic sequences which were transcribed at the mRNA level in mature transgenic sugarcane plants were not translated into functional proteins and therefore resulting in transgenic sugarcane lines unable to biosynthesize these polymers.

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Chapter 5

5.1 Summary, conclusion and future work

The main aim of the work presented in this thesis was to increase lignocellulosic biomass and to improve its characteristics for bio-energy production. This was accomplished by introducing new biosynthetic pathways from organisms such as bacteria, animals and other plant species In the present study a cellulose synthase encoding sequence from a marine invertebrate *Ciona savignyi*, hemicellulosic biosynthetic enzymes encoding sequences from *Cyamopsis tetragonoloba* and an α-1;4-glucan biosynthetic enzyme encoding sequence from *Deinococcus radiodurans* were used for heterologous expression in sugarcane.

5.1.1 Heterologous expression of a cellulose synthase encoding sequence from *C. savignyi* in sugarcane

Transgenic sugarcane plants heterologously expressing a cellulose synthase sequence were produced and characterized (Chapter 3). Cellulose amounts in the transgenic lines expressing the *CsCesA* sequence were increased and the rise in cellulose levels correlated with higher cellulose synthase enzyme activity levels. The elevation in cellulose contents was accompanied by an increase in total soluble sugars (sucrose, glucose and fructose), hexose phosphates (glucose-1-P, glucose-6-P and fructose-6-P) and UDP-glucose in younger internodes and a reduction in starch contents in young and mature internodal tissues of the transgenic lines. The elevation in soluble sugars, hexose phosphates and UDP-glucose could indicate an increased cycling of sucrose to support the increased cellulose biosynthesis in the transgenic lines thereby providing increased levels of UDP-glucose. This assumption is supported by the elevation in levels of the sucrose synthesizing enzymes SPS and SuSy and the catabolic enzymes CWI and SuSy in these lines. Increases in the activities of sucrose metabolic enzymes in other plants is considered to be due to higher demands of UDP-glucose by the cellulose synthase A complex for the biosynthesis of cellulose (Amor *et al*., 1995; Babb and Haigler, 2001; Park *et al*., 2008; Coleman *et al*., 2009). UGPase which is thought to provide UDP-glucose for sucrose synthesis by SPS and SuSy was also observed to be higher in the transgenic lines as compared to untransformed control lines (Coleman *et al*., 2007;

Park *et al*., 2010). Sucrose synthesized by these enzymes is subsequently hydrolyzed by plasma membrane associated pSuSy to produce UDP-glucose, which is then utilized for cellulose biosynthesis by cellulose synthase A (CesA); Amor *et al*., 1995). Hemicellulosic glucose and uronic acids contents were increased in the transgenic lines while lignin was reduced. The mixed linked β-(1,3;1,4)-glucans are synthesized using UDP-glucose via (1,3;1,4)-β-glucan synthase (GS) in grasses (Burton *et al*., 2006; Carpita and McCann, 2010). Therefore increases in the UDP-glucose contents in the transgenic lines may have promoted the biosynthesis of β-(1,3;1,4)-glucans. Uronic acid-containing polysaccharides like pectins, containing glucuronic and galacturonic acid, are synthesized from the precursor UDP-glucuronic acid produced by the UGD enzyme or and by the inositol oxygenation pathway (Loewus *et al*., 1962; Tenhaken and Thulke, 1996). The availability of UDP-glucose and increases in activity of the UGD enzyme in the transgenic plants could have led to increases in uronic acid-containing polysaccharides biosynthesis. The reduction in lignin polymers in the transgenic lines suggests that carbon was diverted away from the lignin biosynthetic pathway to cellulose synthesis. Similar compensations between lignin and cellulose amounts has been observed in other transgenic plants [aspen tree, rice and Arabidopsis] (Hu *et al*., 1999; Joshi *et al*., 2010; Ambavaram *et al*., 2010; Yan *et al*., 2013), when the lignin biosynthetic pathway was silenced. Changes in cell wall lignocellulosic composition led to improved saccharification efficiency of the biomass of the transgenic lines. Improved hydrolysis of the transgenic lignocellulosic biomass is probably due to the increased availability of cellulose for enzymatic accessability.

A hypothetical model to explain the changes in carbon flux towards the biosynthesis of cellulose in the transgenic sugarcane lines heterologously expressing *C. savignyi CsCesA* sequence is suggested in figure 5.1. Increases in the biosynthesis of cellulose, possibly stimulated sucrose hydrolysis by pSuSy in the plasma membrane to provide UDP-glucose for the cellulose synthase A enzyme complex. Demand for sucrose in the sink internodal tissues due to high cellulose biosynthesis might lead to upregulation of photosynthesis in order to synthesise increased amounts of sucrose in source leafs. After sucrose unloading in the sink tissues it is hydrolyzed by INV and CWI to promote further sucrose unloading from source leaves tissues. Hexose sugars (glucose and fructose) derived from this hydrolysis are phosphorylated by hexokinases and then used by UGPase to produce UDP-glucose. To maintain

sucrose demands cytosolic sucrose synthesizing enzymes SuSy and SPS are activated to re-synthesize sucrose. UDP-glucose is subsequently utilized by SuSy and SPS to re-synthesize sucrose to supply pSuSy for the production of UDP-glucose for cellulose synthesis by CesA. Alternatively, the high levels of UDP-glucose are used for the synthesis of hemicellulosic mixed β-(1,3;1,4)-glucans via β-(1,3;1,4)-glucan synthase (GS). UDP-glucose is also utilized by UGD to produce UDP-glucuronic acid (UDP-glucA), which is then used for the synthesis of pectins.

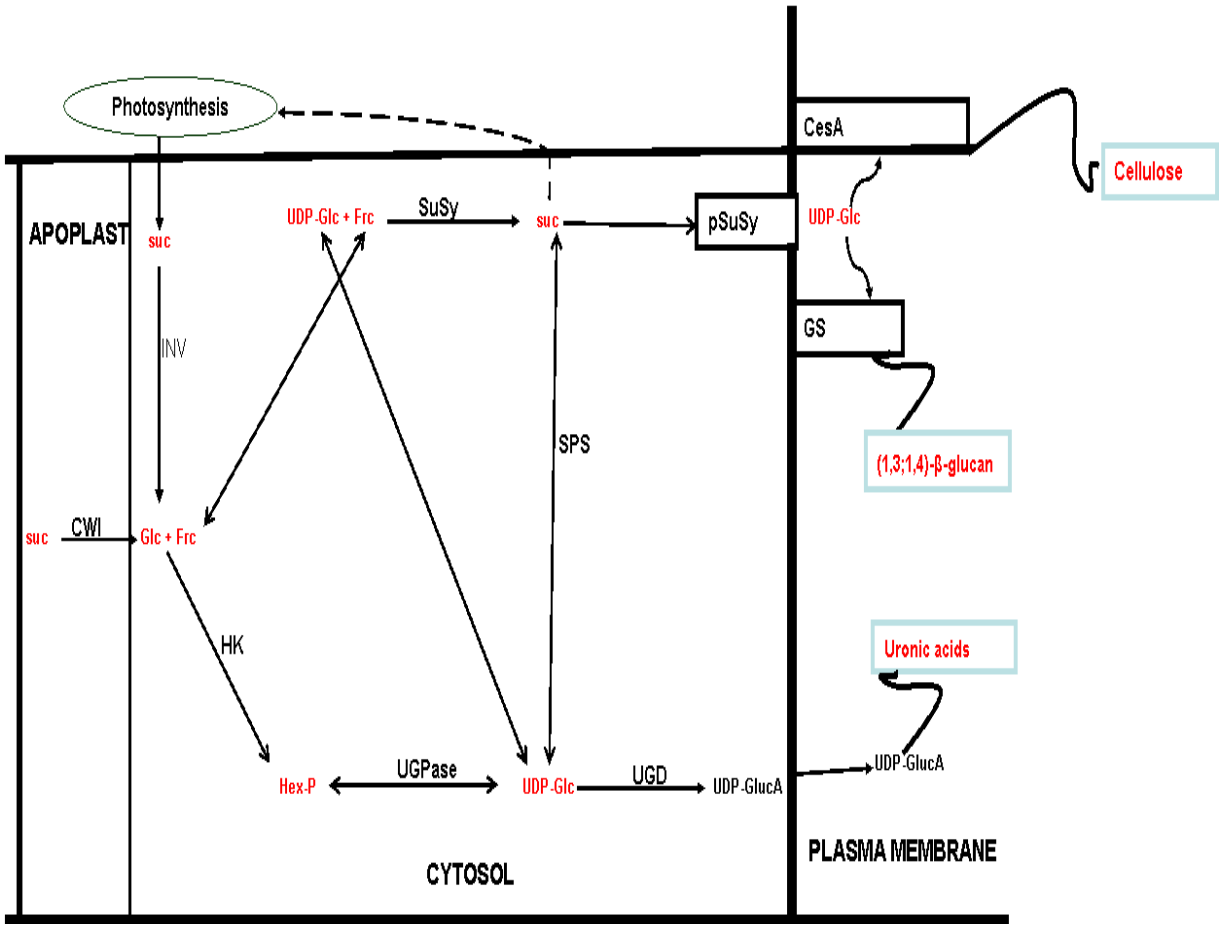

Figure 5.1: Hypothetical model for increased biosynthesis of cellulose in sugarcane transgenic lines expressing *CsCesA* sequence. In red are the increased metabolites and in bold demonstrate up-regulated enzyme activities. Dash arrow represent a feedback mechanism.

The priority for future work is to determine the interaction mechanism of *Ciona savignyi CsCesA* protein with the sugarcane cellulose synthase complex proteins. The transgenic line-derived lignocellulosic polysaccharides will also be investigated in detail using monoclonal antibodies for the profiling of the polysaccharides. The efficiency of bioethanol production from transgenic line-derived lignocellulosic biomass of field grown plants will also be investigated after sucrose extraction, to determine the efficiency of cellulase enzymes in hydrolyzing the lignocellulosic feedstock biomass.

5.1.2 Ectopic expression of sequences encoding *Cyamopsis tetragonoloba* galactomannan and *Deinococcus radiodurans* α-1,4-glucan chain biosynthetic enzymes leads to non-biosynthesis of the polymers mature transgenic sugarcane lines.

Transgenic sugarcane plants heterologously expressing genes encoding galactomannan biosynthesic enzymes and an α-1;4-glucan biosynthetic enzyme targeted to the cytosolic and vacuolar compartments were produced and characterized. Transgenic calli of lines expressing galactomannan synthesis sequences were able to produce galactomannans. However suspension culture cells and mature transgenic line were incapable of biosynthesizing galactomannans, although the sequences were shown to be integrated into the genomic DNA and also to be transcribed at the mRNA level. Enzyme activities of these sequences were also not detected in both suspension cells and mature plants derived of the transgenic lines. The failure of the transgenic lines to biosynthesize galactomannans and the non-detection of mannan synthase and UDP-galactose-dependent galactosyltransferase enzyme activities in these lines may be directly attributed to posttranscriptional mRNA processing.

Expression of the amylosucrase DRAS protein in the cytosol was observed to be detrimental to the growth of transgenic lines. After 18 months growing in the greenhouse, the transgenic lines were observed to have stunted growth. This stunted growth in the transgenic lines has also been observed in transgenic tobacco and sugarcane plants producing sucrose isomers in the cytosol (Börnke *et al*., 2002; Wu and Birch 2007). However targeting DRAS into the vacuole generated transgenic lines, which were able to biosynthesize α-1;4-glucan chains as observed by increases in maltooligosaccharides and which also had high sucrose, glucose and fructose amounts in the leaves when the plants were growing three months in the greenhouse. The differences in internodal tissue maltooligosaccharide, soluble sugar and starch levels however were comparable to untransformed lines after 18 months in the greenhouse. Furthermore, amylosucrase enzyme activity was not detected in leaves and internodal tissues although the *dras* gene was shown to be integrated into the genome and transcribed at the mRNA level. The non-detection of amylosucrase enzyme activity in 3 months old transgenic lines might be due to the acidic and

proteolytic environment in the sugarcane vacuoles (Gnanasambandam and Birch, 2004; Wu and Birch 2007).

The inability of the transgenic lines to biosynthesize galactomannans and α-1;4-glucans albeit transcription of the biosynthetic sequences at the mRNA level in these plants, lead us to propose that the non-biosynthesis of these polymers is due to post-transcriptional protein processing and protein instability in the vacuolar campartment. Future work will focus on under standing the underlying mechanism of posttranscriptional processing of mRNA in the developed transgenic sugarcane lines.

5.2 Conclusion

This study has highlighted the complexity of introducing novel biosynthetic pathways into a polyploid sugarcane genome. Heterologous expression of cellulose synthase encoding sequence from a marine invertebrate *Ciona savignyi CsCesA* resulted in increased cellulose amounts, leading to elevated cellulosic biomass in the mature transgenic sugarcane plants. Transgenic lines lignocellulosic biomass had improved release of fermentable sugars for biofuel production when exposed to cellulose hydrolyzing enzymes. Whereas ectopic expression of galactomannan and α-1;4-glucan biosynthetic sequences from *Cyamopsis tetragonoloba* and *Deinococcus radiodurans* resulted in calli cells and 3 month old transgenic lines producing galactomannans and α-1;4-glucans respectively. Surprisingly, with maturity the transgenic sugarcane plants were unable to biosynthesize these polymers, albeit biosynthetic sequences being transcribed at the mRNA level. Further analysis of these transgenic lines will enhance our understanding of posttranscriptional protein processing in this polyploid grass.

5.3 References

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